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THE DETECTION AND ASSAY OF INTEGRINS AND SOLUBLE INTEGRIN LIGANDS AS AN INDEX OF GRANULOCYTE FUNCTION AND MASS AFTER CHEMOTHERAPY AND STEM CELL TRANSPLANTATION WITH HAEMATOPOIETIC GROWTH FACTOR ADMINISTRATION

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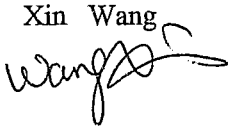
A dissertation submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science (Medicine).

Johannesburg 1998

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science (Medicine) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

Xin Wang



---29th---day of---Nov.---1998

This research was approved by the Committee for Research on Human Subjects, University of the Witwatersrand. (protocol number M 980339).

ABSTRACT

Specific interactions between molecules expressed on the surface of leukocytes and cell adhesion molecules on target cells are important determinants of leukocyte function as well as playing a role in cell - cell communication.Four main groups of adhesion molecules are recognized : the integrin receptor family; the immunoglobulin superfamily; selectins and cadherins. The focus of the present study is on CD11b/CD18 (Mac-1), which belongs to integrin family, and its ligand ICAM-1, which belongs to immunoglobulin family.

The studies which make up the body of this dissertation were designed to investigate:(a) the interaction of neutrophil adhesion molecule CD11b/CD18 and its ligands and (b) to develop a marker of neutrophil recovery and function after severe chemotherapy induced neutropenia following either intensive but unsupported conventional dose consolidation chemotherapy or high dose chemotherapy plus autologous stem cell transplantation. These latter studies focussed on the soluble intercellular adhesion molecule-1 (sICAM-1) .

The results of the studies presented in this dissertation may be summarized as follows :

a) Adhesion of neutrophils to endothelial cells by a CD11b/CD18-dependent mechanism

In this investigation the focus of interest was to study the adhesion of normal neutrophils to vascular endothelial cells. While the factors including the role of CD11/CD18 responsible for the interaction of neutrophils and endothelium had been previously investigated there was still disagreement about the role of CD11b/CD18. In this study an in-vitro method was developed whereby adhesion to the ECV-304 cell line was used to elucidate factors involved in neutrophil adhesion to vascular endothelium. The results showed that adhesion of unstimulated neutrophils to endothelial cells is largely mediated by a CD11b/CD18-dependent cell : cell interaction, probably through its ligand ICAM-1 on endothelial cells . In addition , the results showed that normal serum contains a potent adhesive factor, which promoted this interaction. This factor was identified as complement fragment iC3b.

b) Serum ICAM-1 concentration following conventional dose consolidation chemotherapy for acute myeloid leukemia and after high dose chemotherapy with autologous haematopoietic stem cell rescue

The first study had demonstrated an important role of CD11b/CD18 in neutrophil adhesion. One of the important ligands of CD11b/CD18 is ICAM-1. A soluble form of this ligand has previously been demonstrated. The focus of this study was to elucidate receptor/ligand interactions. In this investigation, serum concentrations of soluble ICAM-1 (sICAM-1) were studied in patients with acute myeloid leukemia (AML) after conventional dose consolidation chemotherapy and in AML and in breast cancer patients following high dose chemotherapy with autologous haematopoietic stem cell transplantation. Investigations were carried out at 3 phases following treatment; during the chemotherapy induced neutropenic phase (neutrophil counts $< 0.5 \times 10^9/l$); during early recovery(neutrophil counts $0.5 \times 10^9/l$ - $1.0 \times 10^9/l$); and at recovery from neutropenia(neutrophil count $1.0 \times 10^9/l$ - $2.5 \times 10^9/l$).

Results showed a significant elevation of serum levels of sICAM-1, above normal, in both groups of patients during the neutropenic phase. A further increase of sICAM-1 was found in conventional dose consolidation chemotherapy treated AML patients during the post-neutropenia recovery phases. By contrast, patients who were treated with high dose chemotherapy plus autologous haematopoietic stem cell transplantation showed a normalisation of sICAM-1 concentration during the post-neutropenic recovery phases. These findings suggest that recovery of

neutrophil function does not coincide with recovery of neutrophil count following intensive but unsupported chemotherapy while there was rapid recovery of neutrophil function occurred among patients who received autologous haematopoietic stem cell support.

c) Expression of CD11b/CD18 on neutrophils in patients undergoing consolidation chemotherapy for acute myeloid leukemia and in patients undergoing high dose chemotherapy and autologous haematopoietic stem cell transplantation

This investigation dealt with the quantitative expression of CD11b/CD18 on neutrophils by flow cytometry in same population of patients investigated in the previous study .

CD11b/CD18 expression on neutrophils during early and full neutrophil recovery was normal in stem cell transplantation supported patients. By contrast CD11b/CD18 expression was markedly decreased in patients who received chemotherapy without stem cell support. These results together with those of the early study indicated that neutrophil abnormalities with potentially important functional implications occur after neutrophil count recovery following chemotherapy and that these defects may be ameliorated by haematopoietic stem cell support.

In summary, the results presented here provide evidence that 1) CD11b/CD18 mediates the interaction of resting neutrophils to endothelial cells, probably by recognizing ICAM-1 on endothelium. Complement fragment iC3b promotes this interaction. 2) recovery of neutrophil function does not coincide with recovery of neutrophil count following intensive chemotherapy. sICAM-1 is considered as a sensitive marker of neutrophil functional recovery.

DEDICATION

I would like to dedicate this to my deeply loved husband :

Qinqiu

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I would like to specially thank my supervisor----Professor W.R. Bezwoda for initiating this study and for his invaluable advice, constant encouragement and endless guidance. I am indebted to him for his careful scrutiny of this study and for reviewing this dissertation, modifying my English countless times.

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LIST OF ABBREVIATION

ADCC	antibody-dependent cellular cytotoxicity
AML	acute myeloid leukemia
ASCT	autologous stem cell transplantation
ATCC	American Type Culture Collection
BSA	bovine serum albumin
BWB	blocking/washing buffer
Ca^{2+}	calcium ion
CO_2	carbon dioxide
CD	cluster differentiation
cDNA	complement deoxyribonucleic acid
Conc.	concentration
CR3	complement receptor type 3
CSF	colony stimulating factor
EDTA	ethylenediamine-tetra-acetic acid
EGTA	ethylenebis(oxyethylenenitriolo) - tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
FITC	fluorescein isothiocyanate
FACS	fluorescence activated cell sorting
FHA	filamentous hemagglutinin
fMLP	N-formyl-methionyl-leucyl-phenyl-alanine

G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GMP-140	granule membrane protein-140
HDC	high dose chemotherapy
HCl	hydrochloric acid
HLA	human leukocyte antigen
HUVEC	human umbilical cord vein endothelial cell
IL	interleukin
ICAM	intercellular adhesion molecule
KD	kilodalton
LAD	leukocyte adhesion deficiency
Lec-CAM	lectin cell adhesion molecule
LFA	leukocyte function associated antigen
LPG	lipophosphoglycan
LPS	lipopolysaccharide
Mg^{2+}	magnesium ion
Mac-1	macrophage antigen-1
MadCAM	mucosal addressin cell adhesion molecule
MCN	mean channel number
MoAb	monoclonal antibbody
mRNA	messenger RNA
MTP	microtiter plate
MW	molecular weight
NK cells	nature killer cells

ng	nanogram
PBS	phosphate buffered saline
PE-CAM	platelet-endothelial cell adhesion molecule
PMN	polymorphonulear neutrophil
POD	peroxidase
RGD	arginine-glycine-asparagine
SLE	systemic lupus erythematosus
SD	standard deviation
SDS-PAGE gel	sodium dodecyl sulfate-polyacrylamide gel
TMB	tetramethylbenzidine
TNF-α	tumour necrosis factor-α
U	unit
VCAM	vascular cell adhesion molecule
VLA	very late antigen
V/V	volume to volume
W/V	weight to volume

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CHAPTER ONE

THE AIM OF THIS DISSERTATION

The aim of the work presented in this dissertation was to develop new methods for the study of neutrophil function both in- vitro, and in-vivo in patients recovering from intensive chemotherapy resulting in severe neutropenia.

There is much clinical evidence to indicate that fatal infection during the neutropenic stage is the major cause of the death in patients receiving high dose chemotherapy. It is thus important to establish sensitive and reliable markers to evaluate functional neutrophil recovery.

While much attention has been given in the past to studies of the relationship of neutrophil numbers and infection, the question of neutrophil function has received less attention. Neutrophil function is however a vital component of host defence and an understanding of the relationship between neutrophil number and neutrophil function on recovery from neutropenia is becoming increasing important.

To achieve this aim , the first study concentrated on the in-vitro investigation of the CD11b/CD18 molecule ---- a neutrophil adhesive receptor. Subsequent investigations included measurements of sICAM-1, the soluble form of the CD11b/CD18 ligand as well as the in-vivo expression of CD11b/CD18 on neutrophils.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical Background

Since the early studies of Metchnikoff in the 1880s, a large body of data has accumulated establishing a crucial role for circulating polymorphonuclear leukocytes (PMN) in host defence against pyogenic infection (Metchnikoff, 1887; Klebanoff and Clark, 1978) . Important leukocyte activities involved in defence against such infection include chemotaxis, cell adhesion and cytotoxicity, all of which functions are crucial for host defence.

Other studies have also documented a pathogenic role for neutrophils in host tissue damage in a number of inflammatory conditions Neutrophil activation and function thus has both potentially beneficial and deleterious effects.

Although significant progress has been made in understanding the underlying basic mechanisms, a critical role for PMN adhesion function and the recognition of specific adhesion molecules as widely expressed families of cell surface receptors with important functions has occurred only in the last 10 years.

2.2 Brief Introduction of Adhesion Molecules

Specific interactions between molecules expressed on the surface to leukocytes (receptors) and those found on the surface of target cells (ligands) are important in cell - cell communication. These membrane associated molecules are collectively referred to as adhesion molecules.

Four main groups of adhesion molecules are recognized : the integrin receptor family, the immunoglobulin superfamily, selectins and cadherins. (Carlos et al, 1990; Larson and Springer, 1990; McEver, 1991; Haskard and lee, 1992; Kovach et al, 1992; Garcia-Barcina et al, 1995; Bruijn et al, 1995). (**Table 1**).

Table 1. Cell Adhesion Molecules

Adhesion Molecules	CD Nomenclature	Family
VLA-1	CD49a	Integrin
VLA-2	CD49b	Integrin
VLA-3	CD49c	Integrin
VLA-4	CD49d	Integrin
VLA-5	CD49e	Integrin
VLA-6	CD49f	Integrin
LFA-1	CD11a/CD18	Integrin
Mac-1	CD11b/CD18	Integrin
P150,95	CD11c/CD18	Integrin
$\alpha_d\beta_2$		Integrin
$\alpha_4\beta_7$	CD49d/β_7	Integrin
$\alpha_{IEL}\beta_7$	CD103	Integrin
Vibronectin and gpIIb/IIIa	CD41/CD61	Integrin
ICAM-1	CD54	IgG superfamily
ICAM-2	CD102	IgG superfamily
ICAM-3	CD50	IgG superfamily
VCAM-1	CD106	IgG superfamily
PECAM-1	CD31	IgG superfamily
MadCAM-1		IgG superfamily

Table 1. Continued

Adhesion Molecules	CD Nomenclature	Family
E-selectin	CD62E	Selectin
P-selectin	CD62P	Selectin
L-selectin	CD62L	Selectin
Cadherins		Cadherins

2.2.1 The Integrin Receptor Family

Integrins are transmembrane proteins made up of 2 chains, the α and the β chains which combine as heterodimers . At least 15 α and 8 β subunits are recognised giving rise to at least 19 different integrins (Ruoslahti et al, 1994;Bruijn and Heer,1995).The most widely studied subfamilies are(1) The β_1 (CD29) or very late activation (VLA) integrins. This group shares the β_1 chain and mainly participates in cell-matrix interactions.(2) The β_2 (CD18) or leukocyte integrins, which share the β_2 chain and participate in cell-cell interactions.(3) The β_3 (CD61) or cytoadhesion integrins, which share the β_3 chain and mediate endothelial and/or platelet-matrix adhesion functions. (4) The β_7 integrins, $\alpha_4\beta_7$ (CD49d) and $\alpha_E\beta_7$ (CD103), sharing β_7 chains and involved in lymphocyte - endothelial cell interactions.

2.2.2 The Immunoglobulin Superfamily

The immunoglobulin superfamily is characterized by immunoglobulin-like domains and comprises(1) leukocyte function antigen-2(LFA-2 or CD2);

(2) leukocyte function antigen-3 (LFA-3 or CD58); (3) intercellular adhesion molecules (ICAMs); (4) vascular cell adhesion molecule-1 (VCAM-1); (5) platelet-endothelial cell adhesion molecule-1 (PE-CAM-1) ; and (6) mucosal addressin cell adhesion molecule-1 (MadCAM-1).

LFA-2 (CD2) is expressed on thymocytes, mature T cells and natural killer (NK) cells (Krensky et al, 1984; Williams et al, 1987). The binding of T lymphocytes to endothelial cells is mediated in part by the interaction of LFA-2 and endothelial LFA-3 (Krensky et al, 1983; Makgoba et al, 1989; Savage et al, 1991).

ICAMs comprise three members : ICAM-1 (CD54); ICAM-2 (CD102) and ICAM-3 (CD50). They are cytokine-inducible adhesion molecules expressed on a variety of cell types, including endothelial cells, fibroblasts and haematopoietic cells . ICAMs help to localize leukocytes to areas of tissue injury.

Vascular cell adhesion molecule-1 (VCAM-1 or CD106) is expressed on activated endothelial cells (Carlos et al. 1990). VCAM-1 may contain

either six or seven immunoglobulin domains of the H type and binds to VLA-4. VCAM-1 regulates adhesion of monocytes, lymphocytes, basophils and eosinophils to endothelial cells activated by several cytokines (Osborn et al, 1989; Graber et al, 1990; Elices et al, 1990; Bochner et al,1991; Dobrina et al, 1991; Lobb,1992; Ruoslahti et al,1994).

Another adhesion molecule is platelet-endothelial cell adhesion molecule-1 (PE-CAM-1, CD31). PE-CAM-1 is expressed in large amounts on endothelial cells at intercellular junctions and to a lesser extent on platelets and on most leukocytes (Vaporeiyan et al, 1993; Benditt and Schwartz, 1994). PE-CAM-1 is involved in the CD18-dependent transmigration of neutrophils across endothelium in vivo.

The mucosal vascular addressin, MadCAM-1, is a novel immunoglobulin-like family member, which is expressed largely by endothelial cells at mucosal sites and is a receptor for both $\alpha_4\beta_7$ and L-selecin (Berg et al, 1993; Berlin et al, 1993).

2.2.3 Selectins

Selectins , also known as lectin cell adhesion molecules (Lec-CAMs) comprise a class of cell adhesion molecules that are expressed on the surface of endothelial cells, leukocytes, and platelets. Slectins have three family members : E-selectin (CD62E) or endothelial cell adhesion molecule-1 (ELAM-1); P-selectin (CD62P) or granule membrane protein-140 (GMP-140); and L-selectin (CD62L) or leukocyte adhesion molecule-1 (LAM-1) (Garcia-Barcina et al, 1995; Tedder et al, 1995). These adhesion molecules influence the localization of circulating leukocytes on the endothelium at the site of inflammation. Selectin-carbohydrate mediated leukocyte adhesion is an early event in adhesion responses and promotes attachment to nonactivated endothelium (Giger et al, 1987).

2.2.4 Cadherins

The maintenance of adult tissue construction largely depends on the structural and functional integrity of cadherins. The cadherins comprise a superfamily of Ca^{2+}-dependent cell-cell adhesion molecules that are

involved in establishing and maintaining intercellular connections(Benditt and Schwartz,1994).Three subclasses are recognized: (1)Epithelial(E)-cadherin or uvomorulin, is expressed in most epithelial cells ; (2) placental (P)-cadherin, is primarily expressed in placenta and in the basal or lower layers of stratified epithelial ; (3) Neural (N)-cadherin, which is mainly localized to adult neural tissues, muscles, kidney and the lens of the eye.

2.3 Classification, Structure and Function of Integrins

Integrins are glycoproteins expressed on the cell surface. All integrins are αβ hetero dimers. The α subunits vary in size between 120 to 180 KD. The β subunit size varies between 90 to 110 KD. In most integrins, the cytoplasmic domains are short , around 50 amino acids or less. The β_4 integrin is a exception, its cytoplasmic domain comprises over 1000 amino acids. The extracellular domains of the integrins are nonconvalently associated to form the αβ hetero dimers.

A number of observations give rise to the models depicted in **Figure 1** . The α subunits all contain a seven-fold repeat of a homologous segment, the last three or four of these repeats contain the sequence (Asp--Asp--Asp--Gly--Asp) or related sequences that appear to contribute to the divalent cation-binding properties of these subunits. Divalent cations are essential for maintaining the molecular structure of integrins and their functional state . Some α subunits are posttranslationally cleaved to give a 25-30 KD transmembrane chain which is disulfide-bonded to a large extracellular chain. Other α subunits contain an extra segment of around 180 amino acids, known as an I domain, which is inserted before the last five homologous repeats containing the cation-binding domains. Functionally, the I domain contributes to ligand-binding.

A characteristic of all β subunits is a four-fold repeat of a cystine-rich segment believed to be internally disulfide bonded (Calvete et al, 1991). Electron microscopic evidence (Burridge et al, 1988) and fluorescence photo-bleaching studies (Duband et al, 1986) suggest that the cytoplasmic domains of the β subunits connect to cytoskeletal proteins and may contribute to extracellular to intracellular signal transduction.

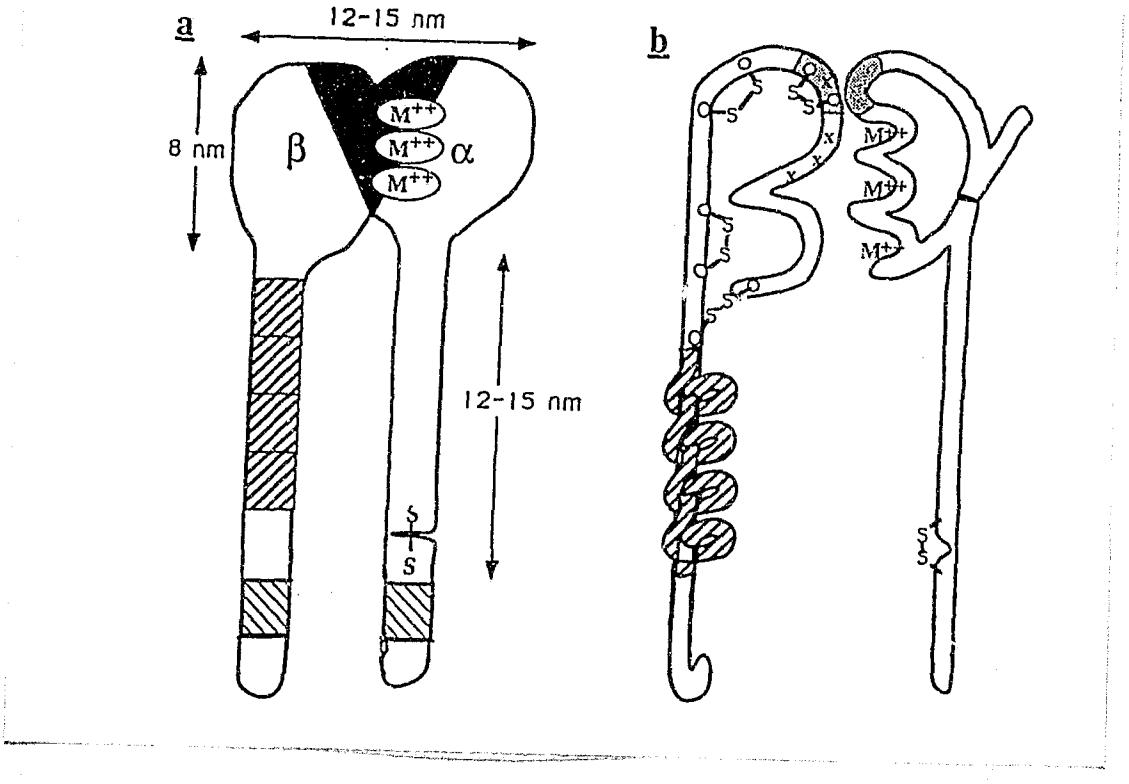

Figure 1 . Structural Features of Integrin Receptors

(a) shows the overall shape, as deduced from electron microscopy, as well as the putative locations of the cystine-rich repeats of the β subunit (crosshatched) and the cation-binding sites in the α subunit (M^{++}). The shaded area represents the ligand-binding region that is known based on cross-linking and binding data to be made up of portions of both subunits. (b) Schematizes the arrangement of the polypeptide chains with cystine repeats internally folded and the head region of the β subunit containing internal disulfide loops. Xs indicate positions of mutations (human β_2 or β_3 subunits) known to affect ligand binding or $\alpha\beta$ dimerization. The position of alternatively spliced segments in Drosophila subunits are shaded.

Integrins participate in a wide range of cell functions. During embryonic development. integrins can impart position-specific information which guides cell migration, localization, and the transfer of information between cells. As cells are triggered to differentiate to form tissues or organs, integrins help to maintain the organization of these structures.

Integrins play an important role in wound healing, host defence, immune response and in the behaviour of malignant cells. Moreover integrins are involved in cell-matrix interactions. These interactions are important for the regulation of cell migration.

2.4 Leukocyte Integrins, CD11/CD18

2.4.1 Nomenclature

The nomenclature of the leukocyte integrins is based on a mixture of functional definition, protein identification and phenotype identification by

antibody reactivity. Some order is being achieved with the use of the cluster designation (CD) system of nomenclature.

LFA-1 is an acronym for leukocyte function-associated antigen-1. Mac-1 is an abbreviation for macrophage antigen-1, also called Mo1, OKM-1, and complement receptor type-3 (CR3). Mac-1 has substantive homology with P150,95 , which also known as the complement receptor type-4 (CR4 or Leu M5). In the CD system of nomenclature LFA-1, Mac-1 and P150,95 have been designated as CD11a, CD11b and CD11c respectively, each of these molecules is noncovalently associated with a common β subunit, designated as CD18 (Kishimoto et al, 1989).

2.4.2 CD11/CD18 Identification

Mac-1 was first defined by monoclonal antibodies (MoAbs) marking myeloid cells (Springer et al, 1979). In 1982, a group of researchers

discovered that Mac-1 is the same molecule as that previously defined as complement receptor type 3 (CR 3) which has the function of binding iC3b. (Beller et al, 1982; Wright et al, 1983). LFA-1 was identified independently of Mac-1 by screening MoAbs for the ability to inhibit cytotoxic T lymphocyte (CTL)- mediated killing of tumour cell targets (Davignon et al, 1981). Analysis of a β-subunit specific MoAb led to identification of a third protein P150,95 (Sanchez-Madrid et al, 1983b; Lanier et al, 1985).

2.4.3 Cellular Distribution of CD11/CD18

CD11/CD18 is expressed only in leukocytes (Carlos and Harlan, 1994). CD11a/CD18 is present on all leukocytes; CD11b/CD18 and CD11c/CD18 are more restricted and are present only on monocytes, macrophages, polymorphonuclear leukocytes and natural killer (NK) cells. CD11c/CD18 is also expressed on some B-cell lines, such as hairy cell leukemia and in

certain cloned, cytotoxic, T- lymphocytes.

2.4.4 CD11/CD18 Biosynthesis

The α and β subunits of CD11/CD18 are synthesized as distinct precursors with molecular weights of 177 KD, 165 KD, 150 KD and 95 KD respectively. After association of α and β subunits , the CD11 and CD18 precursors undergo an increase in their molecular mass due to glycosylation of the N-linked carbohydrates to their complex form. This modification occurs in the Golgi apparatus.

Abundant intracellular storage pools for CD11b/CD18 and CD11c/CD18 exist in the secondary and tertiary granules of granulocytes as well as in intracellular vesicles and in peroxidase-negative granules in monocytes (Arnaout et al , 1984 ; Todd et al . 1984 ; Miller et al,1987 ; Freyer et al, 1988). Little or no CD11a/CD18 is stored intracellularly (Arnaout et al, 1984; Freyer et al. 1988). Upon activation, intracellular granules release

CD11b/CD18 or CD11c/CD18 to the cell surface, resulting in a several fold increased surface density.

2.4.5 Structural and Homology of CD11/CD18

The major structural features of leukocyte integrins were described in the overview section. CD11/CD18 are transmembrane proteins spanning the plasma membrane, with a short C-terminal cytoplasmic region and large N-terminal extracellular domain (**Figure 2**). In the α subunits, CD11a has 1063 amino acids with 12 N-glycosylation sites and a 53 amino acid long cytoplasmic region; CD11b has 1136-1137 amino acids with 19 N-glycosylation sites and a 19 amino acid cytoplasmic region; while CD11c has 1144 amino acids with 10 N-glycosylation sites and a 29 amino acid cytoplasmic region . Among these α subunits, the transmembrane region is highly conserved with about 87% identity. This implies a important functional role of this region perhaps in stabilization of the αβ complex, in signal transduction or in interaction with membrane lipids .

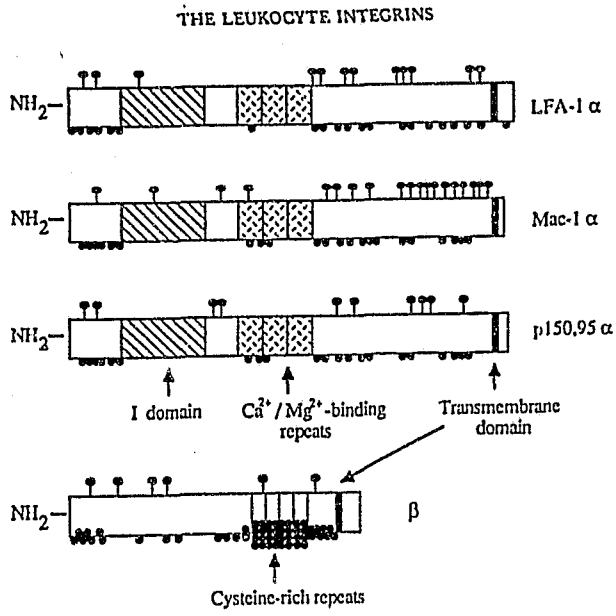

Figure 2. Schematic representation of the primary structure of the CD11a, CD11b and CD11c α subunits and the common CD18 β subunit. Black lollipops and circles represent N-linked glycosylation sites and cysteine residues, respectively.

The extracellular region of CD11/CD18 contains two striking features. The first, a domain consisting of seven homologous tandem repeats, each of which has a putative divalent cation-binding site. These putative metal-binding sites may account for the Mg^{2+} dependency of leukocyte-integrin-mediated adhesion. The second functionally active α domain comprises an insert of 187-200 amino acids and is called the I domain. The I domain is a critical recognition site for ligand binding, including binding of iC3b, fibrinogen and ICAM-1. The I domain is also responsible for MoAb binding and may be involved in cell-cell, cell-matrix adhesion functions . A feature of CD11 α subunits is the lack of a dibasic proteolytic cleavage site present in other integrins , due to a small deletion in the homologous gene region . Previous studies (Fleming et al, 1992) have shown that related, nonleukocyte, α integrin chains, such as fibronectin receptor (Argraves et al, 1986), vitronectin receptor (Suzuki et al, 1986) and gpIIb/IIIa (Poncz et al, 1987) are all proteolytically cleaved at an inserted exon resulting in a larger amino terminal polypeptide which is linked by a disulfide bond to a small carboxyl terminal transmembrane polypeptide. The cDNA of the α chain of the CD11/CD18 integrin family does not include this 28-residue exon containing the proteolytic cleavage site present in other integrin families.

The primary structures of the β chain show, firstly , a short cytoplasmic domain ~ 46 amino acids long, comprising tyrosine, serine and threonine residues, associating with talin and actin-β in the cytoskeleton and responsible for CD18 phosphorylation. Secondly , the β chain of CD18 contains a 23 amino acid transmembrane region. This region may be functionally similar to the corresponding α chain region. A third characteristic feature is an extracellular region of 677 amino acids, consisting of four tandem repeats of an 8-cysteine motif.

Electron microscopic studies have shown the αβ hetero dimer assumes a mushroom head, probably resulting from association of the N-terminal halves of the α and β subunits. Putative metal-binding domains of the α subunits and of the cysteine rich region of the β subunit are presumed to lie in the mushroom head.

The α subunits of CD11/CD18 are highly homologous. They are more related to each other (~40% homology) than to other integrins (~25% to 30% identity). CD11b and CD11c are more closely related to each other (63% identity) than to CD11a (36% homology). The β_2 subunit shares 37%-45% amino acid identity with the other β_{1-7} subunits.

2.4.6 Chromosomal Localization of CD11/CD18

The CD11a subunit gene has been mapped to chromosome 16 by gene complementation in somatic cell hybrids (Marlin et al, 1986).Southern blot analysis of DNA from LFA-1 α hybrid cells has found that CD11b and CD11c genes are on the same chromosome (Corbi et al, 1988a). Further hybridization data show that all three α chain genes occur in a cluster between band p11-p11.2 on chromosome 16 . The sizes of the messenger RNA (mRNA) for CD11a,b,c are 5.5, 4.9, and 4.7 kb, respectively.

The gene for the CD18 was first mapped to chromosome 21 by gene complementation in somatic cell hybrids (Marlin et al, 1986). The corresponding gene has been further localized on band 21q22 by hybridization (Corbi et al, 1988a). The size of CD18 mRNA is 3.2 kb.

2.4.7 Functions of the CD11/CD18 Family

The various functions mediated by CD11 / CD18 are summarized in **Table 2** . CD11a/CD18 was first recognized as a receptor that mediates the

adhesion of T cells to their targets (Springer et al, 1987). In T and B lymphocytes CD11/CD18- dependent functions include mitogen, antigen and alloantigen induced proliferation as well as T cell-mediated cytotoxicity (Trowbridge and Omary,1981; Sanchez-Madrid et al, 1983; Arnaout et al, 1984). B cell aggregation (Mentzer et al, 1985; Patarroyo et al, 1985) and immunoglobulin production (Arnaout et al, 1982) are inhibited by anti-CD11a/CD18 MoAbs. These findings support the hypothesis that CD11a/CD18 is constitutively expressed on these cells and is involved in multiple functions of these cells. Recently, CD11a/CD18 has been shown to play a role in strengthening the adhesion of T lymphocytes to dendritic cells. Both T cells and dendritic cells express CD11a/CD18 (Springer, 1990). All three CD11/CD18 proteins are critical to granulocyte and monocyte adhesion-dependent functions. Apparently, CD11b/CD18 plays the major role in this regard , with variable contributions from other two leukocyte integrins (Dana and Arnaout, 1988). CD11b/CD18 is essential for binding to iC3b and to other ligands, for cell spreading, for homotypic aggregation, for adhesion to endothelial cells, and following migration, for phagocytosis of opsonized particles and the oxidative burst in neutrophils. (Arnaout et al, 1983; Arnaout et al, 1986; Arnaout et al, 1988).

Table 2. Functions of CD11/CD18 on leukocytes

Leukocyte function	Molecules involved
Myeloid series	
Binding to C3bi	CD11b,c/CD18
Adhesion to endothelium	CD11a,b,c/CD18
Aggregation	CD11a,b/CD18
Random migration/chemotaxis	CD11a,b/CD18
Phagocytosis	CD11b/CD18
fMLP-induced oxidative burst	CD11b/CD18
ADCC	CD11a,b,c/CD18
Lymphoid series	
Antigen, mitogen, alloantigen-induced proliferation	CD11a/CD18
T and B cell aggregation	CD11a/CD18
Adhesion to endothelium	CD11a/CD18
Immunoglobulin production	CD11a/CD18

A role for CD11c/CD18 as a complement receptor was first discovered when both CD11b and CD11c were found to elute specifically from a C3bi affinity columns (Micklem and Sim, 1985). These results suggest that CD11c/CD18, like CD11b/CD18 has broad roles and acts as a general adhesion protein, with a iC3b-binding activity on monocytes and tissue macrophages (Te Velde et al , 1987; Myones et al , 1988). Recently CD11c/CD18 expression has been reported on some activated lymphocytes and lymphocytic cell lines. Anti- CD11c/CD18 MoAb was found to inhibit conjugate formation by cytotoxic T lymphocytes (Keizer et al, 1987a).

2.5 Identification and Characterization of Ligands for Leukocyte Integrins

2.5.1 ICAMs

Three members of ICAM formally are recognized : (1) intercellular adhesion molecule-1 (ICAM-1, CD54); (2) intercellular adhesion molecule-2 (ICAM-2, CD102) and (3) intercellular adhesion molecule-3 (ICAM-3, CD50). These proteins all belong to immunoglobulin

supergene family (Rothlein et al, 1986; Springer, 1990). ICAMs help to localize leukocytes to areas of tissue injury(Boyd et al,1988; Dougherty et al,1988). These molecules facilitate the adhesion of leukocytes expressing corresponding receptors such as β_2 integrins.

ICAM-1 (CD54) is expressed on non-haematopoietic cells such as vascular endothelial cells, thymic epithelial cells, certain other epithelial cells, fibroblasts , and on haematopoietic cells such as tissue macrophages, mitogen-stimulated T lymphoblasts as well as on germinal centre dendritic cells in tonsils, lymph nodes, and Peyer's patches. (Dustin et al, 1986; Johnson et al, 1989; Vogetseder et al, 1989). ICAM-1 is normally expressed in low amounts on peripheral blood leukocytes. Phorbol ester-stimulated differentiation of myelomonocytic cell lines greatly increases ICAM-1 expression. ICAM-1 expression on dermal fibroblasts is increased three to fivefold by either interleukin 1 (IL 1) or by interferon-γ over a period of 4 or 10 hours, respectively. The induction is dependent on mRNA and protein synthesis and is reversible . ICAM-1 displays Mr heterogeneity in different cell types with a Mr of 97KD on fibroblasts, 114 KD on the myelomonocytic cell line U937, and 90KD on the B lymphoblastoid cell JY. ICAM-1 biosynthesis involves a Mr~73KD intracellular precursor. ICAM-1 has five immunoglobulin-like constant

region (C) domains. It binds to CD11a/CD18 and CD11b/CD18 present on the cell membranes of neutrophils, T cells and macrophages (Makgoba et al, 1988; Diamond et al, 1990; Diamond et al, 1991; Kavanaugh et al, 1991; Lobb, 1992; Breider, 1993; Fantone and Ward, 1994). ICAM-1 is also an important receptor for rhinovirus (Marlin et al, 1990).

ICAM-2 was cloned by screening a cDNA library from endothelial cells (Law et al, 1987; Rosmarin et al, 1989; Springer et al, 1994). It has two C-like domains most homologous to the two N-terminal domains of ICAM-1. ICAM-2 is constitutively expressed on endothelial cell surfaces and binds to CD11b/CD18. ICAM-2 plays a role in the initial localization of neutrophils to sites of tissue injury.

ICAM-3 consists of five immunoglobulin like domains and binds to CD11a/CD18. ICAM-3 may thus mediate the adherence of leukocytes expressing CD11a/CD18. ICAM-3 is expressed on all leukocytes and is closely related to ICAM-1 but the expression of ICAM-3 on resting leukocytes is low . The highest ICAM-3 expression is on B cells and monocytes/macrophages. ICAM-3 may be the most important ligand for CD11a/CD18 mediated initiation of the immune response.

These ICAM/integrin interactions , such as the interaction of ICAM-1 with CD11a and CD11b, ICAM-2 with CD11a and ICAM-3 with CD11a provide a mechanism for the recruitment of leukocytes in different pathologic situations.

Functionally active , soluble forms of ICAMs (ICAM-1 and ICAM-3) have been described recently (Rothlein et al, 1991; Seth et al, 1991; Martin et al, 1995). Circulating ICAM-1 and ICAM-3 are present in normal human serum and are elevated in some pathologic circumstances, including in patients with leukocyte adhesion deficiency (LAD), Graves's disease, Wegener's granulomatosis, systemic lupus erythematosus and graft rejection (Adams et al, 1989; Rothlein et al, 1991; De Bellis et al, 1994; Mrowka and Sieberth, 1995). Soluble forms of ICAMs(sICAMs) have been detected in the serum of patients with malignancies. Patients with metastatic cancer generally show high levels of sICAM-1 . (Tsujisaki et al, 1991; Banks et al, 1993; Pizzolo et al, 1993).

A number of studies have also reported that overexpression of tissue ICAM-1 is corelated with dissemination of malignancy and a prognostic factor for outcome in non-Hoghkin's lymphoma and childhood acute

lymphoblastic leukemia. (Mielcarek et al, 1997; Terol et al, 1998).

2.5.2 Complement iC3b

The first characterized ligand for cellular adhesion molecules was iC3b. iC3b is a 175KD, C3 complement fragment generated by cleavage of C3b (Beller et al, 1982; Arnaout et al, 1983; Wright et al, 1983). iC3b binds to CD11b/CD18 and to CD11c/CD18 in a divalent cation dependent manner. iC3b contains an RGD sequence , however, it is not as yet clear if the RGD sequence is the only critical peptide for integrin binding.

2.5.3 Factor X and Fibrinogen

Although CD11b/CD18 was originally described as a receptor for iC3b, more recent studies have identified several other ligands, including coagulation factor X and fibrinogen that can bind to this receptor. Surfaces coated with fibrinogen are recognized by CD11b/CD18 (Altieri et al, 1988; Wright et al, 1988) and this receptor may therefore promote the

adhesion of neutrophils to clots and result in the digestion of fibrin. CD11b/CD18 also binds to soluble clotting factor X (Altieri and Edgington, 1988a). Fibrinogen and factor X effectively compete with each other and with iC3b for binding to CD11b/CD18, suggesting substantial identity of the binding site involved. Although these ligands subserve many CD11a/CD18 dependent adhesive functions, they do not account for the entirety of the adhesive interactions. However, the physiologic significance of factor X and fibrinogen binding to CD11b/CD18 remains unclear.

2.5.4 Other Endogenous Ligands

A recent study has found that human leukocyte elastase is an endogenous ligand for CD11b/CD18 (Cai and Wright, 1996). Elastase is thought to play a role in the transmigration of PMN by digesting matrix proteins. However this study found that elastase may act not just to digest extracellular material but also appears to modulate leukocyte detachment by releasing integrins from other substrates and so permit forward motion.

Studies are underway to determine whether elastase maintains its proteolytic activity when bound to CD11b/CD18.

2.5.5 Exogenous Ligands

CD11b/CD18 has been described as a promiscuous receptor for multiple ligands. In addition to the factors described the previous section other ligands have been found. CD11b/CD18 has been implicated in macrophage binding to E.coli and to several other micro-organisms including Histoplasma capsulatum, Bordetella pertussis and Leishmania.CD11b/CD18 also binds to the atypical substrate Zymosan. These binding functions are thought to be due to direct recognitions of lipopolysaccharide (LPS) (Wright and Jong, 1986), filamentous hemagglutinin (FHA) (Relman et al, 1990), lipophosphoglycan (LPS) and gp63 (Russell and Wright, 1988 ; Talamas-Rohana et al, 1990), and β-glucan (Ross et al. 1985a; Ross et al, 1987).

2.6 Clinical Manifestations of Altered CD11/CD18 Expression

2.6.1 Clinical States Associated With Decreased CD11/CD18 Expression

Leukocyte Adhesion Deficiency Type I and II

During the last ten years, deficiency of the CD11/CD18 leukocyte adhesion molecules (LAD) has been described in more than 100 patients worldwide. Leukocyte adhesion molecule deficiency is a rare inherited disease presenting in the first 2 years of life is and characterized by recurrent and often fatal bacterial infections, impaired pus formation, and delayed wound healing. (Dana and Arnaout,1988). In the majority of cases inheritance is as an autosomal recessive trait . The underlying mechanism of deficient surface expression of CD11/CD18 in these patients is a heterogeneous defect in the common CD18 subunit. This defect interferes with normal surface membrane expression of all three hetero dimers (Dana et al, 1987; Kishimoto et al, 1987; Dimanche et al, 1987). Neutrophils have a reduced phagocytic response to bacteria and yeasts, as well as a reduced ability to adhere to various substrates and to migrate to sites of infection (Beller et al, 1982; Arnaout et al, 1982; Arnaout et al, 1984; Beatty et al, 1984; Springer et al, 1986). The recognition of these diseases has apparently increased recently and is probably the result of

newly available diagnostic techniques. The clinical manifestations of the congenital CD11/CD18 deficiency diseases have been classified into two groups (Ross et al, 1985b; Ross, 1986; Styrt, 1989; Ricevuti and Mazzone, 1989) : Type I (partial deficiency) In this group patients have varying clinical features ranging from frequent and long-lasting infective episodes which respond poorly to antibiotics to the occurrence of trivial skin infections. Patients with type I LAD , often survive into adulthood. Type II , (total LAD deficiency) is characterized by frequent infections and severe episodes of bacteremia, sepsis and severe infection of the umbilical stump . S.aureus septicaemia and high mortality often occur in the infantile period.

CD11/CD18 Deficiency in Haematological Disorders

Myelodysplastic disorders are characterized by bone marrow dysmaturation with signs of pathological haematopoiesis in one, two or three cell lines. Clinical features of myelodysplastic disorders include anaemia and peripheral blood cytopenias (Gahmberg et al, 1979). CD11/CD18 expression on the cell surface and in the intracellular pool has been found to be decreased in patients with myelodysplastic disorders and with chronic

myeloid leukemia (Mazzone et al, 1990 ; Mazzone et al, 1993). This abnormality appears to be correlated with nuclear abnormalities and with hypogranulation of the cytoplasm. These findings have given rise to the hypothesis that qualitative and structural changes in CD11/CD18 might be responsible for defects of aggregation and locomotion as well as superoxide release patients with such myeloid disorder.

2.6.2 Clinical Studies in Diseases Associated with Increased CD11/CD18 Expression

Increased expression of CD11/CD18 on circulating leukocytes has been found in several inflammatory disorders associated with neutrophil activation, including patients with burns, sepsis, hemodialysis, systemic lupus erythematosus, inflammatory arthritis, diabetes mellitus (Buyon et al,1983; Setiadi et al, 1987; Emery et al, 1988) and with coronary artery disease .

In diabetic patients, the enhanced expression of fibronectin and CD11/CD18 receptors on diabetic monocytes could lead to increased monocyte adhesion to blood vessel walls (Altieri et al, 1993), resulting in

endothelial cell injury and alteration of vascular permeability (Springer, 1994).

Neutrophil and monocyte adhesion to endothelial cells of the coronary arteries and subsequent leukocyte activation may be involved in the progression and evolution of atherosclerotic coronary disease. Chemotactic factors, including complement fragment C5a, interleukin-8, TNF-α and platelet activating factor, which all enhance CD11b/CD18 expression (Nathan et al, 1989; Yasuda et al, 1990; Rot,1992) are potentially released into the coronary endothelium. Neutrophils may then release proteases, toxic oxygen metabolites and vasoactive substances. These factors acting together may cause endothelial injury, edema, haemorrhage and tissue injury (Baumhuester et al, 1993; Springer. 1994).

Asthma is a chronic inflammatory disease characterized by the accumulation of neutrophils and eosinophils in the airways (Bevilacqua et al. 1994; Adams and Shaw, 1994). In experimental asthma a combination of CD18 monoclonal antibodies plus ICAM-1 and E-selectin monoclonal antibodies completely inhibited the adhesion of inflammatory cells to activated bronchial epithelial cells.

Ischemic reperfusion injury is a disease of uncontrolled autologous tissue damage, probably caused by activated neutrophils. In several animal model studies it has been shown that treatment with MoAb CD11a/CD18 plus MoAb ICAM-1can attenuate such tissue damage (Simpson et al, 1988; Barton et al, 1989; Bevilacqua et al, 1994).

Recent studies have suggested that the administration of anti-CD11a/CD18 MoAb in combination with conventional immunosuppressive therapy may improve the survival of patients undergoing bone marrow or other allogenic organ transplantations. When administered after the onset of acute rejection, anti-CD11a/CD18 MoAb led to reversal of the rejection, mainly by minimizing vascular damage.

CHAPTER THREE

MATERIALS AND METHODS

This section describes the experimental materials , methods and patient data used in the following chapters. Additional methods, particular to specific studies, are described in the appropriate chapters.

Statistical evaluations were carried out using Mann-Whitney U-test for comparisons. Regression analysis was performed in chapter 5. All results were presented in this dissertation as mean values of triplicate analyses.

3.1 Reagents

Human placental collagen type IV, EDTA disodium salt, EGTA, Sepharose CL4B-200, Histopaque-1119 were purchased from Sigma. Bovine serum albumin , NBT-BCIP for Western blot and h-sICAM-1 ELISA kits were from Boehringer Mannheim. Tissue culture media, sera

and typsin/versene solutions were obtained from Highveld Biological Co.. Dextran T 500 was purchased from Pharmacia Fine Chemicals Co..

MoAb MHM23 (against CD11a), MoAb 2LPM19C (against CD11b), MoAb KB90 (against CD11c), MoAb MHM24 (against CD11c) were purchased from DAKO. MoAb HPCA-2 (against CD34) was from Becton Dickinson. Rabbit anti-human C3 antibody was from Serotec . Anti-rabbit Ig-AP antibody was purchased from Beohringer Mannheim. Mo1-FITC (CD11b) clone-94 (Mo1) and isotypic- IgM-FITC clone-R4A3-22-12 were purchased from Coulter .

3.2 Cell line

ECV-304, a spontaneously transformed immortal endothelial cell line established from the vein of an apparently normal human umbilical cord, is able to substitute for HUVEC (human umbilical cord vein endothelial cell) for assays of neutrophil - endothelium adhesion . ECV-304 cells are characterized by a cobblestone monolayer growth pattern, high proliferation potential without any specific growth factor requirement, and anchorage dependency with contact inhibition. Karyotype analysis of this

cell line reveals it to be of human chromosomal constitution with a high trisomic karyotype. These cells are reported to produce pro-urokinase type PA (Pro-u-PA) and express intercellular adhesion molecule 1 (ICAM-1),lymphocyte function associated antigen-3 (LFA-3), vascular cell adhesion molecule 1 (VCAM-1) and granular membrane protein-140 (GMP-140). Interleukin-1 (IL-1) and interferon exert suppressive effects on growth of ECV-304 cells. These cells also produce IL-6 after stimulation with IL-1. ECV-304 cells were obtained originally from ATCC (American Type Culture Collection).

3.3 Neutrophil Isolation

Heparinized blood was obtained from the subjects . Two volumes of blood were diluted with one volume of PBS buffer(PH 7.4). Polymorphonuclear leukocytes (PMN) were isolated by sedimentation of the diluted blood with 0.6% dextran T500 and by centrifugation of the cells recovered from the resulting leukocyte-rich plasma through a Ficoll-Hypaque cushion , followed by hypotonic lysis of residual red blood cells (Tosi and Hammerschlag , 1988). The isolated PMN were washed and suspended in PBS buffer for further experiments . The purity and viability of

neutrophil preparation were consistently greater than 95% as assessed by trypan blue exclusion.

3.4 Plasma and Serum Preparation

Blood was draw from subjects into heparinized tubes and tubes without additives, immediately centrifuged and frozen at -70°C until testing.

3.5 Cell Culture

ECV-304 cells were grown in Medium M199 supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100μg/ml streptomycin at 37°C under an atmosphere containing 5% CO_2 , fed every 2 to 3 days and allowed to grow to confluence. Cells were passaged once weekly after detachment with trypsin/versene solution at 1 : 3 split ratio.

3.6 Adhesion Assays

Wells were precoated with human placental collagen type IV(25 $\mu g/cm^2$) as previously described (Varsano et al , 1988) .

The ECV-304 cells were seeded at a concentration of 1×10^5 cells /well in 16 mm diameter culture wells of 24-well plates. Cell cultures became confluent after 2 to 3 days and then were used for the assays.

To assess adhesion of neutrophils to ECV-304 cells or to other substrates 1×10^6 neutrophils/ml were plated in each well and incubated for 30 minutes at 37°C in a 5% CO_2 humidified incubator . After incubation supernatant plus nonadhered cells were removed by aspiration followed by two washes and reaspiration. Percentage adhesion was calculated according to the formula.

$$\text{Percentage Adhering Cells} = \frac{\text{Number of Cells Plated} - \text{Non-adherent Cells}}{\text{Number of Cells Plated}} \times 100\ \%$$

To test adhesion selectivity , neutrophil adhesion was analysed in confluent ECV-304 cells coated wells ; in wells precoated with human collagen type IV and polystyrene surfaces alone (i.e. naked tissue culture wells). Incubation conditions included 10% autologous serum .

To investigate whether serum adhesion factors were produced or consumed during the blood coagulation cascade and whether heparin might be a cofactor for the plasma adhesion factor, 10% plasma or serum or heparin (10 IU/ml) were added to the culture wells . BSA (10mg/ml) and medium M199 were used as controls . In some experiments testing the thermal stability of plasma/ serum adhesion factor, serum was heated to 56°C for 30 minutes before being used .

To test the requirement for divalent cations in neutrophil adhesion the cultured ECV-304 cells were prefixed with glutaraldehyde [2.5% in Milloning's phosphate buffer (0.1 M NaH_2Po_4 .2 H_2O / 0.1 M NaOH, pH 7.4 for 1hr] and washed twice before incubation with a suspension of neutrophils . 4mM EDTA with various concentrations of Ca^{2+} , Mg^{2+} were added to the neutrophils suspension 25 minutes before the adhesion assay

in the presence or absence of 10% serum respectively .

3.7 Inhibition Assays

Variable dilutions (0.5μg/ml to 10μg/ml) of MoAbs,anti-CD11a (MHM23); anti-CD11b (2LPM19C) ; anti-CD11c (KB90) ; anti-CD18 (MHM24) were used to characterize the CD11/CD18 molecules involved in adhesion of neutrophils to endothelium. Neutrophils were first incubated with the appropriate MoAbs for 1hour , then added into culture cells . Monoclonal anti-CD34 antibody was used as a negative control .

3.8 Preparation of iC3b

3.8.1 Preparation of iC3b using Sepharose Beads (CL4B-200)

Sepharose activates the alternative pathway of the complement cascade (Van Strijp et al., 1993) and this property was exploited to deposit iC3b on a solid matrix . A total of 100μl sepharose beads (CL4B-200)

were incubated for 1h at 37°C in 1 ml pooled human serum supplemented with 4mM $MgCl_2$ and 10mM EGTA . Beads were washed five times in PBS . To analyse the nature and the amount of the C3 fragments deposited , the C3 was released by base hydrolysis of the ester link to sepharose . pH was raised to 11.8 using 0.1M NaOH . After incubation for 30 minutes at 37°C the mixture was centrifuged for 10s at 3000g and the supernatant pH was readjusted to 7.0 .Released proteins in the supernatant were analysed by 6 % SDS-PAGE gel and further characterised by Western blots with anti-human C3c antibody. The amount of protein deposited on these beads was estimated to be around 150μg/100μl beads.

3.8.2 Gel Electrophoresis

The supernatant was analysed by 6% SDS-PAGE gel according to the method of Laemmli (1970). The discontinuous gel system was made up with 4 % (W/V) acrylamide stacking gel with 0.0625 M Tris/ HCL buffer, (pH 6.8), containing 0.1 % (W/V) SDS, and 6 % (W/V) acrylamide resolving gel with 0.375 M Tris/ HCl buffer, (pH 8.8) , containing 0.1 % (W/V) SDS. The supernatant sample was diluted at 1:1 with sample buffer 62.5m M Tris/ HCl buffer, pH 6.8, containing 2 % SDS (W/V) , 5 % (W/V) 2-mercaptoethanol, 10 % (V/V) glycerol and 0.02 ' ')

bromophenol blue. The sample was mixed and boiled for 3 minutes before application. The samples were electrophoresed at 150V for 4-5 hours, 0.025 M Tris/ HCl, 0.192 M glycine buffer, pH 8.3, containing 1 % (W/V) SDS was used as the electrolyte buffer. Gels were stained with silver(11.76 mM $AgNO_3$ solution) and developed with 0.28 M Na_2CO_3 solution containing 0.0037 % formaldehyde (V/V).

3.8.3 Western Blot

One duplicate of each gel was used for the electro-transfer of protein onto the nitrocellulose membranes using BioRad Cell-Trans blot apparatus. Membranes were placed close to the anode with the gel facing the cathode to ensure that the protein would migrate into the nitrocellulose membrane. A constant current of 200 mA was applied overnight at 4°C . After blocking the nitrocellulose membrane with 5 % bovine serum albumin (BSA) at 4 °C overnight, the blot was incubated with anti-human C3c antibody at 2 µl/ml in 1 % BSA/0.2 % tween20/ PBS , pH7.5 (blocking/washing buffer, BWB) for three hours on a rocker platform at room temperature. After three washes with blocking/washing buffer the lane was overlaid with anti-rabbit Ig-alkaline phosphatase (Ap) (1 : 500

in BWB buffer) and incubated for one hour at room temperature. After extensive washing with PBS buffer the blots were developed with a coupled substrate solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) until the colour changed to a visible purple. The reaction was stopped by rinsing several times in distilled water.

3.9 The Role of iC3b in Serum-induced Neutrophil Adhesion

The relevant studies including adhesion and inhibition assays, of iC3b were performed as described previously, using iC3b prepared as described in section 3.8.1

3.10 Patient Populations

All human studies were performed using volunteer subjects. The nature of the investigation was explained to each individual and informed consent was obtained. The Committee for Research on Human Subject of the

Faculty of Medicine of the University of the Witwatersrand approved all studies, which were carried out in conformity with the principles embodied in the Declaration of Helsinki.

The patient studies included two groups : (1) conventional dose consolidation chemotherapy treated acute myeloid leukemia patients and (2) patients undergoing haematopoietic stem cell transplantation. At the time of study all patients with AML were in complete remission following induction therapy (as assessed by bone marrow morphology and phenotypic analysis by flow cytometry). Consolidation therapy for AML patients consisted of cytosine arabinoside 100 mg/m^2 by continuous infusion × 7 days plus idarubicin, 12 mg/m^2 IV daily × 3. ASCT patients were primed with G-CSF (Neupogen, Roche) alone at a dose of 5 μg/ kg/ day s.c. × 5 days preceeding stem cell harvesting. High dose chemotherapy for ASCT patients was with melphalan 140 mg/m^2 plus VP16 1.5g/m^2 given over 6 hours immediately following collection of haematopoietic stem cell products. Re-infusion of haematopoietic stem cells was 24 hours after the end of chemotherapy.

3.11 ELISA Assay for Soluble Serum ICAM-1

An ELISA method (h-sICAM-1 ELISA kit, Boehringer Mannheim, Germany) was used for analysis of serum concentrations of sICAM-1. The assay is based on the quantitative : "sandwich enzyme-immuno-assay" principle, using two monoclonal antibodies, directed against different epitopes of soluble ICAM-1. During the first incubation step sICAM-1 in samples is simultaneously bound by the biotin-labelled antibody and the peroxidase-conjugated detection antibody forming a complex which binds via the biotin-labeled antibody to the streptavidin-coated surface of the microtiter plate (MTP). Subsequent to the washing step the peroxidase bound in the complex is determined photometrically by tetramethylbenzidine as a substrate. All analyses were performed in triplicate and samples were analysed at dilutions resulting in measured concentrations within the range of standard curves. Absorbency was read with a Titertek Multiskan Reader (Flow Laboratories) at 450 nm (measuring wave length) against 690 nm (reference wave length). The developed colour is proportional to the concentration of sICAM-1. Serum samples from 20 healthy individuals were used as normal controls. Patient and control samples were all measured in the same assay using the same kit.

3.12 Quantitative Analysis of CD11b/CD18 Expression

CD11b/CD18 surface antigen density on peripheral blood neutrophils was studied using a flow cytometric technique. In all cases, an isotypic control was run to correct for autofluorescence and nonspecific binding. Specific monoclonal anti- CD11b-FITC (clone 94, Counter) and the corresponding IgM-FITC isotypic monoclonal antibody (clone R4A3-22-12, Counter) were titrated out to obtain an optimal working concentration. For antibody binding 100μl of neutrophil suspension was aliquoted into each of two tubes and 10μl of the either clone 94 or clone 4A3-22-12 antibody , at a dilution of 1: 10 , was added . All samples were incubated for 15 minutes in the dark at room temperature.

For sample analysis the cells were re-suspended in 500μl of wash buffer (0.2% BSA/0.02% NaN_3/ PBS). Light scatter and fluorescent data was acquired using a Counter XL flow cytometer fitted with a 488nm argon laser. Detector gain, voltage and scatter gating was set and saved using the isotypic control. In addition, the isotypic control was used to set the positive threshold for the green fluorescence channel. This was set

between 2 ± 0.2 % positivity. The neutrophils were selectively bit-mapped on a dual parameter histogram by virtue of their light scatter properties [linear forward scatter (FS) vs log side scatter (SS)] using an amorphous gate. The subsequent data was acquired from the gated population using a single parameter histogram (relative count vs FITC/ FL 1). Two parameters of the positive cell population were evaluated: (1) percentage positive cells , (2) mean channel fluorescence , determined by the mean channel number (MCN). The latter parameter was used to evaluate the antigen density or CD11b/CD18 expression. Each experiment was performed in triplicate and results are given as mean± standard deviation from the mean. 20 normal controls were included in this study.

CHAPTER FOUR

ADHESION OF NEUTROPHILS TO ENDOTHELIAL CELLS BY A CD11b/CD18-DEPENDENT MECHANISM

The structure and function of CD11/CD18 as understood so far has been reviewed in Chapter Two.

The aim of this study was to further investigate the role of CD11b/CD18 in neutrophil adhesion to vascular endothelium using the ECV-304 cell line as a model.

4.1 Materials and Methods

The materials and methods used for adhesion study were described in Chapter Three .

For adhesion assays, the isolated neutrophils were resuspended in medium M199 supplemented with 0.1 % BSA and with 5 mM Hepes, pH7.4,and incubated in a humidified CO_2 incubator for 1 hour before use. Autologous serum or plasma and all components used for experiments were prepared freshly on the day of study. Results are presented as mean values of triplicate analyses. Each experiment was repeated 10 times in this study.

4.2 Results

4.2.1 Selectivity and Serum Effect

Addition of unstimulated neutrophils to cultured ECV-304 cells, was 54.3±12.5 %. Coincubation with serum dramatically increased neutrophil adhesion to ECV-304 cells to 86.3 ± 5.6 % ,($p < 0.001$) . In the presence of serum the attachment of neutrophils to individual ECV-304 cells could easily be seen visually (**Figure 3**).

Serum-stimulated adhesion was a surface-dependent phenomenon . Serum was extremely effective in inducing neutrophil adhesion to ECV-304

cells, but serum was not effective in stimulating adhesion to a collagen-coated surface. Percentage neutrophil adhesion to collagen was 27.8±4.7 % with serum and without serum it was 36.3± 3.8 %. This different was not statistically significant . Similarly, serum was not effective in stimulating adhesion to a nonbiologic surface (polystyrene). The adhesion percentage was 49.7± 6.1% with serum and 45.1± 5.7 % without serum (**Figure 4**) .

4.2.2 Coagulation Dependency and Thermosensitivity of Serum Adhesion Factor

Heparinsed plasma also dramatically elevated neutrophil adhesion to ECV-304 cells (84.2±4.8 %), as compared to control (54.3 ± 12.5 %) ($P<0.001$). Heparin alone (10IU/ml) and BSA alone (10mg/ml) had no effect in increasing adhesion which was at 49.5±9.7% and 51.3±13.4% for addition of heparin and BSA respectively.

Denaturation of serum (preheating to 56°C for 30 minutes) significantly decreased its effectiveness as a stimulus to adhesion . Adhesion decreased from 86.3±5.6 % in the presence of 10% autologous serum to 60.5±11.4% when heat denatured serum was used, $p < 0.01$ **(Table 3).**

4.2.3 Divalent Cation Requirement

Calcium and magnesium ions were involved in adhesion of unstimulated neutrophils to ECV-304 cells . 4mM EDTA decreased adhesion from a control level of 60.4±2.4 % to 44.5± 3.5 %, ($p < 0.01$) . 2mM calcium plus 1mM magnesium resulted in reversal of the EDTA effect. However further increases the amounts of cations (6mM Calcium plus 6mM magnesium) had no further effects on adhesion, which remained at 57.6 ± 5.8 % . Ca^{2+} and Mg^{2+} were also critical for serum-induced adhesion. 4mM EDTA diminished the adhesion from 89.5±2.0% to 63.7 ±4.5 %, ($p < 0.001$) . The addition of Ca^{2+} , Mg^{2-} (6mM each) restored the serum-induced adhesion from 63.7 ± 4.5 % to 86.4 ± 4.7 %, ($p < 0.001$) **(Table 4).**

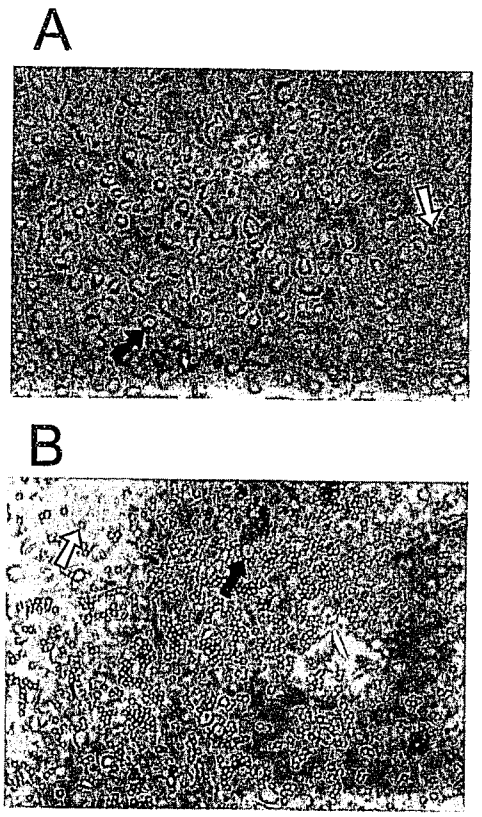

Figure 3. Neutrophils adhesion to endothelium with or without serum

A. Untreated-neutrophils incubated with ECV-304 cells - no rosetting was observed .

B. Neutrophils adhering to ECV-304 cells in presence of 10 % serum. Most neutrophils rosetted round individual endothelial cells .

The open arrow shows a neutrophil, the solid arrow shows an endothelial cell.

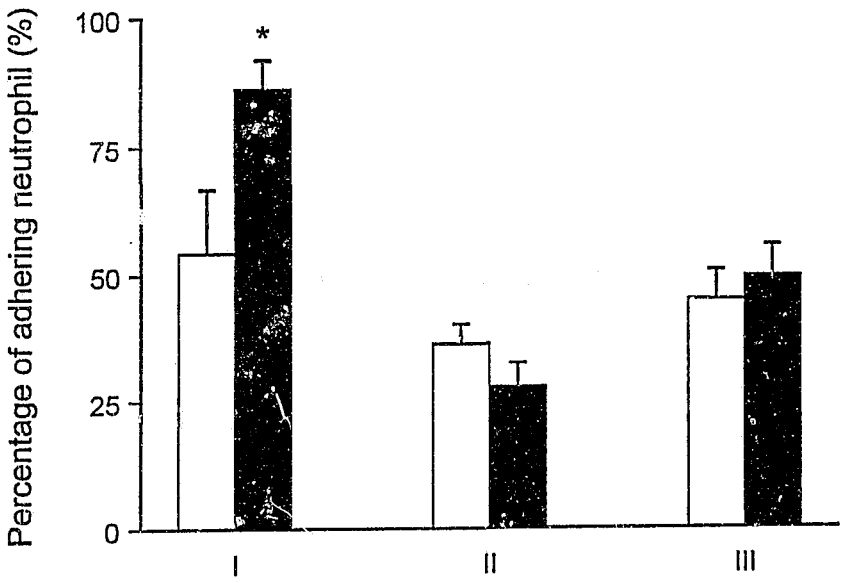

Figure 4. Adhesion of neutrophils to various substrates in the presence or absence of serum

I:ECV-304 cells, II: collagen, III: polystyrene in the absence (open bar) or presence (solid bar) of 10 % serum. * $P < 0.001$

Table 3. Effect of 10 % serum or plasma , heat denatured serum , medium M199, BSA (10 mg/ ml) and heparin (10 IU / ml) on adhesion of neutrophils to ECV-304 cells.

Components In Culture Medium	Percentage of Neutrophils Adhering to Endothelial Cells Mean ± SD
Medium M199 alone	54.3±12.5
10% Plasma*	84.2±4.8
10% Pre-heated serum	60.5±11.4
10 % Serum *	86.3±5.6
Heparin (10IU/ml)	49.5±9.7
BSA(10 mg/ml)	51.3±13.4

* $P < 0.001$

Table 4. Divalent cation requirement for adhesion in the absence or presence of serum

Divalent Cation of Requirement	Percentage of Neutrophils Adhering to Endothelial Cells Mean ± SD
Medium M 199	60.4±2.4
Medium M199 + 4mM EDTA*	44.5±3.5
Medium M199 + 4mM EDTA+ 2mM Ca^{2+} + 1 mM Mg^{2+}	58.2±6.2
Medium M199 + 4mM EDTA + 6mM Ca^{2+} + 6 mM Mg^{2+}	57.6±5.8
10% Serum	89.5±2.0
10% Serum + 4 mM EDTA**	63.7±4.5
10% Serum + 4 mM EDTA + 6mM Ca^{2+} + 6 mM Mg^{2+}	86.4±4.7

EDTA (4 mM), 10 % serum and various concentrations (as shown) of calcium, magnesium were added to neutrophils for 25 minutes before co-incubation with glutaraldehyde-prefixed ECV-304 cells.

* $P < 0.01$, ** $P < 0.001$.

4.2.4 Inhibition by Monoclonal Antibodies

MoAb anti-CD11a and MoAb anti-CD11c failed to inhibit adhesion of either unstimulated neutrophils or of serum-induced adhesion of neutrophils to ECV-304 cells (data not shown). MoAb anti-CD34 was used as a negative control.

MoAb anti-CD11b as well as MoAb anti-CD18 significantly inhibited adhesion of neutrophils to ECV-304 cells in a dose dependent manner both in the presence and in the absence of serum. The antibody concentration required to show the effect in the presence of serum was (≥ 1 μg / ml) **(Table 5 and Figure 5),** while that required in the absence of serum was higher (≥ 2 μg / ml) **(Table 6 and Figure 6).**

4.2.5 The Role of iC3b on Serum-induced Adhesion

Gel and Western blot analysis showed that most of released the protein from CL4B-200 beads was iC3b . The major bands were 75 KD and 62 KD

(**Figure 7**) representing the β chain and a fragment of the α chain of iC3b. In these experiments we failed to detect any 39 KD fragment, a component which has previously been described as another α chain fragment generated by sepharose activation (Van Strijp et al, 1993). iC3b was unstable after preparation and for optimal activity needed to be used within 30 minutes of preparation.

Addition of iC3b (150μg/ml/well) to neutrophils and ECV-304 cells incubated with medium M199 alone showed that iC3b was the major factor in serum responsible for stimulating adhesion . In the presence of iC3b, neutrophil adhesion was 79.1±5.2 %, as compared to 54.3± 12.5 %, adhesion with medium M199 alone ($p< 0.001$). Addition of iC3b stimulated neutrophil adhesion to the same degree as 10 % serum (86.3 ± 5.6 %) (**Figure 8**).

MoAb anti-CD11b (2LPM19C) at 1 μg/ml inhibited the iC3b-stimulated adhesion from 86.3 ± 5.6 % to 57.3 ± 7.2 %, $p< 0.001$, as compared to a nonspecific control (incubated with MoAb anti-CD34)(76.3 ± 6.6 %) .

Table 5. Effect of MoAb anti-CD11b and MoAb anti-CD18 on adhesion of neutrophils to ECV-304 cells in the presence of serum

MoAb Conc. (μg/ml)	Percentage of adhering neutrophil (MoAb anti-CD11b)	Percentage of adhering neutrophil (MoAb anti-CD18)
0	86.3 ± 5.6	86.3 ± 5.6
0.5	89 ± 9.2	76.3 ± 8.1
1 [1*]	52.8 ± 7.1 [1*]	59.3 ± 4.2 [1*]
2	48.3 ± 6.4	50.8 ± 4.7
5	38.5 ± 5.5	42.5 ± 5.1
10 [2*]	26.3 ± 2.6 [2*]	39 ± 3.8[2*]

Results presented here are mean ± SD.

1* : Inhibitory effect of MoAbs was significantly different when concentration (1 μg / ml) was compared to control, $p<0.001$.

2* : Inhibitory effect of MoAbs was significantly different when concentration (10 μg / ml) was compared to (1 μg / ml) , $p<0.001$.

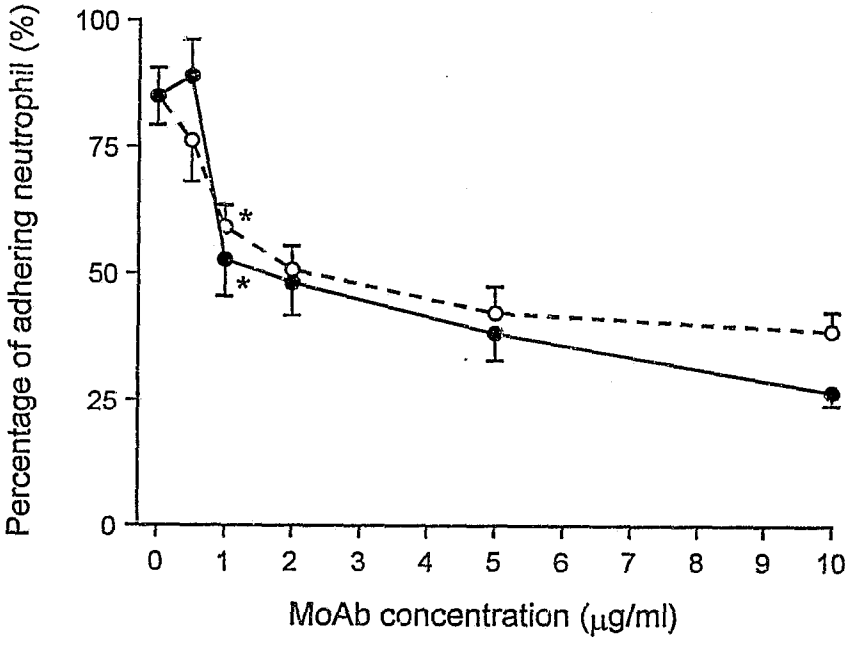

Figure 5. Inhibitory effect of MoAb anti-CD11b (●—●) and MoAb anti-CD18 (O---O) on adhesion of neutrophils to ECV-304 cells in the presence of serum * $P < 0.001$.

Table 6. Effect of MoAb anti-CD11b and MoAb anti-CD18 on adhesion of neutrophils to ECV-304 cells in the absence of serum.

MoAb Conc. (μg / ml)	Percentage of adhering neutrophil (MoAb anti- CD11b)	Percentage of adhering neutrophil (MoAb anti-CD18)
0	54.3 ± 12.5	54.3 ± 12.5
0.5	52.8 ± 9.6	56 ± 6.6
1	55.8 ± 7.2	55 ± 5.4
2 [1*]	37.5 ± 6.1 [1*]	38 ± 4.2 [1*]
5	27.5 ± 4.4	32.5 ± 5.1
10 [2*]	15 ± 3.4 [2*]	25 ± 3.7 [2*]

Results presented here are mean ± SD , * $p < 0.001$.

1* : Inhibitory effect of MoAbs was significantly different when concentration (2 μg / ml) was compared to control, $p<0.001$.

2* : Inhibitory effect of MoAbs was significantly different when concentration (10 μg / ml) was compared to (1μg / ml), $p<0.001$.

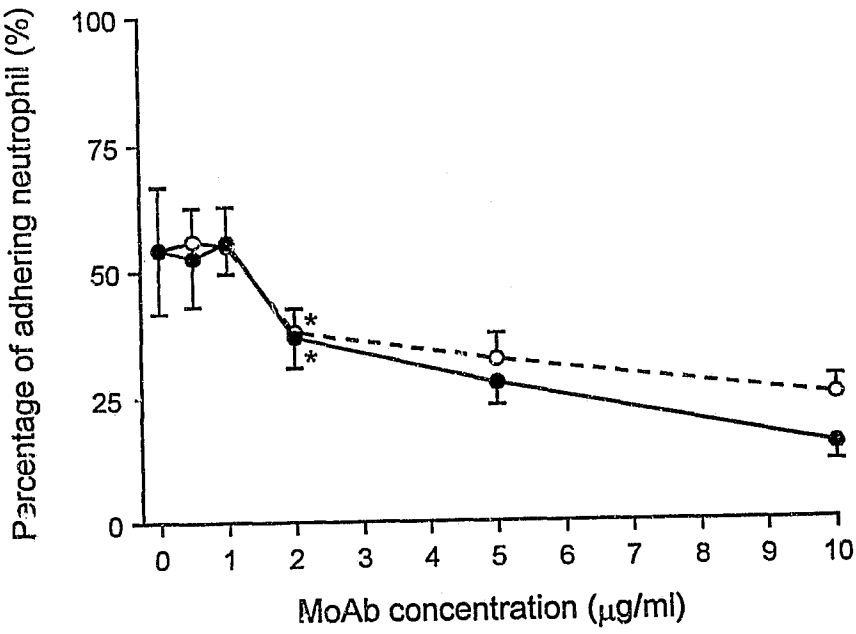

Figure 6. Inhibitory effect of MoAb anti-CD11b (●—●) and anti-CD18 (O---O) on adhesion of neutrophils to ECV-304 cells in the absence of serum * P<0.001.

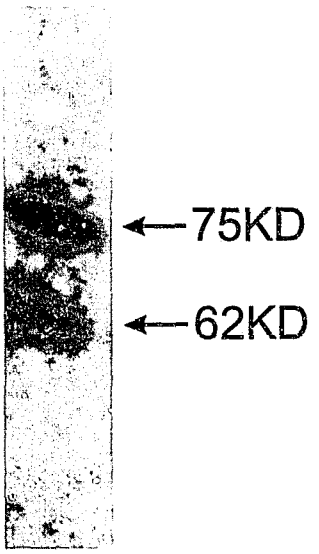

Figure 7. iC3b was analysed by western blot using the rabbit anti-human C3 antibody and 75 KD and 62KD are shown.

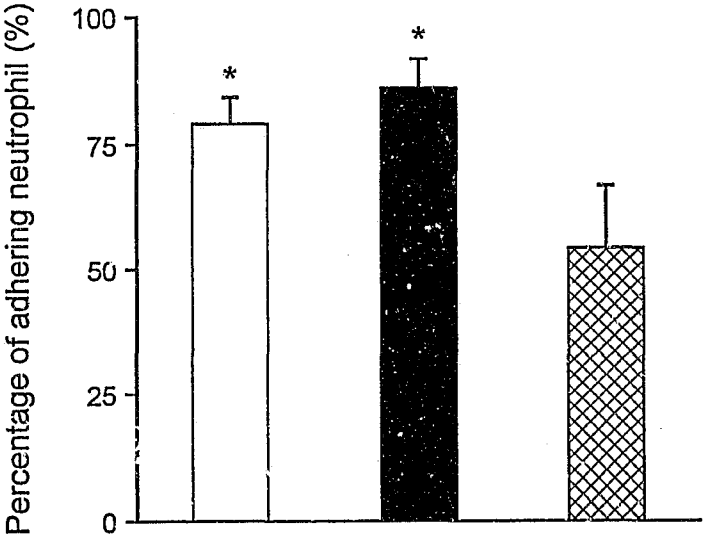

Figure 8 . Effect of iC3b (□) versus 10 % serum (■) and medium M199 (⊠) on adhesion of neutrophils to ECV-304 cells. * P< 0.001.

4.3 Discussion

Interaction with endothelial cells is fundamental to the localized infiltration of neutrophils and monocytes to extravascular inflammatory sites (Colditz,1985; Harlan , 1985) and the homing of lymphocytes to lymphoid organs (Jalkane et al , 1987; Woodruff and Clark , 1987). Leukocytes leave the circulation and enter tissues by migrating across the endothelial lining of blood vessels. Transendothelial migration of leukocytes involves multiple steps, including initial rolling of leukocytes along the endothelium (mediated by the selectin family of adhesion molecules) followed by firm adhesion of the leukocytes (resulting from activation of leukocyte integrins) and ultimately, movement of the leukocytes through the endothelial intercellular junctions (Butcher, 1991; Springer , 1994).

Firm adherence of circulating neutrophils to inflamed vascular endothelium is an essential component of this multiple adhesion cascade and results in the accumulation and eventual migration of neutrophils through the vessel wall. The importance of such interactions is demontrated in leukocyte adhesion deficiency (LAD) patients who suffer a genetic absence of leukocyte integrin (β_2 integrins). These patients have recurrent bacterial

infection and delayed wound healing (Anderson et al , 1984; Anderson and Springer, 1987; Todd III and Freyer, 1988)

The leukocyte integrins comprise a subfamily of related cell-surface glycoproteins that coordinate adhesive functions. CD11/CD18 molecules are broadly expressed on leukocytes and sustain multiple functions, including adhesion, aggregation, chemotaxis, phagocytosis of opsonized particles and initiation of the oxidative burst and (Anderson et al , 1986; Dana et al, 1986; Prieto et al , 1988; Smith et al , 1989a ; Arnaout, 1990; Patarroyo et al, 1990; Shappell et al , 1990; Springer, 1990; Entman et al, 1992) by binding to several cell surfaces and to soluble ligands.

The adhesive glycoprotein CD11b/CD18 is the most abundant member of β_2 integrin family to be found on neutrophils (Arnaout et al, 1984; Anderson et al, 1984; Springer and Anderson, 1986). Stimulation of neutrophils by various chemoattractants, such as f-met-leu-phe (fMLP) and C5a induces a six to tenfold increase of the surface expression of CD11b/CD18 on neutrophils, resulting from a rapid translocation from intracellular storage compartments to the cell surface. (Berger et al, 1984; Bruce et al, 1987).

Recently, a number of studies have identified the role of β_2 integrins in adhesion of neutrophils to endothelium. However the nature of the integrins involved in the interactions is not clear. Lu and co-workers found that CD11a/CD18 (LFA-1) expression was sufficient to mediate neutrophil emigration across endothelium, while CD11b/CD18 played a role in mediating binding of neutrophils to fibrinogen and in neutrophil degranulation. CD11b/CD18 was not found necessary for effective neutrophil emigration (Lu et al , 1997). Smith and co-workers however showed that while unstimulated neutrophils exhibited a CD11a /CD18 (LFA-1) dependent attachment to endothelium, chemotactic stimulation enhanced the attachment of human neutrophils to endothelial cells in a CD11b/CD18 dependent manner (Smith et al , 1989a).Other studies identified leukocyte integrin $\alpha_4\beta_1$ (CD49, VLA 4) as a novel pathway facilitating neutrophil adherence to endothelial cells (Reinhardt et al,1997) In addition , P-selectin (CD31) on platelets has also been shown to act in concert with CD11b / CD18 (Mac-1) during the adhesion cascade (Berman and Muller,1995; Diacovo et al , 1996; Evangelista et al , 1996).

Thus a number of previous studies appeared to show that CD11a rather than CD11b, CD11c played a critical role in adhesion of neutrophils by recognizing ICAM-1 on endothelium (Marlin and Springer, 1987; Arnaout

et al . 1988; Lo et al, 1989; Hakkert et al , 1990; Meerschaet and Furie, 1995; Lu et al , 1997). However, since expression of CD11b on neutrophils is far more abundant than CD11a , CD11c (Myones et al , 1988; Arnaout , 1990). It might appear logical that CD11b should play a major role in attachment to ligands on endothelial cells .

The current study provides direct evidence that attachment of unstimulated neutrophils to endothelium involves a CD11b/CD18 dependent-interaction. Some attempt must, however, be made to explain the apparently discrepant results in different investigations. A number of studies have suggested that chemotactic factors present in conditioned media in tissue cultures could result in the recruitment of CD11b in adherence to endothelial cells in preference to CD11a or CD11c (O'Brien et al , 1984; Mercandetti et al , 1984; Gudewicz et al , 1988). In the current study we could not exclude the possibility that in the ECV-304 cell model a preferential interaction with CD11b occurs. It has also been suggested that the CD11b molecule binds to several ligands other than ICAM-1, these molecules including fibrinogen , iC3b and factor X (Hynes , 1992) . While it has been suggested that fibrinogen promotes CD11b-dependent binding (Languino et al, 1993) this appears unlikely in our system in view of the similar findings with plasma and with serum.

Another reason for the discrepant results could be the use of different MoAbs in different studies. In the current experiments, while MoAb anti-CD18 inhibited as effectively as MoAb anti-CD11b it was unable to induce additive inhibition . These findings appear to substantiate the conclusion that in the ECV-304 model neutrophil-endothelial cell interaction occurs in a CD11b/CD18-dependent manner.

In addition to demonstrating the role of CD11b/CD18 in neutrophil adhesion the current study also demonstrated that serum contains a potent factor able to enhance the adhesion of neutrophils to ECV-304 cells . This adhesion factor was not affected by blood coagulation and acted selectively on ECV-304 cell coated surfaces but not on surfaces coated with collagen or on non-biologic surfaces. The factor was heat sensitive and depended on the presence of divalent cations including calcium and magnesium. In the absence of these cations serum/plasma had no effect on stimulating adhesion . Crucial dependence on the presence of divalent cations is a characteristic feature of several other adhesion phenomena modulated by specific adhesive molecules (Hoover et al , 1980) . These findings suggest that the serum /plasma adhesion factor acts as a bridging molecule between ECV-304 cells neutrophils . Inhibition of serum-induced adhesion by MoAb 2LPM19C. or by MoAb MHM24 suggested that this factor is the

serum complement fragment iC3b which has previously been shown to be a ligand for neutrophil receptor CD11b/CD18. The role of iC3b was confirmed by direct experiments using iC3b generated from normal serum on sepharose beads.

iC3b originates spontaneously in normal plasma and serum by cleavage of C3 into C3a and C3b . Once C3b is formed it attaches to sugars or polysaccharides on the surface of host cells and is further converted into iC3b on these surfaces by plasma factors H and I (Pangburn , 1988) .iC3b is also known as the thermolabile opsonin of serum . The iC3b binding site localizes to the I domain of CD11b/CD18 (Diamond et al, 1993). Mutation of the conserved divalent cation binding amino residues abolishes CD11b/CD18 recognition of iC3b (Michishita and Arnaout , 1993; Ueda et al , 1994; Mc Guire and Bajt , 1995). The primary role of divalent cations in this interaction appears to be to maintain I domain structural and functional integrity.

It is interesting to note that some studies have shown that P150,95(CD11c) also has iC3b-receptor activity (Myones et al, 1988) . In our studies ,

however, MoAb KB90 (anti-CD11c) was unable to inhibit serum-enhanced neutrophil adhesion. This may be due to the fact that only a small amount of P150,95 is expressed on neutrophils . By contrast , macrophages express eight times more P150,95 than neutrophils (Te Velde et al, 1987) suggesting that P150,95 may be an important receptor in adherence of macrophages .

It has also been shown that activated complement fragments potently affect neutrophil functions, including their adhesion, migration , phagocytosis and initiation of the respiratory burst (Cramer et al, 1986; Parkos et al, 1991). iC3b-activated adhesion may therefore be a double edged sword, beneficial by producing phagocyte clumps to entrap microbes but potentially deleterious in that it causes neutrophil autoaggregation and tissue damage (Trond et al, 1990)

CHAPTER FIVE

SERUM ICAM-1 CONCENTRATION FOLLOWING CONVENTIONAL DOSE CONSOLIDATION CHEMOTHERAPY FOR ACUTE MYELOID LEUKEMIA AND AFTER HIGH DOSE CHEMOTHERAPY WITH AUTOLOGOUS HAEMATOPOIETIC STEM CELL RESCUE

The aim of this study was to investigate the usefulness of sICAM-1 as a marker of recovery of neutrophil function following severe chemotherapy induced neutropenia.

The role of intercellular adhesion molecules, particularly of ICAM-1 as ligand for CD11/CD18 dependent neutrophil binding to target cells has been reviewed in previous sections. A circulating form of ICAM-1 (sICAM-1) has been identified and characterised in previous studies (Rothlein et al, 1991; Seth et al, 1991). These studies demonstrated that sICAM-1 retains the ability to bind specifically to LFA-1 and Mac-1. sICAM-1 may be a secretory form of the membrane associated protein. Alternatively, sICAM-1 may be the indirect consequence of inflammation

or tissue damage, being released after proteolytic digestion of membrane associated antigens.

Patients undergoing intensive chemotherapy experience long periods of profound neutropenia. During these periods, the risk of infection is high and patients frequently develop fever, when granulocyte counts are below $0.5 \times 10^9/l$, (Bodey et al, 1966). However, a microbiologically identified causative agent is found in only a proportion of febrile neutropenic patients(Pizzo et al, 1986; Engervall et al, 1992) . In most instances febrile neutropenia settles when neutrophil counts and neutrophil function recover. Because sICAM-1 retains functional activity it was felt that measurement of sICAM-1 levels may be a useful marker of leukocyte mass and function recovery.

5.1 Materials and Methods

Patients

The study consisted of 11 patients with acute myeloid leukemia (AML) undergoing conventional dose consolidation chemotherapy after initial remission induction and 10 patients (5 AML and 5 breast cancer patients) undergoing high dose chemotherapy and autologous haematopoietic stem cell transplantation (ASCT). The clinical data of each patient are given in (**Table 7**).

Sample Collection

Serum samples were collected daily from patients during the chemotherapy-induced neutropenic period (neutrophil counts $< 0.5 \times 10^9/l$) and during early recovery from neutropenia (neutrophil counts. $0.5 \times 10^9/l$ - $1.0 \times 10^9/l$) as well as following full recovery from neutropenia (neutrophil counts $1.0 \times 10^9/l$ - $2.5 \times 10^9/l$). For the ASCT patients blood samples were also collected on the day before the start of G-CSF administration used for priming for stem cell harvest as well as just prior to stem cell harvesting. Blood samples were drawn in 5 ml sterile tubes containing 15% EDTA.

The serum was separated and stored at - 70°C and thawed immediately before analysis.

An ELISA method (h-sICAM-1 ELISA kit, Boehringer Mannheim) used for analysis of serum concentrations of sICAM-1 was described in detail in Chapter Three.

Table 7: *Clinical Characteristics of Patients.*

No.	*Age*	*Sex*	*Race*	*Diagnosis*	*Treatment*	*Duration of Neutropenia* (days)*
1	55	M	B	AML	Consolidation Chemotherapy	12
2	46	F	W	AML	Consolidation Chemotherapy	7
3	19	F	W	AML	Consolidation Chemotherapy	12
4	19	M	B	AML	Consolidation Chemotherapy	11
5	69	F	B	AML	Consolidation Chemotherapy	16
6	22	M	B	AML	Consolidation Chemotherapy	13
7	18	M	B	AML	Consolidation Chemotherapy	17
8	32	F	W	AML	Consolidation Chemotherapy	15
9	28	M	B	AML	Consolidation Chemotherapy	13
10	50	M	B	AML	Consolidation Chemotherapy	14
11	35	F	W	AML	Consolidation Chemotherapy	21
12	38	F	W	Breast Cancer	High Dose Chemotherapy + ASCT	10
13	28	M	B	AML	High Dose Chemotherapy + ASCT	17
14	18	M	B	AML	High Dose Chemotherapy + ASCT	17
15	45	F	W	Breast Cancer	High Dose Chemotherapy + ASCT	16
16	33	F	W	Breast Cancer	High Dose Chemotherapy + ASCT	14
17	25	F	B	Breast Cancer	High Dose Chemotherapy + ASCT	14
18	18	M	B	AML	High Dose Chemotherapy + ASCT	18
19	17	F	W	AML	High Dose Chemotherapy + ASCT	11
20	46	F	B	AML	High Dose Chemotherapy + ASCT	22
21	26	F	B	Breast Cancer	High Dose Chemotherapy + ASCT	10

** Neutropenia defined as neutrophil counts < 0.5 x 10⁹/l*
AML = Acute Myeloid Leukemia

5.2 Results

Levels of sICAM in healthy controls were 241.37 (± SD 71.81) ng/ml.

Acute myeloid leukemia patients with convention consolidation chemotherapy (patients 1-11)

Results in AML patients following consolidation chemotherapy (patient numbers 1-11) are shown in (**Figure 9**). During severe neutropenia (neutrophils < 0.5 x 10^9/l; phase I) the mean serum concentration of sICAM-1 was 328.57 (± SD 38.94) ng/ml. In early recovery (neutrophils 0.5 - 1.0 x 10^9/l; phase II) the mean level of sICAM-1 was 352.58 (± SD 38.92) ng/ml. Following neutrophil recovery (neutrophils 1.0 - 2.5 x 10^9/l; phase III) sICAM levels was 330.7 (± SD 52.85) ng/ml.

sICAM-1 was significantly elevated in AML patients undergoing consolidation chemotherapy during all phases (phase I, phase II and phase III) compared to sICAM healthy controls ($p < 0.01$; $p < 0.001$ and $p < 0.001$, respectively) but with no significant variation related to the level of neutrophil count for this patient group.

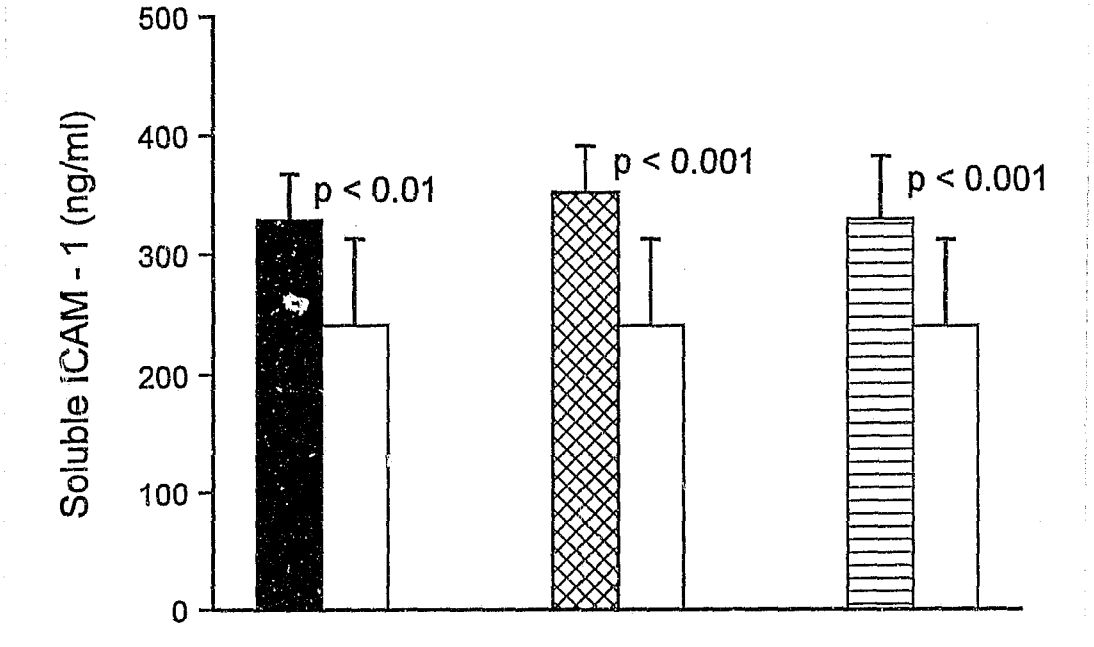

Figure 9 . Comparison of sICAM-1 levels in AML patients treated by conventional dose chemotherapy (patients 1-11) and in healthy controls.

AML patients :

Phase I : neutrophil count < 0.5×10^9 / L (■)

Phase II : neutrophil count: $0.5\text{-}1.0 \times 10^9$ / L (▩)

Phase III : neutrophil count: $1.0\text{-}2.5 \times 10^9$ / L (▤)

Controls : (□)

Autologous Stem Cell Transplantation (patients 12-21)

During phase I, serum sICAM-1 concentration was 355.4 (± SD 46) ng/ml. In phase II sICAM-1 was 233.96 (± SD 55.43) ng/ml. In phase III serum soluble ICAM-1 was 223.04 (± SD 40.22) ng/ml. In this group of patients sICAM concentration was 198.4 (± SD 29.4) ng/ml on the day prior to G-CSF administration and 221.3 (± SD 39.1) ng/ml on the day of stem cell collection, following 5 days of G-CSF administration.

Among patients who underwent ASCT there was a significantly higher level of sICAM-1 during phase I as compared to phases II and III ($p < 0.001$, $p < 0.001$, respectively) (**Figure 10**).

Comparison of the 2 treatment groups i.e: consolidation chemotherapy and autologous haematopoietic stem cell transplantation showed that during phase I, there was no significant difference between AML consolidation chemotherapy patients and ASCT patients. During phase II however, the sICAM-1 level of ASCT patients decreased strikingly to within the normal range (mean 233.96 ± SD 55.4 ng/ml) as compared to AML patients in whom there was a further elevation of sICAM-1 (352.58 ± SD 38.9 ng/ml; $p < 0.001$). During phase III, the sICAM-1 level of ASCT remained within the normal range (mean 223.04 ±SD 40.2 ng/ml) as compared to AML

patients in whom sICAM-1 still remained significantly elevated (mean 330.7 ± SD 52.9 ng/ml), $p < 0.001$ (**Figure 11**).

Regression analysis (**Figure 12**) confirmed the persistent elevation of sICAM following consolidation chemotherapy for AML and the decline of sICAM values to the normal range as neutrophil counts recovered following ASCT.

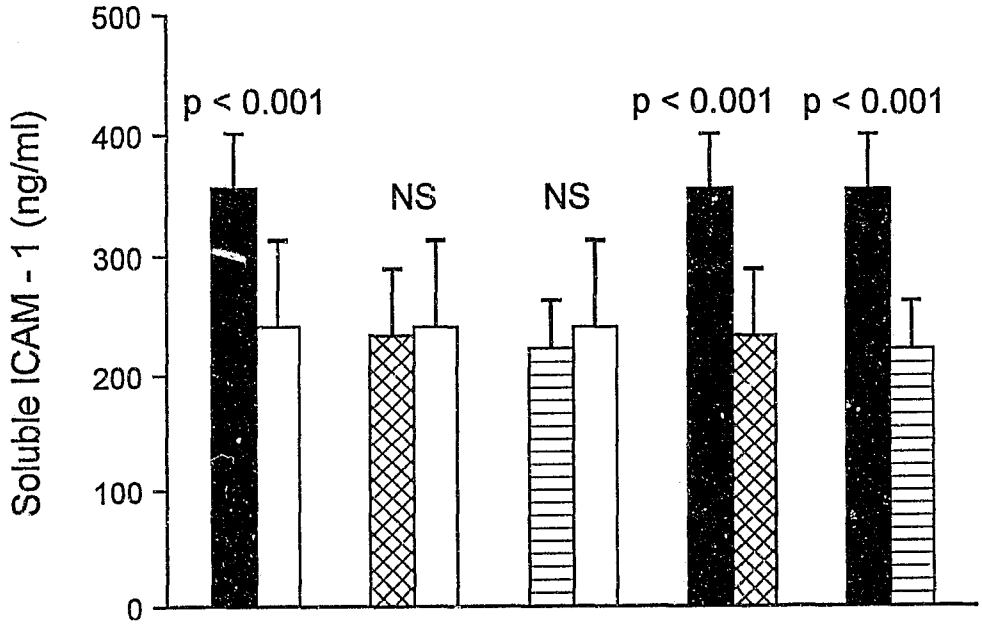

Figure 10 . Comparison of sICAM-1 levels in HDC-ASCT patients (patients 12-21) and in healthy controls. NS : not significant.

HDC-ASCT patients :

Phase I : neutrophil count < 0.5×10^9 / L (■)

Phase II : neutrophil count : $0.5\text{-}1.0 \times 10^9$ / L (▩)

Phase III : neutrophil count : $1.0\text{-}2.5 \times 10^9$/ L (▤)

Controls : (□)

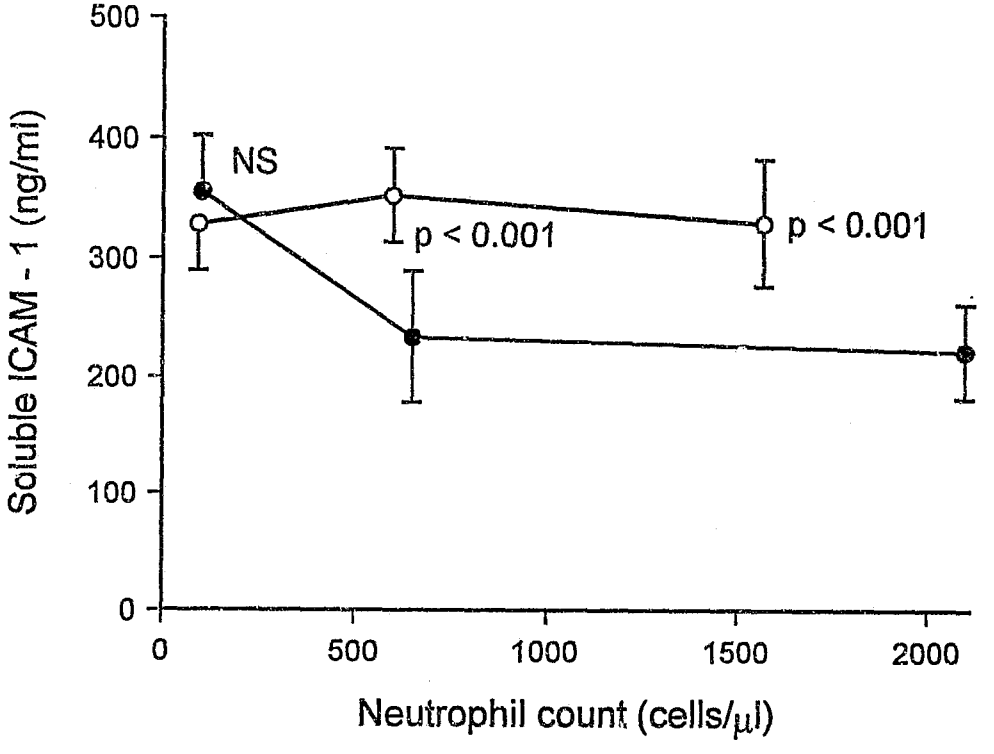

Figure 11. Variation of sICAM-1 levels in AML patients treated by conventional consolidation chemotherapy (○-○) and patients undergoing high dose chemotherapy plus ASCT(●-●). Points represent mean value, line bars represent standard deviation of the mean. Significant levels are showed in the graph. NS: not significant

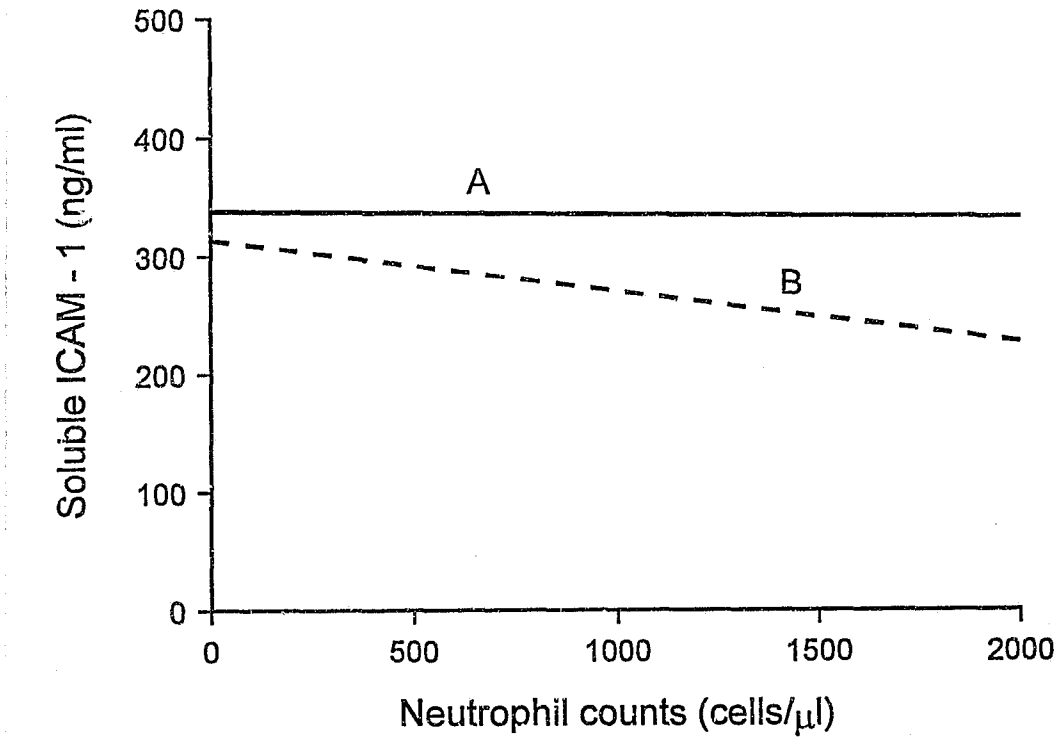

Figure 12 . Regression analysis correlation of sICAM-1 levels and neutrophil count.

A: Consolidation chemotherapy treated AML patients : $y = 338.1-0.002x$, $p>0.05$

B: High dose chemotherapy plus ASCT patients : $y = 313.4-0.043x$, $P < 0.01$.

5.3 Discussion

The current study was undertaken to test the hypothesis that s-ICAM-1 might be a useful marker of neutrophil mass and of neutrophil function in patients undergoing intensive chemotherapy. Patients with AML were investigated during a consolidation cycle of treatment after remission induction in order to avoid confounding effects of aberrant cell surface protein expression or function due to the presence of leukemic cell populations. All leukemic cases were studied during post-remission consolidation as confirmed by bone marrow morphology and FACS analysis. Chemotherapy (either unsupported conventional dose consolidation chemotherapy or high dose chemotherapy with autologous haematopoietic rescue) was given to AML patients, while patients with solid tumours (breast cancer) were studied after high dose chemotherapy resulting in an equivalent degree and duration of leukopenia as that observed following anti-leukemic treatment.

Intensive chemotherapy is associated with a high incidence of neutropenic fever and/or infection. Such indeed was the case in the present series. All patients in the current study had nadir neutrophil counts $< 0.5 \times 10^9$/l (mean $0.11 \pm 0.09 \times 10^9$/l) and all patients developed febrile episodes with fevers

of ≥ 38°C generally lasting for between 3-7 days. Documented infection occurred in 12 cases. These included 10 patients with bacteraemia, one patient with candidemia and one patient with an unidentified yeast found on blood culture. The frequency of infection was similar in the consolidation chemotherapy and ASCT group.

All patients studied had resolution of fever, usually by the time the neutrophil count reached levels of 0.5 - 1.0 x 10^9/l (mean neutrophil count at time of resolution of fever 0.66 ± 0.15 x 10^9/l). The levels of sICAM-1 were followed during the resolution (phase II) and recovery phases (phase III).

The results of the study showed that sICAM-1 levels were elevated to above normal values during the neutropenic phase, both in patients undergoing unsupported consolidation chemotherapy as well as in those receiving autologous stem cell support. These results are similar to those found by Bruserud and co-workers who investigated. 10 patients with acute leukemia found elevated sICAM-1 during bacterial infections. In the Bruserud study although s-ICAM-1 levels declined following resolution of infection it is not clear whether the reduction was to the normal range. The

current study shows that in the AML consolidation chemotherapy group levels of sICAM-1 remained elevated for considerably longer than in the ASCT group.

The elevated s-ICAM levels found during severe neutropenia in the present study suggest that sICAM is derived from cells other than polymorphonuclear leukocytes and furthermore that polymorphonuclear cells may modulate sICAM-1 by binding and sequestration from the serum compartment. Candidate sources for sICAM include tissue monocytes/macrophages, lymphocytes and endothelial cells whose numbers are less affected by chemotherapy than are those of neutrophils. These cells may release sICAM-1, either specifically or non-specifically following stimulation by cytokines such as IL1, IL6, IL8 and TNF ∝. Elevated levels of these cytokines have previously been found in patients with febrile neutropenia following chemotherapy for AML (Dustin et al, 1988; Osterman et al. 1994; Bruserud et al, 1995).

Whichever cytokine is responsible for stimulation of sICAM-1 release the finding of elevated levels following intensive chemotherapy suggests that neutrophils may have a role in control of sICAM-1 levels. Alteration of the

number of ligand-bearing cells may modulate both cellular production and release as well as clearance of these adhesion molecules. Both neutrophil mass as well as neutrophil functional integrity may play a role in this process. Support for the latter hypothesis comes from the finding that patients with leukocyte adhesion deficiency (LAD) have elevated levels of sICAM-1(Rothlein et al, 1991).

In addition to finding elevated levels of sICAM-1 during the phase of severe neutropenia (< 0.5 x 10^9/l) following chemotherapy the pattern of response in the patients differed according to whether chemotherapy induced neutropenia was allowed to resolve spontaneously or whether haematopoietic support was given, irrespective of the underlying diagnosis. Patient undergoing haematopoietic rescue showed a rapid reduction of elevated sICAM-1 during the recovery phases, whereas levels remained persistently elevated even after neutrophil count recovery in patients receiving unsupported chemotherapy.

Interestingly sICAM levels in the ASCT group of patients remained within the normal range following G-CSF administration as priming treatment prior to stem cell collection. Following administration of G-CSF neutrophil counts dramatically increased (mean 28.5 ± 10.7 x 10^9/l). G-CSF can also increase the production of cytokines e.g: IL6 (Ericson et

al, 1998). Activation of the cytokine network has been shown to be able to elevate sICAM in a number of other instances(Osterman et al, 1994; Bruserud et al, 1995) and might have been expected to have a similar effect here. However apart from elevating neutrophil count G-CSF has been shown to also increase the expression of CD11b/CD18 on neutrophils(Arnaout et al, 1986; Devereux et al, 1989; Socinski et al, 1989) so that increased sICAM production may well be counter-balanced by increased binding to its ligand, with no net rise in serum concentration.

Granulocyte colony stimulating factor (G-CSF) was used in these patients as priming for haematopoietic stem cell rescue. There is a potential role for G-CSF to accelerate haematopoietic re-constitution, reducing the risk of infection and enhancing neutrophil recovery (Bronchud et al, 1988; Morstyn et al, 1988; Lindemann et al, 1989). Re-infused progenitor cells can proliferate, differentiate and develop into normal mature neutrophils. We hypothesise that patients undergoing stem cell rescue showed a more rapid recovery of neutrophil mass than evident from peripheral blood counts above and that they may also display greater expression of CD11b/CD18 and more adhesive activity of neutrophils.

We conclude from this study that recovery of leukocyte function might not be always correlated with recovery of leukocyte count. Increase of

leukocyte count could not be the only marker of true recovery of patients who accepted high dose chemotherapy.

It is considered that alteration of sICAM-1 might be a fairly sensitive marker to predict neutrophil adhesive function recovery in these patients. In further studies undertaken in our laboratory, we are investigating the expression of CD11b/CD18 and adhesion function of neutrophils in these two groups of patients, in order to prove this presumption.

CHAPTER SIX

EXPRESSION OF CD11b/CD18 ON NEUTROPHILS IN PATIENTS UNDERGOING CONSOLIDATION CHEMOTHERAPY FOR ACUTE MYELOID LEUKEMIA AND IN PATIENTS UNDERGOING HIGH DOSE CHEMOTHERAPY AND AUTOLOGOUS HAEMATOPOIETIC STEM CELL TRANSPLANTATION

In a previous study, sICAM-1 levels in patients receiving conventional consolidation chemotherapy for acute myeloid leukemia (AML) and those receiving high dose chemotherapy with autologous stem cell transplantation (ASCT) were examined. The results of that study showed different patterns of response of sICAM-1 depending on whether patients were allowed to recover spontaneously from chemotherapy induced neutropenia or whether autologous stem cell rescue was used to promote haematopoietic recovery. In patients receiving consolidation chemotherapy for AML sICAM-1 levels were significantly elevated during neutropenia and remained high even during early neutrophil recovery. By contrast, in ASCT patients, sICAM-1 concentrations were also markedly increased

during neutropenia , but decreased rapidly to normal values with early neutrophil recovery . These findings suggested that sICAM-1 may be a marker of neutrophil function, particularly of the ability of neutrophil integrins to interact with specific ligands during haematopoietic recovery. In order to test this hypothesis CD11b/CD18 expression on post-chemotherapy neutrophils was investigated.

6.1 Materials and Methods

The study population consisted 10 patients with acute myeloid leukemia (AML), in remission, receiving conventional dose consolidation chemotherapy after initial remission induction and 10 patients (5 AML and 5 breast cancer patients) receiving high dose chemotherapy with autologous haematopoietic stem cell transplantation (HDC-ASCT). The clinical data of each patient is given in (**Table 8**).

Heparinized blood was obtained from healthy donors and from patients during recovery from the chemotherapy induced neutropenia. The methods for neutrophil isolation and flow cytometric analysis of neutrophil CD11b/CD18 expression were described in Chapter Three.

Table 8: *Clinical Characteristics of Patients*

No.	*Age*	*Sex*	*Race*	*Diagnosis*	*Treatment*	*Duration of Neutropenia* (days)*
1	31	F	W	AML	Consolidation Chemotherapy	18
2	84	F	W	AML	Consolidation Chemotherapy	14
3	36	M	B	AML	Consolidation Chemotherapy	17
4	30	F	B	AML	Consolidation Chemotherapy	16
5	54	M	B	AML	Consolidation Chemotherapy	13
6	40	M	B	AML	Consolidation Chemotherapy	17
7	32	F	W	AML	Consolidation Chemotherapy	15
8	28	M	B	AML	Consolidation Chemotherapy	20
9	50	M	B	AML	Consolidation Chemotherapy	14
10	35	F	B	AML	Consolidation Chemotherapy	21
11	38	F	W	Breast Cancer	High Dose Chemotherapy + ASCT	10
12	45	F	W	Breast Cancer	High Dose Chemotherapy + ASCT	12
13	33	F	W	Breast Cancer	High Dose Chemotherapy + ASCT	14
14	25	F	W	Breast Cancer	High Dose Chemotherapy + ASCT	13
15	26	F	B	Breast Cancer	High Dose Chemotherapy + ASCT	9
16	28	M	B	AML	High Dose Chemotherapy + ASCT	11
17	18	M	B	AML	High Dose Chemotherapy + ASCT	10
18	21	M	W	AML	High Dose Chemotherapy + ASCT	8
19	13	F	W	AML	High Dose Chemotherapy + ASCT	11
20	50	M	B	AML	High Dose Chemotherapy + ASCT	12

* Neutropenia defined as neutrophil count $< 0.5 \times 10^9$/l. Consolidation therapy for AML patients was cytarabine 100 mg/m^2 x 7 days plus idarubicin 12 mg/m^2 x 3 days. ASCT patients were primed with G-CSF at a dose of 5 μg/kg for 5 days, followed by high dose chemotherapy with melphalan 140 mg/m^2 plus VP16 1.5 mg/m^2 given over 6 hours and started collection of stem cells.

AML = Acute Myeloid Leukemia

6.2 Results

CD11b/CD18 expression (mean channel number/ fluorescence intensity) on neutrophils of AML patients receiving consolidation chemotherapy during early (neutrophil count 0.5-1.0 $\times 10^9$/ L) and full (neutrophil count 1.0-2.5 $\times$ 10^9/ L) neutrophil recovery phases was 52.6±24 and 269.3±25.8 , respectively, compared to controls (375.2±53.2), the values found in the patient population were significantly lower ($P< 0.001$) **(Figure 13 and 14)**.

CD11b/CD18 expression on neutrophils of HDC- ASCT patients at the corresponding times (early and full neutrophil recovery), was 362.5±52.2 and 389.6±37.4 , respectively. These values were not significantly different when compared to controls (**Figure 15 and 16**).

CD11b/CD18 expression on neutrophils of HDC-ASCT patients was significantly higher as compared to that found in conventional dose consolidation chemotherapy treated AML patients for the same levels of neutrophil count ($P < 0.001$) (**Figure 17**).

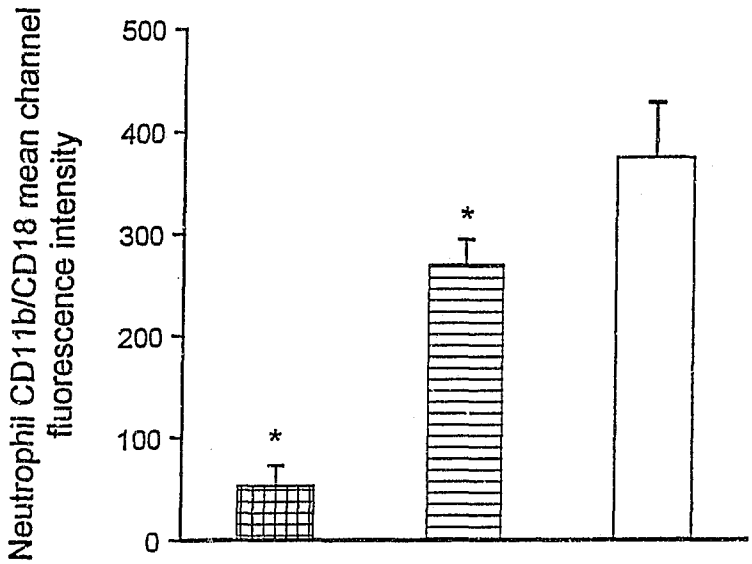

Figure 13 . Comparisons of CD11b/CD18 expression (mean channel fluorescence intensity) in consolidation chemotherapy treated AML patients; early recovery (neutrophil count 0.5-1.0 x 10^9/ L) (▦); full recovery (neutrophil count 1.0-2.5 x 10^9/ L) (▤) and controls (□). * $P < 0.001$.

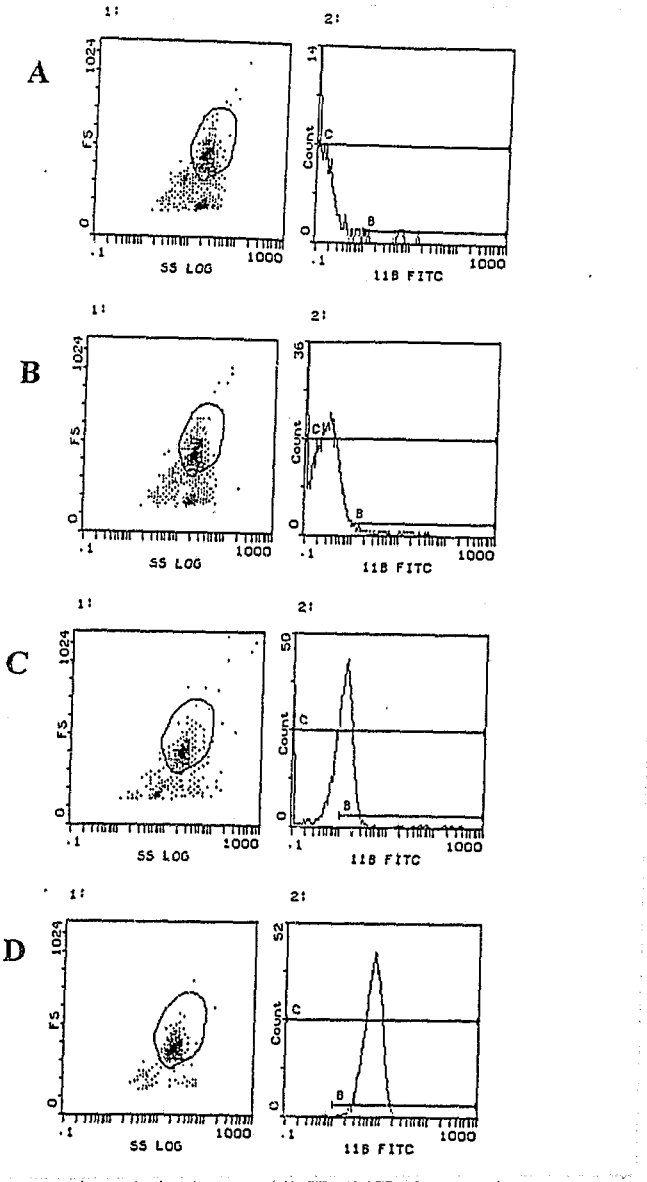

Figure 14 . Flow cytometric analysis of neutrophil CD11b/CD18 expression

Panel 1 : Dot plot of forward scatter (FS) vs log side scatter (SS).The neutrophils population was bit-mapped using an amorphous gate.

Panel 2 : Single parameter (count vs fluorescence) analysis .The data represented in this histogram, related only to the gated population in panel 1. Linear gate B, represents the 0,5-2.0% background positive. Linear gate C provides the mean channel number (fluorescence intensity).

Histogram A : Isotypic control
Histogram B : Results in a consolidation chemotherapy treated AML patient during early neutrophil recovery
Histogram C: Results in a consolidation chemotherapy treated AML patient at full neutrophil recovery.
Histogram D : Control.

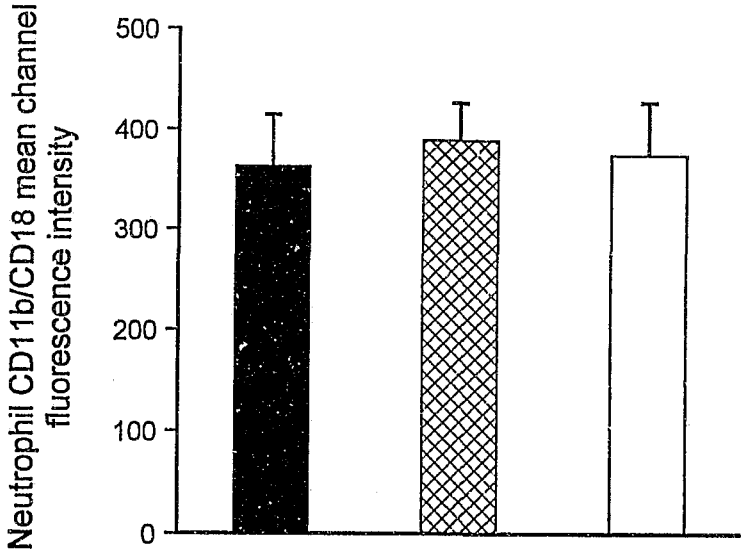

Figure 15 . Comparisons of CD11b/CD18 expression (mean channel fluorescence intensity) in HDC-ASCT patients; early neutrophil recovery (neutrophil count 0.5-1.0 × 10^9) (■) ; full neutrophil recovery (neutrophil count 1.0-2.5 × 10^9) (▩) and controls (□) .

NS : difference not statistically significant

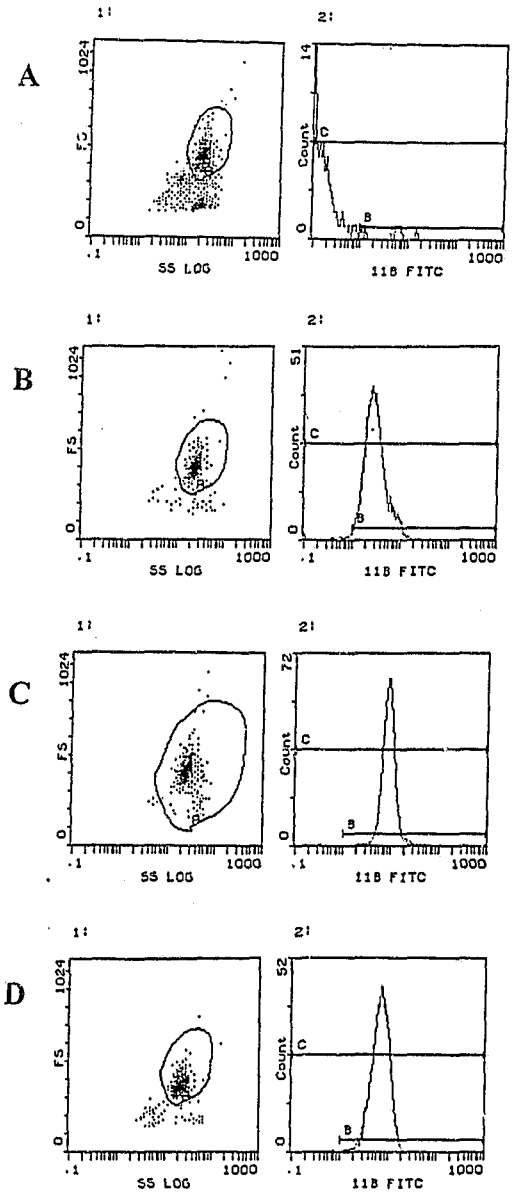

Figure 16 . Flow cytometric analysis of neutrophil CD11b/CD18 expression

Pane 1 : FS vs log SS.

Panel 2 : Single parameter count vs fluorescence analysis. Linear gate C provides the mean channel number (fluorescence intensity).

A : Isotypic control
B : Results in a HDC- ASCT patient during early neutrophil recovery
C : Results in a HDC- ASCT patient at full neutrophil recovery
D : Control.

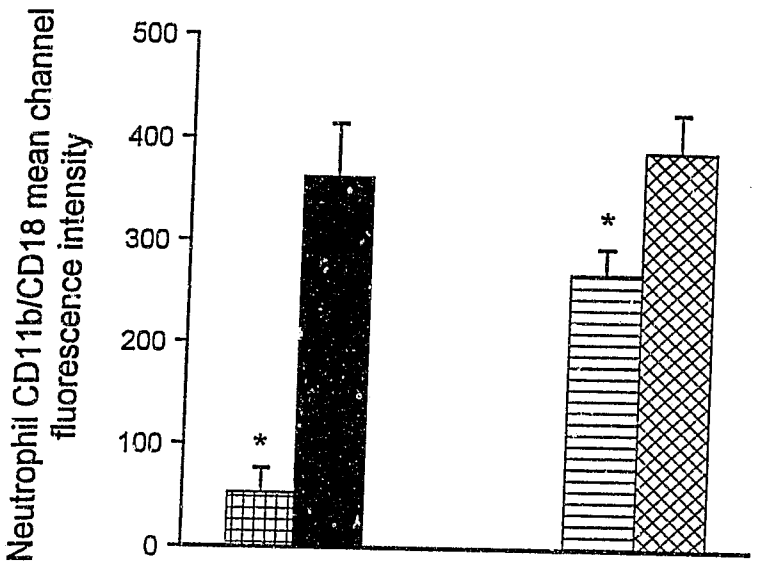

Figure 17 . Comparison of CD11b/CD18 expression in AML patients following conventional dose chemotherapy and in HDC- ASCT patients during early neutrophil recovery (neutrophil count 0.5-1.0 × 10^9) and full neutrophil recovery (neutrophil count 1.0-2.5 × 10^9). * P< 0.001 .

AML patients receiving conventional consolidation chemotherapy :
Early neutrophil recovery (▤)
Full neutrophil recovery (▤) .

HDC-ASCT patients :
Early neutrophil recovery (■)
Full neutrophil recovery (▨).

6.3 Discussion

In a previous study we observed that elevation of serum soluble ICAM-1 levels occurred during chemotherapy induced neutropenia and that during neutrophil recovery there were different patterns of response, depending on the nature of the treatment received. Patients with AML in remission receiving conventional dose consolidation chemotherapy showed persistently elevated sICAM-1 levels despite neutrophil recovery whereas in patients who received HDC with ASCT, sICAM-1 concentrations decreased to the normal range during the neutrophil recovery phase. These results suggested that maintenance of steady state sICAM-1 levels was a balance between production and removal/degradation mediated through binding to CD11b/CD18 on neutrophils. Furthermore evidence has suggested that neutrophil count recovery might not always coincide with neutrophil function recovery.

Previous studies had demonstrated that ICAM-1 is a cytokine-inducible adhesion molecule expressed on cells of multiple lineages at sites of inflammation (Dustin et al 1986; Dustin et al, 1988; Adams et al, 1989; Springer, 1990). ICAM-1 is a ligand for Mac-1 (CD11b/CD18), which is expressed primarily on leukocytes and which is critical for myeloid cell

adhesion to endothelial cell (Lo et al, 1989; Smith et al, 1989b; Arnaout, 1990 ;Yong and Linch, 1992), and for neutrophil homotypic aggregation, and chemotaxis (Anderson et al, 1986). A circulating form of the membrane-bound intercellular adhesion molecule-1 (sICAM-1) was identified and characterized in 1991 (Seth et al, 1991). sICAM-1 contains most of the five extracellular domains of membrane ICAM-1, as well as the ability to bind to CD11b/CD18. In serum from patients with leukocyte adhesion deficiency (LAD), sICAM-1 is elevated from normal levels of around 200 ng/ml to 700ng/ml. The elevated levels of sICAM-1 in LAD serum may be due to an inability to adsorb sICAM-1 to cell-bound CD11b/CD18 (Rothlein et al, 1991).

We postulated that neutrophils of patients recovering from chemotherapy may also be deficient in their ability to bind sICAM-1. To test this hypothesis, we investigated CD11b/CD18 expression by flow cytometry on neutrophils in two groups of patients i.e : patients receiving intensive but conventional dose chemotherapy unsupported by haematopoietic stem cell rescue as consolidation treatment for AML and those undergoing high dose chemotherapy with ASCT. The results showed that neutrophil CD11b/CD18 expression was low following unsupported chemotherapy. However CD11b/CD18 expression in ASCT patients was normal even

during early neutrophil recovery. For this study patients who were receiving consolidation chemotherapy were chosen for investigation in order to avoid confounding effects of aberrant cell surface protein expression or function due to the presence of leukemic cell populations. Recovery of neutrophil CD11b/CD18 expression appeared clearly to be promoted in the ASCT patients.

Proliferation of haematopoietic cells is controlled by growth factors known as colony stimulating factors (CSFs), which in addition enhance haematopoietic cell functions (Arnaout et al, 1986; Devereux et al, 1989; Socinski et al, 1989). While G-CSF administration was considered a possible reason for increased CD11b/CD18 expression in the ASCT patients it should be pointed out that this growth factor was used only for priming and not for post chemotherapy neutropenia. It seems therefore likely that the effect was due to ASCT enhancing neutrophil recovery and function.

This investigation combined with the results of the previous sICAM-1 study provides further evidence that recovery of neutrophil function after chemotherapy is promoted by ASCT.

CHAPTER SEVEN

CONCLUSIONS

CD11/CD18 molecules have been intensively studied for a number of decades. These leukocyte integrins include the leukocyte function-associated antigen-1 LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and P150,95 (CD11c/CD18). CD11/CD18 molecules are expressed on leukocytes. They sustain multiple functions, including adhesion, aggregation, chemotaxis, initiation of the oxidative burst and phagocytosis of opsonized particles. CD11b/CD18 is a key molecule of this family, and plays a crucial role in neutrophil functions related with inflammatory and immune clearance processes by binding to several cell surfaces and soluble ligands, including ICAM-1, iC3b, factor X and fibrinogen. While CD11b/CD18 is widely expressed on haematopoietic cells, expression of CD11b/CD18 is more restricted to neutrophils and monocytes.

The aim of this dissertation was to investigate some aspects of leukocyte integrins and their ligands. The studies included the development of an in-vitro model using cultured ECV-304 cells to analyse neutr ve

function. Previous studies had given conflicting results. Some investigations suggested that CD11a/CD18 was the factor responsible for adhesion of neutrophils to endothelial cells while CD11b/CD18 was responsible for neutrophil binding to other ligands such as fibrinogen and for neutrophil degranulation. Other studies, however, showed that adhesion of unstimulated neutrophils to endothelial cells involved a CD11a/CD18-dependent mechanism, whereas stimulated neutrophils adhered to vascular endothelial cells in a CD11b/CD18- dependent manner. Some studies showed that the integrin $\alpha_4\beta_1$ (VLA 4) and P-selectin (CD31) were involved in mediating the adhesion of neutrophils to endothelial cells. The results presented here, however, show that the adhesion of unstimulated neutrophils to endothelial cells involves CD11b/CD18 , probably by recognizing its ligand ICAM-1 on endothelial cells, rather than CD11a/CD18 or CD11c/CD18. In addition we found that normal serum contains a potent adhesive factor, which is divalent cation dependent and which is thermolabile. This serum factor increased the adhesion of neutrophils to endothelial cells and could be inhibited by anti-CD11b MoAb. The serum adhesive factor was shown to be the complement fragment iC3b, a soluble ligand of CD11b/CD18, which acts as a bridging molecule to induce increased adhesion between neutrophils and endothelial cells.

Neutrophil tethering , rolling and subsequent adhesion to endothelial monolayers appear to be a complex procedure. Under varied conditions, different receptors and ligands exert functions , mediated by various factors. Definition of the role of various CD11/CD18 molecules may help in understanding of a number of inflammatory disorders, and to develop pharmacological strategies to inhibit multiple adhesion molecules.

The following study was to measure sICAM-1 concentrations in two groups of patients. One group consisted of patients with acute myeloid leukemia treated by conventional dose consolidation chemotherapy; the other group included both myeloid leukemia patients and breast cancer patients undergoing high dose chemotherapy plus autologous stem cell transplantation. Interestingly, we found increased sICAM-1 levels in both groups during the chemotherapy-induced neutropenic phase (neutrophil count $<0.5\times10^9$/ L).It was postulated that high levels of soluble cytokines (IL-1 and TNF-α) could have been responsible for the elevated sICAM-1levels. Patients undergoing haematopoietic rescue showed a rapid reduction of sICAM-1 to normal during the recovery phases, whereas the levels remained persistently elevated, even after neutrophil count recovery, in patients receiving unsupported chemotherapy.

Subsequently, quantitative expression of CD11b/CD18 on neutrophils was measured in the same group of patients during the early and full neutrophil recovery phases. The results showed that CD11b/CD18 expression was low following unsupported chemotherapy. However CD11b/CD18 expression in ASCT patients was normal even during early neutrophil recovery. This investigation combined with the previous sICAM-1 study indicated that neutrophil function and the ability to interact with ICAM-1 is defective after intensive but unsupported chemotherapy despite recovery of the neutrophil count. These abnormalities were not found in patients whose chemotherapy was supported by autologous peripheral stem cell rescue.

These investigations indicate that patients undergoing stem cell rescue show a more rapid recovery of neutrophil mass than what is evident from peripheral blood counts and in addition that they also display increased expression of CD11b/CD18 and therefore more functional activity of neutrophils.

It is concluded that the recovery of neutrophil count may not always correlate with functional recovery of these cells. sICAM-1 appear to be a sensitive and reliable marker to assess the neutrophil functional recovery after intensive chemotherapy induced severe neutropenia.

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