

**MODULATION OF THE BIOLOGIC BEHAVIOUR OF
BREAST CANCER; *in vitro* MODELS AND CLINICAL
CORRELATES.**

Lesley Katie Seymour

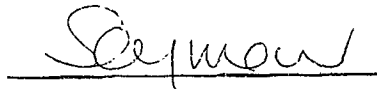
A thesis and original published work submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg 1998

DECLARATION

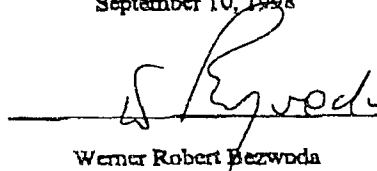
I, Lesley Katie Seymour declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

All papers submitted in this thesis have been previously published with the exception of Paper 13 which is in press (see appendix 1; page 61 for letter of acceptance). With the exception of Papers 5 and 10, the concept, execution, analysis and write-up were predominantly (assistance with routine assays was provided by co-authors), or entirely (Papers 7,9,12,13) my work, with support by my supervisor. For Papers 5 and 10, co-authors performed the serum or plasma assays while I performed the tissue assays and provided assistance with data retrieval, analysis and with the write-up.



Lesley Katie Seymour

September 10, 1998



Werner Robert Bezwoda

Supervisor

September 10, 1998

In memory of my father
Denis Allan William Seymour
1925-1982

ABSTRACT

Breast cancer is a common, devastating disease with an enormous impact on society. It has a complex biology, perhaps more complex than other tumour types, at least in part due to its hormonal control. While this complex biology poses an enormous challenge to researchers investigating breast cancer, it conversely offers the hope of additional interventions, not feasible with other tumours that may have a beneficial impact on outcomes. Important in choosing patients for such interventions are the definition of prognostic indicators that allow the tailoring of therapy to patients, limiting toxic treatments to those patients in whom they may be beneficial. Another potential approach is the modulation of tumour biology to circumvent drug resistance, either constitutional or acquired.

The studies included in this thesis have attempted to translate preclinical hypotheses into clinical investigations and ultimately new management. Hormone receptors, growth factors (TGF- β , PDGF) and markers of growth (Ki67) and oestrogen action (P24) have been demonstrated to have prognostic relevance in the clinic. Proof of principle of the modulation of breast cancer behaviour both in the preclinical and clinical setting has been attempted. These studies include an examination of the effects of oestrogen, given prior to cytotoxic chemotherapy. Biologic effects have been demonstrated although in the clinic a beneficial effect on survival was not seen. In addition, the studies demonstrate that interferon (IFN), given prior to tamoxifen treatment modulates cell behaviour and receptor expression,

potentially improving clinical outcomes. Interferon also modulates multidrug resistance, at least in an *in-vitro* model and may have clinical relevance. The real clinical import of these observations however awaits definitive clinical study, and the development of more effective modulators of drug resistance.

ACKNOWLEDGEMENTS

I would like to offer my sincere appreciation and gratitude to Professor Werner Bezwoda for his support, advice and encouragement.

Assistance was received for the following: hormone receptor assays by radioligand binding assay (Department of Nuclear Medicine, Johannesburg Hospital), pathological sub-typing (Dr G Behr, Dr D Lewis) and some assistance with bulk immunocytochemical assays (Ms D Dajee, Ms K Meyer). Serum assays for c-erbB-2 and PDGF were performed by co-authors (Dr S Ariad, Dr H Kandl).

TABLE OF CONTENTS

DECLARATION	ii
ABSTRACT	iv
ACKNOWLEDGEMENTS	vi
NOMENCLATURE	xi
CHAPTER 1 - INTRODUCTION	
1.1 Epidemiology	1
1.2 Background	1
1.3 Aims of the Research	4
CHAPTER 2 - MATERIALS AND METHODS	6
2.1 Background	6
2.2 Tissue and specimen acquisition and storage	6
2.3 Immunocytochemistry	7
2.3.1 Preparation and fixation	7
2.3.2 Development of methodology	7
2.3.3 Reading and scoring	7
2.3.4 Specific Assays	8
2.4 Tissue culture	8
2.5 Flow Cytometry	8
CHAPTER 3 - DEVELOPMENT OF METHODOLOGY AND PREDICTORS FOR OUTCOME	9
3.1 Paper 1	
ESTIMATION OF PR AND ER BY IMMUNOCYTOCHEMISTRY IN BREAST CANCER. COMPARISON WITH RADIOLIGAND BINDING METHODS. L. Seymour, K. Meyer, J. Esser, P. MacPhail, A. Behr, W.R. Bezwoda. American Journal of Clinical Pathology 94(Suppl 1): S35-S40.1990.	10

3.2	Paper 2 RESPONSE TO SECOND-LINE HORMONE TREATMENT FOR ADVANCED BREAST CANCER. PREDICTIVE VALUE OF PLOIDY DETERMINATION. L. Seymour, W.R. Bezwoda, K. Meyer. Cancer 65:2720-2724, 1990. 11
3.3	Paper 3 DETECTION OF P24 PROTEIN IN HUMAN BREAST CANCER: INFLUENCE OF RECEPTOR STATUS AND OESTROGEN EXPOSURE. L. Seymour, W.R. Bezwoda, K. Meyer, C. Behr. British Journal of Cancer 61:886-890. 1990. 12
3.4	Paper 4 TUMOUR FACTORS PREDICTING FOR PROGNOSIS IN METASTATIC BREAST CANCER. THE PRESENCE OF P24 PREDICTS FOR RESPONSE TO TREATMENT AND DURATION OF SURVIVAL. L. Seymour, W.R. Bezwoda, K. Meyer. Cancer 66:2390-2394.1990. 13
3.5	Paper 5 PLATELET DERIVED GROWTH FACTOR IN PLASMA OF BREAST CANCER PATIENTS: CORRELATION WITH STAGE AND RATE OF PROGRESSION. S. Ariad, L. Seymour, W.R. Bezwoda. Breast Cancer Research and Treatment. 20:11-17. 1991. 14

CHAPTER 4 - CLINICAL MODULATION AND PREDICTORS FOR OUTCOME . . 15

4.1	Paper 6 HORMONE PRIMING IN BREAST CANCER: OESTROGEN PRIMING HAS A DETRIMENTAL EFFECT ON RESPONSE IN OESTROGEN RECEPTOR NEGATIVE PATIENTS. L. Seymour, K. Meyer, W.R. Bezwoda. European Journal of Cancer. 29 (10)1495-1496. 1993. 16
4.2	Paper 7 INTERFERON PLUS TAMOXIFEN TREATMENT FOR ADVANCED BREAST CANCER: In Vivo BIOLOGIC EFFECTS OF TWO GROWTH MODULATORS. L. Seymour, W.R. Bezwoda. British Journal of Cancer. 68:352-356. 1993. 17
4.3	Paper 8 TISSUE PLATELET DERIVED GROWTH FACTOR (PDGF) PREDICTS FOR

	SHORTENED SURVIVAL AND TREATMENT FAILURE IN ADVANCED BREAST CANCER. L. Seymour, D. Dajee, W.R. Bezwoda. Breast Cancer Research and Treatment. 26:247-252. 1993.	18
4.4	Paper 9 POSITIVE IMMUNOSTAINING FOR PLATELET DERIVED GROWTH FACTOR IS AN ADVERSE PROGNOSTIC FACTOR IN PATIENTS WITH ADVANCED BREAST CANCER. L. Seymour, W.R. Bezwoda. Breast Cancer Research and Treatment. 32:229-233.1994.	19
4.5	Paper 10 SOLUBLE C-ERBB-2 FRAGMENT IN SERUM CORRELATES WITH DISEASE STAGE AND PREDICTS FOR SHORTENED SURVIVAL IN PATIENTS WITH EARLY STAGE AND ADVANCED BREAST CANCER. H. Kandl, L. Seymour, W.R. Bezwoda. British Journal of Cancer 70:739-742. 1994.	20
4.6	Paper 11 P-GLYCOPROTEIN IMMUNOSTAINING CORRELATES WITH ER AND WITH HIGH Ki67 EXPRESSION BUT FAILS TO PREDICT ANTHRACYCLINE RESISTANCE IN PATIENTS WITH ADVANCED BREAST CANCER. L. Seymour, W.R. Bezwoda, R.D. Dansey. Breast Cancer Research and Treatment. 36:61-69.1995.	21
CHAPTER 5 - TRANSLATIONAL STUDIES CORRELATING CLINICAL OBSERVATIONS		22
5.1	Paper 12 Sequential Alpha-Interferon and Tamoxifen: in vitro Biologic Effects of Two Growth Modulators L. Seymour, W.R. Bezwoda. International Journal of Oncology 10:1277-1282, 1997.	23
5.2	Paper 13 SEQUENTIAL A-INTERFERON AND TAMOXIFEN: IN VITRO REVERSAL OF MDR-1 MEDIATED MULTIDRUG RESISTANCE IN THE MCF-7 CELL LINE. L. Seymour, W.R. Bezwoda. Breast 1998 - in Press	24
CHAPTER 6 - DISCUSSION AND CONCLUSIONS		25
6.1 Background and biology		25

6.2 Predictors of response and prognosis	26
6.2.1 Oestrogen and Progesterone Receptors	26
6.2.2 Transforming growth factor- β	27
6.2.3 P24	29
6.2.4 C-erbB-2	30
6.2.5 Platelet Derived Growth Factor	31
6.2.6 Ki67	32
6.2.7 PGP	33
6.3 Therapy: the options and the challenges	34
6.3.1 Overcoming resistance	36
6.3.2 Biologic Response Modifiers	38
6.3.3 Other modulatory strategies	39
6.4 Conclusions	42
References	43
Appendix 1	60

NOMENCLATURE

ABC	<i>A</i> -dvanced Breast Cancer
EGF	Epidermal Growth Factor
ER	Oestrogen Receptor
hsp	Heat Shock Protein
IFN	Interferon
kDA	Kilo Dalton
MDR	Multi-Drug Resistance
mRNA	Messenger Ribonucleic Acid
MRP	Multi-Drug Resistance Protein
NK	Natural Killer Cells
PDGF	Platelet-Derived Growth Factor
PGP	P-Glycoprotein
PR	Progesterone Receptor
TGF	Transforming Growth Factor
TMX	Tamoxifen

CHAPTER 1 - INTRODUCTION

1.1 Epidemiology

Breast cancer is the second most common cancer in women worldwide, and is the most common cancer in women in developed countries. One million new cases per year are anticipated by the year 2000, an increase of nearly 40% compared to the 719,000 cases per year reported in 1985 (1). By the year 2000, breast cancer is expected to account for 420,000 deaths per year, with more than 8 million years of life lost per year due to premature death from breast cancer. The incidence of breast cancer rises with increasing age and is uncommon in women younger than 20 years of age. The apparent increase in the worldwide incidence of breast cancer may be related in part to the aging of the population, as well as to earlier diagnosis as a result of mammographic screening, especially in developed countries. Age specific incidence rates vary between developed and developing countries because of variations in population profiles, with age standardised rates in Southern Africa reported as 28.7 per 100,000 compared to 84.8 per 100,000 in North America. Direct medical costs of breast cancer are anticipated to be nearly 8 billion dollars. Breast cancer therefore represents an enormous public health and socioeconomic burden worldwide.

1.2 Background

In the past century, understanding of the biology of breast cancer has evolved dramatically. While initially considered a disease amenable to, and best treated by, surgery, Beatson's

description of the dependency of both breast tissue and breast tumours on ovarian function (2) paved the way for appreciation of the complex biology of breast cancer, and the development of models of the hormonal control of tumour growth.

Beatson's seminal observations led to the use of hormonal intervention for the management of breast (as well as prostate cancer), initially by surgical castration. Although radiation induced ovarian ablation soon replaced surgical oophorectomy, the search for non-operative therapies led to the discovery of testosterone and diethyl-stilboestrol in the 1940's, the definition of the hypophyseal-pituitary axis, the isolation of oestrogen receptors (ER) and progesterone receptors (PR), and, ultimately, the development of anti-oestrogen therapies for breast cancer.

Discovery of the presence of specific hormonal receptors in breast cancer cells, coupled with the demonstration of regression of tumours after oestrogen withdrawal or after similar hormonal manoeuvres allowed the development of a simplistic model of hormonal control of breast cancer. Patients who were ER positive, especially postmenopausal patients, were predicted to and in many instances did, respond to hormonal treatments. Notwithstanding these observations, many patients who had demonstrable ER failed to respond to hormonal therapy, or became resistant to treatment, suggesting that the biologic control of the growth of breast tumours was more complex than originally envisaged. Mechanism of inherent or acquired resistance may include loss of ER due to malignant progression of cells, selection of ER negative clones, or the presence of mutated and abnormal ER among others. Potential

strategies to overcome inherent or acquired resistance to hormonal therapies might include pharmacologic modulation of malignant cells and or manipulation of the expression or function of hormone receptors.

Cytotoxic chemotherapy plays an important role in the management of both early and advanced breast cancer. Pivotal studies have demonstrated the beneficial effects of adjuvant chemotherapy, as well as the palliative benefits of chemotherapy in advanced disease. Despite these advances, it is clear that drug resistance, both inherent and acquired, represents a significant barrier to further progress, as patients frequently either relapse after achieving apparently good response to chemotherapy, or develop disease progression whilst still receiving chemotherapy. Strategies to overcome drug resistance might include further elucidation of the mechanism of drug resistance, including resistance modulated by the efflux pump P-glycoprotein (PGP), the development of drugs capable of overcoming drug resistance, increases in the dosage of drugs in an attempt to overcome drug resistance, or attempted modulation of cellular pharmacology in an attempt to overcome or subvert drug resistance.

A further important strategy for the optimal treatment of breast cancer would be the identification of prognostic factors, or surrogate markers of response to treatment, in an attempt to select patients most likely to respond to specific types of therapy, as well as to gain a better understanding of the biology of the disease. Such prognostic markers might include hormonal receptors (ER and PR), growth factors (transforming growth factor (TGF)- β , platelet

derived growth factor (PDGF), epidermal growth factor (EGF), oncogenes (c-erbB-2), oestrogen related proteins (P24), markers of growth (Ki67, S-phase, ploidy), and markers of drug resistance (PGP).

Despite the significant advances in the understanding and management of breast cancer, major challenges remain. Further elucidation of the biologic behaviour of breast cancer and better understanding of the complex control of growth and interactions between growth factors would hopefully facilitate the development of improved models in which the behaviour of tumour cells is pharmacologically modulated, with the aim of improving therapeutic outcomes. Further, better understanding of the mechanism of intrinsic and acquired drug resistance might define novel means of modulation and reversal of resistance and ultimately, improved therapeutic outcomes.

1.3 Aims of the Research

This research project was undertaken with the following specific aims

- i. to develop the laboratory methodology to examine potential prognostic indicators
- ii. to perform tissue biopsies where possible on patients enrolled into clinical trials to provide a bank of available specimens to validate these potential prognostic indicators as surrogates of prognosis or response
- iii. to confirm *in-vitro* the predictions made from laboratory models using clinical material collected during clinical studies
- iv. to examine whether the anti-tumour effects of chemotherapy and tamoxifen (TMX)

could be beneficially modulated, both in the clinic and in laboratory models

- a. to examine the role of oestrogens in cell cycle synchronisation as a modulator of chemotherapy efficacy in a clinical study with both clinical and translational endpoints
- b. to examine the role of interferon (IFN) as a modulator of TMX both in the clinic and in a laboratory model
- c. to examine the role of IFN and TMX as modulators of drug resistance mediated by PGP

CHAPTER 2 - MATERIALS AND METHODS

2.1 Background

This thesis is based on investigations performed and published as papers between 1990 and 1997. Although the papers were published individually the overall aim of the research was to develop a clearer understanding of breast cancer biology. The main themes of the investigations can be sub-divided into three sections :

Chapter 3: Development of Methodology and Predictors For Outcome

Chapter 4: Clinical Modulation and Predictors For Outcome

Chapter 5: Translational Studies Correlating Clinical Observation.

Each paper is numbered and referred to in subsequent discussion and sections as Paper 1, 2 etc.

2.2 Tissue and specimen acquisition and storage

Human tissue for the studies performed was accessed as part of approved clinical research protocols developed by the author. Fine needle aspirates, Menghini needle biopsy or dermatological punch biopsies were obtained under local anaesthesia. Glass slides for immunocytochemistry were prepared immediately for samples collected by fine needle aspirate, fixed in picric-acid-formaldehyde and stored in storage medium (sucrose/DMSO) at -10°C until used. Fine needle aspirates intended for flow cytometry were collected into alcohol

and stored until use. Tissue cores and punch biopsies were immediately frozen at 135°C after transport in liquid nitrogen. In some early studies, tissue was also obtained from formal surgical biopsy. Tissues for pathology reference were accessed from pathology archives.

2.3 Immunocytochemistry

2.3.1 Preparation and fixation

Multiple glass slides were prepared from each sample either using cytopsin, or by teasing tumour samples onto the slide. Slides were then fixed in picric-acid-formaldehyde for 15 minutes and dehydrated with ethanol. Picric-acid-formaldehyde proved a superior fixative for the range of assays performed, compared to more conventional fixing techniques. Slides were checked for cellularity, and stored until used in a 'specimen storage medium' of sucrose and DMSO at -10°C

2.3.2 Development of methodology

When this research was undertaken, only the assay for ER was commercially available in a kit form. For all the other assays, the antibody was obtained from other researchers, or from commercial source, and the methodology for the assay developed. This involved defining and preparing suitable positive and negative controls, titrating concentrations of primary and secondary antibodies and other reagents. For simplicity, the ABC vectastain system was used where possible.

2.3.3 Reading and scoring

All slides were read and scored by the author, where published using published and validated scoring methods. For assays under development, a scoring system was developed and these are described in the individual published papers.

2.3.4 Specific Assays

Specific methodology for the assays are described in detail in the individual published papers.

- Oestrogen and Progesterone Receptors
- Transforming growth factor- β
- P24
- C-erbB-2
- Platelet Derived Growth Factor
- Ki67
- PGP

2.4 Tissue culture

Standard techniques were used for all tissue culture and were performed by the author.

2.5 Flow Cytometry

Samples included either tissue accessed from fine needle aspirates and stored in alcohol, or fresh frozen tissue stored at -135°C . Tissue was minced and dissociated using standard techniques. Precise methodology is described in detail in the published papers.

CHAPTER 3 - DEVELOPMENT OF METHODOLOGY AND PREDICTORS FOR OUTCOME

- 3.1 Paper 1 - Estimation of PR and ER by Immunocytochemistry in Breast Cancer. Comparison with Radioligand Binding Methods.
- 3.2 Paper 2 - Response to Second-Line Hormone Treatment for Advanced Breast Cancer. Predictive Value of Ploidy Determination.
- 3.3 Paper 3 - Detection of P24 Protein in Human Breast Cancer: Influence of Receptor Status and Oestrogen Exposure.
- 3.4 Paper 4 - Tumour Factors Predicting for Prognosis in Metastatic Breast Cancer. The Presence of P24 Predicts for Response to Treatment and Duration of Survival.
- 3.5 Paper 5 - Platelet Derived Growth Factor in Plasma of Breast Cancer Patients: Correlation with Stage and Rate of Progression.

3.1 Paper 1

ESTIMATION OF PR AND ER BY IMMUNOCYTOCHEMISTRY IN BREAST CANCER. COMPARISON
WITH RADIOLIGAND BINDING METHODS.

I.L. Seymour, K. Meyer, J. Esser, P. MacPhail, A. Behr, W.R. Bezwoda.

American Journal of Clinical Pathology 94(Suppl 1): S35-S40.1990.

Estimation of PR and ER by Immunocytochemistry in Breast Cancer

Comparison with Radioligand Binding Methods

LESLIE SEYMOUR, F.C.P. (SA), KAREN MEYER, B.Sc., JAN ESSER, M.B.B.Ch., A. PATRICK MACPHAIL, Ph.D.,
ANTHONY BEHR, F.F.PATH. (SA), AND WERNER ROBERT BEZWODA, Ph.D.

Immunocytochemical assays for progesterone receptor (PR) using monoclonal antirabbit PR antibodies (PR-ICA) and for estrogen receptor (ER) (ER-ICA) were compared with radioligand binding (dextran-coated charcoal [DCC]) methods for receptor determination in patients with breast cancer. Immunocytochemical staining for PR was exclusively nuclear in localization. In this regard, PR staining is similar to previous findings for ER; PR-ICA showed a sensitivity of 89% and specificity of 100%. ER-ICA was also 89% sensitive and similarly specific. There was good correlation between the degree and intensity of staining and quantitative binding of radioligand. Receptor-positive tumors, however, show considerable variation of immunocytochemical staining, suggesting heterogeneity of cellular PR content. The availability of an immunocytochemical assay for PR increases the discriminatory potential for these methods of receptor determination. (Key words: Estrogen receptor; Progesterone receptor; Immunocytochemistry; Breast cancer) *Am J Clin Pathol* 1990;94(Suppl 1):S35-S40

*Department of Medicine, Division of Haematology/Oncology
and Department of Nuclear Medicine, University of the
Witwatersrand and Johannesburg Hospital,
Johannesburg, South Africa*

Author Biographies

Leslie Seymour is a Medical Oncologist and Consultant to the Breast Clinic, University of the Witwatersrand.

Karen Meyer is a Research Officer at the Division of Medical Oncology, University of the Witwatersrand.

Jan Esser is a Nuclear Medicine Specialist and Head of the Radio and Immunoassay Laboratory, Department of Nuclear Medicine, University of the Witwatersrand.

A. Patrick MacPhail is Associate Professor of Medicine and Medical Oncology and Consultant to the Breast Clinic, University of the Witwatersrand.

Anthony Behr is a Consultant Pathologist, School of Pathology, University of the Witwatersrand and South African Institute for Medical Research.

Werner Robert Bezwoda is Associate Professor of Medicine and Head of the Division of Haematology/Oncology, University of the Witwatersrand.

ESTROGEN (ER) AND PROGESTERONE (PR) receptors are widely used to predict of hormone responsiveness^{7,9,10,23} and for prognostication in breast cancer.^{3,5,15,19,28,32} Radioligand binding (RLBA), using either dextran-coated charcoal (DCC) or density gradient sedimentation to separate bound and free ligand, has until recently been the most frequently used technique for receptor determination.^{2,35} These methods, however, use relatively large amounts of tissue, need immediate collection and storage of samples in liquid nitrogen, and require facilities for handling radioactive materials. Furthermore, radioligand binding methods measure only receptors that have been solubilized. Tightly bound or occupied receptors may thus not be detected. For these reasons, immunochemical methods have been developed to study ER in normal and neoplastic tissues.^{13,14,20,21,29,33,34} ER determination by immunocytochemical methods (ER-ICA) has been shown to correlate

Supported by grants from the National Cancer Association (SA) and Bekker Trust Foundation.

Address reprint requests to Dr. Bezwoda: Department of Medicine, University of the Witwatersrand Medical School, 7 York Road, Parktown 2193, Johannesburg, South Africa.

well with results obtained by RLBA.^{6,11,12,20} Immunocytochemical methods have the advantage that they can be performed on cytologic preparations such as those obtained by fine-needle aspiration^{6,24,31} and can thus be obtained before the operation or on deep-seated tumors not easily accessible to biopsy. Immunocytochemical methods have also led to an appreciation of the heterogeneity of ER expression among different cells constituting the tumor. Although immunologic methods for determining ERs appear to be becoming well established, there are fewer data on PR determination by immunocytochemistry (PR-ICA).

A number of monoclonal antibodies against rabbit PR have recently become available.^{17,25} Two of these monoclonal antibodies to rabbit PR (LET 126 and LET 64 clones) cross-react strongly with human PR.¹⁸ In a small series of 27 patients with breast cancer, good correlation between PR-ICA and PR as measured by ligand binding was reported.²⁶ We have developed a modification of this technique for immunocytochemical detection and localization of PR that can be performed with the use of cytologic aspirates. Because of the ease with which samples can be obtained, this technique can be applied to repeated studies of ER and PR content of tumors during the course of treatment.

Patients and Methods

Thirty-three patients with locally advanced and/or metastatic breast cancer were studied. Cytologic preparations and/or needle biopsies as well as surgical biopsies from primary tumors or soft tissue metastases were obtained before any therapy. The patients ranged in age from 28 to 71 years. Surgical biopsies were trimmed of fat and macroscopically uninvolved tissue. Portions from multiple quadrants of the biopsy specimen were removed for histologic examination. Immediately thereafter, material to be used for ER and PR assay by RLBA was placed in liquid nitrogen. Cytologic preparations were assessed by routine cytomorphologic methods, as well as by immunocytochemistry. Histologic grading of biopsy specimens of the tumors was performed according to the method of Bloom and Richardson.¹

Immunocytochemistry for PR and ER

The monoclonal antibody to PR was obtained from Transbio (Transbio, Paris, France). Avidin-biotin reagent (Vectastain® ABC Kit) was obtained from Vector (Vector Laboratories, Burlingame, CA). Normal mouse immunoglobulin was purchased from Sigma (Sigma Chemical Company, St. Louis, MO).

For PR immunocytochemistry, material obtained by fine-needle aspiration or needle biopsy was ejected or teased onto HC1-ethanol-cleaned slides. Slides were im-

mediately fixed in 4% formalin (10 minutes), rinsed in phosphate-buffered saline (PBS), dipped sequentially in ice-cold methanol and acetone, and then rinsed once more in PBS. Endogenous peroxidase activity was blocked with methanol-hydrogen peroxide. The slides were again rinsed in PBS and then incubated (30 minutes, 18 °C) with normal horse serum followed by incubation (for three hours, 18 °C) with the monoclonal antiprogesterone (primary antibody) at a concentration of 5 µg/mL. This antibody concentration had been found by prior titration to give optimum immunostaining. Biotinylated secondary antibody was applied at a 1:200 dilution. The reaction was developed with diaminobenzidine, and the slides were counterstained with Meyer's hematoxylin, serially dehydrated, mounted with coverslips, and examined at 400× magnification. A negative control following all the steps outlined above but using normal (nonimmune) mouse immunoglobulin instead of specific primary antibody was included with each assay. Positive controls included MCF-7 cells originally obtained from American Type Culture Collection (ATCC) and grown in our laboratories under optimal conditions for hormone receptor expression, as well as strongly PR-positive tumor specimens.

Estrogen receptor immunocytochemistry was performed with the use of the Abbott (Abbott Laboratories) ERICA® kit according to the manufacturer's instructions. The ERICA kit contains both positive and negative controls. The intensity of immunostaining for both ER and PR was graded according to the recommendations for the ERICA. Both intensity of staining and the number of cells stained are included in the assessment. All cells on the slides were evaluated, but specimens had to contain a minimum of 20 intact tumor cells to be deemed evaluable.

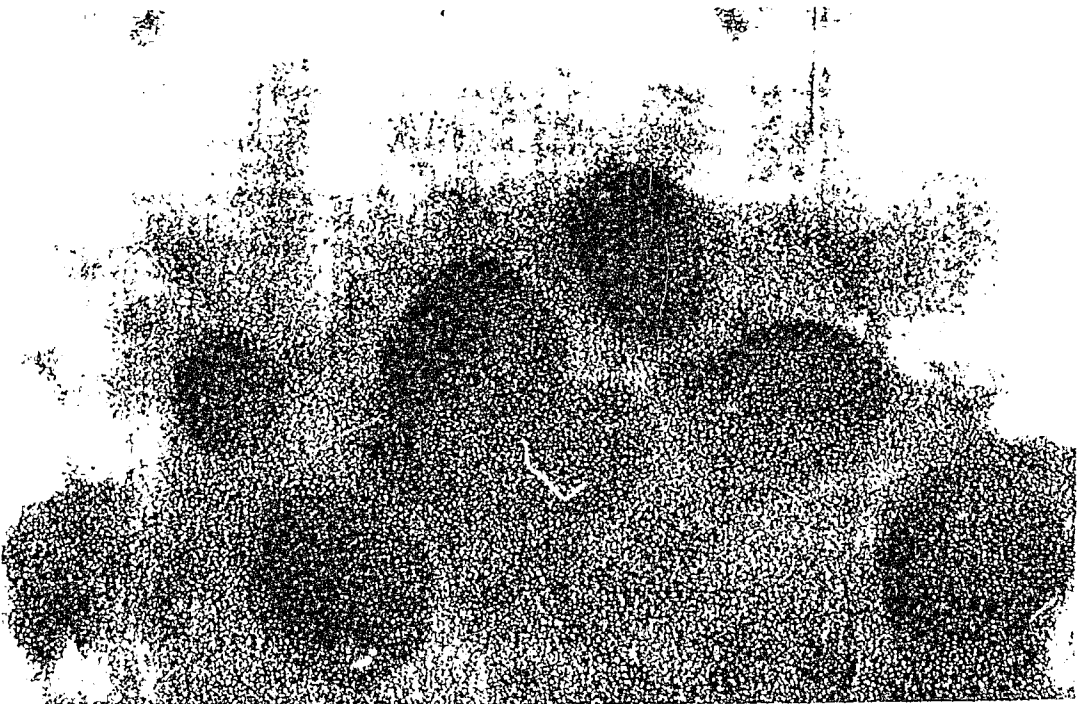
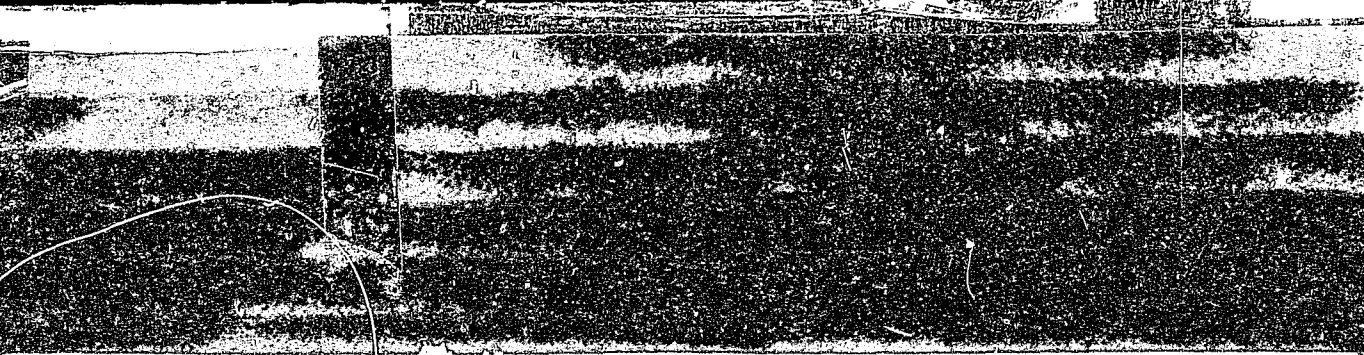
Ligand binding assays for ER and PR content were performed with the use of modifications¹⁶ of the DCC method as described by McGuire and co-workers.²² ER-positive tumors were those with > 10 fmol estrogen binding/milligram protein plus KD > 12. For the PR assay, positives are those samples with > 30 fmol progesterone binding/milligram protein. This laboratory participates in the Eastern Co-operative Oncology Group (ECOG) quality assurance program for hormone receptor determination.

Statistical analysis included chi-square analysis and parametric as well as nonparametric correlation statistics.

All patients who participated in the study gave informed consent, and the study was approved by the Committee for Ethics of Human Experimentation of the University of the Witwatersrand.

Results

Figure 1 shows the pattern of positive staining with monoclonal PR antibody. This staining pattern was sim-



10. The following is a summary of the results of the investigation conducted by the FBI on the matter of the alleged activities of the [redacted] in the [redacted] area of the [redacted] city of [redacted] during the [redacted] period of time.

Table 1. Immunocytochemical and Radioligand Binding Methods of Hormonal Receptor Determination in Breast Cancer

	Number of Tumors	PR-ICA	
		Positive	Negative
PR concentration (fmol/mg protein)			
<10 (negative)	23	0	23
10-30 (borderline)	1	0	1
>30 (positive)	9	8	1
Sensitivity 89%; Specificity 100%			
	Number of Tumors	ER-ICA	
		Positive	Negative
ER concentration (fmol/mg protein)			
<3 (negative)	17	0	17
3-10 (borderline)	0	0	0
>10 (positive)	16	14	2
Sensitivity 89%; Specificity 100%			

ilar for all positive specimens. PR staining was exclusively nuclear, with staining intensity showing considerable heterogeneity within the same sample.

Thirty-three breast cancers were examined (Table 1). Ten of the 33 samples were positive for PR by DCC, with PR concentrations ranging from 15 to 345 fmol/mg protein. Twenty-three had PR concentration < 10 fmol/mg protein. Sixteen were ER positive by DCC. Eight of the nine specimens definitely positive for PR by DCC were also positive by ICA. One patient with a borderline PR value (15 fmol/mg protein) was PR-ICA negative. Although this patient was ER positive by DCC (with a value

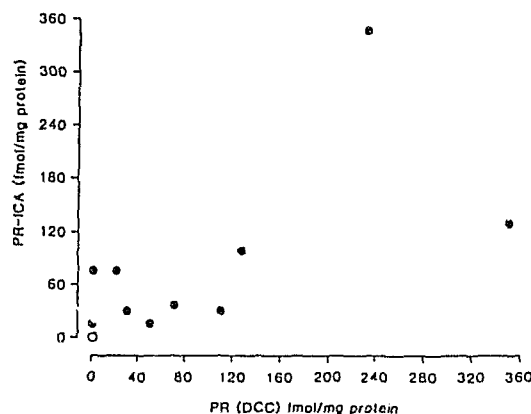


FIG. 2. Quantitation of hormone receptors in breast cancer by radioligand binding and immunocytochemical assays. Progesterone receptor. O, represents tumors that were receptor negative by both methods.

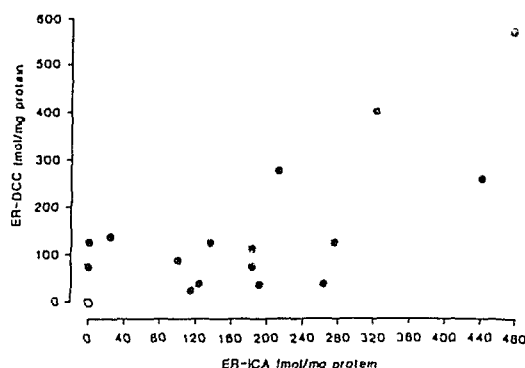


FIG. 3. Quantitation of hormone receptors in breast cancer by radioligand binding and immunocytochemical assays. Estrogen receptor. O, represents tumors that were receptor negative by both methods.

of 75), ERICA was also negative, suggesting either a sampling error or nonreceptor binding. The other tumor that was PR-ICA negative with positive PR binding assay (75 fmol/mg protein) was ER negative by DCC and also ER-ICA negative. All 23 patients negative by DCC were also PR-ICA negative. If a value of 30 fmol/mg protein is taken as being definitely PR positive, then 32 of 33 specimens were concordant for PR.

Fourteen of 16 specimens with positive results for ER by DCC were positive for ICA. All 17 patients negative for ER by DCC were also ER-ICA negative. Thus, 31 of 33 specimens were concordant for ER. Quantitation of receptors by ICA compared with RLBA is shown in Figures 2 and 3 and Table 2.

All 33 tumors were infiltrating duct carcinomas. Two tumors were of histologic grade 1, 12 were graded as grade 2, and the rest²⁰ were grade 3. Correlation between tumor grade and hormone receptor status as measured either by DCC or immunocytochemistry is shown in Table 3. The better-differentiated tumor grades (grades 1 and 2) were significantly more frequently PR positive (by either method of assessment) than poorly differentiated (grade 3) tumors.

Table 2. Correlation Between Hormone Receptor Quantitation by Immunocytochemical and Radioligand Binding Methods

	ER-ICA	PR	PR-ICA
ER	0.84 $P = 0.0001$	0.57 $P = 0.0004$	0.63 $P = 0.0001$
ER-ICA		0.53 $P = 0.014$	0.66 $P = 0.0001$
PR			0.87 $P = 0.0001$

Follow-up data are available for 30 of the 33 patients studied. All 30 patients had advanced disease. Twenty-three have died, and 7 remain alive with a follow-up time of up to 24 months. Survival was significantly related to receptor status (Fig. 4). Median duration of survival for ER-positive patients was 12 months (confidence limits, 10–18 months), whereas for ER-negative patients the median duration of survival was 4 months (confidence limits, 2–6 months). For PR-positive patients the median duration of survival has not been reached but will be in excess of 12 months, whereas for PR-negative patients the median duration of survival was 4 months. In view of the concordance between RIBA and immunocytochemical methods of determining receptor status, the survival graphs using the two methods of assessment are almost superimposable.

Histologic grade was not as accurate as receptor determination in distinguishing prognostic groups. The median duration of survival of patients with grade 1 and 2 tumors was ten months, whereas that of patients with grade 3 tumors was between six and eight months. These differences were not statistically significant.

Discussion

The present study confirms the potential usefulness of PR-ICA for determination of hormonal status in patients with breast cancer, with a sensitivity of 89% and specificity of 100% (as compared with the DCC assay). In addition, we have confirmed the exclusively nuclear localization of PR-ICA as previously described.^{2,6,17} We have also demonstrated positive staining for PR in the MCF-7, a hormonally responsive human breast cancer cell line that has previously been shown to express PR by RLBA.

Because the presence of PR in addition to ER adds significantly to the predictive value of hormonal receptor assays in breast cancer,³ the development of accurate PR-ICA methods is an important step in extending the usefulness of the immunocytochemical techniques for the investigation of patients with potentially hormone-responsive tumors. The overall correlation of combined re-

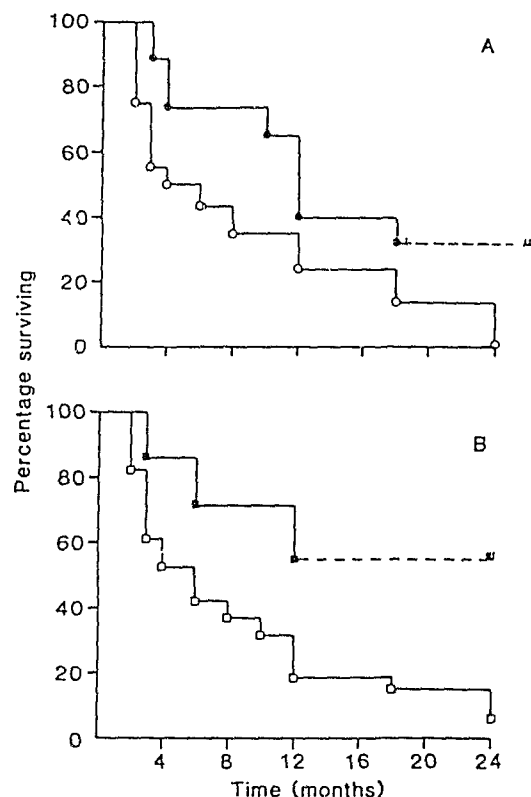


FIG. 4. Actuarial survival by hormone receptor status. A (upper). Estrogen, ○ ER positive; □ ER negative. B (lower). Progesterone receptor, □ PR positive; ○ PR negative.

ceptor determinations by the two methods lends further validity to ICA as a means for receptor determination.

Although ICA and RLBA assays show good correlation, the heterogeneity of PR staining in the same tumor (a phenomenon previously observed with ER immunocytochemistry) may help to further elucidate reasons for the failure of hormone treatment. Such treatment failures still occur in approximately 20% of patients who are ER and PR positive by RLBA. The adaptation of the PR-ICA technique to fine-needle aspiration and needle biopsy as well as to histologic section makes this method useful for sampling deep-seated tumors and for repeated study of the same patients. In the future, combined clinicopathologic studies using such immunocytochemical techniques should give further insight into hormonal control of breast cancer.

References

1. Bloom HJG, Richardson WW. Histologic grading and prognosis in breast cancer. *Br J Cancer* 1957;11:359-377.

Table 3. Correlation of Histologic Grade with Receptor Status

	Grades 1 & 2	Grades 3 & 4	
ER+	9	7	
ER-	4	13	
ER-ICA+	8	6	
ER-ICA-	5	14	
PR+	7	2	
PR-	6	18	$P < 0.05$
PR-ICA+	6	2	
PR-ICA-	7	18	$P < 0.05$

2. Charnness GL, McGuire WL. Steroid receptor assay methods in human breast cancer. In: Thompson EM, Lippman ME, eds. Steroid receptors and the management of cancer, vol 1. Boca Raton: CRC Press, 1979:3-30.
3. Clark GM, McGuire WL, Hubay CA, Pearson OH, Marshall JS. Progesterone receptors as a prognostic factor in stage II breast cancer. *N Engl J Med* 1983;309:1343-1347.
4. Clark GM, McGuire WL. Progesterone receptors and human breast cancer. *Breast Cancer Research* 1983;3:157-163.
5. Cooke T, George D, Shields R, Maynard PV, Griffiths K. Oestrogen receptors and prognosis in early breast cancer. *Lancet* 1979;1:995-997.
6. Coombes R, Berger U, McLelland R, et al. Prediction of endocrine response in breast cancer by immunochemical detection of oestrogen receptor in fine needle aspirates. *Lancet* 1987;2:701-703.
7. De Sombre ER, Carbone PP, Jensen EV, et al. Steroid receptors in breast cancer. *N Engl J Med* 1979;301:1011-1012.
8. De Sombre E, Thorpe S, Rose C, et al. Prognostic usefulness of estrogen receptor immunocytochemical assays for human breast cancer. *Cancer Res* 1986;46(Suppl):4256S-4264S.
9. Horwitz KB, McGuire WL, Pearson OH, Segaloff A. Predicting response to endocrine therapy in human breast: a hypothesis. *Science* 1975;190:726-727.
10. Jensen EV, Block GE, Hyser K, De Sombre ER. Estrogen receptors and breast cancer response to adrenalectomy. Prediction of response in cancer therapy. *NCI Monogr* 1971;34:55-70.
11. Jonat W, Maass H, Stegner H. Immunohistochemical measurement of estrogen receptors in breast cancer tissue samples. *Cancer Res* 1986;46(Suppl):4296S-4298S.
12. King W, De Sombre E, Jensen E, Greene G. Comparison of immunocytochemical and steroid binding assays for oestrogen receptor in human breast tumors. *Cancer Res* 1985;45:293-304.
13. King WJ, Greene GL. Monoclonal antibodies localise oestrogen receptor in the nuclei of target cells. *Nature* 1984;307:745-747.
14. King WL, De Sombre ER, Jensen EV, Green GL. Comparison of immunocytochemical and steroid-binding assays for oestrogen receptor in human breast tumors. *Cancer Res* 1985;45:293-304.
15. Knight WA, Livingston RB, Gregory EJ, McGuire WL. Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. *Cancer Res* 1977;37:4669-4671.
16. Levine J, Kay G, da Fonseca M, Lange M, de Moor NG, Sava M. Oestrogen receptors in tumours of breast cancer patients. *S Afr Med J* 1978;53:577.
17. Logeat F, VuHai MT, Fournier A, Legrain P, Buttin G, Milgrom E. Monoclonal antibodies to rabbit progesterone receptors: cross reaction with other mammalian progesterone receptors. *Proc Natl Acad Sci USA* 1983;80:6456-6459.
18. Logeat F, VuHai MT, Milgrom E. Antibodies to rabbit progesterone receptor: cross reaction with human receptor. *Proc Acad Sci USA* 1981;78:1426-1430.
19. Maynard PV, Blamey RW, Elston CW, Haybittle JL, Griffiths K. Oestrogen receptor assay in primary breast cancer and early recurrence of the disease. *Cancer Res* 1978;39:4292-4295.
20. McCarty K, Svabo E, Flowers J, et al. Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. *Cancer Res* 1986;46(Suppl):4244S-4248S.
21. McClellan MC, West NB, Tacha DE, Greene GL, Brenner RM. Immunocytochemical localization of oestrogen receptors in the Macaque reproductive tract with monoclonal antiestrogens. *Endocrinology* 1984;114:2002-2024.
22. McGuire WL, De La Garza M, Charnness GC. Evaluation of oestrogen receptor assays in human breast cancer tissue. *Cancer Res* 1977;37:637.
23. McGuire WL. Steroid hormone receptors in breast cancer treatment strategy. *Recent Prog Horm Res* 1980;36:135-156.
24. McLelland R, Berger U, Wilson P, et al. Presurgical determination of oestrogen receptor status using immunocytochemically stained fine needle aspirate smears in patients with breast cancer. *Cancer Res* 1987;47:6118-6122.
25. Perrot-Applanat M, Groyer-Picard MT, Logeat F, Milgrom E. Ultrastructural localization of the progesterone receptor using by an immunogold method: effect of hormone administration. *J Cell Biol* 1986;102:1191-1199.
26. Perrot-Applanat M, Groyer-Picard MT, Lorenzo F, et al. Immunocytochemical study with monoclonal antibodies to progesterone receptor in human breast tumors. *Cancer Res* 1987;47:2652-2661.
27. Perrot-Applanat M, Logeat F, Groyer-Picard MT, Milgrom E. Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. *Endocrinology* 1985;116:1463-1484.
28. Pichon MF, Pallud C, Brunet M, Milgrom E. Relationship of presence of progesterone receptors to prognosis in early breast cancer. *Cancer Res* 1980;40:3357-3360.
29. Press MF, Greene WL. Methods in laboratory investigation. An immunocytochemical method for demonstrating estrogen receptor in human uterus using monoclonal antibodies to human estrophilin. *Lab Invest* 1984;50:480-486.
30. Press MF, Holt JA, Herbst AL, Greene GL. Immunocytochemical identification of estrogen receptor in ovarian carcinomas. Localization with monoclonal estrophilin antibodies compared with biochemical assays. *Lab Invest* 1985;53:349-361.
31. Reiner A, Reiner G, Spona J, Teleky B, Kolb R, Holzner J. Estrogen receptor immunocytochemistry for preoperative determination of estrogen receptor status on fine needle aspirates of breast cancer. *Am J Clin Pathol* 1987;88:399-404.
32. Saez S, Pichon MF, Cheix F, et al. Progesterone receptors and prognosis in early breast cancer. The experience of two centres. In: Baudin CS, Milgrom E, Mauvais-Jarvis P, eds. Progesterone and progestins. New York: Raven Press, 1983:355-366.
33. Shimada A, Kimura S, Abe K, et al. Immunocytochemical staining of estrogen receptor in paraffin sections of human breast cancer by use of monoclonal antibody: comparison with that in frozen section. *Proc Natl Acad Sci USA* 1985;82:4803-4807.
34. Skovsgaard Poulsen H, Ozzello L, King WJ, Greene GL. The use of monoclonal antibodies to estrogen receptors (ER) for immunoperoxidase detection of ER in paraffin section of human breast cancer tissue. *J Histochem Cytochem* 1985;33:37-92.
35. Witliff JL. Steroid-hormone receptors in breast cancer. *Cancer* 1983;53:630-643.

3.2 Paper 2

RESPONSE TO SECOND-LINE HORMONE TREATMENT FOR ADVANCED BREAST CANCER.

PREDICTIVE VALUE OF PLOIDY DETERMINATION

L. Seymour, W.R. Bezwoda, K. Meyer.

Cancer 65:2720-2724. 1990.

Response to Second-Line Hormone Treatment for Advanced Breast Cancer

Predictive Value of Ploidy Determination

Leslie Seymour, FCP, Werner Robert Bezwoda, FCP, PhD,
and Karen Meyer, BSc

Twenty-two patients who previously responded to first-line hormonal therapy were evaluated for factors which would predict for response to second hormonal manipulation. Investigations performed at progression after initial hormone response included immunocytochemical estimation of estrogen and progesterone receptors as well as flow cytometric analysis of tumor ploidy. Approximately 50% of patients were found still to be estrogen receptor positive at relapse from first-line hormone treatment. Progesterone receptor had, however, usually become negative. Nine of the 22 patients responded to second-line hormonal therapy. Second hormone responses occurred with equal frequency among hormone receptor-positive and hormone receptor-negative patients. Tumor ploidy, as determined by flow cytometric study did, however, predict for response. Eight of 12 patients with diploid tumors responded to second-line hormone therapy whereas only one of ten with aneuploid tumors responded. Flow cytometric analysis appears to be a promising technique for prediction of second hormone response after relapse from first-line hormone manipulation. *Cancer* 65:2720-2724, 1990.

THERE IS LITTLE INFORMATION regarding the predictive value of hormone receptor determination at the time of relapse from first-line hormonal manipulation. Although there is a fairly good correlation between the hormone receptor status of primary tumors and of simultaneously occurring lymph node metastases, as well as between synchronously occurring metastases in the same patient, discrepant results have been described in as many as 20% of cases.¹⁻⁷ When the results of receptor determinations from primary tumors and in subsequently occurring metastases are compared about one third are found to be discordant, even without intervening therapy. After hormone treatment receptor status appears alter even more frequently,^{2,3,6-9} the change usually being from receptor positive to receptor negative. However, relapse from primary hormone treatment is not invariably associated with a loss of receptor⁹ indicating that loss of

hormone responsiveness is not invariably due to emergence of a receptor-negative tumor cell population.

Since clinical data indicates that initial response to hormone treatment, particularly if the response has been relatively prolonged, is a positive indicator for response to further hormone manipulation, it is of some interest to establish the value of hormone receptor determination at relapse from primary hormone treatment, as well to delineate other factors which may help to predict for response to second-line hormonal manipulation.

Patients and Methods

The study population consisted of 22 post menopausal patients with advanced breast cancer attending the Breast Clinic of the Johannesburg Hospital, Johannesburg, South Africa, between January 1987 and January 1989. At initial presentation with recurrent/metastatic disease the 22 patients were either estrogen receptor (ER) positive or ER unknown. All 22 had either shown an initial objective response to treatment including hormone manipulation or had remained stable for at least 3 months while on hormone therapy (Tables 1 and 2). Seventeen of the 22 patients had received hormone therapy only whereas five patients had received combined hormone therapy plus

From the Department of Medicine, Haematology/Oncology, University of the Witwatersrand, Johannesburg, South Africa.

Supported by grants from Bekker Trust Foundation and National Cancer Association (SA).

Address for reprints: Werner R. Bezwoda, FCP, PhD, Department of Medicine, University of the Witwatersrand, 7 York Road, Parktown 2193, Johannesburg, South Africa.

Accepted for publication December 19, 1989.

TABLE 1. Patient Population

	No.	Percent
Prior hormone therapy for metastatic disease	22	100
Tamoxifen	9	41
Aminoglutethimide	6	27
Medroxyprogesterone acetate	7	22
Initial hormone receptor status		
ER + PR+	10	45.5
ER + PR-	1	4.5
Unknown	11	50
Disease-free interval (median)	4.7 yr	
Time to treatment failure (median)	9 mo	
Metastatic sites		
Soft tissue	22	100
Pleura	5	23
Bone	12	55
Mean no. of metastatic sites per patient	1.9	0.6

ER: estrogen receptor; PR: progesterone receptor; -: negative; +: positive.

Patient population age: 58.3 ± 12.3 years (mean $\pm$ SD).

chemotherapy (cyclophosphamide, methotrexate, 5-fluorouracil [CMF]). The initial hormone receptor determinations were performed on tumor cytosol using dextran-coated charcoal (DCC) ligand binding methods.¹⁰

At the time of entry into the current study all 22 patients

were restaged by means of clinical examination, chest radiographs, bone scans, hepatic ultrasound and computed tomography (CT) scans and hematologic and biochemical investigations including blood count and automated biochemical profile including liver and renal function tests. All 22 had evidence of disease progression with dominant soft tissue and/or bone disease. However, because of initial hormone responsiveness, the favorable sites of recurrent disease and the slow rate of progression, a trial of alternate hormone manipulation was considered justified. At time of entry into the study all 22 patients had at least one site of measurable disease and all 22 had soft tissue metastases accessible for biopsy.

Hormone receptor determination at relapse was performed using immunocytochemical techniques. This was in an attempt to obviate the potential problems of receptor occupancy or nuclear binding when using cytosol ligand binding methods for receptor determination, particularly among patients who had been receiving tamoxifen (TAM).

Specimens were obtained either by surgical biopsy or needle biopsy of accessible tumors. Biopsy specimens were transported on ice and either processed immediately or stored at -135°C until analyzed. Only sections of adequate cellularity, immediately adjacent to areas histologically involved with tumor were used for analysis. Estrogen receptor determination was performed using the Abbott

TABLE 2. Response to Second Hormone Therapy: Clinical and Laboratory Determinants

Initial evaluation and treatment					Evaluation at relapse and second therapy				
ER*	PR*	Hormonal agent	Response	Duration (mo)	ER†	PR†	Ploidy	Hormonal agent	Response
347	12	TAM	PR	14	210	0	A	AG	NR
		TAM	PR	9	400	0	A	AG	NR
		TAM‡	SD	5	100	0	A	AG	Pr
		TAM	PR	14	320	230	A	AG	NR
325	154	TAM‡	PR	17	125	0	D	MPA	Pr
		TAM	SD	7	225	100	D	MPA	Pr
162	12	TAM‡	SD	4	0	0	D	MPA	NR
337	54	TAM‡	PR	12	0	25	A	MPA	NR
370	110	TAM‡	PR	8	320	0	D	MPA	NR
		AG	SD	7	0	0	A	TAM	NR
		AG	SD	8	0	0	D	TAM	Pr
110	65	AG	SD	16	190	0	A	MPA	NR
94	54	AG	PR	7	275	0	D	TAM	NR
		AG	PR	9	0	0	D	TAM	NR
		AG	PR	14	0	0	D	TAM	NR
83	54	MPA	PR	7	40	0	A	TAM	Pr
34	45	MPA	SD	18	0	0	D	TAM	Pr
410	96	MPA	PR	21	275	0	D	AG	CR
		MPA	PR	13	0	0	A	TAM	NR
67	39	MPA	PR	14	0	0	D	AG	Pr
		MPA	SD	4	0	0	D	AG	Pr
		MPA	PR	13	0	0	A	AG	NR

TAM: tamoxifen; AG: aminoglutethimide; MP: medroxyprogesterone; CR: complete response; SD: stable disease; Pr: partial response; NR: no response; A: aneuploid tumor; D: diploid tumor as determined by flow cytometric analysis of DNA distribution; ER: estrogen receptor; PR: progesterone receptor.

* Values are in fmol/mg.

† Values are in histocytochemical scores.

‡ Patients received cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) in addition to hormone therapy.

ER-ICA kit (Abbott Laboratories, North Chicago, IL) according to the manufacturer's instructions.

Progesterone receptor (PR) determination was performed according to previously described methods using a monoclonal antibody to rabbit PR which cross reacts with human PR and has been shown to be highly specific for the receptor.¹¹⁻¹³ A previous study from this institution has shown a linear correlation ($r = 0.90$) between the PR estimations as performed by DCC assay and immunoassay methods.¹⁴ Results of the immunocytochemical assays are expressed as a score based on the percentage of positive cells¹⁴ and intensity of staining, the histocytochemical score.

Flow cytometric analysis was performed using a Coulter Epics cytometer (Coulter Electronics, Hialeah, FL) after enzymatic digestion of minced fresh or frozen tissue fragments. DNA distribution was measured after staining with propidium iodide. Aneuploid tumors were defined as those with DNA indices higher or lower than 1.0 to 1.1 as compared with the standard of human lymphocyte nuclei freshly prepared in similar fashion to the tissue fragments.

Second-line hormone manipulation was with either TAM (20 mg/day), aminoglutethimide (AG, 1000 mg/day) or medroxyprogesterone acetate (MPA, 500 mg/day). Second-line hormone treatment was randomly assigned by an allocation technique adjusted so as to exclude the first-line hormone therapy received by that patient. Patients were followed by means of monthly measurements of measurable tumors and 3-month radiographs and bone scans. Response criteria were those proposed by the Eastern Cooperative Oncology Group (ECOG).¹⁵

The study was performed according to the Principles of the Declaration of Helsinki and was approved by the Ethics Committee of the University of the Witwatersrand.

Results

Eleven of the 22 patients were found to be ER positive at relapse after first-line hormone therapy. Of the 11 patients whose initial ER status was known to be positive, seven were still ER positive as determined by immunoassay at the time of relapse from first-line hormone therapy. Progesterone receptor positivity was, however, significantly less frequent at relapse than was the case for ER. Only two patients had PR values > 30 fmol/mg protein at reevaluation and only one of the ten patients known to be PR positive at initial evaluation remained PR positive at reevaluation.

Nine patients responded to second-line hormone treatment with one complete and eight partial remissions. Responses were seen with all three second-line hormone treatments (AG, four of eight; TAM, three of eight; MPA, one of six) with no significant differences between the response to individual second-line hormonal therapies.

Responses were seen at all sites of involvement including bone but objective evidence of response of bone metastases occurred later than measurable response at soft tissue sites.

Hormone receptor status alone did not discriminate for response to second hormone manipulation. Responses occurred with equal frequency among hormone receptor-negative (five of ten) and hormone receptor-positive patients (four of 12).

Tumor ploidy, however, offered better discrimination. Eight of 12 patients with diploid tumors responded to second-line hormone treatment whereas only one of ten patients with aneuploid tumors responded (chi-square = 5.26, $P = 0.02$). It should be noted, however, that the one patient with the aneuploid tumor who did respond was ER positive. No patients with aneuploid and receptor-negative tumors responded (Table 3).

Time to progression after second-line hormonal treatment was 9 ± 2 months with no significant differences between receptor-positive and receptor-negative patients.

Discussion

There is considerable evidence to show that initial response to hormone therapy correlates with the presence of tumor ER and even more closely with the presence of PR in addition to ER.¹⁶⁻¹⁸ Despite a high initial response rate to hormone treatment in patients with ER-positive, PR-positive tumors, most show further progression of the disease after a variable period of control. One hypothesis regarding escape from initial hormone responsiveness is that growth suppression of a receptor-positive and hormonally dependent tumor cell population allows for the emergence of hormone-insensitive subclones.¹⁹ Whereas a number of investigators have shown that ER content decreases during hormone therapy,^{5,20} Taylor and co-workers⁹ demonstrated that hormone receptor content tends to rise at relapse from hormonal treatment and that loss of receptor is not an invariable feature at relapse.

The current study investigates the hormone receptor status at relapse of a group of patients who had demonstrated clinical responsiveness to hormone manipulation and determines whether response to second-line hormone

TABLE 3. Response to Second Hormone Treatment: Predictive Value of Tumor Ploidy and Hormone Receptor Determination

Receptor status at relapse	No. with CR/Pr			No. with PD	
	No.	Diploid	Aneuploid	Diploid	Aneuploid
ER+ PR+	2	1	—	—	1
ER+ PR—	9	2	1	2	3
ER— PR+	1	—	—	—	1
ER— PR—	10	5	—	2	3

ER: estrogen receptor; PR: progesterone receptor, +: positive; —: negative; Pr: partial response; CR: complete response; PD: progressive disease.

therapy could be predicted by receptor determination or by other tumor parameters at relapse.

The results indicate that a significant proportion of tumors retained ER at the time of relapse from initial endocrine therapy. Although the initial ER status of the entire patient group was not known it should be pointed out that as these patients had previously demonstrated clinical response to hormone manipulation, the inference would be that the majority, if not all, were initially receptor positive. At relapse from initial hormone treatment 50% were found to be ER positive. Data from the subgroups of patients whose ER status was determined both initially and at relapse showed that seven of 11 initially ER-positive tumors remained ER positive at relapse.

The proportion of PR-positive tumors at relapse was, however, very much lower (one of 11, 9%) than was the case at initial evaluation (nine of 11, 82%). These results are consistent with the findings of Waseda and co-workers who described a reduction in PR in patients receiving long-term treatment with TAM.²¹ It should, however, be pointed out that not all patients who became PR negative after first-line hormone therapy were receiving TAM and that loss of PR appeared to be common to a number of different hormonal therapies.

Although a considerable proportion of patients remained ER positive at relapse the hormone receptor status at relapse was a poor predictor for response to second-line hormone manipulation. Response to second-line hormone treatment was observed with equal frequency in receptor-negative as well as in receptor-positive patients. The addition of ploidy data on the other hand was a significant discriminating factor predicting for second-line hormone response.

A number of hypotheses need to be considered as possible explanations for these findings. The first is related to the technical aspects of the hormone receptor determinations and the possibility of false-negative determinations. Immunocytochemical techniques have previously been shown to be a highly sensitive method for hormone receptor determination.^{22,23} Immunocytochemical techniques were employed for determination of hormone receptor status at relapse in order to minimize the theoretical problem of receptor occupancy, particularly by TAM which is known to have a prolonged tissue half-life.^{6,24,25} Furthermore, the use of immunocytochemical study with analysis of sections immediately adjacent to those histologically involved with tumor makes it less likely, than with DCC methods, that the receptor-negative patients who responded to second-line hormone therapy were, in fact, false-negative because of sampling error due to lack to tumor in the specimen analyzed. In this regard the findings of Taylor and co-workers⁹ are of interest since they pointed out that the decrease in ER content of tumors

be explained on the basis of decreased cellularity of the specimens analyzed. In the current investigation only sections of adequate cellularity were analyzed.

Although it remains possible that functional ER was present at a level below the limits of detection by the immunocytochemical technique, the hypothesis that mechanisms other than ER-mediated pathways were responsible for the responses observed needs to be considered. Whereas some of the second-line hormone treatments, e.g., progestogens, act through mechanisms other than ER, there appear, however, to be no differences in response according to the nature of second-line hormone treatment. The response of the ER-negative patients to TAM thus requires some explanation. In this regard it should be pointed out that although ER-negative patients have a low response rate to hormonal therapy, Vogel and co-workers have described what appears to be a significantly higher response rate to TAM among ER-negative patients when a subgroup with clinical features favorable for hormone responsiveness was considered.²⁶ The patients in this study, i.e., previous hormone therapy responders, appear also to be a favorable subgroup for further hormone therapy.

Of interest in this regard is the finding that a high proportion of diploid tumors remained sensitive to hormonal growth control. Aneuploid tumors, on the other hand, particularly when receptor negative in addition, were largely nonresponsive to hormone manipulation. Whereas a relationship between tumor growth fraction and tumor ploidy on the one hand and hormone receptor status on the other has been described, recent investigations indicate that flow cytometric analysis of ploidy and growth fraction are independent predictors of prognosis.^{27,28} Flow cytometric analysis of tumor ploidy appears, in addition, to be useful in defining a group of patients responsive to second-line hormone treatment.

REFERENCES

1. Hawkins RA, Black R, Steele RJC, Dixon JMJ, Forrest APM. Oestrogen receptor concentration in primary breast cancer and axillary node metastases. *Breast Cancer Res Treat* 1975; 1:245-251.
2. Rosen PP, Menendez-Botet CJ, Urban JA, Fracchia A, Schwartz MK. Estrogen receptor protein (ER P) in multiple tumor specimens from individual patients with breast cancer. *Cancer* 1977; 39:2194-2200.
3. Webster DJT, Bronn DG, Minton JP. Estrogen receptor levels in multiple biopsies from patients with breast cancer. *Am J Surg* 1978; 136:337-338.
4. Brennan MJ, Donnyain WL, Appelby DE. The variability of estrogen receptors in metastatic breast cancer. *Am J Surg* 1979; 137:260-263.
5. Allegra JC, Barloch A, Huff KK, Lippman ME. Changes in multiple sequential estrogen receptor determinations in breast cancer. *Cancer* 1980; 45:792-794.
6. Hull D, Clark G, Osborne C, Chamness G, Knight W, McGuire W. Multiple estrogen receptor assays in human breast cancer. *Cancer Res* 1983; 43:413-416.
7. Harland RNL, Barnes DM, Howell A, Ribeiro GG, Taylor J, Sellwood RA. Variation of receptor status in cancer of the breast. *Br J Cancer* 1983; 47:511-515.

therapy could be predicted by receptor determination or by other tumor parameters at relapse.

The results indicate that a significant proportion of tumors retained ER at the time of relapse from initial endocrine therapy. Although the initial ER status of the entire patient group was not known it should be pointed out that as these patients had previously demonstrated clinical response to hormone manipulation, the inference would be that the majority, if not all, were initially receptor positive. At relapse from initial hormone treatment 50% were found to be ER positive. Data from the subgroups of patients whose ER status was determined both initially and at relapse showed that seven of 11 initially ER-positive tumors remained ER positive at relapse.

The proportion of PR-positive tumors at relapse was, however, very much lower (one of 11, 9%) than was the case at initial evaluation (nine of 11, 82%). These results are consistent with the findings of Waseda and co-workers who described a reduction in PR in patients receiving long-term treatment with TAM.²¹ It should, however, be pointed out that not all patients who became PR negative after first-line hormone therapy were receiving TAM and that loss of PR appeared to be common to a number of different hormonal therapies.

Although a considerable proportion of patients remained ER positive at relapse the hormone receptor status at relapse was a poor predictor for response to second-line hormone manipulation. Response to second-line hormone treatment was observed with equal frequency in receptor-negative as well as in receptor-positive patients. The addition of ploidy data on the other hand was a significant discriminating factor predicting for second-line hormone response.

A number of hypotheses need to be considered as possible explanations for these findings. The first is related to the technical aspects of the hormone receptor determinations and the possibility of false-negative determinations. Immunocytochemical techniques have previously been shown to be a highly sensitive method for hormone receptor determination.^{22,23} Immunocytochemical techniques were employed for determination of hormone receptor status at relapse in order to minimize the theoretical problem of receptor occupancy, particularly by TAM which is known to have a prolonged tissue half-life.^{6,24,25} Furthermore, the use of immunocytochemical study with analysis of sections immediately adjacent to those histologically involved with tumor makes it less likely, than with DCC methods, that the receptor-negative patients who responded to second-line hormone therapy were, in fact, false-negative because of sampling error due to lack to tumor in the specimen analyzed. In this regard the findings of Taylor and co-workers⁹ are of interest since they pointed out that the decrease in ER content of tumors

be explained on the basis of decreased cellularity of the specimens analyzed. In the current investigation only sections of adequate cellularity were analyzed.

Although it remains possible that functional ER was present at a level below the limits of detection by the immunocytochemical technique, the hypothesis that mechanisms other than ER-mediated pathways were responsible for the responses observed needs to be considered. Whereas some of the second-line hormone treatments, e.g., progestogens, act through mechanisms other than ER, there appear, however, to be no differences in response according to the nature of second-line hormone treatment. The response of the ER-negative patients to TAM thus requires some explanation. In this regard it should be pointed out that although ER-negative patients have a low response rate to hormonal therapy, Vogel and co-workers have described what appears to be a significantly higher response rate to TAM among ER-negative patients when a subgroup with clinical features favorable for hormone responsiveness was considered.²⁶ The patients in this study, i.e., previous hormone therapy responders, appear also to be a favorable subgroup for further hormone therapy.

Of interest in this regard is the finding that a high proportion of diploid tumors remained sensitive to hormonal growth control. Aneuploid tumors, on the other hand, particularly when receptor negative in addition, were largely nonresponsive to hormone manipulation. Whereas a relationship between tumor growth fraction and tumor ploidy on the one hand and hormone receptor status on the other has been described, recent investigations indicate that flow cytometric analysis of ploidy and growth fraction are independent predictors of prognosis.^{27,28} Flow cytometric analysis of tumor ploidy appears, in addition, to be useful in defining a group of patients responsive to second-line hormone treatment.

REFERENCES

1. Hawkins RA, Black R, Steele RJC, Dixon JMJ, Forrest APM. Oestrogen receptor concentration in primary breast cancer and axillary node metastases. *Breast Cancer Res Treat* 1975; 1:245-251.
2. Rosen PP, Menendez-Botet CJ, Urban JA, Fracchia A, Schwartz MK. Estrogen receptor protein (ER P) in multiple tumor specimens from individual patients with breast cancer. *Cancer* 1977; 39:2194-2200.
3. Webster DJT, Bronn DG, Minton JP. Estrogen receptor levels in multiple biopsies from patients with breast cancer. *Am J Surg* 1978; 136:337-338.
4. Brennan MJ, Donnyan WL, Appelby DE. The variability of estrogen receptors in metastatic breast cancer. *Am J Surg* 1979; 137:260-263.
5. Allegra JC, Barloch A, Huff KK, Lippman ME. Changes in multiple sequential estrogen receptor determinations in breast cancer. *Cancer* 1980; 45:792-794.
6. Hull D, Clark G, Osborne C, Chamness G, Knight W, McGuire W. Multiple estrogen receptor assays in human breast cancer. *Cancer Res* 1983; 43:413-416.
7. Harland RNL, Barnes DM, Howell A, Ribeiro GG, Taylor J, Sellwood RA. Variation of receptor status in cancer of the breast. *Br J Cancer* 1983; 47:511-515.

8. Namer M, Lalane C, Baulieu E. Increase of progesterone receptor content by tamoxifen as a hormonal challenge test in breast cancer. *Cancer Res* 1980; 40:1750-1752.
9. Taylor RE, Powles TJ, Humphreys J *et al*. Effects of endocrine therapy on steroid-receptor content of breast cancer. *Br J Cancer* 1982; 45:577-579.
10. Levin J, Ray G, da Fonseca M, Lange M, de Moor NG, Savage M. Oestrogen receptors in tumours of breast cancer patients. *S Afr Med J* 1978; 53:577-579.
11. Logeat F, Thu Vu Hai M, Fournier A, Legrain P, Buttin G, Milgrom E. Monoclonal antibodies to rabbit progesterone receptors: Cross reaction with other mammalian progesterone receptors. *Proc Natl Acad Sci USA* 1983; 80:6456-6459.
12. Perrot-Applanat M, Logeat F, Groyer-Picard MT, Milgrom E. Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. *Endocrinology* 1985; 116:1473-1484.
13. Perrot-Applanat M, Groyer-Picard M, Lorenzo F *et al*. Immunocytochemical study with monoclonal antibodies to progesterone receptor in human breast tumours. *Cancer Res* 1987; 47:2652-2661.
14. Seymour L, Meyer R, Esser J, MacPhail AP, Behr A, Bezudova WR. Estimation of PR and ER by immunoassay in breast cancer: Comparison with radioligand binding methods. *Am J Clin Pathol* (in press).
15. Hayward JL, Rubens RD, Carbone PP, Heuson J-C, Humaoka S, Segaloff A. Assessment of response to therapy in advanced breast cancer. *Br J Cancer* 1973; 35:292-297.
16. McGuire WL. Hormone receptors: Their role in predicting prognosis and response to endocrine therapy. *Semin Oncol* 1978; 428-433.
17. Jensen EV. Historical perspective steroid receptors in breast cancer. *Cancer* 1980; 46:2759-2761.
18. Manni A, Arafah B, Pearson DH. Estrogen and progesterone receptors in prediction of response of breast cancer to endocrine therapy. *Cancer* 1980; 46:2838-2841.
19. Arafah BM, Gullino PM, Manni A, Pearson OH. Effect of ovariectomy on hormone receptors and growth of N-nitroso-methyl-urea-induced mammary tumours in the rat. *Cancer Res* 1980; 40:4528-4531.
20. Kiang DT, Kennedy BJ. Factors affecting estrogen receptors in breast cancer. *Cancer* 1977; 40:1571-1574.
21. Waseda N, Kato Y, Imura M, Kurata M. Effects of tamoxifen on estrogen and progesterone receptors in human breast cancer. *Cancer Res* 1981; 41:1984-1988.
22. King W, DeSombra E, Jensen E, Greene G. Comparison of immunocytochemical and steroid binding assays for estrogen receptor in human breast tumors. *Cancer Res* 1985; 45:293-304.
23. Thorpe S. Monoclonal antibody technique for detection of estrogen receptors in human breast cancer: Greater sensitivity and more accurate classification of receptor status than the dextran coated charcoal method. *Cancer Res* 1987; 47:6572-6575.
24. Fabian C, Sternson L, El-Serafi M, Cain L, Horne E. Clinical pharmacology of tamoxifen in patients with breast cancer: Correlation with clinical data. *Cancer* 1981; 48:876-882.
25. Sutherland RL, Murphy LC. The binding of tamoxifen to human mammary carcinoma cytosol. *Eur J Cancer* 1980; 16:1141-1143.
26. Vogel CL, East DR, Voight W, Thomsen S. Response to tamoxifen in estrogen receptor-poor metastatic breast cancer. *Cancer* 1987; 60:1184-1189.
27. McGuire WL, Dressler LG. Emerging impact of flow cytometry in predicting recurrence and survival in breast cancer patients. *J Natl Cancer Inst* 1985; 75:405-410.
28. Clark, GM, Dressler LG, Owens MA, Pounds G, Oedakor T, McGuire WL. Prediction of relapse or survival in patients with node-negative breast cancer by DNA flow cytometry. *N Engl J Med* 1989; 320:626-633.

3.3 Paper 3

DETECTION OF P24 PROTEIN IN HUMAN BREAST CANCER: INFLUENCE OF RECEPTOR STATUS
AND OESTROGEN EXPOSURE.

L. Seymour, W.R. Bezwoda, K. Meyer, C. Behr.

British Journal of Cancer 61:886-890. 1990.

Detection of P24 protein in human breast cancer: influence of receptor status and oestrogen exposure

L. Seymour, W.R. Bezwoda, K. Meyer & C. Behr

Department of Medicine, Division of Oncology, University of the Witwatersrand Medical School, 7 York Road, Parktown 2193, Johannesburg, South Africa.

Summary The expression of oestrogen regulated protein, P24, was investigated in 69 breast cancers. At initial evaluation P24 protein was detected significantly more frequently and was present in significantly higher concentration in oestrogen receptor positive than in receptor negative tumours. There was, however, no correlation between P24 staining and progesterone receptor, tumour ploidy or proliferative index. Nineteen patients received a short course of treatment with diethylstilboestrol. Following treatment with oestrogen, P24 staining became positive in 7/13 tumours previously negative for P24, including six tumours which were oestrogen receptor negative. Oestrogen administration also caused an increase of the proliferation index in 12/19 tumours, including 5/7 that were oestrogen receptor positive and 7/12 that were oestrogen receptor negative. In some instances oestrogenic stimulation of proliferation occurred together with increased P24 expression; in other instances proliferation index increased without induction of P24 synthesis. The *in vivo* effects of oestrogen in clinical breast cancer thus appear to show dissociation between enhancement of protein synthesis and cellular proliferation.

Oestrogenic effects in hormone responsive tissues such as the breast include induction of protein synthesis as well as increased proliferation. In addition, oestrogens appear to play an important role in the development, maintenance and growth of breast tumours. The currently held hypothesis is that these oestrogenic effects are mediated through the interaction of hormone and specific nuclear oestrogen receptor (ER). While the presence of specific oestrogen and progesterone (PR) receptors appears to be an important determinant of response to hormone treatment (Whitliff, 1983; Cant *et al.*, 1985; Vollenweider-Zeraigul *et al.*, 1986; Williams *et al.*, 1987), in breast cancer not all receptor positive tumours are amenable to hormonal manipulation. However, absence of receptor is associated with a low probability of response to hormone therapy.

Apart from the utility of receptors as predictors for response to hormonal treatment an important pathophysiological consideration in breast cancer is the influence of endogenous hormones on tumour genesis, promotion and growth. These effects are also thought to be mediated through the receptor mechanism. Steroid receptors are, however, demonstrated only in a proportion of breast cancers (Allegra *et al.*, 1979; Mohla *et al.*, 1982; McGuire *et al.*, 1984). Whether receptor-negative tumours are independent of hormonal influence requires elucidation.

Recently two oestrogen regulated proteins, P24 (Edwards *et al.*, 1981; Ciocca *et al.*, 1982, 1984; Adams *et al.*, 1983; Adams & McGuire, 1985) and P52 (Veith *et al.*, 1983; Garcia *et al.*, 1984, 1985; Rochefort *et al.*, 1987) have been described. The study of such oestrogen regulated proteins may give insight into the mechanisms of hormone action in breast cancer.

In the MCF 7 breast cancer cell line P24 expression appears to be constitutive but with only low levels of P24 being produced in the absence of exogenous oestrogenic stimulation. However, in this experimental model oestrogen exposure results in both new mRNA expression as well as increased P24 protein synthesis, suggesting that the gene is oestrogen inducible. Whether such effects are seen *in vivo* is at present unknown. We have thus chosen to study P24 expression in hormonal regulation of a model of the protein synthesis in human breast cancer.

Materials and methods

Monoclonal IgG antibody to P24 was a generous gift from Dr W. McGuire (University of Texas Health Science Centre). Avidin-biotin reagent (Vectastain ABC Kit) was obtained from Vector (Vector Laboratories, Burlingame, CA, USA). Non-immune mouse IgG and DAB were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA).

Specimens were obtained by either surgical biopsy or needle biopsy (Trucut), under local anaesthetic, of accessible tumours. Most were metastatic tumours at cutaneous sites or primary breast tumours. The specimens were transported on ice and either processed immediately or stored at -135°C for later use. All specimens were examined for the presence of tumour by routine haematoxylin and eosin stained sections. Sections immediately adjacent to those histologically involved with tumour were used for immunocytochemical determinations.

P24 immunocytochemistry

Frozen sections were placed on HCl-ethanol cleaned slides. Specimens were fixed by immersion in 3.7% formalin for 10 minutes followed by ice cold methanol for 4 minutes and then in ice cold acetone for 1-2 minutes. Slides were rinsed with cold phosphate-buffered saline (PBS) and immersed in H_2O_2 -methanol (to block endogenous peroxidase) and then rinsed again. Thereafter slides were incubated for 3 hours with monoclonal anti-P24 antibody ($5\mu\text{g ml}^{-1}$) (Ciocca *et al.*, 1983a) at 20°C . Biotinylated secondary antibody was applied at a 1:400 dilution. The reaction was developed with DAB. Slides were counterstained with Meyer's Haematoxylin, serially dehydrated with graded alcohols and xylene and then mounted with coverslips. All assays were performed with negative controls, substituting non-immune mouse IgG for primary antibody, and with a positive control utilizing MCF-7 cells grown under optimal conditions for hormone receptor and P24 expression. Slides were examined at $400\times$ magnification. Assessment of immunocytochemical staining used for the following scoring system: 0, negative; 1, weak staining, present in 1-10% of cells; 2, moderate staining, present in 11-50% of cells; 3, intense staining, present in 51-90% of cells; 4, intense staining, present in 91-100% of cells.

Immunocytochemical staining for ER and PR

Immunocytochemical staining for ER was performed using the Abbot ER-ICA kit (King *et al.*, 1985; Thorpe, 1987).

(Abbot Laboratories) according to the manufacturers' instructions. Immunocytochemical staining for PR was performed according to previously described methods (Logeat *et al.*, 1983; Perrot-Applanat, 1985, 1987) using a monoclonal antibody to rabbit PR which cross-reacts with human PR and has been shown to be highly specific for the receptor. Assessment of immunocytochemical staining for ER and PR used the same scoring system as for P24.

All immunocytochemical assays were performed analysing a minimum of 10 fields and counting at least 100 cells per field to give an overall score. Only tumour cells were counted for the estimation of degree of positivity. When serial biopsies were performed they were performed from the same tumour area.

Flow cytometric analysis of tumour cell ploidy and proliferative index

Flow cytometry was performed using a Coulter Epics cytometer after enzymatic digestion of minced fresh or frozen tissue fragments and staining with propidium iodide. DNA distribution was compared to a standard of human lymphocyte nuclei as well as normal breast tissue freshly prepared in similar fashion to the tissue fragments. Aneuploid tumours were defined as those with DNA indices lower or higher than 1.0–1.1. Proliferative index (PI) was calculated by summation of cells in S and G₂M. All estimations were performed in triplicate. Only studies where the coefficient of variation was <5% were considered analysable. Significant stimulation of PI following oestrogen exposure was defined as a rise in PI >10% from the pretreatment value.

Patients

A total of 74 patients were studied. Of the 74 patients, 20 were caucasian and 54 were black. All patients had locally advanced primary disease or metastatic disease with tumours accessible for biopsy. The initial evaluations were performed before any hormone therapy or chemotherapy. Sixty-nine patients had evaluable results for all parameters i.e. ploidy, receptor status and P24 staining before therapy.

Apart from the pretreatment investigations 24 patients were investigated serially during the course of a randomised ongoing study of the effects of hormone priming before chemotherapy. Patients were eligible for this study whether ER+ or ER- and were randomly allocated to receive either hormone priming followed by chemotherapy or chemotherapy alone. Nineteen of the 24 patients had been randomised to the hormone priming arm and were evaluated following a short exposure to oestrogen (given as diethylstilboestrol 5 mg day⁻¹ × 5 days). Biopsies were performed immediately before and immediately after hormone administration. Five patients were evaluated before and after chemotherapy without any prior hormonal priming.

The study was approved by the Ethics Committee of the University of the Witwatersrand and was carried out in accordance with the principles of the Declaration of Helsinki.

Results

The results using the histochemical assays were highly reproducible. Inter and intra-observer variation was minimal with a correlation coefficient of >0.9 for single biopsy specimens. When multiple simultaneous biopsies were carried out on the same patient (19 patients had multiple simultaneous biopsies) the overall scores, taking into account 10 fields from each biopsy sample, were also consistent with a correlation coefficient >0.9.

Approximately 50% of samples obtained before therapy showed positive staining for P24 (Table I). There were no significant differences in the proportion showing P24 staining when black or white patients, those with aneuploid or diploid tumours, or PR positive and PR negative tumours were compared. P24 staining was, however, found to be

Table I Pretreatment immunocytochemical staining for P24 protein

Patient characteristics	P24 staining intensity					Total positive (%)
	0	1	2	3	4	
Premenopausal	10	7	1	3	0	11/12 (52)
Post-menopausal	25	8	9	5	1	23/48 (48)
Black	27	12	5	6	1	24/51 (47)
White	8	3	5	2	0	10/18 (55)
Diploid	19	7	4	4	1	16/35 (46)
Aneuploid	16	8	6	4	0	18/34 (53)
ER + PR +	3	2	1	2	1	6/9 (66)
ER + PR -	5	4	2	4	0	10/15 (66)
ER - PR +	1	1	0	0	0	1/2 (50)
ER - PR -	26	8	7	2	0	17/43 (39)
Grades 1 and 2	8	9	5	2	1	17/25 (68)
Grades 3 and 4	27	10	5	2	0	17/44 (41)
Locoregional (stage II) disease	5	2	1	3	1	7/12 (58)
Metastatic disease	30	13	9	5	0	27/57 (47)

significantly, more frequent in ER positive tumours (16/24; 67%) when compared to ER negative tumours (18/45; 40%) ($\chi^2 = 4.50$, $P < 0.05$). Furthermore, the intensity of P24 staining correlated significantly with ER content (Spearman correlation 0.434, $P = 0.001$).

Histological grade also correlated significantly with P24 expression with 17/25 grade 1 and 2 tumours showing P24 positivity, while only 11/44 grade 3 tumours were P24 positive ($\chi^2 = 5.5$, $P < 0.05$).

Results following hormone treatment (DES 5 mg day⁻¹ × 5 days) are shown in Tables II and III. Of the six tumours that were P24 positive before oestrogen exposure four remained P24 positive and two became P24 negative following treatment with DES (Table II). Of the 13 tumours that were P24 negative before hormone treatment seven became positive for P24 after oestrogen administration. P24 induction was noted in 6/9 ER negative as well as 1/4 in ER positive tumours.

Oestrogenic stimulation of cell growth (as defined by an increase of proliferative index >10% from base line values) was seen in 12/19 patients given a short course of diethylstilboestrol. The 12 tumours which showed an increase of proliferation index after oestrogen treatment included 5/7 that were ER positive and 7/12 that were ER negative before hormone administration.

The relationships between P24 expression and alteration of proliferation index were also complex. Proliferation index increased in 3/6 tumours that were initially P24 positive and in 9/13 that were initially P24 negative. Among the tumours that were initially P24 positive, two showed increased proliferation together with loss of P24 expression while one remained P24 positive within increased proliferation following oestrogen. On the other hand, among the nine tumours that were initially P24 negative and which showed an increase in proliferation index, six had P24 induction with increased proliferation while three showed an increase in proliferation with P24 remaining negative. In two instances there was a significant decrease of proliferation index following 5 days of diethylstilboestrol therapy. Both were tumours that were ER+ and PR- and in both instances PR was induced by oestrogen exposure together with the decrease of proliferation index. In addition P24 expression was also induced in one of these tumours.

The effects of oestrogen administration on hormone receptor expression are shown in Table III. Following diethylstilboestrol ER was no longer detectable in 6/7 previously ER positive tumours while in the one tumour that remained ER positive there was a significant decrease in the ER content. At the same time PR either increased in concentration or became positive. In those tumours that were initially ER negative there was, however, no change of either ER or PR status following diethylstilboestrol.

No significant changes in P24, or hormone receptor expression were observed in tumours from those patients who

Table II Effect of oestrogen (diethylstilboestrol) and of chemotherapy on *in vivo* P24 expression and cell proliferation

Hormone receptors	Pretreatment		After oestrogen administration			
	P24 expression			Proliferative index*		
	Positive	Remained positive	Became negative	Up	Down	NC
ER + PR +	1	1	0	0	0	1
ER + PR -	2	1	1	2	0	0
ER - PR -	3	2	1	1	1	1
Total	6	4	2	3	1	2
Hormone receptors	P24 expression			Proliferative index*		
	Negative	Remained negative	Became positive	Up	Down	NC
	Positive	Remained positive	Became negative	Up	Down	NC
ER + PR +	2	1	1	2	0	0
ER + PR -	2	2	0	1	0	1
ER - PR +	1	1	0	1	0	0
ER - PR -	8	2	6	5	1	2
Total	13	6	7	9	1	3

Hormone receptors	Pretreatment		After chemotherapy			
	P24 expression			Proliferative index*		
	Positive	Remained positive	Became negative	Up	Down	NC
ER + PR +	1	1	0	0	0	1
ER + PR -	1	1	0	0	1	0
ER - PR -	1	1	0	0	0	1
Total	3	3	0	0	1	2
Hormone receptors	P24 expression			Proliferative index*		
	Negative	Remained negative	Became positive	Up	Down	NC
	Positive	Remained positive	Became negative	Up	Down	NC
ER - PR -	2	2	0	0	1	1

*Up, increase in proliferative index >10%; down, decrease in proliferative index >210%; no change in proliferative index.

Table III Effects on oestrogen (diethylstilboestrol) and of chemotherapy on *in vivo* hormone receptor expression

Hormone receptors	ER + PR +	ER + PR -	ER - PR +	ER - PR -
Pretreatment				
ER + PR +	3	1	0	0
ER + PR -	4	0	3	1
ER - PR +	1	0	1	0
ER - PR -	11	0	0	11
Total	19	1	6	12
After oestrogen administration				
ER + PR +	1	1	0	0
ER + PR -	1	0	1	0
ER - PR -	3	0	0	3
Total	5	1	1	3
After chemotherapy				

received chemotherapy only. Furthermore no instances of increase in proliferation index were observed following chemotherapy.

Discussion

The 24,000 Da protein, P24, was first detected in MCF-7 cells and appears from protein studies to be an oestrogen regulated (Edwards *et al.*, 1981) secretory protein (Ciocca *et al.*, 1982; Adams *et al.*, 1983). Following these initial investigations P24 has also been found in other oestrogen receptor positive cell lines (Ciocca *et al.*, 1983b) as well as in highly oestrogen responsive target tissues such as human decidua (Ciocca *et al.*, 1983c) and in certain cells of the female genital tract (Ciocca *et al.*, 1983b). Recent DNA sequencing studies have shown that the P24 protein is identical with a human heat shock protein, designated as hsp27, which was first detected in HeLa cells (Fuqua *et al.*, 1990). P24 is generally not found in normal or hyperplastic breast tissue.

While the synthesis of P24 appears to be constitutive in MCF-7 cells, both mRNA expression and synthesis of the protein are selectively increased in MCF-7 by oestrogenic stimulation. Recent investigations have shown that heat shock can also induce P24 synthesis in MCF-7 cells (Hickey *et al.*, 1986). While the function of P24/hsp27 is unknown, the selective tissue expression and its apparent control by

oestrogen suggested that P24 might be a useful marker for the study of oestrogen action in breast cancer, both as an indicator of endogenous hormonal action and possible as a predictor of hormone responsiveness.

The correlation between initial oestrogen receptor status and P24 expression and between histological grade and P24 expression suggest that the expression of these biological tumour markers is in some way linked. In this regard the comparison between black and white patients is of some interest. Previous studies have suggested a lower frequency of receptor positive tumours among black women than among caucasian women (Savage *et al.*, 1980; Mohla *et al.*, 1982; Pegoraro *et al.*, 1986). A previous study from this institution (Dansey *et al.*, 1988) demonstrated, however, that black patients with breast cancer have a significantly younger age distribution, thus raising the possibility of ER masking by endogenous oestrogens when ER estimations are performed by ligand binding methods. The study of P24 might thus offer a means of establishing the presence of endogenous hormone action in apparently ER- tumours. Consistent with this hypothesis the present study showed no significant differences between black and white subjects in regard to P24 expression. It should be pointed out, however, that in the present study, where immunocytochemical methods were used for ER and PR estimation, there were also no significant differences in ER or PR status between the two racial groups.

While the correlation between baseline P24 and ER status was significant, P24 expression was by no means confined to ER positive tumours. The presence of P24 in ER- tumours could be due to constitutive production of the protein by tumour cells or the induction of synthesis by oestrogen in the absence of detectable ER. In this regard the results following exposure to oestrogen have to be taken into account. Following oestrogen administration P24 induction was noted in 7/13 tumours previously negative for P24. There was, however, no correlation, *in vivo*, between ER content and oestrogen induced synthesis of P24.

The influence of diethylstilboestrol on cell proliferation appeared also to be independent of ER with both ER+ and ER- tumours, showing an increase of proliferation as assessed by flow cytometric analysis. The validity of the flow cytometric measurements was confirmed by clinical observations which showed a highly significant correlation between clinical tumour flare and an increase in the proliferative index ($\chi^2 = 17.4$, $P < 0.0001$). Tumour flares following oestrogen administration occurred in ER- as well as in ER+ patients.

The changes observed in hormone receptor expression following oestrogen exposure, on the other hand, did follow a pattern predicted by *in vitro* models, i.e. reduction of ER content and induction of PR expression (Nardulli *et al.*, 1988), which occurred only in ER positive tumours.

That these results were due to the administered oestrogen and not to sampling error is shown by the findings in those

patients receiving chemotherapy only. There were no changes in either P24 or hormone receptor expression following chemotherapy without hormone priming. Furthermore, there was no instance of an increase in proliferation index following chemotherapy alone.

The patterns of response following *in vivo* oestrogen exposure were thus variable and included: (a) induction of new protein synthesis; (b) induction of new protein synthesis together with stimulation of proliferation; (c) stimulation of proliferation occurring without induction of new protein synthesis. While some of these effects, e.g. induction of PR synthesis, appear to be dependent on the presence of specific ER, increased proliferation of P24 synthesis could be induced by oestrogens in the apparent absence of specific ER. In this regard it should be pointed out that since receptor status was determined by demonstration of ER protein by means of immunological rather than ligand binding methods these results are unlikely to be due to receptor masking by high endogenous steroid levels (Thorpe, 1987).

While it remains possible that oestrogenic effects were due to the presence of receptor at concentrations not detectable by current immunological techniques the possibility should be considered that oestrogens can exert significant effects in clinical breast cancer by mechanisms other than binding to specific ER.

Supported by Grants from National Cancer Association (SA) and Bekker Trust Foundation.

References

- ADAMS, D.J., HAJI, H., EDWARDS, D.P., BJERCKE, R.J. & MCGUIRE, W.L. (1983). Detection of Mr 24,000 estrogen-regulated protein in human breast cancer by monoclonal antibodies. *Cancer Res.*, 43, 4297.
- ADAMS, D.M. & MCGUIRE, W.L. (1985). Quantitative enzyme-linked immunosorbent assay for the estrogen regulated Mr 24,000 protein in human breast tumours: correlation with estrogen and progesterone receptors. *Cancer Res.*, 45, 2445.
- ALLEGRA, J.C., LIPMAN, M.E. & THOMPSON, E.B. (1979). Distribution frequency and quantitative analysis of estrogen, progesterone, androgen and glucocorticoid receptors in human breast cancer. *Cancer Res.*, 39, 1447.
- CANT, E., HORSFALL, D. & KEIGHTLEY, D. (1985). Value of hormone receptors in the management of breast cancer. 1. Advanced breast cancer. *Aust. NZ J. Surg.*, 55, 121.
- CIOCCA, D.R., ADAMS, D.J., BJERCKE, R.J., EDWARDS, D.P. & MCGUIRE, W.L. (1982). Immunohistochemical detection of an estrogen-regulated protein by monoclonal antibodies. *Cancer Res.*, 42, 4256.
- CIOCCA, D.R., ADAMS, D.J., BJERCKE, R.J. & 4 others (1983a). Monoclonal antibody storage conditions and concentration effects on immunohistochemical specificity. *J. Histochem. Cytochem.*, 31, 692.
- CIOCCA, D.R., ADAMS, D.J., EDWARDS, D.P., BJERCKE, R.J. & MCGUIRE, W.L. (1983b). Distribution of an estrogen-induced protein with a molecular weight of 24,000 in normal and malignant human tissues and cells. *Cancer Res.*, 43, 1204.
- CIOCCA, D.R., ADAMS, D.J., EDWARDS, D.P., BJERCKE, R.J. & MCGUIRE, W.L. (1984). Estrogen induced 24k protein in MCF-7 breast cancer cells is localised in granules. *Breast Cancer Res. Treat.*, 4, 261.
- CIOCCA, D.R., ASCH, R.H., ADAMS, D.J. & MCGUIRE, W.L. (1983c). Evidence for modulation of a 24k protein in human endometrium during the menstrual cycle. *J. Clin. Endocrinol. Metab.*, 57, 496.
- DANSEY, R.D., HESSEL, P.A., BROWDE, S. & 4 others (1988). Lack of a significant independent effect of race on survival in breast cancer. *Cancer*, 61, 1098.
- EDWARDS, D.P., ADAMS, D.J. & MCGUIRE, W.L. (1981). Estradiol stimulates synthesis of a major intracellular protein in a human breast cancer cell line (MCF-7). *Breast Cancer Res. Treat.*, 1, 209.
- FUQUA, S.A.W., BLUM-SALINGAROS, J. & MCGUIRE, W.L. (1990). Estrogen related 24k protein is induced by heatshock. *Cancer Res.* (in press).
- GARCIA, M., SALAZAR-RETANA, G., RICHTER, G. & 6 others (1984). Immunohistochemical detection of the estrogen regulated 52000 mol wt protein in primary breast cancers but not in normal breast and uterus. *J. Clin. Endocrinol. Metab.*, 59 B, 564.
- GARCIA, M., CAPONY, F., DEROCQ, D., SIMON, D., PAU, B. & ROCHEFORT, H. (1985). Characterisation of monoclonal antibodies to the estrogen regulated M₅₂₀₀₀ glucoprotein and their use in MCF-7 cells. *Cancer Res.*, 45, 709.
- HICKEY, E., BRANDON, W.E. & POTTER, R. (1986). Sequence and organisation of genes encoding the human 17 kDa heat shock protein. *Nucl. Acids Res.*, 14, 2117.
- KING, W., DESOMBRE, E., JENSEN, E. & GREENE, G. (1985). Comparison of immunocytochemical and steroid binding assays for estrogen receptor in human breast tumours. *Cancer Res.*, 45, 293.
- LOGEAT, F., THU VU HAI, M., FOURNIER, A., LEGRAIN, P., BUTTIN, G. & MILGROM, E. (1983). Monoclonal antibodies to rabbit progesterone receptors: cross reaction with other mammalian progesterone receptors. *Proc. Natl. Acad. Sci. USA*, 80, 6456.
- MCGUIRE, W., CARBONE, P., SEARS, H. & ESCHBERG, J. (1984). *Estrogen Receptors in Human Breast Cancer*, p. 15. Raven Press New York.
- MOHLA, S., SAMPSON, C.C., KHAN, T. & 5 others (1982). Estrogen and progesterone receptors in breast cancer in black Americans. *Cancer*, 50, 552.
- NARDULLI, A.M., GREENE, G.L., O'MALLEY, B. & KATZENELLENBOGEN, B.S. (1988). Regulation of progesterone receptor messenger ribonucleic acid and protein levels in MCF-7 cells by estradiol: analysis of estrogen's effects on progesterone receptor synthesis and degradation. *Endocrinology*, 122, 935.
- PEGORARO, R., KARNAN, V., NIRMUL, E. & JOUBERT, S. (1986). Estrogen and progesterone receptors among women of different racial groups. *Cancer Res.*, 46, 807.
- PERROT-APPLANAT, M., LOGEAT, F., GROVER-PICARD, M.T. & MILGROM, E. (1985). Immunocytochemical study of mammalian progesterone receptors using monoclonal antibodies. *Endocrinology*, 116, 1473.
- PERROT-APPLANAT, M., GROVER-PICARD, M., LORENZO, F. & 5 others (1987). Immunocytochemical study with monoclonal antibodies to progesterone receptor in human breast tumours. *Cancer Res.*, 47, 2652.
- ROCHEFORT, H., CAPONY, F., AUGEREAU, F. & 6 others (1987). The estrogen-regulated 52k Intepsin-D in breast cancer from biology to clinical applications. *Int. J. Radiat. Appl. Instrum.*, 14, 377.
- SAVAGE, N., LEVIN, J., DE MOOR, N.G. & LANGE, M. (1980). Cytosolic estrogen receptor content of breast cancer tissue in blacks and whites. *S. Afr. Med. J.*, 59, 807.
- THORPE, S. (1987). Monoclonal antibody technique for detection of estrogen receptors in human breast cancer: greater sensitivity and more accurate classification of receptor status than the dextran coated charcoal method. *Cancer Res.*, 47, 6572.

- VEITH, F., CAPONY, F., GARCIA, M. & 5 others (1983). Release of estrogen induced glycoprotein with a molecular weight of 52,000 by breast cancer cells in primary culture. *Cancer Res.*, 43, 1961.
- VOLLENWEIDER-ZERARGUL, L., BARRELET, L., WONG, Y., LEMARCHAN, D., BERAUD, T. & GOMEZ, F. (1986). The predictive value of estrogen and progesterone concentrations on the clinical behaviour of breast cancer in women. *Cancer*, 57, 1171.
- WHITLIF, J.L. (1983). Steroid hormone receptors in breast cancer. *Cancer*, 53, 630.
- WILLIAMS, M.R., TODD, I., ELLIS, I. & 6 others (1987). Oestrogen receptors in primary and advanced breast cancer, an eight year review of 704 cases. *Br. J. Cancer*, 55, 67.

3.4 Paper 4

TUMOUR FACTORS PREDICTING FOR PROGNOSIS IN METASTATIC BREAST CANCER. THE
PRESENCE OF P24 PREDICTS FOR RESPONSE TO TREATMENT AND DURATION OF SURVIVAL.

L. Seymour, W.R. Bezwoda, K. Meyer.

Cancer 66:2390-2394.1990.

Tumor Factors Predicting for Prognosis in Metastatic Breast Cancer

The Presence of P24 Predicts for Response to Treatment and Duration of Survival

L. Seymour, MBBCh, FCP, W. R. Bezwoda, FCP, PhD,
and K. Meyer, BSc

Fifty-one patients with metastatic breast cancer were investigated to determine tumor parameters with prognostic significance. Investigations included determinations of P24 content by immunocytochemical means using a monoclonal antibody to P24 protein; immunocytochemical analysis of estrogen and progesterone receptors; ploidy analysis by flow cytometry, and histologic grading. There were significant correlations between the presence of P24 and estrogen receptor, between histologic grade and P24 expression, and between estrogen and progesterone receptors. Of the tumor factors investigated only P24 protein was, however, of prognostic significance. Patients with P24-positive tumors had a significantly higher rate of response to treatment as well as more prolonged duration of response and duration of survival from diagnosis of metastatic disease. None of the other variables investigated were significantly predictive of outcome. P24 protein may be a useful predictor of prognosis in metastatic breast cancer.
Cancer 65:2390-2394, 1990.

WHILE HOST-RELATED FACTORS, including performance status as well as the extent of distribution of metastases,^{1,2} are clearly of prognostic importance in patients with metastatic breast cancer, there has been only limited investigation of tumor-specific markers as guides to prognosis. A previous investigation from this institution showed that elevated CEA (carcinoembryonic antigen) and/or serum ferritin levels at initial assessment were associated with a poorer prognosis.³ However, the prognostic significance of these markers probably relates to tumor bulk rather than to tumor biology. While hormone receptor content correlates with response to hormone therapy, the predictive value of this investigation is greatest when excluding patients whose tumors are receptor negative from hormone treatment.⁴⁻¹⁰ Labelling index ap-

peared, in one study, to predict for response to chemotherapy¹¹ but this finding was not confirmed in another investigation.¹² Hormone receptor content was shown in one study¹³ to correlate with response to chemotherapy but again this finding was not confirmed in a number of other investigations.¹⁴⁻¹⁶

Since approximately 50% of patients with breast cancer will ultimately develop metastases, the definition of tumor factors with prognostic significance is of some importance. Previous investigations from this laboratory have dealt with the relationship of a number of tumor factors and the response of metastatic disease to hormone manipulation.^{17,18} The present study aimed to address this question in relation to chemotherapeutic treatment by the investigation of a number of tumor parameters including hormone receptors, tumor ploidy, and detection of P24 protein^{19,20} at the time of diagnosis of metastatic disease.

Patients and Methods

The study population consisted of 51 patients with metastatic breast cancer attending the Breast Clinic of the authors' institution between January 1987 and January 1988. Metastases were confirmed by histologic or cytologic

From the Department of Medicine, Haematology/Oncology, University of the Witwatersrand Medical School and Johannesburg Hospital, Johannesburg, South Africa.

Supported by Grants from Bekker Trust Foundation and the National Cancer Association (South Africa).

Address for reprints: W. R. Bezwoda, FCP, PhD, Department of Medicine, University of the Witwatersrand Medical School, 7 York Rd., Parktown 2193, Johannesburg, South Africa.

Accepted for publication March 28, 1990.

examination of accessible tumors. None of the patients had prior chemotherapy for metastatic disease. Adjuvant chemo- or hormone therapy, when given, had been discontinued at least 2 years prior to entry into the study. All patients included in the study had at least one area of measurable disease and all patients had tumors accessible to biopsy. All patients entered into this study had a performance status ≥ 2 (ECOG). Further patient details are given in Table 1.

In addition to routine histologic/cytologic investigations, biopsy (either needle or surgical biopsy) specimens were obtained for determination of estrogen receptor (ER) and progesterone receptor (PR) content as well as DNA distribution by flow cytometric analysis and for P24 expression. ER, PR, and P24 estimations were performed by immunocytochemical methods as detailed below.

Specimens for immunocytochemical and flow cytometric investigations were transported on ice and were either processed immediately or stored at -135°C for later use.

Monoclonal antibody to P24 was donated by Dr. W. McGuire (University of Texas Health Science Center). Avidin Biotin (Vectastain ABC Kit) was obtained from Vector (Vector Laboratories, Burlingame, CA). Non-immune mouse IgG and diaminobenzidine (DAB) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO).

Immunocytochemical Staining for ER and PR

Immunocytochemical staining for ER²¹ was performed using the Abbott ER-ICA kit (Abbott Laboratories, Chicago, IL) according to the manufacturer's instructions. Immunocytochemical staining for PR was performed according to previously described methods using a mono-

clonal antibody to rabbit PR that cross reacts with human PR and has been shown to be highly specific for PRs.²²⁻²⁴

P24 Protein Immunocytochemistry

Frozen sections were placed on HCl-ethanol-cleaned slides. Specimens were fixed by immersion in 3.7% formalin for 10 minutes followed by ice-cold methanol for 4 minutes, and then ice cold acetone for 1 to 2 minutes. Slides were rinsed with cold phosphate-buffered saline, immersed in H_2O_2 -methanol (to block endogenous peroxidase), and then rinsed again. Thereafter slides were incubated for 3 hours with monoclonal anti-P24 antibody ($5 \mu\text{g}/\mu\text{l}$) at 20°C . The reaction was developed with DAB, slides were counterstained with Meyer's hematoxylin, serially dehydrated with graded alcohols and xylene, and mounted with coverslips. All assays were performed with negative controls substituting non-immune mouse IgG for primary antibody and with a positive control using MCF 7 cells grown under optimal conditions for hormone receptor expression and P24 expression. Slides were examined at $\times 400$ magnification.

P24 staining was considered positive if more than 10% of cells showed cytoplasmic staining in an assay with appropriate findings for positive and negative controls. The scoring system used for assessment of P24 staining has previously been reported¹⁷ and is based on that used for ER-ICA detection. All biopsy specimens were examined by routine histologic methods for the presence of tumor cells. Immunocytochemical assays were performed using sections adjacent to histologically involved areas, and only identifiably tumor cells were counted for immunocytochemical estimations. At least 100 cells were counted per field and at least ten fields were examined.

Flow Cytometric Analysis

Flow cytometric analysis was performed using a Coulter Epics cytometer (Coulter Electronics, Hialeah, FL) after enzymatic digestion of minced fresh or frozen tissue samples and staining with propidium iodide. DNA distribution was compared to a standard of human lymphocyte nuclei freshly prepared in similar fashion to the tissue fragments. Aneuploid tumors were defined as those with DNA indices higher or lower than 1.0 to 1.1, while for diploid tumors the DNA indices fell within this range. All specimens were analyzed in duplicate. Only assays where the coefficient was less than 5% were regarded as analyzable.

Tumor grade was assessed after the method of Bloom and Richardson.²⁵

Clinical assessment prior to treatment included, apart from history and physical examination, chest x-ray, bone scan, CT scan of the liver, full blood count, and automated biochemical profile. Measurable lesions were recorded as the product of the largest bidimensional diameters.

TABLE 1. The Influence of P24 on Breast Cancer Prognosis

Variable	Patients No.	%
Metastatic	51	100
Female	51	100
ER+	20	40
ER-	31	60
PR+	11	21
PR-	40	79
Prior adjuvant hormone treatment (tamoxifen)	20	40
Prior adjuvant chemotherapy	11	21
Histologic grade		
1 and 2	19	37
3	32	63
Tumor cell DNA content (ploidy)		
Diploid	30	58
Aneuploid	21	42
Age	Mean	SD
	47.6	8.4
Number of metastatic sites	1.7	0.9

ER: estrogen receptor; PR: progesterone receptor.

All patients were treated by means of a three-drug combination chemotherapy regimen consisting of mitoxantrone (12 mg/m²), cyclophosphamide (600 mg/m²), and vincristine (1.4 mg/m²). All drugs were given intravenously every 3 weeks. Details of this chemotherapy schedule have been published previously,²⁶ and the treatment regimen gives a response rate and response duration comparable with other combination therapies for metastatic breast cancer. ER-positive patients in addition received tamoxifen (20 mg/day).

Re-evaluation of measurable disease was performed on day 1 of each treatment cycle and included three weekly radiologic examination of the chest and repeat bone scan and CT scan of the liver after every third cycle of treatment where bone or liver lesions were being followed. Assessment of response was as recommended by the Task Force on Breast Cancer of the UICC.²⁷

Statistical methods included chi-square analysis, Student's *t* test, survival analysis using the log rank²⁸ and Kaplan-Meier methods, and multivariate analysis using Cox proportional hazards model.

All patients gave informed consent prior to biopsy, and the study was performed with the approval of the Ethics

TABLE 2. Influence of P24 Expression on Breast Cancer Prognosis: Correlation Between P24, Receptor Status, Tumor Ploidy, and Histologic Grade

Variable	P24+	P24-	P value
ER+	14	6	0.05
ER-	12	19	
PR+	6	5	
PR-	20	20	NS
Ploidy			NS
Aneuploid	12	10	
Diploid	14	15	
Histologic grade			0.02
1 and 2	14	5	
3	12	20	
Variable	ER+	ER-	0.001
PR+	10	1	
PR-	11	29	
Ploidy			NS
Aneuploid	9	13	
Diploid	12	17	
Histologic grade			NS
1 and 2	10	9	
3	11	21	
Variable	PR+	PR-	NS
Ploidy			
Aneuploid	4	18	
Diploid	7	22	NS
Histologic grade			
1 and 2	6	13	
3	5	27	

ER: estrogen receptor; PR: progesterone receptor; NS: not significant.

TABLE 3. The Influence of P24 Expression on Breast Cancer Prognosis: Response Rate

	No.	CR + PR*	No response	P value
P24+	26	20	6	0.01
P24-	25	10	15	
ER+	20	13	8	
ER-	31	17	13	NS
PR+	11	6	5	
PR-	40	23	17	
Diploid	30	20	10	NS
Aneuploid	21	10	11	
Grades 1 and 2	19	13	6	
Grade 3	22	17	15	NS

CR: complete response; PR*: partial response; ER: estrogen receptor; PR: progesterone receptor; NS: not significant.

Committee of the University of the Witwatersrand in accordance with the principles of the Declaration of Helsinki.

Results

Correlations between the tumor variables examined are shown in Table 2. Positive staining for P24 was significantly more frequent in ER-positive as compared to ER-negative tumors. P24 positivity was also significantly more frequent in Grade 1 and 2 tumors as compared to histologic Grade 3 tumors. The only other significant correlation was between ER and PR.

Response to treatment is shown in Table 3. Prior adjuvant therapy did not affect the response rate to treatment for metastatic disease. The response rate for patients who had prior adjuvant tamoxifen, 11 of 20 (55%), and the response rate for those receiving prior adjuvant chemotherapy, 7 of 11 (64%), was similar to the overall response rate, 30 of 52 (59%). The response to CNV (cyclophosphamide, mitoxantrone, vincristine) alone, 17 of 31 (55%), or CNV plus tamoxifen (for ER-positive patients), 13 of 20 (65%), was also not significantly different.

Patients with P24-positive tumors showed a significantly higher rate of response to treatment for metastatic disease than those with P24-negative tumors. There were no significant differences in regard to clinical determinants including performance status, number of metastases, and anatomic site of metastases between P24-positive and P24-negative tumors (Table 4). In a multivariate analysis including P24, ER, PR, tumor ploidy, and histologic grade, only P24 significantly correlated with response.

Duration of response is shown in Figure 1. The response duration of P24-positive tumors (median, 12 ± 3 months) was significantly longer than that of P24-negative tumors (median, 3 ± 2 months; *P* < 0.05). Duration of survival was similarly longer for patients with P24-positive tumors (median, 20 ± 3 months) as compared to those with P24-negative tumors (median, 15 ± 2 months). Neither ER

status nor PR status correlated with duration of response or with overall survival.

Discussion

A number of tumor-related factors including nodal status,²⁹⁻³² receptor content,³³⁻³⁶ ploidy and proliferative index,³⁷⁻⁴⁰ tumor size,⁴¹ and histopathology^{25,42-44} predict for treatment outcome in early breast cancer. Much less is known, however, about tumor factors that may be prognostic in patients with metastatic breast cancer.

The 24KD protein designated P24 is an estrogen-regulated secretory protein first detected by Edwards and co-workers in MCF 7 cells.^{19,20} P24 protein has also been found in other ER-positive cell lines as well as in highly estrogen-responsive target tissues such as human decidua and in certain cells of the female genital tract.^{45,46} P24 is generally not detectable in normal or hyperplastic breast tissue. While the synthesis of P24 appears to be constitutive in MCF 7 cells, both mRNA expression and synthesis of the protein are selectively increased by estrogenic stimulation. It has therefore been suggested that the presence of P24 protein might serve as a cellular marker of estrogen action.

In the present study, cytoplasmic P24 protein was found to be significantly correlated with ER and with histologic grade but not with PR. While these correlations suggest that P24 expression may be related to ER status, the induction of P24 synthesis and PR synthesis may follow different pathways.

Recent DNA sequencing studies have shown that the P24 protein appears to be identical⁴⁷ with a human heat shock designated hsp 27, which was first detected in He La cells,⁴⁸ and that heat shock can also induce P24 in MCF 7 cells. P24 protein may thus be one of a series of proteins responsive to environmental stress, of which estrogen stimulation may be only one factor.

TABLE 4. Tumor Factors Predicting for Response to Chemotherapy in Metastatic Breast Cancer: Correlation Between P24 Status and Patient Determinants

	P24+	P24-	P value
Age (mean $\pm$ SD)	44.8 $\pm$ 9.3	48.4 $\pm$ 10.1	NS
Performance status			
0 and 1	14	13	NS
2	15	15	
No. of metastases (mean $\pm$ SD)	1.7 $\pm$ 0.8	1.6 $\pm$ 0.9	NS
Metastatic sites			
Bone	10	9	
Soft tissue	12	11	
Lungs and pleuras	7	8	
Liver	7	5	
Other	3	3	

NS: not significant.

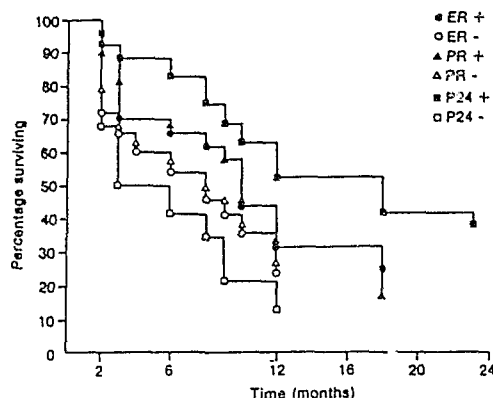


FIG. 1. Influence of P24 expression on survival of patients with metastatic breast cancer treated by chemotherapy.

While the function(s) of the P24/hsp 27 proteins is unknown, it appears to have clinical significance. A recent investigation has demonstrated that the presence of hsp 27 in primary breast cancer is a factor predicting for earlier relapse.⁴⁹ The present study suggests that the finding of P24 protein in tumor cells predicts both response to treatment and duration of response in patients with metastatic breast cancer. While cells that express these proteins may thus have increased metastatic potential and/or growth advantage, such cells appear more sensitive during therapy. Further clinical and biochemical studies may help to further elucidate the significance of P24/hsp proteins as markers of cell behavior.

REFERENCES

- Henderson IC. Chemotherapy for advanced disease. In: Bonadonna G, ed. *Breast Cancer: Diagnosis and Management*. New York: Wiley, 1983; 247-280.
- Valagussa P, Barambilla C, Bonadonna G. Advanced breast cancer: Are the traditional stratification parameters still of value when patients are treated with combination chemotherapy (Abstr)? *Proceedings of the American Association for Cancer Research—American Society of Clinical Oncology* 1978; 19:226.
- Bezwdoda WR, Derman DP, Bothwell TH, MacPhail AP, Levin J. Significance of serum concentrations of carcinoembryonic antigen, ferritin and calcitonin in breast cancer. *Cancer* 1981; 48:1623-1628.
- Jensen EV. Historical perspective steroid receptors in cancer. *Cancer* 1980; 46:2759-2761.
- Byar D, Sears M, McGuire W. Relationship between estrogen receptor values and clinical data in predicting the response to endocrine therapy for patients with advanced breast cancer. *Eur J Cancer* 1979; 15:299-310.
- Cambell FC, Elston CW, Blamey RR et al. Quantitative oestradiol receptor values in primary breast cancer and response of metastases to endocrine therapy. *Lancet* 1981; 2:1317-1319.
- Allegra J, Lippman M, Thompson E et al. Estrogen receptor status: An important variable in predicting response to endocrine therapy in metastatic breast cancer. *Eur J Cancer* 1980; 16:323-331.
- Paridaens R, Sylvester RJ, Ferrazzi E et al. Clinical significance of the quantitative assessment of estrogen receptors in advanced breast cancer. *Cancer* 1980; 46:2889-2895.

status nor PR status correlated with duration of response or with overall survival.

Discussion

A number of tumor-related factors including nodal status,²⁹⁻³² receptor content,³³⁻³⁶ ploidy and proliferative index,³⁷⁻⁴⁰ tumor size,⁴¹ and histopathology^{25,42-44} predict for treatment outcome in early breast cancer. Much less is known, however, about tumor factors that may be prognostic in patients with metastatic breast cancer.

The 24KD protein designated P24 is an estrogen-regulated secretory protein first detected by Edwards and co-workers in MCF 7 cells.^{19,20} P24 protein has also been found in other ER-positive cell lines as well as in highly estrogen-responsive target tissues such as human decidua and in certain cells of the female genital tract.^{45,46} P24 is generally not detectable in normal or hyperplastic breast tissue. While the synthesis of P24 appears to be constitutive in MCF 7 cells, both mRNA expression and synthesis of the protein are selectively increased by estrogenic stimulation. It has therefore been suggested that the presence of P24 protein might serve as a cellular marker of estrogen action.

In the present study, cytoplasmic P24 protein was found to be significantly correlated with ER and with histologic grade but not with PR. While these correlations suggest that P24 expression may be related to ER status, the induction of P24 synthesis and PR synthesis may follow different pathways.

Recent DNA sequencing studies have shown that the P24 protein appears to be identical⁴⁷ with a human heat shock designated hsp 27, which was first detected in He La cells,⁴⁸ and that heat shock can also induce P24 in MCF 7 cells. P24 protein may thus be one of a series of proteins responsive to environmental stress, of which estrogen stimulation may be only one factor.

TABLE 4. Tumor Factors Predicting for Response to Chemotherapy in Metastatic Breast Cancer: Correlation Between P24 Status and Patient Determinants

	P24+	P24-	P value
Age (mean $\pm$ SD)	44.8 $\pm$ 9.3	48.4 $\pm$ 10.1	NS
Performance status			
0 and 1	14	13	NS
2	15	15	
No. of metastases (mean $\pm$ SD)	1.7 $\pm$ 0.8	1.6 $\pm$ 0.9	NS
Metastatic sites			
Bone	10	9	
Soft tissue	12	11	
Lungs and pleuras	7	8	
Liver	7	5	
Other	3	3	

NS: not significant.

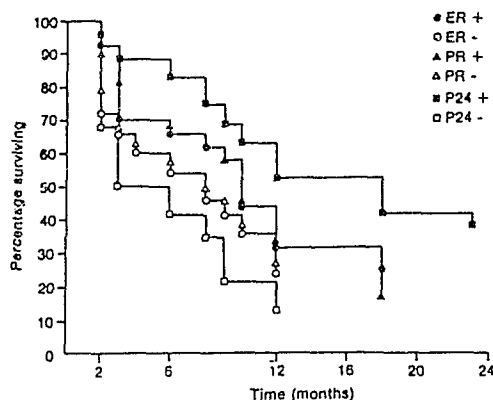


FIG. 1. Influence of P24 expression on survival of patients with metastatic breast cancer treated by chemotherapy.

While the function(s) of the P₂₄/hsp 27 proteins is unknown, it appears to have clinical significance. A recent investigation has demonstrated that the presence of hsp 27 in primary breast cancer is a factor predicting for earlier relapse.⁴⁹ The present study suggests that the finding of P24 protein in tumor cells predicts both response to treatment and duration of response in patients with metastatic breast cancer. While cells that express these proteins may thus have increased metastatic potential and/or growth advantage, such cells appear more sensitive during therapy. Further clinical and biochemical studies may help to further elucidate the significance of P24/hsp proteins as markers of cell behavior.

REFERENCES

- Henderson IC. Chemotherapy for advanced disease. In: Bonadonna G, ed. *Breast Cancer: Diagnosis and Management*. New York: Wiley, 1983; 247-280.
- Valagussa P, Barambilla C, Bonadonna G. Advanced breast cancer: Are the traditional stratification parameters still of value when patients are treated with combination chemotherapy (Abstr)? *Proceedings of the American Association for Cancer Research—American Society of Clinical Oncology* 1978; 19:226.
- Bezwdoda WR, Derman DP, Bothwell TH, MacPhail AP, Levin J. Significance of serum concentrations of carcinoembryonic antigen, ferritin and calcitonin in breast cancer. *Cancer* 1981; 48:1623-1628.
- Jensen EV. Historical perspective steroid receptors in cancer. *Cancer* 1980; 46:2759-2761.
- Byar D, Sears M, McGuire W. Relationship between estrogen receptor values and clinical data in predicting the response to endocrine therapy for patients with advanced breast cancer. *Eur J Cancer* 1979; 15:299-310.
- Cambell FC, Elston CW, Blamey RR et al. Quantitative oestradiol receptor values in primary breast cancer and response of metastases to endocrine therapy. *Lancet* 1981; 2:1317-1319.
- Allegra J, Lippman M, Thompson E et al. Estrogen receptor status: An important variable in predicting response to endocrine therapy in metastatic breast cancer. *Eur J Cancer* 1980; 16:323-331.
- Paridaens R, Sylvester RJ, Ferrazzi E et al. Clinical significance of the quantitative assessment of estrogen receptors in advanced breast cancer. *Cancer* 1980; 46:2889-2895.

9. Manni A, Arrafat B, Pearson OH. Estrogen and progesterone receptors in prediction of response of breast cancer to endocrine therapy. *Cancer* 1980; 46:2838-2841.
10. Vogel CA, East DR, Voight W, Thorsen S. Response to tamoxifen in estrogen receptor-poor metastatic breast cancer. *Cancer* 1987; 60:1184-1189.
11. Sulkes A, Livingston RB, Murphy WK. Tritiated thymidine labelling and response in human breast cancer. *J Natl Cancer Inst* 1979; 62:513-515.
12. Wolberg WH, Ansfield FJ. The relation of thymidine labelling index in human tumours in vitro to the effectiveness of 5-fluorouracil. *Chemotherapy and Cancer Research* 1971; 31:448-450.
13. Lippman ME, Allegra JC, Thompson EB *et al*. The relation between estrogen receptors and response rate to cytotoxic chemotherapy in metastatic breast cancer. *N Engl J Med* 1978; 29:1223-1228.
14. Rosner D, Nemoto T, Wibe J. Estrogen receptors and responses to combination chemotherapy in metastatic breast cancer: Results of a prospective randomised study. *Current Chemotherapy and Infectious Disease* 1980; 2:1672-1673.
15. Rosner D, Nemoto T, Lane WW. A randomized study of intensive versus moderate chemotherapy programs in metastatic breast cancer. *Cancer* 1987; 59:874-883.
16. Frennings DH, Kennedy BJ, Volska GJ *et al*. Correlation of estrogen receptors and response to chemotherapy in advanced breast cancer (Abstr). *Proceedings of the American Society of Clinical Oncology* 1978; 319:347.
17. Seymour L, Bezwoda WR, Meyer K, Behr A. Detection of P24 protein in human breast cancer: Influence of receptor status and oestrogen exposure. *Br J Cancer* 1990; 61:886-890.
18. Seymour L, Bezwoda WR, Meyer K. Response to second line hormone treatment for advanced breast cancer: Predictive value of ploidy determination. *Cancer* 1990; 65:2720-2721.
19. Edwards DP, Adams DJ, McGuire WL. Specific protein synthesis regulated by estrogen in human breast cancer. *Cancer Res* 1986; 46:6034-6040.
20. Ciocca DR, Adams DJ, Edwards DP *et al*. Estrogen-induced 24K protein in MCF-7 breast cancer cells is localized in granules. *Breast Cancer Res Treat* 1984; 4:261-268.
21. Thorpe S. Monoclonal antibody technique for detection of estrogen receptors in human breast cancer: Greater sensitivity and more accurate classification of receptor status than the dextran coated charcoal method. *Cancer Res* 1987; 47:6572-6575.
22. Perrot-Applanat M, Groyer-Picard M, Lorenzo F *et al*. Immunocytochemical study with monoclonal antibodies to progesterone receptor in human breast tumors. *Cancer Res* 1987; 47:2652-2661.
23. Seymour L, Meyer R, Esser J *et al*. Estimation of PR and ER by immunoassay in breast cancer: Comparison with radioligand binding method. *Am J Clin Pathol* (in press).
24. Perrot-Applanat M, Logeat F, Groyer-Picard MT, Milgrom E. Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. *Endocrinology* 1985; 116:1473-1484.
25. Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer: A study of 1409 cases of which 359 have been followed for 20 years. *Br J Cancer* 1957; 9:359-377.
26. Bezwoda WR, Dansey R, Seymour L. First line chemotherapy of advanced breast cancer with mitoxantrone, cyclophosphamide and vincristine. *Oncology* 1988; 46:4-9.
27. Hayward J, Carbone PP, Heuson JC *et al*. Assessment of response to therapy in advanced breast cancer: A project of the panel of clinical oncology of the International Union Against Cancer. Geneva: Switzerland. *Cancer* 1977; 39:1289-1294.
28. Peto R, Pike MC, Armitage P *et al*. Design and analysis of randomised clinical trials requiring prolonged observation of each patient II. *Br J Cancer* 1977; 35:1-39.
29. Haagenson CD. Diseases of the Breast. 2nd ed. Philadelphia: WB Saunders, 1971.
30. Fisher B, Slade N, Katriuch D, Wolmark N. Ten year follow up of breast cancer patients in a co-operative clinical trial evaluating surgical adjuvant chemotherapy. *Surg Gynecol Obstet* 1975; 140:528.
31. Valagussa P, Banadonna G, Veronesi U. Patterns of relapse and survival following radical mastectomy. *Cancer* 1978; 41:1170.
32. Breast Cancer Study Group. Identification of breast cancer patients with high risk of early recurrence after radical mastectomy. *Cancer* 1978; 42:2809-2826.
33. Thorpe SM, Rose C, Rasmussen BB *et al*. Steroid hormone receptors as prognostic indicators in primary breast cancer. *Breast Cancer Res Treat* 1986; 7 (Suppl): 1-7.
34. Papatestas AE, Miller SR, Pertsemilidis D *et al*. Association between prognosis and hormone receptors in women with breast cancer. *Cancer Detect Prev* 1986; 9:303-310.
35. Russo J, Frederick J, Ownby HE *et al*. Predictions of recurrence and survival of patients with breast cancer. *Am J Clin Pathol* 1987; 88:123-131.
36. Moot SK, Peters GN, Cheek JH. Tumor hormone receptor status and recurrence in premenopausal node negative breast carcinoma. *Cancer* 1987; 60:382-385.
37. Ewer S-B, Lanström E, Baldetrop B, Killander D. Flow cytometric DNA analysis in primary breast carcinoma and clinicopathologic correlations. *Cytometry* 1984; 33:441-445.
38. Coulsen PB, Thornthwaite JR, Wolley TW *et al*. Prognostic indicators including DNA histogram type, receptor content and staging related to breast cancer survival. *Cancer Res* 1984; 44: 117-1196.
39. McGuire WL, Meyer JS, Barlogie S, Kate TE. Impact of flow cytometry on predicting recurrence and survival in breast cancer patients. *Breast Cancer Res Treat* 1985; 5:117-128.
40. Thorad E, Fossa SD, Vaage S *et al*. Primary breast cancer: Flow cytometric DNA pattern in relation to clinical and histopathological characteristics. *Cancer* 1986; 57:808-811.
41. Fisher B, Slack NM, Bross IDJ *et al*. Cancer of the breast: Size of neoplasm and prognosis. *Cancer* 1969; 24:1071-1080.
42. Rockette H, Fisher B, Fisher E *et al*. Prognostic significance of tumor estrogen and progesterone receptor (ER, PR) nuclear and histologic grade (NG, HG) in node negative breast cancer patients: Findings from NSABP B-06. *Proc Am Soc Clin Oncol* 1987; 6:63.
43. Van der Linden HC, Baak JP, Lindeman J *et al*. Morphometry and breast cancer II. Characterisation of breast cancer cells with high malignant potential in patients with spread to lymph nodes: Preliminary results. *J Clin Pathol* 1986; 39:603-609.
44. Fisher ER, Sass R, Fisher B. Pathologic findings for the national surgical adjuvant project for breast cancer (protocol No. 4): Discriminants for 10 years treatment failure. *Cancer* 1984; 53:712-723.
45. Ciocca DR, Adams DJ, Edwards DP *et al*. Distribution of an estrogen-induced protein with a molecular weight of 24,000 in normal and malignant human tissues and cells. *Cancer Res* 1983; 42:1204-1210.
46. Ciocca DR, Asch RH, Adams DJ, McGuire WL. Evidence for modulation of a 24K protein in human endometrium during the menstrual cycle. *J Clin Endocrinol Metab* 1983; 57:496-499.
47. Fuqua SAW, Blum-Salingaros M, McGuire WL. Estrogen related "24K" protein is induced by heat shock. *Cancer Res* (in press).
48. Hickey E, Brandon WE, Potter R *et al*. Sequence and organisation of genes encoding the human 27 K DA heat shock protein. *Nucleic Acids Res* 1986; 14:4127-4145.
49. Chamness GC, Ruiz A, Fucher L *et al*. Estrogen inducible heat shock protein hsp 27 predicts recurrence in node negative breast cancer. *Proc Am Assoc Cancer Res* 1989; 30:252.

3.5 Paper 5

PLATELET DERIVED GROWTH FACTOR IN PLASMA OF BREAST CANCER PATIENTS:

CORRELATION WITH STAGE AND RATE OF PROGRESSION.

S. Ariad, L. Seymour, W.R. Bezwodá.

Breast Cancer Research and Treatment. 20:11-17. 1991.

Report

Platelet-derived growth factor (PDGF) in plasma of breast cancer patients: Correlation with stage and rate of progression

S. Ariad, L. Seymour and W.R. Bezwoda

Department of Medicine, Hematology/Oncology Unit,
University of the Witwatersrand Medical School and Johannesburg Hospital, Johannesburg, South Africa

Key words: platelet-derived growth factor, plasma concentrations, immunoassay, breast cancer, prognosis, tumor progression

Abstract

Plasma levels of platelet-derived growth factor (PDGF) were measured in 58 female patients with breast cancer and in 9 normal female control subjects by means of a specific radioimmunoassay. Levels in normal control subjects were all below the lower limits of detection by the assay (1.56 fmol/100 μ l plasma). Two of 17 (12%) patients with stage 2 breast cancer had detectable plasma levels. Among patients with Stage 4 breast cancer 13/41 (32%) had significantly elevated levels (> 2 times the lower limit of sensitivity of the assay). Patients with elevated PDGF levels had a significantly greater degree of metastatic involvement and significantly shorter survival. Apart from being a marker of aggressive high bulk breast cancer, PDGF may be involved in the acceleration of growth of some metastatic breast tumors.

Introduction

The production and secretion of polypeptide growth factors is an important mechanism in the growth and differentiation of a number of cell types [1–3]. These growth factors exert their effects on cells by interaction with specific receptors on the cell surface. The genes coding for a number of these growth factors or their receptors have been identified as being cellular oncogenes [4–7]. While the regulated production and secretion of such growth factors is a normal physiologic feature, aberrant production of growth factors has been identified in a number of tumor tissues and cell lines [8–10]. Autocrine and/or paracrine stimulation of cell proliferation by such tumor derived growth factors is thought to be an important mechanism in tumor growth and progression [2].

Breast cancer cell lines have been shown *in vitro* to produce and to be able to respond to a number of growth factors including transforming growth factor α and β (TGF α and TGF β) [11–13] as well as platelet-derived growth factor (PDGF) [14, 15]. *In vivo* studies which suggest that growth factor activities may be important for tumor growth are the studies demonstrating the prognostic importance of EGF receptor expression [16, 17] by breast cancers and the observation that increased levels of HER-2/neu oncogene expression are also associated with a worse prognosis [18]. The protein product of this oncogene has recently been identified as a growth factor receptor [19].

While the mechanism of autologous growth stimulation in tumor cells does not always involve extracellular transport of putative growth factors [1], it is known that in breast cancer cell lines a number

Report

Platelet-derived growth factor (PDGF) in plasma of breast cancer patients: Correlation with stage and rate of progression

S. Ariad, L. Seymour and W.R. Bezwoda

Department of Medicine, Hematology/Oncology Unit,

University of the Witwatersrand Medical School and Johannesburg Hospital, Johannesburg, South Africa

Key words: platelet-derived growth factor, plasma concentrations, immunoassay, breast cancer, prognosis, tumor progression

Abstract

Plasma levels of platelet-derived growth factor (PDGF) were measured in 58 female patients with breast cancer and in 9 normal female control subjects by means of a specific radioimmunoassay. Levels in normal control subjects were all below the lower limits of detection by the assay (1.56 fmol/100 μ l plasma). Two of 17 (12%) patients with stage 2 breast cancer had detectable plasma levels. Among patients with Stage 4 breast cancer 13/41 (32%) had significantly elevated levels (> 2 times the lower limit of sensitivity of the assay). Patients with elevated PDGF levels had a significantly greater degree of metastatic involvement and significantly shorter survival. Apart from being a marker of aggressive high bulk breast cancer, PDGF may be involved in the acceleration of growth of some metastatic breast tumors.

Introduction

The production and secretion of polypeptide growth factors is an important mechanism in the growth and differentiation of a number of cell types [1–3]. These growth factors exert their effects on cells by interaction with specific receptors on the cell surface. The genes coding for a number of these growth factors or their receptors have been identified as being cellular oncogenes [4–7]. While the regulated production and secretion of such growth factors is a normal physiologic feature, aberrant production of growth factors has been identified in a number of tumor tissues and cell lines [8–10]. Autocrine and/or paracrine stimulation of cell proliferation by such tumor derived growth factors is thought to be an important mechanism in tumor growth and progression [2].

Breast cancer cell lines have been shown *in vitro* to produce and to be able to respond to a number of growth factors including transforming growth factor α and β (TGF α and TGF β) [11–13] as well as platelet-derived growth factor (PDGF) [14, 15]. *In vivo* studies which suggest that growth factor activities may be important for tumor growth are the studies demonstrating the prognostic importance of EGF receptor expression [16, 17] by breast cancers and the observation that increased levels of HER-2/neu oncogene expression are also associated with a worse prognosis [18]. The protein product of this oncogene has recently been identified as a growth factor receptor [19].

While the mechanism of autologous growth stimulation in tumor cells does not always involve extracellular transport of putative growth factors [1], it is known that in breast cancer cell lines a number

of the growth factors are indeed secreted and that in turn such cells can respond to exogenously added growth factors [20, 21]. The ability to measure growth factors in the minute concentrations present in the plasma has added a new dimension to our potential understanding of the mechanisms regulating breast cancer growth. The aim of this investigation was to determine whether growth factor activities could be detected in the plasma of patients with malignancies. The present study concentrates on the detection of PDGF activity in patients with breast cancer.

Patients and methods

The study population consisted of 58 patients with histologically proven breast cancer attending the Breast Clinic of the Johannesburg Hospital. Seventeen of the patients had Stage 2 breast cancer and were either receiving or had completed adjuvant chemotherapy following primary treatment for local disease. Forty-one patients were suffering from Stage 4 metastatic breast cancer for which they were being treated either by means of chemotherapy or by hormone therapy. Blood for PDGF estimation was taken at the time of diagnosis of metastatic disease. Further patient details are shown in Tables 1 and 2. In addition to the patients with breast cancer, 8 normal individuals were studied in order to provide an internal control for the assay. PDGF should be undetectable in the plasma of normal individuals. These 8 control subjects were all female and the age range was similar to that of the patient population.

Blood samples for PDGF estimations were drawn at 08h00 into vacutainer tubes containing sodium citrate (0.105 M) which had been prechilled to 4°C. Blood samples were rapidly separated by centrifugation at 10,000 g for 30 minutes, and the platelet-poor plasma was stored at -20°C until analysis. Plasma PDGF estimations were performed by means of a commercially obtainable immunoassay (Amgen Biologicals, Amersham International, Amersham, U.K.) which utilises a high specific activity ¹²⁵I-PDGF (C-sis, recombinant) tracer together with a highly specific and sensitive anti-

serum. Separation of the antibody-bound from the free fraction is achieved by addition of a donkey anti-sheep second antibody/PEG mixture followed by centrifugation. This assay system was used according to manufacturer's instructions and is capable of detecting PDGF in a range of 1.56–200 fmol per tube. All samples were assayed in duplicate using separate blood samples obtained at the same phlebotomy, and only samples that agreed within 5% of each other were considered as valid. The intra-assay coefficient of variation for the purified standards was <2%. Percentage cross-reactivity of this assay for other growth factors is as follows: PDGF B-B homodimer 79%; PDGF human purified 61%; EGF (human) <0.0004%, TGFβ (human) <0.08%; B thromboglobulin (human) <0.03%; platelet factor 4 (human) <0.06%.

All patients included in the study were staged according to accepted criteria. Minimum staging procedures included history and full physical examination, chest x-ray, radioisotope bone scan, to-

Table 1. Patient details, Stage 2 disease

	Number	%
Pathology		
Infiltrating duct cancer	15	88
Other	2	12
Primary		
T1	7	41
T2	10	58
Nodes (number positive)		
0	1	6
1–3	7	41
> 3	9	53
Hormone receptor status		
ER +	3	17
ER –	4	24
ER unknown	10	59
Treatment		
Prior CMF	7	41
Currently Recurring CMF	9	53
Hormonal therapy	1	6
Age		
mean ± SD	42	± 11
range	30	– 64
Platelet count		
mean ± SD	222	± 49
range	82	– 314

gether with x-rays of any areas of increased isotope uptake, hepatic ultrasound, and automated biochemical profile analysis (Technikon 18/60) including electrolytes, calcium, and hepatic function tests. Patients who were suspected of having hepatic metastases either by clinical examination, by ultrasonography, or because of biochemical abnormalities had the presence of metastases confirmed by computerised tomography of the liver. Blood counts including hemoglobin estimation, white cell count, and differential count and platelet counts were performed at the same time as phlebotomy for PDGF estimation.

Details of previous and present therapy, prior hormone receptor determinations on primary or metastatic tumors, and clinical course of the disease were abstracted from the clinical records. Initial hormone receptor determinations were performed by means of the ligand-binding dextran-coated charcoal (ER DCC) methods after McGuire and co-workers. In addition a number of

patients who had metastatic disease accessible for biopsy had needle biopsies performed at the time of PDGF assay, for histologic examination as well as for hormone receptor determination by immunocytochemistry using previously described methods.

Disease-free survival (from time of original diagnosis to first evidence of metastases) and survival from diagnosis of metastatic disease were calculated according to the log rank method of Peto and Pike. Other statistical tests included Students *t* test and χ^2 analysis.

The study was performed in accordance with the principles of the Declaration of Helsinki. All patients participating in the study gave informed consent, and the study was approved by the Committee on Ethics of Human Research of the University of the Witwatersrand.

Results

None of the normal sera contained measurable (> 1.56 fmol) levels of PDGF in plasma. In Stage 2 breast cancer, 2 patients were found to have levels of PDGF above the lower limit of measurability for the assay (Fig. 1). Data from these two patients was reviewed at latest follow-up 4 months from the time the assay was performed. One of the two had a strong family history of breast cancer, but both are still classified as having Stage 2 disease.

Among patients with Stage 4 breast cancer, 13 of the 41 patients had PDGF values > 3.2 fmol ($2 \times$ greater than the lower limit of detection of the assay); the highest level was 7.8 fmol (Fig. 1). Disease-free survival (from time of original diagnosis to first detection of metastatic disease) was significantly shorter (DFS 13 months) for patients with PDGF values > 3.2 fmol than for patients with stage 4 disease who had PDGF values < 3.2 (DFS 40 months).

There was no specific pattern of metastasis that was related to PDGF value. Metastatic involvement at all sites was seen in patients with PDGF values < 3.2 fmol as well as among those with PDGF values > 3.2 fmol. Patients with PDGF values > 3.2 fmol did, however, have significantly

Table 2. Patient details, Stage 4 disease

	Number	%
Pathology		
Infiltrating duct cancer	34	83
Other	7	17
Metastatic sites involved		
Skin and soft tissue	26	63
Bone	23	56
Lung & pleura	19	46
Liver	18	44
Other	9	22
Hormone receptor status		
ER +	8	20
ER -	12	29
ER unknown	21	51
Treatment		
Chemotherapy	32	78
Hormone treatment	9	22
Age		
mean $\pm$ SD	47	$\pm$ 14
range	29	- 70
Platelet count ($\times 10^9/l$)		
mean $\pm$ SD	212	$\pm$ 47
range	78	- 327
Metastatic sites/patient		
mean $\pm$ SD	1.6	$\pm$ 0.4

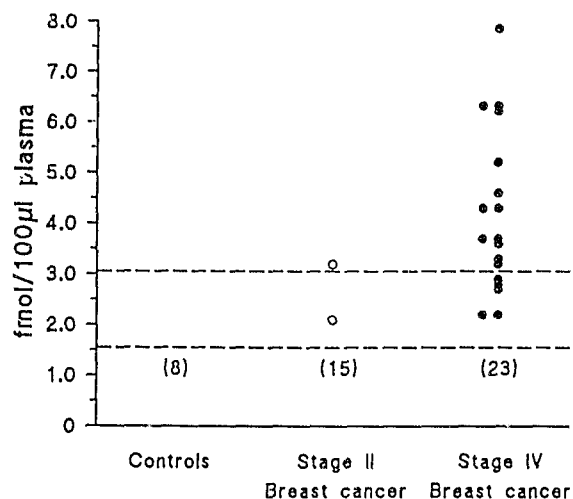


Fig. 1. Distribution of plasma PDGF values in patients with breast cancer. The value of 1.56 fmol/100 µl plasma represents the lower limit of detection of the assay. The upper dotted line (3.2 fmol/100 µl plasma) is 2 × the lower limit of sensitivity of the assay.

more metastatic involvement (median number of metastatic sites involved per patient = 4) than those with PDGF values of < 3.2 fmol (median number of metastases per patient = 2) ($p < 0.05$) (Table 3).

There was no correlation between PDGF value and platelet count or other hematologic indices. Three of the patients with metastatic disease and one patient receiving CMF chemotherapy had mild elevation of fibrin degradation products (FDP) in the serum. These did not, however, correlate with elevated PDGF values. The PDGF values for these patients were 2.3, 2.3, 3.4, and 2.2 fmol respectively.

Seven of 13 patients with PDGF values > 3.2 fmol have died during the 4 month observation period since the assays were done, as compared to 3/28 deaths amongst patients with PDGF values of < 3.2 fmol (χ^2 8.95, $p < 0.01$). Survival from the time of diagnosis of metastatic involvement was significantly shorter ($p < 0.05$) for patients with PDGF values > 3.2 fmol than for patients with PDGF values < 3.2 fmol (Fig. 2).

Discussion

Platelet-derived growth factor (PDGF) is a family of cationic glycoproteins initially identified as a platelet product [22]. PDGF is found in the granules of platelets and can be released from these cells on exposure to thrombin and collagen. PDGF is a potent mitogen and has been shown to stimulate proliferative activity of untransformed fibroblasts and glial cells, and as such may be involved in normal tissue repair processes after injury [23].

Mitogenically active PDGF is a dimer of two polypeptide chains which have been identified as PDGFA (or PDGF-1) and PDGFB (or PDGF-2) linked together by disulphide bonds. Mitogenic activity appears to reside mostly in the B chains, since both PDGF AB and PDGF BB are highly mitogenic, while PDGF AA has only low mitogenic activity [24]. The biologic effects of PDGF are mediated by specific cell surface receptors with an extracellular domain and intracellular tyrosine kinase activity [25].

Under normal circumstances, the *in vivo* mitogenic activity of PDGF is confined to sites of injury or repair. Normal human plasma does not contain detectable levels of PDGF. Secreted PDGF is quickly bound to circulating binding proteins such as β 2-macroglobulin [26, 27], and such binding inhibits the interaction of PDGF and its receptor.

Table 3. Relationship of plasma PDGF values with clinical and pathological variables

Number	PDGF < 3.2 fmol	PDGF > 3.2 fmol	P value
Totals	28	13	
Pathology			
Infiltrating duct cancer	22	10	NS
Other	6	3	
Hormone receptor status			
ER +	6	0	NS
ER -	5	3	
ER unknown	17	10	
Number of metastatic sites			
≤ 2	23	5	< 0.05
> 2	5	8	
Median disease-free interval (months)	13	40	< 0.05

Elevated levels of PDGF have, however, been found in a number of disease states including myeloproliferative disorders [28]. In the present investigation, elevated levels of PDGF were found in a significant proportion of patients with advanced breast cancer, particularly those with more extensive metastatic involvement.

The association of elevated levels of PDGF with high bulk tumors suggests either production and secretion of PDGF from tumor cells themselves, or production and release of PDGF by tumor associated elements such as stroma or even by circulating platelets which have in some way been activated by the presence of tumor [29]. It should be noted at this point that none of the patients with markedly elevated PDGF levels had evidence of disseminated intravascular coagulation (DIC) or other platelet related abnormalities. Whatever the origin, the association of elevated PDGF levels with more aggressive disease suggests that PDGF may play a role in tumor progression.

While PDGF synthesis and secretion was initially found in platelets, other cell types such as monocytes/macrophages, endothelial cells, vascular smooth muscle cells, embryonic cells, and megakaryocytes [2, 3] have been found to produce this growth factor. PDGF synthesis and release has also been found in a number of leukemic cell lines and in HTLV-infected lymphocytes [30–32]. The finding that the simian sarcoma virus (SSV) encoded V-sis protein is homologous to the β -chain of PDGF has led to the suggestion that PDGF may be intimately involved in the process of retrovirus-induced neoplasia [5]. More recently it has been suggested that the cellular C-sis homolog may play a similar role in non-virally induced neoplasia. Production of PDGF has also recently been found in a number of malignant non-hemopoietic cell lines including mesothelioma, Wilms tumor, melanoma, and prostatic and ovarian cancer, as well as in breast cancer cell lines [33–36]. PDGF has been shown in a number of these *in vitro* systems to be mitogenic for neoplastic cell lines [37]. In other instances, PDGF appears to sensitize cells to the growth stimulatory effects of EGF.

While it is tempting to ascribe the elevated PDGF levels found in these patients to release by

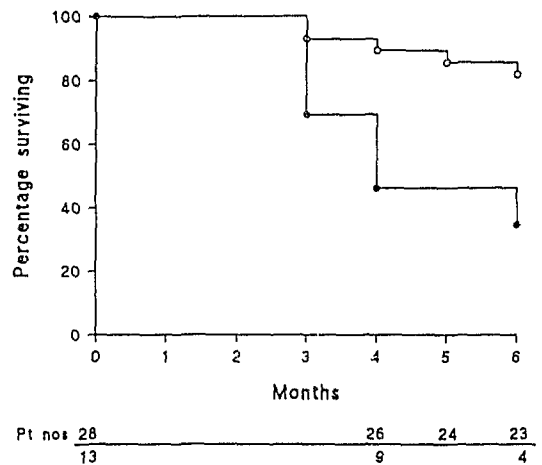


Fig. 2. Survival from diagnosis of metastatic disease. ○ PDGF < 3.2 fmol/100 µl plasma, ● PDGF > 3.2 fmol/100 µl plasma.

tumor cells themselves and to speculate on a role for PDGF as an autocrine/paracrine growth factor, there is at present no clear proof that PDGF was involved in the mechanism of the progression. A clearer understanding of these findings may well come from future studies of the PDGF and PDGF receptors in breast tumor tissue.

Acknowledgements

Supported in part by a grant from the National Cancer Association (S.A.).

References

1. Heldin C-H, Westermark B: Growth factors: mechanism of action and relation to oncogenes. *Cell* 37: 9–20, 1984
2. Antoniades HN, Owen AJ: Growth factors and regulation of cell growth. *Ann Rev Med* 33: 445–463, 1988
3. Ogaswara M, Subuska DA: A new serum-free method of measuring growth factor activities for human breast cancer cells in culture. *In Vitro Cell Devel Biol* 24: 911–920, 1988
4. Doolittle RF, Hankapiller M, Hood L, Devare S, Robbins K, Aaronson SA, Antoniades HN: Simian sarcoma virus oncogene, V-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. *Science* 221: 275–276, 1983
5. Waterfield M, Scrase G, Whittle H, Stroobant P, Johnsson

- A, Wasteson A, Westermarck B, Heldin C-H, Huang J, Deuel T: Platelet-derived growth factor is structurally related to the putative transforming protein p28^{src} of simian sarcoma virus. *Nature* 304: 35-39, 1983
6. Delarco J, Todaro GJ: Growth factors from murine sarcoma virus-transformed cells. *Proc Natl Acad Sci USA* 75: 4001-4005, 1978
7. Downward J, Yarden Y, Mayes E, Scrace G, Totty M, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD: Close similarity of epidermal growth factor receptor and V-erb-B oncogene protein sequences. *Nature* 307: 521-527, 1984
8. Hasselbacker GK, Irminger JC, Zapf J, Ziegler WH, Humbel RE: Insulin-like growth factor II in human adrenal pheochromocytomas and Wilms tumors: expression at the mRNA and protein levels. *Proc Natl Acad Sci USA* 84: 1104-1106, 1987
9. Peres R, Betscholtz C, Westermarck B, Heldin CH: Frequent expression of growth factors for mesenchymal cells in human mammary carcinoma cell lines. *Cancer Res* 47: 3421-3429, 1987
10. Ohmura E, Okuda M, Onoda N, Kamiya Y, Murakami H, Tsushima T, Shizume K: Insulin-like growth factors and transforming growth factor α as autocrine growth factors in pancreatic cancer cell growth. *Cancer Res* 50: 103-107, 1990
11. Solomon DS, Zwiebel JA, Bano D, Losonczy I, Fehnel P, Kidwell WR: Presence of transforming growth factor in human breast cancer cells. *Cancer Res* 44: 4069-4077, 1984
12. Sporn MB, Roberts AB, Waterfield LM, Assosia RK: Transforming growth factor β : biological function and chemical structure. *Science* 233: 532-534, 1986
13. Knabbe C, Lippman ME, Waterfield LM, Flanders KV, Kasid A, Derynck R, Dickson RB: Evidence that transforming growth factor β is a hormonally regulated negative growth factor in human breast cancer cells. *Cell* 48: 417-418, 1987
14. Bronzert DA, Pantazis P, Antoniadis HN, Kasid A, Davidson N, Dickson RB, Lippman ME: Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. *Proc Natl Acad Sci USA* 84: 5763-5767, 1987
15. Kasid A, Lippman ME: Estrogen and oncogene mediated growth regulation of human breast cancer cells. *J Steroid Biochem* 27: 465-470, 1987
16. Sainsbury JRC, Farndon JR, Needham GK, Malcolm AJ, Harris AL: Epidermal growth factor receptor as a predictor of early recurrence and death from breast cancer. *Lancet* i: 1398-1402, 1987
17. Spitzer E, Grosse R, Kunde D, Schmirer HE: Growth of mammary epithelial cells in breast cancer biopsies correlates with EGF binding. *Int J Cancer* 39: 279-282, 1987
18. Patterson AH, Lees AW, Jamil N, Dietrich KD, Huson J, Fournier RM, Salmon DJ, Patterson MC, Danyluk J: Comparison of Her-2/neu oncogene amplification with other prognostic factors in node negative patients with breast cancer. *Proc ASCO* 8: 99, 1989
19. Lupu R, Colomer R, Zugmaier G, Sarup J, Shepard M, Slamon D, Lippman ME: Direct interaction of a ligand for the erbB₂ oncogene product with the EGF receptor and p185erbB₂. *Science* 249: 1552-1555, 1990
20. Westphal M, Branchen M, Rhode E, Herrman HD: Growth factors in cultured human glioma cells: differential effects of FGF, EFG, and PDGF. *Cancer Lett* 38: 283-296, 1988
21. Bates SE, McManaway M, Lippman ME, Dickson RB: Characterisation of estrogen responsive transforming activity. *Cancer Res* 46: 1707-1713, 1986
22. Pantazis P, Goustin AS, Nixon J: Platelet-derived growth factor and its receptor in blood cell differentiation and neoplasia. *Eur J Haematol* 45: 127-138, 1990
23. Ross R, Raines EW, Bowen-Pope DF: The biology of platelet-derived growth factor. *Cell* 46: 155-169, 1986
24. Nister M, Hammacher A, Mellstrom K: A glioma-derived PDGF A chain homodimer has different functional activities from a PDGF heterodimer purified from human platelets. *Cell* 52: 791-799, 1988
25. Yarden Y, Escobedo JA, Kuang W-J, Yang-Feng TL, Daniel TO, Tremble PM, Chen EY, Ando ME, Harkins RN, Francke U, Freed VA, Ullrich A, Williams L: Structure of the receptor for platelet-derived growth factor helps to define a family of closely related receptors. *Nature* 323: 226-232, 1986
26. Bowen-Pope DF, Malpass TW, Foster DM, Ross R: Platelet-derived growth factor *in vivo*: levels, activity, and rate of clearance. *Blood* 64: 458-469, 1984
27. Raines EW, Bowen-Pope EF, Ross R: Plasma binding protein for platelet-derived growth factor that inhibit its binding to cell-surface receptors. *Proc Natl Acad Sci USA* 81: 3424-3438, 1984
28. Bernabei PA, Arcangeli A, Casini M, Grossi A, Padovani R, Rossi Ferrini P: Platelet-derived growth factor(s) mitogenic activity in patients with myeloproliferative disease. *Brit J Haematol* 63: 353-357, 1986
29. Mannucci PM, Cattaneo M, Canciani MT, Manniezzo M, Vaglini M, Cascinelli N: Early presence of activated (exhausted) platelets in malignant tumors (breast adenocarcinoma and malignant melanoma). *Eur J Cancer Clin Oncol* 25: 1413-1417, 1989
30. Pantazis P, Sariban E, Kufe D, Antoniadis HN: Induction of C-sis gene expression and synthesis of platelet-derived growth factor in human myeloid leukemia cells during monocytic differentiation. *Proc Natl Acad Sci USA* 83: 6455-6459, 1986
31. Papayannopoulou T, Raines E, Collins S, Nakamoto B, Tweedale M, Ross R: Constitutive and inducible secretion of platelet-derived growth factor analogs by human leukemia cell lines co-expressing erythroid and megakaryocytic markers. *J Clin Invest* 79: 859-866, 1987
32. Eva A, Robbins KS, Anderson PR: Cellular genes analogous to retroviral oncogenes are transcribed in human tumor cells. *Nature* 295: 116-119, 1982
33. Gerwin BI, Lechner JF, Reddel RR, Roberts AB, Robbins

- KC, Gabrielson FW, Harris CC: Comparison of production of transforming growth factor-beta and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. *Cancer Res* 47: 6180-6184, 1987
34. Fraizer GE, Bowen-Pope DF, Vogel AM: Production of platelet derived growth factor by cultured Wilms tumor cells. *J Cell Physiol* 133: 169-174, 1987
35. Rodeck U, Koprowski H: Human melanoma cell lines of primary and metastatic origin express the genes encoding the chains of platelet-derived growth factor (PDGF) and produce a PDGF-like growth factor. *Proc Natl Acad Sci USA* 83: 7197-7200, 1986
36. Sitaras NM, Saibon E, Bravo M, Pantazis P, Antoniades HM: Constitutive production of platelet-derived growth factor like proteins by human prostate-carcinoma cell lines. *Cancer Res* 48: 1930-1935, 1988
37. Golombick T, Bezwoda WR: Long term growth of a new ovarian cancer cell line UWOV2 (pf) in tissue culture in the absence of exogenous serum factors, proteins transferrin and insulin: a potential model for autonomous growth and tumor progression. *Eur J Cell Biol* (in press)

CHAPTER 4 - CLINICAL MODULATION AND PREDICTORS FOR OUTCOME

- 4.1 Paper 6 - Hormone Priming in Breast Cancer: Oestrogen Priming Has a Detrimental Effect on Response in Oestrogen Receptor Negative Patients.
- 4.2 Paper 7 - Interferon plus Tamoxifen Treatment for Advanced Breast Cancer: In Vivo Biologic Effects of Two Growth Modulators.
- 4.3 Paper 8 - Tissue Platelet Derived Growth Factor (PDGF) Predicts for Shortened Survival and Treatment Failure in Advanced Breast Cancer.
- 4.4 Paper 9 - Positive Immunostaining for Platelet Derived Growth Factor is an Adverse Prognostic Factor in Patients with Advanced Breast Cancer.
- 4.5 Paper 10 - Soluble C-erbB-2 Fragment in Serum Correlates with Disease Stage and Predicts for Shortened Survival in Patients with Early Stage and Advanced Breast Cancer.
- 4.6 Paper 11 - P-Glycoprotein Immunostaining Correlates with ER and with High Ki67 Expression but Fails to Predict Anthracycline Resistance in Patients with Advanced Breast Cancer.

4.1 Paper 6

HORMONE PRIMING IN BREAST CANCER: OESTROGEN PRIMING HAS A DETRIMENTAL EFFECT
ON RESPONSE IN OESTROGEN RECEPTOR NEGATIVE PATIENTS.

L. Seymour, K. Meyer, W.R. Bezwoda.

European Journal of Cancer. 29 (10)1495-1496. 1993.

Eur J Cancer, Vol. 29A, No. 10, pp. 1495-1496, 1993.
Printed in Great Britain
0964-1947/93 \$6.00 + 0.00
© 1993 Pergamon Press Ltd

Letters

Hormone Priming in Breast Cancer: Oestrogen Priming Has a Detrimental Effect on Response in Oestrogen Receptor-negative Patients

Lesley Seymour, Karin Meyer and
Werner R. Bezwoda

POTENTIAL THERAPEUTIC benefits from hormonal recruitment of neoplastic cells into the cell cycle has been suggested in a number of preclinical observations and has also been reported in clinical studies [1-4]. Results have, however, not been uniformly favourable [5, 6]. We undertook a randomised controlled trial comparing treatment with combination chemotherapy with or without diethylstilbestrol (DES) priming. Eligibility criteria included pre- and postmenopausal patients with histologically documented recurrent or progressive breast cancer with at least one measurable site of disease. Oestrogen receptor-positive (ER+) and oestrogen receptor-negative (ER-) patients were included in the study. Receptor status was determined at the start of the study by biopsy of accessible metastatic lesions (usually cutaneous), the determinations being carried out immunochemically using a commercially available kit (ER-ICA, Abbott Laboratories) according to the manufacturer's instructions. Additional histological material was utilised for determination of progesterone receptor, proliferative index (PI), assessed by fluorescence-activated cell sorter, and the expression of the proliferation-associated antigen Ki67 [7-9]. Patients with accessible tumour were rebiopsied at the end of oestrogen priming and again at the end of the first cycle of chemotherapy.

The chemotherapy regimen used in both arms of the study was identical and consisted of a combination of cyclophosphamide 600 mg/m², mitoxantrone 12 mg/m² and vincristine 1.4 mg/m² given intravenously (iv) once every 28 days (CNV). Patients randomised to the hormone priming arm (DES-CNV) received DES 5 mg orally daily for 5 days starting on day 1 of each chemotherapy cycle. Postmenopausal patients on the hormone priming arm received, in addition, aminoglutethimide 250 mg twice daily orally throughout the entire treatment period. Randomisation was by the random number closed envelope technique and all patients gave informed consent. The study was

Correspondence to W.R. Bezwoda.

The authors are at the Hematology/Oncology Division, Department of Medicine, University of Witwatersrand Medical School, York Road, Parktown, 2193, Johannesburg, South Africa..

Revised 11 Feb. 1993; accepted 1 Apr. 1993.

Table 1. Hormone priming in breast cancer. Patients' characteristics

	DES-CNV	CNV
No. patients entered	23	25
Mean age (range)	48 (29-69)	47 (28-71)
Inevaluable	4	5
Reason for inevaluability for response		
Death prior to initiation of treatment	1	2
Lost after 1st dose of treatment	3	3
Evaluable patients	19	20
Premenopausal	11/19	9/20
ER positive	5/19	5/20
ER negative	14/19	15/20
Metastatic sites		
Soft tissue only	3	6
Bone and local	3	6
Viscera and local	12	10
Inflammatory	5	3

approved by the Ethics Committee of the University of Witwatersrand and was carried out in accordance with the principles of the Declaration of Helsinki.

A total of 48 patients were eligible and randomised. 9 (19%) patients defaulted from treatment during the first course and were considered to be unevaluable for response and time to treatment progression. Survival analysis, however, includes these patients up to the time of last follow-up. Further patient details are shown in Table 1.

Of the 19 evaluable patients who received DES-CNV, 5 (26%) achieved a response with 2 (11%) complete responses. In the CNV treatment arm the objective response rate was 11/20 (55%) with 5 (25%) complete responders (Table 2). This difference in overall response rate approached statistical significance ($P = 0.06$) and was significant for the subgroup of ER- patients ($P = 0.02$), with 8/15 (53%) of ER- patients responding to CNV alone vs. 2/14 (14%) responding to DES-CNV. Among the patients receiving CNV alone, there was no significant difference in response rate according to ER status. Premenopausal patients had a significantly higher response to CNV (7/9, 77%) than did postmenopausal patients (4/11, 36%) ($P = 0.05$).

Neither progesterone receptor, tumour ploidy or percentage positivity for the proliferation-associated antigen Ki67 had any impact on response rate. Both ER+ (4/6, 67%) as well as ER- (10/14, 71%) patients who were rebiopsied after DES priming showed an increase in PI and Ki67 expression.

Time to treatment failure (median 200 days for patients receiving DES-CNV, 300 days for patients receiving CNV), as well as overall survival (median 410 days, DES-CNV and 450 days, CNV), were not significantly different between the two treatment groups.

The present study showed a significantly lower response rate among ER- patients receiving DES-CNV. That this difference is due to the oestrogen priming is suggested by the lack of significant differences in response to chemotherapy alone when ER+ and ER- patients are compared.

Table 2. Hormone priming in breast cancer: response to treatment

	DES-CNV	CNV
Response rate	5 (26%)	11 (55%)
Complete response	2 (10%)	5 (25%)
Partial response	3 (16%)	6 (30%)
No response	14 (74%)	9 (30%)

Reasons for the adverse effect of oestrogen priming need to be considered. While the DES dose used was relatively large and the duration of oestrogen treatment (5 days) was somewhat longer than that described in other studies, it should be pointed out that one of the objects of oestrogen priming, namely recruitment of cells into the growth cycle, as evidenced by increase in PI and Ki67 expression, was in fact achieved with this regimen, irrespective of ER. A number of previous studies have shown that pharmacological doses of oestrogen can increase the proliferation of tumour cells even in the absence of specific receptor protein. It may well be the ability of oestrogen to stimulate ER- breast tumours that was responsible for the adverse effect on response rate since there is no evidence to suggest that oestrogens have any effect on chemotherapy drug uptake or efflux.

The results of this study suggest that hormonal recruitment is unlikely to play a major role in improving results of chemotherapy for advanced breast cancer and in ER- subgroups may well have a deleterious effect.

- Dubik D, Dembinski T, Shiu R. Stimulation of *c-myc* oncogene expression associated with estrogen-induced proliferation of human breast cancer cells. *Cancer Res* 1987, 47, 6517-6521.
- Epstein R, Smith P. Estrogen-in-luciferase potentiation of DNA damage and cytotoxicity in human breast cancer cells treated with topoisomerase II-interactive antitumour drugs. *Cancer Res* 1988, 48, 297-303.
- Hug V, Johnston D, Finders M, Hortobagyi G. Use of growth stimulatory hormones to improve the in vitro therapeutic index of doxorubicin for human breast tumours. *Cancer Res* 1986, 46, 147-152.
- Conte P, Pronetto P, Rubagatti A, et al. Conventional versus cytotoxic polychemotherapy with estrogenic recruitment in metastatic breast cancer: results of a randomised cooperative trial. *J Clin Oncol* 1987, 5, 339-347.
- Marini G, Simoncini E, Marnicati P, et al. Premarin priming before prednisone, high dose folinic acid and 4-fluorouracil as salvage chemotherapy for advanced breast cancer. *Chemioterapia* 1988, 7, 130-132.
- Eisenhauer E, Bowman D, Pritchard K, Paterson A. Tamoxifen and conjugated oestrogens (premarin) followed by sequenced methotrexate and 5-FU in refractory advanced breast cancer. *Cancer Treat Rep* 1984, 68, 1421-1422.
- Seymour L, Meyer K, Esser J, MacPhail P, Behr A, Bezwod WR. Estimation of PR and ER by immunocytochemistry in breast cancer. *Am J Clin Path* 1990, 94 (Suppl. 1), 35-40.
- Seymour L, Bezwod WR, Meyer K, Behr C. Detection of P24 protein in human breast cancer: influence of receptor status and oestrogen exposure. *Br J Cancer* 1990, 61, 886-890.
- Walker RA, Campjohn RS. Comparison of monoclonal antibody Ki-67 reactivity with grade and flow cytometry of breast carcinoma. *Br J Cancer* 1988, 57, 281-283.
- Dao T, Sinha D, Nemoto T, Patel J. Effect of estrogen and progesterone on cellular replication of human breast tumours. *Cancer Res* 1982, 42, 359-362.

4.2 Paper 7

INTERFERON PLUS TAMOXIFEN TREATMENT FOR ADVANCED BREAST CANCER: *In Vivo*

BIOLOGIC EFFECTS OF TWO GROWTH MODULATORS.

L. Seymour, W.R. Bezwoda.

British Journal of Cancer. 68:352-356, 1993.

Interferon plus tamoxifen treatment for advanced breast cancer: *in vivo* biologic effects of two growth modulators

L. Seymour & W.R. Bezwoda

Division of Clinical Hematology and Medical Oncology, Department of Medicine, University of the Witwatersrand, South Africa.

Summary The effects of interferon- α (IFN) plus tamoxifen (TMX) in the treatment of advanced breast cancer were assessed. Changes of *in vivo* biologic determinants including hormone receptors, P24 protein, Ki-67 and growth factor expression were evaluated. Seven patients with advanced, heavily pretreated, breast cancer with accessible disease, underwent biopsy prior to and after sequential treatment with IFN and IFN plus TMX. Clinically 4/7 patients responded to treatment with one complete and three partial remissions. Apart from the favourable response rate the sequential *in vivo* changes in expression of tumour variables were of considerable interest.

IFN treatment consistently increased the expression of the estrogen receptor (ER) and of the estrogen regulated protein P24 while decreasing the expression of the proliferation associated antigen Ki-67. Addition of TMX on the other hand resulted in a reduction of ER expression to pre-IFN levels and a rise in progesterone receptor (PR) expression.

When the effect of either IFN or IFN plus TMX on the expression of two growth factors was assessed they were found to be somewhat variable. While PDGF expression tended to be suppressed, there was no clinical correlation with response to therapy. TGF β expression was found in all patients prior to treatment and while all non-responders showed reduction of TGF β following treatment, the alterations were variable amongst responders (including two patients with increased expression, one with no change, and one with decreased expression).

It is concluded that both IFN and TMX exert multiple effects on the expression of tumour biologic variables and that while the study confirmed some of the predictions from *in vitro* models, the *in vivo* effect are more complex than has been appreciated from the models. From the clinical point of view, it might be expected that treatment which enhances the expression of ER in tumours should have a positive effect on the response to TMX.

Patients with advanced or metastatic breast cancer continue to pose major therapeutic dilemmas for the practising oncologist. Despite advances in the detection and treatment of early stage disease, patients with advanced breast cancer almost invariably die of the illness despite fairly frequent responses to various treatment modalities (Henderson, 1987). Additions to and substitutions of one or other chemotherapeutic agent/s in conventional dose combination chemotherapy has moreover failed to produce any further substantial improvement in either response rate or response duration (Coates *et al.*, 1987).

Since the aim of treatment for patients with metastatic disease is palliative, an important consideration is therapy related toxicity. Amongst the palliative therapies, there is no doubt that hormonal manipulation remains the approach with the least treatment related morbidity and mortality. Hormonally based treatment is however limited both as to the proportion of patients responding and the duration of response (Byer *et al.*, 1979; Powles *et al.*, 1984; Bezwoda *et al.*, 1991). While a number of new hormonal agents have become available over the last few years it seems unlikely that there will be major differences among any of them in regard to either of these limitations. It would seem reasonable however to explore approaches which might increase the effectiveness of hormone based treatment from either the response rate or response duration point of view.

Although interferon appears to have limited clinical effect as a single agent it has interesting *in vitro* effects on various breast cancer derived, estrogen responsive, cell lines including MCF7 and ZR 75 cells. Alpha interferon has been shown to increase estrogen receptor (ER) expression, and to have a synergistic effect together with tamoxifen (TMX) in inhibiting MCF-7 cell growth (Bezwoda & Meyer, 1990). Other authors

have reported similar effects with α interferon using the ZR 75 cell line (Van den Berg *et al.*, 1987) and with β -interferon using other breast cancer derived cell lines (Sica *et al.*, 1987).

In view of the potentially favourable effects of alpha-interferon on both ER expression and response to tamoxifen we performed a small pilot trial utilising interferon with tamoxifen in patients with locally advanced metastatic breast cancer. This study gave the opportunity to examine the effects of these agents on *in vivo* tumour biology.

Methods

Premenopausal and postmenopausal patients were considered eligible for entry to the trial if they had informed consent, had evaluable disease, metastatic skin or soft tissue lesions suitable for biopsy, had failed conventional cytotoxic chemotherapy, and had no prior exposure to hormonal manipulation. Recruitment began in June 1990 and ended 12 months later.

All patients underwent initial biopsy by means of a dermatological punch biopsy or cytologic examination by fine needle aspiration and were then started on interferon- α 2b (Intron A - Schering S.A.; IFN α) 3 million units subcutaneously three times per week. Fourteen days later patients underwent a second biopsy, and tamoxifen 20 mg per day by mouth was added to the treatment. Fourteen days later a third biopsy was performed.

Response was assessed on the basis of criteria proposed by the Eastern Co-operative Oncology Group (Hayward *et al.*, 1979). Toxicity assessment was by WHO criteria. Therapy with both agents was continued until treatment failure occurred.

Biopsy specimens were flash frozen and stored at -135°C until use. ER immunocytochemistry, using the Abbot (Abbott Laboratories) ER-ICA kit was performed according to the manufacturers instructions. The monoclonal antibody to PR was obtained from Transbio (Transbio, Paris, France). P24 antibody was a kind gift from Dr W. McGuire, San Antonio.

Texas. The methods used and specificity of the staining reactions have been previously described (Seymour *et al.*, 1990a; Seymour *et al.*, 1990b; Seymour *et al.*, 1990c).

Monoclonal antibody to platelet derived growth factor bb (PDGF BB) was obtained from Promega (Promega Laboratories, Madison Wis., USA). The anti-PDGF aa antibody was a monospecific, polyclonal antibody (British Biotechnology, Abingdon, UK).

The antibody against TGF α was a polyclonal antibody (British Biotechnology) which has neutralising activity against TGF β_1 and TGF β_2 and which reacts with both isoforms of this growth factor on Western blotting. This antibody has been shown in our laboratories to give consistent staining of MCF7 cells (with the intensity of the staining increasing on exposure of the cells to tamoxifen). The neutralising activity of this anti-TGF β antibody has also been confirmed in our laboratories and hence staining is thought to represent an active form of TGF β . This antibody does not show any cross reactivity with acidic or basic fibroblast growth factors (FGFs), platelet derived growth factor (PDGF) or epidermal growth factor (EGF).

The immunocytochemical techniques used a standard avidin-biotin technique (ABC kit, Vectastain Laboratories). All immunocytochemical determinations were performed in a single run, using picric acid-formaldehyde fixed preparations. Positive controls included tissues processed in the same manner as the experimental tissue and known to stain positively for the appropriate determinant. Negative controls followed all the steps used for the immunocytochemical determinations but substituting normal (non-immune) immunoglobulin from the appropriate species for the primary antibody.

Specimens were examined by light microscopy and scored according to number of cells positive and intensity of staining. The scoring system used for ER, PR and P24 was the H-score method as recommended by the manufacturers of the ER-ICA kit. TGF β and PDGF bb were graded on a scale of 0–5, and PDGF aa on a scale of 0–500. Ki 67 was expressed as a percentage of cells showing positive staining.

In all cases there was concordance between multiple specimens from the same tumour area. Repeat biopsies were carried out from the same anatomical area as the original biopsy, avoiding, however, the site previously subjected to biopsy induced trauma.

Results

Seven patients were eligible for entry to the trial. Six patients were premenopausal and had previously failed conventional cytotoxics, and three of the six had failed second line chemotherapy as well. One patient was post menopausal and was deemed suitable for primary hormonal manipulation. The mean age of the patients was 44 years with range 25–53 years. All patients had locally recurrent disease, usually extensive and four had concurrent bone metastases. One patient had extensive hepatic metastases. No patient had any significant drug related symptoms or morbidity related to biopsies. Further patient details and tumour biologic variables at baseline assessment are shown in Table I.

Four of the seven patients (57%) responded, of which one

(14%) was a complete response. Two of the four responders had early minor responses to IFN prior to addition of TMX to the treatment regimen. The response duration ranged from 4 weeks to 32 weeks. Three patients had no initial response to therapy. Toxicity from IFN was minimal at the doses used (Table II). Flu-like symptoms and fever when they occurred were readily controlled with the use of paracetamol.

At baseline evaluation 5/7 patients were ER positive and four were PR positive as well. All of the responses occurred among the ER positive patients, although one patient who was both ER and PR positive failed to respond. All of the ER positive patients, including the non-responder, showed an increase in the intensity of ER staining after IFN α . Of the three responders who were PR positive, one increased, one decreased and one showed no change in PR expression after interferon, but all increased PR expression after TMX (Table III).

All of the responding patients were also initially P24 positive and all showed an increase in P24 immunostaining after interferon administration. Two of the non-responders showed positive immunostaining for P24 prior to IFN therapy, and both became negative after IFN.

Two of the four responders had elevated Ki 67 levels (18–25%), which decreased to low levels after the initiation of IFN α therapy. Of the three non-responders, only one had an elevated Ki 67 (19%), which failed to decrease after either interferon or tamoxifen. This patient was both ER and P24 negative.

Although immunostaining for TGF β could be demonstrated in all patients, levels in ER positive patients were higher (mean 2.7) than those in ER negative patients (mean 1.5). Of the four responders, three had no change and one decreased the degree of staining. All the non-responders decreased TGF β levels after interferon and or tamoxifen (Table IV).

All of the patients showed positive staining for PDGF aa, but there was no clear pattern of change after either interferon or tamoxifen that was correlated with response. Only two patients had clear positive immunostaining for PDGF bb, and both of these were responders (1PR, 1CR). Responding patients thus had a significantly higher level of PDGF bb than non-responders (1.02 vs 0.2) (Table IV).

When all the variables were examined for an association with response to treatment only pretreatment ER positivity and an increase in P24 expression after IFN treatment correlated significantly with response (Table V).

Discussion

In vitro studies suggest that the interferons should be useful in the treatment of breast cancer, modulating both tumour

Table II Toxicity from IFN α and IFN α plus tamoxifen

	Number	Per cent	Worst grade at any time (WHO)
Flu-like symptoms	2	29	I
Fever	1	14	I
Hematologic	0	0	0

Table I Patient and biologic determinants and response to treatment with IFN and TMX

	Age	ER	PR H-score	P24	TGF β	PDGF aa score	PDGF bb	Ki 67 %	Response to treatment
1	25	0	0	100	2	150	0	1	NR
2	39	100	0	100	2	300	2.5	18	PR
3	40	130	125	200	5	400	0	6	PR
4	38	0	0	0	1.5	150	0	19	NR
5	45	130	30	100	2	250	1.5	16	CR
6	46	110	110	120	3	250	0	1	NR
7*	53	125	150	200	2	100	0	1	PR

*Postmenopausal.

Table III *In vivo* effect of IFN and TMX on tumour ER and PR expression by immunocytochemical assay (ICA)

	After IFN			After addition of TMX		
	Increase	Decrease	No change	Increase	Decrease	No change
<i>Responders</i>						
ER-ICA	4	0	1	0	2	2
PR-ICA	1	1	2	3	0	1
<i>Non-responders</i>						
ER-ICA	1	0	2	0	1	2
PR-ICA	0	1	2	1	0	2

Table IV *In vivo* effect of IFN and TMX on tumour cell expression. The estrogen regulated protein P24, the proliferation associated antigen Ki 67 and of two growth factors

	After IFN			After addition of TMX		
	Increase	Decrease	No change	Increase	Decrease	No change
<i>Responders</i>						
P24	4	0	0	0	3	1
Ki 67	0	2	2	0	1	3
TGFβ	0	1	3	1	1	2
PDGF	2	1	0	1	2	1
<i>Non-responders</i>						
P24	0	2	1	1	0	2
Ki 67	0	0	3	0	0	3
TGFβ	0	2	1	0	1	2
PDGF	1	0	2	0	1	2

Table V *In vitro* biologic predictors of response to treatment with IFN and TMX

	Responders	Non-responders
Pretreatment		
ER +	4/4	1/3 ^a
Post therapy		
P24 ^b	4/4	0/3 ^a

^aSignificant at 0.05 Fishers exact probability test). ^bIncrease occurring after IFN administration.

cell growth as well as the expression of a number of functional proteins (such as hormone receptors), the expression of which may, in turn, offer opportunities for the more effective use of other growth modulating drugs. In this pilot investigation, alterations in the expression of ER, the estrogen regulated protein P24, and the proliferation associated antigen Ki 67, as well as that of two growth factors, PDGF and TGFβ were studied. The changes observed were related to predictions from *in vitro* models (Knabbe *et al.*, 1987), as well as to clinical results.

Serial biopsies showed that ER expression consistently increased after IFN administration. This increase was expected on the basis of *in vitro* models (Van den Berg *et al.*, 1987; Bezwoda & Meyer 1990). In addition the expression of the estrogen regulated protein P24 [which has been found to have prognostic importance in breast cancer (Seymour *et al.*, 1990c)] also showed consistently increased expression in tumour cells of responding patients after IFN administration. P24 has been shown to be an estrogen regulated secretory protein (Edwards *et al.*, 1981; Adamis *et al.*, 1983; Ciocca *et al.*, 1984) expressed in hormone receptor positive cell lines as well as in estrogen responsive target tissues. Because of its regulation by estrogens it was thought that investigation of P24 would provide an additional index of the hormone receptor-new protein synthesis pathway. Although the functional significance of P24 is not fully understood it is thought to be related to the heat shock protein, hsp 27 (Fuqua *et al.*,

1990). Whether the alteration in P24 expression was directly related to IFN therapy or a consequence of increased ER expression is not certain. Interferons have previously been reported to directly influence the production of other estrogen regulated proteins, e.g. PS 2/BCE1 (Solary *et al.*, 1991) although in this instance the effects of IFNα are to cause a reduction of the rate of synthesis of this protein.

In addition to showing the effects of drug treatment on tumour proteins the present study confirmed the relationship between P24 and ER. P24 staining was moreover, equivalent to ER in predicting response, the same patients demonstrating increments of both ER and P24 and response to treatment.

The addition to showing the effects of drug treatment on tumour proteins the present study confirmed the relationship between P24 and ER. P24 staining was moreover, equivalent to ER in predicting response, the same patients demonstrating increments of both ER and P24 and response to treatment.

The alterations induced by IFN pretreatment on the expression of the proliferation associated antigen Ki 67, which has previously been shown to correlate with mitotic index, 'S' phase fraction and prognosis in breast cancer (Barnard *et al.*, 1987; Walker & Campeljoh, 1988), were those which were predicted from the *in vitro* anti-proliferative effects of IFN. Both the patients who had initial high Ki 67 expression, showed significant reduction after IFN and both responded to therapy.

The *in vitro* demonstration of PDGF in breast cancer cells was also of some interest. The presence of both PDGF and its receptor in a number of soft tissue tumours suggest that PDGF may have an auto-stimulatory role in the growth of these cancers (Perosio & Brooks, 1989). PDGF occurs in three isoforms. While the bb isoform has the most mitogenic activity, the aa isoform is most commonly observed in tumours. We have previously demonstrated that increased plasma levels of PDGF are of prognostic importance in breast cancer, and that elevated levels correlate with tumour bulk (Ariad *et al.*, 1991). PDGF aa staining was found to be positive prior to treatment in the majority of breast cancers

studied in this investigation, although no correlation could be found between level of expression and any other variable, including response to treatment in this small study. PDGF bb, however, was positive in only 2/7 patients, both of whom were ER positive and both of whom responded to therapy. Neither patient showed any significant change in PDGF bb immunostaining after IFN or IFN and TMX. Although PDGF has previously been detected in breast cancer cell lines (Perosio & Brooks, 1989), the present study appears to be the first in which PDGF has been demonstrated in clinical breast cancer specimens.

The *in vivo* pattern of TGF β expression was the most difficult to reconcile with the predictions from the *in vitro* models. TGF β has been extensively studied using the hormonally responsive MCF-7 cell line as a model. In the MCF 7 model TMX has been shown to induce both the synthesis as well as secretion of TGF β , which then acts as an autocrine growth inhibitor. Although TGF β has mostly been characterised as an inhibitory growth factor (Knabbe *et al.*, 1987; Arrick *et al.*, 1990), there is evidence which suggests that TGF β may also be growth stimulatory to cells derived from mammary epithelium (Welch *et al.*, 1990). It should also be pointed out that the majority of patients investigated here showed easily detectable TGF β immunostaining prior to treatment. The question thus arises as to why tumours which demonstrated the presence of an apparently inhibitory growth factor (TGF β) have such aggressive and clinically progressive disease, as had the patients in this study. While it is possible that functionally active TGF β levels may not correlate with TGF-beta immunostaining [since TGF β has been shown to exist in a precursor form (Wakefield *et al.*, 1989), and the mere presence of TGF β immunostaining in cells does not necessarily indicate the presence of active or secreted growth factor], it should be pointed out that the antibody used for detection of TGF β has neutralising activity and that in our hands, changes in TGF β immunostaining have correlated with the expected increase in synthesis and secretion of TGF β in MCF 7 cells following treatment with TMX. It is thus believed that an active form of TGF β is being detected although, because of cross reactivity were unable to distinguish between the TGF β_1 and TGF β_2 isoforms. While the changes in TGF β immunostaining following treatment were variable (Table IV), it might be pointed out that the non-responders all decreased staining after IFN and/or TMX.

It would appear that for both TGF β and for PDGF, the relationship between the presence of growth factor in tumour cells and the influence on tumour growth is probably more complex than has been appreciated from *in vitro* studies. The possibility of multiple interactions between malignant cells and stromal cells which can both respond to, and in turn produce their own growth controlling signals (many of which are probably as yet unidentified) makes it probable that the end result is a balance between multiple interactions.

From the clinical point of view, it is difficult to draw

conclusions regarding the effectiveness of the combination of IFN plus tamoxifen as compared to tamoxifen alone. The prime object of this pilot study was to determine the *in vivo* tumour biologic changes resulting from exposure to each of the drugs. In this regard, while the present study confirmed the relationship between ER expression and response to therapy (Bczwoda *et al.*, 1991), it should be pointed out that responses in 4/7 (57%) occurring in a group of premenopausal, heavily pretreated patients may well be higher than expected except among a strongly ER positive group, and that IFN did significantly increase ER concentration.

Against this, however, is a previous study by Macheldt and co-workers (1991) using a combination of IFN and TMX (either from the outset or by adding IFN to patients not responding to TMX) which came to the conclusion that IFN neither contributed to the response rate observed nor was it able to reverse established TMX resistance. While this question remains unresolved the results of the present study show that IFN (under appropriate conditions) is able to induce *in vivo* changes in breast tumour determinants which would be expected to result in a synergistic effect with tamoxifen. Moreover, while previous *in vitro* studies showed that the effects of IFN on ER content were induced fairly rapidly, the present *in vivo* study demonstrates a sustained effect (lasting for at least 2 weeks) on the increment of ER expression.

Since the present study also showed that some of the effects of TMX and IFN on tumour cells appeared to be opposed (with the addition of TMX causing a down-regulation of both ER and P24) the results may also indicate that any therapeutic plan aimed at modulation of biologic influence on tumour cell growth may well have to be approached more subtly than mere empiric combination of the two agents. The optimum method of combining the two therapies may well be by the use of alternating periods of treatment. Such an approach might help to extend the period of hormonal responsiveness amongst patients whose tumours are potentially responsive to tamoxifen through the ER related mechanism of action.

In addition the present study appears to indicate a complex relationship between response to treatment and changes in growth factor expression, which were variably influenced by both interferon and by tamoxifen again with sometimes opposing effects. These studies should caution against a simplistic model of control of tumour cell proliferation derived from a single *in vitro* model and indicate the need for further research. While such studies may well be complex, the fact that a number of predictions made from *in vitro* models have been able to be tested and confirmed *in vivo*, points to the importance of this type of investigation and should provide a stimulus for further research.

This work was supported by a grant from the National Cancer Association (S.A.).

References

- ADAMS, D.J., HAJJ, H., EDWARDS, D.P., BJERCKE, R.J. & MCGUIRE, W.L. (1983). Detection of Mr 24,000 estrogen-regulated protein in human breast cancer by monoclonal antibodies. *Cancer Res.*, 43, 4297.
- ARIAD, S., SEYMOUR, L. & BEZWODA, W.R. (1991). Platelet derived growth factor (PDGF) in plasma of breast cancer patients: Correlation with stage and rate of progression. *Breast Cancer Res. Treat.*, 20, 11-17.
- ARRICK, B., KORC, M. & DERYNCK, R. (1990). Differential regulation of expression of three transforming growth factor beta species in human breast cancer cell lines by estradiol. *Canc. Res.*, 50, 299-303.
- BARNARD, N.J., HALL, P.A., LEMOINE, N.R. & KADAR, N. (1987). Proliferative index in breast carcinoma determined *in situ* by Ki 67 immunostaining and its relationship to clinical and pathological variables. *J. Pathol.*, 52, 287-295.
- BEZWODA, W.R., ESSER, J.D., DANSEY, R., KESSEL, I. & LANGE, M. (1991). The value of estrogen (ER) and progesterone (PR) receptor determination in advanced breast cancer: ER level but not PR correlates with response to tamoxifen. *Cancer*, 68, 867-870.
- BEZWODA, W.R. & MEYER, K. (1990). Effect of alpha-interferon, 17 β -oestradiol and tamoxifen on oestrogen receptor concentration and cell cycle kinetics of MCF 7 cells. *Canc. Res.*, 50, 5387-5391.
- BYER, D.P., SEARS, M.E. & MCGUIRE, W.L. (1979). Relationship between estrogen receptor values and clinical data in predicting the response to endocrine therapy for patients with advanced breast cancer. *European J. Cancer*, 15, 299-310.
- CIOCCA, D.R., ADAMS, D.J., EDWARDS, D.P., BJERCKE, R.J. & MCGUIRE, W.L. (1984). Estrogen induced 24 K protein in MCF-7 breast cancer cells is localised in granules. *Breast Cancer Res. Treat.*, 4, 261-000.

- COATES, A., GEBSKI, V. & BISHOP, J.F. (1987). Improving the quality of life during chemotherapy for advanced breast cancer. A comparison of intermittent and continuous treatment strategies. *New England J. Med.*, **317**, 1490-1495.
- EDWARDS, D.P., ADAMS, D.J. & MCGUIRE, W.L. (1981). Estradiol stimulates synthesis of a major intracellular protein in human breast cancer cell line (MCF 7). *Breast Cancer Res. Treat.*, **1**, 209.
- FUQUA, S.A.W., BLUM-SALINGAROS, J. & MCGUIRE, W.L. (1990). Estrogen regulated 24 K protein is induced by heatshock. *Cancer Res.*, **50**, 3631-0000.
- HAYWARD, J.L., RUBENS, R.D., CARBONE, P.P., HEUSON, J.C., KUMAOKA, S. & SEGALOFF, A. (1977). Assessment of response to therapy in advanced breast cancer. *Br. J. Cancer*, **35**, 292-298.
- HENDERSON, I.C. (1987). Chemotherapy for advanced breast cancer. In Harris J.R., Hellman, S., Henderson, I.C., Kinne, D.W. (eds). *Breast Diseases*, **1**, 428-479.
- KNABBE, C., LIPPMAN, M.E., WAKEFIELD, L., FLANDERS, K., KASID, A., DERYNCK, R. & DICKSON, R. (1987). Evidence that transforming growth factor- β is a hormonally regulated negative growth factor in human breast cancer cells. *Cell*, **48**, 417-428.
- MACHELDT, J.E., BUDZAR, A.U., HORTOBAGYI, G.N., FRYE, D.K., GUTTERMAN, J.U. & HOLMES, F.A. (1991). Phase II evaluation of interferon α added to tamoxifen in the treatment of metastatic breast cancer. *Breast Cancer Res. Treat.*, **18**, 165-170.
- PEROSIO, P. & BROOKS, J. (1989). Expression of growth factors and growth factor receptors in soft tissue tumours. Implications for an autocrine hypothesis. *Lab. Invest.*, **60**, 245-253.
- POWLES, T.J., FORD, H.T. & NASH, A.G. (1984). Treatment of disseminated breast cancer with tamoxifen, aminoglutethimide, hydrocortisone and danazol in combination or sequentially. *Lancet*, **1**, 1369-1373.
- SEYMOUR, L., MEYER, K., ESSER, J., MACPHAIL, P., BEHR, A. & BEZWODA, W.R. (1990a). Estimation of PR and ER by Immunocytochemistry in Breast Cancer. *Amer. J. Clin. Path.*, **94**, 35-40.
- SEYMOUR, L., BEZWODA, W.R., MEYER, K. & BEHR, C. (1990b). Detection of P24 protein in human breast cancer: influence of receptor status and oestrogen exposure. *Br. J. Cancer*, **61**, 886-890.
- SEYMOUR, L., BEZWODA, W.R. & MEYER, K. (1990c). Tumour factors predicting for prognosis in metastatic breast cancer. *Cancer*, **66**, 2390-2394.
- SICA, G., NATOLI, V., STELLA, C. & DEL BIANCO, S. (1987). Effect of natural beta-interferon on cell proliferation and steroid receptor level in human breast cancer cells. *Cancer*, **60**, 2419-2423.
- SOLARY, E., PRUD'HOMME, J.F., GAUVILLE, C., MAGDENELAT, H. & CALVO, F. (1991). Modulation of proliferation, estradiol receptors and oestrogen regulated protein PS2/BCE1 in human breast cancer cell lines by gamma interferon. *J. Biol. Regul. Homeost. Agents*, **5**, 98-106.
- VAN DEN BERG, H.W., LEAHEY, W.J., LYNCH, M., CLARKE, R. & NELSON, J. (1987). Recombinant human interferon alpha increases oestrogen receptor expression in human breast cancer cells (ZR-75-1) and sensitises them to the anti-proliferative effects of tamoxifen. *Br. J. Cancer*, **55**, 255-257.
- WAKEFIELD, L., THOMPSON, N., FLANDERS, K., O'CONNOR-MCCOURT, M. & SPORN, M. (1988). Transforming Growth Factor Beta: Multifunctional regulator of cell growth and phenotype. *Annals N.Y. Acad. Sci.*, **551**, 290-297.
- WALKER, R.A. & CAMPLEJOHN, R.S. (1988). Comparison of monoclonal antibody Ki 67 reactivity with grade and flow cytometry of breast carcinomas. *Br. J. Cancer*, **57**, 281-283.
- WELCH, D., FABRA, A. & NAKAJIMA, M. (1990). Transforming growth factor- β stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc. Natl Acad. Sci. USA*, **87**, 7678-7682.

4.3 Paper 8

TISSUE PLATELET DERIVED GROWTH FACTOR (PDGF) PREDICTS FOR SHORTENED
SURVIVAL AND TREATMENT FAILURE IN ADVANCED BREAST CANCER.

L. Seymour, D. Dajee, W.R. Bezwoda.

Breast Cancer Research and Treatment. 26:247-252. 1993.

Report

Tissue platelet derived-growth factor (PDGF) predicts for shortened survival and treatment failure in advanced breast cancer

L. Seymour, D. Dajee and W.R. Bezwoda

Division of Clinical Haematology and Medical Oncology, Department of Medicine, University of the Witwatersrand, South Africa

Key words: platelet derived growth factor, breast cancer, immunohistochemistry, prognosis, tumor progression

Abstract

In a study of plasma and tissue platelet derived growth factor (PDGF) concentration in patients with breast cancer, elevated levels of plasma PDGF were found in a significant proportion, 11/37 (30%), of patients. Sixteen patients (43%) had tumors which expressed PDGF-AA and 6 patients had tumors which in addition expressed the BB isoform of PDGF. All patients with elevated plasma levels of platelet derived growth factor had tumors which expressed the growth factor on immunohistochemical staining of tumor cells. Furthermore there was a significant correlation between plasma levels of platelet derived growth factor and the intensity of tissue staining. Patients with stage four breast cancer with tumors which were positive for platelet derived growth factor had a significantly lower response rate to chemotherapy as well as significantly shorter duration of survival. In addition, patients with stage four breast cancer who had elevated plasma PDGF levels had a significantly shorter survival. These results indicate that elevated plasma levels of platelet derived growth factor in patients with breast cancer are derived from the tumor cells and suggest that platelet derived growth factor may play a significant role in control tumor cell growth.

Introduction

Growth factors, especially those with homology to cellular oncogenes, have attracted extensive research in an attempt to understand both tumorigenesis as well as the biologic control of cancer cell growth. One such growth factor is platelet derived growth factor (PDGF) which has been implicated both in virus induced tumorigenesis [1] as well as autocrine control of tumors [2].

In addition to being a potent mitogenic and chemotactic factor for fibroblasts, platelet derived growth factor as well as receptors for PDGF have

been found in various cancers, including colon carcinomas [3] and mesenchymal and glial tumors [4, 5].

Little is known, however, of the role that PDGF plays in breast cancer. Although breast cancer cell lines have been shown *in vitro* to produce PDGF [6], clinical correlates are unavailable. However, we have recently described that elevated plasma PDGF levels predict shorter survival times in patients with breast cancer and may be a marker of patients with aggressive or high bulk disease [7]. In this study we have extended our previous investigation by examining tissue expression of various iso-

forms of PDGF in patients with breast cancer in an attempt to elucidate the role PDGF plays, if any, in the biologic control of breast cancer.

Methods

Representative sections from the histological specimens of 37 patients in whom plasma PDGF levels had been measured previously [7] were obtained. The method used for plasma PDGF assay has been described in a previous publication [7], and measures plasma levels of PDGF in a range of 1.56–2.00 fmol. PDGF is undetectable in normal plasma using this method. All patients had had a histologic diagnosis of breast cancer and all had been staged according to accepted criteria.

The sections were examined for PDGF-AA and PDGF-BB expression using standard immunohistochemical techniques. Briefly, sections were dewaxed and rehydrated, endogenous peroxidase was blocked by immersion in methanol/peroxide, and then blocking antibody, primary antibody, and secondary antibody were sequentially layered onto the sections. Monoclonal anti-PDGF-BB (Promega) and polyclonal anti-PDGF-AA (British Biotechnology) was used, and the Vectastain-ABC kit (Vector Lab Burlingame) was used for visualisation. The slides were then dehydrated and mounted with coverslips, and examined under light microscopy for the presence of staining, which was scored according to both the intensity of staining and the number of tumor cells showing staining. PDGF-AA was scored by assessing both intensity (*i*) of staining (*i* = 0–4) and degree of staining (%), and calculating AA-score from the formula $AA = [1 + i] \times \% \text{ positive cells}$. PDGF-BB was scored in a similar fashion, except that the BB-score was divided by 100.

Statistical analysis was performed using SAS software (release 6.03). Survival curves and statistics were computed using SAS.STAT and the life-test procedure.

Results

Tissue was available for examination in 37 patients

who had had plasma PDGF levels measured. 1 patient had an adenosquamous histology, 2 patients lobular carcinoma, 1 patient medullary, and the remaining 33 patients had histological findings consistent with a diagnosis of infiltrating ductal carcinoma. The mean age of the patients was 50 years (range 33–79 sd ± 12.6). Further clinical details of the patients studied are shown in Table 1.

At the time of sampling for plasma PDGF, 11 (30%) patients had stage 2 disease, and 26 (70%) had stage 4 disease. 24 of the 26 patients with stage 4 disease received primary combination chemotherapy consisting of a standard 3 drug combination chemotherapy regimen; CNV (cyclophosphamide, mitoxantrone, vincristine). Only 2 patients received hormonal treatment. Both were negative for plasma and for tissue PDGF.

Of the 37 patients 11 (30%) had elevated plasma levels of PDGF and 16 (43%) had significant tissue expression of PDGF-AA. These 16 tissue PDGF-AA positive patients included all 11 who had elevated plasma levels of PDGF. Six (16%) patients showed significant tissue expression of PDGF-BB. Again all PDGF-BB positive patients showed con-

Table 1. Platelet-derived growth factor in breast cancer: patient characteristics

	Number	Percentage
Stage		
Two	11	30
Four	16	70
ER		
Positive	3	8
Negative	6	16
Unknown	28	75
Menopausal status		
Pre	22	59
Post	15	41
Disease sites		
Local	6	20
Lung	3	10
Liver	4	13
Bone and local	3	10
Widely disseminated	10	33
Bone	4	13
Treatment for metastatic disease		
Chemotherapy	24	92
Hormone therapy	2	8

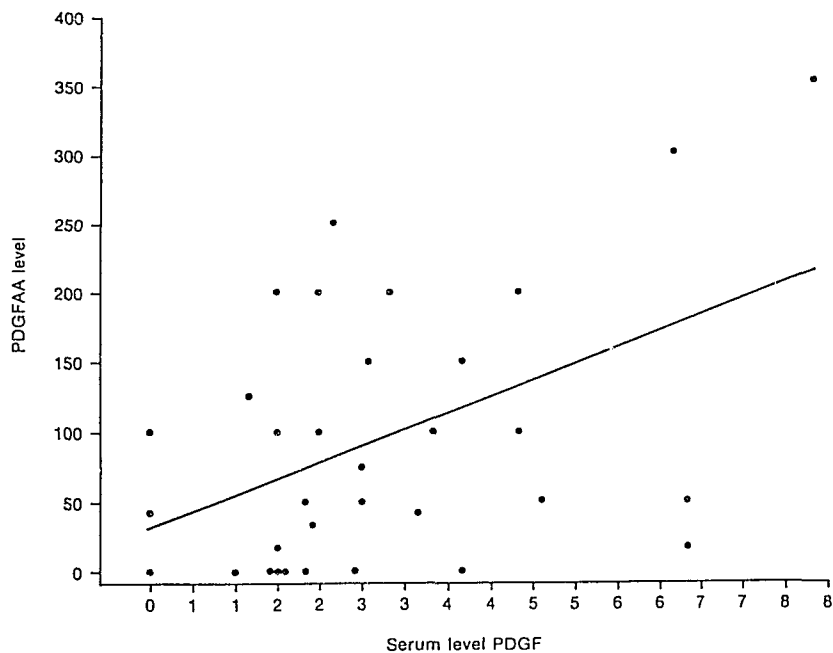


Fig. 1a. Correlation of serum PDGF and tissue PDGF-AA.

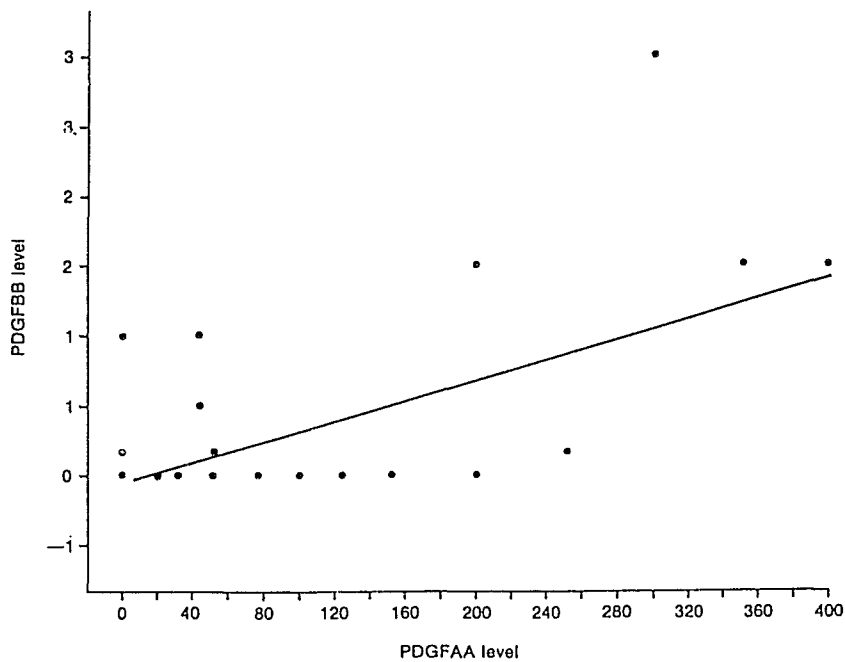


Fig. 1b. Correlation of tissue PDGF-BB and PDGF-AA.

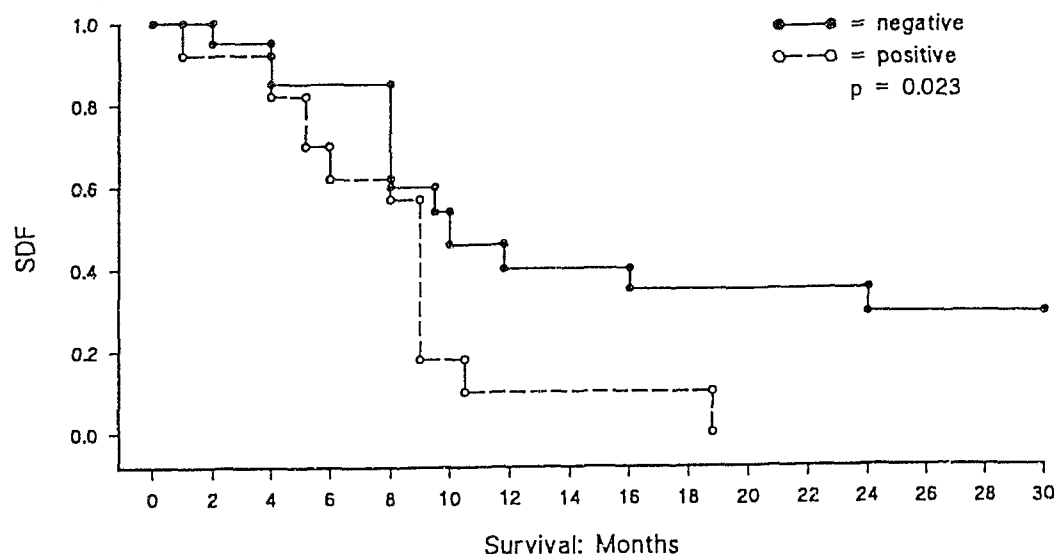


Fig. 2. Impact of tissue PDGF-AA on survival in stage 4 breast cancer.

comitant expression of PDGF-AA and elevated plasma levels. Both PDGF-AA and PDGF-BB expression was confined to tumour cells, connective tissue and stromal cells being uniformly negative. Staining was moreover cytoplasmic in localisation.

There was a significant correlation between plasma PDGF and tissue PDGF-AA levels as well as between tissue PDGF-AA and PDGF-BB levels using regression analysis (Figs 1a and 1b, Table 2). Amongst the patients with stage 4 disease, those with bulky disease had significantly higher tissue levels of PDGF-AA (172 vs 40) and PDGF-BB (0.6 vs 0.1).

In addition, patients who had stage 4 disease at sampling had a significantly shorter survival when immunostaining for PDGF-AA was detected [Table 4 & Fig. 2, (log rank $p = 0.02$, Wilcoxon $p = 0.05$)]. Furthermore, only 1 patient out of 11 with

stage 4 disease who had positive tissue PDGF-AA expression responded to chemotherapy, whereas a response rate of 7/13 was seen in those patients who did not express PDGF-AA ($p = 0.019$ [Fishers exact] Table 4).

The prognostic significance of plasma PDGF was further confirmed in the group of patients with stage 4 breast cancer, as elevated levels of plasma PDGF had a significant negative impact on survival (log rank $p = 0.03$, Wilcoxon $p = 0.05$) (Fig. 3).

Discussion

Platelet derived growth factor (PDGF) is a 30 kilodalton cationic glycoprotein composed of disulfide bonded dimers of A and B peptide chains. A and B

Table 2. Correlation between plasma and tissue PDGF in breast cancer patients

	Correlation coefficient	p-value
PDGF-plasma: tissue PDGF-AA	0.413	0.0122
PDGF-plasma: tissue PDGF-BB	0.37448	0.0244
Tissue pdgf-aa: tissue PDGF-BB	0.58387	0.0002

Table 3. Survival in stage 4 breast cancer: correlation with plasma and tissue PDGF

	Median survival months		
	Negative	Positive	p-value
Plasma PDGF	11.6	6.7	0.03
Tissue PDGF-AA	13.7	7.9	0.023
Tissue PDGF-BB	11.9	7.7	0.41

chains have 60% sequence homology and three forms are thus possible [8]. The AA isoform has been predominantly isolated from tumors, while AB and BB are isolated predominantly from alpha granules of platelets and are a major mitogenic constituent of plasma.

PDGF has two distinct forms of receptors which are cell surface receptors between 175 and 185 kilodaltons. α -receptors recognise all 3 isoforms of PDGF with high affinity, whereas β -receptors recognise BB with higher affinity than AB or AA [9].

PDGF-BB is homologous to p28v-sis, and is the normal cellular homologue of the simian sarcoma virus protein. As v-sis leads to the formation of mesenchymal and glial tumors when injected into newborn marmosets [1] it is postulated that v-sis may bind to PDGF receptors and activate the process leading to neoplasia. Its normal cellular homologue C-sis may play a similar role in non-virally induced cancer.

In addition, activation of these oncogenes has been postulated to result in autostimulation of cell growth, and may thus play a role in the clinical progression of tumors as well as in the development of cancer. Certainly PDGF and its receptors may play a role in autocrine control of soft tissue tumors [2].

In breast cancer cell lines, growth factors and their receptors (such as TGF- α and TGF- β) are believed to play an important role in the control of growth [10] and may have a role in the control of

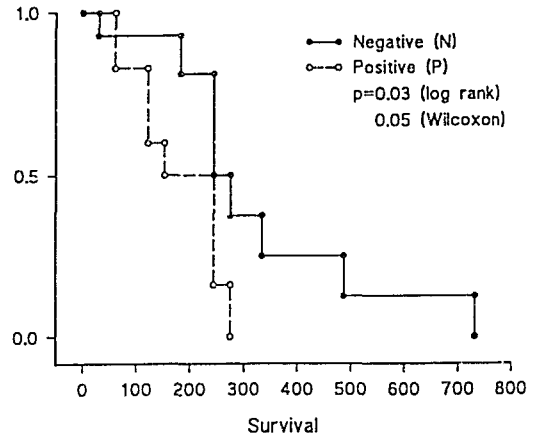


Fig. 3. Impact of plasma PDGF on survival in stage 4 breast cancer.

clinical breast cancer. Little is known about PDGF in breast cancer. *In-vitro* studies indicate that while PDGF is secreted by a number of breast cancer cell lines, PDGF receptor has not been identified in these systems, leading to the suggestion that any effects resulting from PDGF synthesis and secretion may be paracrine rather than autocrine [6, 11]. While interaction of growth factors with tumour stroma may well be important, no information was, however, previously available as to the presence or absence of PDGF or its receptor in *in-vivo* breast cancer.

Our previous study of plasma PDGF levels [7] in patients with breast cancer suggested that high levels of circulating PDGF correlated both with bulk of disease and prognosis in these patients. In that study the source of the increased levels of PDGF in plasma was not identified and may have been due either to production of PDGF by the tumor cells themselves, production by surrounding stromal cells, or PDGF released from platelets activated by contact with tumor cells [13].

The present study extends our previous findings in a number of important ways. Firstly, the significant correlation between plasma PDGF and expression of PDGF in tumor cells suggests that the origin of the elevated blood levels is probably the tumor cells. Furthermore, the finding that PDGF expression by tumour cells has an adverse effect on response to chemotherapy and on survival suggests

Table 4. Impact of PDGF on response to chemotherapy for stage 4 breast cancer

	Response to chemotherapy		p-value
	Complete & partial response (%)	No response (%)	
Tissue PDGF-AA			
positive	1 (4)	10 (42)	0.019
negative	7 (29)	6 (25)	
Tissue PDGF-BB			
positive	1 (4)	3 (13)	0.6
negative	7 (29)	13 (54)	
PDGF-plasma			
positive	4 (17)	4 (17)	0.27
negative	4 (17)	12 (50)	

that PDGF may be implicated as a resistance factor as well as affecting the growth and progression of breast cancer. Whether the biologically more aggressive behaviour of those tumours that express PDGF is mediated via an effect on stroma (by a paracrine mechanism) or via the tumor cells themselves (through an autocrine mechanism) requires further elucidation.

The present study also set out to investigate the tissue expression of the various forms of PDGF by using antibodies specific for each of the isoforms. Not surprisingly PDGF-AA expression was found to occur more frequently than BB since previous studies have shown that AA is the isoform found most commonly in tumor cells [5]. The lack of predictive value (either in terms of disease bulk, response to therapy or prognosis) of PDGF-BB may be due to the low frequency of expression of this isoform or to a lower mitogenic activity of PDGF-BB. It should however be pointed out that in a number of tumors expressing PDGF-AA there was concurrent expression of PDGF-BB while in no instance was there expression of PDGF-BB alone. What the function of PDGF-BB in those tumors expressing both isoforms is could not be elucidated in the present study.

In conclusion, the present study confirms and extends our previous study and extends our previous observations on PDGF in breast cancer and suggests a possible role for therapy aimed at inhibition of the activity of this growth factor.

Acknowledgements

Supported by a grant from the National Cancer Association (S.A.).

References

1. Deinhardt F: Biology of primate retroviruses. In: Klein G (ed) *Viral Oncology*. Raven, New York, 1980
2. Perosio PM, Brooks JJ: Expression of growth factors and growth factor receptors in soft tissue tumors: implications for the autocrine hypothesis. *Lab Invest* 60: 245-253, 1989
3. Ito M, Yoshida K, Kyo E, Ayhan A, Nayakama H, Yasui W, Ito H, Tahara E: Expression of several growth factors and their receptor genes in human colon carcinomas. *Virchows Arch* 59: 173-178, 1990
4. Wang JL, Nister M, Hermansson M, Westermark B, Ponten J: Expression of PDGF β -receptors in human meningioma cells. *Int J Cancer* 46: 772-778, 1990
5. Leveen P, Claesson-Welsh L, Heldin CH, Westermark B, Betsholtz C: Expression of messenger RNA's for platelet derived growth factor and its receptors in human sarcoma cell lines. *Int J Cancer* 46: 1066-1070, 1990
6. Bronzert DA, Pantazis P, Antoniadis HN, Kasid A, Davidson N, Dickson RB, Lippman ME: Synthesis and secretion of platelet derived growth factor by human breast cancer cell lines. *Proc Natl Acad Sci* 84: 5763-5767, 1987
7. Ariad S, Seymour L, Bezwoda WR: Platelet derived growth factor (PDGF) in plasma of patients with breast cancer: correlation with stage and rate of progression. *Breast Cancer Res Treat* 20: 11-17, 1991
8. Heldin CH, Betsholtz C, Johnsson A, Westermark B: Role of PDGF-like growth factors in malignant transformation. *Cancer Rev* 2: 34-39, 1986
9. Pantazis P, Goustin AS, Nixon J: Platelet derived growth factor and its receptor in blood cell differentiation and neoplasia. *Eur J Haematol* 45: 127-138, 1990
10. Wakefield L, Thompson N, Flanders K, O'Connor-McCourt M, Sporn M: Transforming growth factor-beta: multifunctional regulator of cell growth and phenotype. *Ann NY Acad Sci*: 290-297, 1989
11. Lippman ME, Dickson RB, Gelmann EP, Rosen N, Knabbe C, Bates S, Bronzert D, Huff K, Kasid A: Growth regulation of human breast cancer occurs through regulated growth factor secretion. *J Cell Biochem* 35: 1-19, 1987
12. Mannucci PM, Cattaneo M, Canciani MT, Manniczzo M, Vaglini M, Cascinelli N: Early evidence of activated (exhausted) platelets in malignant tumors (breast adenocarcinoma and malignant melanoma). *Eur J Cancer Clin Oncol* 25: 1413-1417, 1989

4.4 Paper 9

POSITIVE IMMUNOSTAINING FOR PLATELET DERIVED GROWTH FACTOR IS AN ADVERSE
PROGNOSTIC FACTOR IN PATIENTS WITH ADVANCED BREAST CANCER.

L. Seymour, W.R. Bezwoda.

Breast Cancer Research and Treatment. 32:229-233.1994.

Brief communication

Positive immunostaining for platelet derived growth factor (PDGF) is an adverse prognostic factor in patients with advanced breast cancer

L. Seymour and W.R. Bezwoda

Division of Clinical Haematology and Medical Oncology, Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa

Key words: platelet derived growth factor, PDGF, breast cancer, immunohistochemistry, prognosis

Abstract

Previous studies suggest a prognostic role for PDGF in patients with breast cancer, with patients with high plasma PDGF levels or positive response to therapy. We have examined a further 58 patients with advanced breast cancer for the presence of tissue PDGF immunostaining. Patients displaying positive tissue immunostaining for PDGF had a highly significant shorter survival ($p = 0.002$) than patients with no immunostaining. In addition PDGF positive patients treated with combination chemotherapy had a significantly lower response rate ($p = 0.05$) than PDGF negative patients. These results confirm our previous findings that PDGF may be an important indicator of shortened survival and treatment failure in patients with advanced breast cancer.

Introduction

PDGF is a ubiquitous growth factor comprising three isoforms (AA, AB and BB) and has specific dimeric receptors (PDGFR's) on target cells. PDGF shares 90% homology with the transforming protein of the simian sarcoma virus (P28v-sis) and may play a role as a cellular oncogene in tumourigenesis. In addition it may play a role in growth control of certain tumours either by means of an autocrine loop [1], or by complex paracrine interactions between stromal and mesenchymal cells.

The role of PDGF in breast cancer is still controversial. While some investigators have found both PDGF and PDGFR's in human breast cancer cell lines [25], others find either no mitogenic activity of PDGF, or fail to find PDGFR.

We have previously described PDGF to be a

prognostic indicator in patients with advanced and early stage breast cancer [1, 2]. We present here further data which confirms the prognostic import of PDGF in advanced breast cancer.

Methods

Material and methods

The study population comprised 58 patients with advanced breast cancer. Tissue was obtained by dermatological punch biopsy of accessible cutaneous lesions under local anaesthesia prior to initiation of treatment for recurrent or metastatic disease. Specimens were flash frozen and stored at -135° until assay. Frozen sections were fixed in picric-acid-paraformaldehyde, and then immunos-

tained for PDGF-AA utilising a polyclonal anti-PDGF-AA antibody (British Biotechnology) and for PDGF BB (monoclonal anti-PDGF-BB antibody – Promega) and the Vectastain-ABC kit (Vector Lab Burlingame). Both the method and scoring system have been described in a previous publication [3]. PDGF-AA scores of ≥ 300 were deemed strongly positive. Specimens were also examined for the presence of oestrogen (ER) and progesterone receptors (PR) and the presence of P24 (an oestrogen regulated protein), TGF- β , and Ki67 according to previously described methods [4, 5].

Response criteria

Response was assessed on the basis of criteria proposed by the Eastern Cooperative Group [6].

Ethical considerations

Informed consent was obtained prior to enrollment in all patients. The study was approved by the Committee on Ethics of Human Research of the University of the Witwatersrand and was carried out in accordance with the principles of the declaration of Helsinki.

Results

Patient characteristics

58 patients with advanced breast cancer were evaluated. The mean age of the patients was 46 years (sd ± 10.26 , range 24–71 years). Twenty four (41%) patients were postmenopausal. 43 (74%) received combination chemotherapy as their initial treatment, while 15 (26%) received hormonal manipulation. Further patient details are shown in Table 1.

PDGF immunostaining

14 (24%) tumours expressed strong PDGF-AA immunostaining, while 13 (25%) expressed PDGF-BB.

There was no correlation between expression of PDGF-AA and ER ($p = 0.16$), PR ($p = 0.46$), P24 ($p = 0.7$), TGF- β ($p = 0.4$), Ki67 ($p = 0.5$), menopausal status ($p = 0.2$), or PDGF-BB expression ($p = 0.1$).

Response to treatment

Thirty three (57%) of patients showed no response to therapy, 9 (16%) achieved a complete response (CR), while 15 (26%) had a partial response (PR). PDGF-AA expression was not predictive ($p = 0.22$) for response when the entire study population was analysed. However, on subset analysis, patients who were PDGF positive and who received combination chemotherapy had a significantly poorer response rate (20%) to this form of treatment than patients who were negative for PDGF (RR = 54%; $p = 0.05$). The presence of PDGF immunostaining had no effect on response in patients who received hormonal therapy.

Survival

Median survival of the group was 8 months. The

Table 1. Patient details

	Number	Percentage
Site of disease		
Loco-regional	25	43%
Lung	11	19%
Bone	8	14%
Hepatic	7	12%
Inflammatory breast cancer		
Menstrual status		
Post menopausal	24	41%
Pre menopausal	34	59%
Oestrogen receptor		
Positive	20	35%
Negative	38	64%
Progesterone receptor		
Positive	16	28%
Negative	41	70%
Unknown	1	2%
Treatment		
Chemotherapy	43	74%
Hormonal	15	26%

presence of PDGF-AA immunostaining had a highly significant negative impact on survival ($p = 0.0023$, Fig. 1). The median survival of PDGF-AA positive patients was 4.9 months versus 11.9 months for patients displaying no immunostaining. The presence of PDGF-BB had no impact on survival.

Discussion

Platelet Derived Growth Factor (PDGF), a homo or heterodimer of disulfide linked A or B chains, has three possible combinations, i.e. AA, AB, and BB. The different isoforms have differing activities dependent on the presence of specific receptors on target cells. In a similar fashion PDGF receptors (PDGFR's) exist as dimers of either α or β subunits. PDGF-AA binds only $\alpha\alpha$ receptors, BB activates all three receptors, while AB is capable of activating both $\alpha\alpha$ and $\alpha\beta$ receptors [7]. In addition there is some evidence that PDGF-AB can bind to β receptors with high affinity via the B chain [8].

PDGF may be an important growth factor in the initiation and control of malignant tumours. Cellular oncogene products have been identified in a number of human tumours [9], and may play a role in tumourigenesis. P28v-sis (the transforming protein of the simian sarcoma virus) is 90% homologous to the B chain of PDGF and is able to induce

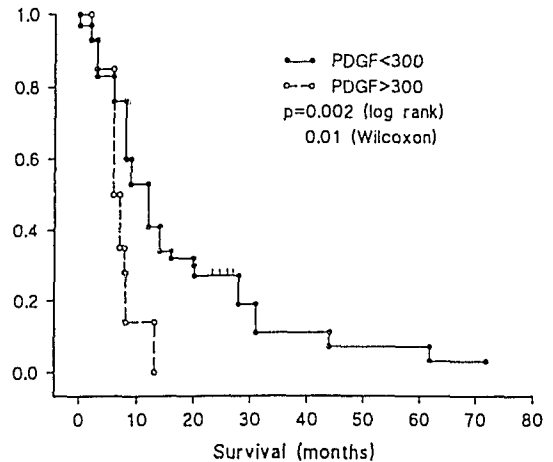


Fig. 1. Relationship of positive tissue immunostaining for PDGF to survival of patients with advanced breast cancer.

tumours and transform cells which express PDGFR's [10].

The precise role of PDGF in malignancy is, however, difficult to define as it is an ubiquitous growth factor, found not only in a range of tumours, but also in stromal and mesenchymal cells, suggesting a complex regulatory system. Some effects of PDGF are thought to be mediated by an autocrine loop, with cells both producing PDGF and expressing PDGFR. Fleming and co-workers [11] elegantly describe activated PDGFR's in cell lines in which no exogenous ligand is present, suggesting the presence of an activated PDGF autocrine pathway. Definition of the action of PDGF is further complicated by the demonstration of internal autocrine pathways for PDGF, with active PDGF present in cells together with receptors (PDGFR's) which are, however, not expressed on the surface membrane [12, 13]. Such autocrine loops may also be co-regulated by other growth factors, such as Transforming Growth Factor- β (TGF- β), which has been shown to mediate its growth stimulatory effects on cultured smooth muscle cells via a PDGF autocrine loop [14]. Similar relationships between TGF- β and PDGF autocrine loops have also been demonstrated in other cell lines [15].

While it is clear that PDGF also has functional activity in various malignant cells such as melanoma and mesothelioma cell lines [16, 17, 18] the pre-

Table 2.

	Number	Percentage
PDGF-AA expression		
Positive	14	24%
Negative	44	76%
PDGF-BB expression		
Positive	22	38%
Negative	30	52%
Unknown	6	10%
p24 expression		
Positive	35	60%
Negative	23	40%
TFG- β expression		
Positive	48	83%
Negative	10	17%
Ki67 expression		
> 20%	29	50%
< 20%	29	50%

cise role of PDGF in breast cancer is as yet undefined. Animal models suggest a role for PDGF [19, 20]. However, Bronzert and co-workers [21] demonstrated growth inhibition of normal human mammary cells by TGF- β , with a concomitant increase in PDGF mRNA and secretion, while Lippman and co-workers described an oestrogen regulated PDGF-like molecule in breast cancer cell lines suggesting a possible paracrine role for PDGF in breast cancer, but failed to demonstrate PDGFR's [22, 23].

Other workers have demonstrated intracellular PDGFR in cell lines previously thought to be PDGFR negative [24]. In addition, Ginsburg and Vonderhaar demonstrated a mitogenic action of PDGF on T47D and MCF cell lines as well as the presence of specific PDGFR in T47D cells [25], and suggest that previous investigators may have seen the mitogenic effects of PDGF when utilising culture systems with non-charcoal stripped bovine or foetal calf serum, both of which contain high levels of endogenous PDGF.

We have previously described a significant correlation between high plasma levels of PDGF and disease bulk and prognosis in patients with advanced and early stage breast cancer [26], and shortened survival in patients with advanced breast cancer who demonstrate tissue immunostaining for PDGF [2]. The present study examines a further 58 patients, and confirms the highly significant correlation between PDGF-AA immunostaining and survival in patients with breast cancer, with survival of PDGF positive patients being only 4.9 months, compared to 11.9 months for PDGF negative patients ($p = 0.002$). In addition, the presence of positive PDGF immunostaining predicted for lack of response to chemotherapy ($p = 0.05$).

While these findings confirm our previous observations that PDGF expression appears to be an important prognostic factor in breast cancer, it is still unclear precisely how PDGF exerts its effect, i.e. whether it acts in a paracrine or autocrine fashion. We have confirmed that cells such as MCF-7's and other human tumour cell lines displaying positive PDGF-AA immunostaining are also positive for PDGF mRNA when examined by in-situ hybridisation (unpublished data). However, this does not clarify the site of action of PDGF in clinical breast cancer.

It is possible that PDGF produced by breast tumours not only acts in an autocrine loop, but also exerts paracrine effects on stromal cells, including fibroblasts, which are known to express PDGFR's [27] and which may in turn produce other growth factors, with potential growth stimulatory effects on tumour cells such as fibroblast growth factor (FGF) and insulin-like growth factor (IGF). Fibroblast growth factors (FGFs) may be important mediators of angiogenesis and growth regulation in tumours [28].

PDGF is also produced by stromal mesenchymal cells, and PDGF effects on stromal tissue surrounding tumours may play a role in growth regulation [29]. Stroma contains, in addition to cells, extracellular matrix consisting of proteins such as collagen and fibronectin as well other glycoproteins and proteoglycans which may modulate the effects of growth factors [30, 31, 32].

Further studies are required to elucidate the precise function of PDGF in breast cancer including a search for PDGFR's (both internal and cell surface expressed) in tumour specimens. The possible paracrine interactions between tumour and stroma will also need to be investigated using sophisticated *in vitro* tissue culture methods, and may be more difficult to define.

References

1. Ariad S, Seymour L, Bezwoda WR: Platelet derived growth factor (PDGF) in plasma of patients with breast cancer: correlation with stage and rate of progression. *Breast Cancer Res Treat* 20: 11-17, 1991
2. Seymour L, Dajee D, Bezwoda WR: Tissue platelet derived growth factor (PDGF) predicts for shortened survival and treatment failure in advanced breast cancer. *Breast Cancer Res Treat* 26: 247-252, 1993
3. Seymour L, Dajee D, Bezwoda WR: Tissue platelet derived growth factor (PDGF) predicts for shortened survival and treatment failure in advanced breast cancer. *Breast Cancer Res Treat* 26: 247-252, 1993
4. Seymour L, Meyer K, Esser J, MacPhail AP, Behr A, Bezwoda WR: Estimation of PR and ER by immunocytochemistry in breast cancer. *Am J Clin Path* 94, 35-40, 1990
5. Seymour L, Bezwoda WR, Meyer K: Tumour factors predicting for prognosis in metastatic breast cancer. *Cancer* 66: 2390-2394, 1990
6. Hayward JL, Rubens RD, Carbone PP, Heuson JC, Kumao-

- ka S, Segaloff A: Assessment of response to therapy in advanced breast cancer. *Br J Cancer* 35: 292-298, 1977
7. Heidaran MA, Beeler JF, Yu J-C, Ishibashi T, LaRochelle WJ, Pierce JH, Aaronson SA: Differences in substrate specificities of α and β platelet derived growth factor (PDGF) receptors. *J Biol Chem* 268: 9287-9295, 1993
8. Fretto LJ, Snape AJ, Tomlinson JE, Seroogy JJ, Wolf DL, LaRochelle WJ, Giese NA: Mechanism of platelet derived growth factor (PDGF) AA, AB, and BB binding to α and β PDGF receptor. *J Biol Chem* 268: 3625-3631, 1993
9. Slamon DJ, DeKernion JB, Verma IM, Cline MJ: Expression of cellular oncogenes in human malignancies. *Science* 224: 256-259, 1984
10. Leal F, Williams L, Robbins K, Aaronson S: Evidence that the v-sis gene product transforms by interaction with the receptor for PDGF. *Science* 230: 327-330, 1985
11. Fleming TP, Matsui T, Heidaran MA, Molloy CJ, Artrip J, Aaronson SA: Demonstration of an activated Platelet Derived Growth Factor autocrine pathway and its role in human tumour cell proliferation *in vitro*. *Oncogene* 7: 1355-1359, 1992
12. Silver BJ: Platelet Derived Growth Factor in human malignancy. *Biofactors* 3: 217-227, 1992
13. Lokeshwar VB, Huang SS, Huang JS: Intracellular turnover, novel secretion, and mitogenically active intracellular forms of v-sis gene product in simian sarcoma virus-transformed cells. Implications for intracellular loop autocrine transformation. *J Biol Chem* 265: 1665-1675, 1990
14. Bategay EJ, Raines EW, Selfert RA, Bowen-Pope DF, Ross R: TGF- β induces bimodal proliferation of connective tissue cells via complex control of an autocrine loop. *Cell* 63: 515-524, 1990
15. Haberstroh U, Zahner G, Disser M, Thaiss F, Wolf G, Stahl RAK: TGF- β stimulated rat mesangial cell proliferation in culture role of PDGF β -receptor expression. *Am J Physiol* 264: F199-F205, 1993
16. Behl C, Bogdahn U, Winckler J, Apfel R, Brysch W, Schlingsiepen K-H: Autoinduction of Platelet Derived Growth Factor (PDGF) A chain mRNA expression in a human malignant melanoma cell line and growth inhibitory effects of PDGF-A chain mRNA-specific antisense molecules. *Biochem Biophys Res Comm* 193: 744-751, 1993
17. Van de Meeren A, Seddon MB, Betsholtz CA, Lechner JF, Gerwin BI: Tumorigenic conversion of human mesothelioma cells as a consequence of Platelet Derived Growth Factor A chain overexpression. *Am J Respir Cell Mol Biol* 8: 214-221, 1993
18. Langerak AW, Dirks RP, Versnel MA: Splicing of the Platelet Derived Growth Factor A chain mRNA in human malignant mesothelioma cell lines. *Eur J Biochem* 208: 589-596, 1992
19. Elliott B, Ostman A, Westermarck B, Rubin K: Modulation of growth factor responsiveness of murine mammary carcinoma cells by cell matrix interactions: Correlation of cell proliferation and spreading. *J Cell Physiol* 152: 292-301, 1992
20. Taverna D, Groner B, Hynes NE: Epidermal Growth Factor Receptor, Platelet Derived Growth Factor Receptor and c-erbB-2 receptor activation all promote growth but have distinctive effects upon mouse mammary epithelial cell differentiation. *Cell Growth Differ* 2: 145-154, 1991
21. Bronzert DA, Bates SE, Sheridan JP, Lindsey R, Valverius EM, Stampfer MR, Lippman ME, Dickson RB: Transforming Growth Factor- β induces Platelet Derived Growth Factor (PDGF) messenger RNA and PDGF secretion while inhibiting growth in normal human mammary epithelial cells. *Mol Endocrinol* 4: 981-989, 1990
22. Lippman ME, Dickson RB, Gelmann EP, Rosen N, Knabbe C, Bates S, Bronzert D, Huff K, Kasid A: Growth regulation of human breast cancer occurs through regulated growth factor secretion. *J Cell Biochem* 35: 1-16, 1987
23. Bronzert DA, Pantazis P, Antoniadis HN, Kasid A, Davidson N, Dickson RB, Lippman ME: Synthesis and secretion of platelet derived growth factor by human breast cancer cell lines. *Proc Natl Acad Sci* 84: 5763-5767, 1987
24. Rakowicz-Szulczynska EM, Koprowski H: Expression of A-type PDGF receptor in cytoplasm of tumor cell lines synthesising PDGF. *Exp Mol Pathol* 51: 171-178, 1989
25. Ginsburg E, Vonderhaar BK: Stimulation of growth of human breast cancer cells (T47D) by Platelet Derived Growth Factor. *Cancer Lett* 58: 137-144, 1991
26. Ariad S, Seymour L, Bezwoda WR: Platelet derived growth factor (PDGF) in plasma of patients with breast cancer: correlation with stage and rate of progression. *Breast Cancer Res Treat* 20: 11-17, 1991
27. Tingstrom A, Reuter Dahl C, Lindahl P, Heldin C-H, Rubin K: Expression of Platelet Derived Growth Factor- β receptors on human fibroblasts. *J Immunol* 148: 546-554, 1992
28. Wellstein A, Lupu R, Zugmaier G, Flamm SL, Cheville AL, Delli Bovi P, Basilio C, Lippman ME, Kern FG: Autocrine growth stimulation by secreted Kaposi fibroblast growth factor but not by endogenous basic fibroblast growth factor. *Cell Growth Differ* 1: 63-71, 1990
29. Funa K, Papanicolaou V, Juhlin C, Rastad J, Akerstrom G, Heldin C-H, Oberg K: Expression of Platelet Derived Growth Factor β -receptors on stromal tissue cells in human carcinoid cells. *Cancer Res* 50: 748-753, 1990
30. Bissell MJ, Hall HG, Parry G: How does the extracellular matrix direct gene expression? *J Theor Biol* 99: 31-68, 1982
31. Brooks RF, Howard M, Leake DS, Riddle PN: Failure of Platelet Derived Growth Factor plus insulin to stimulate sustained proliferation of Swiss 3T3 cells. Requirement for hydrocortisone, prostaglandin E, lipoproteins, fibronectin and an unidentified component derived from serum. *J Cell Sci* 97: 71-78, 1990
32. Golombick T, Dajee D, Bezwoda WR: Cell extracellular matrix interactions 2: Extracellular matrix structure is important for growth factor localisation and function. (In press)

4.5 Paper 10

SOLUBLE C-erbB-2 FRAGMENT IN SERUM CORRELATES WITH DISEASE STAGE AND
PREDICTS FOR SHORTENED SURVIVAL IN PATIENTS WITH EARLY STAGE AND ADVANCED
BREAST CANCER.

H. Kandl, L. Seymour, W.R. Bezwoda.

British Journal of Cancer 70:739-742. 1994.

Soluble c-erbB-2 fragment in serum correlates with disease stage and predicts for shortened survival in patients with early-stage and advanced breast cancer

H. Kandl, L. Seymour & W.R. Bezwoda

Division of Clinical Haematology and Medical Oncology, Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa.

Summary Seventy-nine patients with advanced breast cancer were tested for the presence, in serum, of a 110 kDa soluble c-erbB-2 fragment. Thirty-nine patients were seropositive. There was no correlation between seropositivity and menopausal status, or with oestrogen status. In addition, no correlation could be found between tissue c-erbB-2 immunostaining for the external domain of the c-erbB-2 receptor and the presence of soluble c-erbB-2 in serum. The presence of serum soluble c-erbB-2, however, had a significant impact on survival of patients with advanced disease, suggesting that this test may become a useful independent prognostic indicator.

Breast cancer is a major health problem, affecting one in nine women in western countries. A particularly important goal is the early identification of poor-risk patients who may benefit from aggressive intervention with intensive chemotherapy.

While many tumour factors, including hormone receptor status, ploidy and growth fraction, and the expression of various oncogenes and proto-oncogenes by the tumour cells have been proposed as prognostic indicators, the results, to date, have been equivocal in a number of instances. Recent investigations into the role of amplification of the c-erbB-2 gene, the product of which is a transmembrane protein with extensive homology to the epidermal growth factor (EGF) receptor, have also appeared to give somewhat contradictory results. Gene amplification and increased c-erbB-2 expression have been reported in approximately 20% of patients with primary breast cancer (Clark & McGuire, 1991). Both gene amplification and increased expression of the gene product have been associated with a poorer prognosis in some studies. The discriminant power may, however, be confined to specific subsets of patients. Moreover, in a number of studies the prognostic significance of c-erbB-2 expression appears to be lost 5 or more years from diagnosis.

There has also been considerable interest, of late, in soluble forms of cell-surface receptors. Circulating soluble receptors include soluble forms of the insulin receptor and of the interleukin 2 receptor. Serum levels of soluble interleukin 2 receptor (IL-2R) can be shown to correlate with disease activity in autoimmune disorders (Rubin & Nelson, 1990) and with tumour bulk in certain lymphomas. A soluble, 100 kDa, c-erbB-2 fragment has been detected in the serum and effusions of patients with breast cancer (Mori *et al.*, 1990; Leitzel *et al.*, 1992 and may provide prognostic information in this disease.

We have undertaken a study of 79 patients with both early- and advanced-stage breast cancer in an attempt to evaluate the prognostic significance of elevations of serum soluble c-erbB-2.

Materials and methods

Methods

Blood samples were obtained from 79 patients attending the Breast Clinic of the Johannesburg Hospital between 1986 and 1993. Serum was stored at -20°C until assay. Sampling was

performed at the time of diagnosis of recurrent or metastatic disease. The 110 kDa, serum, soluble c-erbB-2 fragment was measured using a serum c-erbB-2, enzyme-linked immunosorbent assay kit (Triton Diagnostics, Alameda, CA, USA). Briefly, monoclonal anti-c-erbB-2 antibody conjugates were added to aliquots of serum, incubated for 2 h, followed by addition of linking solution and then chromogen substrate. Absorbance was read in a spectrophotometer at 450 nm. Control and calibrator samples were run with each assay. Controls included samples from 24 healthy women falling into the same age range as the patients with breast cancer. The amount of c-erbB-2 protein was calculated from a standard curve. Results are expressed as units per ml of serum. Serum levels $\geq 10 \text{ u ml}^{-1}$ were deemed positive. This level was chosen as being two standard deviations above the mean for healthy women and was also the upper limit for the negative controls supplied with the kit. The antibody has no significant cross-reactivity with epidermal growth factor (EGF), and reacts only with the external domain of the c-erbB-2 molecule. Western blotting of samples with elevated levels confirmed the presence of a 100 kDa protein in serum which showed reactivity with this antibody.

Oestrogen receptor (ER) status and tissue c-erbB-2 were also determined when suitable specimens were available. ER was measured using the ERICA kit (Abbott Laboratories) method according to the manufacturer's instructions. Tissue c-erbB-2 determination was by means of immunohistochemistry using a monoclonal antibody to the external domain of c-erbB-2 (Triton Diagnostics) and a standard avidin-biotin procedure. Briefly, endogenous peroxidase was blocked using methanolic peroxide, and then blocking antibody, primary and control antibodies, secondary antibody, ABC (Vectastain) and diaminobenzidine (DAB) were layered on sequentially. Specimens were deemed positive if clear membrane immunostaining was observed. Suitable positive and negative controls were incorporated into each assay procedure.

Statistical analysis

Disease-free survival, overall survival and survival from disease progression were analysed using SAS statistical software. Additional variables analysed included age, sex, site of disease, initial stage of disease, menopausal status and ER status (where available). Survival curves were generated using the method of Kaplan and Meier (1958), and were compared using the log-rank statistic.

Ethical considerations

All patients gave informed consent prior to entry into the study. The study was approved by the Committee on Ethics

Correspondence: W.R. Bezwoda, Department of Medicine, University of the Witwatersrand Medical School, York Road, Parktown 2193 Johannesburg, South Africa.

Received 9 February 1994; and in revised form 6 May 1994.

of Human Research of the University of the Witwatersrand and was carried out in accordance with the principles of the Declaration of Helsinki.

Results

Patient characteristics

Forty-four out of 79 (56%) patients were post-menopausal at time of diagnosis. The mean age at presentation was 51.4 ± 13.1 (range 24–85) years. Further patient characteristics are shown in Tables I and II.

Serum soluble c-erbB-2

Serum levels of the soluble fraction of c-erbB-2 ranged from 2 to 278 with a mean of 35 ± 57.6 u ml⁻¹. Intra- and inter-assay variation was <2%. Intra-patient variation of serum c-erbB-2 levels, when levels were tested in blood samples from 17 patients who had clinically stable disease and who had two or more separate blood samples taken at intervals of 14 to ≤ 42 days, was also <2%.

Thirty-nine patients (49%) had serum soluble c-erbB-2 levels of ≥ 10 u ml⁻¹. There was no correlation between the presence of elevated serum soluble c-erbB-2 level and menstrual status ($P = 0.66$). There was, however, a significant correlation between serum level and the type of treatment chosen for patients with stage IV disease [27 of 42 (64%) patients receiving chemotherapy were seropositive compared with 11 of 32 (34%) receiving hormonal therapy] (Table II). There was no correlation between the presence of raised serum soluble c-erbB-2 level and any specific site of relapse.

Tissue c-erbB-2 immunostaining

Twenty-four patients had contemporaneous tumour tissue and serum samples available for c-erbB-2 determination. Tis-

sue immunostaining did not correlate with the presence of soluble c-erbB-2 in serum (Table III). The presence of positive tissue immunostaining had no impact on overall survival, time to relapse or on survival from progression ($P = 0.26$).

Oestrogen receptor levels

There was no significant correlation between oestrogen receptor expression among 37 patients of known receptor status and serum soluble c-erbB-2 levels ($P = 0.6$).

Survival and time to relapse

The median overall survival of this cohort of patients from time of initial diagnosis was 44 ± 7.4 months (range 1–254 months). Among the 74 patients who either presented with or who had progressed to stage IV disease, the median survival time from progression was 19 ± 24.7 months (range 1–158). The presence of soluble c-erbB-2 fragment in serum at the time progression was diagnosed had a significant impact on overall survival of these patients. Seropositive patients had a median survival of 21 months vs 64 months for seronegative patients ($P = 0.03$) (Figure 1). The prognostic impact of soluble c-erbB-2 on survival was lost when the analysis was confined to ER-positive patients, possibly because of the low number of such patients.

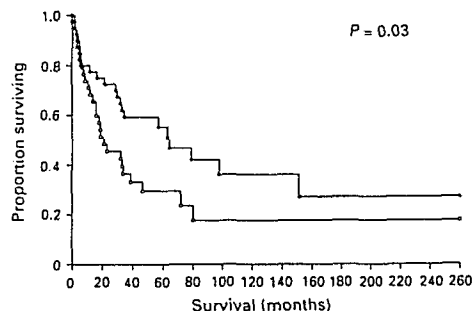


Figure 1 Influence of the presence of soluble c-erbB-2 fragment in the serum on prognosis of patients with breast cancer. Overall survival. ●, Patients with serum soluble c-erbB-2 < 10 u ml⁻¹; ○, patients with serum soluble c-erbB-2 ≥ 10 u ml⁻¹.

Table I Serum soluble c-erbB-2: patient characteristics and seropositivity rate

Patient characteristics	Soluble c-erbB-2				P-value
	Number (%)	Positive Number (%)	Negative Number (%)		
Menopausal status					
Pre	35 (44)	17 (49)	18 (51)		NS
Post	44 (56)	22 (50)	22 (50)		
Stage at presentation					
I	4 (5)	1 (25)	3 (75)		
II	31 (39)	11 (35)	20 (65)		
III	19 (24)	10 (53)	9 (47)		
IV	25 (32)	17 (68)	8 (32)		
Stage at sampling					
IV	74 (94)	39 (49)	40 (51)		
ER status					
Positive	15 (20)	7 (47)	8 (53)		NS
Negative	25 (33)	12 (48)	13 (52)		
Unknown	39 (47)	20 (51)	19 (49)		

NS, not significant.

Table III Breast tumour tissue expression of c-erbB-2 protein and the presence of serum soluble c-erbB-2 fragment

	Serum soluble c-erbB-2 positive	Serum soluble c-erbB-2 negative
Tissue immunostaining		
c-erbB-2 positive	6	4
c-erbB-2 negative	6	8

Table II Serum soluble c-erbB-2: patient characteristics and response to therapy for stage IV disease

	Soluble c-erbB-2				P-value
	Number (%)	Positive Number (%)	Negative Number (%)		
Treatment for stage IV disease					
Hormonal	32 (42)	11 (34)	21 (66)		$P < 0.05$
Chemotherapy	42 (48)	27 (64)	15 (34)		
Response to first-line therapy					
Complete and partial response	40 (52)	20 (50)	20 (50)		NS
No response	34 (43)	18 (53)	16 (47)		

NS, not significant.

Response to therapy

Serum soluble c-erbB-2 had no influence on response to either initial ($P = 0.64$) or salvage treatment for stage IV disease ($P = 0.78$).

Discussion

The c-erbB-2 protein is a 185 kDa transmembrane protein with tyrosine kinase activity. It comprises both an extracellular and an intracellular domain. While the extracellular domain has ligand-binding activity, the ligand has yet to be clearly defined (Maguire & Green, 1989), but is thought to act as a growth factor (Perez *et al.*, 1993). Antibodies to c-erbB-2 have been shown to inhibit both anchorage-dependent and anchorage-independent growth *in vivo* (Xu *et al.*, 1993).

c-erbB-2 has been found to be amplified in 20–30% of primary breast cancers, and gene amplification correlates with oncoprotein overexpression. c-erbB-2's impact on prognosis is, however, somewhat controversial. A number of investigators have reported a correlation between c-erbB-2 amplification and survival in node-positive primary breast cancer (Tandon *et al.*, 1989; Borg *et al.*, 1990). However, both Zhou *et al.* (1989) and Toikkanen *et al.* (1992) failed to demonstrate any impact of c-erbB-2 expression on survival in node-positive patients. Conflicting results have also been reported in node-negative patients (Wright *et al.*, 1989; Paterson *et al.*, 1991). Allred *et al.* (1992) found a highly significant correlation between disease-free survival and c-erbB-2 expression, but only in specific subsets of patients (small tumour size, ER positive and no significant *in situ* component), so-called 'low-risk patients'. Furthermore, while Gusterson *et al.* (1992) found c-erbB-2 immunostaining to have an overall prognostic impact only in patients with node-positive disease, c-erbB-2-positive, node-negative patients receiving adjuvant chemotherapy fared less well in their study than those who were c-erbB-2 negative. These findings tended to suggest that, whatever influence the presence of c-erbB-2 expression has on the biology of breast cancer, this effect is confined to the earlier clinical phases of the illness.

In addition, it has been suggested that c-erbB-2 amplification and protein expression correlate both with poor histological grade and lack of ER expression (Cline *et al.*, 1987; Schroeter *et al.*, 1992). Poller *et al.* (1991) demonstrated that overexpression of c-erbB-2 is significantly correlated with S-phase and proliferative index in ductal carcinoma *in situ* ($P = 0.001$), as well as in early invasive duct carcinoma ($P = 0.04$).

There is also evidence to suggest that c-erbB-2 overexpression may be preferentially associated with certain histological subtypes of breast cancer. Van de Vijver *et al.* (1988) described a high incidence of c-erbB-2 overexpression in large-cell, comedo-type ductal carcinoma *in situ* as compared with invasive ductal carcinoma, suggesting either that the invasive ductal carcinomas that are c-erbB-2 positive are derived from a specific type (large-cell comedo) of ductal carcinoma *in situ* or that c-erbB-2 expression may be lost during tumour invasion and progression. Evidence supporting the first theory is provided by Maguire *et al.* (1992), who found that while tumours with c-erbB-2-negative *in situ* components had

immunonegative invasive components, tumours with immunopositive comedo-type *in situ* components had immunopositive invasive ductal carcinoma. It should be pointed out, however, that the immunostaining was frequently more intense in the *in situ* components than in the invasive carcinomas.

Soluble forms of the c-erbB-2 protein have been reported in the serum of patients with breast cancer, and in addition have been shown to correlate with disease bulk as well as with tissue overexpression in an animal model (Langton *et al.*, 1991). No reports have, however, been published to date on the prognostic significance of soluble c-erbB-2 in patients with breast cancer.

The present study examined a group of 79 women with advanced breast cancer. The method used in this study measured a 100 kDa c-erbB-2 antigen fragment, which is not detected in the serum of normal controls or in patients with benign breast disease (Teramoto *et al.*, 1991). A surprisingly high frequency of elevated serum soluble c-erbB-2 levels was found, possibly because this study included mainly patients with aggressive disease, with 25 patients presenting with advanced breast cancer and all but five of the remainder having progressed to stage IV disease. In addition, there was a statistically significantly higher incidence of seropositivity in patients given chemotherapy as first-line therapy for progressive disease – indicative of the perception that these patients were suffering from aggressive disease. In addition, the presence of serum soluble c-erbB-2 fragment concentrations of $>10 \text{ u ml}^{-1}$ had a significant impact on overall survival from diagnosis of metastatic disease.

The lack of correlation between seropositivity and tissue expression of c-erbB-2 raises some interesting possibilities. While the lack of correlation may be due to low sample number, definite tissue staining was demonstrated in 10 of the 24 patients with tumour samples available for examination. This frequency was, again, a relatively high rate of c-erbB-2 expression. While it may be argued that lack of tissue immunostaining is related to the sensitivity of the method, both tissue-positive and serum-negative as well as tissue-negative and serum-positive cases were found. Among the serum soluble c-erbB-2-negative patients with negative tissue expression there were three patients with extremely high serum levels (range $120\text{--}160 \text{ u ml}^{-1}$) and with extensive disease, while all four patients with negative serum soluble c-erbB-2 tissue and positive tissue staining demonstrated strong positive staining and also had extensive disease. Since both the serum and tissue assays were performed using a monoclonal antibody specific for the external domain of c-erbB-2, these findings suggest the possibility that loss of tissue expression may result from proteolytic cleavage, with release of the external domain and transfer into the blood, rendering the tissue negative to reaction with the antibody used, or that the serum component represents an alternatively spliced variant lacking the membrane domain. This question will be addressed in future studies by using antibodies to both the internal domain as well as to the external domain of the c-erbB-2 molecule.

Whatever the pathophysiological explanation, assay for soluble c-erbB-2 in serum is a relatively simple test, requiring only a blood sample rather than tissue. Soluble c-erbB-2 may offer prognostic information with seropositivity being a predictor of shorter survival in patients with breast cancer.

References

- ALLRED, D.C., CLARK, G.M., TANDON, A.K., MOLINA, R., TORMEY, D.C., OSBORNE, C.K., GILCHRIST, K.W., MANSOUR, E.G., ABELOFF, M., EUDY, L. & MCGUIRE, W.L. (1992). HER-2/neu in node negative breast cancer: prognostic significance of overexpression influenced by the presence of in-situ carcinoma. *J. Clin. Oncol.*, **10**, 1049–1056.
- BORG, A., TANDON, A.K., SIGURDSSON, H., CLARK, G.M., FERNÖ, M., FUQUA, S.A., KILLANDER, D. & MCGUIRE, W.L. (1990). HER-2/neu amplification predicts poor survival in node-positive breast cancer. *Cancer Res.*, **50**, 4332–4337.
- CLARK, G.M. & MCGUIRE, W.L. (1991). Follow-up study of HER-2/neu amplification in primary breast cancer. *Cancer Res.*, **51**, 944–948.
- CLINE, M.J., BATTINFORA, H. & YOKOTA, J. (1987). Proto-oncogene abnormalities in human breast cancer: correlation with anatomic features and clinical course of disease. *J. Clin. Oncol.*, **7**, 999.

- GUSTERSON, B.A., GELBER, R.D., GOLDBIRSH, A., PRICE, K.N., SÄVE-SÖDERBORGH, J., ANBAZHAGAN, R., STYLES, J., RUDENSTAM, C.M., GOLOUHI, R. & REED, R. (1992). Prognostic importance of c-erbB-2 expression in breast cancer. *J. Clin. Oncol.*, **10**, 599-605.
- KAPLAN, E.L. & MEIER, P. (1958). Non-parametric estimations from incomplete observations. *J. Am. Stat. Soc.*, **53**, 457-481.
- LANGTON, B.C., CRENSHAW, M.C., CHAO, L.A., STUART, S.G., AKITA, R.W. & JACKSON, J.E. (1991). An antigen immunologically related to the external domain of gp 185 is shed from nude mouse tumours overexpressing the c-erbB-2 (HER-2/neu) oncogene. *Cancer Res.*, **51**, 2593-2598.
- LEITZEL, K., TERAMOTO, Y., SAMPSON, E., MAUCERI, J., LANGTON, B.C., DEMERS, L., PODCZASKI, E., HARVEY, H., SHAMBAUGH, S., VOLAS, G., WEAVER, S. & LIPTON, A. (1992). Elevated soluble c-erbB-2 antigen levels in the serum and effusions of a proportion of breast cancer patients. *J. Clin. Oncol.*, **10**, 1436-1443.
- MAGUIRE, H.C. & GREEN, M.I. (1989). The neu (c-erbB-2) oncogene. *Semin. Oncol.*, **16**, 148-155.
- MAGUIRE, H.C. Jr, HELLMAN, M.E., GREENE, M.I. & YEH, I. (1992). Expression of c-erbB-2 in in-situ and in adjacent invasive ductal adenocarcinomas of the female breast. *Pathobiology*, **60**, 117-121.
- MORI, S., MORI, Y., MUKAIYAMA, T., YAMADA, Y., SONOBE, Y., MASUSHITA, H., SAKAMOTO, G., AKIYAMA, T., OGAWA, M. & SHIRAIISHI, M. (1990). In-vitro and in-vivo release of soluble erbB-2 protein from human carcinoma cells. *Jpn. J. Cancer Res.*, **81**, 489-494.
- PATERSON, M.C., DIETRICH, K.D., DANYLUK, J., PATERSON, A.H., LEES, A.W., MAJIE, N., HANSON, J., JENKINS, H., KRAUSE, B.E. & MCLAIN, W.A. (1991). Correlation between c-erbB-2 amplification and risk of recurrent disease in node negative breast cancer. *Cancer Res.*, **51**, 556-567.
- PEREZ, C., CHO, C., NERI, C., LIPPMAN, M.E. & LUPU, R. (1993). Characterization and cloning of the gp30 ligand for the c-erbB-2 receptor, from human breast cancer cells (abstract). *Proc. Am. Assoc. Cancer Res.*, **34**, 97.
- POLLER, D.N., GALEA, M., PEARSON, D., BELL, J., GULLICK, W.J., ELSTON, C.W., BLAMEY, R.W. & ELLIS, I.O. (1991). Nuclear and flow cytometric characteristics associated with overexpression of the c-erbB-2 oncoprotein in breast carcinoma. *Breast Cancer Res. Treat.*, **20**, 3-10.
- RUBIN, L.A. & NELSON, D.L. (1990). The soluble interleukin-2 receptor: biology, function and clinical application. *Ann. Intern. Med.*, **113**, 619-627.
- SCHROETER, C.A., DE POTTER, C.R., RATHSMAN, K., WILLIG-HAGEN, R.G.J. & GREER, J.C. (1992). C-erbB-2 positive tumours behave more aggressively in the first years after diagnosis. *Br. J. Cancer*, **66**, 728-734.
- TANDON, A.K., CLARK, C.M., CHAMNESS, G.C., TANDON, A.K., CLARK, G.M., CHAMNESS, G.C., ULRICH, A. & MCGUIRE, W.L. (1989). HER-2/neu oncogene protein and prognosis in breast cancer. *J. Clin. Oncol.*, **7**, 1120-1128.
- TERAMOTO, Y., WALLINGFORD, S., MAUCERI, J. & SAMPSON, E. (1991). Serum enzyme immunoassay kit for the detection of c-erbB-2 oncoprotein. Proceedings of the Annual American Association of Cancer Research Meeting No. 32, 243 (A1446). Waverly Press: Baltimore.
- TOIKKANEN, S., HELIN, H., ISOLA, J. & JOENSUU, H. (1992). Prognostic significance of HER-2 oncoprotein expression in breast cancer: a 30 year follow-up. *J. Clin. Oncol.*, **10**, 1044-1048.
- VAN DE VIJVER, M.J., PETERSE, J.L., MOOI, W.J., WISMAN, P., LOMANS, J., DALESIO, O. & NUSSE, R. (1988). Neu-protein overexpression in breast cancer. *N. Engl. J. Med.*, **319**, 1239-1245.
- WRIGHT, C., ANGUS, E., NICHOLSON, S., SAINSBURY, J.R., CAIRNS, J., GULLICK, W.J., KELLY, P., HARRIS, A.L. & HORNE, C.H. (1989). Expression of c-erbB-2 oncoprotein: a prognostic indicator in human breast cancer. *Cancer Res.*, **49**, 2087-2090.
- XU, F., LUPU, R., RODRIGUEZ, G.C., WHITAKER, R.S., BOENTE, M.P., BERCHUCK, A., YU, Y., DESOMBRE, K.A., BOYER, C.M. & BAST Jr, R.C. (1993). Antibody induced growth inhibition is mediated through immunochemically and functionally distinct epitopes on the extracellular domain of the c-erbB-2 (HER-2/neu) gene product p185. *Int. J. Cancer*, **53**, 401-408.
- ZHOU, D.J., AHUJA, H. & CLINE, M.J. (1989). Proto-oncogene abnormalities in human breast cancer: c-erbB-2 amplification does not correlate with recurrence of disease. *Oncogene*, **4**, 105-108.

4.6 Paper 11

P-GLYCOPROTEIN IMMUNOSTAINING CORRELATES WITH ER AND WITH HIGH Ki67
EXPRESSION BUT FAILS TO PREDICT ANTHRACYCLINE RESISTANCE IN PATIENTS WITH
ADVANCED BREAST CANCER.

L. Seymour, W.R. Bezwoda, R.D. Dansey.

Breast Cancer Research and Treatment. 36:61-69.1995.

Report

P-glycoprotein immunostaining correlates with ER and with high Ki67 expression but fails to predict anthracycline resistance in patients with advanced breast cancer

Lesley Seymour, Werner Robert Bezwoda, Roger Delemere Dansey

Division of Clinical Haematology and Medical Oncology, Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa

Key words: breast cancer, p-glycoprotein, estrogen receptor, Ki67, drug resistance

Summary

In an attempt to further define the clinical utility of p-glycoprotein immunostaining in breast cancer, we examined 101 specimens from patients with advanced breast cancer. There was a significant correlation between estrogen receptor status and p-glycoprotein expression but only for low levels of p-glycoprotein. Premenopausal status appeared to correlate with increased p-glycoprotein expression, but this probably reflects patient selection as premenopausal patients had higher prior exposure to anthracyclines and were more likely to have received chemotherapy as initial treatment. P-glycoprotein expression was highly significantly correlated with expression of the proliferation related antigen Ki67, suggesting that p-glycoprotein expression may well be cell cycle dependent, with overexpression occurring in rapidly cycling cells. These findings may explain reported findings of modulation of p-glycoprotein expression by agents such as anti-oestrogens. P-glycoprotein positive staining did not, however, predict chemotherapy treatment failure or survival duration.

Introduction

Recurrent or metastatic breast cancer remains an incurable disease. Although many patients with advanced breast cancer show clinical response to cytotoxic chemotherapy, these responses are often of short duration. In addition the clinical course is usually marked by the development of acquired resistance to previously effective cytotoxic drugs, usually culminating in refractory or progressive disease which is ultimately fatal.

This acquired or progressive multidrug resistance may be mediated by overexpression of a transmembrane transport protein, p-glycoprotein [1, 2].

the presence of which can be evaluated by immunohistochemical methods.

The occurrence of p-glycoprotein overexpression has been linked to the development of drug resistance in some haematologic malignancies such as acute non-lymphoblastic leukaemia [3]. High levels of expression have also been reported in inherently drug resistant tumours such as cervix cancer [4]. In addition, Chan and co-workers [5] found that p-glycoprotein positivity is a significant negative prognostic factor in children with soft tissue sarcomas.

The role of p-glycoprotein in human breast cancer is unclear. While some workers report that p-glycoprotein immunostaining in patients with locally advanced breast cancer predicts failure to re-

spond to chemotherapy as well as a shortened survival [6, 7], others have failed to demonstrate any utility for p-glycoprotein immunostaining [8]. All of these studies, so far, have involved quite small (20–40) numbers of patients.

We have undertaken a study of p-glycoprotein immunostaining in a cohort of 101 patients with advanced breast cancer, in an attempt to more clearly define the prognostic value of this investigation.

Material and methods

Immunohistochemical methods

Tissue was obtained by punch biopsy of accessible, recurrent/metastatic lesions and flash frozen at -135°C until use. Frozen sections were prepared and fixed in picric-acid-paraformaldehyde. Specimens were then washed, immersed in methanol-peroxide to block endogenous peroxidase, and then routinely immunostained using the p-glycoCHEK C219 kit (Centocor Diagnostics). This kit uses a mouse monoclonal antibody directed against an epitope located on the inner aspect of the cell membrane, thus requiring tissue fixation to allow antibody access. Staining is demonstrated using biotinylated anti-mouse antibody and an avidin-biotin horseradish peroxidase staining system. Specimens were ex-

amined under light microscopy for the presence of immunostaining, and scored for the purposes of this study as percentage of cells displaying clear membrane immunostaining. Positive controls included human ovarian cancer cell lines known to express p-glycoprotein. Negative controls consisted of:

(a) samples where non-immune mouse serum was substituted for specific C219 antibody

(b) normal non-lactating breast tissue which has, in our hands, given consistently negative staining.

To be considered p-glycoprotein positive, $\geq 5\%$ of malignant cells from a total of 20 high power fields examined had to show clear membrane staining. This level was chosen as the cut off value since repeated blind assessments showed that inter and intra observer variation was $< 5\%$ (range 2–4%). It was thus considered that $\geq 5\%$ provided a reliable index of definite positive staining. While an attempt was made to assess the intensity of staining, it was found that staining intensity correlated so closely with percentage of cells showing staining that the single index, percentage positive cells, was utilised for subsequent analyses.

In addition, sections from the same specimens used for p-glycoprotein assessment were examined immunohistochemically for oestrogen receptor (ER), progesterone receptor (PR), and Ki67 using previously described methods [9, 10]. Data regard-

Table 1. P-glycoprotein expression in advanced breast cancer: patient characteristics at biopsy

	Number	Percentage
Patients with local recurrence/metastatic disease	101	100%
Menopausal status		
Pre	40	40%
Post	57	56%
Male	4	4%
Histology		
Inflammatory breast cancer	13	13%
Infiltrating ductal cancer	48	47%
Adenocarcinoma n.o.s.	40	40%
Previously untreated	56	55%
Prior adjuvant treatment	45	45%
Postmenopausal		
Tamoxifen	32	56
Anthracycline containing adjuvant chemotherapy regimen	4	7%
Premenopausal		
Adjuvant tamoxifen + anthracycline containing adjuvant chemotherapy regimen	13	32%

ing correlations between these variables are thus contemporaneous.

Patient characteristics

101 patients with breast cancer were studied at the time of first recurrence/metastasis (Table 1). Of these, 57 patients (56%) were post menopausal and 40 patients were premenopausal. There were 4 male patients. 56 patients (56%) were previously untreated, while 45 patients were receiving adjuvant tamoxifen treatment at the time of recurrence and biopsy. Seventeen (38%) of these 45 patients who were receiving hormonal therapy at the time of biopsy had in addition had previous exposure to adjuvant chemotherapy containing anthracyclines. Further details of adjuvant therapy are shown in Table 1.

Postmenopausal patients had a significantly lower prior exposure to anthracyclines (4 of 57, 7%) than did premenopausal patients (13 of 40, 32%, $p = 0.01$), and were more likely (32 of 57, 56%) to have

received hormonal therapy than premenopausal patients (13 of 40, 32%, $p = 0.02$). The 4 male patients were all previously untreated.

Following the diagnosis of recurrence/metastasis and biopsy assessment, 90 patients received systemic treatment (Table 2). This included systemic chemotherapy in 52 patients and second line hormonal treatment in 38. Response to treatment for recurrent/metastatic disease was assessable in 4 patients. Reasons for unassessability were lost follow-up (2 patients) and no assessable disease after biopsy (4 patients).

Response criteria

Response was assessed on the basis of criteria proposed by the Eastern Co-operative Oncology Group [11].

Table 2. P-glycoprotein expression in advanced breast cancer: disease sites and treatment

	Number	Percent	Number responding	Percent responding
Assessable patients receiving systemic treatment	84	100		
Prior adjuvant therapy	44	55		
Previously untreated	40	45		
Chemotherapy	48	57	20	42
Adriamycin + Vinca alkaloid	20	24	9	45
CNV ^a	18	21	8	44
MTF ^b	6	7	2	33
CMF ^c	4	5	1	25
Hormonal therapy	36	43	10	28
Aminoglutethamide	10	12	5	30
Gestagens	20	24	5	25
Testosterone	6	7	2	33
Sites of recurrence/metastasis				
Soft tissue	84	100	30	35
Bone	18	21	4	22
Lung	12	14	6	50
Liver	8	10	2	25
CNS ^d	1	1	-	-

^a CNV = cyclophosphamide, mitoxantrone, vincristine.

^b MTF = mitomycin C, thiotepa, 5 fluoro-uracil.

^c CMF = cyclophosphamide, methotrexate, 5 fluoro-uracil.

^d CNS = CNS lesion not assessable due to concurrent radiation therapy.

Table 3. Immunocytochemical detection of p-glycoprotein in patients with advanced breast cancer: degree of staining of malignant cells

Percentage of malignant cells showing immunostaining	Number of patients	p-glycoprotein phenotype
< 5%	45 (45%)	negative
5–10	6 (6%)	low
10–33	6 (6%)	
34–65	28 (28%)	midrange
66–100	16 (16%)	high

Statistical analyses

Statistical analyses were performed using SAS statistical software. Statistical tests employed were χ^2 analyses, parametric (Pearson correlation coefficient) and non parametric correlation analyses (Mann-Whitney U), and analyses of time to treatment failure and survival. Variables examined for relationship to p-glycoprotein expression and to survival included age, sex, site of disease, menopausal status, hormone receptor status, previous anthracycline exposure, and subsequent treatment. Survival curves were generated using the method of Kaplan-Meier and were compared using the log-rank statistic.

Ethical considerations

All patients gave informed consent to participation and biopsy, prior to entry into the study. The study was approved by the Committee on Ethics of Human Research of the University of the Witwatersrand and was carried out in accordance with the principles of the Declaration of Helsinki.

Results

P-glycoprotein immunostaining

56 (55%) of the patients studied displayed $\geq 5\%$ malignant cells showing immunostaining for p-glycoprotein. Further analysis of the distribution of staining intensity suggested 3 groups of patients with positive immunostaining for p-glycoprotein: low positive ($< 1/3$ of cells showing staining); mid

range (with between $1/3$ – $2/3$ of malignant cells showing positivity); and high positive (with $> 2/3$ cells showing positive staining). The majority were mid range positive. Only 16 (16%) of tumours had $> 2/3$ positive cells (Table 3), and only 6 had 100% positive cells.

There was a significant overall correlation between ER positivity and p-glycoprotein positivity ($p = 0.027$) (Table 4). There was, however, no corre-

Table 4. Immunocytochemical detection of p-glycoprotein in patients with advanced breast cancer: overall correlation with patient determinants

	P-glycoprotein		p value
	positive	negative	
Oestrogen receptor			
Positive	29	13	0.027
Negative	27	32	
Menopausal status			
Pre	22	18	0.038
Post	34	23	
Progesterone receptor			
Positive	16	7	N.S.
Negative	36	35	
Unknown	4	3	
Prior adjuvant anthracyclines			
Yes	13	4	N.S.
No	43	41	
Ki67 expression			
$< 20\%$	37	33	N.S.
$> 20\%$	19	12	
Prior adjuvant tamoxifen			
Yes	28	17	N.S.
No	26	30	
Histologic type			
Infiltrating duct cancer	25	23	N.S.
Adenocarcinoma n.o.s.	23	17	
Inflammatory	8	5	

N.S. = Not significant.

lation between the level of p-glycoprotein staining and ER expression (Table 5). There was also no significant quantitative relationship between progesterone receptor levels and percentage p-glycoprotein staining ($p = 0.12$). Premenopausal patients had a significantly higher frequency of p-glycoprotein staining than postmenopausal patients ($p = 0.038$). Interestingly, all 4 male patients displayed no immunostaining for p-glycoprotein.

In addition to the findings in the 101 patients at the time of presentation with recurrent/metastatic disease, there were 9 patients who were initially p-glycoprotein positive, were treated with hormone therapy, who had follow-up biopsies which allowed

Table 5. Immunocytochemical detection of p-glycoprotein in advanced breast cancer: correlation of extent of immunostaining among p-glycoprotein positive ($\geq 5\%$ cells) tumours and other patient determinants

	Oestrogen receptor		p-value
	positive	negative	
P-glycoprotein positivity			
Low	10	2	NS
Mid	12	16	
High	7	9	
	Menopausal status		p-value
	pre	post	
Low	4	8	NS
Mid	13	15	
High	7	9	
	Prior anthracycline		
	yes	no	
Low	0	12	NS
Mid	1	27	
High	12	4	
	Ki67		
	low ($< 20\%$)	high ($\geq 20\%$)	
Low	6	6	$p = 0.038$
Mid	21	7	
High	4	12	

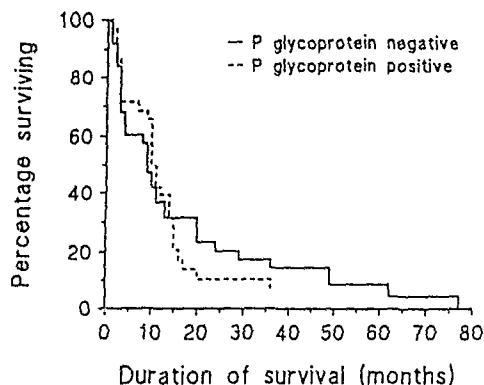


Fig. 1. Survival duration in metastatic breast cancer: — p-glycoprotein negative; --- p-glycoprotein positive patients.

for determination of whether hormone therapy (tamoxifen) affected p-glycoprotein staining. In 4 of these patients (44%) the degree of p-glycoprotein expression was reduced and in 5 (56%) it remained the same.

Patients who had been previously exposed to anthracyclines showed a trend for increased frequency of p-glycoprotein expression (13 of 17 (76%) vs. 43 of 84 (51%) not previously exposed to anthracyclines but this did not reach statistical significance ($p = 0.09$).

There was no overall correlation between Ki67 levels and p-glycoprotein staining. However, very high Ki67 ($\geq 20\%$) expression correlated significantly with high ($> 2/3$ cells positive) p-glycoprotein expression (Table 5).

Response to treatment

84 patients were evaluable for response to further therapy. Post biopsy treatment included chemotherapy in 48 patients, including 20 patients treated with anthracyclines, the others mostly receiving a combination of cyclophosphamide, mitoxantrone, and vincristine (CNV; 18 patients), while 6 received mitomycin C, thiotepa, plus 5 fluoro-uracil, and 4 received cyclophosphamide, methotrexate, and 5 fluoro-uracil (CMF). Thirty six patients received hormone treatment (aminoglutethamide 20; pro-

gestogens 10; testosterone 6). Thirty patients (35%) responded to treatment, of whom 12 (14%) were complete responders. Response to the chemotherapy was seen in 20/48 (42%; 7 CR plus 13 PR). Response to hormone therapy occurred in 10/36 (28%; 6 CR and 4 PR). P-glycoprotein immunostaining at any level failed to impact on overall response to treatment ($p = 0.34$), or on response to chemotherapy ($p = 0.86$) or to hormonal therapy ($p = 0.14$). Specifically, p-glycoprotein immunostaining did not predict failure to respond to anthracycline-containing chemotherapy ($p = 0.65$). When response to chemotherapy was analysed according to both ER status and p-glycoprotein staining the results showed 9/11 (82%) of ER+, p-glycoprotein positive patients to be responders whereas 11/14 (78%) ER-, p-glycoprotein positive patients failed to respond ($p < 0.01$). There was insufficient data on p-glycoprotein negative patients who received chemotherapy to determine the influence of ER status on response to chemotherapy in this subgroup.

Expression of Ki67 at $> 20\%$ correlated significantly with failure of response to therapy, with only 3/24 (12.5%) of patients with Ki67 expression $> 20\%$ achieving a response as compared to a 27/60 (45%) response rate in patients with $< 20\%$ Ki67 expression ($p < 0.01$).

Survival

The median survival of this cohort of patients was 8.9 months. The presence or absence of p-glycoprotein immunostaining had no impact on survival ($p = 0.77$) (Fig. 1). Further analysis showed no effect on survival or on time to treatment failure of either low, intermediate, or high p-glycoprotein staining, nor did p-glycoprotein staining of any degree influence the survival of, specifically, those patients who received cytotoxic combination chemotherapy ($p = 0.82$), of those who received anthracycline-containing chemotherapy ($p = 0.67$), or of those who received hormonal therapy ($p = 0.92$).

Ki67 expression ($\geq$ or $< 20\%$ had a significant influence on time to treatment failure (TTF for patients with Ki67 $> 20\% = 4.1$ months; TTF of patients with Ki67 $< 20\% = 9.3$ months). Ki67 expres-

sion did not, however, significantly influence survival.

Discussion

Intrinsic and/or acquired drug resistance is a major therapeutic problem limiting the treatment of patients with advanced or metastatic breast cancer. While many patients do respond to combination chemotherapy, the responses are frequently short-lived, and clinical drug resistance emerges. One of the causes of this emergence of multidrug resistance may be overexpression of the *mdr-1* gene encoding p-glycoprotein.

The transmembrane transport protein p-glycoprotein has ATPase activity, and is thought to act as a cellular efflux pump for a number of chemotherapeutic agents as well as for other potentially toxic substances. It is encoded in human cells by the *mdr1* gene [12, 13]. Mammalian p-glycoprotein is partially homologous with other membrane transport related proteins such as the gene associated with chloroquine resistance in *Plasmodium falciparum* [14]. P-glycoprotein overexpression mediates classical multidrug resistance, which is marked by the development of resistance to anthracyclines, vinca alkaloids, and the epipodophyllotoxins.

The precise prognostic role of p-glycoprotein in human breast cancer remains to be defined. Verrelle and co-workers [6] reported that p-glycoprotein immunostaining, in patients with locally advanced breast cancer, predicts failure to respond to chemotherapy as well as a shortened survival. A similar prediction of chemotherapy resistance was reported by Ro and co-workers [7].

However Dixon and co-workers [8] failed to demonstrate any utility for p-glycoprotein immunostaining in a group of 26 patients with locally advanced breast tumours because of a failure to find convincing positive staining in any patient.

While p-glycoprotein can be demonstrated in vitro to be associated with drug resistance, there may be other potentially important associations, which may be open to manipulation. For example, p-glycoprotein has been reported to be associated with reduced expression of steroid hormone receptors

and increased expression of the epidermal growth factor receptor. Clarke and co-workers [15] found, however, that while transduction of the *mdr-1* gene into MCF cells result in neither cross resistance to anti-oestrogens nor loss of steroid hormone receptor expression. Kirk and co-workers [16] report that anti-oestrogens may partially reverse the multidrug resistant phenotype in p-glycoprotein positive cell lines, and Lonn and co-workers [17] report that the development of *mdr-1* overexpression is reduced in patients receiving anti-oestrogens when compared to patients who receive other hormonal manipulation. These findings accord with the clinical results found in patients treated by means of hormone therapy in this study. Extensive work has also been done on the reversal of p-glycoprotein mediated drug resistance using calcium channel antagonists [18]. It would thus seem that a therapeutic potential exists for *in vivo* manipulation of the multidrug resistant phenotype using agents such as tamoxifen or verapamil.

The present study attempted to define the role of immunostaining for p-glycoprotein expression in predicting both response to chemotherapeutic agents (particularly the anthracyclines but also other agents) as well as the relationship between p-glycoprotein and other prognostic determinants such as ER, menstrual status, and measures of proliferation.

Of note in this study was the relatively high proportion of patients (55%) who showed positive staining for p-glycoprotein. This proportion appears higher than the positivity rates (approximately 15–20%) found in patients with sarcomas and in paediatric patients. Such high rates of positive p-glycoprotein staining may relate to the nature of the disease being studied. The patients included in this study were all patients with advanced disease. Botti and co-workers [20] using the same antibody also found a high proportion (76%) of patients with positive p-glycoprotein staining in patients with locally advanced breast cancer. Although these workers used different percentage positive values to define low, medium, and high p-glycoprotein staining groups, it should be pointed out that in their study, paraffin fixed sections were used while the present study looked at fresh frozen tissue. Our experience

has been that paraffin fixation reduces the intensity of positive staining but not the overall proportion of patients showing positive p-glycoprotein expression.

Despite these differences in methods, however, the proportion of patients with low, medium, and high frequency positive staining appears to be very similar in the two studies. Both studies thus show a high frequency of p-glycoprotein staining in advanced breast cancer. The difference between the present study and that of Botti and co-workers is in the relationship between p-glycoprotein staining and response to treatment. Botti and co-workers found an inverse relationship between p-glycoprotein staining and response to chemotherapy which was not found in this study. The present study does, however, suggest that ER negative p-glycoprotein positive patients fail to respond to chemotherapy. Since there was no overall correlation between response to chemotherapy and P-glycoprotein expression, the results in this particular subset of patients would appear to indicate some interaction between ER expression and P-glycoprotein function. It should be pointed out in this regard that the patients of Botti and co-workers were selected for ER negativity, which possibly introduced a selection bias.

Somewhat unexpectedly, p-glycoprotein immunostaining was found to show an overall correlation with the presence of the ER. This correlation appeared to be at variance with the finding that patients who were postmenopausal at time of biopsy were significantly less likely to express p-glycoprotein than premenopausal patients. One possible explanation of the lower frequency of p-glycoprotein staining in postmenopausal patients is the fact that postmenopausal patients are in general less likely to receive chemotherapy (either as first line or as adjuvant treatment), and indeed, in this cohort of patients, postmenopausal women had a significantly lower incidence of previous exposure to chemotherapy and in particular to anthracyclines. This may have led to less selection pressure on the tumour cell population. However, while a higher proportion of patients who had previously been exposed to anthracycline therapy expressed p-glyco-

protein than patients not so exposed, this difference did not reach statistical significance.

On further analysis, however, the correlation between p-glycoprotein expression and ER was lost when the results were expressed quantitatively or semi quantitatively. The precise significance of the relationship between low frequency p-glycoprotein expression and ER is thus unclear.

Another interesting finding was the highly significant correlation between high levels of p-glycoprotein expression in tumours displaying a high Ki67 staining index. Ki67 is a murine monoclonal antibody that reacts with a human nuclear antigen present in proliferating cells. Ki67 antigen is expressed during G1, S, G2 and mitosis, but not in G0, and represents the growth fraction of the tumour cell population [19]. This finding suggests that high expression of p-glycoprotein in tumour cells may be cell cycle and growth fraction related. This may be a possible explanation for the reported finding of reduced p-glycoprotein expression in patients treated with anti-oestrogens, as such agents are known to reduce the growth fraction of hormone responsive tumour cells [21].

The question arises as to why there was this lack of correlation between p-glycoprotein immunostaining and any of the clinical parameters of response, since there is no doubt that cellular retention of chemotherapeutic drugs is one of the determinants of chemosensitivity and since moreover a number of studies using cell lines have demonstrated a correlation between p-glycoprotein staining and accelerated rates of drug efflux. One possible reason may be the nature of the antibody used. It should be pointed out, however, that high positive staining with the C219 antibody does correlate, in our laboratory, with cell lines showing the multidrug resistant phenotype, and p-glycoprotein staining with C219 was present in cell lines demonstrating more rapid rates of efflux of doxorubicin and vincristine when tested *in vitro*. These findings suggest that immunologic demonstration of the presence of p-glycoprotein by the C219 antibody may not be sufficient to define functional activity *in vivo*. Functional activity may well be modulated by additional influences such as hormonally induced perturbations of the cell membrane as well as the prolifer-

ative state of the cells in question. A number of such factors probably interact *in vivo* and these together with the other factors that modulate chemotherapy drug sensitivity make the mere immunologic demonstration of p-glycoprotein staining alone an unreliable method of assessing the potential response to treatment.

In conclusion, we have found that while patients who have had prior exposure to anthracyclines may have a higher frequency of expression of p-glycoprotein as detected by immunologic means, p-glycoprotein staining per se does not predict treatment failure, and offers only limited additional prognostic information. When coupled with ER, p-glycoprotein staining may, however, be more significant, suggesting that the functional activity of this protein is affected by hormone receptor status. In addition, p-glycoprotein immunoreactivity appears to be cell cycle dependent, with overexpression occurring in rapidly cycling cells. Both of these observations may explain reported findings of modulation of p-glycoprotein expression by agents such as anti-oestrogens.

References

1. Beck-Hansen NT, Till JE, Ling V: Pleiotropic phenotype of colchicine resistant CHO cells: cross resistance and collateral sensitivity. *J Cell Phys* 88: 23-32, 1976
2. Bhalla K, Hindenburg A, Taub RN, Grant S: Isolation and characterisation of an anthracycline resistant human leukaemic cell line. *Cancer Res* 45: 3657-3662, 1985
3. Ma DDF, Davey RA, Harman DH, Isbister JP, Scurr RD, Makertich SM, Dowden G, Bell DR: Detection of a multidrug resistant phenotype in acute non-lymphoblastic leukaemia. *Lancet* i: 135-137, 1987
4. Schneider J, Efferth T, Mattern J, Rodriguez-Escudero FJ, Volm M: Immunohistochemical detection of the multi-drug resistance marker p-glycoprotein in uterine cervical carcinomas and normal cervical tissue. *Am J Obstet Gynaecol* 166: 825-829, 1992
5. Chan HS, Thorner PS, Hadda G, Ling V: Immunohistochemical detection of p-glycoprotein: prognostic correlation in soft tissue sarcoma of childhood. *J Clin Oncol* 8: 689-704, 1990
6. Verrelle P, Meissonnier F, Fonck Y, Feillel V, Dionet C, Kwiatkowski F, Plagne R, Chassagne J: Clinical relevance of immunohistochemical detection of multidrug resistance p-glycoprotein in breast carcinoma. *J Natl Cancer Inst* 83: 111-116, 1991

7. Ro J, Sahin A, Ro H, Fritsche H, Hortobagyi G, Blick M: Immunohistochemical analysis of p-glycoprotein expression correlated with chemotherapy resistance in locally advanced breast cancer. *Human Pathol* 21: 787-791, 1990
8. Dixon AR, Bell J, Ellis IO, Elston CW, Blamey RW: P-glycoprotein expression in locally advanced breast cancer treated by neoadjuvant chemotherapy. *Brit J Cancer* 66: 537-541, 1992
9. Seymour L, Meyer K, Esser J, MacPhail P, Behr A, Bezwoda WR: Estimation of PR and ER by immunocytochemistry in breast cancer. *Am J Clin Pathol* 94: 35-40, 1990
10. Seymour L, Bezwoda WR, Meyer K: Tumour factors predicting for prognosis in metastatic breast cancer. *Cancer* 66: 2390-2394, 1990
11. Hayward JL, Rubens RD, Carbone PP, Heuson JC, Kamaoka S, Segaloff A: Assessment of response to therapy in advanced breast cancer. *Brit J Cancer* 35: 292-298, 1977
12. Hamada H, Tsuruo T: Purification of the 170-180 kilodalton membrane glycoprotein associated with multidrug resistance. 170-180 kilodalton membrane glycoprotein is an ATPase. *J Biol Chem* 263: 1454-1458, 1988
13. Chen C, Chin JE, Ueda K, Clark D, Pastan I, Gottesman M, Roninson I: Internal duplication and homology with bacterial transport proteins in the *mdr-1* (P-glycoprotein) gene from multidrug resistant human cells. *Cell* 47: 381-389, 1986
14. Wilson CM, Serrano AE, Wasley A, Bogenschutz MP, Shankar A, Wirth DF: Amplification of a gene related to mammalian *mdr* genes in drug resistant *Plasmodium falciparum*. *Science* 224: 1184-1186, 1989
15. Clarke R, Currier S, Kaplan D, Lovelace E, Boulay V, Gottesman M, Dickson RB: Effect of p-glycoprotein expression on sensitivity to hormone in MCF-7 human breast cancer cell lines. *J Natl Cancer Inst* 84: 1506-1512, 1992
16. Kirk J, Houlbrook S, Stuart NS, Stratford IJ, Harris AL, Carmichael J: Differential modulation of doxorubicin toxicity to multidrug and intrinsically drug resistant cell lines by anti-oestrogens and their major metabolites. *Brit J Cancer* 67: 1189-1195, 1993
17. Lonn U, Lonn S, Stenkvist B: Reduced occurrence of *mdr-1* amplification in stage IV breast cancer patients treated with tamoxifen compared with other endocrine patients. *Int J Cancer* 53: 574-578, 1993
18. Dalton WS, Grogan TM, Meltzer PS: Drug resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of p-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *J Clin Oncol* 7: 415-424, 1989
19. Gerdes J, Li L, Schlueter C, Duchrow M, Wohlenberg C, Gerlach C, Stahmer I, Kloth S, Brandt E, Flad H: Immunobiochemical and molecular biologic characterization of the cell proliferation associated nuclear antigen that is defined by monoclonal antibody Ki67. *Am J Pathol* 138: 867-873, 1991
20. Botti G, Chippetta G, D'Aluto G, De Angelis E, de Matteis A, Montella M, Picone A, Cascione F: PCNA/cyclin and p-glycoprotein as prognostic factors in locally advanced breast cancer: an immunohistochemical, retrospective study. *Tumori* 79: 214-218, 1993
21. Darbre P, Curtis S, King RJB: Effects of estradiol and tamoxifen on human breast cancer cells in serum free culture. *Cancer Res* 44: 2790-2793, 1984

CHAPTER 5 - TRANSLATIONAL STUDIES CORRELATING CLINICAL OBSERVATIONS

- 5.1 Paper 12 - Sequential Alpha-Interferon and Tamoxifen: in vitro Biologic Effects of Two Growth Modulators
- 5.2 Paper 13 - Sequential α -interferon and Tamoxifen: in vitro Reversal of MDK-1 Mediated Multidrug Resistance in the MCF-7 Cell Line.

5.1 Paper 12

SEQUENTIAL ALPHA-INTERFERON AND TAMOXIFEN: IN VITRO BIOLOGIC EFFECTS OF TWO

GROWTH MODULATORS

L. Seymour, W.R. Bezwoda.

International Journal of Oncology 10:1277-1282, 1997.

Sequential α -interferon and tamoxifen: *in vitro* biologic effects of two growth modulators

L. SEYMOUR¹ and W.R. BEZWODA

Division of Clinical Haematology and Medical Oncology, Department of Medicine,
University of the Witwatersrand, South Africa

Received February 24, 1997; Accepted April 10, 1997

Abstract. Tamoxifen and α -interferon have interesting and complementary biologic effects which may be relevant for breast cancer growth and regression. The hormone responsive cell line MCF-7 was used as a model to study the biologic effects of sequential administration of these two agents. Interferon had a significant antiproliferative effect and increased ER and TGF- β expression. The combination had at least additive antiproliferative effects as well as effects on expression of ER, TGF- β , *c-erbB-2*, P24 and Ki67. α -IFN modulates TMX induced biologic effects and the sequential administration of α -IFN and TMX may lead to potentially important modulation of biologic endpoints. Further studies are appropriate.

Introduction

Advanced breast cancer (ABC) remains a largely incurable disease, although effective palliation can frequently be achieved using either hormonal or combination cytotoxic chemotherapy. Nonetheless most patients ultimately progress and die from their disease. Modulation of standard therapy in an attempt to improve outcomes is an attractive idea and has received considerable attention. Earlier attempts at therapeutic modulation included estrogen/anti-estrogen synchronization (1,2) and estrogenic recruitment (3-5) to potentiate the effect of conventional cytotoxic chemotherapy. These approaches have generally not proven successful.

Tamoxifen (TMX) is widely used in the management of ABC, and appears an attractive agent for modulation because of its diverse cellular effects. α -interferon (α -IFN) in addition has been shown to have a number of interesting biologic effects in hormone responsive breast cancer cell lines (MCF-7,

ZR-75). These include enhancement of TMX induced growth inhibition as well as an increase in estrogen receptor (ER) expression. Clinical and preclinical (6,7) data suggest that the combination of α -IFN and TMX may offer a therapeutic advantage in the treatment of patients with ABC. We have previously shown that treatment with α -IFN consistently increased the ER and P24 expression *in vivo* (8). P24, or hsp27, is an oestrogen related protein that appears to play a role in thermotolerance and may play a role in drug resistance (9), as well as predicting for response and survival (10). TMX and α -IFN have also been reported to have effects on the expression and function of a diverse number of growth factors, including transforming growth factor (TGF) α and β which appear to have relevance for breast cancer growth and regression.

In the current study the hormone responsive, ER positive cell line MCF-7 was used as a model to study the biologic effects of sequential administration of TMX and α -IFN. Expression of ER, PR, P24, *c-erbB-2*, TGF- β , PDGF- $\alpha\alpha$ and Ki67 was measured. Ki67 recognizes a nuclear antigen expressed in cycling cells. Ki67 expression is absent in resting cells and is thus a marker of growth fraction, shown to correlate with S-phase fraction (11), while the expression of the other factors measured have all been correlated with outcome and prognosis in ABC and may reflect biologically significant changes in cell physiology or indeed may themselves be suitable targets for therapeutic intervention.

Materials and methods

Materials. The MCF-7 cell line, indicator free (if) RPMI and fetal calf serum (FCS) were obtained from Highveld Biologicals S.A., TMX from ICI Pharmaceuticals, Cheshire, UK, α -IFN (Intron A) from Scherag SA. The ERICA kit was purchased from Abbott Laboratories, the PR monoclonal antibody from Transbio (Paris, France), the PDGF- $\alpha\alpha$ polyclonal antibody and the TGF- β monoclonal antibody from British Biotechnology (Abingdon, UK), the *c-erbB-2* monoclonal antibody from Triton Diagnostics (Alameda, CA), the Ki67 monoclonal antibody from Dakopatts, (Copenhagen), the p-glycoCHEK C219 kit from Centocor Diagnostics. The DAB was purchased from Sigma, and the Vectastain ABC kit from Vector Laboratories, Burlingame CA. The P24 monoclonal antibody was a generous gift from the late Dr W. McGuire.

Correspondence to: Dr L. Seymour, ¹Present address: NCIC Clinical Trials Group, Queens University, 18 Barrie Street, Kingston, Ontario K7L 3N6, Canada

Key words: breast cancer, tamoxifen, interferon, MCF-7, modulation, growth factors, estrogen receptor

Cell culture and harvesting. MCF-7 cells were cultured in *if*-RPMI and 3% heat inactivated FCS that had been stripped (s-FCS) using the dextran coated charcoal method to remove steroid hormones. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cultures were fed every 3 days, and passaged when confluent. After 4 weeks, MCF-7 cells were plated into 24-well culture plates at a plating density of 5×10^4 cells per well in *if*-RPMI/3% s-FCS. After 24 h a baseline sample was harvested, and the medium replaced with either fresh medium alone or with fresh medium containing α -IFN at a concentration of 100, 500 or 1,000 units/ml. At day 4 the cells were fed again with fresh medium ($\pm$ α -IFN) and TMX at 10^{-8} M. Control wells with medium alone, with α -IFN alone and with TMX alone were maintained. Samples were harvested at 2 day intervals until either confluence was attained, or the cells died. All experiments were conducted in triplicate.

Assay procedures. Harvested cells were counted, and then washed in sterile normal saline. The triplicate samples were pooled for immunocytochemical analysis, and cytospun onto glass slides. The slides were then fixed in picric-acid-formaldehyde, and frozen at -20°C in a storage medium containing sucrose and DMSO until analysis. ER, PR, P24, TGF- β , PDGF- $\alpha\alpha$, Ki67 assays were performed according to previously described methods (12-14). Specimens were examined by light microscopy and scored according to the intensity of staining and the number of cells displaying positive immunostaining. At least 100 cells per field and four fields were examined. All findings except Ki67 were assigned a score of 0-500 dependent on the degree and intensity of staining; Ki67 is presented as percentage of cells showing positive staining.

c-erbB-2 determination was by means of a similar immunocytochemical method using a monoclonal antibody to the external domain of c-erbB-2 and a standard avidin-biotin procedure. Briefly, the stored specimens were washed in phosphate buffered saline (PBS), endogenous peroxidase was blocked using methanolic peroxide, and then blocking antibody, primary and control antibodies, secondary antibody, ABC (vectastain) and DAB were layered on the specimen sequentially. Specimens were deemed positive if clear membrane immunostaining was observed.

Specific analyses were performed in a single run, and positive (MCF-7, ZR-75, postmenopausal ER positive breast cancer and A549 cells) and negative controls (A549) were included in each run. All runs included samples in which non-immune mouse serum was substituted for primary antibody as further negative controls.

Results

Growth kinetics. Cells in the control conditions reached confluence by day 8; growth was most rapid in the first 4 day period (logarithmic growth phase) compared to the second 4 day period (plateau phase). As shown in Fig. 1, TMX and all concentrations of α -IFN led to a statistically significant inhibition of growth when compared to controls. There was a suggestion of a dose-response for α -IFN but the difference between concentrations did not reach statistical significance.

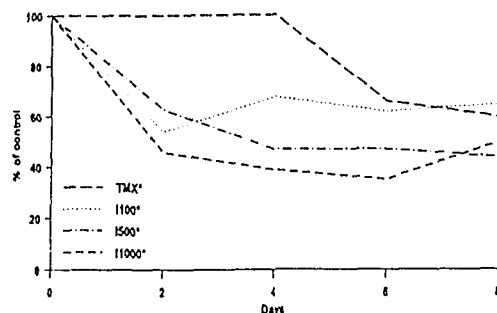


Figure 1. The effect of TMX alone and α -IFN alone on growth of MCF-7 cells. Cells were plated at 4×10^5 cells per well in indicator free (*if*) RPMI and 3% stripped heat inactivated FCS. At 24 h medium was replaced with either fresh *if*-RPMI and FCS or together with various concentrations of α -IFN or TMX 10^{-8} M. Cells were fed again with either medium alone or medium and IFN $\pm$ TMX on day 4.

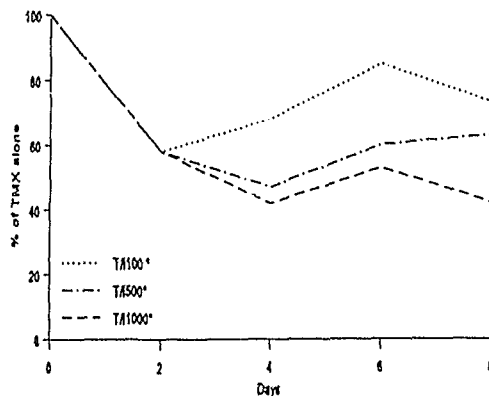


Figure 2. The effect of addition of α -IFN to TMX (10^{-8} M) on growth of MCF-7 cells. Cells were grown under the conditions described in the text. α -IFN at concentrations of 100, 500 and 1,000 U/ml were added together with TMX (10^{-8} M) to *if*-RPMI + 3% SFCS. Cell numbers were calculated as a percentage of growth following the addition of TMX (10^{-8} M) alone.

The effects of combinations of various concentrations of α -IFN and TMX, as compared to TMX alone, are shown in Fig. 2. The addition of α -IFN at all concentrations to TMX appeared to result in at least additive growth inhibition. The increased growth inhibition by the combination was statistically significant when compared to TMX alone. Again there appeared to be a trend for increasing effect with increasing dose of α -IFN. Ki67 expression in the control group peaked by day 4, and then decreased substantially by day 8, as cells approached confluence. Expression of Ki67 was significantly decreased (Fig. 3) by α -IFN and α -IFN $\pm$ TMX. Although cell growth was inhibited by TMX, Ki67 expression remained similar in control and TMX exposed cells, suggesting that TMX exposed cells are still potentially cycling cells.

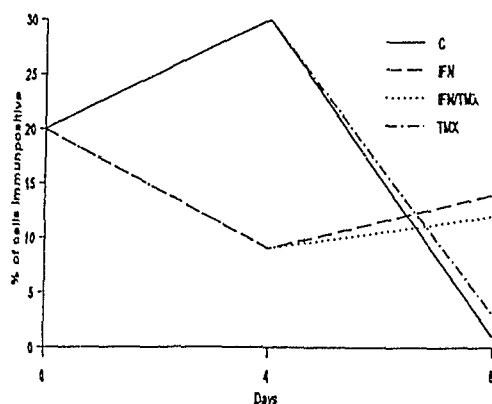


Figure 3. The effect of α -IFN (100 U/ml) and of TMX (10^{-8} M) on Ki67 expression MCF-7 cells.

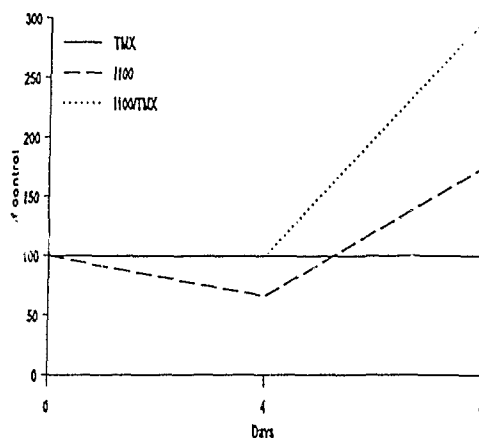


Figure 5. The effect of TMX (10^{-8} M), of α -IFN (100 U/ml), and of the combination on ER expression in MCF-7 cells.

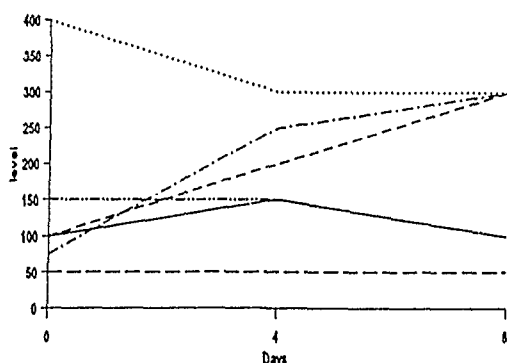


Figure 4. Temporal expression of hormone receptor and growth factor levels during growth of MCF-7 cells in if-RPMI + SFCS. Days 1-4 represent log phase of growth. Cells reach confluence at day 8. Hormone receptor and growth factor expression were assayed by quantitative immunocytochemistry as described in the text.

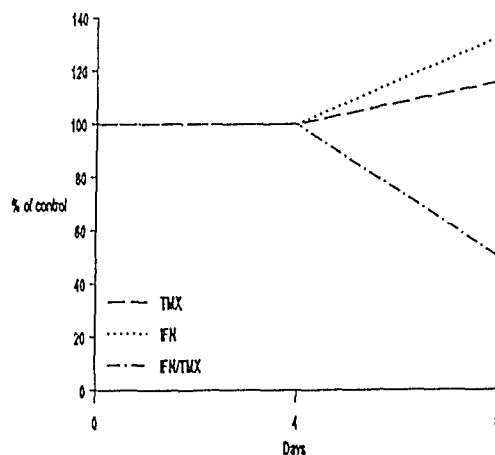


Figure 6. The effect of TMX (10^{-8} M), of α -IFN (100 U/ml), and of the combination (TMX and α -IFN) on PDGF-aa expression in MCF-7 cells.

Expression of growth factors and estrogen related proteins.

In general all 3 concentrations of α -IFN gave comparable results in the immunocytochemical analyses. Results are presented for α -IFN 100 $\pm$ TMX, and for other concentrations where differences were observed. Temporal expression of hormone receptors and growth factors in control cells is shown in Fig. 4. ER expression in control cells increased modestly during the logarithmic growth phase. PR and *c-erbB-2* expression appeared to remain largely unchanged. P24 and TGF- β expression increased during both the growth and plateau phases of growth, while PDGF-aa expression initially declined and then remained unchanged. Expression of ER, P24, PDGF-aa and TGF- β appears thus to be cell cycle related.

ER expression was unchanged following exposure to TMX alone, but was increased by α -IFN $\pm$ TMX (Fig. 5).

MCF-7 cells have high constitutive PDGF-aa expression at baseline, and the decrease in expression seen in control cells was potentiated by α -IFN/TMX, but essentially unchanged as compared to controls when either agent was used alone (Fig. 6). Expression of TGF- β was similar in TMX treated cells as compared to controls, while α -IFN $\pm$ TMX resulted in a modest increase in expression of TGF- β during the growth phase (Fig. 7).

c-erbB-2 expression was unchanged as compared to control cells in the growth phase, while expression was increased during the plateau phase by TMX as well as α -IFN $\pm$ TMX. There was evidence of a dose-response effect for α -IFN (Fig. 8). Some increase in P24 was noted after

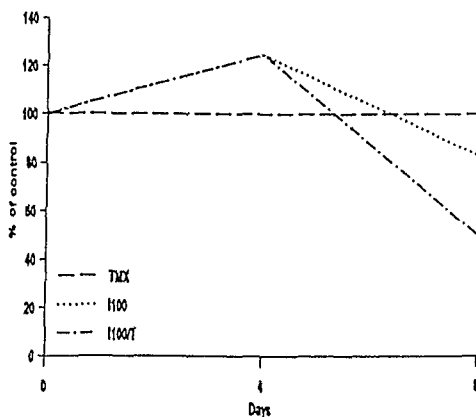


Figure 7. The effect of TMX (10^{-8} M), of α -IFN (100 U/ml), and of the combination (TMX and α -IFN) on TGF- β expression in MCF-7 cells.

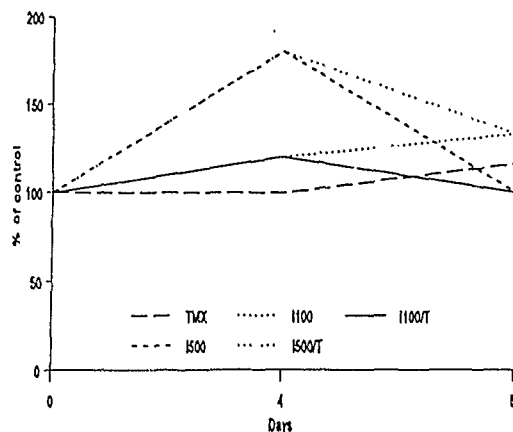


Figure 9. The effect of TMX (10^{-8} M), of α -IFN (100 U/ml and 500 U/ml) and of the combinations (TMX and α -IFN) on P24 expression in MCF-7 cells.

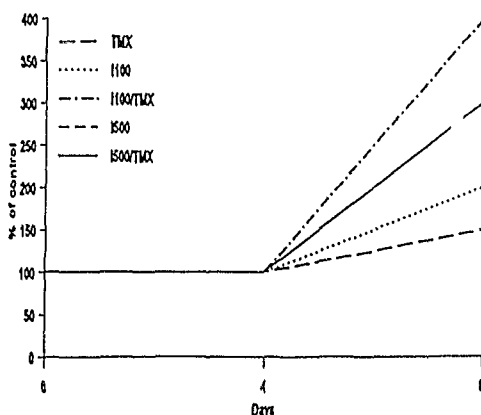


Figure 8. The effect of TMX (10^{-8} M), of α -IFN (100 U/ml and 500 U/ml) and of the combinations (TMX and α -IFN) on c-erbB-2 expression in MCF-7 cells.

exposure of cells to either TMX or α -IFN $100 \pm$ TMX. For P24 there was a dose-response effect, with α -IFN $500 \pm$ TMX leading to significant increase in expression compared to controls (Fig. 9). PR was expressed at a low, but constant level, and no change in expression was observed (data not presented).

Discussion

TMX is widely used in the management of patients with breast cancer, and its effects on growth hormone receptors and the expression of various growth factors and oncogenes have been well described both *in vitro* and *in vivo*. Many of these effects are presumed to be of potential therapeutic importance, e.g. increase in TGF- β and alteration of ER expression. Strategies modulating these effects may thus be of clinical interest.

The current studies have largely confirmed the previously reported effects of TMX on growth and expression of ER. TMX induced TGF- β expression may be time and cell cycle related (15), possibly explaining the failure to demonstrate induction of TGF- β expression in this model. Alternatively the failure to demonstrate increased intracellular levels of TGF- β may be due to secretion of this growth factor as previous studies have demonstrated that conditioned medium obtained from TMX treated MCF-7 cells is growth inhibitory to the same cell line. Interestingly, c-erbB-2 expression was increased modestly by TMX, an effect not previously described. Previous studies have demonstrated estrogen mediated downregulation of c-erbB-2, although clinical studies examining the effects of TMX on tissue expression of c-erbB-2 have shown no obvious changes (16). The implications of these observations are not clear.

Of note, expression of a number of growth factors, including TGF- β , P24, ER, and PDGF- $\alpha\alpha$, appeared to be related to cell kinetics. The effect of growth and the cell cycle on ER expression is well described (17,18). TGF- β is growth inhibitory in MCF-7 cells and has been implicated in apoptotic cell death induced by TMX (19). Increasing expression as cells reach confluence is consistent with an inhibitory autocrine function. Similar effects have been described for other growth factors such as IGF-1 (20). The demonstrated changes in c-erbB-2 expression as cells approach confluence is relevant to the interpretation of clinical results, where expression has been shown to correlate with proliferative activity and to inversely correlate with ER expression (21-23). *In vitro*, c-erbB-2 is known to be suppressed by ER/estrogen and our demonstration of declining levels of c-erbB-2 through the plateau phase, as ER expression increases, may be consistent with this finding (24).

α -IFN increases ER expression and is antiproliferative. We have demonstrated a significant antiproliferative effect of α -IFN, even at low dose, and modest, but interesting increases in ER and TGF- β expression. α -IFN thus appears to be a potential modulator of TMX induced biologic effects.

The combination of these two agents which have similar and complementary effects has been investigated to some extent *in vitro*. Both we and other workers have reported that the combination of α -IFN and TMX has at least additive antiproliferative effects (25) *in vitro* although variable effects on ER expression have been reported (26). The current studies demonstrate that the effects of TMX or α -IFN alone were increased by the combination of these two agents following pretreatment of cells with α -IFN which appears to be indicative of a priming effect. ER and TGF- β expression with the combination was increased, but in addition PDGF- $\alpha\alpha$ expression was decreased. PDGF expression has been shown to correlate with vascular counts and micro vessel density and may be an important angiogenic factor in clinical breast cancer (27). In addition, PDGF- $\alpha\alpha$ stimulation of cell proliferation (28,29) and aromatase activity *in vitro* has been demonstrated (30). *c-erbB-2* expression, compared to controls was also increased and may be mediated by effects on transcription (31).

While these *in vitro* observations are interesting, as induction of ER and TGF- β as well as reduction in proliferation and PDGF- $\alpha\alpha$ expression would, intuitively, seem to offer benefit, the question arises as to the clinical relevance of a model based on *in vitro* growth of cells which demonstrate changes in expression of biologic determinants according to growth phase. In this regard, it should be pointed out that there is a known heterogeneity of cell cycle distribution and ER expression in *in vivo* human breast cancers. Not all cells may therefore be susceptible to the effects of these two growth modulators when administered to patients. However no potentially detrimental interactions have been demonstrated. Indeed, pilot clinical studies (8) with sequential α -IFN and TMX in patients with ABC suggested that the combination does increase ER and TGF- β expression *in vivo*. The modulation of TMX appears an attractive strategy, and such *in vitro* investigations offer insight into the biology and pharmacology of ABC. It might further be pointed out that the changes in expression of factors influencing cell biology were seen even with low dose of α -IFN, although there may be evidence of a dose response for effects on proliferation, P24 and *c-erbB-2*. Given the potential toxicities and cost of α -IFN, low dose α -IFN would appear to be an attractive option for use in clinical studies. Optimal sequence and timing of administration of the two agents require further elucidation. The cyclic administration of these 2 agents *in vivo* may or may not be appropriate.

In summary, the sequential administration of α -IFN and TMX appears to lead to potentially important modulation of biologic endpoints, and further *in vitro* and clinical studies examining schedule and sequencing issues, as well as clinical outcomes, are appropriate.

Acknowledgements

This work was supported in part by the National Cancer Association (SA).

References

- Allegria JC: Methotrexate and 5-fluorouracil following tamoxifen and premarin in advanced breast cancer. *Semin Oncol* 10: 23-28, 1983.
- Benz C, Gandara D, Miller B, Drakes T, Monroe S, Wilbur B and DeGregorio M: Chemoendocrine therapy with prolonged estrogen priming in advanced breast cancer: endocrine pharmacokinetics and toxicity. *Cancer Treatment Rev* 71: 283-289, 1987.
- Ingle JN, Foley JF, Mailliard JA, Krook JE, Hartmann LC, Jung S-H, Veeder MH, Gesme DH, Hatfield AK and Goldberg RM: Randomised trial of cyclophosphamide, methotrexate and 5-fluorouracil with or without estrogenic recruitment in women with metastatic breast cancer. *Cancer* 73: 2337-2343, 1994.
- Seymour L, Meyer K and Bezwod WR: Hormone priming in breast cancer: oestrogen priming has a detrimental effect on response in oestrogen receptor negative patients. *Eur J Cancer* 29: 1495-1496, 1993.
- Conte PF, Pronzato P, Rubagotti A, Alama A, Amadori D, Demicheli R, Gardin G, Gentilini P, Jacomuzzi A, Lionetto R, Monzeglio C, Nicolini A, Rosso R, Sismondi P, Sussio M and Santi L: Conventional versus cytotoxic polychemotherapy with estrogenic recruitment in metastatic breast cancer: results of a randomised cooperative trial. *J Clin Oncol* 5: 339-347, 1987.
- van den Berg HW, Leahey WJ, Lynch M, Clark R and Nelson J: Recombinant human interferon alpha increases oestrogen receptor expression in human breast cancer cells (ZR-75-1) and sensitises them to the anti-proliferative effects of tamoxifen. *Br J Cancer* 55: 255-257, 1987.
- Bezwod WR and Meyer K: Effect of α -interferon, 17-oestradiol and tamoxifen on estrogen receptor concentration and cell cycle kinetics of MCF-7 cells. *Cancer Res* 50: 5387-5391, 1990.
- Seymour L and Bezwod WR: Interferon plus tamoxifen treatment for advanced breast cancer: *in vivo* biologic effects of two growth modulators. *Br J Cancer* 68: 352-356, 1993.
- Ciocca DR, Oesterreich S, Charness G, McGuire W and Fuqua S: Biological and Clinical Implications of heat shock protein 27,000 (hsp27): a review. *J Natl Cancer Inst* 85: 1558-1569, 1993.
- Seymour L, Bezwod WR and Meyer K: Tumour factors predicting for prognosis in metastatic breast cancer: the presence of P24 predicts for response to treatment and duration of survival. *Cancer* 66: 2390-2394, 1990.
- Gasparini G, Boracchi P, Verderio P and Bevilacqua P: Cell kinetics in human breast cancer: comparison between the prognostic value of the cytofluorimetric S-phase fraction and that of the antibodies to Ki-67 and PCNA antigens detected by immunocytochemistry. *Int J Cancer* 57: 822-829, 1994.
- Seymour L, Meyer K, Esser J, MacPhail AP, Behr A and Bezwod WR: Estimation of PR and ER by immunocytochemistry in breast cancer. Comparison with radioligand binding methods. *Am J Clin Pathol* 1 (Suppl): S35-S40, 1990.
- Seymour L, Bezwod WR, Meyer K and Uehr C: Detection of P24 protein in human breast cancer: influence of receptor status and oestrogen exposure. *Br J Cancer* 61: 886-890, 1990.
- Seymour L, Dajee D and Bezwod WR: Tissue platelet derived growth factor (PDGF) predicts for shortened survival and treatment failure in advanced breast cancer. *Breast Cancer Res Treat* 26: 247-252, 1993.
- Perry RR, Kang Y and Greaves BR: Relationship between tamoxifen induced transforming growth factor β_1 expression, cytostasis and apoptosis in human breast cancer cells. *Br J Cancer* 72: 1441-1446, 1995.
- Soubeyran I, Quenel N, Mauriac L, Durand M, Bonichon F and Coindre J-M: Variation of hormonal receptor, p52, *c-erbB-2* and GSTp1 contents in breast carcinomas under tamoxifen: a study of 74 cases. *Br J Cancer* 73: 735-743, 1996.
- Brooks SC, Hansen ER, Saunders DE, Batelli MG and Shafie SM: Effect of growth on the estrogen receptor levels in MCF-7 cells. *Cancer Res* 44: 3724-3729, 1984.
- Rostagno P, Caldani C and Lahlou B: Cell cycle expression of steroid receptors determined by image analysis on human breast cancer cell lines: a hypothesis on the effects of antiestrogens. *Breast Cancer Res Treat* 37: 77-87, 1996.
- Chen H, Tritton TR, Kenny N, Absher M and Chiu JF: Tamoxifen induces TGF- β 1 activity and apoptosis of human MCF-7 breast cancer cells *in vitro*. *J Cell Biochem* 61: 9-17, 1996.
- Noguchi M, Thomas M, Koyasaki N, Ohta N, Taniya T, Kitagawa H, Miyazaki I and Mizukami Y: Influence of hormones on tumor growth, cell kinetics, estrogen receptor and insulin-like growth factor-I-related protein of human breast cancer (MCF-7) cells transplanted in nude mice. *Mol Cell Endocrinol* 92: 69-76, 1993.

21. Keshgegian AA: *c-erbB-2* oncoprotein overexpression in breast carcinoma: inverse correlation with biochemically and immunohistochemically determined hormone receptors. *Breast Cancer Res Treat* 35: 201-210, 1995.
22. Sirvent JJ, Santafe M, Salvado MT, Alvaro T, Raventos A and Palacios J: Hormonal receptors, cell proliferation fraction (Ki-67) and *c-erbB-2* amplification in breast cancer. Relationship between differentiation degree and axillary lymph node metastases. *Histol Histopathol* 9: 563-570, 1994.
23. Piffanelli A, Dittadi R, Catozzi L, Gion M, Capitano G, Gelli MC, Brazzale A, Malgutti R, Pelizzola D, Menegon A, Giovannini G and Gardini G: Determination of *c-erbB2* protein in breast cancer tissues by different methods. Relationships with other biological parameters. *Breast Cancer Res Treat* 37: 267-276, 1996.
24. Grunt TW, Saceda M, Martin MB, Lupu R, Dittrich E, Krupitza G, Harant H, Huber H and Dittrich C: Bidirectional interactions between the estrogen receptor and the *c-erbB-2* signalling pathways: heregulin inhibits estrogenic effects in breast cancer cells. *Int J Cancer* 63: 560-567, 1995.
25. Porzolt F, Otto AM, Trauschel B, Buck C, Wawer AW and Schonenberger H: Rationale for combining tamoxifen and interferon in the treatment of advanced breast cancer. *J Cancer Res Clin Oncol* 115: 465-469, 1989.
26. Goldstein D, Bushmeyer SM, Witt PL, Jordan VC and Borden EC: Effects of type I and II interferons on cultured human breast cells: interaction with estrogen receptors and with tamoxifen. *Cancer Res* 49: 2698-2670, 1989.
27. Anan K, Morisaki T, Katano M, Ikubo A, Kitsuki H, Uchiyama A, Kuroki S, Tanaka S and Torisu M: Vascular endothelial growth factor and platelet derived growth factor are potential angiogenic and metastatic factors in human breast cancer. *Surgery* 119: 333-339, 1996.
28. Leal JA, Gangrade BK, Kiser JL, May JV and Keel BA: Human mammary tumor cell proliferation: primary role of platelet derived growth factor and possible synergism with human alpha-fetoprotein. *Steroids* 56: 247-251, 1991.
29. Ginsburg E and Vonderhaar BK: Stimulation of growth of human breast cancer cells (T47D) by platelet derived growth factor. *Cancer Lett* 58: 137-144, 1991.
30. Ryde CM, Nicholls JE and Dowsett M: Steroid and growth factor modulation of aromatase activity in MCF-7 and T47D breast carcinoma cell lines. *Cancer Res* 52: 1411-1415, 1992.
31. Taverna D, Antoniotti S, Maggiora P, Dati C, de Bortoli M and Hynes N: *c-erbB-2* expression in estrogen-receptor-positive breast tumour cells is regulated by growth-modulatory reagents. *Int J Cancer* 56: 522-528, 1994.

5.2 Paper 13

SEQUENTIAL A-INTERFERON AND TAMOXIFEN: IN VITRO REVERSAL OF MDR-1 MEDIATED
MULTIDRUG RESISTANCE IN THE MCF-7 CELL LINE.

L. Seymour, W.R. Bezwoda.

Breast 1998 - In Press

**SEQUENTIAL α -INTERFERON AND TAMOXIFEN: IN-VITRO REVERSAL OF
MDR-1 MEDIATED MULTIDRUG RESISTANCE IN THE MCF-7 CELL LINE**

L. SEYMOUR¹, FCP
W.R. BEZWODA, PhD, FRCP

Division of Clinical Hematology and Medical Oncology, Department of Medicine,
University of the Witwatersrand, South Africa.

Running Title: α -interferon and tamoxifen reverse MDR-1 phenotype in MCF-7
cells.

Address for offprints and correspondence, and current address¹

Lesley Seymour
NCIC Clinical Trials Group
Queen's University
Kingston, ONTARIO K7L 3N6, CANADA
Tel: 613 5456430
Fax: 613 5452411
e-mail: seymourl@ncic.ctg.queensu.ca

This study was supported in part by the National Cancer Association (South Africa).

SUMMARY

Chemotherapy drug resistance in breast cancer is mediated in part by the MDR-1 gene product, P-glycoprotein (PGP). Tamoxifen (TMX) is able to modulate the MDR-1 phenotype in vitro. Interferons modulate other biologic effects of TMX and may enhance the effect of TMX on PGP function. We have investigated the effects of combinations of α -interferon (IFN) and TMX on the MDR-1 phenotype and function in MCF-7 cells. IFN and TMX were both growth inhibitory to MCF-7 cells. Preincubation with IFN potentiated the antiproliferative effect of TMX even at low concentrations. Immunocytochemically detected staining for the external domain of PGP was increased by both TMX and IFN and the effect of the two agents appeared to be synergistic. Despite the increased expression of the external domain of PGP, in functional studies, as assessed by inhibition of ^3H vinblastine (VBL) efflux, TMX partially reversed the MDR-1 phenotype in both parental MCF-7 and a multidrug resistant subline MCF-7^{mdr}. Prolonged exposure to IFN during culture, followed by TMX + IFN resulted in a further and significant decrease of VBL efflux in this cell line as compared to after TMX alone. TMX and IFN both have antiproliferative as well as anti-PGP functional effects in this model. Further studies with these two agents in combination, in the setting of clinical drug resistance, appear to be warranted.

Keywords: breast cancer, MCF-7, in vitro, modulation, MDR, p-glycoprotein, interferon, tamoxifen.

INTRODUCTION

Although advanced breast cancer (ABC) is frequently chemotherapy sensitive, resistance to available treatment usually develops and the patient ultimately succumbs to the disease. In some patients with ABC the tumor is primarily refractory to cytotoxic agents. Both intrinsic and acquired multidrug resistance (MDR) present a formidable challenge for the oncologist treating breast cancer. MDR is mediated at least in part by P-glycoprotein (PGP), a glycosylated transmembrane protein of 170 kDa, encoded by the MDR-1 gene, which acts as an energy dependent drug efflux pump, decreasing intracellular drug accumulation¹. Overexpression of PGP is associated in vitro, with a drug resistant phenotype^{2,3}. PGP expression and/or function therefore is an attractive pharmacologic target for modulation.

A number of agents have been shown to affect PGP function in vitro, including calcium channel blockers⁴, calmodulin inhibitors⁵ and agents with effects on membrane fluidity, or APTase function. Many of the currently available MDR modulators are unsuitable for use in the clinic, being associated with significant toxicity^{6,7}, although newer molecules in early clinical development may have an improved therapeutic index^{8,9}.

Tamoxifen (TMX) is an active agent in breast cancer. It is well tolerated and has been shown to have a number of interesting additional biologic actions apart from hormone receptor blockade and growth factor modulation. Among these are the demonstration of a relationship between resistance to TMX and PGP expression¹⁰ and the recent findings that TMX is able to reverse MDR¹¹, by interaction with PGP. Reversal of the MDR-1 phenotype is accompanied by an increase in intracellular chemotherapy drug accumulation¹².

Alpha-interferon (IFN) has a number of modulatory biologic effects in hormone responsive breast cancer cell lines (MCF-7, ZR-75) including enhancement of TMX growth inhibition and increased expression of estrogen receptor (ER)¹³. Similar effects are seen in vivo. The administration of IFN in combination with TMX to patients with ABC increases the expression of tumour ER and of PR¹³ as well as that of oestrogen

regulated proteins such as P24¹⁴. Both TMX and IFN decrease the expression of the proliferation related antigen Ki67^{15,16}. In addition, to these potentially beneficial effects in vitro studies have suggested that combinations of TMX and IFN have synergistic and schedule dependent modulatory effects on doxorubicin (DOX) resistance¹⁷. Similar effects have been described with medroxyprogesterone acetate (MPA)¹⁸ and for TMX modulation of mitoxantrone cytotoxicity.¹⁹

The present study examines the biologic effects of sequential TMX and IFN on cell growth as well as PGP expression and function in the hormone responsive, ER positive breast cancer cell line MCF-7, as a model for PGP modulation by these agents.

MATERIALS AND METHODS

Materials:

The MCF-7 cell line, indicator free (if) RPMI and fetal calf serum (FCS) were obtained from Highveld Biologicals S.A., TMX from ICI Pharmaceuticals, Cheshire, UK, IFN (Intron A) from Scherag SA. The Ki67 monoclonal antibody was obtained from Dakopatts, (Copenhagen) and the p-glycoCHEK C219 kit from Centocor Diagnostic DAB and ^3H -vinblastine (VBL) was purchased from Sigma, and the Vectastain ABC kit from Vector Laboratories, Burlingame CA.

Cell Culture and Harvesting:

MCF-7^{mdr} doxorubicin resistant cells were selected by exposing cells to increasing concentrations of doxorubicin (DOX), initially using DOX at a concentration of 0.05 μM , a concentration leading to <10 % inhibition of growth. At subsequent passages the DOX concentration was doubled until ≥ 50 % growth inhibition was obtained, at which point the concentration was maintained until growth inhibition fell to ≤ 25 % . After further passages (approximately 40) a stable subline emerged in which a concentration of 20 μM DOX produced ≤ 10 % growth inhibition. The resulting subline is 4 fold resistant for DOX and 6 fold resistant for vincristine (VCR) and vinblastine (VBL) as compared to the parent line. MCF-7^{mdr} express high levels of p-glycoprotein but are otherwise similar in growth characteristics and response to tamoxifen as wild type MCF-7. MCF-7 and MCF-7^{mdr} cells were cultured in if-RPMI and 3 % heat inactivated FCS that had been stripped (s-FCS) using the dextran coated charcoal method to remove steroid hormone. All cultures were maintained at 37°C in a humidified atmosphere of 5 % CO_2 . Cultures were fed every 3 days, and passaged when confluent. After 4 weeks, MCF-7 cells were plated into 24 well culture plates at a plating density of 5×10^4 cells per well in if-RPMI/3 % s-FCS. After 24 hours, a baseline sample was harvested, and the medium replaced with either fresh if-RPMI/s-FCS or with fresh medium containing IFN at 100, 500 or 1000 units/ml. At day 4, the medium was replaced with fresh ($\pm$ IFN) and TMX at 10^{-8} M was added. Control wells with medium alone, with IFN

alone and with TMX alone were maintained. Samples were harvested at 2 day intervals until either confluence was attained, or the cells died. All experiments were conducted in triplicate. IFN was diluted in if-RPMI/3% s-FCS while TMX was initially dissolved in 5ul methanol before further dilution in if-RPMI/3% s-FCS to achieve a final concentration of 10^{-6} M. Culture conditions were otherwise identical between experiments.

Harvested cells were counted, and then washed in sterile normal saline. The triplicated samples were pooled for ICA analysis, and cytospun onto glass slides. The slides were then fixed in picric-acid-formaldehyde, and frozen at -20°C in a storage medium containing sucrose and DMSO until analysis.

ICA Procedures:

All immunocytochemical (ICA) analyses were performed in a single run, and positive and negative controls (MCF-7, ZR-75, postmenopausal ER+ breast cancer, A549) were included in each run. In addition, each run included a negative control where non-immune serum was substituted for the appropriate primary antibody. Specimens were examined by light microscopy and scored according to the intensity (I) of staining (0=negative, 1=weak, 2=intermediate, 3=strong, 4=intense) and the number of cells displaying positive immunostaining (Pi 0 - 100 %). At least 100 cells per field and four fields were examined. All findings except Ki67 were assigned a score of 0 - 500 derived by summing the percentage of cells staining at each intensity ($\sum Pi [I+1]$); Ki67 expression was assessed as percentage of cells staining positively.

ER, Ki67 and PGP assays were performed according previously described methods.^{20,21} Briefly, the stored specimens were washed in phosphate buffered saline (PBS), endogenous peroxidase was blocked using methanolic peroxide, and then blocking antibody, primary and control antibodies, secondary antibody, ABC (vectastain) and DAB were layered on the specimen sequentially.

Functional PGP Assay Procedures:

Functional assays of PGP were performed including uptake and efflux studies in whole cells and in isolated membrane vesicle preparations, using membrane vesicles prepared from MCF-7^{mdr} cells. Briefly, for uptake studies, cells were exposed to ³H-VBL dissolved in RPMI at 37°C, incubated and cooled rapidly to 4 °C at either time 0, 10, 20, 30 and 40 minutes, cooled rapidly to 4°C at the stated times and then washed to remove extracellular ³H-VBL. Intracellular ³H-VBL was measured by scintillation counting of cytosol preparations. For efflux studies, cells were preincubated with ³H-VBL for 60 minutes, then washed with HBSS and rapidly cooled to 4°C at time 0, 10, 20, 30 and 40 minutes. Intracellular ³H-VBL (VBL retention) was measured by scintillation counting of cytosol preparations. For each experiment corrections were made for non-specific leakage of ³H-VBL from cells maintained at 4°C after VBL exposure. Studies were performed using either short term (2 hour) exposure to TMX and IFN, exposure to TMX and/or IFN after preincubation for 48 hours with TMX, and exposure to TMX and IFN after preincubation for 48 hours with IFN.

Membrane vesicles were prepared from cells grown with or without IFN (2000 u/ml) for 48 hours. Cells were harvested and suspended in TRIS buffer with phenylmethylsulphonyl fluoride (1 mM), benzamidine (1 mM), aprotinin (1 µg/ml) and EDTA (1 mM), disrupted by nitrogen cavitation at 1500 ps for 20 minutes, then the homogenate centrifuged to remove debris. The supernatant was layered over a 35 % sucrose cushion, vesicles at the interface were collected, diluted in TRIS-buffered saline (TBS) and then resuspended. The vesicle preparation (40 µg protein/ml) was suspended in nucleotide buffer with ATP (control - AMP) TMX 10⁻⁹ - 10⁻⁶ was then added, followed by ³H-VBL (55 mM) and incubated for 60 minutes at 25 °C. Ice cold TBS was added and the vesicle suspension filtered through 0.25 µm nitrocellulose filters. Filters were added to scintillant vials and counted. Results were corrected for non-specific binding in the presence of a 2000 fold excess of unlabelled drug.

Statistical Methods:

All studies were performed in triplicate. For growth studies and functional PGP assays, means and standard errors of the mean were calculated for all time points. For ICA assays, cells were pooled. Statistical comparisons were performed using Students t-test and analysis of variance and co-variance. The significance level was defined as $p < 0.05$.

RESULTS

Growth Kinetics:

Cells in control conditions reached confluence by day 8 with growth being most rapid in the first 4-day period (logarithmic growth phase; doubling time 31.4 hours) compared to the second 4 day period. Growth kinetics of MCF-7^{mdr} were not significantly different with doubling time during the log phase of 34.6 ± 5.6 hours and confluence being reached at day 8.

TMX and all concentrations of IFN were growth inhibitory in MCF-7 cells. There was a trend for increasing doses of IFN to result in increasing inhibition of growth ($p = 0.09$) (figure 1). When IFN at any concentration was added to TMX there was a clear and significant ($p < 0.01$) potentiation of growth inhibition (figure 2), with evidence of a dose response with increasing concentrations of IFN ($p = 0.03$). In parallel with growth inhibition, expression of Ki67 was decreased, most markedly with IFN and IFN in combination with TMX (data not shown). ER expression increased modestly during logarithmic growth phase and appeared to be cell cycle related. While ER expression was not altered by TMX alone, there was some increase in expression after TMX plus IFN.

Expression of PGP:

The expression of PGP in control cells appeared to be growth dependent, with expression decreasing as confluence was approached. The results of ICA analyses for PGP using IFN 1000 were comparable to the results obtained with IFN500, and results are therefore presented for IFN100 and IFN500 only. PGP expression was increased by TMX and by all concentration of IFN and IFN + TMX (figure 3). These effects were evident after day 4, except with both concentrations of IFN + TMX, where the increase in expression peaked at day 4. In control cells, levels of PGP were highest at baseline with a modest decrease

as cells approached confluence.

Effects of IFN/TMX on PGP function:

Exposure of MCF-7^{mdr} cells to either TMX, IFN, or the combination of TMX and IFN had no effect on ³H-VBL uptake. Short term (2 hours) exposure to TMX however significantly increased VBL retention (decreased VBL efflux) in the MCF-7^{mdr} cell line ($p < 0.05$) (figure 4). No such effect was seen with short term exposure of the cells to IFN (figure 4) or following prolonged exposure, during culture, of the cells to either IFN, or to TMX followed after washing by IFN exposure (data not shown). Short term exposure to the combination of TMX plus IFN was not significantly different to TMX alone (data not shown). However, prolonged exposure to IFN during cell culture, followed by TMX + IFN just prior to cytotoxic drug exposure significantly increased VBL retention in this cell line as compared to TMX alone ($p < 0.01$) (figure 5). The effect of IFN preincubation on TMX + IFN induced modulation of VBL efflux was evident at all concentrations tested (100, 500 and 1000 u/ml), but were most significant at the 1000 u/ml concentration.

Membrane vesicles prepared from MCF-7^{mdr} cells and exposed short term to TMX demonstrated a dose related reduction in ATP dependent ³H-VBL accumulation (figure 6). Accumulation of ³H-VBL by isolated membrane vesicles was not affected by short term incubation with IFN alone. The effect of TMX plus IFN was not significantly different to TMX alone in this system, at any of the concentrations of IFN and TMX studied. There was a modest, albeit significant, ($p = 0.05$) shift of the curve of ³H-VBL accumulation in membrane vesicles prepared from MCF7^{mdr} after exposure to IFN (1000 u) for 48 hours when these vesicles were incubated with TMX at concentrations from 10^{-9} to 10^{-6} M. The shape of the curve was, however, unaffected.

DISCUSSION

Therapy of ABC is challenging as relentless progression to drug resistance almost invariably ensues despite achievement of meaningful responses with chemotherapy. MDR-1 is believed to play a role in clinical drug resistance. TMX, commonly used in the management of hormone responsive ABC appears at least in vitro, to modulate the MDR-1 phenotype, TMX has recently been shown to be an effective substrate for PGP, and is a potent activator of PGP ATPase²². These observations are not inconsistent with the role of TMX as a modulator of multidrug resistance²³, since molecules such as steroids which bind to PGP with high affinity interfere with drug efflux catalysed by PGP^{12,24,35}. Similar effects have been reported for medroxyprogesterone acetate²⁶. However, the efficacy of TMX as a modulator of drug resistance at clinically achievable concentrations has not been demonstrated in the clinical setting. It is also not clear whether a complex biologic phenomenon such as drug resistance will be sufficiently influenced by a single modulator so as to give clinically meaningful results.

IFN modulates a number of biologic effects of TMX and may also modulate the interaction of TMX and PGP. In the current study, both IFN and TMX were clearly growth inhibitory to MCF-7 cells. Preincubation with IFN potentiated the growth inhibitory effects of TMX even at low concentrations. These effects have been documented previously^{13,15,16}. In addition to the growth modulation obtained, we explored the potential of these two agents to modulate PGP expression and function. In this model, membrane bound, ICA detected, expression of PGP was increased by incubation with either IFN or with TMX alone. The combination of the two agents appeared to have an additional effect on increasing PGP expression as compared to either agent alone. However, functional studies demonstrated that despite the apparent increase of membrane protein expression, TMX could partially reverse the MDR-1 phenotype, and that this effect was potentiated by preincubation with IFN.

Previous studies which suggests that both IFN and TMX can reverse the MDR phenotype include those of Kang and Perry¹⁷. These authors noted that while TMX alone was able to enhance the growth inhibitory effect of doxorubicin in a multidrug resistant Chinese-hamster ovary cell line, pretreatment of these cells with IFN was even more effective in reducing PGP mediated MDR. These findings were apparent despite the fact that IFN alone had no effect on PGP expression.

Both the study by Kang and Perry and the current investigation, suggest that the effect is schedule dependent. However, a significant difference between the two studies were the findings in regard to PGP expression as detected by immunocytochemistry. In the current investigation, the expression of the external domain of PGP increased during prolonged culture with both TMX and IFN. Kang and Perry failed to detect any such alteration in ICA detected PGP expression. These differing results are likely multifactorial, and may be due to use of different cell lines in the two studies, or due to different incubation times with the respective agents. Furthermore, although the present study demonstrates a difference in PGP expression after both TMX and IFN treatment, both the magnitude and the timing of the increase differed for different incubation conditions. IFN exposure appeared to result in fairly rapid increase in ICA PGP expression, while the same changes occurred only with relatively prolonged (> 4 days) TMX exposure.

The effect of the two agents on PGP function also differs. TMX treatment was associated with a rapid modulation of PGP function with increased whole cell retention of VBL after a 2 hour exposure. The findings from the membrane vesicle studies suggest that the effects of TMX are due to rapid, direct binding of TMX to PGP. No direct effect of IFN on VBL retention was demonstrable in the isolated membrane vesicle studies. The change noted in membrane vesicles prepared from cells pre-incubated with IFN are consistent with an increased membrane expression of PGP following IFN exposure but no basic alteration in the mechanism of PGP/TMX interaction. These results as well as the results obtained with whole cells are consistent with the interpretation that IFN and TMX have independent effects on PGP function.

In the studies by Kang and Perry, ER was undetectable both under control conditions and when treated with modulators. In the current study, although the cells expressed ER, TMX had no effect on expression, while IFN and TMX had a modest effect. These data suggest that the modulation of PGP by IFN and by TMX are not directly related to the effects of either of these agents on ER expression. Although the mechanism of IFN enhancement of TMX modulation of PGP function has not been fully elucidated it would appear to be possibly related to perturbation of membrane structure or distribution of PGP enhancing the ability of TMX to bind to the active ATP binding site.

TMX is known to have diverse biologic effects among which is inhibition of protein kinase C (PKC). PKC inhibitors such as staurosporine and dexamethasone have been shown to modulate PGP function at least in part through effects on PKC²⁷. PKC may be involved in the phosphorylation of the multidrug transporter²³, and some, but not all inhibitors of PKC may be substrates of PGP²⁸. Drugs such as dexamethasone have, however, been shown to decrease the ICA expression of PGP while decreasing multidrug resistance²⁹, suggesting that the effects of TMX on PGP are not mediated via PKC.

One further aspect of PGP expression that deserves comment is the issue of the relationship between PGP expression and cell cycle distribution. Ramachandran and co-workers³⁰ have demonstrated that PGP mRNA expression is highest while intracellular accumulation of drugs affected by PGP is lowest during the S-phase of the cell cycle. The current study demonstrated that ICA detectable PGP increases as cells reached confluence following exposure to both IFN and TMX, suggesting that while cell cycle distribution may have an influence on drug sensitivity, the effects observed after exposure to the two agents in this model are not merely related to traverse of cells through the cell cycle.

Clinically, the addition of IFN to TMX has been investigated in small pilot studies^{13,31,32}, usually using regimens designed to reverse constitutive or acquired hormone resistance. These studies have not, however, examined the role of IFN and TMX in the modulation of

multidrug resistance. The demonstration that TMX can reverse the MDR-1 phenotype, and that these effects can be substantially potentiated by the addition of IFN, especially when administered prior to the initiation of TMX, suggests a potential role for these two agents in the reversal of multidrug resistance.

Clinical studies of MDR-1 reversing agents are challenging in design and the definition of relevant endpoints: nonetheless further studies with these two agents in combination in the setting of clinical drug resistance appear to be indicated.

REFERENCES

1. Gottesman M, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann Rev Biochem* 1993; 62:385-427.
2. Chan HS, Grogan T, Haddad G, DeBoer G, Ling V. P-glycoprotein expression: critical determinant in the response to osteosarcoma chemotherapy. *J Natl Cancer Inst* 1987;89:1706-1715.
3. Mechetner E, Kyshtoobayeva A, Zonis S, Kim H, Stroup R, Garcia R, Parker R, Fruehauf J. Levels of multidrug resistance (MDR-1) p-glycoprotein expression by human breast cancer correlate with in vitro resistance to Taxol and doxorubicin. *Clin Canc Res* 1998 4:389-398.
4. Tsuruo T, Iida H, Tsukagoshi S, Sukurai Y. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* 1983; 41: 1967-1972.
5. Ganapathi R, Grabowski D. Enhancement of adriamycin sensitivity in resistant P388 leukemia by the calmodulin inhibitor trifluoperazine. *Cancer Res* 1983; 43: 3696-3699.
6. Bartlett N, Lum B, Fisher G, Brophy N, Ehsan M, Halsey J, Sikic B. Phase I trial of doxorubicin with cyclosporine as a modulator of multidrug resistance. *J Clin Oncol* 1994 12;4:835-842
7. Warner E, Hedley D, Andrulis I, Myers R, Trudeau M, Warr D, Pritchard K, Blackstein M, Goss P, Franssen E, Roche K, Knight S, Webster S, Fraser R, Oldfield S, Hill W, Kates R. Phase II study of dexverapamil plus anthracycline in patients with metastatic breast cancer who have progressed on the same

anthracycline regimen. Clin Canc Res 1998 4: 1451-1457.

8. Dantzig A, Shepard R, Cao J, law k, Ehlhardt W, Baughman T, Bumol T, Starling J. Reversal of p-glycoprotein mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator LY335979. Canc Res 1996. 56:4171-4179.
9. Barbarics E, Kronauge J, Cogen D, Davison A, Jones A, Croop J. Characterization of p-glycoprotein transport and inhibition in-vivo. Canc Res 1998 58:276-282.
10. Keen JC, Miller EP, Bellamy C, Dixon JM, Miller WR. P-glycoprotein and resistance to tamoxifen. Lancet 1994 23:1047-1048.
11. Leonessa F, Jacobson M, Boyle B, Lippman J, McGarvey M, Clarke R. Effect of tamoxifen on the multidrug resistant phenotype in human breast cancer cells: isobologram, drug accumulation and M_r170,000 glycoprotein (gp170) binding studies. Cancer Res 1994 54:441-447.
12. Callaghan R, Higgins CF. Interactions of Tamoxifen with multidrug-resistance p-glycoprotein. Br J Cancer 1995; 71: 294-299.
13. Seymour L, Bezwoda WR. Interferons plus tamoxifen treatment for advanced breast cancer: in vivo biologic effects of two growth modulators. Br J Cancer 1993; 68: 352-356.
14. Seymour L, Bezwoda WR, Meyer K, Behr C. Detection of P24 protein in human breast cancer: influence of receptor status and oestrogen exposure, BR J Cancer 1995; 61: 886-890.
15. Van den Berg HW, Leahy WJ, Lynch M, Clark R, Nelson J. Recombinant human interferon alpha increases oestrogen receptor expression in human breast cancer

- cells (ZR-75-1) and sensitises them to the anti-proliferative effects of tamoxifen. *Br J Cancer* 1987; 55: 255-257.
16. Bezwoda WR, Meyer K. Effect of α -interferon, 17-estradiol and tamoxifen on oestrogen receptor concentration and cell cycle kinetics of MCF-7 cells. *Cancer Res* 1990; 50: 5387-5391.
 17. Kang Y, Perry R. Modulatory effects of tamoxifen and recombinant human α -interferon on doxorubicin resistance. *Cancer Res* 1993; 53: 3040-3045.
 18. Zibera, Gibelli N, Maestri L, Della Cuna GR. Medroxyprogesterone acetate reverses the MDR phenotype of the CG5-doxorubicin resistant human breast cancer cell line. *AntiCancer Res* 1995; 15: 745-750.
 19. Damle B. Effect of tamoxifen on mitoxantrone cytotoxicity in drug sensitive and multidrug resistant MCF-7 cells. *Cancer Chemotherapy Pharm* 1995; 36: 368-372.
 20. Seymour L, Meyer K, Esser J, MacPhail AP, Behr A, Bezwoda WR. Estimation of PR and ER by immunocytochemistry in breast cancer. Comparison with radioligand binding methods. *Am J Clin Path* 1993; Suppl 1: S35-S40.
 21. Seymour L, Dajee D, Bezwoda WR. Tissue platelet derived growth factor (PDGF) predicts for shortened survival and treatment failure in advanced breast cancer. *Breast Cancer Res Treat* 1993; 26: 247-252.
 22. Subrahmanyeswara Rao U, Fine R, Scarborough G. Antioestrogens and steroid hormones: substrates of the human p-glycoprotein: *Biochem Pharm* 1994; 48: 287-292.
 23. Trunp DL, Smith D, Ellis P, Rogers M, Schold S, Winer E, Panella T, Jordan V, Fine

- R. High dose oral tamoxifen, a potential multidrug-resistance-reversal agent: phase 1 trial in combination with vinblastine. *J Natl Cancer Inst* 1992; 84: 1811-1816.
24. Yang CP-H, Cohen D, Greenberger L, Hsu Si-H, Horwitz S. Differential transport properties of two mdr gene products are distinguished by progesterone. *J Biol Chem* 1990; 265: 10282-10288.
25. Ueda K, Okamura N, Hirai M, Tanigawara Y, Saeki T, Kioka N, Komano T, Hori R. Human P-glycoprotein transports cortisol, aldosterone and dexamethasone, but not progesterone. *J Biol Chem* 1992; 367: 24248-24252.
26. Claudio JA, Emerman JT. The effects of cyclosporin A, tamoxifen, and medroxyprogesterone acetate on the enhancement of adriamycin cytotoxicity in primary cultures of human breast epithelial cells. *Breast Cancer Res Treat* 1996; 41: 111-122.
27. Sha EC, Sha MC, Kaufmann SH. Evaluation of 2,6-diamino-N-([1-(1-oxotridecyl)-2-piperidinyl]methyl) hexanamide (NPC 15437), a protein kinase C inhibitor, as a modulator of P-glycoprotein mediated resistance in vitro. *Investigational New Drugs* 1996; 13: 285-294.
28. Sachs CW, Ballas LM, Mascarella SW, Safa AR, Lewin AH, Loomis C, Carroll FI, Bell RM, Fine RL. Effects of sphingosine stereoisomers on p-glycoprotein phosphorylation and vinblastine accumulation in multidrug resistant MCF-7 cells. *Biochem Pharm* 1996; 52: 603-612.
29. Patterson KK, Beckman BS, Klotz DM, Mallia CM, Jeter JR. Dexniguldipine hydrochloride, a protein-kinase-C specific inhibitor, affects the cell cycle, differentiation, p-glycoprotein levels, and nuclear protein phosphorylation in Friend erythroleukemia cells. *J Cancer Res Clin Oncol* 1996; 122: 465-475.

30. Ramachandran C, Mead D, Wellham LL, Sauerteig A, Krishan A. Expression of drug resistance associated mdr-1, GSTpi, and topoisomerase II genes during cell cycle traverse. *Biochem Pharm* 1995; 49: 545-52.
31. Macheledt J, Buzdar A, Hortobagyi G, Frye D, Gutterman J, Holmes F. Phase II evaluation of interferon added to tamoxifen in the treatment of metastatic breast cancer. *Br Cancer Res Treat* 1991. 18:165-179.
32. Recchia F, Sica Gigiola, De Filippis S, Discepoli S, Rea S, Torchio P, Frati L. Interferon-beta, retinoids and tamoxifen in the treatment of metastatic breast cancer: a phase II study. *J Interferon Cytokine Res* 1995. 15:605-610.

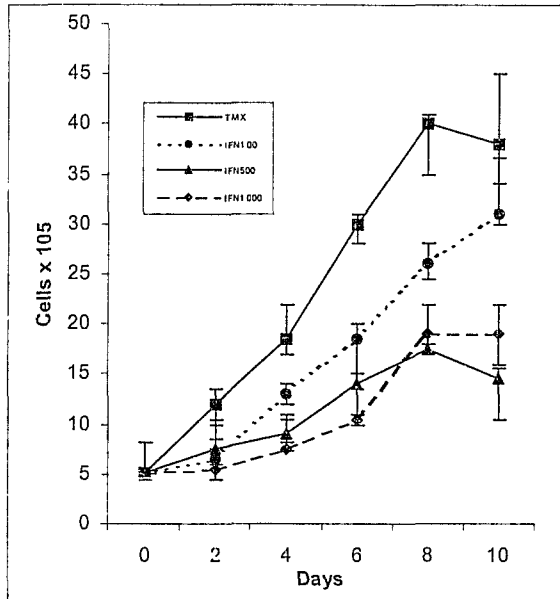


Figure 1: The effect of TMX and IFN alone on growth of MCF-7 cells. Cells were plated at 4×10^5 cells per well in indicator free RPMI and 3 % stripped heat inactivated FCS. At 24 hours medium was replaced with either fresh if-RPMI and FCS or together with IFN 100 u/ml (IFN100), IFN 500 u/ml (IFN500), IFN 1000 u/ml (IFN1000) or TMX 10^{-8} M (TMX). Cells were fed with medium alone or medium and IFN or TMX on day 4. All experiments were conducted in triplicate; mean values are presented. Bars represent standard errors of the mean.

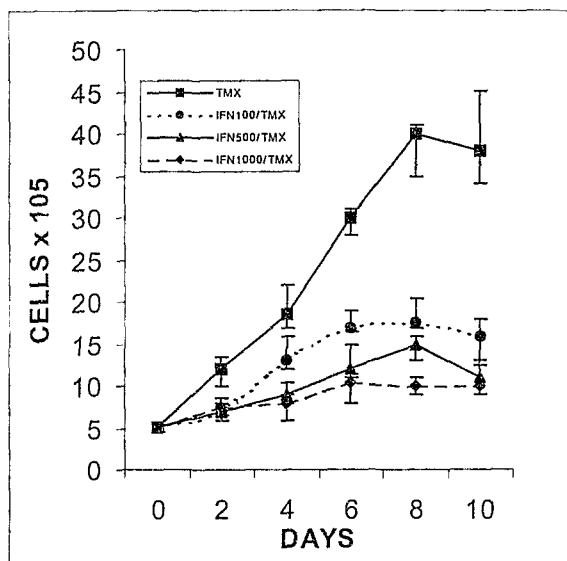


Figure 2: The effect of addition of IFN to TMX (10^{-8} M) on growth of MCF-7 cells. Cells were plated at 4×10^5 cells per well in indicator free RPMI and 3 % stripped heat inactivated FCS. At 24 hours medium was replaced with either fresh if-RPMI and FCS or together with TMX 10^{-8} M alone or with IFN 100 u/ml (IFN100/TMX), IFN 500 u/ml (IFN500/TMX), IFN 1000 u/ml (IFN1000/TMX). Cells were fed again with medium alone or medium and IFN $\pm$ TMX on day 4. All experiments were conducted in triplicate; mean values are presented. Bars represent standard errors of the mean.

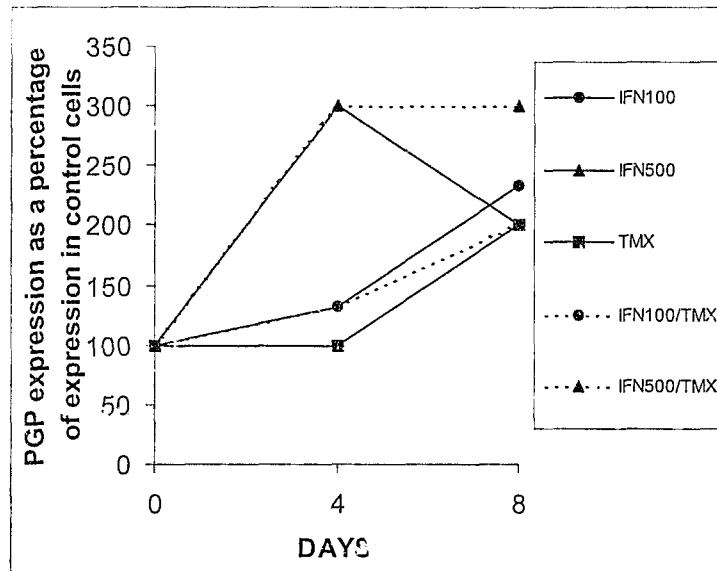


Figure 3: The effect of addition of IFN to TMX (10^{-8} M) and of IFN alone on the expression of the external domain of PGP in MCF-7 cells. Cells were plated at 4×10^5 cells per well in indicator free RPMI and 3 % stripped heat inactivated FCS. At 24 hours medium was replaced with either fresh if-RPMI and FCS with TMX 10^{-8} M alone (TMX), or together with IFN 100 u/ml (IFN100/TMX), IFN 500 u/ml (IFN500/TMX), IFN 1000 u/ml (IFN1000/TMX), or IFN alone at various concentrations (IFN100, IFN500, IFN1000). Cells were fed again with medium alone or medium and IFN $\pm$ TMX on day 4. All experiments were conducted in triplicate; cells were pooled for ICA analysis. Data are presented as a percentage of PGP expression in control cells.

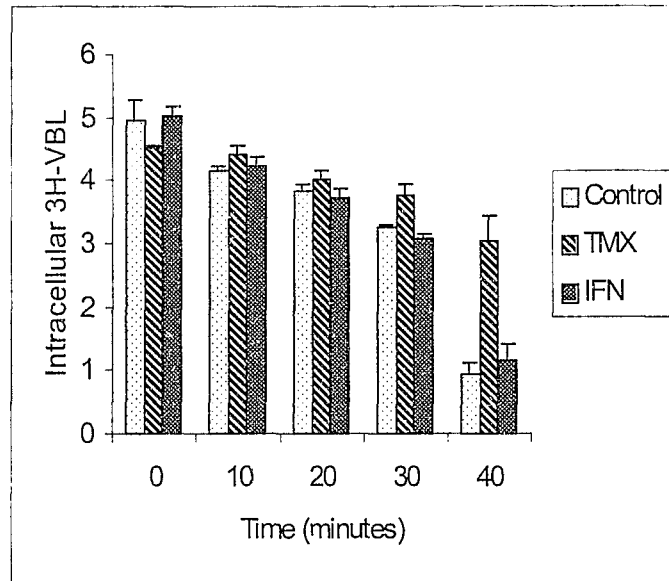


Figure 4: Inhibition of ^3H -VBL efflux: The effect of 2 hour exposure of IFN (1000 U/ml) and TMX 10^{-8} on ^3H -VBL retention in MCF-7^{mdr} cells. Cells were exposed to ^3H -VBL, cooled to 4°C at the times indicated, washed to remove extracellular ^3H -VBL. Retained intracellular ^3H -VBL was measured by scintillation counting of cytosol preparations. All studies were performed in triplicate. Columns and bars represent means $\pm$ SE's. Corrections were made for non-specific loss of ^3H -VBL from cells maintained at 4°C following pre-incubation with ^3H -VBL

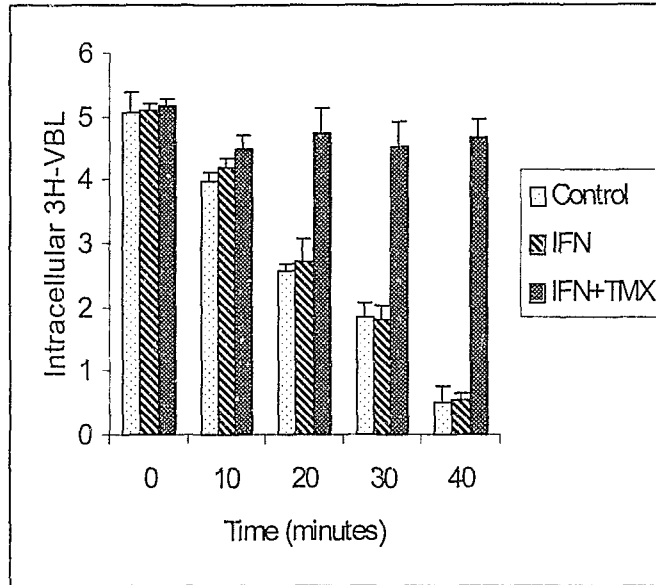


Figure 5: Inhibition of ^3H -VBL efflux: The effect of 2 hour exposure to IFN (1000 U/ml) and TMX 10^{-8} (IFN + TMX) on ^3H -VBL retention in MCF-7^{mdr} cells, after preexposure to IFN for 48 hours. Cells were exposed to ^3H -VBL, cooled to 4°C (4C) at the times indicated, washed to remove extracellular ^3H -VBL. Retained intracellular ^3H -VBL was measured by scintillation counting of cytosol preparations. All studies performed in triplicate. Columns and bars represent means $\pm$ SE's. Corrections were made for non-specific loss of ^3H -VBL from cells maintained at 4°C following pre-incubation with ^3H -VBL.

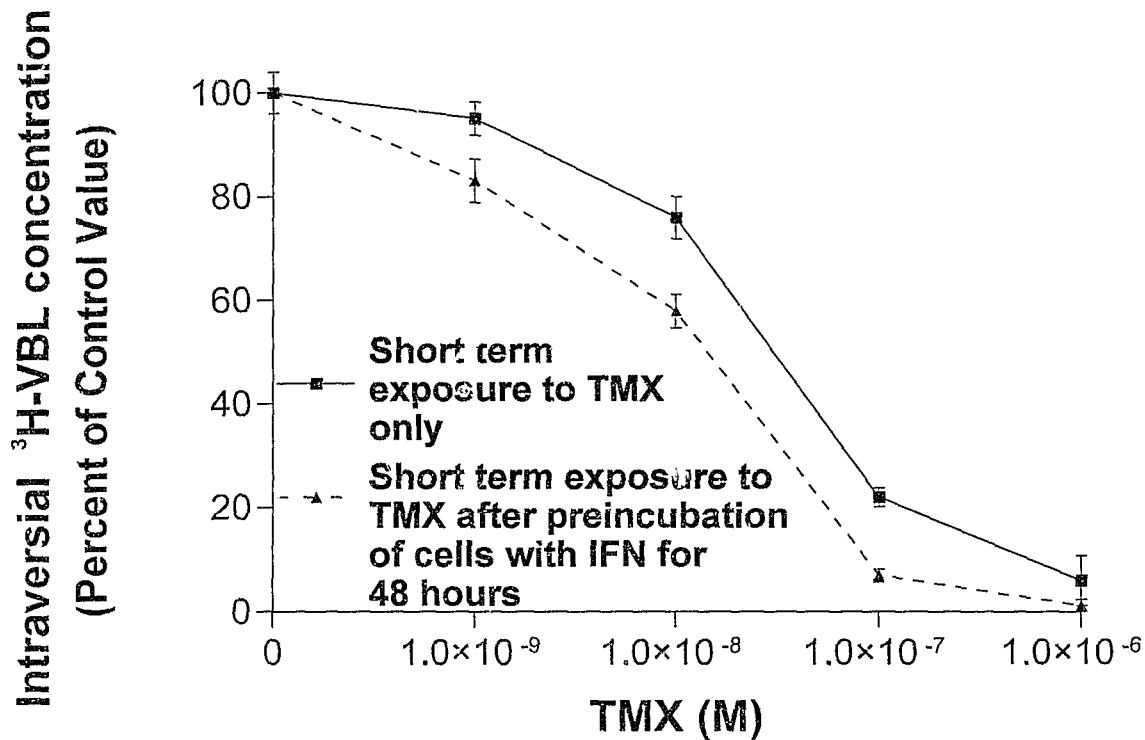


Figure 6: The effect of TMX on ATP dependent ^3H -VBL accumulation in membrane vesicle preparations from MCF-7^{mdr} cells grown with or without IFN (2000 u/ml) for 48 hours. Bars represent standard errors of the mean.

CHAPTER 6 - DISCUSSION AND CONCLUSIONS

6.1 Background and biology

Breast cancer is the most common malignancy affecting women in developed countries, and has enormous public health and socioeconomic effects worldwide. Appreciation of the complex biology of breast cancer has progressed markedly in the past decades but full understanding remains elusive. Research into the hormonal control of tumour cells has advanced knowledge substantially, but the challenge remains: the presence of ER does not necessarily predict for response to hormonal manipulation, nor for sustained response to these manoeuvres. Similarly, while breast cancer is a chemotherapy sensitive disease, as evidence by the beneficial effects of cytotoxic chemotherapy in the adjuvant and palliative treatment of breast cancer, intrinsic and or acquired drug resistance is frequently encountered and remains a major obstacle to effective therapy.

Clearly, the biologic control of breast cancer growth is complex. The observation that cancer cells in tissue culture require less exogenous growth factor support than their normal counterparts, and that conditioned media has growth stimulatory effects, suggests that malignant cells synthesize, and respond to, endogenous growth factors (3), in addition to exogenous factors. Factors that normally regulate growth and cellular differentiation appear to be fundamentally involved in the development and progression of a number of cancers, including breast cancer (4). Dysregulation of the balance between positive and negative growth factors may be important in malignant progression (5). Clearer definition of the

biology of breast cancer is important both in defining prognostic groups as well as the development of new therapeutic strategies.

6.2 Predictors of response and prognosis

Response to treatmentⁱ, and prognosis both in early and advanced breast cancer (ABC) has been correlated with histologic grade, mitotic index, tumour ploidy, receptor status (both ER and PR) (6), and a number of growth factors, growth factor receptors and oncogenes, including c-erbB-2 (7), TGF- β (8) and PDGF. These factors may have prognostic relevance in their own right, or may be surrogate markers of tumour biology as the expression of some appears to be related to the cell cycleⁱⁱ. The assessment of such surrogate or biological markers may have a role not only in furthering understanding of the disease, but also potentially in predicting treatment outcomes and planning therapy. The ability to measure many of these markers by immunocytochemistry, on small biopsy or needle biopsy specimens has facilitated the correlation of these markers with treatment outcomes.

6.2.1 Oestrogen and Progesterone Receptors

Quantitative measurement of ER has been shown to predict response to endocrine therapy in patients with breast cancer (9). The reliability of this prediction appears to be enhanced if progesterone receptors are detectable (10) in addition. The ER has three domains : the N-

ⁱsee paper 2 - "Response to Second-Line Hormone Treatment for Advanced Breast Cancer. Predictive Value of Ploidy Determination"

ⁱⁱ see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: in vitro Biologic Effects of Two Growth Modulators".

terminal domain containing a transcriptional activation region, a DNA binding domain and a C-terminal oestrogen binding domain. Once ligand binding has occurred, the receptor dimerizes, binds with high affinity to DNA and initiates transcription. Expression may be related to cell cycleⁱ and to doubling times, with expression most marked in cells in G₁ and G₂, and in slowly proliferating cells (11). It appears possible to pharmacologically modulate expression of ERⁱⁱ ⁱⁱⁱ. The detection of hormone receptors and the correlation with clinical endpoints has been facilitated by the use of immunocytochemical methods of detection^{iv}.

6.2.2 Transforming growth factor- β

The TGF- β family of growth factors consists of three isoforms, all highly homologous. Signalling occurs via type I and II receptors, and it appears that both types of receptor are required for TGF- β activity on cells. These receptors are transmembrane serine/threonine kinases (12). TGF- β has long been known to be secreted in a biologically active form by breast cancer cells in culture (13), with secretion being induced by exposure to anti-oestrogens. We have shown that expression may be cell cycle related^v. We have also demonstrated induction of TGF- β expression in tumour cells by IFN and IFN plus TMX. Similar effects

ⁱ see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: in vitro Biologic Effects of Two Growth Modulators"

ⁱⁱ see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: in vitro Biologic Effects of Two Growth Modulators"

ⁱⁱⁱ see paper 7 - "Interferon plus Tamoxifen Treatment for Advanced Breast Cancer: in vitro Biologic Effects of two Growth Modulators"

^{iv} see paper 1 - "Estimation of PR and ER by Immunocytochemistry in Breast Cancer. Comparison with Radioligand Binding Methods"

^v see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: in vitro Biologic Effects of Two Growth Modulators"

have been noted for anti-progestins such as onapristone (14). There is some evidence that secretion of TGF- β mediates cell death by inducing apoptosis (15). Similar effects, of anti-oestrogen induced TGF- β expression have been demonstrated in fibroblasts (16). For the majority of human breast cancer cell lines, TGF- β appears to be growth inhibitory (17). TGF- β however appears to also have a potential role in carcinogenesis and tumour growth (18), and in some models can be shown to be growth stimulatory and increase metastatic potential (19).

In vivo secretion of TGF- β has also been documented. Most clinical breast tumours appear to express TGF- β and mRNA can be demonstrated in most breast cancer specimens, although there may be differences in isoform expression dependent on stage of disease (20). In keeping with *in-vitro* data, induction of TGF- β expression in stromal cells by anti-oestrogens can be demonstrated (21). In a small translational pilot study, we found variable effects of IFN and TMX on TGF- β expressionⁱ. Immunohistological studies suggest that the presence of TGF- β staining in tumour cells may predict for progression of disease (22), or to be associated with nodal metastases (23). Further evidence of a role for TGF- β in predicting treatment outcomes comes from the observation that elevation in serum levels correlates with response to TMX (24). Further studies are required to reconcile some of the *in-vitro* effects with *in-vivo* findings.

ⁱ see paper 7 - "Interferon plus Tamoxifen Treatment for Advanced Breast Cancer : *In vivo* Biologic Effects of Two Growth Modulators"

6.2.3 P24

Investigators in San Antonio initially identified the presence of a cytoplasmic oestrogen regulated 24k protein, and its mRNA, in both unstimulated as well as oestrogen stimulated MCF-7 cells (25), and later developed a monoclonal antibody to this protein (26). P24 is located in cytoplasmic granules in the apical cytoplasm, and is detected in receptor positive cell lines, but not receptor negative cell lines (27). P24 can also be demonstrated in normal oestrogen target organs such as uterus, vagina, oviduct and breast (28) (29). This protein has subsequently been identified as a heat shock protein - hsp27. The expression of heat shock proteins may be increased by various forms of stress, but many are constitutively expressed and may play critical roles in cellular functioning, as well as functioning in protection of the cell from various stresses such as thermal. Six families of heat shock proteins have been identified based on molecular weight: hsp100, hsp90, hsp70, hsp60, hsp27, and ubiquitin (30). Hsp27 expression has been shown to be induced by heat shock, and to induce doxorubicin resistance, by a mechanism other than mdr-1 (31). Hsp27 also appears to have a role in mediation of both anchorage dependent and anchorage independent growth (32).

In human tumours, the presence of P24 correlates with the presence of hormone receptors, especially the ER (33), and may predict for sensitivity to hormonal manipulation (34). We have shown that the expression of P24 can be correlated with ER expression, and is increased after exposure to oestrogens, even in ER negative tumours¹. We have also demonstrated that

¹ see paper 3 - "Detection of P24 Protein in Human Breast Cancer: Influence of Receptor Status and Oestrogen Exposure"

expression may be related to the cell cycle and can be increased *in vitro*ⁱ and *in vivo*ⁱⁱ by IFN plus TMX. The expression of this oestrogen regulated protein has also been shown to be correlated with increased objective response rates and prolonged duration of response and overall survivalⁱⁱⁱ.

P24 therefore appears to be a constitutive oestrogen regulated protein which appears to function in growth regulation and protection from various insults including exposure to doxorubicin.

6.2.4 C-erbB-2

The M_{185,000} transmembrane receptor tyrosine kinase c-erbB-2 is activated in a third of human breast cancers, either by gene amplification or overexpression of the protein (35). In ER positive cells expression of c-erbB-2 appears to be controlled by many factors, including growth as well as exposure to oestrogens and anti-oestrogens (36) (37). We have demonstrated that expression can be increased by IFN and TMX^{iv}. Overexpression of c-erbB-2 appears to be inversely correlated with the expression of both ER and PR (38). In patients the overexpression of c-erbB-2 may be associated with a poor outcome (39) (40) (41) (42),

ⁱ see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: *in vitro* Biologic Effects of Two Growth Modulators"

ⁱⁱ see paper 7 - "Interferon plus Tamoxifen Treatment for Advanced Breast Cancer: *In vivo* Biologic Effects of Two Growth Modulators"

ⁱⁱⁱ see paper 4 - "Tumour Factors Predicting for Prognosis in Metastatic Breast Cancer. The Presence of P24 Predicts for Response to Treatment and Duration of Survival"

^{iv} see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: *in vitro* Biologic Effects of Two Growth Modulators"

expression may be related to the cell cycle and can be increased *in vitro*ⁱ and *in vivo*ⁱⁱ by IFN plus TMX. The expression of this oestrogen regulated protein has also been shown to be correlated with increased objective response rates and prolonged duration of response and overall survivalⁱⁱⁱ.

P24 therefore appears to be a constitutive oestrogen regulated protein which appears to function in growth regulation and protection from various insults including exposure to doxorubicin.

6.2.4 C-erbB-2

The M_{185,000} transmembrane receptor tyrosine kinase c-erbB-2 is activated in a third of human breast cancers, either by gene amplification or overexpression of the protein (35). In ER positive cells expression of c-erbB-2 appears to be controlled by many factors, including growth as well as exposure to oestrogens and anti-oestrogens (36) (37). We have demonstrated that expression can be increased by IFN and TMX^{iv}. Overexpression of c-erbB-2 appears to be inversely correlated with the expression of both ER and PR (38). In patients the overexpression of c-erbB-2 may be associated with a poor outcome (39) (40) (41) (42),

ⁱ see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: *in vitro* Biologic Effects of Two Growth Modulators"

ⁱⁱ see paper 7 - "Interferon plus Tamoxifen Treatment for Advanced Breast Cancer: *In vivo* Biologic Effects of Two Growth Modulators"

ⁱⁱⁱ see paper 4 - "Tumour Factors Predicting for Prognosis in Metastatic Breast Cancer. The Presence of P24 Predicts for Response to Treatment and Duration of Survival"

^{iv} see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: *in vitro* Biologic Effects of Two Growth Modulators"

although a study conducted by the South West Oncology Group did not confirm this (43). The presence of the a soluble c-erbB-2 fragment in serum has been shown in this unit to correlate with both stage of disease and survivalⁱ, although the presence of serum c-erbB-2 could not be correlated with tissue expression, and tissue expression showed no correlation with prognosis. Subsequently, other investigators have confirmed the prognostic importance of plasma c-erbB-2 (44). Recent studies suggest that overexpression of c-erbB-2 may be associated with acquired resistance to anti-oestrogens (45), possibly via upregulation of bcl-2 and bcl-X_L and subsequent protection against apoptosis (46), and be associated with increased tumour invasiveness (47). In non-small cell lung cancer cell lines, overexpression of c-erbB-2 may be associated with resistance to doxorubicin and etoposide (48). Further, antibodies targeting c-erbB-2 appear to have antitumour activity in the clinical setting (49).

6.2.5 Platelet Derived Growth Factor

PDGF is a bivalent disulfide linked homo- or hetero-dimer of A and B polypeptide chains, which exerts its effect by binding to tyrosine kinase surface receptors. Three isoforms exists - PDGF-AA, PDGF-AB and PDGF-BB, which bind with different affinities to two receptors, types α , and β , which also dimerise. A chains appear to bind only to α receptors, whereas B chains appear to bind to either α or β receptors (50). Human breast cancer cell lines secrete PDGF in response to oestrogenic stimulation (51), and PDGF can be shown to be mitogenic

ⁱ see paper 10 - "Soluble c-erbB-2 Fragment in Serum Correlates with Disease Stage and Predicts for Shortened Survival in Patients with Early Stage and Advanced Breast Cancer"

in these cell lines (52). Expression may be cell cycle relatedⁱ. Other than direct autocrine effects, PDGF may have a paracrine role on stromal tissues, and PDGF receptors have subsequently been demonstrated in the periepithelial stroma of breast cancer specimens (53). We have been able to demonstrate that PDGF in the plasma of breast cancer patients has prognostic significance and can be correlated to both stage and rate of progressionⁱⁱ, and that patients with detectable serum PDGF have tissue PDGF expressionⁱⁱⁱ. Further studies confirmed the prognostic significance of tissue PDGF in patients with advanced breast cancer, demonstrating a significant negative correlation with response to cytotoxic chemotherapy and overall survival^{iv}.

6.2.6 Ki67

Proliferative index and ploidy can be correlated to the presence of hormone receptor, response to therapy and prognosis in breast cancer (54) (55) (56) (57), although some studies suggest that ploidy and S-phase fraction may not be independent prognostic variables (58). Ki67 is a mouse monoclonal antibody which recognises a non-histone nuclear antigen present in proliferating cells in all phases except G₀ (59). The amount of antigen detected varies, with increased expression in G₂ and M phases. Immunostaining appears to correlate with S-phase

ⁱ see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: *in vitro* Biologic Effects of Two Growth Modulators"

ⁱⁱ see paper 5 - "Platelet Derived Growth Factor in Plasma of Breast Cancer Patients: Correlation with Stage and Rate of Progression"

ⁱⁱⁱ see paper 8 - "Tissue Platelet Derived Growth Factor (PDGF) Predicts for Shortened Survival and Treatment Failure in Advanced Breast Cancer"

^{iv} see paper 9 - "Positive Immunostaining for Platelet Derived Growth Factor is an Adverse Prognostic Factor in Patients with Advanced Breast Cancer"

as defined by flow cytometry (60), and has been shown to be an independent prognostic variable in lymphoma (61). Although apparently modestly overestimating the growth fraction, Ki67 appears to be a reasonable surrogate marker for cell proliferation (62), and we have been able to demonstrate growth stimulatory effects of oestrogenⁱ, the growth inhibitory effects of IFNⁱⁱ ⁱⁱⁱ, as well as a correlation of expression of Ki67 with PGP expression, failure of response to treatment and time to treatment failure, although no impact on overall survival could be demonstrated^{iv}.

6.2.7 PGP

PGP is a plasma membrane protein of 170 kDa which acts as an energy dependent drug efflux pump. It is encoded by the MDR-1 gene, and is expressed in normal human tissues, including colon, kidney and blood vessels, and has also been described in many malignant tumour types. In vitro studies suggest that expression may correlate with drug resistance for taxol and doxorubicin (63). In a cohort of 101 patients, 55% demonstrate PGP immunostaining, which can be correlated with ER positivity. Premenopausal patients and patients with prior exposure to anthracycline chemotherapy have a somewhat higher expression of PGP, but the presence

ⁱ see paper 6 - "Hormone Priming in Breast Cancer: Oestrogen Priming has a Detrimental Effect on Response in Oestrogen Receptor Negative Patients"

ⁱⁱ see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: *in vitro* Biologic Effects of Two Growth Modulators"

ⁱⁱⁱ see paper 7 - "Interferon plus Tamoxifen Treatment for Advanced Breast Cancer: *In Vivo* Biologic Effects of two Growth Modulators"

^{iv} see paper 11 - "P-Glycoprotein Immunostaining Correlates with ER and high Ki67 Expression but Fails to Predict Anthracycline Resistance in Patients with Advanced Breast Cancer"

of PGP failed to predict for subsequent anthracycline failureⁱ. Other investigators have confirmed higher expression of MDR-1 in younger, premenopausal patients (64). We have also demonstrated a similar lack of predictive correlation of PGP in patients with follicular lymphoma (65). Modulation of drug resistance utilising agents known to reverse MDR-1 phenotype in vitro has yet to demonstrate clinical benefit (66).

6.3 Therapy: the options and the challenges

A number of anti-oestrogens of steroidal or non-steroidal origin have been synthesised and developed for use in the clinic, the most commonly used one being the triphenylethylene TMX. Although TMX was initially believed to function purely as an antagonist of oestrogen function with effects mediated via binding to the ER, the observation that the anti-oestrogenic actions of TMX and its metabolites could not be completely reversed by oestrogen suggested alternative mechanisms of action (67). Subsequently, TMX has been demonstrated to have a number of alternate effects: reduction in ER expression (68) likely by selection of ER negative clones (69) (70); modulation of expression of and response to growth factors (71) (72) and their receptors; modulation of oncogene expression (73) ; and effects on signal transduction (74) (75). Data showing a beneficial effect of TMX even in patients who are ER negative in the adjuvant setting (76), further suggest that TMX may mediate some of its effects in non-receptor positive cells, including stromal cells and tumour vasculature (77). Resistance to anti-oestrogens, either primary or acquired, may be related to mutations in the

ⁱ see paper 11 - "P-Glycoprotein Immunostaining Correlates with ER and high Ki67 Expression but Fails to Predict Anthracycline Resistance in Patients with Advanced Breast Cancer"

ER (78) (79), abnormalities in the activating pathways, loss of ER, alteration in the expression of growth factors or their receptors, bypassing of signal transduction pathways by cross-talk, development of predominantly agonist effects (80) or to alterations in the metabolism (81) or the cellular pharmacology of ER itself (82).

Resistance to cytotoxic chemotherapies also represents a formidable challenge. Although adjuvant chemotherapies confer a survival advantage, many patients so treated subsequently relapse, presumably because of the failure to eradicate residual tumour cells. Overall response rates to chemotherapy administered in the setting of advanced disease are relatively encouraging, but most, if not all, patients relapse after therapy, or progress on treatment, and responses to second line chemotherapy are discouragingly low. Few if any patients are cured of their disease once relapse has occurred.

MDR is one of the most widely studied mechanisms of anticancer drug resistance. Cells selected for resistance to doxorubicin, one of the most active and widely used drugs in combination regimens for breast cancer, are typically cross resistant to other cytotoxic agents. The MDR-1 gene codes for the efflux pump PGP. PGP transports a wide variety of hydrophobic drugs out of the cell, including a number of anticancer drugs as well as endogenous compounds, including steroids (83). Another gene product which may be involved in drug resistance is MRP, an ATP-binding cassette drug transporter (84), also postulated to play a role in clinical drug resistance. Other potential mediators of drug resistance include decreased topoisomerase II activity and changes in glutathione levels (85).

Clinical drug resistance thus appears multifactorial. While MDR-1 is only one of a number of described drug resistance mechanisms, data suggest a role for PGP in the clinical setting, both in terms of prediction of poor outcome (86) and clinical drug resistance (87) (88). Further, the emergence of drug resistance mediated by PGP can be circumvented in vitro by co-administration of MDR-modulators (89). We have shown that the immunocytochemically detected expression of PGP is correlated with Ki67 and may be cell-cycle related. We could not however demonstrate a correlation between PGP expression and response to anthracycline chemotherapy or prognosis¹. Thus, while preclinical models of drug resistance are persuasive and well defined, the evidence for a direct correlation of MDR-1/PGP expression with acquired or intrinsic drug resistance in the clinical setting is less well defined. For endometrial cancer, the expression of MDR-1 does not appear to correlate with intrinsic drug resistance in this tumour type (90).

6.3.1 Overcoming resistance

In a laboratory setting, a number of agents have been shown to modulate PGP activity. Agents such as calcium channel blockers, calmodulin inhibitors, cyclosporin, steroids and anti-oestrogens can be shown to have such activity. Novel molecules such as LY335979 also have activity and are entering clinical development (91). Steroid hormones and anti-oestrogens may be substrates of PGP (92), or merely modulate the effects of PGP (73).

¹ see paper K - "P-Glycoprotein Immunostaining Correlates with ER and high Ki67 Expression but Fails to Predict Anthracycline Resistance in Patients with Advanced Breast Cancer"

Clinical drug resistance thus appears multifactorial. While MDR-1 is only one of a number of described drug resistance mechanisms, data suggest a role for PGP in the clinical setting, both in terms of prediction of poor outcome (86) and clinical drug resistance (87) (88). Further, the emergence of drug resistance mediated by PGP can be circumvented in vitro by co-administration of MDR-modulators (89). We have shown that the immunocytochemically detected expression of PGP is correlated with Ki67 and may be cell-cycle related. We could not however demonstrate a correlation between PGP expression and response to anthracycline chemotherapy or prognosisⁱ. Thus, while preclinical models of drug resistance are persuasive and well defined, the evidence for a direct correlation of MDR-1/PGP expression with acquired or intrinsic drug resistance in the clinical setting is less well defined. For endometrial cancer, the expression of MDR-1 does not appear to correlate with intrinsic drug resistance in this tumour type (90).

6.3.1 Overcoming resistance

In a laboratory setting, a number of agents have been shown to modulate PGP activity. Agents such as calcium channel blockers, calmodulin inhibitors, cyclosporin, steroids and anti-oestrogens can be shown to have such activity. Novel molecules such as LY335979 also have activity and are entering clinical development (91). Steroid hormones and anti-oestrogens may be substrates of PGP (92), or merely modulate the effects of PGP (73).

ⁱ see paper K - "P-Glycoprotein Immunostaining Correlates with ER and high Ki67 Expression but Fails to Predict Anthracycline Resistance in Patients with Advanced Breast Cancer"

Indomethacin and D,L-BSO have been demonstrated to reverse MRP mediated drug resistance and drug efflux in MRP overexpressing cell lines (93) (94).

TMX has been shown to potentiate the cytotoxicity of doxorubicin (95) in doxorubicin resistant murine leukemia cell lines, an effect mediated by increased retention of doxorubicin, and to reverse MDR (96). Similar effects have been described for other anti-oestrogens as well as for medroxyprogesterone acetate (97). IFN has been demonstrated to have similar effects in human melanoma and colon lines. In addition to the effects of the two agents alone, the combination of IFN and TMX may have synergistic beneficial effects on the reversal of PGP mediated doxorubicin resistance (98). We have shown that in addition to the growth inhibitory effects of TMX and IFN aloneⁱ, the agents in combination have modulatory effects on PGP, with an increase in immunocytochemical expression of PGP but partial reversal of the drug resistant phenotype of multidrug resistant MCF-7 sublines, suggesting a potential clinical role for the two agents in the clinicⁱⁱ.

Experience with modulators of drug resistance in the clinic is however limited. Clinical studies are complicated by the difficulty of achieving effective plasma levels of earlier PGP modulators and the effects of many modulators of PGP on the pharmacokinetics of coadministered doxorubicin or other cytotoxic agents; cyclosporin and other PGP modulators

ⁱ see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: *in Vitro* Biologic Effects of Two Growth Modulators"

ⁱⁱ see paper 13- " Sequential α -Interferon and Tamoxifen: *in Vitro* Reversal of MDR-1 Mediated Multidrug Resistance in the MCF-7 Cell Line"

has been shown to inhibit the clearance of both doxorubicin and doxorubicinol, making interpretation of data from clinical trials difficult (99). To date, no clear benefit has been demonstrated in clinical trials examining the effects of first generation modulators (100), however it must be noted that comparative clinical trials of the more specific and potent PGP modulators are currently ongoing (79).

6.3.2 Biologic Response Modifiers

Other therapeutic agents such as the biologic response modifiers have been investigated, both as treatments for ABC and modulators of biologic behaviour. Interferons comprise 4 main families, with 2 types : type I IFN includes α , β and ω IFN's, all of which share some antigenic similarities and which bind to the same receptor. IFN γ is a type II IFN, and is produced only by T lymphocytes and NK cells, and has a unique receptor, present on nearly all cells. IFN's are known to have a range of immunomodulatory, anti-proliferative and biologic actions, including reduction in c-erbB-2 phosphorylation (101), modulation of p53 expression and modulation of drug resistance (102). Although a number of breast and ovarian cell lines demonstrate growth inhibition to IFN (103), clinical trials using IFN as a single agent in ABC have been disappointing (104). There appears to be some evidence of synergism of the effects of retinoids and IFN (105).

In vitro data suggest that IFN (both α and β) and TMX may have synergistic effects on growth and ER expression (106) (107) (108). In the MCF-7 cell line we have demonstrated the antiproliferative effects of IFN alone and interesting effects on ER and TGF- β expression

as well as at least additive effects of the combination of IFN and TMX on growth and the expression of ER (1), TGF- β (1), P24(1), c-erbB-2 (1) and Ki67 (1)ⁱ. The biologic effects of the combination of IFN and TMX were confirmed in a translational pilot study suggesting that the model is a reasonable one. This study demonstrated that IFN increases expression of ER and P24 while decreasing expression of Ki67, and that the addition of TMX decreases expression of ER but increased expression of PRⁱⁱ. Variable effects on PDGF and TGF- β were demonstrated. Other small phase II studies have in some instances confirmed these effects, although it is currently unclear whether the combination offers a real clinical benefit (109).

6.3.3 Other modulatory strategies

The combination of hormonal and cytotoxic treatment is potentially desirable, as hormonal agents may selectively kill cells sensitive to hormonal therapy, while cytotoxics may preferentially kill ER negative cells. However, pharmacologic interactions are an important consideration. Initial observations on the interactions between these two classes of agents suggested that some demonstrated pharmacologic interactions might ultimately lead to beneficial modulation of the biologic behaviour of breast cancer tumours. Conversely, the potential also exists for antagonism between a cytostatic and a cytotoxic agent.

Actively proliferating cells are thought to be most responsive to the effects of cytotoxic

ⁱ see paper 12 - "Sequential Alpha-Interferon and Tamoxifen: *in vitro* Biologic Effects of Two Growth Modulators"

ⁱⁱ see paper 7 - "Interferon plus Tamoxifen Treatment for Advanced Breast Cancer: *In Vivo* Biologic Effects of two Growth Modulators"

chemotherapy (110). Early *in vitro* work suggested that breast cancer cells treated with anti-oestrogens accumulate in early G phase, and that these effects are reversed by oestrogen (111). Other *in vitro* studies have demonstrated that growth stimulatory hormones, such as oestrogen, EGF, insulin and glucocorticoids can improve cell kill with cytotoxic chemotherapy (112). These effects of oestrogen were also seen in ER negative cell lines (113). Epstein demonstrated however that this potentially beneficial effect was not necessarily seen with all cytotoxic agents of similar class (114).

Nonetheless, these preclinical studies led to the initiation of clinical trials testing the hypothesis that anti-oestrogen /oestrogen pretreatment of tumours may be associated with superior efficacy (115). A randomised controlled clinical trial performed in this unit demonstrated biologic effects of oestrogen, with increases in Ki67 and P24 even in ER negative patients, but a lower objective response rate in ER negative patients who underwent oestrogen priming, although this did not ultimately have an adverse effect on survivalⁱ. These stimulatory effects of oestrogen have also been demonstrated in ER negative tumours by other researchers (116) leading to the hypothesis that this was mediated by a paracrine effect with secretion of a growth stimulatory growth factor from neighbouring ER positive cells, even if these ER positive cells were present in infrequent numbers (117). Although biologic effects are generally demonstrable using this approach, the impact on efficacy outcomes is mixed, with some studies describing superior efficacy in terms of increases in response rates (118)

ⁱ see paper 6 - "Hormone Priming in Breast Cancer: Oestrogen Priming has a Detrimental Effect on Response in Oestrogen Receptor Negative Patients"

(119) or in certain subsets of patients such as those who had prior adjuvant chemotherapy (120), with overall, little impact on survival.

In recent years, a number of workers have also demonstrated effects of cytotoxic agents on ER expression. Yang reported that this downregulation of ER was accompanied by a reduction in the rate of protein synthesis (121). Others have demonstrated a decrease in ER expression without any discernable effect on overall protein synthesis (122). These data suggest that cytotoxics may have a potentially negative impact on response to hormonal agents. More sophisticated and relevant preclinical models need to be developed to allow clinical studies to test robust hypotheses rather than merely testing empiric mixes of hormonal and chemotherapy approaches.

6.4 Conclusions

Breast cancer is a common but devastating disease with an enormous impact on society. It has a complex biology, perhaps more complex than other tumour types, given its hormonal control. While this complex biology poses an enormous challenge to researchers investigating breast cancer, it conversely offers the hope of additional interventions, not feasible with other tumours that may have a beneficial impact on outcomes. Such interventions may include the definition of important prognostic indicators that might allow the tailoring of therapy to patients, limiting toxic therapies to those patients in whom they are beneficial, or the modulation of the biology of the tumour to circumvent drug resistance, either constitutional or acquired.

These studies have attempted to translate preclinical hypotheses into clinical investigations and ultimately define new management. Growth factors and markers of growth and oestrogen action have been demonstrated to have prognostic relevance in the clinic. We have shown proof of principle in the attempted modulation of breast cancer both in the preclinical and clinical setting; the ultimate investigation of the real clinical import of these observations awaits definitive clinical study, and the development of more effective modulators of drug resistance.

References

1. Forbes JF. The incidence of breast cancer: the global burden, public health considerations. *Semin Oncol* 24: S1-20 -S1-35 1997.
2. Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *Lancet* ii: 104-107, 162-165 1896.
3. Sporn MB, Todaro GJ. Autocrine secretion and malignant transformation of cells. *N Engl J Med* 303:878-880 1980.
4. Dickson RB, Lippman ME. Molecular determinants of growth, angiogenesis and metastases in breast cancer. *Semin Oncol* 19: 286-298 1992.
5. Brattain MG, Howell G, Sun L, Wilson JKV. Growth factor balance and tumor progression. *Current Opinion in Oncology* 6:77-81 1994.
6. Robertson JFR, Ellis IO, Pearson D, Elston CW, Nicholson RI, Blamey RW. Biological factors of prognostic significance in locally advanced breast cancer. *Breast Cancer Res Treat* 29: 259-264 1994.
7. Makris A, Powles TJ, Dowsett M, Osborne CK, Trott PA, Fernando N et al. Prediction of response to neoadjuvant chemoendocrine therapy in primary carcinomas. *Clin Cancer Res* 593-599 1997.
8. Gorsch SM, Memoli VA, Stukel TA, Gold LI, Arrick BA. Immunohistochemical staining

for transforming growth factor β -1 associates with disease progression in human breast cancer. Cancer Res 52: 6949-6953 1992.

9. McGuire WL, De La Garza M. Improved sensitivity in the measurement of estrogen receptor in human breast cancer. J Clin Endocrinol Met. 37: 986-989 1973.

10. Clark GM, McGuire WL, Hubay CA, Pearson OH, Marshall JS. Progesterone receptors as a prognostic factor in Stage II breast cancer. N Engl J Med 309: 1343-1347 1983.

11. Jakesz R, Smith CA, Aitken S, Huff K, Schuette W, Shackney S et al. Influence of cell proliferation and cell cycle phase on expression of estrogen receptor in MCF-7 breast cancer cells. Cancer Res 44:619-625 1984.

12. Massague J. TGF β signalling : receptors, transducers and Mad proteins. Cell 85: 947-950 1996.

13. Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R et al. Evidence that transforming growth factor- β is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48: 417-428 1987.

14. Dannecker C, Possinger K, Classen S. Induction of TGF- β by an anti-progestin in the human breast cancer cell line T-47D. Ann Oncol 7:391-395 1996.

15. Chen H, Tritton TR, Kenny N, Absher M, Chiu J-F. Tamoxifen induces TGF- β 1 activity and apoptosis of human MCF-7 breast cancer cells in vitro. J Cell Biochem 61: 9-17

16. Colletta AA, Wakefield LN, Howell FV, van Roozendaal KEP, Danielpour D, Ebbs SR et al. Antiestrogens induce the secretion of active TGF- β from human fetal fibroblasts. *Br J Cancer* 62 405: 409 1990.
17. Zugmaier G, Lippman M. Effects of TGF beta on normal and malignant mammary epithelium. *Ann New York Acad Science* 593: 272-275 1990.
18. Roberts AB, Thompson NL, Heine U, Flanders C, Sporn MB. Transforming growth factor- β : possible roles in carcinogenesis. *Br J Cancer* 57: 594-600.
19. Welch DR, Fabra A, Nakajima M. Transforming growth factor β stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc Natl Acad Sciences* 87: 7678-7682 1990.
20. MacCallum J, Bartlett JMS, Thompson AM, Keen JC, Dixon JM, Miller WR. Expression of transforming growth factor beta mRNA isoforms in human breast cancer. *Br J Cancer* 69: 1006-1009 1994.
21. Butta A, MacLennan K, Flanders KC, Sacks NPM, Smith I, McKinna A et al. Induction of transforming growth factor β 1 in human breast cancer in vivo following tamoxifen treatment. *Cancer Res* 52: 4261-4264 1992.
22. Gorsch SM, Memoli VA, Stukel TA, Gold LI, Arrick BA. Immunohistochemical staining for transforming growth factor β 1 associates with disease progression in human breast cancer. *Cancer Res* 52: 6949-6952 1992.

23. Walker RA, Dearing SJ , Gallacher B. Relationship of transforming growth factor β 1 to extracellular matrix and stromal infiltrates in invasive breast carcinoma. *Br J Cancer* 69: 1160-1165 1994.
24. Kopp A, Jonat W, Schmahl M, Knabbe C. Transforming growth factor β 2 levels in plasma of patients with metastatic breast cancer treated with tamoxifen. *Cancer Res* 55: 4512-4515 1995.
25. Edwards DP, Adams DJ, McGuire WL. Estradiol stimulates synthesis of a major intracellular protein in a human breast cancer cell line (MCF-7). *Breast Cancer Res Treat* 1: 209-223 1981.
26. Adams DJ, Hajj H, Bjercke RJ, McGuire WL. Detection of a Mr 24,000 estrogen-regulated protein in human breast cancer by monoclonal antibodies. *Cancer Res* 43: 4297-4301 1983.
27. Ciocca DR, Adams DJ, Edwards DP, Bjercke BJ, McGuire WL. Estrogen induced 24K protein in MCF-7 breast cancer cells is localised in granules. *Breast Cancer Res Treat* 2: 261-268 1984
28. Ciocca DR, Adams DJ, Edwards DP, Bjercke BJ, McGuire WL. Distribution of an estrogen-induced protein with a molecular weight of 24,000 in normal and malignant human tissues and cells. *Cancer Res* 43: 1204-1210 1983.
29. Ciocca DR, Asch RH, Adams DJ, McGuire WL. Evidence for modulation of a 24K

protein in human endometrium during the menstrual cycle. J Clin Endocrinol Metabol 57: 496-499 1983.

30. Conroy SE, Latchman DS. Do heat shock proteins have a role in breast cancer? Br J Cancer 74: 717-721 1996.

31. Ciocca DR, Fuqua SAW, Lock-Lim S, Toft DO, Welch WJ et al. Response of human breast cancer cells to heat shock and chemotherapeutic drugs. Cancer Res 52: 3648-3654 1992.

32. Oesterreich S, Weng C-N, Qiu M, Hilsenbeck SG, Osborne CK, Fuqua SAW. The small heat shock protein hsp27 is correlated with growth and drug resistance in human breast cancer cell lines Cancer Res 53: 4443-4448 1993.

33. Adams DJ, McGuire WI. Quantitative enzyme-linked immunoabsorbent assay for the estrogen-regulated Mr24,000 protein in human breast tumours: correlation with estrogen and progesterone receptors. Cancer Res 45:2445-2449 1985.

34. Love S, King JJB. A 27kDA heat shock protein that has anomalous prognostic powers in early and advanced breast cancer. B J Cancer 69: 743-748 1994.

35. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:172-182 1987.

36. Taverna D, Antoniotti S, Maggiora P, de Bortoli M, Hynes NE. ErbB-2 expression in estrogen receptor-positive breast-tumour cells is regulated by growth-modulatory reagents. *Int J Cancer* 56:522-528 1994.
37. Grunt TW, Saceda M, Martin MB, Lupu R, Dittrich E, Krupitza G et al. Bidirectional interactions between the estrogen receptor and the c-erbB-2 signalling pathways: heregulin inhibits estrogenic effects in breast cancer cells. *Int J Cancer* 63:560-567 1995.
38. Keshgegian AA. ErbB-2 oncoprotein overexpression in breast carcinoma: inverse correlation with biochemically and immunohistochemically-determined hormone receptors. *Breast Cancer Res Treat* 35: 201-210 1995.
39. Gullick WJ. The role of the epidermal growth factor receptor and the c-erbB-2 protein in breast cancer. *Int J Cancer* 5:55-61 1990.
40. Bianco AR, De Laurentiis M, Carlomagno C, Lauria R, Petrella G, Panica L et al. 20 year update of the Naples GUN trial of adjuvant breast cancer therapy: evidence of an interaction between c-erbB-2 expression and tamoxifen efficacy. *Proc Am Soc Clin Oncol* 373: 1998
41. Sjogren S, Inganas M, Lindgren A, Holmberg L, Bergh J. Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and in combination with other prognostic markers. *J Clin Oncol* 16: 462-469 1998.
42. Andrulis IL, Bull SB, Blackstein ME, Sutherland D, Mak C, Sidlofsky S et al. Neu/erbB-2 amplification identifies a poor prognostic group of women with node negative breast cancer.

J Clin Oncol 16: 1340-1349 1998.

43. Elledge RM, Green S, Ciocca D, Pugh R, Allred DC, Clark GM et al. HER-2 expression and response to tamoxifen in estrogen receptor positive breast cancer: a South West Oncology Group study. Clin Cancer Res 4: 7-12 1998.

44. Mehta R, McDermott JH, Hieken TJ, Marler KC, Patel MK, Wild LD et al. Plasma c-erbB-2 levels in breast cancer patients: prognostic significance in predicting response to chemotherapy. J Clin Oncol 16: 2409-2416 1998.

45. Borg A, Baldetorp B, Ferno M, Killander D, Olsson H, Ryden S et al. ERBB2 amplification is associated with tamoxifen resistance in steroid receptor positive breast cancer. Cancer Lett 81: 137-144 1994.

46. Kumar R, Mandal M, Lipton A, Harvey H, Thompson CB. Overexpression of HER2 modulates bcl-2, bcl-X_L, and tamoxifen induced apoptosis in human breast cancer cells. Clin Cancer Res 2: 1215-1219 1996.

47. Xu F-J, Stack S, Boyer C, O'Brian K, Whitaker R, Mills GB et al. Heregulin and agonistic anti-p185^{c-erbB2} antibodies inhibit proliferation but increase invasiveness of breast cancer cells that overexpress p185^{c-erbB2}: increased invasiveness may contribute to poor prognosis. Clin Cancer Res 3: 1629-1634 1997

48. Tsai C-M, Chang K-T, Wu L-H, Chen J-Y, Gazdar AF, Mitsudomi T et al. Correlations between intrinsic chemoresistance and HER-2/neu gene expression, p53 gene mutations and

cell proliferation characteristics in non-small cell lung cancer cell lines. *Cancer Res* 56: 206-209 1996.

49. Slamon D, Leyland-Jones B, Shak S, Paton V, Bajamonde A, Fleming T et al. Addition of Herceptin (humanised anti-HER2 antibody) to first line chemotherapy for HER2 overexpressing metastatic breast cancer (HER2+/MBC) markedly increases anticancer activity: a randomised multinational controlled phase III trial. *Proc Am Soc Clin Oncol* 377: 1998

50. Fretto LJ, Snape AJ, Tomlinson JE, Seroogy JJ, Wolf DL, LaRochelle WJ et al. Mechanism of platelet derived growth factor (PDGF) AA, AB, and BB binding to α and β PDGF receptor. *J Biol Chem*. 268:3625-3631 1993.

51. Bronzert DA, Pantazis P, Antoniades HN, Kasid A, Davidson N, Dickson RB et al. Synthesis and secretion of platelet derived growth factor by human breast cancer cell lines. *Proc Natl Acad Sciences* 84:5763-5767 1987.

52. Ginsburg E, Vonderhaar BK. Stimulation of growth of human breast cancer cells (T47D) by platelet derived growth factor. *Cancer Lett* 58: 137-144 1991.

53. Bhardwaj B, Klassen J, Cossette N, Sterns E, Tuck A, Deeley R et al. Localisation of platelet derived growth factor β receptor expression in the periepithelial stroma of human breast carcinoma. *Clin Cancer Res* 2: 773-782 1996.

54. Kute TE, Muss HB, Hopkins M, Marshall R, Case D, Kammire L. Relationship of flow

cytometry results to clinical and steroid receptor status in human breast cancer. *Breast Cancer Res Treat* 6:113-121 1985.

55. Baildam AD, Zaloudik J, Howell A, Barnes DM, Turnbull L, Swindell R et al. DNA analysis by flow cytometry, response to endocrine treatment and prognosis in advanced carcinoma of the breast. *Br J Cancer* 55:553-559 1987.

56. Hedley DW, Rugg CA, Gelber RD. Association of DNA index and S-phase fraction with prognosis of node positive early breast cancer. *Cancer Res* 47: 4729-4735.

57. Gasparini G, Boracchi P, Verderio P, Bevilacqua P. Cell kinetics in human breast cancer: comparison between the prognostic value of the cytofluorometric S-phase fraction and that of the antibodies to Ki-67 and PCNA antigens detected by immunocytochemistry. *Int J Cancer* 57:822-829 1994.

58. Witzig TE, Ingle JN, Scaid DJ, Wold LE, Barlow JF, Gonchoroff NJ et al. DNA ploidy and percent S-phase as prognostic factors in node positive breast cancer: results from patients enrolled in two prospective randomized trials. *J Clin Oncol* 11:351-359 1993.

59. Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 133: 1710-1715 1984.

60. Walker RA, Camplejohn RS. Comparison of monoclonal antibody Ki-67 reactivity with grade and DNA flow cytometry of breast carcinomas. *Br J Cancer* 57:281-283.

61. Grogan TM, Lippman SM, Spier CM, Slymen DJ, Rybski JA, Rangel CS et al. Independent prognostic significance of a nuclear proliferation antigen in diffuse large cell lymphomas as determined by the monoclonal antibody Ki-67. *Blood* 71:1157-1160 1988.
62. Scott RJ, Hall PA, Haldane JS, van Noorden S, Price Y, Lane DP et al. A comparison of immunohistochemical markers of cell proliferation with experimentally determined growth fraction. *J Path* 165:173-178 1991.
63. Mechetner E, Kyshtoobayeva A, Zonis S, Kim H, Stroup R, Garcia R et al. Levels of multidrug resistance (MDR-1) P-glycoprotein expression by human breast cancer correlate with in vitro resistance to Taxol and doxorubicin. *Clin Cancer Res* 4:389-398 1998.
64. Dexter DW, Reddy RK, Geles KG, Bansal S, Myint MA, Rogakto A et al. Quantitative reverse transcriptase-polymerase chain reaction measured expression of MDR1 and MRP in primary breast carcinoma. *Clin Cancer Res* 4: 1533-1542 1998.
65. Seymour L, Davidge-Pitts M, Lewis D, Bezwoda WR. P-glycoprotein: low constitutional expression does not correlate with treatment failure in follicular lymphomas. *Int J Oncology* 2: 1067-1070 1993.
66. Samuels BL, Hollis DR, Rosner GL, Trump DL, Shapiro CL, Vogelzang NJ et al. Modulation of vinblastine resistance in metastatic renal cell carcinoma with cyclosporine A or tamoxifen: a Cancer and Leukemia Group B study. *Clin Cancer Res* 3: 1977-1984 1997.
67. Sutherland RL, Watts CKW, Reunitz PC. Definition of two distinct mechanisms of action

of antioestrogens on human breast cancer cell proliferation using hydroxytriphenylethylenes with high affinity for the oestrogen receptor. *Biochem Biophys Res Comm* 140:523-529 1986.

68. Lundgren S, Soreide JA, Lea OA. Influence of tamoxifen on the tumour content of steroid hormone receptors (ER, PgR and AR) in patients with primary breast cancer. *Anticancer Res* 14:1313-1316 1994.

69. Kodama F, Greene GL, Salmon SE. Relation of estrogen receptor expression to clonal growth and antiestrogen effects on human breast cancer cells. *Cancer Res* 45:2720-2724 1985.

70. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 56:2321-2330 1996.

71. Perry RR, Kang Y, Greaves BR . Relationship between tamoxifen-induced transforming growth factor β_1 expression, cytostasis and apoptosis in human breast cancer cells. *Br J Cancer* 72:1441-1446 1995.

72. Lippman ME, Dickson RB. Mechanisms of growth control in normal and malignant breast epithelium. *Rec Prog Hormone Res* 45:383-440 1989.

73. Johnston SRD, MacLennan KA, Sacks NPM, Salter J , Smith IE , Dowsett M. Modulation of bcl-2 and Ki-67 expression in oestrogen receptor-positive human breast cancer by tamoxifen. *Eur J Cancer* 30:1663-1669 1994.

74. O'Brian CA, Liskamp RM, Solomon DH, Weinstein IB. Inhibition of protein kinase C by tamoxifen. *Cancer Res* 45:2462-2465 1985.
75. Cabot MC, Zhang Z-C, Giuliano A. Tamoxifen elicits rapid transmembrane lipid signal responses in human breast cancer cells. *Breast Cancer Res Treat* 36:299-306 1995.
76. Medical Research Council Scottish Trial Office. Adjuvant tamoxifen in the management of operable breast cancer. *Lancet* ii:171-175 1988.
77. Haran EF, Maretzek AF, Goldberg I, Horowitz A, Degani H. Tamoxifen enhances cell death in implanted MCF-7 breast cancer by inhibiting endothelium growth. *Cancer Res* 54:5511-5514 1994.
78. Jordan VC, Catherino WL, Wolf DM. Drug resistance to tamoxifen: mutant estrogen receptors as a potential mechanism of tamoxifen-stimulated tumor growth. *Endocrine-Related Cancer* 2:45-51 1995.
79. Fuqua SAW. Estrogen receptor mutagenesis and hormone resistance. *Cancer* 74:1026-1029. 1994.
80. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in thymic mice after long-term antiestrogen administration. *Cancer Res* 48:5183-5187 1988.
81. Wiebe VJ, Osborne CK, McGuire WL, DeGregorio MW. Identification of estrogenic

tamoxifen metabolite (s) in tamoxifen-resistant human breast tumours. *J Clin Oncol* 10:990-994 1992.

82. Osborne CK. Tamoxifen metabolism as a mechanism for resistance. *Endocrine-Related cancer* 2: 53-58 1995.

83. Gruol DJ, Bourgeois S. Chemosensitising steroids; glucocorticoid receptor agonists capable of inhibiting P-glycoprotein function. *Cancer Res* 54: 720-727 1997.

84. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CR, Almquist KC et al. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 258:1650-1654 1992.

85. Beck WT. Unknotting the complexities of multidrug resistance: the involvement of DNA topoisomerases in drug action and resistance. *J Natl Cancer Inst* 81:1683-1685. 1989.

86. Linn SC, Honkoop AH, Hoekman K, van der Valk P, Pinedo HM, Giaccone G. P53 and P-glycoprotein are often co-expressed and are associated with poor prognosis in breast cancer. *Br J Cancer* 74:63-68 1996.

87. Sanfillipo O, Ronchi E, De Marco C, Di Fronzo G, Silvestrini R. Expression of p-glycoprotein in breast cancer tissue and in vitro resistance to doxorubicin and vincristine. *Eur J Cancer* 7:155-158 1991.

88. Veneroni S, Zaffaroni N, Daidone MG, Benini E, Villa R, Silvestrini R. Expression of P-

glycoprotein and In vitro or In vivo resistance to doxorubicin and cisplatin in breast and ovarian cancers. *Eur J Cancer* 30A; 1002-1007 1994.

89. Sikic BL. Pharmacologic approaches to reversing multidrug resistance. *Sem Hematol* 34:40-47 1997.

90. Kuo D, Mallick S, Shen H-J, DeVictoria C, Jones J, Fields AL et al. Analysis of MDR-1 expression in normal and malignant endometrium by reverse transcription polymerase chain reaction and immunocytochemistry. *Clin Cancer Res* 2:1981-1992 1996.

91. Dantzig AH, Shepard RL, Cao J, Law KL, Ehlhardt WJ, Baughman TM et al. Reversal of P-glycoprotein mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. *Cancer Res* 56: 4171-4179 1996.

92. Rao US, Fine RL, Scarborough GA. Anti-oestrogens and steroid hormones: substrates if the human p-glycoprotein. *Biochem Pharmacol* 48:287-292 1994.

93. Draper MP, Martell RL, Levy SB. Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein. *Br J Cancer* 75:810-815 1997.

94. Vanhoefer U, Cao S, Minderman H, Toth K, Skenderis II BS, Slovak ML et al. D,L-Buthionine-(S,R)-sulfoximine potentiates in vivo the therapeutic efficacy of doxorubicin against multidrug resistance protein expressing tumors. *Clin Cancer Res* 2: 1961-1968 1996.

95. Kirk J, Syed SK, Harris AL, Jarman M, Roufogalis BD, Stratford IJ et al. Reversal of P-glycoprotein mediated drug resistance by pure anti-oestrogens and novel tamoxifen derivatives. *Biochem Pharmacol* 48: 277-285 1994
96. Callaghan R, Higgins CF. Interaction of tamoxifen with the multidrug resistance P-glycoprotein. *Br J Cancer* 72:294-299 1995.
97. Claudio JA, Emerman JT. The effects of cyclosporine A, tamoxifen and medroxyprogesterone acetate on the enhancement of Adriamycin cytotoxicity in primary cultures of human breast epithelial cells. *Breast Cancer Res Treat* 41: 111-112 1996.
98. Kang Y, Perry RR. Modulatory effects of tamoxifen and recombinant human α -interferon on doxorubicin resistance. *Cancer Res* 53:3040-3045 1993.
99. Bartlett NL, Lum BL, Fischer GA, Brophy NA, Ehsan MN, Halsey J et al. Phase I trial of doxorubicin with cyclosporin as a modulator of multidrug resistance. *J Clin Oncol* 12:835-842 1994.
100. Wood L, Palmer M, Hewitt J, Urtansan R, Bruera E, Rapp E, Thael JF. Results of a phase III, double blind, placebo-controlled trial of megestrol acetate modulation of P-glycoprotein-mediated drug resistance in the first line management of small cell lung carcinoma. *Br J Cancer* 77:627-631 1998.
101. Mishra S, Hamburger AW. Interferon gamma-induced reduction in erbB-2 tyrosyl phosphorylation in human ovarian carcinoma cells. *Int J Cancer* 58:538-542 1994.

102. Davol PA, Goulette FA, Frackelton AR, Darnowski JW. Modulation of p53 expression by human recombinant interferon- α 2a correlates with abrogation of cisplatin resistance in a human melanoma cell line. *Cancer Res* 56: 2522-2526 1996.
103. Coradini D, Biffi A, Pirronello E, di Fronzo G. The effect of alpha-, beta- and gamma-interferon on the growth of breast cancer cell lines. *Anticancer Res* 14: 1779-1784 1994.
104. Nethersell A, Smedley H, Katrak M, Wheeler T, Sikora K. Recombinant interferon in advanced breast cancer. *Br J Cancer* 49 615-619 1984.
105. Daxenbichler G, Widschwendter M, Marth C. Sensitivity of breast and ovarian cancer cells for interferons (IFN's) and retinoids. *Ann New York Acad Sciences* 784:294-303 1996.
106. Bezwoda WR, Meyer K. Effect of α -interferon, 17β -estradiol and tamoxifen on estrogen receptor concentration and cell cycle kinetics of MCF 7 cells. *Cancer Res* 50: 5387-5391 1990.
107. van den Berg HW, Leahey WJ, Lynch M, Clarke R, Nelson J. Recombinant human interferon alpha increases oestrogen receptor expression in human breast cancer cells (ZR-75-1) and sensitises them to the anti-proliferative effects of tamoxifen. *Br J Cancer* 55: 255-257 1987.
108. Di Martino L, Demontis B, Iotti GS, Murenu G. In vivo effect induced by interferon beta on steroid receptor status, cell kinetics and DNA ploidy in operable breast cancer patients. *Anticancer Res* 15:537-542 1995.

109. Sica G, Iacopino F, Recchia F . Interferon and hormone sensitivity of endocrine related tumors. *Anti-Cancer Drugs* 7: 150-160 1996.
110. Valeriote F, van Putten L. Proliferation-dependent cytotoxicity of anticancer agents: a review. *Cancer Res* 35:2619-2724 1975.
111. Osborne CK, Boldt DH, Estrada P. Human breast cancer cell cycle synchronisation by estrogens and antiestrogens in culture. *Cancer Res* 44: 1433-1439. 1984
112. Hug V, Johnston D, Finders M, Hortobagyi G. Use of growth stimulatory hormones to improve the in vitro therapeutic index of doxorubicin for human breast tumours. *Cancer Res* 46: 147-152 1986.
113. Reddel RR, Sutherland. Effects of pharmacologic concentrations of estrogens on RL proliferation and cell cycle kinetics of human breast cancer cell lines in vitro. *Cancer Res* 47: 5323-5329 1987.
114. Epstein RJ, Smith PJ. Estrogen induced potentiation of DNA damage and cytotoxicity in human breast cancer cells treated with topoisomerase II interactive antitumour drugs. *Cancer Res* 48: 297-303 1988.
115. Benz C, Gandara D, Miller B, Drakes T, Monroe S, Wilbur B et al. Chemoendocrine therapy with prolonged estrogen priming in advanced breast cancer: endocrine pharmacokinetics and toxicity. *Cancer Treat Rep* 71: 283-289 1987.

116. Conte PF, Fraschini G, Drewinko B. Estrogen induced expansion of the growth fraction in receptor negative human breast cancer. *J Steroid Biochem.* 23: 1169-1172 1985.
117. Conte PF, Alama A, Bertelli G, Canavese G, Carnino F, Catturich A et al. Chemotherapy with estrogenic recruitment and surgery in locally advanced breast cancer: clinical and cytokinetic results. *Int J Cancer* 40: 490-494 1987.
118. Allegra JC. Methotrexate and 5-fluorouracil following tamoxifen and premarin in advanced breast cancer. *Sem Oncol* 10 (2 Suppl 2) : 23-28 1983.
119. Ingle JN, Foley JF, Maillard JA, Krook JE, Hartmann LC, Jung S-H et al. Randomized trial of cyclophosphamide, methotrexate and 5-fluorouracil with or without estrogenic recruitment in women with metastatic breast cancer. *Cancer* 73: 2337-2343 1994.
120. Conte PF, Pronzato P, Rubagotti A, Alama A, Amadori D, Demicheli R et. Conventional versus cytokinetic polychemotherapy with estrogenic recruitment in metastatic breast cancer: results of a randomised cooperative trial. *J Clin Oncol* 5: 339-347 1987.
121. Yang KP, Samaan NA, Reduction of oestrogen receptor concentration in MCF-7 cells following exposure to chemotherapeutic drugs. *Cancer Res* 43: 3534-3538 1983.
122. Clarke R, Morwood J, van den Berg HW, Nelson J, Murphy RF. Effect of cytotoxic drugs on ER expression and response to tamoxifen in MCF-7 cells. *Cancer Res* 46: 6116-6119 1986.

Please reply to :

Mr J. Michael Dixon

Edinburgh Breast Unit
Western General Hospital
Crewe Road South
Edinburgh EH4 2XU, UK

Tel : + 44 (0)131 537 2653

Fax : + 44 (0)131 537 2653

email: ebu@dial.pipex.com

THE BREAST

AN INTERNATIONAL JOURNAL

24th August 1998

Dr Lesley Seymour
NCIC Clinical Trials Group
Queen's University
Kingston, Ontario K7L 3NG

Dear Dr Seymour,

Manuscript No: 13/98

**Title: Sequential Interferon and Tamoxifen: In Vitro Reversal of
MR-1 Mediated Multidrug Resistance in the MCF-7 Cell Line**

Thank you for submitting your paper to *The Breast*. It is acceptable for publication and will now be edited and sent for typesetting. You will receive proofs in due course.

With kind regards,

Yours sincerely,



Mr J Michael Dixon
Managing Editor

Chairman
Professor Sir Patrick Forrest
Professor Umberto Veronesi

Managing Editor
Mr J Michael Dixon

US Editor
David L Page MD

Author Seymour L K

Name of thesis Modulation Of The Biologic Behaviour Of Breast Cancer: In Vitro Models And Clinical Correlates Seymour L K 1999

PUBLISHER:

University of the Witwatersrand, Johannesburg

©2013

LEGAL NOTICES:

Copyright Notice: All materials on the University of the Witwatersrand, Johannesburg Library website are protected by South African copyright law and may not be distributed, transmitted, displayed, or otherwise published in any format, without the prior written permission of the copyright owner.

Disclaimer and Terms of Use: Provided that you maintain all copyright and other notices contained therein, you may download material (one machine readable copy and one print copy per page) for your personal and/or educational non-commercial use only.

The University of the Witwatersrand, Johannesburg, is not responsible for any errors or omissions and excludes any and all liability for any errors in or omissions from the information on the Library website.