Chapter 4: Discussion

Oesophageal cancer is a disease with a high and ever increasing incidence worldwide (Munoz, 1993). No single factor has been linked to the cause of the disease and it is therefore believed to be a multifactorial disease. There is ongoing research to develop a model on how oesophageal carcinogenesis develops or progresses. There are many genes that have been shown by numerous studies to play a role in the carcinogenesis of oesophageal tumours, but no evidence showing how the tumour develops. Many researchers believe it to be the human papilloma virus (HPV) that initiates the development of the tumour, but more research is needed to prove that it occurs in all oesophageal carcinoma cases and not only in a small percentage, as have occurred (Matsha et al., 2002). Others believe it to be the mutation of apoptosis regulating genes, tumour suppresser genes and oncogenes that are involved in the initiation and development of oesophageal cancer; a gene directly related to oesophageal cancer has yet to be identified, for an effective therapeutic strategy to be determined (Cui et al., 2003). Oesophageal cancer develops in a sequential form from normal to malignant tissue in four stages; metaplasia, dysplasia (low and high grade), carcinoma in situ, and then finally an invasive carcinoma stage.

This study was carried out to determine the expression patterns of three genes, 1-ACBP, B-ACBP and PBR in oesophageal carcinoma, and determine their roles in the development of oesophageal carcinogenesis. ACBP has five isoforms that are very similar and the two focused on in this study was the general isoform that is found in most tissues, and the second isoform was brain specific. 1-ACBP was found expressed in many diseased tissues and so the expression pattern in oesophageal cancer was considered to be

determined. ACBPs bind to PBR and play role in steroidogenesis, this interaction is also believed to play a role in apoptosis.

4.1 PBR, 1-ACBP and B-ACBP expression and localisation in oesophageal cancer.

4.1.1 In situ hybridisation

In situ hybridisation is a method of localising and detecting specific mRNA sequences in morphologically preserved tissue sections or cells, by hybridising the complimentary strand of a nucleotide probe to the sequence of interest. Digoxigenin (DIG) is a steroid isolated from the digitalis plant and as the blossoms and leaves are the only known source of digoxigenin, the anti-DIG antibodies are not likely to bind to other biological material. The digoxigenin is linked by a spacer arm containing 11 carbon atoms to the C-5 position of the uridine nucleotide. The advantage of using a DIG labelled probe is that it can be detected with antibodies conjugated to a number of different labels such as alkaline phosphatase, which results in a blue precipitate when the enzyme is incubated in the presence of the substrate NBT/BCIP (Tetrazoliun salt/ 5-bromo- 4-chloro- 3 idolyl-phosphate) or becomes a fluorescent label when incubated with HNPP (2hydroxy-3naphthoic acid-2'phenylanilide phosphate) or FITC.

All three genes showed substantial upregulation within the malignant tissue sections compared to normal oesophageal sections, within the lamina propria, muscularis mucosa, submucosa, connective tissue and longitudinal muscle layers. All three transcripts localised specifically and mainly to plasma cells and few lymphocytes in diseased tissue

sections, with an increase in expression as the disease progressed and plasma cells increased.

The ACBP genes also localised to endothelial cells in arteries within the connective tissue of diseased submucosa, but no localisation of these genes were found in these cells in the normal oesophageal submucosa. B-ACBP localised to the nucleus in epithelial cells of the transformed epithelium, but no localisation of this gene was found when compared to the normal epithelial cells. PBR was also found localised to tumour islands in invasive squamous carcinomas. Since all three genes are localising to plasma cells and some lymphocytes, there is a possibility that these genes may be interacting with each other in some way to play a role in the immunological response to cancer cells. These roles could be pro- or antiapoptotic and therefore it would be useful to determine the exact role these genes play in immunology, so that potential therapeutic strategies can be determined to combat this disease. Quantitative RT-PCR illustrated that PBR expression levels were the highest compared to the ACBP genes expression in tumours, with the ACBP genes having comparative levels of transcripts.

Since 1-ACBP, B-ACBP and PBR all are localised to mainly plasma cells in response to cancer cells invading the body, they are believed to play a role in the production of antibodies needed to combat the tumour cells. The main functions of ACBPs are to stimulate transport of cholesterol from the outer to inner mitochondrial membrane, by binding to PBR, playing a role in steroidogenesis. Plasma cells are mature B-lymphocytes that are specialised for antibody production. These cells consist of a nucleus that is eccentric and oval in shape, cytoplasm that contains plentiful ribosome-studded or rough

endoplasmic reticulum, and also many mitochondria which produce the energy required for a highly metabolically active cell. Mature plasma cells are often oval or fan shaped, measuring 8-15 μm. Plasma cells are rarely found in the peripheral blood. They comprise from 0.2% to 2.8% of the bone marrow white cell count. The bone marrow (BM) is well known to be the major site of Ig production in secondary immune responses; thus, the microenvironment of BM is considered to be essential for final differentiation of plasma cells. The figure below shows the maturation and locations of the B-lymphocyte to mature plasma cell.

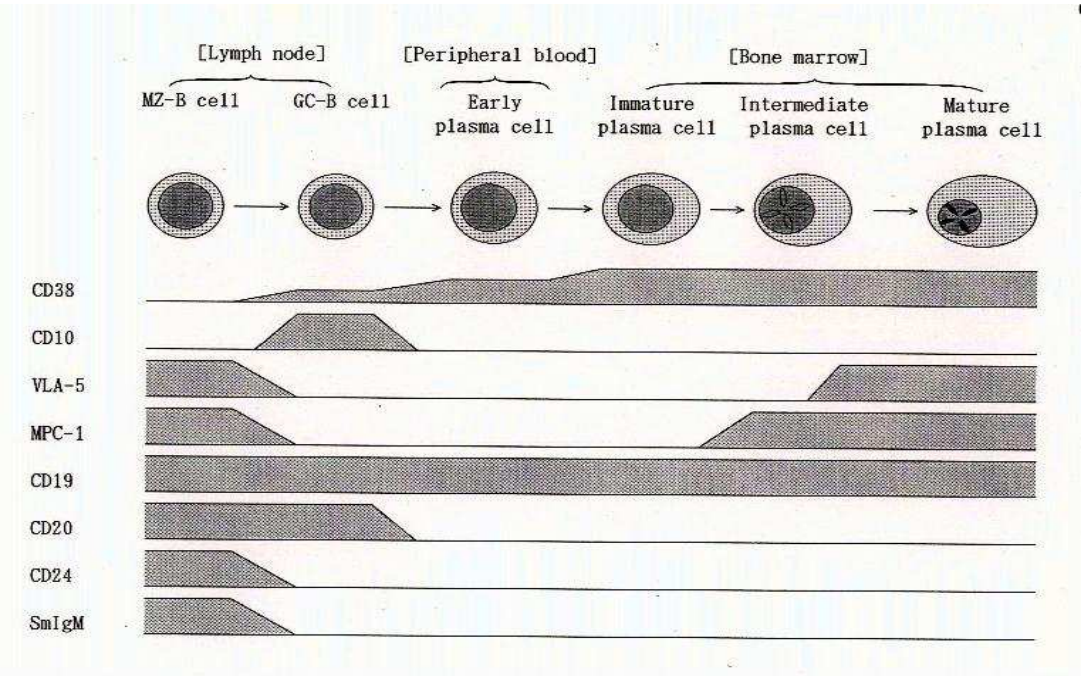

Figure 62: Maturation B-lymphocytes to mature plasma cells from (Kawano et. al, 1995).

As seen in figure above, the germinal centre B lymphocyte (GC-B cell) found in the lymph node, divides and develops into plasma cells, where the early plasma cell is found in the peripheral blood but the final maturation stages of the plasma cell takes place in the

bone marrow. From the ISH micrographs, 1-ACBP, B-ACBP and PBR are highly expressed in the mature plasma cell. This is expected, since antibodies are produced by the mature plasma cell and therefore a high amount of energy is required at this stage and therefore an increase in these proteins are observed.

1-ACBP and B-ACBP were found expressed in endothelial cells of malignant oesophageal tissue sections, B-ACBP was localised to epithelial nuclei, and no expression was found when compared to the normal oesophagus. PBR was also highly expressed in the islands of tumours.

1-ACBP and B-ACBP localised to endothelial cells of arteries in high-grade squamous carcinomas. Endothelial cells are involved in the inhibition or promotion of angiogenesis, which is the development of new blood vessels for the blood and nutrient supply for tumour survival. Endothelial cell proliferation and migration promote angiogenesis, whereas apoptosis of endothelial cells leads to vessel regression. It has been found that stimulation of endothelial cell apoptosis results in tumour degeneration, due to the tumour blood supply being withdrawn. A study has shown that inhibition of VEGF (Vascular endothelial growth factor) in tumours has led to endothelial cell detachment, apoptosis, vascular collapse, haemorrhage, and tumour necrosis (Dimmeler and Zeiher, 2000). ACBP functions in endothelial cells are believed to be that of steroidogenesis, due to the high-energy requirement of angiogenesis for tumour survival in high-grade squamous carcinomas.

B-ACBP has been found expressed in the nuclei of epithelial cells in high-grade stratified epithelium of well-differentiated squamous cell carcinoma tissue sections. In an

aggressive human breast cancer cell line, PBR along with 1-ACBP was found highly expressed, and PBR was found responsible for the increased cholesterol transport into the nuclei of the cell line (Harwick et. al, 1999). Increased expression in the nuclei of the epithelial cells could be due to the increase of cholesterol transport into the nuclei of the epithelial cells, mediated by some other factor other than PBR, due to PBR expression not found in epithelial cells.

PBR was also found overexpressed in the islands of tumours in invasive squamous cell carcinoma tissue sections. Tumour islands consist of compressed tumour cells and therefore, in these cells are high levels of PBR and therefore a high localisation level is observed in these islands. No localisation for ACBP molecules occurs in these islands due to these molecules having short to half life spans.

It has been found that discrepancies between micromolar concentrations of PBR ligands inhibit cell growth, whereas nanomolar concentrations promote cell growth. ACBPs have a high affinity for PBR and displace benzodiazepines with an inhibitory constant of 1-2uM (Papadopuoulos and Brown, 1995). High concentrations of PBR and correlating ACBP concentrations have been found in many tumours and therefore are believed to play a role in the pathogenesis of malignancy.

In this study however, high expression of these genes were found in plasma cells and an interaction between these genes are believed to take place for high-energy production, for the generation of antibodies. PBR, 1-ACBP and B-ACBP from these findings therefore suggest an immunological role of these three genes in plasma cells, in response to oesophageal cancer. There has been localisation of the ACBP genes to endothelial cells

and nuclei of epithelial cells, but no PBR expression was found in these locations. These findings therefore suggest that the ACBP genes could have a potentially pathogenic role in oesophageal carcinoma, without the help of PBR, suggesting that other factors may be involved in tumour development of the oesophagus.

4.1.2 Protein expression of 1-ACBP, B-ACBP and PBR:

The ORFs of these three genes were synthesised and cloned into pGEX vectors for protein expression and antibody production for immunocytochemistry analysis.

Two sets of primers were designed for each ORF, the first set was designed to adjacently flank the regions of the ORF of the gene, and the second set was designed to have restriction enzyme sites flanking the ends of the primers that when the gene was amplified via PCR, restriction enzyme sites would flank the genes and could be cloned and subcloned in to pGEM T Easy and pGEX vectors respectively. Once the desired PCR product was obtained it was cloned initially into the pGEM T Easy vectors and once the ORFs were successfully cloned they were sent for sequencing at Inqaba biotech (Pretoria, South Africa) to determine whether the desired sequence was obtained and whether the gene with restriction enzyme sites were still intact. Once all three ORFs were successfully cloned, sequenced and the correct sequence determined, the ORFs were digested with Bam HI and Xho I and subcloned into pGEX. At this stage only two ORFs were successfully cloned into pGEX for expression, which was PBR and B-ACBP. Once these ORFs were successfully cloned, the clones were sent to Inqaba biotech for sequencing again and only the desired sequences of B-ACBP were obtained.

Further experiments were carried out to express the two proteins, which entailed growing the clone in LB containing ampicillin, and later inducing the expression of the genes with IPTG for a 16-hour period at a temperature of 25 degrees Celsius. The culture was then freeze thawed to release the desired protein, and loaded onto a SDS page gel to determine whether the desired proteins were obtained. The expected result was recombinant proteins of GST bound to PBR and B-ACBP proteins, resulting in a size of

approximately 37 kDa and 40kDa respectively, but the result was not as expected. The proteins observed in the experimental sample were the same as that in the crude extract, but at a lower concentration. The experimental sample was then further analysed by performing GST-protein purification, using the GST-magnetic purification system from Promega™ and no recombinant GST-proteins were detected, which means that no GST-recombinant protein was synthesised or that these proteins became embedded in the membrane of the *E*.coli.

Documentation supporting this latter statement shows evidence for ACBP binding to the membrane in E.coli cells (Simonsen et al, 2003). ACBPs bind acyl-CoA as well in E.coli, and these acyl-CoA molecules bind to and forms aggregates on the membrane, depending on the concentration of acyl-CoA in the bacterium. Once ACBP is induced or exposed in the E.coli cell, ACBP binds to the acyl-CoA aggregates on the membrane and extracts it and binds to the entire membrane itself. ACBPs in some cases, when the densities of the acyl-CoA molecules are too high and embedded in the membrane, bind to the CoA group of the molecules without extracting it from the membrane (Simonsen et al, 2003).

PBR is also a membrane-associated protein and therefore could be embedded or localised to the membrane in the *E*.coli.

A solution would be to synthesise a smaller section of the ORFs of the proteins, where an antigen to the particular protein will still be expressed and antibodies can be raised. A smaller portion of the proteins will result in easier cloning of fragment due to its size, and the expressed products will not interact with *E*.coli cellular activities, and therefore expression will be simpler as well.

4.1.3 Quantitative gene analysis of 1-ACBP, B-ACBP and PBR in oesophageal Cancer, using the Roche LightCycler:

Quantification of the expression of 1-ACBP, B-ACBP and PBR was carried out using the LightCycler to compare the expression levels of the genes with relative quantification graphs. These graphs are produced by the measure of SYBR green dye that binds to the product generated and so the amount of fluorescence emitted is directly proportional to the amount of double stranded DNA produced. This system was also used to confirm that the probes of the three genes showed similar results to the open reading frames of the three genes. Relative quantification was also used to compare the expression levels of the the three genes' open reading frames in oesophageal cancer cell line SNO 1.

The expression levels of 3' and 5' 1-ACBP showd a substantial difference in concentration o fthe amplicons generated, which was not expected and believed to be due to the primer annealing to the template cDNA. The 5' 1-ACBP was also compared to the open reading frame of 1-ACBP, it showed a compareable PCR product amplification, which was expected, as the same gene was amplified and a similar expression level should be seen (View figure 60). 3'1-ACBP primer annealing may therefore be much more efficient in binding to the CDNA template increasing the amount of product generated.

Another consideration to the 3'1-ACBP alteration in expression level could be due to a higher concentration of an altered mRNA transcript. It has been found that PBR has two transcripts in diseased tissue, one that lacks exon 2 and one that contains exon 2. The one that lacks exon 2 has been found expressed at a much higher level in diseased tissue and

this transcript is not translated into protein (Gavish et al. 1999). The transcript that contains exon 2 is transcribed into a normal protein even in diseased tissue. It could therefore be a possibility that the same result occurs in the same case of the 3' 1-ACBP, where an alteration in the 3' end of this gene, resulting in a much higher transcript concentration but it is not translated into protein.

The 3' and 5' B-aCBP gene expression was analysed and a considerable difference in amplicon concentration was also observed which was not expected. 5'B-ACBP when compared to the open reading frame of B-ACBP showed similar results indicating the expression levels of the same gene was detected (View figure 61), which was expected. 3'B-ACBP therefore when compared to the 5' and open reading frame of B-ACBP, it showed a very low concentration and thought to be due to the inefficient binding of the primers, therefore yielding a much lower concentration of product generated, as the primers does not anneal efficiently to the altered product, or a much lower concentration of an unaltered mRNA transcript occurs and a higher concentration of the 3' altered product occurs and the primers do not anneal to this altered cDNA.

The PBR sequence that contains part of the exon 1 and exon 2 compared to the open reading frame sequence of PBR generated comparable amplicon concentrations in the diseased cDNA as expected, (View Figure 62).

The open reading frames of 1-ACBP, B-ACBP and PBR concentrations were then compared and the results showed comparable expression levels with B-ACBP having the

highest concentration, PBR second highest and 1-ACBP the lowest, (View Figure 63). Since B-ACBP is expressed in squamous epithelial cells, endothelial cells and plasma cells, it is probably expected that a higher concentration of this gene should occur. And since B-ACBP and PBR have the highest concentrations compared to 1-ACBP, it is most likely that B-ACBP binds more often to PBR having cell proliferation effects on the cell, or it could have apoptotic effects on the cell. Further analysis would have to be carried to determine the exact roles of these genes in the development of cancer, and also mutation analysis would determine whether these genes are mutated in the diseased oesophagus.

Chapter 5 Conclusion:

1-ACBP, B-ACBP and PBR have all been localised mainly to plasma cells in the tissue sections of the diseased oesophagus. This data supports other findings that PBR and ACBP ligands might have an immunomodulation role. The ACBP genes were found expressed in endothelial cells of the diseased oesophagus and are believed to play a role in the development of new blood vessels for tumour survival. B-ACBP was found expressed in the nuclei of epithelial cells of the diseased epithelium, suggesting a role played in the transformation of epithelial cells to a neoplastic nature, in the development of squamous cell carcinomas. PBR localised to tumour islands indicates a high level of the gene localises to tumour cells, and supports other findings where PBR is upregulated in other cancers.

Though ACBP and PBR have been documented to interact with each other in diseased tissue, no interaction seemed to have occurred between these genes in the development of the disease. Further analysis would have to be carried out to confirm whether there are any interactions between the ACBP molecules and PBR in the development of oesophageal cancer. The only potential interaction by location of these proteins in this study was found in plasma cells and lymphocytes, suggesting a role in immunomodulation and antibody production. Further studies would have to be carried out to determine the exact role these genes play in the plasma cells so a potential therapeutic strategy could be determined to increase the effectiveness of the plasma cells and antibody production and combat the fight against oesophageal cancer.

Protein expression of these genes was not successful, but the *E.*coli bacteria are believed to be partly responsible for many grievances experienced with the cloning of these ORFs.

Since protein expression studies of these genes in oesophageal cancer are of great interest, it is suggested that a smaller segment of the protein is synthesised instead, that which still contains an antigen of the desired protein for antibodies to be generated against, but will not be recognised by the bacterium as its own. This would result in easier cloning and expression studies of the ACBP and PBR ORFs.

For further therapeutic strategies to be determined, the exact functional role of 1-ACBP, B-ACBP and PBR has to be elucidated in the normal and the cancerous cell. From this study the ACBP genes show to play a role in the development and progression of the cancers, by endothelial cell localisation, suggesting the potential role of assisting in the process of angiogenesis, by steroidogenesis. It would be a thought to cut off the energy supply in this process and therefore block tumour development. But since all cells need ACBP to function, these ACBP inhibiting molecules would have to have specific targets to those of tumour cells. Once the tumour cell ACBPs are targeted, the effect on the cell can be observed to determine whether direct ACBP inhibition results in energy starvation, and hopefully cell death.

It has recently been shown that a decrease of the mitochondrial membrane potential is a prerequisite for PBR-ligand induced apoptosis (Sutter, 2002), and induction of apoptosis is dependent on the activation of the p38 mitogen-activated kinase (MAPK) pathway (Sutter, 2004). A drastic decrease in energy supply for neoplastic cells to proliferate combined with the PBR-ligand-induced apoptosis pathway, could be a potential strategy to combat the development and progression of cancer.