PROTHYMOSIN ALPHA,

A GENE DIFFERENTIALLY EXPRESSED IN CD34^{+} CELLS

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ABSTRACT

Haemopoietic stem and progenitor cells from bone marrow and cord blood are well characterised with respect to their phenotype, growth in clonal assays, responsiveness to cytokine stimulation, receptor profile and their ability to sustain multilineage engraftment of receptive hosts in animal models of transplantation and of course, clinically in the treatment of some haemopoietic and immunological disorders. It is generally accepted that cells bearing the $CD34^+$ phenotype are enriched for the most primitive of haemopoietic stem cells that possess the cardinal features of self-renewal and multipotency. However, the molecular mechanisms, the spectrum of expressed genes that give rise to the physical characteristics of haemopoietic progenitor cells are not well understood. Furthermore, although $CD34^+$ cells from different sources (bone marrow, cord blood, mobilised peripheral blood) share many common features, there are also significant differences.

The purpose of this study was to identify and characterise genes differentially expressed in $CD34^+$ cells from CB and BM, exposed to cord or adult sera. This protocol was chosen to exploit the known ability of cord serum to enhance proliferation of primitive progenitors, effects that are more pronounced in progenitors derived from cord blood than other sources, suggesting differences in the genetic program of cord blood and bone marrow progenitors. The greater proliferative response of cord blood compared to bone marrow progenitors to combinations of known cytokines also indicates that cord blood progenitors innately express a different program of gene expression compared to adult bone marrow progenitors. Therefore the gene discovery protocol was designed to investigate differences in gene expression between: $CD34^+$ cells compared to the more mature $CD34^-$ phenotype; cord blood progenitors compared to bone marrow progenitors; and, cells exposed to pooled cord sera compared to pooled adult sera.

Using dd-PCR and real time PCR we identified prothymosin alpha (PTA) as more highly expressed in $CD34^+$ cells compared to $CD34^-$ cells in bone marrow and cord blood. Published data indicated that in other cell systems up-regulation of PTA was associated with active proliferation, however $CD34^+$ cells are inherently quiescent.

During the course of this study program other groups have also found differential expression of PTA in haemopoietic progenitors from human and mouse but there are no published data relating to the regulation of this gene in this important group of cells. We undertook a detailed analysis of the regulation of gene expression in $CD34^+$ cells exposed to a variety of cytokines, conducted a comprehensive assessment of gene expression in mature subsets of human haemopoietic cells and extended previous work on the expression of PTA receptors on various cell types, including $CD34^+$ cells. Our data indicates that the expression of PTA in $CD34^+$ cells is not solely related to proliferation and down-regulation of the gene occurs with differentiation. This suggests that maintenance of high levels of PTA may be necessary to maintain an immature cell type, which could have implications for expansion protocols, designed to increase cell number while retaining a primitive phenotype.

DECLARATION

This is to certify that

(i) the thesis comprises only my original work towards the PhD except where indicated in the Preface,

(ii) due acknowledgement has been made in the text to all other material used,

(iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Caryll M. Waugh

Signed: ……..Date: ……

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TABLE OF CONTENTS

CHAPTER TWO - MATERIALS AND METHODS

CHAPTER FOUR - PROTHYMOSIN α LITERATURE REVIEW

LIST OF TABLES, FIGURES AND ILLUSTRATIONS

CHAPTER ONE

A BRIEF REVIEW OF HAEMOPOIESIS AND PROJECT OUTLINE

INTRODUCTION

Haemopoietic stem cells (HSC) possess the hallmark features of self-renewal and multi-lineage developmental potentials. It is estimated that adult humans produce in excess of 10^{11} mature blood cells per day and haemopoiesis or blood cell production is sustained throughout the lifetime of the individual by the carefully regulated balance of these hallmark features, self-renewal and multi-lineage differentiation. The complex mechanisms that regulate this process are largely unknown but extensive investigations have been on-going for several decades to characterise both HSC and the regulatory molecules, humoral and cell-associated that comprise the haemopoietic system. During this time an appreciation has grown of the points of distinction between HSC from different sources which has (quite apart from the intellectual challenges that have been generated) broadened the therapeutic choices available to the clinician. Even more recently, within the last 10 years, many investigators have turned their attention to the molecular mechanisms, the array of gene expression, that distinguish HSC from functionally mature haemopoietic cells and that distinguish HSC (with measurable different proliferative potentials) from different sources.

Most of the interest in characterising HSC has arisen from the clinic and the need to better tailor therapies for patients who require a stem cell transplant. Haemopoietic stem cell transplantations are used to restore normal haemopoiesis to the patient in a variety of clinical settings, including some forms of leukaemia, apastic anaemia and inherited diseases of the bone marrow. There are three sources of tissue regularly used to harvest stem cells: adult bone marrow (BM), mobilised peripheral blood (mPB) and umbilical cord blood (CB)[1]. Of these, bone marrow, as an intuitive source of stem cells, has the longest clinical history, with bone marrow transplantation (BMT) used for the treatment of a variety of life-threatening disorders since the 1950's (and sporadic

[1] HSC are found in tissues other than these, most notably the embryonic yolk sac, foetal liver and foetal BM, but this discussion will be restricted to those tissues that are most commonly used for stem cell transplantation.

documentation of its use since 1891) (Santos 1983). The early history of BMT records an unpredictable and controversial clinical practice used mainly in patients considered *in extremis*. BMT did not gain favour as a controllable therapeutic modality and thus a useful clinical tool until the early to mid-1970's. Throughout the 1950's and particularly the 1960's an appreciation developed of the complexity of interactions between the patient (the host), the transplant (the graft) and the underlying disease. Considerable resources were devoted to the investigation of tissue types and immune tolerance, graft rejection and most importantly, the nature and effects of "runt's disease" or graft versus host disease (GvHD). Accordingly, the history of BMT is intimately linked to that of the immunology of blood cells, an understanding of tissue types, the pharmacokinetics of chemotherapeutic agents and the development of blood products tailored to the needs of acutely immune deficient and cytopaenic patients (Little and Storb 2002). During these decades BMT was gradually transformed from an experimental therapy to a central therapeutic regimen, along with chemotherapy and radiotherapy, for the treatment of childhood and adult malignancies.

Cross circulation experiments conducted in animal models in the 1950's and 60's showed that haemopoietic stem cells (HSC) also occurred in the peripheral blood (Mangan 1995). In the steady state however these cells comprised too small a population to be considered a useful source for clinical trials. In the mid-1970's, it was noted that the rebound recovery of bone marrow following chemotherapy produced a sharp increase in the numbers of circulating HSC (Mangan 1995, To 1997). Later, growth factors were discovered that produced the same effect, notably granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF), and these became available for clinical use in the 1980's. Refinement of mobilisation regimens eventually made it feasible to stimulate movement of HSC from the BM into the peripheral blood (PB) so that the cells could then be 'harvested' using automated apheresis techniques. Although the use of mPB as a source of HSC lagged behind that of BM by 15 years, utilising mPB became routine practice during the 1980's such that by the early 1990's mPB overtook BM as the preferred source of HSC for haemopoietic rescue (To 1997). This shift in clinical practice has been due largely to a trend to use autologous, rather than allogeneic sources of HSC and has been noted in the data from the International Bone Marrow Transplant Registry, the

Europe Blood and Bone Marrow Group and the Australian Bone Marrow Transplant Registry (To 1997).

The presence of haemopoietic progenitor cells in umbilical cord blood was noted by Soren Knudtzon in 1974 (Knudtzon 1974) when he compared the colony forming capacity of CB with that of PB from healthy adults. Knudtzon found that CB contained between 40-100 times the number of granulocyte-macrophage colony forming units (CFU-GM) of PB and that these numbers were comparable to that found in human BM. He concluded that CB might prove a viable alternative to BM for the restoration of BM function in humans.

Throughout the ensuing years various groups investigated the stem and progenitor cell content of CB, culminating in the work by Broxmeyer *et al* (Broxmeyer 1989) who specifically looked at the potential of CB as an alternative to BM in a transplantation setting. This work resulted in an international and multi-institutional collaboration to perform the first CB transplant, which was undertaken in a child with Fanconi's Anaemia in 1988. The success of this pioneering clinical work precipitated a huge increase in interest in CB as an alternative to BM for transplantable stem cells and prompted the establishment of the first CB bank in New York in 1992. Since then many other CB banks have been set up throughout the world and currently there are more than 100,000 CB units available for transplantation and more than 2000 CB transplants have been performed (Broxmeyer*, et al* 2003).

CB presents an attractive alternative to BM (or mPB) for several reasons. As a by-product of birth, which has hitherto been discarded, CB represents an easily procured source of stem cells for storage. Disease screening of the mother and tissue typing of the CB, permit the collection of units that, once listed on a CB register, are immediately available for use and the potential exists to significantly reduce the incidence of infectious disease transmission. In addition, the immune naivety of CB should lessen the incidence of GvHD in HLA-matched transplants and/or permit the use of mis-matched donor CB units. An immense amount of work has been done to characterise and compare the haemopoietic stem cell content of CB, BM and mPB. A summary of the major findings relating to the comparative analyses of these sources of HSC is presented below.

CLINICAL DATA

The first comprehensive assessment of CB as a source of stem cells for transplantation was reported by Broxmeyer *et al* in 1989 (Broxmeyer 1989). In this review the investigators assessed more than 100 collections of CB for their nucleated cell number and their *in vitro* colony forming activity. They also assessed methods for short and long-term storage of CB units and the suitability of separation procedures on volume reduction and progenitor cell enrichment. They concluded that CB could serve as a source of stem cells in either the autologous or allogeneic setting. In the same year Gluckman *et al* (Gluckman 1989) and Broxmeyer *et al* (Broxmeyer 1990) reported the successful engraftment of a boy with Fanconi's anaemia transplanted with CB collected from his HLA-compatible sibling. These reports heralded the 'arrival' of CB as an alternative to BM or mPB as source of stem cells. In the decade following, the number of CB transplants (CBT) attempted increased steadily each year with most of these being performed in children.

In 1998 Rubinstein *et al* (Rubinstein 1998) reported the outcomes of the first 562 transplantations that were supplied by the New York Blood Center. In 2000, Eliane Gluckman (Gluckman 2000), on behalf of the Eurocord Netcord Registry, reported the outcomes of 527 CBT performed between 1988 and 2000. These two reports comprise the most extensive assessment of the efficacy of CBT to date. Both reports note the delayed time to engraftment for both neutrophils and platelets and in the case of myeloid engraftment, attribute this mainly to the lower cell dose generally administered in CBT compared to BMT. An association between time to platelet engraftment and any of the multiple variables assessed was more difficult to determine, however, patient age, infection post-transplantation and GvHD were implicated (Rubinstein 1998).

As expected, due to the immune naivety of the neonate, the incidence of severe acute GvHD and chronic GvHD was less in CBT recipients than in comparable studies of BMT recipients (Gluckman 2000). This held true in a multicentre analysis of children with acute leukaemia undergoing either unrelated CBT or unrelated BMT. During the first 100 days of transplant, the children receiving CBT had significantly less incidence of acute GvHD than those children receiving unmanipulated BM even though the donor was HLA-mismatched in 92% of the CBT compared to 18% of the BMT. At two years post transplant, the relapse rate for both groups was similar (CBT:38% v BMT:39%)

however the survival rate was 35% in the CB recipients (95% confidence interval 25-45) and 49% in the BM recipients (95% CI 43-55).

Both studies concluded that CB was a useful source of stem cells in the allogeneic setting with the *caveat* that more research was required to reduce the time to full haemopoietic restoration.

PHENOTYPIC ANALYSIS OF HAEMOPOIETIC PROGENITORS

The CD34 antigen

CD34 is the antigen most often used to identify, enumerate and enrich haemopoietic progenitors. The CD34 antigen was discovered in 1984 as a consequence of a strategy to develop monoclonal antibodies against immature subsets of bone marrow (Krause 1996). It is present on approximately 1-3% of nucleated cells in BM, ~1% of nucleated CB and 0.1% of steady-state PB and is expressed on virtually all stem and committed progenitors. It also occurs on small-vessel endothelial cells and some tissue fibroblasts (Greaves M.F. 1995, Krause 1996)

CD34 is a heavily glycosylated transmembrane protein, rich in O-linked carbohydrates and sialic acid. The negatively charged extracellular part of the protein has a putative role in cell-cell adhesion (Healy, *et al* 1995). The intracellular domain is a target for phosphorylation by activated protein kinase C and so may have a role in the transduction of cell signalling pathways. The human CD34 gene is localised to chromosome 1q32.

The purification of CD34 cells from BM, CB or PB causes a co-enrichment of colony forming units (CFU)[2] of all types ranging from the lineage committed to the most primitive identifiable by *in vitro* techniques and encompasses a HSC capable of long-term marrow engraftment (Baum 1992, Bhatia, *et al* 1997, Hogan 1997, Wang 1997).

[2] The 'colony forming unit' is an operational term describing a cell that can produce a colony of cells (a colony forming cell that undergoes clonal expansion) when cultured in a semi-solid media (such as agar or methylcellulose) enriched with colony stimulating factors (CSF). Different colony types can be identified based on cell morphology, the size of the colonies, the time delay prior to proliferation and the ability to produce secondary colonies upon re-plating of the dispersed cells of a primary colony. Assuming that culture conditions are optimal, primitive cells produce colonies comprised of several cell types (multi-lineage, e.g. CFU-GEMM colony forming units for granulocytes, erythroid cells, monocytes and megakaryocytes) while a cell that is more developmentally advanced or committed will produce a colony with fewer cell types and the most mature cell will produce a colony of a single cell type (lineage restricted e.g. CFU-G colony forming unit for granulocytes). *In vitro* colony assays detect only committed and multipotent progenitors.

Thus, although the CD34 antigen is present on only a minor population of cells from BM, CB or PB, $CD34^+$ cells represent a heterogeneous population of haemopoietic progenitors which display a wide spectrum of proliferative capacities and lineage commitment.

Somewhat surprisingly, there is also compelling evidence that the most primitive haemopoietic progenitor, identifiable by surface antigen expression, lacks the CD34 antigen ($CD34^-$) and is also devoid of lineage markers, (Lin^-). The gold standard definition for HSC is the ability to produce haemopoietic reconstitution in lethally irradiated animals. To date comparisons of $CD34^+$ populations with $CD34^-Lin^-$ in animal models has produced conflicting results with some groups describing haemopoietic reconstitution with $CD34^-$ cells (Bhatia, *et al* 1998, Goodell 1996, Osawa, *et al* 1996) and others describing no engraftment or engraftment but no reconstitution (Andrews, *et al* 2000, Kim, *et al* 1999). Recently a 'stem cell cycle' has been proposed which describes the circulation of HSC between the BM and the circulating PB (Huss 1998, Huss 2000). The theory postulates that $CD34^+$ cells circulate in the PB, home to the BM where they become quiescent and down-regulate CD34 expression ($CD34^-$) before responding to micro-environmental stimuli, re-expressing CD34 and either cycling as before or differentiating along a haemopoietic pathway. In any case, the $CD34^+$ phenotype describes a cell with well-documented capabilities to produce *in vivo* long-term haemopoietic reconstitution and therefore the enumeration and enrichment of cells expressing CD34 is a useful strategy for comparing the relative proliferative capacity of haemopoietic cells from different sources.

Lineage Markers

In comparisons of lineage markers on $CD34^+$ enriched populations it has been shown that CB and mPB have greater proportions of early myeloid progenitors and very low levels of B-cell progenitors compared to BM (Fritsch, *et al* 1996, Steen, *et al* 1994), (Bender, *et al* 1994, De Bruyn, *et al* 1995). The T-lymphoid marker CD7 is expressed at negligible levels on $CD34^+$ cells from all sources (Bender 1991, Fritsch, *et al* 1996).

Other antigens commonly used in conjunction with CD34 to assess primitive haemopoietic populations are CD38, HLA-Dr, Thy-1 (CD90) and c-kit (CD117). Of these, CD38 and HLA-Dr are probably the antigens most often used to describe

primitive haemopoietic cells. The more primitive $CD34^+$ cells do not express CD38 ($CD34^+CD38^-$) and these cells comprise about 1% of the $CD34^+$ population in BM and about 4% of the $CD34^+$ population from CB (Mayani 1998, Terstappen 1991). It should be noted that some investigators (Bender, *et al* 1994, De Bruyn, *et al* 1995, Payne, *et al* 1995) have reported much higher levels of $CD34^+CD38^-$ subsets in CB and BM, although the general pattern is the same, with $CD34^+CD38^-$ population of CB being significantly greater than that of BM. The evaluation of CD34 expression and that of its subsets is not standardised and differing cell manipulations can make direct comparisons of the findings of different laboratories very difficult.

Whereas it has been demonstrated that the $CD34^+HLA\text{-}Dr^-$ subset of BM is enriched for long-term culture initiating cells (LTC-IC) and is thus more primitive than its $CD34^+HLA\text{-}Dr^+$ counterpart, the opposite appears true in CB. Traycoff *et al.* found that the CB cells capable of producing long-term cord blood cultures (LTCBC, the equivalent of BM LTC-IC) resided in the $CD34^+HLA\text{-}Dr^+$ fraction (Payne, *et al* 1995, Traycoff 1994). This contrast in functionality of HLA-Dr populations from CB and BM compromises the usefulness of comparing the relative distribution of this antigen on $CD34^+$ cells from different sources as subsets with similar phenotypes possess quite disparate proliferative potentials.

The function of Thy-1 (CD90) is unknown, although it may act to inhibit cell proliferation (Mayani 1994). Baum *et al.* (Baum 1992) have shown that the cells capable of reconstituting human haemopoiesis in SCID mice are $CD34^+Thy\text{-}1^+$. Approximately 20-30% of bright $CD34^+$ cells co-express Thy-1 in both BM and CB (Craig, *et al* 1993, Mayani 1994). It has also been shown in BM and mPB that most of the $CD34^+Thy\text{-}1^+$ cells are quiescent and conversely that the majority of $CD34^+$ that are cycling (in S/G2/M) have low levels of Thy-1 (Uchida 1997).

The c-kit antigen (CD117) is a transmembrane receptor with tyrosine kinase activity and is expressed on haemopoietic progenitor cells. The ligand for the c-kit antigen is the early acting cytokine stem cell factor (SCF), also known as steel factor or mast cell growth factor. Approximately 60% of CB $CD34^+$ cells co-express c-kit (De Bruyn, *et al* 2000, de Wynter, *et al* 1999 quotes Reisbach, Mayani 1998 quotes Anderson) as do $CD34^+$ cells from adult BM (De Bruyn, *et al* 2000, Steen, *et al* 2000), whereas about 43% of $CD34^+$ cells from mPB co-express CD117 (De Bruyn, *et al* 2000). In an analysis of the CD38 subsets of $CD34^+$ cells, de Bruyn *et al* (De Bruyn, *et al* 2000)

found that the proportion of cells co-expressing CD117 was less in the $CD34^+CD38^-$ subset compared to the corresponding $CD34^+CD38^+$ subset for CB and mPB. Similarly, the density of SCF receptors was lower on the more primitive $CD34^+CD38^-$ cells compared to their $CD34^+CD38^+$ counterparts in CB, BM and mPB. In addition the receptor density on the immature $CD34^+CD38^-$ cells from mPB was lower than the same population from CB or BM.

CELL CYCLE STATUS

In general, primitive progenitors are more quiescent than committed progenitors from the same tissue but there are also differences in the cycling status of $CD34^+$ cells from CB, BM and mPB.

It has been shown that $CD34^+$ cells from CB and mPB are more quiescent than BM $CD34^+$ cells with a greater proportion of cells in G0/G1, the 'non-cycling' part of the cell cycle, (>94% for CB and >99% for mPB and ~88% for BM) (De Bruyn*, et al* 2000, Gothot*, et al* 1997, Ladd 1997, Lucotti*, et al* 2000). Table 1 summarises a selection of published studies on the cell cycle status of phenotypically defined HSC from CB, BM and mPB. Several of the studies show that $CD34^+$ cells from mPB are almost completely quiescent and this phenomenon has been confirmed in animal studies (Donahue*, et al* 1996, Roberts and Metcalf 1995, Uchida 1997). The quiescence of mPB $CD34^+$ cells seems incongruous given that mobilisation treatments cause extensive proliferation of HSC and in mouse models, increased cycling of HSC in the BM and spleen. Not only are $CD34^+$ from mPB more quiescent than those from BM, a greater proportion of them are deeply dormant (in G0) with very low levels of RNA. Interestingly, in the G0 and G1 $CD34^+$ cells from mPB there is only a two-fold difference in the frequency of LTC-IC. The equivalent populations from BM show a nine-fold difference with most of the LTC-IC residing in the G0 population (Gothot*, et al* 1997).

Why mPB HSC possess these attributes is unclear and may be related to an altered stromal environment following mobilisation therapy or a signal that induces dormancy in mobilised stem cells (Uchida 1997) or perhaps inherent differences in the ability of cells in distinct parts of the cell cycle to mobilise into the bloodstream.

Although the $CD34^+$ population of CB has a greater proportion of cells in G0/G1 compared to BM, the CB cells are able to cycle out of G0/G1 more quickly than BM cells (Traycoff, *et al* 1994). This effect was evident in cultures containing the early acting cytokine SCF and in cultures containing CB plasma. When interleukin-3 (IL-3) or SCF and IL-3 were added to BM cells in culture their exit from G0/G1 was comparable to that of CB.

Table 1.1 Summary of some of the published data relating to HSC and the cell cycle

	Approximate percentage of cells in G0/G1			
Phenotype	**CB**	**BM**	**mPB**	**Author (year)**
$CD34^+$	96			Lucotti *et al*, (2000)
$CD34^+$		89		Ladd *et al*, (1997)
$CD34^+$		87	99	Gothot *et al*, (1997)
$CD34^+$ $CD34^+CD38^+$ $CD34^+CD38^-$	94 77 98	88 80 94	98 82 99	de Bruyn *et al*, (2000)
$CD34^+CD38^+$ $CD34^+CD38^-$		85 96		Reems and Torok-Storb, (1995)
$CD34^+HLA\text{-}Dr^+$ $CD34^+HLA\text{-}Dr^-$	97 97	89 92		Traycoff *et al*, (1994)
$CD34^+HLA\text{-}Dr^-$		75		Leemhuis *et al*, (1996)
$CD34^+Lin^-$ $CD34^+Lin^{lo/-}$ $CD34^+Thy^+Lin^-$ $CD34^+Thy^-Lin^-$		89 78 93 93	 >99 >99	Uchida *et al*, (1997)

CHEMOKINE RECEPTORS ON HAEMOPOIETIC PROGENITORS

Chemokines play a role in diverse cell functions, including the homing, proliferation and differentiation of primitive haemopoietic precursors. In a study by Rosu-Myles *et al* (Rosu-Myles, *et al* 2000), it was found that the chemokine receptor profile of $CD34^{+}CD38^{-}Lin^{-}$ and $CD34^{-}CD38^{-}Lin^{-}$ sub-populations derived from CB was significantly different to that from similar populations derived from foetal blood (FB), adult BM and G-CSF mPB. These stringently purified populations from FB, BM and mPB did not express detectable levels of the chemokine receptors CXCR1, CCR5 and CXCR2. In contrast, the same cell populations in CB expressed these receptors on 5-19% of cells.

The CXCR4 receptor was detectable on the $CD34^{+}CD38^{-}Lin^{-}$ and $CD34^{-}CD38^{-}Lin$ sub-populations from all sources. The percentage of cells expressing this receptor in FB, BM or mPB was 6-17% with the exception of the $CD34^{-}CD38^{-}Lin^{-}$ population of mPB, which expressed CXCR4 on about 45% of cells. However, within the equivalent populations from CB, the receptor was expressed at levels of 82% in the $CD34^{+}CD38^{-}Lin^{-}$ sub-fraction and 63% in the $CD34^{-}CD38^{-}Lin^{-}$ sub-fraction. CB cells also expressed CCR7 on the $CD34^{+}CD38^{-}CD7^{-}Lin^{-}$ and $CD34^{-}CD38^{-}CD7^{-}Lin^{-}$ populations, while CCR8 and Bonzo were detected on the $CD34^{-}CD38^{-}CD7^{-}Lin^{-}$ cells only and BOB was not detected in either primitive fraction of HSC.

These findings suggested two things to the authors. Firstly, that CXCR4 signaling was more prevalent in early and late haemopoietic sources of FB, BM and mPB; and secondly, that the distinct chemokine profile ($CXR1^{+}$, $CCR5^{+}$, $CXR2^{+}$ and $CXC4^{+++}$) of highly enriched CB progenitors may be indicative of an expanded role of chemokine signaling in CB.

The results for FB were very similar to those for BM and mPB, whereas one might expect that the chemokine receptor profile of the foetus might more closely match that of CB. Furthermore, one might predict a logical hierarchy of chemokine receptor expression from the 'youngest' source of HPC to the 'oldest'. It is worth noting that the source of FB used was from 16-22 week abortus. It is well documented that at this stage of foetal development the main sites of haemopoiesis are in the liver and spleen. Examination of foetal HPC from these tissues may have yielded different, possibly more relevant, results.

Chemokine receptor expression on mature human blood types is well documented. The same is not true for chemokine receptor expression on HPC. Whereas there is a significant body of work on the clonogenic properties of haemopoietic cells (defined by phenotypes largely restricted to primitive progenitors e.g. $CD34^{+}lin^{-}rho^{lo/-}$ etc), there is a dearth of literature on the clonogenic characteristics of primitive progenitors based on their chemokine receptor expression. Given the role of chemokines in migratory and proliferative signals, it is reasonable to assume that such investigations may help in the analysis of clonogenicity and proliferative potential of HPC from different sources.

CORD BLOOD PLASMA

Several lines of evidence indicate that CB plasma contains factors that enhance proliferation of primitive progenitors and that CB cells are intrinsically pre-programmed to be more responsive to these and other cytokines than BM mononuclear cells.

Carow *et al* (Carow 1993) have reported an increase in the size of primary CB CFU-GEMM (colony forming unit- for granulocytes, erythroid, monocytes and megakaryocytes) grown in CB plasma plus erythropoietin (Epo) and SCF (mean $\pm$ SD: 202,925 $\pm$ 21,460 cells) compared to the size of colonies grown with these cytokines in either adult PB plasma (63,977 $\pm$ 7,410 cells) or foetal bovine serum (FBS) (93,920 $\pm$ 9,433 cells). The ability of primary colonies, picked and dispersed as a single cell suspension in supportive media, to give rise to secondary colonies is referred to as the re-plating potential and has been used as an estimate of self-renewal capacity. Compared to PB plasma or FBS, CB plasma also increased the re-plating potential of CB cells, the number of secondary colonies grown was greatest when CB plasma was present in the primary and secondary cultures and CB plasma enhanced the production of secondary CFU-GEMM, rather than the more mature CFU-GM (colony forming unit for granulocytes and macrophages) or BFU-E (burst forming unit-erythroid). None of the early acting cytokines IL-1, IL-3, IL-6, IL-11, G-CSF, GM-CSF and FBS, nor endothelial cell conditioned media (ECCM)±FBS could re-produce the CFU-GEMM replating potential of CB plasma even though ECCM±FBS produced ~2-fold more primary CFU-GEMM.

CB plasma also exerted an effect on BM cells, increasing the re-plating potential, increasing the number of secondary colonies grown and enhancing the growth of

secondary CFU-GEMM over that of CFU-GM and BFU-E (Carow 1993). Whereas CB plasma (compared to PB plasma or FBS) drastically increased the size of primary CFU-GEMM derived from CB cells (~202,925 cells) it did not have an effect on the size of primary CFU-GEMM grown from BM cells (~29,960 $\pm$ 5,045 cells/primary CFU-GEMM grown in CB plasma, Epo, SCF v 28,407 $\pm$ 6,115 cells/primary CFU-GEMM grown in FBS, Epo, SCF). Thus although factor/s in CB plasma effected a change on the growth characteristics of CB and BM MNC, the CB cells clearly also had a more profound response than BM MNC.

Traycoff *et al* (Traycoff*, et al* 1994) demonstrated the differential ability of CB plasma to increase the cycling of CB but not BM progenitor cells. They found that CB 34^{+}HLA-Dr^{+} cells exit G0/G1 more rapidly when cultured for 36-48 hours in 10% CB plasma plus either SCF or IL-3 (55% cells in G0/G1) than when in serum-free media with SCF of IL-3 alone (83% cells in G0/G1). Combining SCF with IL-3 in serum-free media produced a response similar to that achieved when CB plasma was added to the culture, with about 50% of cells in G0/G1 by 48 hours of culture. In contrast, addition of CB plasma to the culture of BM 34^{+}HLA-Dr^{+} cells made no appreciable difference to the rate of exit of cells from G0/G1. This data supports the notion that cord blood $CD34^{+}$ cells are more reactive to a factor/s in CB plasma than are $CD34^{+}$ cells from BM.

Ruggieri *et al* (Ruggieri*, et al* 1994) compared the effects of CB plasma, adult PB plasma and FBS on CB $CD34^{+}$ cells, with and without exogenous growth factors, in both suspension culture and clonogenic assays. The addition of any plasma alone to a suspension of $CD34^{+}$ cells was insufficient to maintain survival, let alone expansion, with viability falling to about 20-36% by day 7. The addition of any of the plasmas with SCF or PIXY (a GM-CSF/IL-3 fusion protein) or, SCF and PIXY, or IL-3, IL-6 and IL-1 combined, caused a net increase in cell number for up to 28 days. In all cases, plasma in combination with SCF and PIXY caused the greatest expansion of total nucleated cell number and the effect of CB plasma was greater than PB plasma, which was better than FBS. Cell proliferation was associated with differentiation, mostly into the late myeloid lineage. Cultures containing CB plasma with SCF and PIXY also supported the production of erythroblasts, a cell type that was virtually unseen in the other cultures.

In cultures of plasma plus SCF and PIXY the absolute number of $CD34^+$ cells peaked at day 7 and the percentage of $CD34^+$ cells in all conditions was similar (~17%). However as the greatest fold-increase in cell number occurred in the cultures containing CB plasma, SCF and PIXY these conditions also yielded the greatest number of $CD34^+$ cells. By day 14 the percent of $CD34^+$ cells in any culture was less than 1%.

In clonogenic assays the greatest raw (non-cumulative) increase in primitive progenitors was evident in the mean 11-fold expansion of CFU-GEMM colonies from cells cultured in suspension for 7 days in the presence of CB plasma, SCF and PIXY. This was better than the combination of PB plasma, SCF and PIXY, which produced on average a 6-fold expansion in CFU-GEMM colonies. Both plasmas showed at least a 5-fold enhancement over that of FBS, SCF and PIXY. However, the enhancing effects of CB plasma over PB plasma were not evident in CFU-GEMM production from suspension cultures at later time-points (day 14, 21 or 28), nor at any time-point for other combinations of cytokines (plasma + IL-3, IL-6 and IL-1; plasma + PIXY alone; plasma + SCF alone). Furthermore, PB plasma was at least as good as CB plasma at expanding the number of CFU-GM colonies.

The re-plating potential of CFU-GEMM colonies expanded by suspension culture in CB plasma, SCF and PIXY was compared to that of un-expanded CFU-GEMM colonies to assess the maintenance of self-renewing cells in the colonies. Ruggieri *et al* (Ruggieri, *et al* 1994) found that the re-plating potential was greatest in those colonies that had not been expanded by liquid culture. Of the colonies that had undergone expansion in liquid culture, those expanded for 7 days showed some re-plating potential, while colonies that had undergone expansion for 14 or 21 days in liquid culture did not grow secondary colonies. This would suggest that expansion of colonies was associated with lineage commitment rather than maintenance of self-renewal.

Finally with respect to expansion of primitive progenitors it has been noted that serum is required for the initiation of expansion conditions and that in this respect CB plasma was at least as good as, if not superior to, FBS in supporting the expansion of CB $CD34^+$ cells (Gilmore, *et al* 2000).

CLONOGENICITY AND *EX VIVO* EXPANSION

Results of clonogenic assays indicate that the more primitive ontogenic sources of HSC contain the highest proportion of primitive progenitors (Holyoake, *et al* 1999, Michejda 1996, Wu, *et al* 1999). It has also been shown that the source of growth factors used (eg conditioned media or recombinant cytokines) in colony forming assays can have a dramatic effect on the both the detection of and fold-increase in colonies grown (Broxmeyer 1992).

Compared to BM, mononuclear cells from CB produce similar numbers of CFU-GM, BFU-E, CFU-Mk and CFU-GEMM per 10^5 cells plated (De Bruyn, *et al* 1995, DeBruyn, *et al* 1993, Hows 1992, Wu, *et al* 1999) although some investigators have found greater numbers of CFU-GM and BFU-E in CB and mPB compared to BM (Broxmeyer 1992, Lansdorp, *et al* 1993, Mayani 1998, Steen, *et al* 1994). Additionally, several reports indicate that the $CD34^+$ fraction of CB has a greater concentration of CFU-GM and BFU-E than that of BM, that the colonies produced are larger, (Kim, *et al* 1999, Steen, *et al* 1994, Theilgaard-Monch, *et al* 1999, van den Oudenrijn, *et al* 2000) as are the primary CFU-GEMM (Carow 1993) indicating a greater proliferative potential in CB $CD34^+$ cells. Echoing these results, it has also been reported that the $CD34^+CD38^-$ subset of CB exhibits greater cloning efficiency than the equivalent cells from BM, that the onset of proliferation of the CB cells is earlier than that of the BM cells and that the colonies produced contain seven-fold more cells (Hao, *et al* 1995). Of more relevance, it has been shown that CB contains a greater proportion of very primitive precursors as measured by high proliferative potential-colony forming cells[3] (HPP-CFC) and secondary re-plating potential (Broxmeyer 1992, Carow, *et al* 1991, Carow 1993, Kim, *et al* 1999, Lu, *et al* 1993), LTC-IC (Hows 1992) and cobblestone area-forming cells[4] (CAFC) (Pettengell, *et al* 1994, Theilgaard-Monch, *et al* 1999). Although mPB has a greater concentration of CAFC than does CB (Pettengell, *et al* 1994) transplantation models in mouse have shown that CB $CD34^+$ cells have ~100-fold higher engraftment potential over $CD34^+$ cells from mPB with persistence of human cells in the BM and spleen of animals up to 20 weeks post-transplantation (Leung, *et al*

[3] The HPP-CFC describes a cell capable of producing macroscopic colonies of macrophages either with or without stroma. This cell types is relatively quiescent and has some replating potential (reviewed in Gordon, M.Y. (1993) Human Haemopoietic Stem Cell Assays. *Blood Reviews,*, **7,** 190-197.).

[4] The CAFC describes a cell type that produces stroma-adherent colonies of cells that have the appearance of cobblestones. These colonies have limited self renewal and replating potential giving rise to more mature CFU. (reviewed in Ibid.).

1999). Similarly it has been shown that CB contains more SCID repopulating cells[5] than BM or mPB (Holyoake, *et al* 1999, Wang 1997) and more competitive repopulating units than does BM (Holyoake, *et al* 1999).

In addition to these parameters, it has been shown that CB progenitors possess an equivalent or superior capacity for *in vitro* expansion compared to BM (Lansdorp, *et al* 1993, Lewis and Verfaillie 2000, Ng, *et al* 2002, Traycoff 1995, van den Oudenrijn, *et al* 2000) and that both BM and CB have superior expansion potential to that of mPB (Benboubker, *et al* 2002, Gilmore, *et al* 2000, Ng, *et al* 2002). This capacity has been demonstrated in the fold-increase in total cell number, $CD34^+$ cells, $CD34^+CD38^-$ cells, LTHC-IC, natural killer–initiating cell (NK-IC) and production of megakaryocyte progenitors.

GENES EXPRESSION IN HAEMOPOIETIC PROGENITORS

Already there exists a large body of work comparing stem cells derived from CB, BM and mPB and quantitating differences using parameters such as phenotype, cell cycle status and capacity for proliferation and differentiation under myriad conditions. Ultimately these differences will be due to the way in which genes are regulated in the stem cell population throughout the life cycle. Efforts to elucidate differences in gene expression between primitive haemopoietic populations from various sources should help to identify the mechanisms by which stem cells are triggered into proliferation, differentiation and self-renewal. Several strategies exist to address the issue of global gene profiles specific for HSC and include comparative molecular analyses to identify conserved mechanisms among diverse developmental systems; generation of expression profiles of at least partially purified HSC with or without subtractive hybridisation of mature blood cells; and screening for changes in gene expression in identical populations of HSC subjected to different stimuli (such as cytokine exposure to promote proliferation versus no exposure).

[5] The SCID repopulating cell (SRC) describes a cell that can engraft and restore haemopoiesis when injected into a nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse. As an *in vivo* functional assay it provides a gold standard by which candidate HSC can truly be said to exhibit the hallmark features of self-renewal and multipotency Bhatia, M., Wang, J.C., Kapp, U., Bonnet, D. & Dick, J.E. (1997) Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A,* **94,** 5320-5325, Wang, J.D., M; Dick, JE (1997) Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. *Blood,* **89,** 3919 - 3924..

The underlying premise of comparative analyses is that fundamental regulatory mechanisms will be shared by stem cells from different tissues (Lemischka 1999, Lemischka 2001). Implicit to this notion is that comparisons of stem cells from diverse tissues (such as human embryonic stem cells (ESC), neural stem cells (NSC) and HSC) as well as cross-species comparisons (eg mouse versus man versus baboon) will reveal basic molecular programs essential for the shared features of "stemness", most notably, proliferation and self-renewal. Criticisms of these assumptions are that:

- uncommitted stem cells may exhibit low level expression of genes associated with mature cell types, thus these genes would not be unique to a stem cell profile (Lemischka 1999);
- problems exist with defining a suitable phenotype (quite apart from achieving suitable purities) and phenotypically similar cells from different sources may have quite different biological properties (Lemischka 1999) (eg $CD34^{+}HLA\text{-}Dr^{+}$ CB cells are biologically closer to $CD34^{+}$ $HLA\text{-}Dr^{-}$ BM cells than to the phenotypically similar $CD34^{+}HLA\text{-}Dr^{+}$ BM cell) (Traycoff 1994);
- the expression of stem cell genes may be only relatively elevated compared to differentiated cells (Fortunel, *et al* 2003);
- stem cells from diverse origins "may use different gene networks to achieve self-renewal and multipotency" (Fortunel, *et al* 2003); and
- the transience of expression of essential gene programs may make them "elusive" to capture thus hampering comparisons of two stem cell sets (Evsikov and Solter 2003, Fortunel, *et al* 2003).

Some of these criticisms apply equally to the other strategies commonly used to generate global gene profiles of stem cells. Obviously the phenotype selected to define a candidate stem cell for purification and molecular analysis is of vital importance to interpreting the data that is generated. For instance, transplantable HSC capable of engraftment can be purified from primary haematopoietic tissue based on phenotype, however the same is not true of ESC which are derived from cultured cell lines and may have altered genetic programs (Ramalhos-Santos M. 2003). Furthermore, even a highly purified HSC population is functionally heterogeneous (Lemischka 1999) and so presumably would express a promiscuous gene profile reflective of the relative maturities of the purified cells. In a further complication, if uncommitted stem cells

express genes associated with mature cell types, then strategies relying on subtractive hybridisation of libraries generated from mature cell types will inevitably result in some gene combinations being omitted inappropriately from a stem cell global gene profile. On the other hand exhaustive sequencing of stem cell libraries would require an input of time and resources beyond the capacity of most laboratories and indeed would necessitate a collaborative effort not unlike that required for the Human Genome Project. Because of these considerable complexities there is no single strategy or methodological protocol to compensate for, or satisfy all, criticisms. Ultimately approaching the issue from several directions and utilising a wide variety of methodological strategies will elucidate the molecular basis of some if not all stem cell programs.

DNA array technologies represent powerful tools for the simultaneous analysis of the patterns of gene expression of thousands of genes. The high throughput nature of these techniques requires sophisticated robotics and microgram quantities of starting material to produce customised high density samples immobilised onto suitable substrate. Alternatively commercially produced arrays are available, at a cost, which can be probed with labelled cDNA derived from the cells of interest. Both require hybridisation of a labelled probe, equipment to detect the hybridisation signal and appropriate software to quantitate the image analysis. DNA array technologies have been used successfully to identify genes in adult murine HSC (de Haan, *et al* 2002, Park, *et al* 2002), murine foetal liver stem cells (Phillips, *et al* 2000), to compare gene expression in human $CD34^+$ HSC from mPB and adult BM (Steidl, *et al* 2003) and cross-species analyses between human and baboon $CD34^+$ cells (Gomes, *et al* 2001), to compare gene expression between embryonic stem cells (ESC), neural stem cells (NSC) and HSC of mouse (Ramalho-Santos, *et al* 2002), to compare gene expression profiles of murine foetal, murine adult and human HSC and to compare these with that of murine ESC and NSC (Ivanova, *et al* 2002).

Differential display also is a technique employed to compare the pattern of gene expression between different cell populations. Although it lacks the power of array technologies and is somewhat labour-intensive, differential display does not require sophisticated robotic or imaging systems, can accommodate very small quantities of RNA (nanogram amounts) making it ideal for the investigation of gene expression in small populations of cells and thus presents a relatively cheap alternative for gene

discovery on a small scale. It has been used to identify genes differentially expressed between human $CD34^{+}38^{hi}$ and $CD34^{+}38^{lo}$ cells (Graf 1995) and in this laboratory has been used profitably to compare gene expression in human BM and CB HSC (Gregorio-King, *et al* 2001, Gregorio-King, *et al* 2002).

The gene expression profiles of $CD34^{+}$ cells from CB (Mao, *et al* 1998), BM (Gu, *et al* 2000, Zhang, *et al* 2000) and leukaemic origins (Gu, *et al* 2000) have also been evaluated using PCR based protocols to construct libraries which have been cloned, partially sequenced to produce expressed sequence tags (EST) and then analysed against public databases to identify known and reveal novel gene products. Serial analysis of gene expression has been used to identify and quantitate gene expression in human $CD34^{+}$ HSC (Zhou, *et al* 2001).

A library of genes derived from CB CD34+ cells consisted of 9866 ESTs, of which ~74% were homologous to known genes in the Genbank database or to ESTs in a public database, ~14% were undescribed and the remaining were mitochondrial DNA, ribosomal RNA or repetitive sequences (Mao, *et al* 1998). Of the known genes (855 in total), ~26% were associated with gene/protein expression, 18% with metabolism, 19% were unclassified and the remaining were associated with hemopoiesis (8%), cell division (8%), cell signalling (10%), cell structure/defence (5%) and cell/organism defence (5%). A study of BM CD34+ cells identified 622 known genes, 522 genes with EST homology and 486 novel sequences (Gu, *et al* 2000). The genes associated with hemopoiesis were categorised into differentiation antigens, cytokines and chemokines (including Prothymosin α), receptor and signaling, transcription regulation, lymphoid, erythroid, granulocytic or megakaryocytic differentiation and leukemogenesis and "others". A comparison of the BM and CB libraries revealed that ~57% of the known genes were expressed in the $CD34^{+}$ cells from both sources. Noted differences in expression included the respiratory chain component COXII in CB and COXVIc in BM; high expression of HLA-Dr antigens in CB but not BM; the abundance of cytokines thymosin β4 and IL-8 in BM but not CB; and the high expression of γ and β globin in CB but not BM, which accords with the switch in globin synthesis from $\alpha 2\gamma 2$ in the foetus to $\alpha 2\beta 2$ in adults (Gu, *et al* 2000). A study comparing the gene expression profiles of BM CD34+ and mPB found that genes involved in cell cycle regulation, DNA synthesis and replication were more highly expressed in BM, consistent with their higher cycling activity compared to mPB (Steidl, *et al* 2003). In contrast genes

associated with apoptosis were more highly expressed in mPB. Finally, an analysis of gene expression in BM $CD34^+$ cells using SAGE generated over 100,000 SAGE tags (Zhou, *et al* 2001). The majority of these (76%) were single copy number, approximately 21% of tags had 2-9 copies and only 0.2% had in excess of 100 copies. Of the ~42,000 unique SAGE tags about half matched ESTs (including 3,687 known genes) and the remaining 20,854 tags were novel genes which tended to be expressed at lower levels than the known. In spite of the low specificity of the SAGE tags (one third of the tags matched more than one sequence located in different UniGene clusters) it was evident that known genes accounted for only 9% of the total unique SAGE tags and about 18% of the matched tags, indicating that the majority of genes expressed in haemopoietic progenitor cells have not been identified or studied (Zhou, *et al* 2001).

These strategies have produced abundant information regarding the global gene and comparative profiles of haemopoietic cells from human, monkey and mouse. The cataloguing of the data is an immense task and involves categorising sequences according to their homology to ESTs in public databases and if known, by protein type (or family). Several public databases now exist which can be "mined" for information and used to direct research projects.

No strategy or technique is flawless and the selection of which cell type to analyse and which method to use is often restricted by pragmatic considerations of available resources (such as access to raw materials, exploiting established laboratory protocols, cost) as it is driven by legitimate and reasoned analysis and questioning of biological systems.

AIMS OF THIS STUDY

Although a heterogeneous cell type, the $CD34^+$ population is of great importance because of its clinical utility in stem cell transplantation. It is generally accepted that cellular product for transplantation should undergo minimal manipulation and often total mononuclear cells are used rather than purified populations of stem cells for the restoration of normal haemopoiesis. This is due to the significant cell losses that occur as a consequence of many purification techniques and, more recently, to the implementation of codified practices of Good Manufacturing Practice (GMP). However, in all cases it is the $CD34^+$ concentration that is used to assess the suitability

of a product for infusion. The repertoire of conditions which can be treated with transplanted cells is gradually increasing and include the restoration of normal haemopoiesis following myelo-ablative treatment for some haematological malignancies, the amelioration of acute neutropaenia following dose escalation of chemotherapy for non-haematological conditions and gene transfer protocols for inherited disorders of lympho-haemopoiesis. Comparative studies of $CD34^+$ cells from different ontological sources have revealed striking differences in the proliferative capabilities, responsiveness to cytokine stimulation and content of very primitive subsets as measured by *in vitro* and *in vivo* techniques. Therefore we chose to use the $CD34^+$ phenotype as the basis for a comparative study of gene expression.

In response to the literature detailing differences in the way in which $CD34^+$ cells from CB and BM respond to factor/s in CB plasma we wished to establish a protocol that might provide insights into the molecular mechanisms of the amplified proliferative response of CB cells. To achieve these outcomes we had established protocols for the purification and culture of $CD34^+$ cells from CB and BM, differential display PCR for gene comparison/discovery and real time PCR for confirmation of differential expression.

In the current study we used these techniques to compare gene expression in $CD34^+$ purified HSC from CB and BM with that of $CD34^-$ cells. We cultured purified cells for 24 hours in either 10% pooled CB serum or pooled adult serum to examine the effect/s of exposure of cells to cord and adult serum. The aims of the project were:

1. To identify genes differentially expressed between $CD34^+$ and $CD34^-$ cells.
2. To identify genes with differential expression between CB and BM $CD34^+$ cells.
3. To identify genes with differential expression in CB and BM $CD34^+$ cells following exposure to cord or adult sera.
4. To investigate possible functions of the protein/s encoded by the genes identified.
5. To investigate regulation of identified genes in physiologically relevant assays.

CHAPTER 2

MATERIALS AND METHODS

ETHICS APPROVAL

All peripheral blood, cord blood and bone marrow samples were collected from donors at Geelong and St John of God hospitals with appropriate institutional ethics approval (Barwon Health ethics approval no. 97/14 and St John of God ethics approval no. UCB project 139).

SAMPLE COLLECTION

Cord Blood

CB samples were collected following uncomplicated vaginal or caesarean section deliveries. The umbilical cord was clamped and cut and blood from the placenta drained into 50ml sterile tubes containing 200 units of heparin (David Bull Laboratories, Australia) in 5ml of alpha-minimal essential media (α-MEM) (Thermo Trace, Australia). Alternatively, following delivery of the placenta the umbilical cord was clamped and cut and the main umbilical vein venesected and blood drained via gravity into a blood collection bag containing 35 ml of citrate phosphate dextrose (CPD) anticoagulant (Baxter, Mexico).

Cord Serum

Serum from cord blood was collected using the same method as for cord blood collection with the exception that blood was collected into sterile tubes without anticoagulant. Blood was allowed to coagulate fully, the tubes were centrifuged and the serum decanted and aliquoted into sterile freeze vials. Serum was stored at –80^{o}C until needed. For use, a single vial of serum from 5-10 donors was thawed and pooled and used in experiments immediately.

Bone Marrow

BM cells were collected as cell scrapings from the femoral shaft at hip-replacement surgery. Scrapings were suctioned into 50ml sterile tubes containing 200 units of heparin in 20ml of α-MEM. To prepare the samples for density gradient separation the tubes were shaken vigorously to dislodge cells from the bony fragments then the contents allowed to settle for a few minutes. After the larger bone fragments had settled, the cellular supernatant was removed to a sterile collection tube and the BM tube refilled with phosphate buffered saline (PBS) (Sigma-Aldrich, USA) containing 0.5% bovine serum albumin (BSA) (Sigma Aldrich, USA) and 2mM ethylene diamine tetra-acetic acid (EDTA) (Sigma Aldrich, USA). This process continued with pooling of the supernatant fractions until most of the cellular content had been collected (the bony fragments became progressively whiter and the supernatant clearer).

The pooled cellular fractions were centrifuged (480g, 10mins, 18-20^{o}C), supernatant removed and the cell pellets pooled and resuspended in ~40ml of PBS/ BSA/ EDTA.

Adult Serum

Blood from ten healthy volunteers was collected into sterile tubes without anticoagulant. The blood was allowed to coagulate, the tubes were centrifuged and the serum decanted, pooled and aliquoted into freeze vials. Serum was stored at –80^{o}C until needed.

SAMPLE PROCESSING

Density gradient separation of mononuclear cells

CB and BM samples were diluted in twice their volume of sterile PBS, layered onto an appropriate volume of Ficoll-Hypaque (Amersham Biosciences, Sweden) and centrifuged for 25 mins at 440g, 18-20^{o}C as per the manufacturer's recommendations. Following centrifugation, MNC at the ficoll-plasma interface were collected into sterile tubes and washed twice in at least double their volume of PBS (10 mins, 480g, 18-20^{o}C).

Red Cell Lysis

Following density enrichment of MNC, contaminating red blood cells (RBC) were removed by resuspending the cells in 0.83% ammonium chloride lysing solution. Cells were incubated at room temperature (RT), 5-10 min, then centrifuged (5 min, 480g, 18-20^{o}C) and the lyse solution removed. Cells were washed once in PBS/ BSA/ EDTA prior to further processing.

Immunomagnetic separation of CD34^{+} cells

Magnetic-activated cell selection (MACS) was used to isolate CD34^{+} cells from CB and BM MNC. Following density separation and RBC lysis, MNC were resuspended in PBS/BSA/EDTA at a concentration of 10^{8} cells per 300µl in accordance with the manufacturer's recommendations (Miltenyi Biotec, Germany). An appropriate volume of FcR blocking reagent and colloidal microbeads conjugated to monoclonal mouse anti-human CD34 antibody (clone QBEND/10) were added to the cells. Cells were incubated (30 mins, 4^{o}C) then washed, resuspended in PBS/BSA/EDTA solution and applied to a column (MS^{+} column and miniMACS magnet for 2 x 10^{8} cells or VS^{+} column and varioMACS magnet for > 2 x 10^{8} cells). Unlabelled cells (CD34^{-} fraction) were washed through the column thoroughly by repeated applications of PBS/BSA/EDTA. The column was removed from the magnetic field and the immunomagnetically labelled CD34^{+} cells eluted with 2-5ml PBS/BSA/EDTA or α-MEM media. Viability and cell number of the CD34 enriched and depleted populations were assessed using trypan blue (TB) (Sigma Aldrich, USA) exclusion and manual cell counts.

Immunomagnetic separation of CD14^{+} cells

MACS was used to isolate CD14^{+} cells from CB and PB MNC. Following density separation with or without RBC lysis, MNC were resuspended in PBS/BSA/EDTA at a concentration of 10^{7} cells per 80µl, in accordance with the manufacturer's recommendations (Miltenyi Biotec, Germany). An appropriate volume of FcR blocking reagent and colloidal microbeads conjugated to monoclonal mouse anti-human CD14 antibody (clone not specified; part no. 130-050-201) were added to the cells. Cells were

incubated at 4°C for 15min, washed, resuspended in PBS/BSA/EDTA solution and applied to a column (MS^+ column and miniMACS magnet for 2 x 10^8 cells or VS^+ column and varioMACS magnet for > 2 x 10^8 cells). Unlabelled cells ($CD14^-$ fraction) were washed through the column thoroughly by repeated applications of PBS/BSA/EDTA. Immunomagnetically labelled cells were eluted by removing the column from the magnetic field and flushing with 2-5ml PBS/BSA/EDTA or α-MEM media. Viability and cell number of the enriched and depleted populations were assessed using trypan blue exclusion and manual cell counts.

Immunomagnetic separation of lymphocyte subsets

Ficoll separated PB MNCs underwent three sequential MACS enrichments to isolate $CD3^+$ T cells (clone not specified; part no. 130 050 101), $CD19^+$ B cells (clone not specified; part no. 130 050 301) and $CD56^+$ natural killer cells (clone AF12-7H3.6.11; part no. 130 050 401) (Miltenyi Biotec, Germany). Cell preparation and incubation protocols were identical to that for the MACS isolation of $CD14^+$ cells. Viability and cell number of the enriched and depleted populations were assessed using trypan blue exclusion and manual cell counts. An aliquot of each enriched population and the final depleted MNC was reserved for FACS analysis of cell purities and the remaining cells processed for RNA extraction and gene expression studies.

CELL CULTURE TECHNIQUES

Culture of $CD34^+$ and $CD34^-$ populations with cord or adult sera for 24 hours

In initial experiments CD34 enriched ($CD34^+$) and depleted ($CD34^-$) cells were split into two fractions and cultured for 24 hr at 37oC in an atmosphere flushed with 5% CO_2 in air at a concentration of 5 x 10^5 cells/ml in α-MEM with 2mM L-glutamine (JRH Biosciences, USA) and either 10% pooled cord or pooled adult serum. After 24 hours incubation the cells were pelleted by centrifugation, the supernatant removed and the cells resuspended and homogenised in TRIzol (Life Technologies, USA). Samples were stored at -80°C until RNA extraction was performed.

Ex vivo proliferation and differentiation of $CD34^+$ cells cultured for 7 days

MACS CD34 enriched cells were cultured for 7 days (37^oC, humidified, 5% CO_2 in air), at a concentration of 5 x 10^5 cells/ml in α-MEM with 20% foetal calf serum (FCS) (CSL Biosciences, Australia), 2mM L-glutamine (JRH Biosciences, USA), 100 U/ml penicillin/streptomycin (P/S) (Sigma Aldrich, USA), supplemented with SCF, IL-1β, IL-3, IL-6 and G-CSF at the final concentrations indicated in Table 2.1 (media formulations). At the end of the culture period, viability and cell number were assessed using TB exclusion and manual cell counting. Cells were centrifuged, homogenized in TRIzol and stored at -80^oC until RNA extraction was performed

Ex vivo proliferation and differentiation of $CD34^+$ cells cultured for 24 hours with four different cytokine cocktails

MACS CD34 enriched cells were split into 5 equal aliquots and cultured for 24 hours (37^oC, humidified, 5% CO_2 in air), at a concentration of 1 x 10^5 cells/ml in a base media of α-MEM, FCS, L-glutamine and P/S supplemented with one of 5 cytokine cocktails: (i) no growth factors (media alone added to control culture); (ii) expansion cocktail :SCF, IL-3, TPO and F3L; (iii) expansion cocktail plus MIP-1α; (iv) expansion cocktail plus TGF-β; (v) differentiation cocktail: G-CSF, GM-CSF, IL-3. The final concentrations of base media components and cytokine supplements are listed in Table 2.1 (media formulations). At the end of the culture period cells were centrifuged, homogenized in TRIzol and stored at -80^oC until RNA extraction was performed.

Effect of single growth factors on PTA gene regulation in $CD34^+$ cells

MACS CD34 enriched cells were split into 5 equal aliquots and cultured for 2 hours (37^oC, humidified, 5% CO_2 in air), at a concentration of 1 x 10^5 cells/ml in a base media of α-MEM, FCS, L-glutamine and P/S supplemented with one of 4 single cytokines: SCF, IL-3, F3L or GM-CSF. Control cultures were supplemented with media alone. The final concentrations of base media components and cytokine supplements are listed in Table 2.1 (media formulations). At the end of the culture period cells were centrifuged, homogenized in TRIzol and stored at -80^oC until RNA extraction was performed.

Gene regulation by single growth factors (as above) was also assessed in a time-course where cells were cultured under identical conditions to those stated above with RNA taken at time points 0, 2, 6 and 10 hours.

PHA stimulation of PB MNC

Ficoll density gradient separation was used to purify MNC from the blood of ten normal donors. Cells were cultured (37^{o}C, humidified, 5% CO_2 in air) at a concentration of 10^6/ml for 4 days, in α-MEM supplemented with 10% FCS, 2mM L-glutamine, P/S and 2.5 µg/ml phytohemagglutin leukoagglutinin (PHA) (ICN Biomedicals, USA). At time-points day 0, 1, 2, 3 and 4 cells were assessed for viability, cell cycle and gene expression. The PHA had previously been titrated against the PB MNC of three normal donors in a 96 hour assay to determine the optimal concentration for stimulation.

Separation of PB MNC by plastic adherence

Ficoll separated PB MNC were re-suspended at a concentration of 10^6/ml in RPMI supplemented with 20% FCS and incubated (37^{o}C, humidified, 5% CO_2 in air) for 2 hours in tissue culture flasks. Following the incubation the non-adherent cells were removed, an aliquot reserved for FACS analysis and the remaining cells spun down and disrupted in TRIzol solution for gene studies. The morphology of the adherent fraction was established by inspecting the cells under an inverted microscope. Cells were washed twice with PBS, the supernatant thoroughly aspirated, then TRIzol added directly to the adherent layer to solubilise cells in preparation for total RNA extraction.

Colony forming assay

CB MNC (10^6 cells/culture) were suspended in 1 ml Methocult GF H4534 (StemCell Technologies, USA) containing recombinant human (rh) SCF at 50 ng/ml, rhGM-CSF at 10 ng/ml and rhIL-3 at 10 ng/ml in 35 mm dishes and incubated (37^{o}C, humidified, 5% CO_2 in air) for 10 days. At the end of the incubation period, the contents of each dish were washed in PBS and cells stained with TB for cell count and viability.

Table 2.1 Media formulations for culture of $CD34^+$ cells.

Component	Abbreviation	Final concentration	Source	
Alpha minimal essential media	α-MEM	N/A	Thermo-Trace, Australia	**BASE MEDIA**
Foetal Calf Serum	FCS	10 %	CSL Biosciences, Australia	
Penicillin and Streptomycin	P/S	100 U/ml	Sigma Aldrich, USA	
L-glutamine	L-glut	2 mM	JRH Biosciences, USA	
Stem Cell Factor	SCF	50 ng/ml	Chemicon, USA	
Interleukin-6	IL-6	10 ng/ml	Endogen, USA	
Interleukin-3	IL-3	50 ng/ml	Chemicon, USA	
Interleukin-1β	IL-1β	10 ng/ml	Endogen, USA	
Thrombopoietin	TPO	20 ng/ml	Chemicon, USA	
Flt 3 ligand	F3L	10 ng/ml	Chemicon, USA	
Macrophage inflammatory factor 1α	MIP-1α	50 ng/ml	Endogen, USA	
Transforming growth factor β	TGF-β	5 ng/ml	Chemicon, USA	
Granulocyte colony stimulating factor	G-CSF	10 ng/ml	AMRAD, Australia	
Granulocyte-macrophage colony stimulating factor	GM-CSF	10 ng/ml	Chemicon, USA	

Osteoclast formation assay

Pooled colonies from the colony forming assay were seeded (4 x 10^4 cells/well) into 96 well tissue culture plates containing slices of sperm whale dentine (4 x 4 x 0.1 mm). The cells were cultured in 200µl MEM containing 10% FCS, non-essential amino acids, 50U/ml penicillin, 50µg/ml streptomycin, 2mM L-glutamine, 25ng/ml macrophage-CSF (M-CSF) and 125ng/ml soluble receptor activator of nuclear factor kappaB ligand (sRANKL) either with or without Prothymosin alpha (PTA) (generous gift of Dr Milan Pesic, Biofactor, Germany). PTA was titrated at final concentrations of 0.1, 0.3, 1, 3 and 10µg/ml. The cultures were refreshed twice weekly by replacing additives in one half volume of media. At 14 days, the cells were fixed in 1% formalin and reacted for tartrate resistant acid phosphatase (TRACP) activity. The formation of osteoclasts (OC) was assessed by transmission light microscopy and quantified using microcomputer image analysis software (MCID-Imaging Research Inc, Canada).

Osteoclast resorption assay

Cells were removed from dentine slices by brief sonication in chloroform:methanol 2:1. Xylene-free black ink was applied to the resorbed surface of each slice, and residual ink was removed by wiping against absorbent paper. Resorption was assessed by transmission light microscopy, and the percentage area resorbed was quantified using MCID software.

FLOW CYTOMETRY PROTOCOLS

Direct staining of surface antigens

For direct labelling of surface antigens, 0.25 x 10^6 cells were resuspended in 50µl PBS and 5µl of appropriate fluorochrome conjugated monoclonal antibody test or isotype control. Cells were mixed gently and incubated at room temperature (RT), 10 min, in the dark, then washed in PBS (1500rpm, 5min). Supernatant was aspirated and cells resuspended in 200µl PBS or in PBS plus 5µl (0.25µg) of 7-amino-actinomycin D (7-AAD) (BD Pharmingen, USA). 7-AAD is a nucleic acid dye that can be used in place of propidium iodide (PI) for exclusion of nonviable cells in flow cytometric analyses. Its emission spectra exhibits minimal spectral overlap with fluorescein isothiocyanate

(FITC) or phycoerythrin (PE) fluorescence emissions. Prior to analysis samples were stored at 4°C, protected from the light. All analyses were performed within 1 hour.

Combinations of conjugated antibodies used were: for purity of $CD34^+$ MACS cells either α-CD45-FITC (clone T-200, Chemicon, USA.) or α-CD45-PerCP (clone 2D1, BD Biosciences, USA) and α-CD34-PE (clone HPCA-2, BD Biosciences, USA); for purity of $CD14^+$ MACS cells, either α-CD3-FITC (clone UCHT1, Cymbus Biotechnology, UK) + α-CD19-FITC (clone SJ25-C1, Cymbus Technology) / α-CD14-PE (clone MΦP9, BD Biosciences, USA) / α-CD45-PerCP or α-CD3-FITC / α-CD14-PE / 7-AAD; and for purity of MACS isolated lymphocyte subsets, α-CD45-PE was used in combination with α-CD3-FITC or α-CD19-FITC or α-CD56-FITC (clone MEM188, Cymbus Biotechnology, UK), as appropriate. Equivalent numbers of cells labelled with α-IgG_1-FITC (CBL 600F, Cymbus Biotechnology, UK) and α-IgG_1-PE (clone X40, BD Biosciences, USA) were used as negative controls for the analysis.

Affinity crosslinking and indirect staining of surface antigens with biotinylated PTA

Cells (~10^6) were resuspended in PBS and 20 μg biotinylated PTA (1mg/ml) and incubated for 30 min at RT. Control tubes where incubated with carrier (PBS). Where crosslinking was performed, the agent used was 1 mM bis(sulfosuccinimidyl) suberate (BS^3) (Pierce Biotechnology, USA) for 30 min, 4°C and the reaction quenched by the addition of 1 M Tris-HCl, 15 min, RT. After this procedure (and where crosslinking was not performed), cells were washed three times in ice cold PBS, resuspended in PBS and 10 μl streptavidin-PE (Molecular Probes, USA), incubated for 30 min, at 4°C, and washed three times prior to resuspending in PBS for analysis by flow cytometry.

Indirect staining procedure for intracellular antigens

Cells (~10^6) were fixed by resuspending in 875 μl of cold PBS, gently mixed and 125 μl cold 2% formaldehyde solution added followed by thorough vortexing. Cells were incubated for 30-60 min at 4°C, centrifuged (440g for 5 min), the supernatant removed and the cells resuspended in 1 ml of PBS containing 0.2% Tween 20 (Sigma-Aldrich, USA) (PBS/Tw20). Cells were incubated for 15 min, 37°C, PBS added and cells centrifuged for 5 min, 250g. The supernatant was aspirated and cells resuspended in

~80 µl of PBS supplemented with 0.5% bovine serum albumin (PBS/BSA) and 20 µg biotinylated PTA (1mg/ml). Cells were mixed and incubated for 20 min, 4°C and washed twice in PBS/Tw20. Cells were resuspended in 90 µl PBS/BSA and 10 µl streptavidin-Alexa Fluor® 488 (1 mg/ml) (Molecular Probes, USA) and incubated for 20 min, 4°C, in the dark, washed twice in PBS/Tw20 and resuspended in PBS for analysis by flow cytometry.

Fluorescence Activated Cell Sorting of $CD34^+CD38^-$ subsets

In selected experiments, immunomagnetically separated CB $CD34^+$ cells were labelled with α-CD38-FITC (clone T16, Beckman Coulter, USA), α-CD34-PE and α-CD45-PerCP according to the manufacturer's instructions and the $CD34^+CD38^-$ cells sorted using the cell concentrator module of the FACSCalibur. Sort gates were established by analyzing a sample of MACS $CD34^+$ cells labeled with α-IgG1-FITC / α-CD34-PE / α CD45-PerCP. After sorting, viability and cell number were assessed using trypan blue exclusion and manual cell counting. Cells were then immediately homogenized in TRIzol (Life Technologies, USA) reagent according to the manufacturer's instructions and stored at -80°C until RNA extraction.

Flow cytometric analysis of cell cycle

For analysis of cell cycle, cells were fixed in 70% cold ethanol added dropwise to the cell pellet while vortexing. Cells were incubated at 4°C for at least 30 min, washed twice in PBS, and re-suspended in 15 µg/ml propidium iodide (Molecular Probes, USA) plus 2µg/ml RNase (DNase-free) (Roche, USA). Cells were incubated at RT for 1 hour or for several hours at 4°C.

Flow cytometric hardware and software

Samples were analysed on a FACSCalibur cytometer (BD Biosciences, USA) using CELLQuest™ (BD Biosciences, USA) or ModfitLT v2.0 (Verity Software House, USA) software for analysis of cell cycle. ISHAGE guidelines (Sutherland 1996) with minor modifications were used for the analysis of $CD34^+$ populations. Equivalent

numbers of cells labelled with appropriate isotype controls were used to verify specificity of labelling and to set gating boundaries.

MOLECULAR BIOLOGY TECHNIQUES

Total RNA isolation and DNase treatment

Total RNA was extracted from TRIzol treated cells by addition of chloroform, centrifugation and removal of the aqueous phase with subsequent precipitation of RNA using isopropanol (Sigma-Aldrich, USA). RNA was washed twice in 75% ethanol, air-dried and dissolved in RNAse-free water (RFW) (Promega, USA). Quantitation of RNA was determined by spectrophotometry (260nm).

Prior to reverse transcription PCR (RT-PCR) all RNA samples underwent DNase digestion. One unit of RQ1 RNase-free DNase (Promega, USA) was used per microgram of RNA in a 10µl reaction volume. DNase was inactivated by the addition of stop solution and incubation at 65^{o}C for 10 min.

Reverse transcription and differential display PCR

DNA-free RNA was reverse transcribed using standard PCR conditions and either random hexamers and AMV (Promega, USA) or a one-base anchored primer (anchor A) and SuperScript II RT (Life Technologies, USA).

cDNA from the latter RT-PCR reaction was used as a template for the differential display PCR (dd-PCR). Briefly, 2µl of cDNA was amplified by Taq DNA polymerase (Qiagen, Australia) and primed by Anchor A (5'-AAGCT$_{11}$A-3') and one of 30 possible arbitrary primers (Pacific Oligos, Australia) and incorporating dATP^{33}. Amplified cDNAs were separated on a 6% denaturing polyacrylamide gel (Beckman Coulter, USA) using a Sequi-Gen^{TM} Sequencing Gel apparatus (BioRad, USA) according to the manufacturer's instructions. Gels were transferred to chromatography paper and dried in a gel dryer (Model 583) (Bio-Rad, USA). Bands were visualised by exposure of the chromatography paper to Kodak Biomax MR1 film (Eastman Kodak, USA) for 1-4 days.

cDNA generated by AMV and primed with random hexamers was used in Real Time PCR reactions.

Excision of cDNA bands, re-amplification and sequencing

Bands of cDNA that appeared to be differentially expressed in multiple comparisons were excised from the chromatography paper. The cDNA was recovered by boiling the slice of paper in water and precipitating the cDNA from the supernatant in a mixture of sodium acetate, ice-cold ethanol and glycogen (Boehringer Mannheim, Germany). The recovered cDNA was dissolved in nuclease-free water.

Amplification was performed in a 40µl PCR reaction using Taq DNA Polymerase and the anchored and arbitrary primers that had been used in the original RT-PCR reaction. Cycling parameters and PCR conditions were as specified in RNAimage Differential Display System (GenHunter). PCR products were visualised by standard agarose gel electrophoresis in a low melting point TAE gel with ethidium bromide staining. Bands to be sequenced were excised, purified using a Qiaquick Gel Extraction Kit (Qiagen, Australia) and sequencing reactions carried out using ABI Prism BigDye^{TM} Terminator Cycle Sequencing Ready reaction kit (PE Applied Biosystems, USA) according to the manufacturer's instructions. PCR products were ethanol precipitated, air dried and sequenced by the DNA Sequencing Laboratory, WEHI, Melbourne using an ABI PRISM^{TM} 377 DNA Sequencer (PE Applied Biosystems, USA).

Real-Time PCR semi-quantitation of differentially expressed genes

cDNA that had been reverse transcribed with AMV and random hexamers to prime the reaction were used for real-time PCR. All primers and probes (Table 2.2) for use in real-time PCR reactions were designed using Taqman Primer Express^{TM} software (PE Applied Biosystems, USA) and optimised as recommended by the manufacturer (Table 2.3). Real-time PCR amplification was carried out using either Taqman^{TM} Universal master mix or SYBR^{TM} Green PCR master mix on an ABI PRISM^{TM} 7700 Sequence Detection System following the manufacturer's guidelines (PE Applied Biosystems, USA).

Gene expression was quantitated relative to expression of a housekeeper gene (β-actin) using Sequence Detector Software and the comparative Ct method (PE Applied Biosystems User Bulletin No. 2).

The specificity of SYBR™ Green reactions was checked by one of two methods. Initially, the PCR product from test wells chosen at random and the no template control (NTC) wells was run on a 1.5% agarose gel and the bands visualised with ethidium bromide staining. Results were considered satisfactory if the NTC product did not exhibit a band and if the test product displayed a single band of the expected size. Appropriate DNA ladders were run with every gel. When the software became available, SYBR™ Green reactions were checked by performing a dissociation curve analysis after the Real Time PCR reaction was complete. Data were analysed using the Dissociation Curves software (PE Applied Biosystems, USA) following the manufacturer's guidelines (SDS Compendium 7700 v4, PE Applied Biosystems, USA).

PROTEIN PREPARATION AND ANALYSIS

Preparation and analysis of calf thymus PTA

Sterile, lyophilised bovine thymus PTA was a kind gift of Dr Milan Pesic, Biofactor GmbH, Germany. The alignment of the human, bovine (and rat) nucleotide coding and amino acid sequences is given in Chapter 4 (Fig 4.1). Each vial was reconstituted in sterile saline, aliquoted and stored at -20^{o}C. Reversed-phase HPLC and electrospray-mass spectrometric analysis confirmed the purity of the protein with the expected molecular weight (expected mass: 11982.72 Da; observed mass 11983.69 Da). ESI-MS analysis was performed on a Micromass QTOF II fitted with W-optics (courtesy of Dr Robert Moritz, Joint ProteomicS Laboratory, Ludwig Institute for Cancer Research and The Walter and Eliza Hall Institute of Medical Research, Australia).

Biotinylation protocol

Protein biotinylation was carried out using the FluoReporter® Mini-Biotin –XX protein labelling kit (Molecular probes, USA) in accordance with the manufacturer's procedure. Briefly, 12 µl of biotin-XX solution was added to PTA (1 mg) in 1 ml PBS plus 100 µl of 1 M sodium bicarbonate (pH 8.5) and incubated for 1.5 hours, RT with constant stirring. To separate free biotin from the conjugated protein the solution was filtered through a Vivaspin 500 concentrator (Vivascience, Germany), molecular weight cut off 5000, and the biotinylated protein recovered in PBS.

Table 2.2 Real-time PCR primer and probe sequences

Gene	Forward primer	Reverse primer	Probe
β-actin	5' – gac agg atg cag aag gag att act – 3'	5' – tga tcc aca tct gct gga agg t – 3'	fam – atc att gct cct cct gag cgc aag tac tc - tamra
GAPDH	5 ' – cca cat cgc tca gac acc at – 3'	5' – cca ggc gcc caa tac g – 3'	fam – aag gtg aag gtc gga gtc aac gga ttt g – tamra
PTA	5' – gga agt tgt gga aga ggc aga a – 3'	5' – tgc tcc cca ttt tcc tca tta g – 3'	Not applicable
c-myc	5' – cag ccc cga gcc cct – 3'	5' – gtt cct cct cag agt cgc tgt – 3'	Not applicable

Table 2.3 Optimised Real-time PCR primer and probe concentrations

	Final concentration in reaction mix (nM)			
Gene	**Forward primer**	**Reverse primer**	**Probe**	**Real Time chemistry**
β-actin	600	600	100	Taqman™
GAPDH	600	600	100	Taqman™
PTA	200	100	Not applicable	SYBR™ Green
c-myc	200	100	Not applicable	SYBR™ Green

STATISTICS

All gene expression results were calculated from duplicate determinations and are expressed in arbitrary units (mean ± SEM) relative to β-actin.

Where data displayed parametric distribution, comparison of the means was done using either paired samples T-test or ANOVA (more than two groups) with post-hoc analysis by Tukey's honestly significant difference (HSD) (equal variance assumed) or Tamhane's T2 (equal variance not assumed) and a family error rate of $\alpha=0.05$.

For non-parametric data, analysis was performed with either the Wilcoxon Signed Ranks test (two related samples) or the Friedman test (more than two related samples, this is equivalent to the repeated measures or within–subjects ANOVA).

Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) version 10.1 (Fullerton, USA) or Minitab Release 14 (USA). In all instances $p \leq 0.05$ was considered statistically significant.

BIOINFORMATICS

Nucleic acid sequences were analysed using the National Center for Biotechnology Information (NCBI) database and associated software. Additional sequence alignment and graphical presentation of data were performed with Bioedit Sequence Alignment Editor version 5.0.9 (Tom Hall, North Carolina State University, USA) (Hall 1999).

CHAPTER 3

GENE DISCOVERY AND CONFIRMATION OF DIFFERENTIAL EXPRESSION

INTRODUCTION

Differential display PCR (dd-PCR) was used to assess gene expression in target populations of $CD34^+$ cells enriched by magnetic separation. Although somewhat labour intensive, differential display is relatively inexpensive, requires minimal amounts of $poly(A)^+$ RNA, does not require prior knowledge of target gene sequences and has been used successfully in the identification of genes from diverse organisms reviewed in (reviewed in Ding and Cantor 2004, Sambrook, *et al* 1989). In recent years this laboratory has used dd-PCR to identify genes differentially expressed in $CD34^+$ cells (compared to $CD34^-$) from CB and BM and in HL60 cells that are resistant to the apoptotic effects of oxysterols compared to the non-resistant parent line (Gregorio-King, *et al* 2001, Gregorio-King, *et al* 2002). One of the major drawbacks of dd-PCR is the high rate of false positives that are generated (bands that falsely appear to be differentially expressed) and to overcome this problem we confirmed the differential expression of candidate genes by real time PCR.

RESULTS

Magnetic Activated Separation (MACS) of $CD34^+$ cells

For the dd-PCR a total of ten BM and 13 CB samples were enriched for $CD34^+$ cells by magnetic separation. The purity of samples was analysed by flow cytometry using a modified ISHAGE gating strategy. A representative analysis of a MACS CD34 enriched sample is depicted in Fig 3.1. After magnetic separation the purity (mean ± SEM) of the $CD34^+$ populations were: BM 71% ± 4.3; CB 69% ± 4.3. These figures represented an enrichment of 80-fold in BM and 140-fold in CB. The percentage of $CD34^+$ cells remaining in the depleted ($CD34^-$) populations were: BM 0.9% ± 0.25; CB 0.1% ± 0.02.

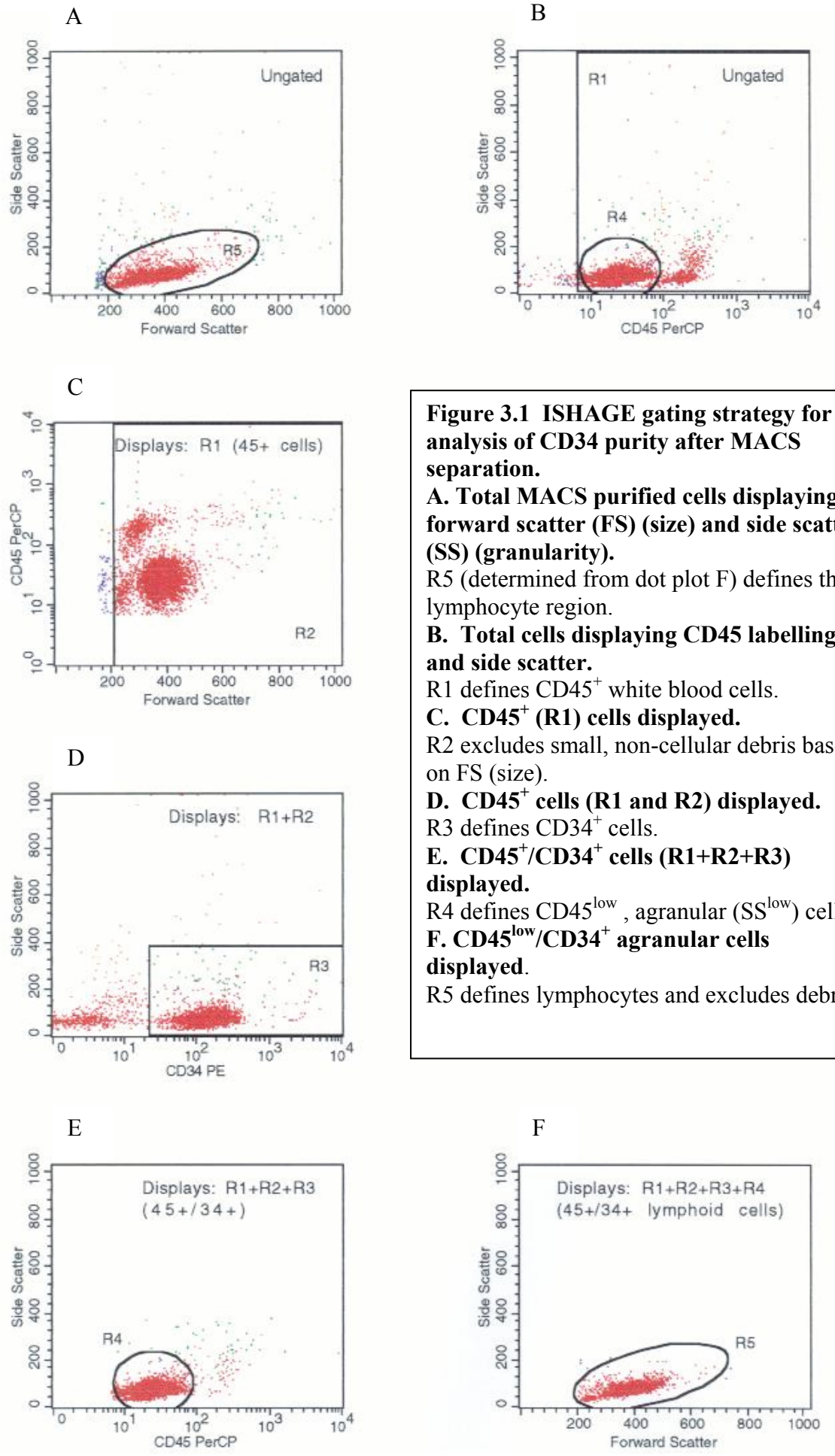


Figure 3.1 ISHAGE gating strategy for analysis of CD34 purity after MACS separation.

A. Total MACS purified cells displaying forward scatter (FS) (size) and side scatter (SS) (granularity).
R5 (determined from dot plot F) defines the lymphocyte region.

B. Total cells displaying CD45 labelling and side scatter.
R1 defines $CD45^+$ white blood cells.

C. $CD45^+$ (R1) cells displayed.
R2 excludes small, non-cellular debris based on FS (size).

D. $CD45^+$ cells (R1 and R2) displayed.
R3 defines $CD34^+$ cells.

E. $CD45^+/CD34^+$ cells (R1+R2+R3) displayed.
R4 defines $CD45^{low}$, agranular (SS^{low}) cells.

F. $CD45^{low}/CD34^+$ agranular cells displayed.
R5 defines lymphocytes and excludes debris.

Differential display PCR

Differential display PCR (dd-PCR) was used to compare the pattern of gene expression between:

- $CD34^+$ and $CD34^-$ cells from CB and BM; and
- $CD34^+$ and $CD34^-$ cells exposed to adult or cord sera.

Ten bands that appeared to be differentially expressed were excised, nine of which were successfully sequenced. The sequences were analysed using the Basic Local Alignment Search Tool (BLAST) of the NCBI public database. Two sequences identified the same gene (an uncharacterised clone located on chromosome 18), five were genes described in the NCBI database (a K^+/Cl^- transporter, centromere protein C1, Prothymosin alpha, a translation initiation factor eIF-3 p110 subunit and ribosomal protein L27a), one other was an uncharacterised gene with multiple EST homologies (which was later found to be a zinc finger protein) and one was unknown. Real time PCR confirmed the differential expression of two of the sequences, both of which were known genes: *Homo sapiens* centromere protein C1 (Accession No. NM 001812.1); and *Homo sapiens* prothymosin *alpha* gene (Accession No. NM 002823.1).

Confirmation of Differential Expression

The expression pattern of the centromere C1 gene appeared to be related to sera source rather than cell type so confirmation of differential expression by real time PCR was conducted in samples of $CD34^+$ cells from CB and BM cultured with either CS or AS. There was no significant difference in its expression in CB $CD34^+$ cells cultured either with cord or adult sera (T-test, NSD, n=4) (Fig 3.2A). In BM $CD34^+$ cells the gene was more highly expressed in cells cultured with adult compared to cord sera (T-test, $p \leq 0.007$, n=4) (Fig 3.2B). The gene and protein function in mitosis but also exhibit cyclic behaviour in the cell cycle with greatest abundance in G1 (Knehr*, et al* 1996). Because its differential expression in BM $CD34^+$ cells cultured with adult sera probably reflected the higher number of cycling cells in BM $CD34^+$ cells compared to the more quiescent CB $CD34^+$ cells no further work was pursued with this gene.

Differential expression of PTA was confirmed in populations identical to that used for the original gene discovery, that is $CD34^+$ and $CD34^-$ cells exposed to adult or cord sera, and was also assessed in uncultured $CD34^+$ and $CD34^-$ cells.

As the data were not normally distributed non-parametric tests were used to assess significance. CB $CD34^+$ cells express higher levels of PTA than CB $CD34^-$ cells (Fig 3.3A), irrespective of exposure to cord (Wilcoxon Signed Ranks, $p \leq 0.012$, n=13) or adult sera (Wilcoxon Signed Ranks, $p \leq 0.019$, n=13). Similarly, BM $CD34^+$ cells exposed to cord sera express significantly higher levels of PTA compared to their counterpart ($CD34^-$ exposed to cord sera) (Wilcoxon Signed Ranks, $p \leq 0.05$, n=9) (Fig 3.3B). However, there was no significant difference between the levels of PTA in BM cells exposed to adult sera ($CD34^+$ versus $CD34^-$, NSD, n=9).

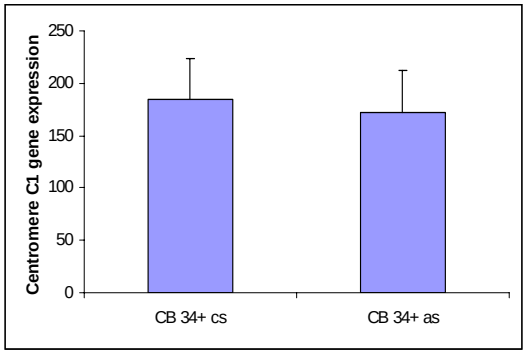
In uncultured cells, PTA expression was elevated in $CD34^+$ cells compared to $CD34^-$ cells in both CB (Fig 3.4A) (~3-fold, Wilcoxon Signed Ranks, $p \leq 0.001$, n=25) and BM (Fig 3.4B) (~4.5-fold, Paired Samples T-test, $p \leq 0.001$, n=8).

Figure 3.2 Mean Centromere C1 expression (relative to β-actin) in CB and BM CD34$^+$ populations cultured for 24 hours with either pooled cord (CS) or adult sera (AS).

CB CD34 enriched and depleted populations were cultured at a concentration of 0.5x10^6 cells/ml in α-MEM supplemented with 2mM L-glutamine and either 10% pooled cord sera (cs) or pooled adult sera (as). Cells were cultured for 24 hours at 37^{o}C, 5% CO_2 in a humidified incubator.

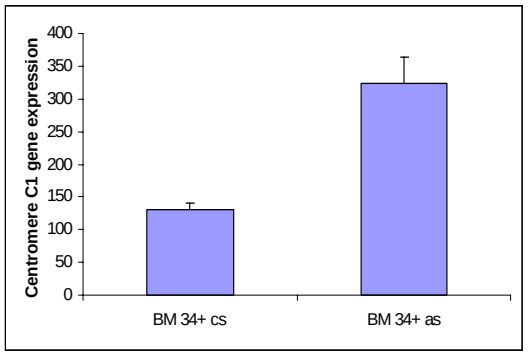
There was no significant difference in the expression of centromere C1 in CB CD34$^+$ cells cultured with cord or adult sera. In BM, gene expression was significantly up-regulated in CD34$^+$ cells cultured with AS compared to CS. Gene expression levels are shown in arbitrary units relative to β-actin.

A. Cord Blood (n=4)



T-test, NSD

B. Bone Marrow (n=4)



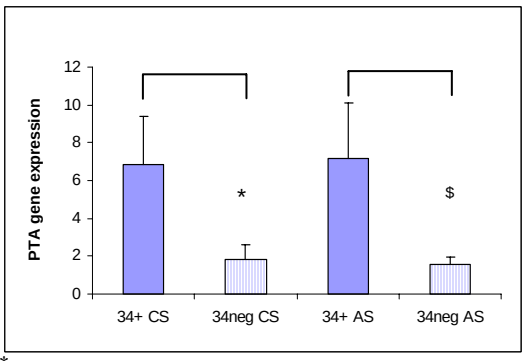
T-test, $p \leq 0.007$

Figure 3.3 Mean PTA expression (relative to β-actin) in CB and BM CD34$^+$ and CD34$^-$ populations cultured for 24 hours with either pooled cord (CS) or adult sera (AS).

CB CD34 enriched and depleted populations were cultured at a concentration of 0.5x10^6 cells/ml in α-MEM supplemented with 2mM L-glutamine and either 10% pooled cord sera or pooled adult sera. Cells were cultured for 24 hours at 37^{o}C, 5% CO_2 in a humidified incubator.

PTA gene expression was significantly up-regulated in CB CD34$^+$ cells cultured with cord or adult sera compared to their paired CD34$^-$ cells. In BM PTA gene expression was significantly up-regulated in CD34$^+$ cells cultured with CS compared to paired CD34$^-$ cells. PTA gene expression was not significantly different in BM CD34$^+$ and CD34$^-$ cells cultured with adult sera. Gene expression levels are shown in arbitrary units relative to β-actin.

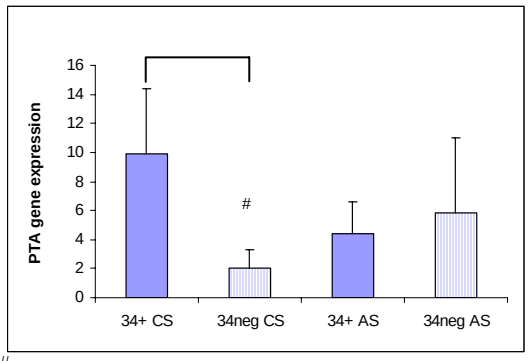
A. Cord Blood (n=13)



* Wilcoxon Signed Ranks, $p \leq 0.012$

$ Wilcoxon Signed Ranks, $p \leq 0.019$

B. Bone Marrow (n=9)



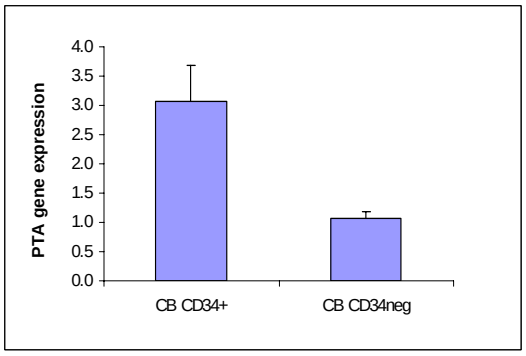
Wilcoxon Signed Ranks, $p \leq 0.05$

Figure 3.4 Mean PTA expression (relative to β-actin) in uncultured cord blood and bone marrow $CD34^+$ and $CD34^-$ populations.

CB and BM MNC were immunomagnetically labelled with a $CD34^+$ microbead and separated using MACS. CD34 enriched and depleted populations were immediately spun down and resuspended in TRIzol. Samples were stores at –80°C until RNA extraction.

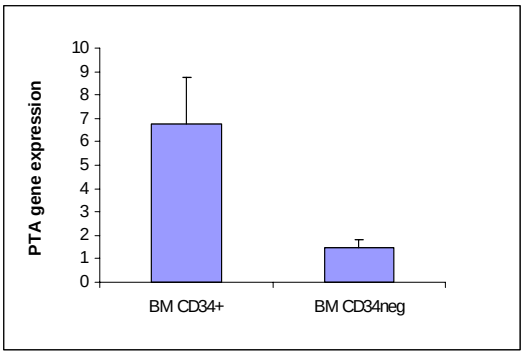
PTA gene expression was significantly up-regulated in $CD34^+$ cells compared to $CD34^-$ cells in both CB and BM. Gene expression levels are shown in arbitrary units relative to β-actin.

A. Cord Blood (n=25)



Wilcoxon Signed Ranks, $p \leq 0.001$

B. Bone Marrow (n=8)



Paired T-test, two-tailed, $p \leq 0.001$

DISCUSSION

The test conditions chosen were designed to exploit well characterised differences in the proliferative potential of $CD34^+$ cells from CB and BM. These two populations provided the basic source material for the gene discovery experiments. Although the CD34 antigen is expressed on cells with a wide range of proliferative and regenerative capabilities, including a population of lineage committed cells that lack repopulating ability, enumeration of the number of $CD34^+$ cells is the most useful clinical indicator of the suitability of a product for stem cell transplantation. In fact, a minimum dose of $CD34^+$ cells (~$2x10^6$ $CD34^+$ cells per kg bodyweight of recipient) is used to calculate whether mobilisation and harvesting regimes have been adequate. It is because of the clinical and biological importance of this population of cells that they were considered a suitable starting point for the investigation of genes of importance in haemopoietic stem and progenitor cell development. Several groups have used the same population to construct libraries for global gene analysis (Mao, *et al* 1998, Zhang, *et al* 2000, Zhou, *et al* 2001), for comparative analyses of normal and disease states (Gu, *et al* 2000), to determine functional distinctions in gene expression of $CD34^+$ derived from different sources of human and monkey tissue (Gregorio-King, *et al* 2001, Gregorio-King, *et al* 2002, Lu, *et al* 2002, Steidl, *et al* 2003) and in cross species analyses (Gomes, *et al* 2001). Libraries have been constructed from PCR-based protocols (using commercial construction kits) and serial analysis of gene expression (SAGE). Comparative analyses often use the combination of commercial cDNA technology and real time PCR or semi-quantitative PCR and as the cost of arrays declines the utility of this powerful gene discovery and analysis technique increases. Although it would be premature to say these approaches have provided exhaustive detail with respect to the spectrum of genes expressed in haemopoietic progenitor cells they undoubtedly have provided a wealth of information that not only requires comprehensive cataloguing but will direct research efforts for many years to come.

Differential display, although the least powerful of the techniques now available to do comparative studies, has the major benefits of being relatively cheap, does not require particularly sophisticated equipment and requires nanogram quantities of mRNA, thus making it suitable for small-scale discovery and experimental protocols with multiple comparisons, such as the program detailed in this thesis. While using all possible combinations of anchored and arbitrary primers should generate a more comprehensive

expression profile, this approach would necessitate performing in excess of 150 separate PCRs and running ~20 gels, which illustrates the labour-intensive nature of the differential display technique (Sambrook, *et al* 1989). We generated a limited set of mRNA sequences by restricting the number of anchored and arbitrary primers used while increasing the groups for comparison.

In our study, in addition to the differences between the $CD34^+$ and negative sub-populations we wished also to assess the effects of cord and adult sera on these groups. It is possible that not all of the differences in growth potential between $CD34^+$ and negative cells and between $CD34^+$ cells from CB and BM are attributable to inherent differences in gene expression between CB and BM. Several studies have found that CB plasma (compared to PB plasma or FBS) enhances the size and number of primary and secondary colonies grown form CB MNC (Carow 1993, Ruggieri, *et al* 1994) and the expansion of total cells and haemopoietic progenitors in liquid culture (Ruggieri, *et al* 1994). The effect of CB plasma on adult BM $CD34^+$ cells is less profound although it has been shown to increase the re-plating potential of BM progenitors (Carow 1993). It is not known whether the enhancement of proliferation caused by exposure to CB plasma is attributable to novel or known growth factors.

To broaden the scope of the gene discovery experiments, $CD34^{+/-}$ cells from CB and BM were exposed to pooled cord and adult sera for 24 hours prior to extraction of RNA. Thus, when analysing the DD-PCR gels, comparisons were made between:

- CD34 positive and negative populations, to look for global differences in gene expression between these groups;
- $CD34^+$ cells from CB and BM, to look for differences in gene expression that might relate to differences in their ability to proliferate and differentiate; and
- Cells exposed to cord sera versus cells exposed to adult sera, particularly the $CD34^+$ populations, to look for changes in gene expression resulting from the culture conditions.

A total of ten bands were excised from five polyacrylamide gels and yielded eight individual sequences. Five of these were described in the NCBI database, two had multiple EST homologies but were otherwise uncharacterised and one band was unknown. Although real time PCR confirmed the differential expression of only two

bands the high false positive rate is an acknowledged disadvantage of the dd-PCR technique necessitating the use of alternative methods for confirming differential expression (Liang 1995, Liang 1993). It has also been demonstrated that dd-PCR shows a strong bias towards the amplification and identification of high copy number mRNA's (Bertioli, *et al* 1995) so it was not surprising that one of the genes differentially expressed, PTA, is one of the most abundantly expressed genes known (Adams, *et al* 1995). Furthermore, two of the known genes (human translation initiation factor eIF-3 and ribosomal protein L27a) that were determined not to be differentially expressed were amongst the top 60 genes expressed in $CD34^+$ cells determined by SAGE analysis of a human BM CD34+ library (Zhou, *et al* 2001).

From the data displaying the cultured CB and BM (Fig 3.3) there are two points of interest. The first is that CB $CD34^+$ cells express higher levels of PTA than CB $CD34^-$ cells, irrespective of exposure to cord ($p=0.012$) or adult sera ($p= 0.019$). In contrast, only BM $CD34^+$ cells exposed to cord sera express significantly higher levels of PTA than their counterpart ($CD34^-$ cells exposed to cord sera) ($p=0.05$). There was no significant difference between the levels of PTA in BM cells exposed to adult sera ($CD34^+$ versus $CD34^-$, NSD). PTA gene expression was also assessed in uncultured $CD34^{+/-}$ cells from CB and BM. The results of real time-PCR confirmed the differential expression of PTA which was up-regulated in the $CD34^+$ populations compared to the $CD34^-$, of both CB ($p \leq 0.001$) and BM ($p \leq 0.001$).

Together these results suggest the presence of an inhibitory factor/s present in adult sera capable of causing the down-regulation of PTA. If this is the case then the second point of interest is that the inhibitory effect is not apparent in CB $CD34^+$ cells cultures with adult sera. This result may be explained in a number of ways. Perhaps the most obvious explanation is that CB $CD34^+$ cells may lack receptors for the inhibitor/s in question. However, other hypotheses are possible. CB and BM may share similar signal transduction pathways of proliferation but in CB there may be greater amplification of these pathways which may override inhibitory signals; and/or, CB $CD34^+$ cells may have alternative signalling networks for proliferation that are lost as part of the aging process. Alternatively, CB $CD34^+$ cells may produce proteins that bind and inactivate inhibitors. Before any of these hypotheses could be given serious consideration it would seem essential to assess the effect of known growth factors and growth inhibitors on regulating PTA expression in CB $CD34^+$ cells.

Following the discovery and confirmation of the differential expression of PTA in CB and BM $CD34^+$ cells, a search was conducted of the Stem Cell Database (SCDB) maintained by Princeton University, USA. The SCDB was created as a joint project of the laboratories of Ihor Lemischka (Princeton University) and G. Christian Overton (University of Pennsylvania) from libraries of subtracted cDNA clones of day 14 mouse foetal liver cells. The subtracted libraries were created from cells that were selectively enriched or depleted for haemopoietic activity. In support of our finding that PTA was up-regulated in human haemopoietic progenitors, the SCDB also lists PTA as being differentially expressed in foetal mouse haemopoietic progenitors. (http://stemcell.princeton.edu; PTA identifier SC | B4-42 | 13153.)

During the course of this project three groups have published findings pertaining to the expression of PTA in haemopoietic progenitor cells. In 2000, Gu *et al* listed PTA in a catalogue of genes associated with haemopoiesis derived from a human adult BM $CD34^+$ cell library (Gu, *et al* 2000) and the results of the Princeton group, curators of the SCDB, were also published (Phillips, *et al* 2000). Two years later Park *et al* published their findings related to the differential gene expression profile of adult murine haemopoietic stem cells (Park, *et al* 2002). Park's findings complement those of the SCDB group in that they found PTA to be expressed in populations of adult murine haemopoietic progenitors that displayed both multipotency and long-term re-populating ability.

A preliminary literature search of PTA revealed the gene appeared to be essential for proliferation with conflicting views about its role in differentiation. Addition of the protein to mitogen stimulated MNC was reported to augment the proliferation of the cells. There was a strong bias in the literature towards elucidating the immuno-regulatory properties of the protein and its role as a prognostic indicator in some malignancies, primarily as a consequence of it association with proliferation. To date, as mentioned above, there have been only cursory references to its up-regulation in haemopoietic progenitor cells and no publications relating to its regulation or function in this important cell group.

Because an ability to undergo massive proliferation is one of the key differences between haemopoietic progenitors (represented by the $CD34^+$ sub-set) and mature haemopoietic cells ($CD34^-$) and as PTA has been found to be significantly up-regulated in $CD34^+$ cells in both humans and mice by several different groups we decided PTA

warranted closer investigation. A thorough literature review was performed (see next chapter) and several key areas were identified for initial experimental investigation.

CHAPTER 4

PROTHYMOSIN α LITERATURE REVIEW

INTRODUCTION

The thymus is an organ of the immune system and is generally accepted as the site of T-cell maturation.[6] It also produces humoral factors with immuno-regulatory properties. In the mid-1960s the purification of the humoral elements resulted in the production of a standardised fraction of calf thymus known as thymosin fraction V (TFV) (Goldstein, *et al* 1966, Low, *et al* 1979). TFV is comprised of 30-40 polypeptides that can be subdivided according to their isoelectric point: α-thymosins, pI<5.0; β-thymosins, pI 5-7; and γ-thymosins, pI>7. In early preparations of TFV a short peptide was isolated, designated thymosin α_1. Modifications to the purification process that prevented proteolysis resulted in the isolation of a longer molecule, of which the first 28 amino acids were identical to thymosin α_1. The longer molecule was presumed to be the precursor of thymosin-α_1 and so was called *pro*thymosin α (PTA). Subsequent work however, suggested that PTA is the biologically active molecule and that thymosin α_1 is a by-product of its degradation.

The gene for PTA is ubiquitously expressed in mammals and recently identified in frog (Aniello, *et al* 2002) and zebrafish (Ton, *et al* 2000). In a survey of human cDNA libraries it rated amongst the most abundantly expressed genes, present in 26 of 30 tissues (Adams, *et al* 1995). The thymus is the richest source of PTA protein and within the first decade of life protein levels are 15 to 32 fold higher than in samples from older patients (Tsitsiloni, *et al* 1993). Thymic levels decline exponentially during the first years of life which is consistent with thymic involution and fatty infiltration that occurs after the first year of life. The spleen also shows a decline in PTA cross-reactive peptides with age, while liver has lower but steady levels of protein (Tsitsiloni, *et al* 1993).

[6] It should be noted that there is a body of work that suggests the thymus is the site for all lymphopoiesis (T and B-cell). This theory draws on comparative evolutionary biology, phylogeny and the ontogeny of the components of the immune system in the

With a pI of 3.5 PTA is one of the most acidic proteins known. Across species its amino acid sequence is highly conserved including conservation of a bi-partite nuclear localisation signal. Its ubiquity is suggestive of an essential role in cellular function and metabolism; however PTA has proven a uniquely difficult protein to characterise, both physically and functionally. Its unusual amino acid sequence, structural features, regulation and efforts to determine its function will be discussed below.

PTA GENE FAMILY AND PROTEIN PRODUCTS

The human PTA gene family consists of one unprocessed gene (containing exons and introns) located on chromosome 2q35-q36 and at least 5 processed (intronless) pseudogenes which share 85-95% sequence homology with the parental gene (Manrow, *et al* 1992). The rat PTA gene family also consists of several processed pseudogenes and one expressed gene (Gaubatz, *et al* 1994).

Manrow *et al* conducted an exhaustive analysis of human PTA and its pseudogenes, finding that four of the five have relatively intact reading frames that code for PTA-like polypeptides; a TATA-like sequence in the 5' untranslated region; a Kozak consensus sequence immediately upstream of a potential ATG start codon and a conserved polyadenylation sequence (Manrow, *et al* 1992).

Pseudogene 1 shows the least homology at the protein level sharing 16 of the first 20 amino acids, then only one of the next 29 whereupon the peptide terminates. It also has an ATG site embedded upstream of the recognised ATG start site which potentially introduces 35 amino acids 5' of the usual start codon (Manrow, *et al* 1992). Pseudogene 2 bears the greatest likeness to the authentic PTA, differing in only two positions: a deletion at amino acid 68 and a substitution of a threonine for a serine at position 84 (Manrow, *et al* 1992). Manrow *et al* used restriction enzyme digestion with Acc1 to identify diagnostic bands that could discriminate between products of PTA and its pseudogenes but could find no evidence of transcribed products from any pseudogene in normal human tissues (placenta, liver, thyroid, striated muscle, colon, stomach, kidney) ovarian carcinoma or three libraries derived from normal skin fibroblasts, myeloma and teratocarcinoma cells (Manrow, *et al* 1992).

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developing embryo. An informative commentary on this theory is given at Anastassova-Kristeva, M. (2003) The origin and development of the immune system with a view to stem cell therapy. *J Hematother Stem Cell Res*, **12,** 137-154..

AMINO ACID SEQUENCE AND STRUCTURAL FEATURES OF PTA PROTEIN

The complete coding sequence for human PTA was published independently by two groups in 1986 based on clones identified in cDNA libraries derived from spleen (Goodall, *et al* 1986, Pan, *et al* 1986) and mitogen stimulated human lymphocytes and SV40 transformed fibroblasts (Eschenfeldt and Berger 1986). Two splice variants were identified differing in one glutamic acid residue created by alternative splicing of adjacent AG couplets at the boundary of intron 2 and exon 3 (Eschenfeldt, *et al* 1989, Manrow and Berger 1993, reviewed in Pineiro, *et al* 2000). The mRNA transcripts (and presumably the protein) exist in the ratio of ~9:1 (shorter: longer) and do not display evidence of tissue specificity. The splice junction has been conserved in the African Monkey *Colobus* and a study of 22 different human individuals failed to find any substitutions at the end of intron 2 which would suggest active protection of the splice site (Manrow and Berger 1993). Figure 4.1 shows the nucleotide code and amino acid sequence of human, cow and rat PTA.

Human PTA is 109-110 amino acids long, is extremely acidic with ~50% acidic residues (35 glutamates, 19 aspartates) and lacks sulphur containing amino acids (methionine and cysteine) and aromatic amino acids (phenylalanine, tyrosine, tryptophan). As a consequence of its unusual primary structure the protein does not absorb at 280nm, partitions to the aqueous phase of a phenol extraction (Evstafieva, *et al* 1995, Sburlati, *et al* 1990) and due to its high negative charge adopts a random coil-like conformation (Gast, *et al* 1995). Some evidence exists to suggest that at pH<5.5 PTA adopts a partially folded conformation (Gast, *et al* 1995, Uversky, *et al* 1999) and a similar effect inducing substantial secondary structure occurs at neutral pH when PTA binds Zn^{2+} but not Ca^{2+} or Mg^{2+} (Uversky, *et al* 1999 ; reviewed in Pineiro, *et al* 2000; and Hannappel and Huff, 2003). However, whether the shape change is associated with function is unknown.

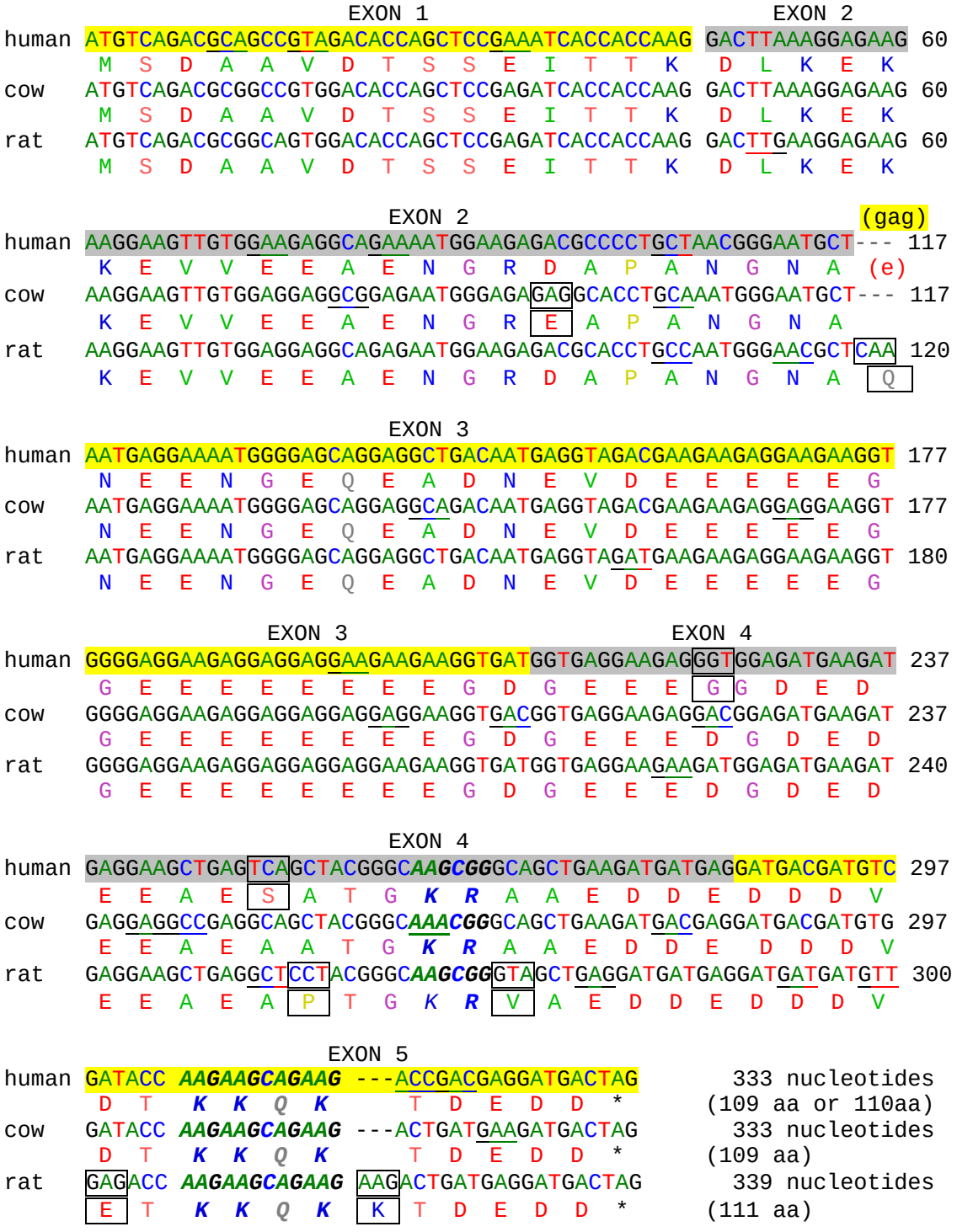
PTA does not have a secretory signal peptide (Eschenfeldt and Berger 1986, Goodall, *et al* 1986) and is extremely hydrophilic making transport of the protein across the hydrophobic cell membrane problematic. Eschenfeldt *et al* (Eschenfeldt, *et al* 1989) found that in myeloma cells, PTA mRNA was exclusively associated with free

polyribosomes rather than polysomes bound to the membrane of the endoplasmic reticulum as would be expected if the protein were secreted. This data suggests it is unlikely that the protein is secreted.

The protein does however have a bi-partite nuclear localisation signal (NLS) (Pavlov, *et al* 1995, Rubtsov, *et al* 1997) at the carboxyl terminus (italicised and bold in Fig 4.1) and this will be discussed more fully in the next section.

Figure 4.1 Alignment of complete coding sequences (with one letter translation) of human (NM002823), cow (AV605031) and rat (NM021740) PTA.

Exons of human sequence are indicated by alternate yellow and grey shading. The insertion point for the extra (gag) nucleotide at the exon 2/3 border which is present in the long splice variant in human is also noted. Nucleotide variations that conserve the amino acid sequence are underlined, while variations that change the amino acid sequence are boxed. The bi-partite NLS is italicized and bold.



CELLULAR LOCALISATION AND NUCLEAR LOCALISATION SIGNAL (NLS)

Gomez-Marquez and Segade (Gomez-Marquez and Segade 1988) hypothesised that PTA would function as a nuclear protein based on structural features of the protein and its similarity to other nuclear proteins. They recognised that an amino acid sequence (DT**KKQK**T) in the carboxyl terminal of PTA had significant homology to the recognised nuclear localisation signal of the large T antigen of SV40, (PK**KKRK**V). In addition to large T antigen and PTA, they reviewed several other published karyophilic signals in proteins from *Xenopus,* hamster and human and proposed a consensus sequence for a nuclear localisation signal: X B/Thr Lys Lys Z Lys X, where X is any residue, B is a basic residue and Z is a polar residue (Gomez-Marquez and Segade 1988).

Experimental evidence for the nuclear localisation of PTA was provided by Watts *et al* (Watts*, et al* 1989) by injecting radiolabelled bovine PTA into the oocytes of the African clawed toad, *Xenopus laevis*. A time course study comparing the amount of radioactive material in the enucleated and separately pooled cytoplasmic and nuclei fractions showed that PTA accumulated in the nucleus over a 48 hour period. Similarly, monitoring intact protein on polyacrylamide gels showed an increase in the nuclear band that peaked at 12 hours and remained stable for at least 24 hours (Watts*, et al* 1989).

Contrary evidence was produced by Tsitsiloni *et al* (Tsitsiloni*, et al* 1989) who used a radio-immunoassay and polyclonal antibodies directed against the thymosin α_1 fragment of PTA (amino acids 1-28 of the NH_2-terminus) to assess the cellular localisation of PTA in the cytoplasmic and nuclear fractions of calf liver and thymus. Almost all the recovered activity resided in the cytoplasm, although there was considerable loss of material in the nuclear and cytoplasmic extracts compared to the whole cell control extracts.

Conteas *et al* (Conteas*, et al* 1990) used a polyvalent sera raised against thymosin α_1 and immunoelectron microscopy to study the localisation of thymosin immunoreactive peptides (TIP). They found that in the rat small intestinal crypt cell line (IEC-6) TIP were located in the nucleus and to a lesser extent in the cytoplasm.

Other investigators have shown that in disrupted cells PTA partitions into the cytoplasmic fraction (Manrow, *et al* 1991, Sburlati, *et al* 1990). Sburlati *et al* exploited this behaviour to develop a method of purifying the protein from post-nuclear supernatants. Manrow *et al* (Manrow, *et al* 1991) examined the localisation of PTA in transfected COS cells. Initially, PTA appeared in the cytoplasmic fractions of detergent-lysed, transfected cells, however leakage of nuclear proteins was apparent in that 20% of SV40 large T antigen, a nuclear protein, was found also in the cytoplasm of the cells. When cells were treated with cytochalasin B, which depolymerises actin filaments that anchor nuclei in cells, PTA was predominantly located in the nuclei, rather than the cytoplasm (Manrow, *et al* 1991). In addition, this group used constructs to create fusion proteins of PTA and β-galactosidase (a protein normally resident in the cytoplasm) and determined the localisation of the fusion protein *in situ* by using mouse monoclonal antibodies to β-galactosidase. They found that the fusion protein was located almost exclusively in the nucleus. To determine which part of the amino acid sequence of PTA was essential for nuclear localisation, further studies were performed in COS cells transfected with constructs that produced a truncated PTA fused to β-galactosidase. The carboxyl terminus (amino acids 31-111), localised to the nucleus whereas the amino terminus (amino acids 2-53) fusion protein remained in the cytoplasm indicating that the NLS was located in the carboxyl end of PTA (Manrow, *et al* 1991). This work was supported by the findings of another group (Clinton, *et al* 1991) who stably transfected HeLa S3 with constructs that produced chimeras of PTA linked to human growth hormone (hGH) a protein not normally produced by the HeLa cell line. In this model the hGH-PTA chimera also located to the nucleus. In support of the concept of PTA providing active passage into the nucleus (as opposed to passive diffusion) it was noted that neither the native hGH (which contains a secretory signal) nor a truncated hGH lacking the secretory signal were found in the nucleus. The native hGH was located in the perinuclear region (consistent with being located on the endoplasmic reticulum and the golgi apparatus) and detected in the culture medium while the truncated protein was diffusely distributed in the cytoplasm (Clinton, *et al* 1991).

In a series of exacting experiments Enkemann *et al* (Enkemann, *et al* 2000b) extended the work of Manrow *et al* using constructs to produce three chimeric proteins: PTA-β-galactosidase fusion (as above), PTA tagged with poly-histidine residues at the carboxy

terminal and PTA with an amino-terminal FLAG tag. Protein localisation was studied in COS and NIH-3T3 cells and confirmed that the protein was predominantly nuclear although with traces in the cytoplasm except in mitotic cells where the protein could be found in the cytoplasm before the nuclear membrane degraded and after it reformed. In transfected cells treated for 2 hours with cycloheximide to prevent protein production, or actinomycin D to prevent RNA synthesis, the distribution of PTA was unaffected with traces of PTA still apparent in the cytoplasm. The results suggest that either PTA protein can remain in the cytoplasm for more than 2 hours following its production and/or that PTA moves into and out of the nucleus. Evidence to support the mobility of PTA was produced in transfected COS cells cooled to 0^oC for up to 2 hours to inhibit energy dependent movement into the nucleus then re-warmed to 37^oC. Under these conditions the behaviour of PTA was similar to that of an endogenous protein Ran that is known to shuttle between the cytoplasm and the nucleus. Initially (at 37^oC) both proteins were predominantly nuclear, then following 2 hours at 0^oC substantial amounts of protein had accumulated in the cytoplasm but after 30 mins at 37^oC most of the protein had again re-located to the nucleus. The exception to this pattern was with the PTA-β-galactosidase chimera which remained nuclear after 2 hours at 0^oC, presumably because the large β-galactosidase moiety prevented diffusion of PTA back across the nuclear membrane. An alternative model of inhibition of energy–dependent nuclear import produced similar results. Transfected COS cells permeabilised with α-toxin and treated with ATPγS and GTPγS showed the cytosolic accumulation of significant amounts of PTA and Ran. These result are consistent with PTA being a nuclear protein but able to shuttle between the cytoplasm and nucleus (Enkemann, *et al* 2000b).

The general consensus from these various experimental models is that PTA is predominantly a nuclear protein but with traces of cytoplasmic localisation that increase immediately before and after cell division. Its movement into the nucleus is an energy dependent phenomena that can be abrogated by shutting down energy production systems either by cooling cells to 0^oC or using non-hydrolyzable ATP and GTP analogues to competitively inhibit energy-dependent enzyme systems. These data also support the notion that diffusion of PTA from the nucleus back to the cytoplasm is energy passive.

The finding that wild-type human PTA when overexpressed in yeast caused inhibition of yeast growth and that a mutant lacking the NLS did not (Pavlov, *et al* 1995)

prompted a series of experiments that used the inhibitory effect as a screening tool for mapping the NLS of PTA. Deletion of the last 9 amino acids from the carboxy-terminus of PTA produced a protein that did not inhibit yeast growth nor did it localise to the nucleus in either yeast or human embryonic kidney 293 (HEK293) cells. (Rubtsov, *et al* 1997). Presumably localisation to the nucleus is a necessary pre-requisite for the inhibition of yeast cell growth. Thus mutants that permitted yeast cell growth would most likely contain mutations in the NLS. Using this model system several functionally important residues were identified, mutations of which abrogated the nuclear localisation of PTA in yeast and HEK293 cells, including lysine-87 (Rubtsov and Vartapetian 1996), lysine 101 (Rubtsov, *et al* 1997) as well as amino acids 101-109 (Pavlov, *et al* 1995, Rubtsov, *et al* 1997). To confirm the location of the NLS in the carboxy terminus a mutant protein comprised of residues 82-109 fused to GFP was located exclusively in the nucleus of HEK293 cells although it was diffusely spread in yeast cells possibly indicating a requirement for an additional signal upstream of the NLS for nuclear targeting in yeast (Rubtsov, *et al* 1997). Rubtsov *et al* concluded that the NLS of PTA is bi-partite and comprised of two blocks of basic amino acids separated by 12 amino acids (see Fig 4.1). In this respect it is similar to the bi-partite NLS of nucleoplasmin which is composed of two basic blocks separated by 10 amino acids (Rubtsov, *et al* 1997).

With regard to the inhibition of yeast cell growth an interesting finding was that a point mutation in threonine-105 permitted localisation of PTA into the nucleus of yeast cells but did not inhibit cell growth. This is clearly suggestive of the fact that threonine-105 is essential for the inhibitory phenotype in yeast (Rubtsov, *et al* 1997).

PTA EXPRESSION IN MAMMALIAM SYSTEMS

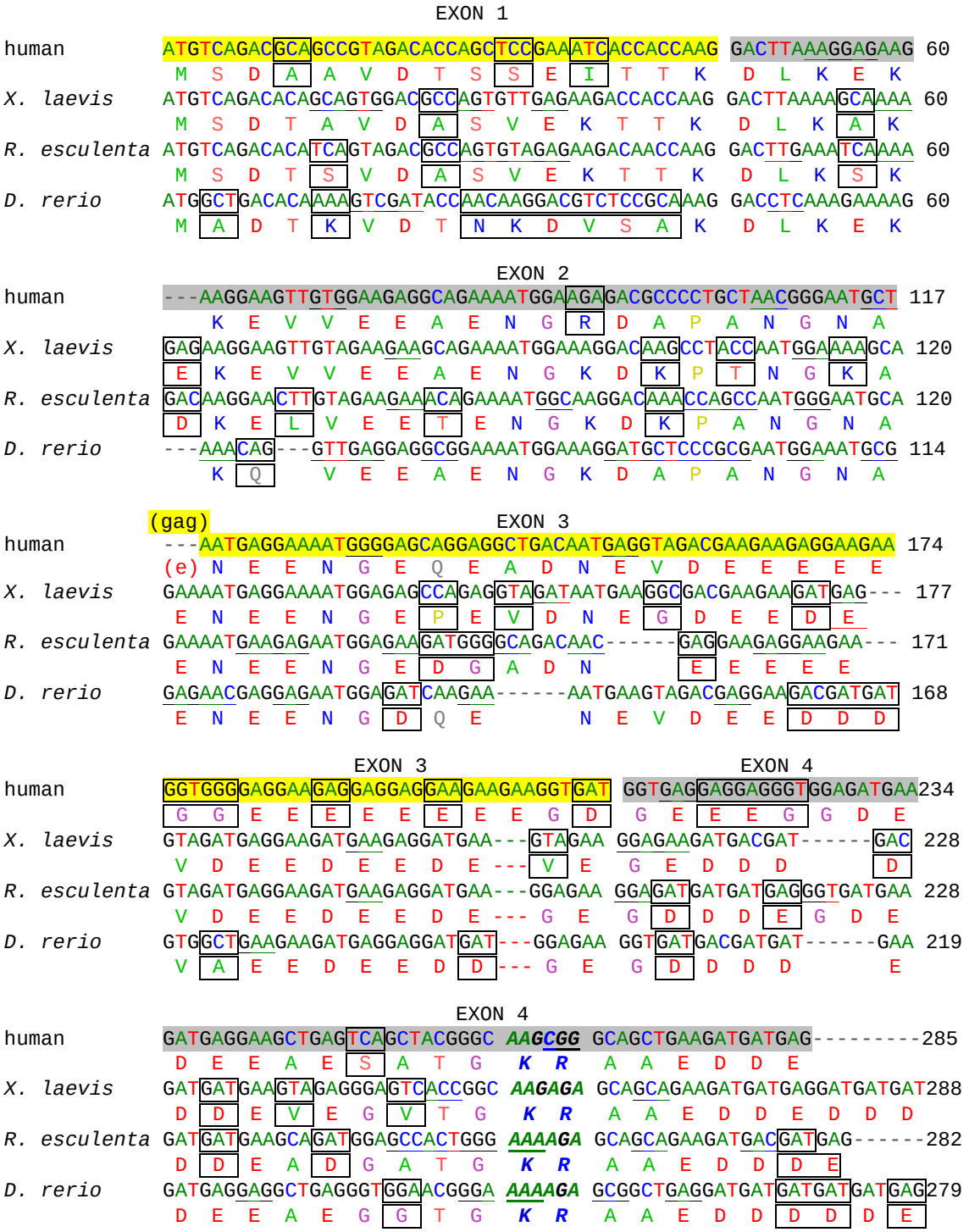
In 1989 Makarova *et al* (Makarova, *et al* 1989) reported the isolation of PTA from yeast. The PTA was reported to be covalently linked to RNA and was estimated to be 1000-fold less concentrated in yeast than in mammalian cells. This finding was thoroughly refuted by Trumbore *et al* (Trumbore, *et al* 1998) who used several approaches, unsuccessfully, to isolate or otherwise identify a PTA homologue in yeast, including a search of the *S. cerevisiae* genome on the NCBI database using the human PTA amino acid and protein sequences.

A PTA homologue has been isolated from the testis of the frog, *Rana esculenta* (Aniello, *et al* 2002). The similarity between human and frog PTA protein is about 70% but lower at the nucleotide level. The expression of PTA in the testis was highly variable with high levels coinciding with the annual spermatogenic cycle and thereafter falling to very low levels. The frog PTA homologue was also detected at high levels in brain and kidney tissue and at much lower levels in frog muscle, liver and harderian gland (Aniello, *et al* 2002).

PTA transcripts have also been isolated from the zebrafish, *Danio rerio* (NCBI accession no.: NM 194376) (Ton, *et al* 2000), and the African clawed frog, *Xenopus laevis* (BC 054174). Figure 4.2 illustrates the alignment of the PTA cDNA and corresponding protein translation for human, frogs and zebrafish. The degree of conservation of PTA protein across these species (~70%) only serves to reinforce the view that this small acidic protein must perform a function/s essential to cellular metabolism.

Figure 4.2 (next page) Alignment of complete coding sequence (with one-letter translation) of PTA homologues for human (NM002823), African clawed frog (*Xenopus laevis*, BC054174), edible frog (*Rana esculenta*, AJ312835) and zebrafish (*Danio rerio*, NM194376).

Exons of human sequence are indicated by alternate yellow and grey shading. The insertion point for the extra (gag) nucleotide at the exon 2/3 border which is present in the long splice variant in human is also noted. Nucleotide variations that conserve the amino acid sequence are underlined, while variations that change the amino acid sequence are boxed. The bi-partite NLS is italicized and bold.


EXON 1
human ATGTCAGACGCAGCCGTAGACACCAGCTCCGAAATCACCACCAAG GACTTAAAGGAGAAG 60
M S D A A V D T S S E I T T K D L K E K
X. laevis ATGTCAGACACAGCAGTGGACGCCAGTGTTGAGAAGACCACCAAG GACTTAAAAGCAAAA 60
M S D T A V D A S V E K T T K D L K A K
R. esculenta ATGTCAGACACATCAGTAGACGCCAGTGTAGAGAAGACAACCAAG GACTTGAAATCAAAA 60
M S D T S V D A S V E K T T K D L K S K
D. rerio ATGGCTGACACAAAAGTCGATACCAACAAGGACGTCTCCGCAAAG GACCTCAAAGAAAAG 60
M A D T K V D T N K D V S A K D L K E K
EXON 2
human ---AAGGAAGTTGTGGAAGAGGCAGAAAATGGAAGAGACGCCCCTGCTAACGGGAATGCT 117
K E V V E E A E N G R D A P A N G N A
X. laevis GAGAAGGAAGTTGTAGAAGAAGCAGAAAATGGAAAGGACAAGCCTACCAATGGAAAAGCA 120
E K E V V E E A E N G K D K P T N G K A
R. esculenta GACAAGGAACTTGTAGAAGAAACAGAAAATGGCAAGGACAAACCAGCCAATGGGAATGCA 120
D K E L V E E T E N G K D K P A N G N A
D. rerio ---AAACAG---GTTGAGGAGGCGGAAAATGGAAAGGATGCTCCCGCGAATGGAAATGCG 114
K Q V E E A E N G K D A P A N G N A
(gag) EXON 3
human ---AATGAGGAAAATGGGGAGCAGGAGGCTGACAATGAGGTAGACGAAGAAGAGGAAGAA 174
(e) N E E N G E Q E A D N E V D E E E E E
X. laevis GAAAATGAGGAAAATGGAGAGCCAGAGGTAGATAATGAAGGCGACGAAGAAGATGAG--- 177
E N E E N G E P E V D N E G D E E D E
R. esculenta GAAAATGAAGAGAATGGAGAAGATGGGGCAGACAAC------GAGGAAGAGGAAGAA--- 171
E N E E N G E D G A D N E E E E E
D. rerio GAGAACGAGGAGAATGGAGATCAAGAA------AATGAAGTAGACGAGGAAGACGATGAT 168
E N E E N G D Q E N E V D E E D D D
EXON 3 EXON 4
human GGTGGGGAGGAAGAGGAGGAGGAAGAAGAAGGTGAT GGTGAGGAGGAGGGTGGAGATGAA234
G G E E E E E E E G D G E E E G G D E
X. laevis GTAGATGAGGAAGATGAAGAGGATGAA---GTAGAA GGAGAAGATGACGAT------GAC 228
V D E E D E E D E --- V E G E D D D
R. esculenta GTAGATGAGGAAGATGAAGAGGATGAA---GGAGAA GGAGATGATGATGAGGGTGATGAA 228
V D E E D E E D E --- G E G D D D E G D E
D. rerio GTGGCTGAAGAAGATGAGGAGGATGAT---GGAGAA GGTGATGACGATGAT------GAA 219
V A E E D E E D D --- G E G D D D E
EXON 4
human GATGAGGAAGCTGAGTCAGCTACGGGC AAGCGG GCAGCTGAAGATGATGAG---------285
D E E A E S A T G K R A A E D D E
X. laevis GATGATGAAGTAGAGGGAGTCACCGGC AAGAGA GCAGCAGAAGATGATGAGGATGATGAT288
D D E V E G V T G K R A A E D D E D D D
R. esculenta GATGATGAAGCAGATGGAGCCACTGGG AAAAGA GCAGCAGAAGATGACGATGAG------282
D D E A D G A T G K R A A E D D D E
D. rerio GATGAGGAGGCTGAGGGTGGAACGGGA AAAAGA GCGGCTGAGGATGATGATGATGAG279
D E E A E G G T G K R A A E D D D D D E
EXON 5
human GATGACGATGTCGATACC AAGAAGCAGAAG ACCGACGAGGATGACTAG 333
D D D V D T K K Q K T D E D D *
X. laevis GATGATGATGTTGAAATA AAGAAGCAGAAA ACAGATGAAGATGACTAG 336
D D D V E I K K Q K T D E D D *
R. esculenta GATGATGACGTTGATGCA AAGAAGCAGAAA ACAGACGATGACGACTAG 330
D D D V D A K K Q K T D D D D *
D. rerio GATGAC---GTTGATCCT AAGAAGCAAAAG ACTGATGTGTAA 318
D D V D P K K Q K T D V *

PTA AND BINDING PARTNERS

Freire *et al* (Freire, *et al* 2001) used PTA-affinity chromatography and immunoblotting to identify binding partners for PTA in NC37 human B lymphoma cells. Affinity chromatography and immunoblotting identified core histones (H2A, H2B, H3 and H4) in the nuclear membrane, cytosolic and nucleoplasmic fractions while the protein kinase that phosphorylates PTA was found only in the cytosolic fraction (Freire, *et al* 2001). This group had previously demonstrated binding of PTA to all 5 histones (H1, H2A, H2B, H3 and H4) demonstrating greatest affinity of PTA for H3 and H4 (Diaz-Jullien, *et al* 1996). Extending these studies, immunoblotting directed against proteins important in nuclear transport identified strong binding with karyopherin β_1, karyopherin α (both of which stimulate the nuclear transport of proteins containing a NLS), Ran and RCC1, weak binding to NTF2, nucleoporin p62, and heat shock protein 70 and no binding to transportin (which mediates the transport of proteins lacking a NLS), CAS and Ran BP1 (Freire, *et al* 2001). Immunoblotting of cell proliferation-associated proteins showed strong binding to PCNA, cdk2 and cyclin A, weak binding to cdc2, cdk4 and cyclin B and no binding to cyclin D1, cyclin D3, Cip1, and Kip1 (Freire, *et al* 2001). Supporting a role for PTA in chromatin re-modelling and histone binding, nucleosome assembly was induced in the presence of PTA, purified thymocyte cytosol and ATP (Diaz-Jullien, *et al* 1996). Polyglutamic acid also showed some nucleosome assembly activity providing preliminary evidence that the histone binding properties of PTA were due to its acidic region (Diaz-Jullien, *et al* 1996).

In contrast to these findings, chromosomes incubated with PTA underwent decondensation, an effect inhibited by preincubation of PTA with histone H1 but not other basic peptides (Boan, *et al* 2001). Also, stably transfected HL60 cells over-expressing PTA showed increased accessibility to chromatin in micrococcal nuclease digestion assays where it was inferred from the size of the nucleosome multimer that the chromatin was depleted of H1 (Gomez-Marquez and Rodriguez 1998). Other studies using ligand blotting, immunoaffinity chromatography and PTA covalently linked to agarose beads have conflicted with Freire's data (discussed above) and shown that PTA binds histone H1 but not H2A, H2B, H3 and H4 (Karetsou, *et al* 1998, Papamarcaki and Tsolas 1994). Furthermore, PTA could not bind DNA or H1-DNA, and the presence of PTA during chromatin assembly assays did not affect incorporation of H1 into the nucleosome (Karetsou, *et al* 1998). H1 bound to chromatin at physiological levels

could not be dissociated by PTA but if excess H1 was incorporated this could be detached by PTA and PTA also prevented the incorporation of excess H1 in chromatin assembly assays (Karetsou, *et al* 1998). Thus, as a result of these findings it was suggested that PTA may act as an acceptor molecule for H1 stripped from chromatin by other means (Karetsou, *et al* 1998). Supporting these findings, in transiently transfected COS1 cells, PTA did not co-localise with H1, nor strongly with chromatin (Enkemann, *et al* 2000b). There was evidence of some co-localisation with RNA polymerase II while, with proteins involved in transport through the nuclear pore, PTA showed partial co-localisation with Ran and extensive co-localisation with transportin and karyopherin β (Enkemann, *et al* 2000b).

Immunofluorescence labeling and confocal scanning laser microscopy have generated data showing fine punctuate nuclear distribution of PTA with exclusion from the nucleoli and from the chromosomes during mitosis (Vareli, *et al* 2000). These studies also found that PTA formed dense clumps or domains often at the periphery of a nucleolus. Dual labeling showed that the PTA domains did not co-localise with coiled bodies or nuclear domains rich in heterogeneous ribonucleoprotein particles (hnRNP-I) both of which bind pre-mRNA's and assist in processing; but PTA did co-localise with PML bodies and cleavage bodies, the latter rich in proteins involved in cleavage and polyadenylation of RNA (Vareli, *et al* 2000). This data suggested a role for PTA in transcription processes and gene expression, an association strengthened by the finding that PTA colocalised at transcription sites in RNA run-on transcription assays, visualised by the incorporation of bromodeoxyuridine (BrdU) into nascent RNA strands labeled with anti-BrdU antibodies (Vareli, *et al* 2000). PTA has also been found covalently linked to RNA in mouse Krebs II cells (Makarova, *et al* 1989) and to possess multiple binding sites for tRNA at both termini of the protein when rat PTA was produced in *E coli* (Lukashev, *et al* 1999). In support of this data, Trumbore and Berger used run-on transcription assays in nuclei isolated from NIH3T3 and COS-1 cells to show that RNA transcription was enhanced when PTA protein was present (Trumbore and Berger 2000). This effect was not solely dependent on the negatively charged regions of glutamate in PTA, as both polyglutamic acid and a glutamate:alanine:tyrosine polymer failed to enhance transcription, nor was it attributable to PTA targeting a specific polymerase, prompting this group to suggest that PTA acted as a "highly diffusible, concentrated salt" chaperoning positively charged molecules through the

channels of the highly charged nuclear milieu (Trumbore and Berger 2000). The non-specificity of such a role is rather appealing as it accommodates the rather perplexing array of molecules to which PTA seems to bind.

Kubota *et al* (Kubota, *et al* 1995) found that PTA bound the leucine-motif activation domains of HTLV-I Rex and HIV-1 Rev proteins. These viral proteins transport incompletely spliced mRNAs out of the nucleus and their association with PTA suggested an involvement in splicing or nuclear/cytoplasmic trafficking. (see (Chichkova, *et al* 2000, Enkemann, *et al* 2000b, Trumbore and Berger 2000). In contrast, Enkemann *et al* (Enkemann, *et al* 2000b) failed to find convincing evidence of co-localisation of PTA with "speckled domains" in NIH3T3 cells. These domains consist of interchromatin granule clusters (storage areas for splicing factors) and perichromatin fibrils (sites of active transcription) (Spector 1996).

In addressing the manner in which two negatively charged biomolecules (PTA and tRNA) could bind to each other, Chichkova *et al* hypothesized that divalent cations might be required and found that PTA could bind Zn^{2+} and Ca^{2+} but not Mg^{2+}, Ni^{2+} or Co^{2+} (Chichkova, *et al* 2000). Furthermore, PTA binding to the viral protein, Rev, occurred in the absence of zinc ions but was dramatically enhanced in its presence, while Mg^{2+} had no effect and Ca^{2+} an intermediate effect. None of the divalent cations had any enhancing potential on the interaction of PTA with histone H1 (Chichkova, *et al* 2000).

Cotter *et al* (Cotter and Robertson 2000) found that nuclear antigen 3C from Epstein-Barr virus (EBNA3C), an essential protein in the immortalization of EBV infected primary human B-lymphocytes, binds PTA and exists in a complex with PTA, p300 (histone acetyltransferase) and histone H1 in EBV-transformed human B-lymphocytes. They suggest that PTA may be important in recruiting p300 or other histone acetyltransferases to the nucleosome resulting in increased acetylation and transcription of cellular and viral genes in EBV-infected lymphocytes. As a result EBNA3C is expressed and exerts a negative effect on transcription, possibly by displacing p300, PTA or other co-activators (Cotter and Robertson 2000). p300 and CBP (**c**AMP-responsive element-binding protein **b**inding **p**rotein) are highly homologous transcriptional coactivators that are present with RNA polymerase IIa in a subset of PML bodies (von Mikecz, *et al* 2000). Like p300, CBP has intrinsic histone acetylase activity and modulates the activity of many disparate DNA-bound transcription factors,

many of which associate with the protein via its cysteine and histidine (CH) rich domains (Newton, *et al* 2000). The CH domains bind zinc ions in novel modules called "zinc bundles" rather then through any zinc-binding modules characterized in "zinc fingers" (Newton, *et al* 2000). Recently Karetsou *et al* (Karetsou, *et al* 2002) used immunofluorescent labeling and confocal microscopy to show that CBP and PTA co-localised in HeLa cells. GST-pull down experiments with peptides of PTA provided evidence that the polyglutamic acid stretches in PTA mediated the PTA-CBP interaction (Karetsou, *et al* 2002). Transfection of HeLa cells with PTA and a luciferase reporter gene that bound the CBP-dependent transcription factor AP1 showed that PTA increased transcriptional activity in a dose dependent manner (Karetsou, *et al* 2002). Furthermore, PTA and CBP acted synergistically to enhance the transcriptional activity of AP1, and another CBP target, NF-κB, but not MHC class II promoter activity (Karetsou, *et al* 2002). Thus these authors postulated that PTA might be directed to promoter regions by CBP, where PTA could promote chromatin decondensation possibly by interacting with H1, thereby enhancing the access of RNA polymerase II (Karetsou, *et al* 2002).

Overall PTA exhibits considerable heterogeneity in the range of proteins to which it binds and appears to have a non-specific nuclear role promoting transcription, possibly by binding histones and enhancing chromatin accessibility, and/or by facilitating movement of RNA (and other charged molecules) through the nucleus. Undoubtedly as a consequence of its promiscuous nature, several studies have produced conflicting data which has made assigning a definitive function to PTA extremely problematic. As function is derived from structure, ultimately, any assignation of function must accommodate the peculiar features of the PTA protein, including its acidity derived from its polyglutamic and aspartate residues, and its lack of tertiary structure.

PHOSPHORYLATION OF PTA

Initial investigations in PTA identified phosphorylation sites at the N-terminal acetylated serine residue (Sburlati, *et al* 1993) and a threonine (residue 7, 12 or 13) within the first 14 amino acids (Barcia, *et al* 1993). Later work cast doubt on whether either of these were the site of phosphorylation *in vivo* and suggested that they were the by product of hydrolysis that occurred during the purification procedure (Trumbore, *et*

al 1997). It would seem that PTA possesses phosphorylated glutamate residues between amino acids 48-87 and that it is the only mammalian protein known to do so (Trumbore, *et al* 1997). The loss of phosphates during cell lysis cannot be inhibited by the addition of phosphatase inhibitors to the lysing buffer (Trumbore, *et al* 1997), phosphorylation positively correlates with cellular proliferation (Barcia, *et al* 1993) and it has been suggested that lability of the phosphate groups correlates with PTA activity (Tao, *et al* 1999) as the phosphates are energy-rich and thus the protein could supply energy for energy-dependent processes in the nucleus (Tao, *et al* 1999, Trumbore, *et al* 1997, Wang, *et al* 1997). More specifically it has been proposed that hydrolysis or transfer of the phosphate groups to cationic molecules might briefly neutralise their charge and promote movement of macromolecules along highly charged nuclear channels and surfaces (Trumbore and Berger 2000).

REGULATION OF PTA GENE EXPRESSION

The relationship between the c-myc proto-oncogene and PTA has been extensively studied and is somewhat contentious. Eilers *et al* (Eilers, *et al* 1991) found that PTA expression could be regulated in rat fibroblast cells (RAT1A) carrying a construct consisting of the hormone-binding domain of the human oestrogen receptor and the human myc gene (MYC-ER). The product of the chimeric gene was a protein consisting of the oestrogen-binding domain fused to the myc protein which bound oestrogen conferring hormone dependence on the function of the myc protein (Eilers, *et al* 1989, Eilers, *et al* 1991). A subtractive cDNA library was generated from these cells grown to quiescence then split in two, one half induced with oestrogen, the other half with carrier. In this system PTA expression was significantly up-regulated even with low concentrations of oestrogen. PTA gene expression was not induced by the addition of oestrogen to non-recombinant rat fibroblasts. Furthermore, when cells were exposed to cycloheximide, to prevent protein synthesis, then induced with oestrogen, PTA expression was still up-regulated. This data led the investigators to believe that the myc protein directly activated transcription of the PTA gene and that both genes were implicated in proliferative responses in cells.

Further investigations utilising reporter constructs revealed that the PTA promoter was not regulated by c-myc but that an enhancer element (E-box) containing a consensus

myc-binding domain (CACGTG) existed within the first intron of PTA and could mediate a response to myc and max and activate MYC-ER chimeras (Desbarats, *et al* 1996, Gaubatz, *et al* 1994).

In contrast, Mol *et al* failed to find any convincing evidence that E-box elements were necessary for PTA transcription (Mol, *et al* 1995) although they confirmed binding of myc to E-box elements within the promoter region and the first intron. This group created constructs of the entire PTA promoter region and progressive deletions of same to drive expression of chloramphenicol acetyltransferase (CAT) and found that the presence or absence of the E-box had no effect on the expression of the CAT gene. The addition of the first intron of PTA to the CAT construct similarly failed to reveal any dependence on c-myc. A double mutant of the PTA gene with both E-boxes disrupted and co-transfected with a plasmid bearing the myc gene was well-expressed, suggesting that the E-box binding sites were not necessary for PTA transcription.

Recent reports have shown enhancement of oestrogen receptor (ER) transcriptional activity by PTA (Martini, *et al* 2000) and regulation of PTA gene expression by oestrogen in breast cancer cells that express ER (Martini and Katzenellenbogen 2001). PTA bound and sequestered the protein, repressor of ER activity (REA), preventing REA from binding to the oestrogen-ER complex which could then bind co-activators, such as steroid receptor co-activator 1 (SRC-1), and become transcriptionally active (Martini and Katzenellenbogen 2003). Within 1-2 hours of exposure to oestrogen, PTA levels increase in the neuroblastoma cell line SK-ER3 (which expresses the oestrogen receptor) (Garnier, *et al* 1997) and in the ER-positive breast cancer cell lines MCF-7 and MDA-MB-231 (Martini and Katzenellenbogen 2001). The addition of cycloheximide to the breast cancer cell lines did not affect PTA up-regulation indicating a direct effect of oestrogen on PTA transcription. In support of this data it was found that the PTA promoter region contains two consensus half-palindromic repeats (TGACC) of the oestrogen–responsive element (ERE) which mediate PTA gene expression in a dose-dependent manner (Garnier, *et al* 1997); (Martini and Katzenellenbogen 2001); (Martini and Katzenellenbogen 2003). Anti-oestrogens successfully competed with oestrogen to block the induction of PTA (Martini and Katzenellenbogen 2001). In this model of induction of PTA gene expression an ER with an intact DNA-binding domain is essential for transcriptional activation.

The PTA promoter region also contains multiple sites responsive to the nuclear transcription factor, Sp1 (Martini and Katzenellenbogen 2001, Martini and Katzenellenbogen 2003). Transfection studies in Drosophila Schneider cells, deficient in Sp1, and co-transfected with an Sp1 expression vector or an ER expression vector and a PTA-CAT reporter, demonstrated that PTA promoter activity could be stimulated by the oestrogen-ER complex or by Sp1 but when both were present their effects were additive (Martini and Katzenellenbogen 2001). An alternative model of synergistic action between the oestrogen-ER complex and Sp1 has been proposed in which the unliganded ER associates with the DNA-binding domain of the Sp1 protein (Saville, *et al* 2000). In these studies, on addition of oestrogen, reporter gene (CAT) activity significantly increased in several different cell lines assayed. Similar results were obtained with oestrogen antagonists, hydroxytamoxifen and ICI 182,780, which bind the receptor but do not induce its transcriptional activity (this activity was receptor sub-type specific). These results are consistent with a model in which transcriptional activity is transduced by the action of the Sp1 protein binding promoter DNA, but regulated through binding of oestrogen to the ER-Sp1 protein complex. These authors hypothesised a conformational change in the AF-1 domain of the ER occurring upon ligand binding which then permitted interactions with co-activators for transcriptional activity via Sp1 sites (Saville, *et al* 2000).

The fact that oestrogen responsiveness linked to the ER-Sp1 complex, where only Sp1 protein binds DNA, has been demonstrated for several genes (Porter, *et al* 1997, Saville, *et al* 2000), coupled with the finding that the PTA promoter has multiple Sp1 sites responsive to Sp1, introduces the possibility that this pathway, rather than c-myc signalling was responsible for the up-regulation of PTA in transfected rat fibroblasts reported by Eilers *et al* (Eilers, *et al* 1991) (and referred to above). As the rat fibroblasts were engineered to constitutively produce an ER-myc fusion protein it is possible that endogenous Sp1 protein bound the ER domain and that addition of exogenous oestrogen caused up-regulation of PTA expression driven from the Sp1 sites in the PTA promoter. In this way addition of ligand to the cells would have caused direct up-regulation of PTA gene expression without the requirement of any intermediate protein production.

Another gene causing up-regulation of PTA expression is E6 of the human papilloma viruses (HPV's) (Kinoshita, *et al* 1997). In these studies a mouse fibroblast cell line

was transfected with a conditional E6 allele of HPV-16 under the control of the human metallothionein IIa promoter which was induced by heavy metal ions. When ZnSO4 was added to cultures of these cells the induction of HPV-16 E6 caused up-regulation of PTA message. Furthermore, in transient transfections of H358 cells (a p53 deficient human lung cancer cell line), it was shown that an HPV-16 E6 expression vector transactivated a rat PTA-CAT reporter construct, but co-transfection of the E6 vector with a PTA-CAT construct bearing a disrupted myc E-box greatly decreased induction of CAT expression. In this system it would seem that the regulation of PTA was independent of p53 and that the myc E-box was important for PTA regulation. In support of this hypothesis it was demonstrated that in the ion-inducible mouse system, addition of zinc caused up-regulation of c-myc within 45 minutes and that up-regulation of a myc-CAT reporter occurred in H358 cells co-transfected with the E6 vector, suggesting that c-myc regulation was also independent of p53 pathways.

A consensus sequence (TTTGCCGC) which binds the cell cycle transcription factor E2F occurs between -323 and -316 in the 5' flanking region of PTA (Vareli, *et al* 1996). Vareli *et al* (Vareli, *et al* 1996) have shown that transiently transfected NIH3T3 cells co-transfected with a PTA-CAT reporter and an E2F expression vector show strong induction of CAT activity. E2F binding sites have been found in the promoters of genes that are important in the regulation of DNA synthesis and cell proliferation (Nevins 1992), thus implicating a role for PTA in the regulation of these activities.

Thus a variety of proteins appear important in the regulation of PTA expression and several support a role for PTA in augmenting cell proliferation. The regulation of PTA by c-myc has been extensively studied, in part, because a direct interaction between the two is intellectually satisfying, given the association of each with proliferation and differentiation. Although there is conflicting evidence about the direct link, it is undoubtedly true that the two are very closely associated making it likely that they are effectors in the same signal transduction pathways.

REGULATION OF PTA GENE EXPRESSION DURING THE CELL CYCLE

Several authors have investigated the regulation of PTA expression during the cell cycle producing evidence that its transcription is not cell cycle regulated (Zalvide, *et al* 1992); (Bustelo, *et al* 1991), that levels peak during mid-G1 (Szabo, *et al* 1992); (Conteas, *et al*

1990), G1/S transition (Bustelo, *et al* 1991), S/G2 (Vareli, *et al* 1996) or that the protein is active during G2 (Sburlati, *et al* 1991). The discrepancies possibly arise from differences in the models used to analyse proliferation which fall into three broad categories: (i) synchronisation of cells in G0/G1 by serum starvation then stimulating cells to re-commence proliferation; (ii) synchronisation of cells by techniques that do not induce quiescence but permit continuous proliferation; and (iii) mitogen stimulation of cells.

Synchronisation of cells by serum starvation

In some studies where cells have been serum deprived to induce quiescence, PTA mRNA falls to very low levels, then upon serum stimulation mRNA induction is detectable within 3-14 hours depending on the cell line used. In serum-deprived NIH3T3 mouse fibroblasts PTA mRNA levels were barely detectable in the quiescent cells and induced at 10-14 hours (G1/S) after serum stimulation (Eschenfeldt and Berger 1986, Vareli, *et al* 1996, Zalvide, *et al* 1992) peaking during G1/S transition coincident with induction of histone H4 (Zalvide, *et al* 1992) or at the end of S/G2 (estimated by tritiated thymidine incorporation) (Vareli, *et al* 1996). Additionally, in serum starved NIH3T3 cells addition of cycloheximide at the time of serum-stimulation inhibited PTA mRNA production for up to 22 hours. This data suggested that mRNA induction was not a direct effect of serum but required the synthesis of intermediary proteins for the induction of PTA expression (Zalvide, *et al* 1992).

Serum stimulation of quiescent CV1 monkey kidney cells, like that of the fibroblasts, also showed initial very low levels of mRNA, a significant increase at ~15 hours in mid S-phase, peaking at 25-31 hours coincident with cyclin B expression (G2/M). Similarly, U937 cells (human lymphoma) had low but detectable levels of PTA mRNA at the time of serum re-feeding, showed mild mRNA induction at ~3 hours after serum stimulation which was maintained for at least 24 hours, peaking at ~34 hours coincident with peak expression of cyclin B (Vareli, *et al* 1996).

Chemical induction of cell synchronisation

PTA mRNA expression has also been studied in models where the mode of synchronisation permitted the cells to continue cycling rather than inducing quiescence

(nocodazole arrest in metaphase and thymidine double block which synchronises cells at G1/S) (Zalvide, *et al* 1992); (Vareli, *et al* 1996). In contrast to their results using serum starved NIH3T3 cells, Zalvide *et al* found that, in chemically synchronised cells, PTA mRNA levels were invariant throughout the cell cycle (Zalvide, *et al* 1992). Other studies have found maximal levels of PTA expression correlate with the S/G2 phase (Vareli, *et al* 1996).

Mitogen stimulation of cells

Bustelo *et al* (Bustelo, *et al* 1991) used concanavalin A (conA) or the calcium ionophore A23187 to stimulate rat splenic T-cells and found that PTA mRNA was readily detectable throughout the cell cycle with moderate increments of gene expression at the G1/S transition. An *in vivo* model of proliferation with regenerating rat liver produced similar results (Bustelo, *et al* 1991). In contrast, mouse splenic T-cells induced to proliferate via signalling through the T-cell receptor (with anti-CD3 monoclonal antibodies) showed a substantial increase in PTA mRNA in mid-G1 and mouse splenic B-cells induced with LPS showed a similar, though less dramatic, pattern of gene expression (Szabo, *et al* 1992).

PTA protein and cell cycle

Attempts have been made to correlate PTA protein expression with progression through the cell cycle although these are somewhat compromised by a lack of antibodies of sufficient titre and specificity to unambiguously identify PTA. Conteas *et al* (Conteas, *et al* 1990) developed a surrogate PTA ELISA assay which detected thymosin immunoreactive peptides (TIP). They found that stimulation of serum-deprived IEC-6 cells by re-feeding caused levels of TIP to rise more than 3-fold within 1 hour. Correlation of this data with thymidine uptake revealed the peak of TIP levels occurred within 3 hours of re-feeding and well ahead of S-phase which occurred at ~9 hours, suggesting a role for TIP in G1 rather than S-phase.

Vareli *et al* measured thymosin protein levels (the equivalent of TIP) as a surrogate indicator of PTA and also assessed cellular localisation in serum-deprived/serum stimulated NIH3T3 cells (Vareli, *et al* 1996). They found protein levels peaked at the S/G2/M phases of the cell cycle and confirmed the presence of PTA protein in the

nucleus of cells at all stages of the cell cycle, with lowest levels in G0/G1 and highest at S-phase.

Finally, indirect evidence of cell cycle dependent functioning of PTA was inferred from a study of the role of PTA in the cell division of serum starved RPMI 8226 human myeloma cells (Sburlati, *et al* 1991). Anti-sense oligomers were added to stationary phase myeloma cells with fresh medium to induce cell proliferation and oligomer uptake. At the time of addition, all cells had low levels of PTA mRNA. In control cultures (sense oligomers) PTA mRNA was detectable at 6 hours (S-phase) after addition of fresh serum and mitosis occurred at approximately 17 hours (G2/M) and untreated controls behaved in an identical manner. This data indicated that PTA levels were not critical for the transition from quiescence (G0/G1). In cultures containing PTA antisense oligomers, protein was not depleted until ~10 hours after serum stimulation, with lowest levels at ~18 hours (at which time control cells were undergoing mitosis, G2/M). The anti-sense treated cells failed to divide until ~36 hours following treatment. Although not a direct examination of the expression of PTA gene expression throughout the cell cycle, the authors inferred from this data that PTA functioned in G2.

In summary, the results of analyses of PTA regulation in the cell cycle are somewhat contradictory which may reflect the different models of cell proliferation used and also variations in PTA induction in different cell types. For instance Vareli *et al* (Vareli, *et al* 1996) noted lower basal levels of PTA in fibroblast cell lines (NIH3T3 and CV1) compared to lymphoma cells (U937). However, taken together these results indicate that although it is possible to regulate PTA gene expression, in continuously cycling cells PTA mRNA induction is not tightly aligned to any phase of the cell cycle. It would also appear that its induction with serum in quiescent cells is reliant on the production of intermediary proteins.

PTA EXPRESSION AND PROLIFERATION/DIFFERENTIATION EVENTS

Many studies have shown induction of PTA gene expression in proliferating cells although the length of time and degree to which the gene is up-regulated is highly variable. Eschenfeldt and Berger (Eschenfeldt and Berger 1986) found that mitogen stimulated human lymphocytes showed up to a 15-fold increase in gene expression after

72 hours of mitogen treatment (although induction was evident as early as 2 hours) and remained elevated for up to 96 hours. In contrast, others have shown gene induction of shorter duration (~48 hours) and more moderate increase (as high as 6-fold, although more often 2 to 3-fold) in mitogen stimulated rat thymocytes, splenic lymphocytes (Bustelo, *et al* 1991), mouse splenic T-cells stimulated with α-CD3 monoclonal antibody and splenic B-cells stimulated with lipopolysaccharide (Szabo, *et al* 1992). This data indicated a more modest induction of PTA expression following mitogen stimulation and that the results were independent of the type of lymphoid cell analysed.

In models where quiescence has been induced through serum starvation some investigators report marked down-regulation of PTA mRNA in NIH3T3 (Vareli, *et al* 1996, Zalvide, *et al* 1992) and CV1 cells (Vareli, *et al* 1996), while others have noted slight or moderate down-regulation in U937 (Vareli, *et al* 1996) and HL60 cells (Dosil, *et al* 1993).

Physiological models of PTA mRNA up-regulation have also shown a wide range of gene induction. Bustelo *et al* (Bustelo, *et al* 1991) used an *in vivo* rat model of physiological cell proliferation following partial hepatectomy and demonstrated moderate increases in PTA mRNA in regenerating liver, detectable at 6 hours and maximal (2 to 4-fold increase) at 12-32 hours after surgery. Marked proliferation and high expression of PTA were demonstrated in the spatial distribution of PTA in developing mouse embryos coincident with areas of extreme mitotic activity in undifferentiated mesenchyme and the apical ectodermal ridge of the forelimb bud (Franco del Amo and Freire 1995); and high levels of PTA mRNA occurred in primary and secondary spermatocytes during the seasonal spermatogenesis cycle of the frog *Rana esculenta* (Aniello, *et al* 2002). It has also been reported that considerable overlap exists between the tissue specific expression of PTA, c-myc and N-myc in mouse embryos and that, with c-myc particularly, this corresponded with areas of proliferation (Moll, *et al* 1996). Interestingly, in the neocortical layers of the mouse brain, both N-myc and PTA were expressed in differentiating, non-proliferating neurons and in some tissues PTA levels remained high when c-myc levels had declined, possibly reflecting either differences in mRNA stabilities, or a lack of dependence on c-myc for PTA expression (Moll, *et al* 1996).

Studies of malignant cell growth have been informative. PTA mRNA was up-regulated in leukaemic lymphocytes (Gomez-Marquez, *et al* 1989) in hepatocellular cancer (Wu,

et al 1996) compared to normal tissue and in neuroblastoma where levels correlate with that of N-myc (Sasaki, *et al* 2001). Protein levels were higher in colon cancer cell lines compared to normal colon mucosal cells (where it has been suggested that PTA may be a useful biomarker of colon cancer (Shiwa, *et al* 2003), >2-fold higher in intestinal cancer tissue (Tsitsiloni, *et al* 1993) and 6 to 18-fold higher in breast cancer tissue compared to neighbouring healthy tissue (Tsitsiloni, *et al* 1993, Tsitsilonis, *et al* 1998). Patients with tumours with low or moderate PTA levels (<100 ng/mg of protein) had significantly decreased rate of tumour recurrence compared to those with high levels (>100 ng/mg of protein) who had 2.1 times the relative risk of dying (Magdalena 2000). Transgenic mice over-expressing either c-myc, c-neu or v-ras oncogenes and subsequently developing mammary tumours also showed up-regulation of PTA which was not solely dependent on signalling through c-myc, as demonstrated by the up-regulation in the c-neu and v-ras transfected animals (Loidi, *et al* 1999). Furthermore, the down-regulation of c-myc expression with antisense oligomers in the breast cancer cell line MCF7 inhibited cell proliferation but had no effect on PTA mRNA levels (Loidi, *et al* 1999), although again this may reflect greater stability of the PTA mRNA compared to that of c-myc.

The up-regulation of PTA mRNA and protein in malignancy may simply reflect the increased mitotic activity of the malignant clone or may reflect oncogenic properties of the protein. Rat-1 fibroblasts over-expressing PTA showed increased proliferation, loss of contact inhibition, anchorage independent growth, transformed morphology in soft agar colonies and reduced serum reliance (Orre, *et al* 2001) supporting a role for the protein in oncogenesis.

In studies of stably transfected cells it has been shown that over-expression of PTA increases the proportion of cells in S-phase, decreases the proportion in G0/G1 and maintains the proportions in G2/M (Rodriguez, *et al* 1998, Wu, *et al* 1997). Under proliferative stimulants (serum starvation followed by re-feeding and double synchronisation) transformed NIH3T3 cells had a shortened cell cycle time due to a shortened G1-phase (Wu, *et al* 1997), while Rat-1 cells (Orre, *et al* 2001) and HL60 cells (Rodriguez, *et al* 1998) showed a higher multiplication rate. Rodriguez *et al* (Rodriguez, *et al* 1998) transformed HL60 cells with a dexamethasone–inducible vector and examined the effect of over-expression of PTA on differentiation. HL60 cells cultured with dexamethasone (to induce over-expression of PTA) and either PMA or

retinoic acid (to induce differentiation) showed more than 50% inhibition of differentiation compared to controls. Thus, in these models PTA over-expression increased proliferation of the cells and inhibited differentiation.

In support of these data, PTA antisense oligomers inhibited proliferation in RPMI 8226 human myeloma cells (Sburlati, *et al* 1991) and the human promyelocytic cell line HL60 (Rodriguez, *et al* 1999). In the latter study, down-regulation of PTA also triggered apoptosis, an effect not seen in the sense-treated control and thus not attributable to non-specific toxicity of the treatment (Rodriguez, *et al* 1999).

As noted above, many studies have shown proliferating cells express PTA and that it is significantly down-regulated or non-detectable in mature, non-proliferating cells. Immature, non-proliferating cells, such as serum-deprived or hydroxyurea treated HL60 (Bustelo, *et al* 1991, Dosil, *et al* 1993) and U937 cells (Vareli, *et al* 1996), showed only marginal down-regulation of PTA mRNA. In contrast serum-deprived and quiescent NIH3T3 cells, (Vareli, *et al* 1996, Zalvide, *et al* 1992) and CV1 cells (Vareli, *et al* 1996) showed significant down-regulation of PTA expression. Because proliferation and differentiation are so intricately connected and designing experiments to clearly delineate both events is difficult, there has been some debate about whether PTA expression is critical for differentiation or whether it is primarily required for proliferation. In an attempt to resolve this question, gene expression was studied in HL60 cells cultured with IFN-γ to stimulate proliferation concurrent with monocytic differentiation (Smith, *et al* 1993). Under these conditions PTA expression was similar to that of untreated cells supporting the hypothesis that gene expression correlated with proliferative events rather than differentiation (Smith, *et al* 1993). This is consistent with studies showing marked down-regulation of PTA mRNA in differentiated cells where terminal differentiation coincides with a loss of proliferative activity (Dosil, *et al* 1993) and with the data produced by Rodriguez et al showing that induction of PTA expression retarded differentiation (Rodriguez, *et al* 1999).

In IEC-6 cells cultured under normal permissive conditions both the total TIP levels and cell number increased steadily until day 4, at which time both reached a plateau (confluence). Because the rate of increase in cell number was greater than the rate increase in TIP levels, TIP/10^6 cells decreased over the same time and continued to do so until day 6, at which time cells were stimulated by re-feeding. Within 24 hours of re-feeding total TIP levels, TIP/10^6 cells and cell number increased significantly and

continued to increase for 48 hours before reaching a plateau (Conteas, *et al* 1990). This data indicates that, like PTA mRNA expression, PTA protein levels correlate with proliferation, decreasing as cells reach confluence and undergoing marked increase when cells are actively proliferating.

In spite of prodigious efforts over many years it is still unclear whether PTA exerts its main effect on proliferation or differentiation. As the processes of proliferation, differentiation and apoptosis are inextricably bound it is not surprising to find that, in experimental models, it is difficult to unequivocally disentangle these pathways, while in biological systems there is functional overlap of proteins active in these areas. The induction of PTA in many models of cell proliferation provide convincing evidence of its importance in this event, however there is equally persuasive evidence that it functions in differentiation and emerging data supporting a role in apoptosis.

IMMUNOSTIMULATING PROPERTIES OF TFV, THYMOSIN α_1 AND PTA

As discussed in the introduction to this chapter, increasingly sophisticated methods of isolating the active agents in extracts of calf thymus (thymosin) led to the identification of thymosin fraction V (TFV) and thymosin α_1 ($T\alpha_1$). The crude extract, thymosin, stimulated the incorporation of ^{3}H-thymidine into mesenteric lymph node cells *in vitro* and into the axillary, brachial and inguinal lymph nodes of mice *in vivo* (Goldstein, *et al* 1966), activities that, through further refinements, were found to be attributable to TFV and $T\alpha_1$. Thymosin α_1 was found to be 10-1000 times more potent than TFV in E-rosetting assays, stimulating production of migration inhibitory factor (MIF), augmenting mitogen-stimulated proliferation and early T-cell surface marker induction but not mixed lymphocyte reactions (MLR), antibody production or terminal deoxynucleotidyltransferase (TdT) induction assays (Low and Goldstein 1979, Low, *et al* 1979) and about 30 times more potent in its ability to protect mice challenged with *C. albicans* infection (Caldarella, *et al* 1983). Surprisingly, in some instances, bovine TFV but not thymosin α_1 could augment the production of IL-2 by PHA-stimulated lymphocytes from normal donors within 24 hours although the effect was evident in only 48% of donors by 72 hours (Zatz, *et al* 1984). Further investigation of the induction of IL-2 receptors (IL-2R) on PBL stimulated with optimal amounts of PHA (2 μg/ml) showed that induction could be divided into two categories: optimal induction

(essentially defined as the percent of IL-2R$^+$ cells being equal to or better than the overall mean value) and sub-optimal induction. TFV had no effect on the group with optimal IL-2R induction, although it did increase the IL-2R expression in ~62% of donors in the sub-optimal response group increasing both the number of IL-2R$^+$ cells and the receptor density (Sztein, *et al* 1986). When sub-optimal amounts of PHA (0.2 μg/ml) were used TFV had a stimulatory effect on IL-2R response in ~75% of cases, as did $T\alpha_1$. Signaling through the T-cell receptor (TCR) produced similar results with TFV exerting an influence only in those cases where optimal anti-TCR concentrations produced sub-optimal IL-2R responses in the cells, or when sub-optimal doses of antibody where used (Sztein, *et al* 1986). When sub-optimal doses of PHA or anti-TCR antibodies were used the increase in IL-2R$^+$ cells and antigen density were accompanied by increased proliferation of the cells but not increased IL-2 production (Sztein, *et al* 1986).

Employing methods to prevent proteolytic degradation the entire PTA peptide was isolated from rat thymus by Haritos *et al* (Haritos, *et al* 1984a) and found to be a more potent regulator of immunity in mice challenged with *C. albicans* infection than thymosin α_1 (Haritos, *et al* 1985). The wide tissue distribution of PTA led the authors to suggest that it was not a thymic hormone but that it might act as a cytokine released following tissue injury (Haritos, *et al* 1985). Interestingly, human PTA proved to be much less effective than rat PTA in conferring protection on mice challenged with *C. albicans* and in the *in vivo* production of MIF (Pan, *et al* 1986). As PTA was more immunologically active than the shorter $T\alpha_1$, and as rat and human PTA had greatest disparity (16%) in amino acid sequence in the last 37 residues of the carboxyl terminal, the data suggested that the carboxy terminal determined species differences in biological activity.

The non-degraded PTA protein was more effective than $T\alpha_1$ in augmenting the proliferation of human PBMNC stimulated with sub-optimal doses of PHA, but had no effect on the PHA stimulation of purified lymphocytes (Cordero, *et al* 1990, Czarnecki 1995). Optimal proliferative activity was restored when monocytes were added back to the PHA culture of purified lymphocytes and the addition of PTA augmented the response (Cordero, *et al* 1990). Monocyte supernatant had a partially restorative effect but this was not affected by the presence of PTA suggesting that contact between monocytes and PTA was required for cellular proliferation (Cordero, *et al* 1990). The

addition of recombinant IL-1 (rIL-1) and PTA to purified lymphocytes had a synergistic effect, increasing proliferation but less than that of rIL-2 which showed no synergy with PTA and none of these was as effective as monocytes (Cordero, *et al* 1991, Cordero, *et al* 1990, Czarnecki 1995). The PTA/rIL-1 synergy could be abrogated by adding an anti-IL-2 antibody to the culture suggesting that PTA might augment proliferation by modulating IL-2R expression (Cordero, *et al* 1990). With regard to IL-2R expression, PTA increased expression but only in specific sub-groups of PHA-stimulated cells, mimicking the earlier findings with TFV. Thus, PB MNC were stimulated with either an optimal dose of PHA (5μg/ml) or a sub-optimal dose (0.5 μg/ml) and in both cases cells either responded well or poorly. PTA enhanced the lectin stimulated expression of IL-2R only in those cells that responded poorly to optimal stimulation, or to those cells that responded well to sub-optimal lectin stimulation (Cordero, *et al* 1991). The synergistic effect of PTA with PHA was not mediated by IL-2 production (which can up-regulate IL-2R expression) and was accompanied by an increase in cellular proliferation.

Baxevanis *et al* (Baxevanis, *et al* 1990) found that addition of PTA increased IL-2 production and IL-2R expression in optimally stimulated PHA and mixed lymphocyte cultures however, the effect was variable requiring titration of PTA for each donor tested to determine the optimal concentration. PTA (appropriately titrated for each donor or cell line) or IFN-γ (at a constant dose of 100 U/ml) but not $T\alpha_1$ could enhance the expression of MHC Class II surface antigen (HLA-Dr) on HLA-Dr^+ B-cell lines (RAJI, GES, JCOT) and HLA-Dr^{neg} monocytic cell line (U937) and enhanced HLA-Dr expression and mRNA production in human monocytes (Baxevanis, *et al* 1992). The lack of activity of $T\alpha_1$ indicated that the active sequence in PTA was not located within the first 28 amino acids. Mice given intra-peritoneal injections of PTA for 7 days showed up-regulation of mRNA and marginal enhancement of splenic Iα (MHC Class II) antigen expression (Baxevanis, *et al* 1992).

Monocytes cultured with PTA, but not control cultures with added $T\alpha_1$, showed increased RNA synthesis and release of $T\alpha_1$ into the supernatant which could be abrogated by the addition of either actinomycin D or cycloheximide to prevent transcription or translation, respectively (Frillingos, *et al* 1992). The authors suggested that monocytes might have receptors for the carboxy-terminal of PTA, activation of which caused proteolysis and release of $T\alpha_1$, probably from pre-existing intracellular

stores of PTA, in a process requiring RNA and protein synthesis (Frillingos, *et al* 1992). It is pertinent to note that others have found no evidence of PTA or $T\alpha_1$ in the supernatants of a variety of cell lines, including human monocytes (THP1), cultured under normal conditions. Furthermore, using an extraction method that prevented proteolysis, only PTA, not $T\alpha_1$ was detected inside the cell (Vareli, *et al* 1996).

PTA increased the oxidative response of neutrophils that were stimulated with sub-optimal doses of phorbol dibutyrate $P(Bu)_2$, but had no effect on optimally stimulated neutrophils (Wykretowicz 1994). It also effected a slight but statistically significant increase in the adherence of neutrophils to HUVEC (Wykretowicz 1994).

Recombinant protein was shown to be as effective as native PTA in stimulating proliferation in mouse thymocytes cultured with Con A or in augmenting E-rosetting and phagocytosis by peritoneal mouse macrophages (Evstafieva, *et al* 1995).

PTA has been shown to partially restore the defective *in vitro* immune responses of patients with cancer and systemic lupus erythematosis (SLE). Garbin *et al* (Garbin, *et al* 1997) found that patients with colorectal cancer had impaired anti-tumour responses when their PB MNC were challenged with SW620 carcinoma cells compared to normal controls. Pre-incubation of monocytes with PTA or interferon-γ (IFN-γ) or both significantly augmented the anti-tumour activity of both cancer and normal donors, such that the effect of PTA and IFN-γ > IFN-γ > PTA > media alone. However, the synergistic effect of PTA and IFN-γ on the anti-tumour activity of PB MNC from colon cancers did not restore their activity to that of PB MNC from normal donors incubated in media alone (Garbin, *et al* 1997). TGF-β decreased the anti-tumour activity of both normal donors and colon cancer patients and this effect was ameliorated by the presence of PTA in both groups (Garbin, *et al* 1997). PTA or IFN-γ alone, and in combination with IFN-γ, was able to stimulate TNF-α and IL-1β production and reduce the secretion of prostaglandin E2 (PGE_2) and TGF-β. The effects were generally more pronounced in PB MNC from patients compared to normal donors and of the patients, those who had had chemotherapy tended to have greater impairment of monocyte function and were more responsive to treatment. In patients with a variety of solid tumours the addition of PTA (the concentration optimized for each individual) increased cell mediated lympholysis (CML), MLR and NK activity, in part, by increasing IL-2 production and reducing PGE_2 (Baxevanis, *et al* 1993). Interestingly, PTA was found to increase the activity of purified NK cells from normal donors, not by increasing secretion of IL-2,

but by facilitating cytoskeletal re-arrangement (thought to relate to granule release), increasing expression of p70 IL-2R and increasing internalization of IL-2 (Cordero, *et al* 1992).

PTA (titrated for each individual) increased the MLR of SLE patients to both auto- and allo-antigens and this effect was greatest amongst those who had active disease (Baxevanis, *et al* 1987). Normal controls showed no significant enhancement.

In general the immuno-potentiating effects of PTA are most effective in situations of significant defective cellular immunity or contrived instances of sub-optimal *in vitro* stimulation. PTA alone does not exert an effect but augments the action of other immuno-regulators of PB MNC. The target cell appears to be the monocyte with PTA potentiating proliferation, IL-2R and HLA-Dr expression. It should also be noted that the effect of PTA pre-supposes a mechanism for stimulating monocytic activity which presumably involves binding to a receptor. This concept is somewhat contradictory for a molecule which has been found in every cell type tested, which does not have a recognized secretory signal and which has a nuclear site of action.

PTA RECEPTORS

Despite lacking a recognised secretory signal PTA has an apparent biological activity as an immuno-regulator when assayed under *in vivo* and *in vitro* conditions. Although it is a poorly immunogenic molecule which has hampered efforts to produce high titre specific antibodies, within the last ten years several papers have been published describing receptors for PTA on PB MNC (Cordero, *et al* 1994), and lymphocytes (Cordero, *et al* 1996, Pineiro, *et al* 2001). Cordero *et al* (Cordero, *et al* 1994) exposed PB MNC to iodinated PTA protein and separated the cell-bound radioactivity from free radiopeptide by centrifuging the cells through cold FCS and then measured the radioactivity of the cell pellet in a gamma counter. They also performed equilibrium and competitive binding assays and their data indicated the presence of two receptors: a high affinity receptor with 883-2,257 sites per cell and a low affinity receptor with 42,822-58,457 sites per cell. Extending this work Pineiro *et al* (Pineiro, *et al* 2001) cross-linked ^{125}I-PTA to PHA-activated lymphocytes and isolated a major complex of ~36 kDa and a minor complex of ~45 kDa. To further characterise the receptor-ligand complex a biotinylated PTA was crosslinked to cells and revealed in addition to the 36

and 45kDa complexes three other bands of mass 43, 57 and 75 kDa. From this data the authors surmised that, assuming a one-to-one stoichiometry of protein to receptor, the receptor was composed of three molecules with masses of ~31, 29 and 22 kDa (the minor bands of 75 and 57 kDA were assumed to be accessory or non-specific proteins). Competitive binding assays with native PTA displaced the biotinylated protein from PHA-activated lymphocytes and affinity chromatography of membrane extracts confirmed the detection of three bands with molecular mass of 31, 29 and 19 kDa, thus supporting the assumption of the one-to-one stoichiometry of the receptor-ligand complexes.

PTA AND CELL DEATH

Apoptosis or programmed cell death is the process whereby, in response to a death signal, cytochrome c is released from the mitochondria into the cytosol where it binds apoptotic protease activating factor 1 (APAF1), inducing a conformational change that permits stable interaction with dATP/ATP. This in turn drives assembly of the APAF1-cytochrome c monomers into the heptameric apoptosome which binds and cleaves procaspase-9 to caspase-9. The enzyme activates caspases-3, -6 and -7, then degrade many intracellular substrates and eventually lead to cell death. Coincident with these processes are the hallmark physiological features of apoptotic cells, namely, chromatin condensation, cytoplasmic shrinkage, plasma membrane blebbing and phosphatidylserine exposure which promotes phagocytosis of the dead/dying cell (Jiang and Wang 2000, and reviewed in Leist and Jaattela 2001, Wang 2001).

In the last decade several papers have been published associating PTA with the apoptotic process. Furuya and Isaacs (Furuya and Isaacs 1993) used castrated rats as a model of programmed cell death, with testosterone replacement one week later to stimulate proliferation, and studied changes in the regulation of several genes, including PTA and c-myc. C-myc expression was enhanced in both the apoptotic phase and androgen induced proliferation of prostatic glandular cells whereas PTA expression was enhanced only during prostatic cell death (Furuya and Isaacs 1993). They hypothesised that due to its high negative charge PTA might have a role in DNA fragmentation by binding to and unwinding genomic DNA thus increasing its accessibility to endonucleases (Furuya and Isaacs 1993). Likewise, induction of apoptosis by retinoic

acid exposure to sensitive T-cell lymphoma cell lines revealed that PTA was up-regulated within 6 hours of treatment (Wang, *et al* 2000). The morphologic features of apoptosis (cell shrinkage, chromatin condensation, cytoplasmic blebbing) became evident at ~12 hours of treatment, flow cytometric analysis of DNA revealed a normal profile for the first 9 hours followed by a sub-G1 peak, typical of apoptotic cells and the disappearance of the G2M peak by 12 hours, DNA laddering was faint at 3-6 hours but readily recognisable at 12 hours. Studies with cycloheximide to prevent the synthesis of apoptosis-related proteins could inhibit apoptosis for up to 6 hours as measured by the appearance of DNA fragmentation ladders, however addition of cycloheximide after 12 hours of RA treatment was ineffective, indicating that this time corresponded to the phase of irreversible commitment to programmed cell death. The up-regulation of PTA at 6 hours thus coincided with an early stage of apoptosis (Wang, *et al* 2000).

In two independent studies apoptosis was induced by a variety of methods in HeLa-B cells, a sub-line of HeLa S3 cells (Evstafieva, *et al* 2000) and in HeLa S3 (Enkemann, *et al* 2000a) where, along with the cardinal features of programmed cell death, PTA protein underwent cleavage by caspase-3 and -7 but not caspase-8 (Enkemann, *et al* 2000a, Evstafieva, *et al* 2000, Evstafieva, *et al* 2003) or -6 (Enkemann, *et al* 2000a). Both groups found that (1) the site/s of cleavage corresponded to the caspase cleavage recognition site designated by the amino acid motif D-x-x-D located in the carboxyl end of the protein bisecting the NLS; (2) mass spectrometry of the truncated protein and its tryptic peptides confirmed they were derived from PTA; and (3) the truncated protein re-located to the cytoplasm of the affected cells (Enkemann, *et al* 2000a, Evstafieva, *et al* 2000, Evstafieva, *et al* 2003). In addition, the truncated protein was distinctly under-phosphorylated (Enkemann, *et al* 2000a). The data indicate that the de-phosphorylation of the protein was an early event in the apoptotic process compromising its function and normal cellular location (Enkemann, *et al* 2000a) raising the possibility that fragmentation of the protein might be a first step in converting the nuclear protein into an externalised bioactive peptide, although the physiological significance of such a process was and is unknown (Evstafieva, *et al* 2000). Evidence supporting the externalisation of the truncated protein was recently reported when surface labelling with fluorescent anti-PTA monoclonal antibodies produced a positive signal in apoptotic, but not normal, Hep2 and HeLa-B cells (Evstafieva, *et al* 2003). Significantly, considerable overlap was observed between the anti-PTA labelling and

that of phosphatidylserine, a specific marker of apoptotic cell death (Evstafieva, *et al* 2003).

There is evidence that the up-regulation of PTA may have a protective (anti-apoptotic) effect as PTA appears to inhibit apoptosome formation thus preventing the activation of caspase-9 and ensuing cascade of effector caspases-3, -6 and -7 which ultimately cause apoptotic cell death (Jiang, *et al* 2003). When HeLa cells were depleted of PTA mRNA by RNA interference the cells became more sensitive to UV-induced apoptosis exhibiting ~70% cell death compared to 25% in controls, and this correlated with increased caspase-3 activity (Jiang, *et al* 2003). A small molecule α-(trichloromethyl)-4-pyridineethanol (PETCM) antagonised the effect of PTA thus promoting programmed cell death (Jiang, *et al* 2003). Also, treatment of HL60 cells with PTA anti-sense oligomers induced apoptosis after 3 days of culture (Rodriguez, *et al* 1999) and over-expression of wild-type PTA or various mutants either resistant to caspase cleavage or lacking a NLS and thus located in the cytosol, reduced caspase activation and chromatin condensation in HeLa-B cells (Evstafieva, *et al* 2003). Somewhat surprisingly the PTA mutants sensitive or resistant to caspase cleavage with either nuclear or cytoplasmic localization were equally effective in their anti-apoptotic effects.

In contrast to these anti-apoptotic activities, *in vitro* both polyglutamate and truncated PTA can bind cytochrome c severely restricting its anti-oxidant activity by curtailing its role in the respiratory chain, preventing the conversion of O_2 to H_2O. As a result the concentrations of intracellular O_2 and reactive oxygen species increase, enhancing pro-apoptotic processes. However, physiological levels of KCl and $MgCl_2$ abrogate this effect by preventing formation of the cytochrome c –truncated PTA complex thus making it difficult to assign an effective physiological role to this interesting *in vitro* phenomenon (Markova, *et al* 2003).

The apparent contradictory effects of PTA in the apoptotic pathway may reflect a dose dependent role, such that, below a critical level PTA may bind cytochrome c abrogating its role in the respiratory conversion of O_2 to H_2O thus promoting oxidation and apoptosis. Conversely, above a critical level truncated PTA binding cytochrome c might sequester the molecule from APAF1 preventing apoptosome formation (Markova, *et al* 2003). Additionally or perhaps alternatively, cellular localization may determine the function of the protein, such that in non-apoptotic cells PTA may primarily function in proliferation, whereas caspase-cleavage of the protein and subsequent relocalisation

to the cytosol followed by externalization may trigger immune recognition and phagocytosis in a manner similar to that of phosphatidylserine (Evstafieva, *et al* 2003).

Aside from programmed cell death, other common pathways of cell death exist, including that of autophagic cell death (ACD). The characteristics of the alternative death pathways are less well known but do not involve caspase activation and generally lack some features of classical apoptosis such as tight chromatin condensation, DNA laddering and cytoplasmic membrane blebbing (Leist and Jaattela 2001). PTA is up-regulated in the neurons of transgenic mice affected with Huntington's disease, a condition marked by slow, non-apoptotic neurodegeneration caused by the production of a toxic, mutant protein containing many glutamine residues (Iannicola, *et al* 2000). Speculation has arisen that in this disease up-regulation of PTA may suppress caspase-dependent death but may also eventually switch the mode of cell death from apoptosis to autophagy by binding cytochrome c and promoting its pro-oxidant features (Piacentini, *et al* 2003).

DISCUSSION

PTA is in many respects a difficult entity with which to work; its structure has been known for almost 20 years but its function and regulatory pathways are not well understood. Its extremely acidic properties, negative charge and lack of tertiary shape make it a promiscuous binding partner with conflicting accounts of its primary nuclear sites of action. Then again the degree of evolutionary conservation of these unusual traits make it likely that its role in cellular metabolism is essential and perhaps lies at the hub of many diverse pathways that require a common step involving a protein able to act promiscuously in the maze of cell signalling.

Transfection studies indicate that over-expression of PTA increases the proportion of cells in S-phase, decreases the proportion of cells and time spent traversing G0/G1 (Rodriguez, *et al* 1998, Wu, *et al* 1997) and inhibits differentiation (Rodriguez, *et al* 1998). These data are consistent with anti-sense studies where down regulation inhibits proliferation (Rodriguez, *et al* 1999, Sburlati, *et al* 1991). Clearly PTA functions in promoting proliferation rather than differentiation and it does so possibly through an involvement in promoting chromatin re-modelling and nucleosome assembly (Diaz-Jullien, *et al* 1996) or by sequestering histone H1 thereby increasing DNA accessibility

for transcriptional purposes (Boan, *et al* 2001, Gomez-Marquez and Rodriguez 1998, Karetsou, *et al* 1998). It may also have a more direct role in transcription through its interaction with the histone acetyltransferases and transcriptional co-activators CBP and p300, possibly also RNA polymerase II. Yet it seems also to have a role in the antithesis of these processes, cell death. The gene is up-regulated in apoptotic (Furuya and Isaacs 1993, Wang, *et al* 2000) and non-apoptotic (Iannicola, *et al* 2000) cell death and in various experimental models it can exert either anti- (Evstafieva, *et al* 2003, Jiang, *et al* 2003, Rodriguez, *et al* 1999) or pro-apoptotic (Markova, *et al* 2003) effects. This contrary behaviour may reflect a functional dose-dependence (Markova, *et al* 2003) and/or location-dependence (nuclear vs cytosol) (Evstafieva, *et al* 2003) and/or a switching mechanism between competing cell death programs (apoptosis vs autophagy) (Piacentini, *et al* 2003).

Early in apoptosis the protein undergoes caspase cleavage and re-locates to the cytosol (Enkemann, *et al* 2000a, Evstafieva, *et al* 2000, Evstafieva, *et al* 2003) where it is externalised on apoptotic cells (Evstafieva, *et al* 2003). This is as yet the only experimental evidence of externalisation of the protein which otherwise lacks any classical secretory signal (Eschenfeldt and Berger 1986, Goodall, *et al* 1986). Its externalisation in apoptosis is similar to that of phosphatidylserine and may trigger phagocytosis by the immune system (Evstafieva, *et al* 2003). Interestingly and somewhat suggestively, receptors for PTA have been described on PB monocytes (Cordero, *et al* 1994) and lymphocytes (Cordero, *et al* 1996, Pineiro, *et al* 2001).

Immunomodulatory effects have been attributed to PTA protein in both *in vivo* mouse models (Caldarella, *et al* 1983, Goldstein, *et al* 1966, Haritos, *et al* 1985) and *in vitro* assays of normal human PB MNC (Baxevanis, *et al* 1990, Cordero, *et al* 1990, Czarnecki 1995); and neutrophils (Wykretowicz 1994); immune impaired PB MNC (Baxevanis, *et al* 1987, Garbin, *et al* 1997) HLA-Dr $^{+ \text{ and } -}$ cell lines (Baxevanis, *et al* 1992); and normal mouse thymocytes (Evstafieva, *et al* 1995). The immuno-potentiating effects of PTA are quite subtle and require either titration of the protein to determine an effective dose for each and every donor blood or cell line (Baxevanis, *et al* 1990, Baxevanis, *et al* 1987, Baxevanis, *et al* 1992) or sub-optimal stimulation of cells and dissection of the results into responders and non-responders (Cordero, *et al* 1991, Cordero, *et al* 1990, Czarnecki 1995, Wykretowicz 1994). Furthermore, in patients with significant immune impairment its immune-potentiating effectiveness is generally

inversely correlated with baseline cellular responsiveness (Baxevanis, *et al* 1987, Garbin, *et al* 1997). The externalisation of the truncated protein during tissue injury and apoptosis may provide a mode of egress for the peptide where it might act as a cytokine as was suggested by Haritos *et al* almost 20 years ago (Haritos, *et al* 1985), however due to its ubiquity across tissue and cell types it is doubtful that its subtle enhancement of immune responses is the primary role of PTA in cells of the immune system.

PTA and haemopoiesis

We have found that PTA gene expression is up-regulated in CB $CD34^+$ cells cultured for 24 hours with either cord or adult sera compared to their $CD34^-$ counterpart and also in BM $CD34^+$ cells cultured in cord sera. In uncultured $CD34^+$ cells PTA gene expression is up-regulated in both CB and BM, a finding that has been confirmed independently by other groups in mouse foetal liver (Phillips, *et al* 2000), mouse bone marrow hemopoietic progenitors (Park, *et al* 2002) and human adult BM $CD34^+$ cells (Gu, *et al* 2000). To date no further assessment of the role or regulation of PTA in haemopoietic cells has been published. Given its association with promoting proliferation, retarding differentiation and its ambiguous role in apoptosis the up-regulation of PTA in haemopoietic progenitors raises the intriguing possibility that it may have a fundamental role in maintaining/regulating the haemopoietic stem and progenitor cell pool. These attributes justify further evaluation of the protein and gene and the regulation of its expression and function in immature and mature subsets of haemopoietic cells.

There is evidence that PTA is regulated by c-myc in some models (Desbarats, *et al* 1996, Eilers, *et al* 1991, Gaubatz, *et al* 1994) and the expression of both genes correlates in embryogenesis (Moll, *et al* 1996). This coincidence of expression and suggestive regulatory role of c-myc is particularly relevant to haemopoiesis and HSC as c-myc plays a crucial role in haemopoietic homeostasis, cell cycle regulation, differentiation, apoptosis and adhesion (reviewed in Hoffman, 2002). C-myc must be repressed for terminal differentiation as does, apparently, PTA (Rodriguez, *et al* 1998) and dysregulation of c-myc expression is associated with haematological malignancies (reviewed in Hoffman, 2002). It is thus relevant to investigate the association of c-myc and PTA expression in HSC to supplement similar research in other cells systems.

Finally, in light of existing evidence regarding receptors for PTA on PB MNC and the monocyte being a likely target of PTA when added to culture, it is pertinent to note that $CD14^+$ monocytes are thought to be precursors of osteoclasts (Nicholson, *et al* 2000), cells that function in remodelling bone. Recently published data indicate that human CFU-GM derived from CB MNC have a high osteoclastogenic potential that is enhanced by short term exposure to GM-CSF (Hodge, *et al* 2004). It is thus worthwhile to investigate the effect of PTA on the osteoclastic potential of CB-derived CFU-GM to determine if the protein has any modulating activity.

Therefore the aims of this project were:

1. To examine the regulation of PTA gene expression by single and multiple growth factors known to stimulate expansion or differentiation in CD34+ cells;
2. To compile a comprehensive profile of PTA gene expression in a variety of mature unstimulated and activated haemopoietic subsets;
3. To monitor the expression of c-myc to determine its association with PTA expression in a diverse range of conditions;
4. To assess the receptor profile of PTA in haemopoietic cells from CB and adult PB;
5. To assess the effects of addition of PTA to the culture of cells using osteoclastogenesis from cytokine stimulated CFU-GM as a model of haemopoietic cell proliferation and maturation.

CHAPTER 5

GENE EXPRESSION STUDIES IN CD34$^+$ CELLS

INTRODUCTION

Haemopoietic progenitors as defined by the CD34$^+$ phenotype are a clinically and biologically important population of cells that are multipotent and possess a great capacity for proliferation. They are an inherently quiescent population of cells with ~88% of BM and ~96% of CB CD34$^+$ cells residing in the "resting" G0/G1 phase of the cell cycle (De Bruyn, *et al* 2000, Gothot, *et al* 1997, Ladd 1997, Lucotti, *et al* 2000). In the literature PTA expression is associated with active proliferation and its down-regulation correlates with the decline in proliferation that occurs with terminal differentiation (Dosil, *et al* 1993, Smith, *et al* 1993). Some data suggests that down-regulation of PTA is necessary for differentiation to proceed (Rodriguez, *et al* 1998). As previously noted, aside from our own findings, three other groups (Gu, *et al* 2000, Park, *et al* 2002, Phillips, *et al* 2000) have identified up-regulation of PTA expression in human and murine haemopoietic progenitor cells, however, there are no published data concerning the regulation of PTA gene expression in this important subgroup of cells. Therefore, the aims of this series of experiments were to establish an expression profile of PTA in CD34$^+$ subsets and in response to cytokine stimuli. Specifically, three areas were identified for analysis: gene expression in a primitive subset of CD34$^+$ cells; gene regulation in CD34$^+$ cells cultured in conditions designed to promote proliferation and/or differentiation; and, gene regulation in response to single cytokine stimuli.

Gene expression in primitive subsets of CD34$^+$ cells

A number of surface markers can be used to identify primitive subsets of haemopoietic progenitors within the CD34$^+$ population. One that is frequently used is the CD38 antigen, an activation marker, that is absent on primitive progenitors (CD34$^+$CD38$^-$) (de Wynter, *et al* 1999). Less than 10% of CD34$^+$ cells lack the CD38 antigen, but the CD34$^+$CD38$^-$ fraction possesses extensive proliferation capacity and can produce sequential generations of blast cell colonies from single CD34$^+$CD38$^-$ cells, a property

that is lost with increasing CD38 antigen density (Terstappen 1991). In accord with the extremely primitive phenotype, the $CD34^+CD38^-$ fraction is also more quiescent than the total $CD34^+$ population with more than 98% of CB and more than 96% of BM $CD34^+CD38^-$ cells in G0/G1 (De Bruyn, *et al* 2000, Reems and Torok-Storb 1995). We sub-fractionated the $CD34^+$ population in order to assess the expression of PTA in a distinct subset of highly quiescent, primitive, haemopoietic progenitors.

Gene regulation in $CD34^+$ cells in conditions designed to promote proliferation and/or differentiation

The strong association in the literature of induction of PTA mRNA in response to proliferative stimuli prompted a series of experiments to assess regulation of gene expression in proliferating $CD34^+$ cells. $CD34^+$ cells were cultured with combinations of cytokines and gene expression was assessed at two time-points, 7 days and 24 hours. The 7 day culture was designed to assess gene regulation in cultures that had undergone significant differentiation, while the shorter time-point was designed to analyse acute gene regulation. The combinations of cytokines were well defined with respect to their ability to promote proliferation and differentiation and, in select experiments, also to inhibit expansion of progenitors.

Gene regulation in response to single cytokine stimuli

The cytokines involved in haemopoiesis display a marked degree of redundancy, in that, the formation of most colony types can be stimulated by more than one growth factor and the action of growth factors is usually not restricted to a single type of target cell (Metcalf 1993). For example, differentiating neutrophilic/granulocytic colonies can be stimulated by G-CSF, GM-CSF, IL-3 and SCF; and, IL-3 exerts an effect on precursors of erythrocytes, granulocytes, megakaryocytes and mast cells (Metcalf 1993). When growth factors are combined the synergies involved increase the complexity of the ensuing molecular signalling and the magnitude of the response. With respect to the interpretation of colony forming assays, Metcalf has suggested one strategy to reduce the complexities of synergism and redundancy of action is to assess the effects of single regulators acting alone (Metcalf 1993). We applied the same strategy to assess the ability of single cytokines to modulate PTA expression. The single growth factors

examined (SCF, IL-3, GM-CSF and F3L) have been shown to promote viability and/or cell division and/or differentiation and we wished to determine whether these outcomes where in part, a result of modulation of PTA expression.

RESULTS

PTA gene expression in the $CD34^+CD38^-$ subset

$CD34^+$ cells that are negative for CD38 are a primitive subset of haemopoietic progenitors (De Bruyn, *et al* 1995, Fritsch, *et al* 1996, Ng, *et al* 2002, Payne, *et al* 1995, Theilgaard-Monch, *et al* 1999). They are highly quiescent, but when induced to cycle have a prodigious output as measured by the LTC-IC and CFU assays. We had found that PTA was highly expressed in uncultured, $CD34^+$ cells and wished to assess its expression in a more primitive subset of progenitor cells. $CD34^+$ cells from CB were MACS purified, labelled with CD34-PE and CD38-FITC and enriched for the $CD34^+$ $CD38^-$ population using the concentrator module on a FACSCalibur flow cytometer. Figure 5.1 depicts the modified ISHAGE gating strategy that was used to isolate $CD34^+CD38^-$ cells.

The results of the real time PCR are depicted in Figure 5.2. The $CD34^+CD38^-$ sorted subsets did not express PTA as highly as the total $CD34^+$ population; however this difference was not significant. In contrast, both the total $CD34^+$ fraction and the sorted subset exhibited significantly higher expression compared to the $CD34^-$ population (ANOVA $p \leq 0.002$, Tukey's HSD: $CD34^+$ v $CD34^-$, $p \leq 0.002$; $CD34^-$ v $CD34^+CD38^-$, $p \leq 0.023$; $CD34^+$ v $CD34^+CD38^-$, NSD).

Figure 5.1 Modified ISHAGE gating protocol for selection of $CD34^+CD38^-$ cells.

Magnetically selected $CD34^+$ cells were labelled with CD34-PE and CD38-FITC and the $CD34^+CD38^-$ subset (CD34 sort) selected using the concentrator module of a FACSCalibur flow cytometer. Dot plots depict the sorting strategy based on a modified ISHAGE gating protocol.

Dot plot A: Forward scatter (size) v side scatter (granularity). Total MACS purified cells displayed. R4 (determined from dot plot E defines the lymphocyte area.

Dot plot B.: CD45 (white blood cells) v side scatter. Total MACS purified cells displayed. R1 defines $CD45^+$ white blood cells.

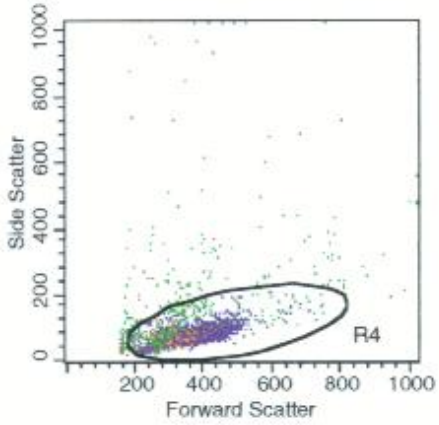
Dot plot C.: CD34 (haemopoietic progenitors) v side scatter. $CD45^+$ (R1) white blood cells only displayed. R2 defines $CD34^+$ haemopoietic progenitors.

Dot plot D.: CD45+ v side scatter. Combination of R1 and R2 displayed, $CD34^+$ white blood cells. R3 defines $CD45^{lo}$ side $scatter^{lo}$ progenitors.

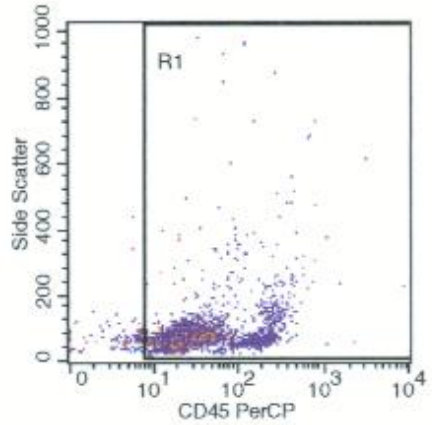
Dot plot E.: Forward scatter v side scatter. Combination of R1+R2+R3 displayed. R4 defines lymphoid area, excluding debris.

Dot plot F.: CD38 v CD34. Combination of R1+R2+R3+R4 displayed. R5 defines the sort gate used to select $CD34^+/CD38^-$ progenitors.

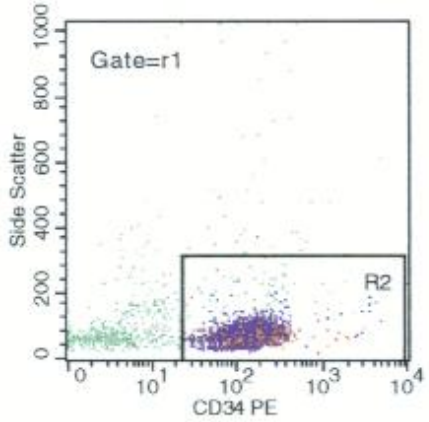
A.


Side Scatter
Forward Scatter
R4

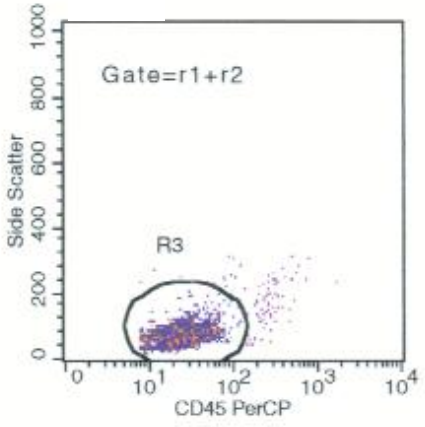
B.


Side Scatter
CD45 PerCP
R1

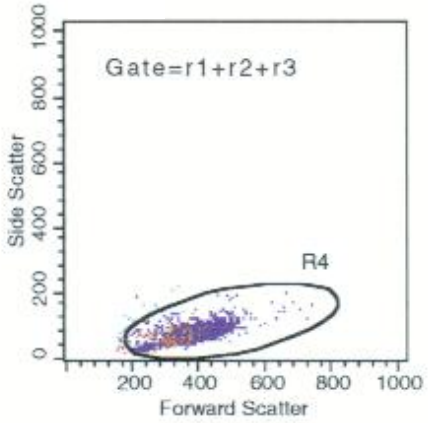
C.


Gate=r1
Side Scatter
CD34 PE
R2

D.


Gate=r1+r2
Side Scatter
CD45 PerCP
R3

E.


Gate=r1+r2+r3
Side Scatter
Forward Scatter
R4

F.

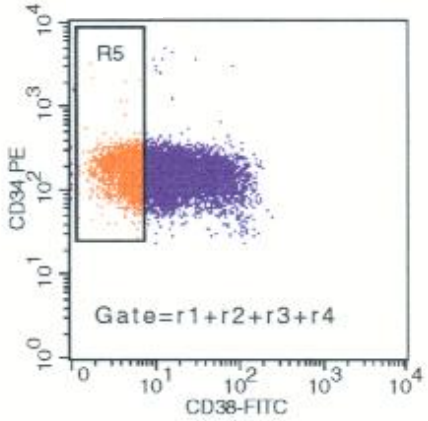
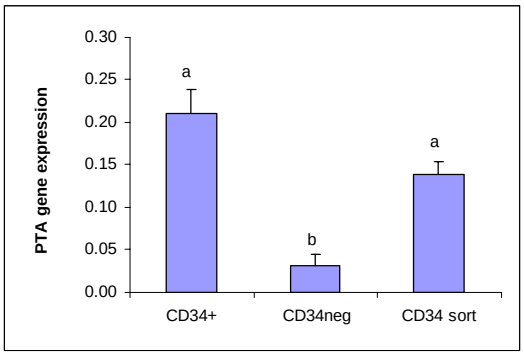

R5
CD34 PE
Gate=r1+r2+r3+r4
CD38-FITC

Figure 5.2 PTA gene expression in CB $CD34^{+}CD38^{-}$ cells.

PTA gene expression was assessed by real time PCR in magnetically selected $CD34^{+}$ and $CD34^{-}$ cells and in sorted $CD34^{+}CD38^{-}$ cells (CD34+ sort) from cord blood. Mean PTA gene expression is shown in arbitrary units relative to β-actin. Groups are "lettered" to indicate similarity in gene expression.



ANOVA, $p \leq 0.002$

Ex vivo proliferation and differentiation of $CD34^+$ cells cultured with multiple cytokines for 7 days

We had found that PTA, a gene associated with active proliferation, was highly expressed in $CD34^+$ cells compared to $CD34^-$ cells in both CB and BM. Published data indicated that PTA mRNA levels were usually low in quiescent cells and strongly induced in proliferating cells (Eschenfeldt and Berger 1986, Vareli, *et al* 1996, Zalvide, *et al* 1992). To assess gene regulation in proliferating haemopoietic progenitors, CD34 enriched cells were cultured for one week in a combination of SCF, G-CSF, IL-1β, IL-6 and IL-3, a combination designed to potentiate proliferation and differentiation. Gene expression was assessed using real-time PCR.

As illustrated in Table 5.1, after one week in culture the number of $CD34^+$ cells decreased significantly in both CB and BM reflecting the process of differentiation apparent in the cultured samples (Paired Samples Test, 2-tailed, $p \leq 0.001$ for both). The total cell number increased, however the change reached significance in CB only ($p \leq 0.03$).

Figure 5.3 depicts the effects on PTA gene expression of long-term exposure to this combination of growth factors. In both CB and BM, the initially high levels of PTA expression dropped after one week in culture to levels similar to that in the $CD34^-$ population (Paired Samples Test, 2-tailed CB $p \leq 0.006$ and BM $p \leq 0.001$).

Table 5.1 Change in phenotype and number of cells after one week in culture.

$CD34^+$ cells were cultured for 7 days at a concentration of 5 x 10^5 cells/ml in α-MEM, 20% FCS, 2mM L-glutamine, P/S, SCF, IL-1β, IL-3, IL-6 and G-CSF. $CD34^+$ enumeration was performed by flow cytometry. Cell number and viability was assessed by trypan blue staining.

	Time	**CB (n=14)**	**BM (n=11)**
$CD34^+$ %	**Day 0**	56 +/- 1.4	60 +/- 6
	Day 7	33 +/- 3.3[#]	40 +/- 40[#]
Viability %	**Day 0**	94	94
	Day 7	92	90
Cell No x10^6	**Day 0**	2.08 +/- 0.5	0.62 +/- 0.2
	Day 7	2.21 +/- 0.5[$]	0.87 +/- 0.2[*]

[#] $p \leq 0.001$

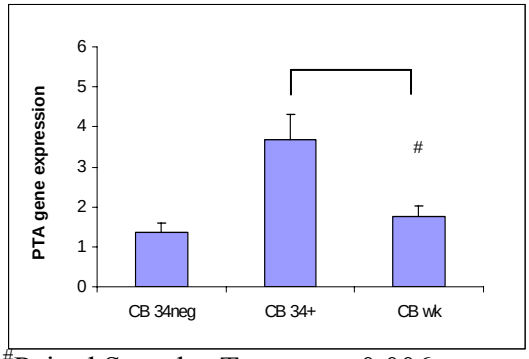
[$] $p \leq 0.003$

[*] NSD

Figure 5.3 Mean PTA gene expression (relative to β-actin) in $CD34^+$ cells from CB and BM after one week in culture.

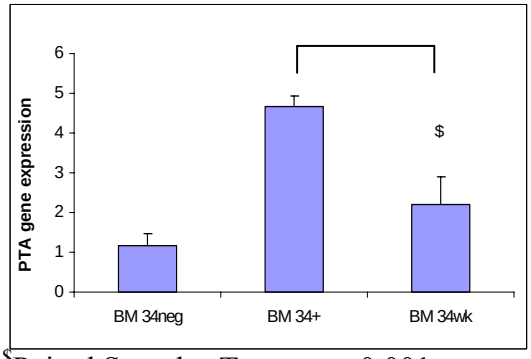
$CD34^+$ cells were cultured for 7 days as described in the text of Table 5.1. PTA gene expression was assessed in $CD34^+$ cells, $CD34^-$ cells and $CD34^+$ cells after 7 days of culture (wk). Mean gene expression is shown in arbitrary units relative to β-actin.

A. Cord Blood (n=10)



#Paired Samples T-test, $p \leq 0.006$

B. Bone Marrow (n=5)



$Paired Samples T-test, $p \leq 0.001$

Ex vivo proliferation and differentiation of $CD34^+$ cells cultured for 24 hours with four different cytokine combinations

The previous experiment had shown that PTA expression decreased in proliferating and differentiating cells, which is consistent with published data in other cell types. In cells undergoing mitogen induced proliferation it has been shown that PTA expression is up-regulated within hours of the proliferative stimulus, is maintained for up to 72 hours, then declines with terminal differentiation and loss of proliferative capacity (Bustelo, *et al* 1991, Eschenfeldt and Berger 1986). Accordingly, the protocol used in these experiments was designed to test the hypothesis that growth stimulation in $CD34^+$ cells would cause an initial increase in PTA gene expression followed by the down-regulation associated with differentiation that was evident in $CD34^+$ cells after one week in culture.

The combinations tested were designed to promote expansion (E) (SCF, IL-3, TPO and F3L); antagonise expansion by adding an inhibitor to the expansion cocktail, such as TGFβ (ET) or MIP-1α (EM); or promote differentiation and proliferation (D) (G-CSF, GM-CSF and IL-3).

$CD34^+$ cells from CB were MACS purified and cultured at 1 x 10^5/ml for 24 hours with each of the combinations mentioned above. Control cultures consisted of $CD34^+$ cells cultured for the same period with base media (α-MEM, 10% FCS, L-glutamine and penicillin/streptomycin) but no added growth factors.

The results of PTA gene expression are illustrated in Figure 5.4A. The PTA expression data were not normally distributed so the Friedman test of non-parametric data was used to assess significance. Using this experimental design, no combination of cytokines significantly changed the expression of PTA (Friedman Test, NSD, n=5).

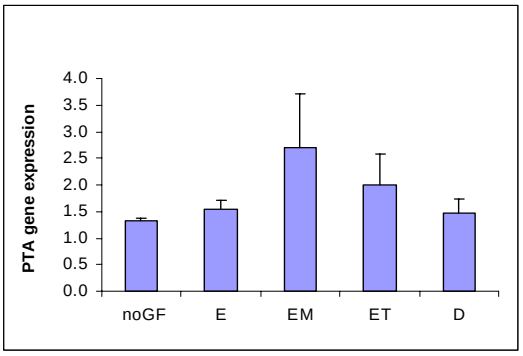
In addition to assessing PTA expression levels the expression of c-myc was also assessed. As has been noted, c-myc expression positively correlates with proliferation (Dang 1999), it is a well-characterised regulator of haemopoiesis *in vivo* (Hoffman, *et al* 2002) and some investigators have suggested that it directly induces PTA expression (Eilers, *et al* 1991). Figure 5.4B demonstrates that each of the "expansion" combinations significantly up-regulated the expression of c-myc (ANOVA, $p \leq 0.001$, n=5) when compared to control (noGF) (Tukey's HSD: no GF v E, $p \leq 0.007$; noGF v EM, $p \leq 0.003$; noGF v ET, $p \leq 0.003$). The group exposed to cytokines to enhance

differentiation showed an intermediate level of gene induction. However, there was no significant difference in gene expression between the control group and the group exposed to differentiation cytokines (D) (Tukeys HSD: noGF v D, $p \leq 0.099$), nor was there a difference between the differentiation group and any of the expansion groups.

Figure 5.4 Effect of growth factor cocktails on the expression of PTA and c-myc in CB CD34$^+$ cells after 24 hours of culture.

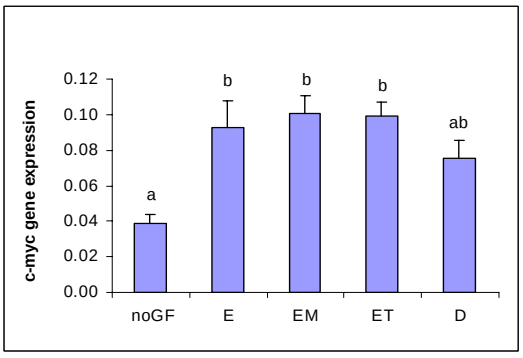
Cord blood CD34$^+$ cells were cultured at a concentration of 10^5 cells/ml in α-MEM, 10% FCS, L-glutamine and penicillin/streptomycin supplemented with: no added growth factors (noGF); an expansion (E) cocktail (SCF, IL-3, TPO and F3L); the expansion cocktail with MIP-1α (EM); the expansion cocktail with TGFβ (ET); or with a differentiation (D) cocktail (G-CSF, GM-CSF and IL-3). Mean gene expression levels are shown in arbitrary units relative to β-actin. Where necessary groups are "lettered" to indicate similarity in gene expression. (NSD = no significant difference)

A. PTA gene expression (n=5)



Friedman's, NSD

B. c-myc gene expression (n=5)



ANOVA, $p \leq 0.001$

Effect of Single Growth Factors on PTA gene regulation: SCF, IL-3, F3L, GM-CSF

Various cytokines have been described that are known to affect the proliferation and survival of haemopoietic progenitors. Many act synergistically, increasing the complexity of the nuclear signalling and the magnitude of the physiological result, however, single factors also can act directly on enriched populations of haemopoietic progenitors. To minimise the complexity of nuclear signalling we adopted a strategy of culturing $CD34^+$ cells with single growth factors to determine whether any individual cytokine affected PTA mRNA expression. The cytokines tested (SCF, IL-3, F3L, GM-CSF) were chosen because collectively they had a broad sphere of action.

CD34 enriched cells from CB were cultured for two hours in media supplemented with one of the four cytokines. Control cells were cultured in base media alone. The short-term exposure to each growth factor was used to determine whether any single factor produced a signal transduction resulting in regulation of PTA or c-myc gene expression. The pattern of expression of PTA (Figure 5.5A) and c-myc (Figure 5.5B) is very similar. Although each growth factor seemed to up-regulate gene expression, especially the early acting cytokines, SCF, IL-3 and F3L, the responses were extremely variable and did not become statistically significant using ANOVA analysis.

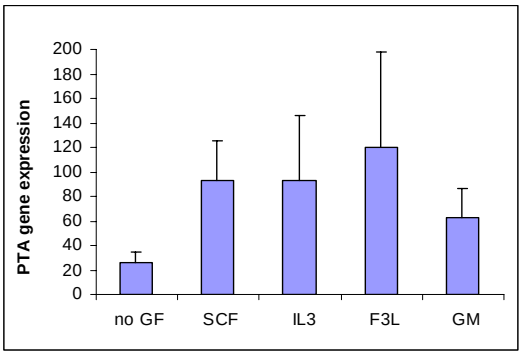
As the evidence from other cell proliferation systems points to up-regulation of PTA mRNA within 2-24 hours of proliferative stimuli, a short-term exposure time-course was performed to ensure that no acute gene regulation was missed. Cells were prepared and cultured in the same way as for the 2-hour exposure. RNA was extracted from cells at time points 0, 2, 6 and 10 hours in culture and assessed for gene expression (Figure 5.6) As the data showed predominantly non-parametric distribution, a Friedman test was used for analysis of significance. Notably, again there was significant variation between replicate experiments, such that although there appears to be some regulation in responses to some growth factors, at no point did this reach statistical significance.

Figure 5.5 Effect on gene expression of exposure to single growth factors for 2 hours

$CD34^+$ cells were cultured at a concentration of 10^5 cells/ml in α-MEM, 10% FCS, L-glutamine, penicillin/streptomycin, and either SCF, IL3, F3L or GM-CSF for 2 hours. Results are expressed in arbitrary units as mean PTA expression relative to β-actin.

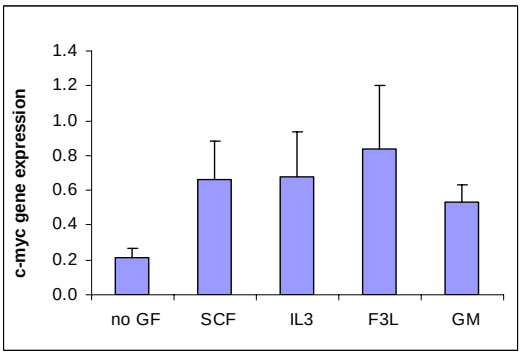
There was no significant difference in the expression of PTA or c-myc after 2 hours of culture with each of the cytokines tested. (NSD = no significant difference)

A. PTA expression (n=4)



ANOVA, NSD

B. c-myc expression (n=4)



ANOVA, NSD

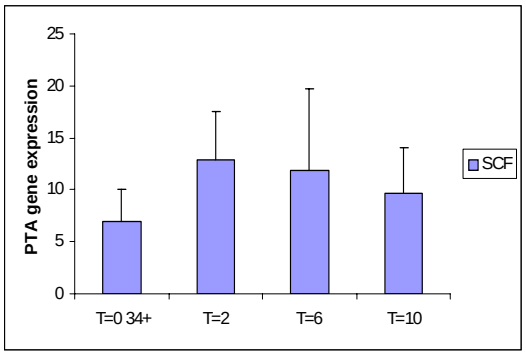
Figure 5.6 Time course of exposure to single growth factors.

CD34 enriched cells were cultured at a concentration of 10^5 cells/ml in α-MEM, 10% FCS, L-glutamine, penicillin/streptomycin, and either SCF, IL3, F3L or GM-CSF for 0, 2, 6 or 10 hours as indicated.

Results are expressed in arbitrary units as mean PTA expression relative to β-actin.

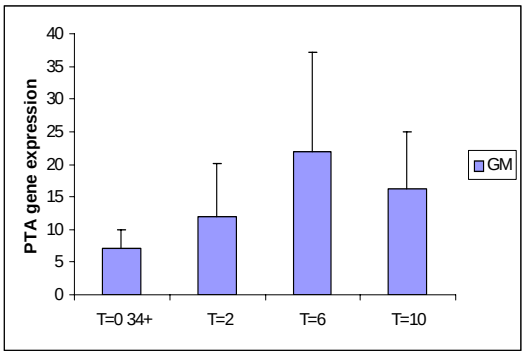
There was no significant difference in the expression of PTA at any time-point for each of the cytokines tested. (NSD = no significant difference)

A. Stem Cell Factor (n=7)



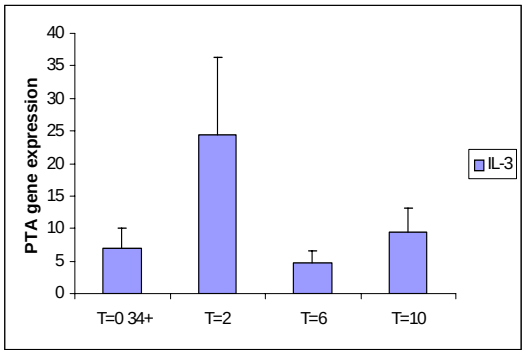
Friedman test, NSD

B. GM-CSF (n=6)



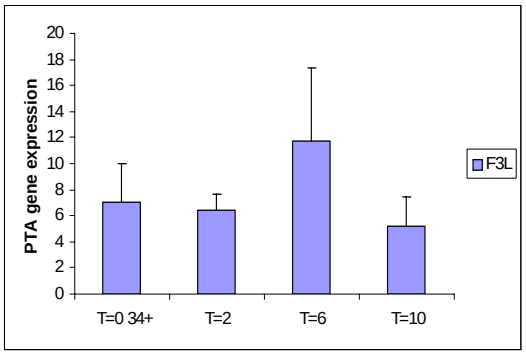
Friedman test, NSD

C. IL-3 (n=7)



Friedman test, NSD

D. F3L (n=7)



Friedman test, NSD

DISCUSSION

The differential expression of PTA in $CD34^+$ cells from human and mouse has been noted by several groups, however, a systematic study of the regulation of this gene in $CD34^+$ cells has not been published. We studied expression of the gene in a primitive population of $CD34^+$ cells and attempted to find evidence of modulation of gene expression under conditions designed to promote expansion and maturation of haemopoietic precursors and in response to single growth factors to determine if their effect was transduced by modulation of PTA expression.

The $CD34^+$ population is a heterogeneous pool of haemopoietic cells with a wide spectrum of proliferative potentials which decline as the degree of lineage commitment increases. Many studies have shown that the cells capable of sustained multi-lineage engraftment *in vivo* are contained within the more primitive subsets of $CD34^+$ cells. These primitive subsets are narrowly defined according to their phenotype and in human are considered to be $CD34^{bright}$, negative for lineage markers ($CD33^-$, $CD38^-$, $CD45RA^-$, $CD71^-$, HLA-Dr^-), positive for other markers with a very restricted distribution ($CD90^{lo/+}$, $AC133^+$) and can exclude dyes (such as rhodamine or Hoechst 33342) due to high level expression of the multidrug resistant gene (MDR), another indicator of "stemness". Isolation of such subsets requires the use of cytometers with sophisticated sorting capabilities and these were not available during the term of this study. However, the simple $CD34^+CD38^-$ phenotype describes a highly quiescent subset of primitive, haemopoietic progenitors. This phenotype is commonly used to define a more primitive subset of haemopoietic progenitors that produce multi-lineage colonies, have a higher content of cobble-stone area forming cells and long-term culture initiating cells and exhibit features of quiescence, having more fastidious cytokine requirements and longer delay prior to proliferation, compared to the total $CD34^+$ population (Bhatia, *et al* 1997, de Wynter 1996, Hao, *et al* 1995, Ng, *et al* 2002, Reems and Torok-Storb 1995). We used this simple phenotype to isolate and enrich cells with a profound ability to proliferate in order to assess the baseline levels of PTA expression. We found that there was a non-significant decline in PTA expression in the primitive subset compared to the total $CD34^+$ population, however, like the parent population, the $CD34^+CD38^-$ subset showed significantly higher levels of expression of PTA compared to the $CD34^-$ population. The data again demonstrates that PTA can be highly expressed in non-proliferating, immature cells. The up-regulation of PTA in quiescent populations is

unusual, as the induction of quiescence (through serum starvation) in a variety of cell lines is associated with slight to marked down-regulation of gene expression (Dosil, *et al* 1993, Vareli, *et al* 1996, Zalvide, *et al* 1992). It was not, however, entirely unexpected as there have been other reports of PTA mRNA induction in a quiescent population, co-incident with N-myc expression in differentiating, non-proliferating neurons of the neocortical layers of embryonic mouse brains (Moll, *et al* 1996), and in normal, non-proliferating liver (Bustelo, *et al* 1991). In the latter case, partial hepatectomy in rats caused induction of PTA mRNA which was evident at 6 hours post surgery and reached maximal levels (2 to 4-fold increase) between 12-32 hours after surgery.

Although expression of PTA in quiescent cells seems incongruous given its reported association with proliferation, it was relevant to determine whether induction of gene expression above baseline levels occurred in proliferating $CD34^+$ cells.

The culture of $CD34^+$ cells for 7 days with a combination of cytokines known to promote proliferation and differentiation had a marked effect on the expression of PTA, causing significant down-regulation. This result is consistent with the known capacity of these cytokines to promote proliferation and differentiation and with published data describing the down-regulation of PTA in terminally differentiated cells that have reduced proliferative capacity.

Given the preponderance of literature describing significant up-regulation of PTA expression in response to growth stimuli, such as addition of fresh serum to serum starved, quiescent cell lines (Conteas, *et al* 1990, Eschenfeldt and Berger 1986) and mitogen stimulated primary cultures {Bustelo, 1991 #3057; (Eschenfeldt and Berger 1986), we hypothesised that exposure of $CD34^+$ cells to factor/s known to promote proliferation would produce a similar response in PTA expression. An experimental protocol was designed to assess gene expression in response to multiple factors after a shorter exposure (24 hours). For the 24 hour culture, conditions were chosen that would provide several different kinds of stimuli to the cells in culture and so provoke a range of signal transduction pathways. An expansion cocktail of SCF, IL-3, TPO and F3L was selected for its broad scope in inducing proliferation in a range of primitive and committed progenitors. SCF promotes survival of primitive stem cells (Ashman 1999, Broudy 1997b) but not more mature cells. Alone it can hasten recruitment of cells into the cell cycle (Broudy 1997b) however it acts synergistically to promote proliferation in

primitive and lineage-committed cells (Ashman 1999, Broudy 1997b, Lyman 1998). IL-3 acts on primitive and lineage committed cells and promotes proliferation. TPO (or c-mpl ligand) was originally thought to be a megakaryocyte lineage specific cytokine. Sufficient data has accumulated to show that it also stimulates proliferation of HSC in human and mouse and has profound synergistic action with F3L and SCF on the expansion of primitive haemopoietic progenitors (Luens, *et al* 1998, Ng, *et al* 2002, Piacibello 1997). F3L can recruit quiescent cells into cycle.

In two of the groups the expansion cocktail was supplemented with TGFβ or MIP-1α. TGF-β inhibits proliferation of early high proliferative progenitors by down-regulating c-kit receptors (the receptors for SCF), in part by reducing the stability of c-kit mRNA (Ashman 1999, Broudy 1997a, Dubois, *et al* 1994) and by inducing quiescence (Batard, *et al* 2000). MIP-1α has a myeloprotective effect on primitive progenitors by inhibiting cell cycle progression in a reversible manner (Dunlop, *et al* 1992, Ritter, *et al* 1995). Its target population is at the more primitive end of the haemopoietic spectrum and includes those cells capable of forming colony forming units-spleen (CFU-S), a measure of *in vivo* re-populating ability (Dunlop, *et al* 1992).

For the culture conditions designed to promote differentiation the combination used was G-CSF, GM-CSF and IL-3. GM-CSF is a potent stimulant for expansion and differentiation acting on lineage committed cells. IL-3 supports the survival of primitive progenitors, an effect that is augmented by the presence of G-CSF and GM-CSF (Bodine 1991). It also promotes differentiation and expansion of a broad range of cell types compared to the more restricted effects of G-CSF and GM-CSF (Metcalf 1993).

Many studies have been performed on the expansion capabilities of $CD34^+$ cells. Most notably, Piacibello using $CD34^+$ cells showed prodigious expansion of cells grown in liquid culture containing F3L, SCF and TPO for up to 6 months (Piacibello, *et al* 1998, Piacibello 1997). More modest but nonetheless significant expansion of $CD34^+$ cells has been reported by other authors (de Wynter 1996, Denning-Kendall, *et al* 1998, Gilmore, *et al* 2000) and most of the variation in outcomes are attributable to differences in the cytokine combinations tested as investigators strive to determine which cytokines are most clinically relevant. Furthermore, published data describe significant increases in PTA gene expression at 24 hours following induction of proliferation (Bustelo, *et al* 1991, Conteas, *et al* 1990, Eschenfeldt and Berger 1986,

Rodriguez, *et al* 1998). It would seem, therefore, that with the culture conditions used in this study it was reasonable to expect some degree of regulation in a gene that is reported to be associated with proliferation, such as PTA. However, none of the combinations showed any significant effect on the modulation of PTA gene expression at 24 hours. Therefore, we conclude that PTA is highly expressed in a quiescent population of $CD34^+$ cells, but conditions designed to promote proliferation do not induce further gene expression at 24 hours.

The expression of PTA in quiescent cells has been noted in some studies. For instance, Dosil *et al* (Dosil, *et al* 1993) found that in a human promyelocytic cell line (HL60) proliferation arrest could be effectively induced (without a commitment to differentiation) by treating cells with hydroxyurea or via serum deprivation but, only a slight decrease in PTA expression was noted at 12 hours or 4 days, respectively. Thus PTA expression was detectable in non-proliferating cells, however, if HL60 cells were induced to divide and differentiate PTA gene expression was significantly down-regulated within 24-72 hours depending on the agent used (Dosil, *et al* 1993, Smith, *et al* 1993). This data appears to indicate that the decrease in PTA gene expression in HL60 cells was associated with differentiation. In contrast, where differentiation was associated with continuing proliferation, such as HL60 cells exposed to interferon-γ, PTA gene expression was maintained at high levels for at least 48 hours (Smith, *et al* 1993) implicating an association between gene expression and proliferation. Using a different perspective to address the same issue, it has also been reported that over-expression of PTA enhances proliferation (Rodriguez, *et al* 1998, Wu, *et al* 1997), impairs differentiation (Rodriguez, *et al* 1998), and that PTA antisense oligomers inhibit cell division in human myeloma cells (Sburlati, *et al* 1991), and in HL60 cells (Rodriguez, *et al* 1999), the latter case associated with increased apoptosis.

These results are somewhat contradictory and difficult to resolve with the current studies on PTA gene expression in proliferating $CD34^+$ cells. It is currently accepted that proliferating cells show up-regulation of PTA gene expression and indeed in many documented cases there is an increase (2-4 fold) in the levels of PTA mRNA in proliferating cells over basal levels. The accumulated data also suggest that cells that have a proliferative potential, regardless of whether or not they are actively proliferating, express PTA. When such cells are driven to exhaust their proliferative potential, often co-incident with terminal differentiation to a mature cell type, they lose

expression of PTA mRNA. To extend this argument it has also been shown that a differentiated cell that retains a significant proliferative potential will still express high levels of PTA message. Therefore, it would seem that detectable levels of PTA are synonymous with an ability to proliferate; that cells which can proliferate *ipso facto* express PTA. This line of reasoning re-focuses the current paradigm with respect to the relationship of PTA gene expression and proliferation/differentiation and permits a less contradictory understanding of the plethora of data relating to the regulation of PTA gene expression.

The association between PTA and c-myc expression was also studied in this experiment. Levels of c-myc mRNA were significantly increased in response to the three expansion cytokine mixtures and this result is consistent with its role in cell proliferation. The induction of c-myc and lack of response of PTA gene expression also indicate that in this setting at least, c-myc does not play a direct role in the activation of PTA gene expression. Eilers *et al* (Eilers, *et al* 1991) found that induction of a MYCER construct in RAT1A cells resulted in the up-regulation of PTA expression within one hour that was sustained for at least 20 hours. If c-myc had a direct role in the induction of PTA gene expression in $CD34^+$ cells it is likely that the increase in myc expression seen in the multi-cytokine expansion experiments should have resulted in an increase in PTA expression. It would seem that under these conditions c-myc is not a direct inducer of PTA in $CD34^+$ cells. This finding of a lack of association is not conclusive evidence that a direct regulatory mechanism does not exist between c-myc and PTA, but is consistent with other work that has demonstrated in different cell proliferation models a similar lack of association, albeit in the opposite direction with maintenance of high levels of PTA mRNA and marked down-regulation of c-myc (Dosil, *et al* 1993, Smith, *et al* 1993).

Many cytokines act synergistically, increasing the magnitude of the cellular response and broadening the range of responding (target) cells. A simplified approach to characterise the effect of cytokines is to use single growth factors in clonal assays (Metcalf 1993). We employed a similar strategy, culturing cells with single growth factors, to simplify the ensuing signal transduction pathways and to assess modulation of gene expression. The individual responses to each of the cytokines tested exhibited marked variability with respect to the induction of PTA or c-myc expression at 2 hours of culture. Both genes followed a similar pattern of up-regulation that was more

marked with the early acting cytokines than with GM-CSF. However, no single factor was found that caused a statistically significant regulation of either gene. A more detailed analysis of PTA expression was performed at 2, 6 and 10 hours of culture. Again, the results showed quite marked variability and a lack of statistical significance. The suggestive increases in mean gene expression for both PTA and c-myc, particularly in response to SCF, IL-3 and F3L, may indicate that a more primitive subset of $CD34^+$ cells does modulate expression of these genes in response to some cytokines. We cannot exclude the possibility that, for instance, the $CD34^+CD38^-$ sub-population, that showed a non-significant decrease in PTA expression compared to the total $CD34^+$ population, may exhibit induction of gene expression in response to early acting cytokines. As the $CD34^+CD38^-$ subset comprise less than 10% of the total population such an effect could be lost in assays of total $CD34^+$ cells.

To summarise, the studies of gene expression and regulation in $CD34^+$ cells showed that PTA was highly expressed in a more immature and quiescent subset of $CD34^+$ cells, the $CD34^+CD38^-$ subset. Combinations of cytokines known to cause proliferation of $CD34^+$ cells did not produce further induction of PTA within 24 hours of stimulus, although they did produce consistent and significant induction of c-myc. This data does not disprove a direct effect of c-myc on the expression of PTA, but it does suggest that such an effect is not always transduced. Exposure of $CD34^+$ cells to proliferative stimuli for 7 days caused significant differentiation of cells (evident from the loss of CD34 antigen) and down-regulation of PTA. This result is consistent with published data showing down-regulation of PTA in cells that have lost proliferative capacity due to terminal differentiation. Our data support the notion that PTA expression is a determinant of proliferative capacity (hence, its up-regulation in a quiescent, immature population of cells with high proliferative capacity) and in this respect is consistent with published data showing over-expression of PTA caused increased cell proliferation (Rodriguez*, et al* 1998, Wu*, et al* 1997) and inhibited differentiation (Rodriguez*, et al* 1998), and that down-regulation of expression by anti-sense oligomers inhibits cell division (Rodriguez*, et al* 1999, Sburlati*, et al* 1991). Our studies with single growth factors exhibited wide variation in gene induction of both PTA and c-myc, but no evidence of a consistent and significant response. It is possible that studies on more primitive populations may yield different results, but this approach would require high

speed sorting to produce sufficient cells to conduct such studies and was beyond the scope and time restraints of this project.

Although several papers have published Northern blots of gene expression in rat and human tissues, including total lymphocytes and thymic T-cells, to the best of our knowledge no-one has undertaken a detailed analysis of PTA expression in mature subsets of blood cells. Details of this and further studies of the effects of mitogen stimulation of lymphocytes are given in the next chapter.

CHAPTER 6

PTA EXPRESSION IN SUBSETS OF MATURE HAEMOPOIETIC CELLS AND PHA-STIMULATION AS AN ALTERNATIVE MODEL OF PROLIFERATION.

INTRODUCTION

The experiments detailed in this chapter were designed to address two aspects of PTA expression that had not previously been studied: gene expression in unstimulated, mature haemopoietic cells and a detailed appraisal of PTA and c-myc expression in proliferating cells compared to non-proliferating cells.

Several studies have examined PTA levels in mitogen stimulated rat splenic thymocytes (Bustelo, *et al* 1991, Gomez-Marquez, *et al* 1989), mouse splenic lymphocytes (Szabo, *et al* 1992), human PB MNC (Eschenfeldt and Berger 1986) and lymphocytes from primary human leukaemias (Gomez-Marquez, *et al* 1989) but a comprehensive assessment of baseline gene expression in unstimulated subsets of normal human PB MNC is lacking. The mononuclear cells of peripheral blood are a composite of monocytes, T cells, B cells and natural killer (NK) cells that can be isolated and enriched by several common methods. However, as some cell manipulations can cause modulation of gene expression, we compared separation techniques to assess their suitability prior to commencing the main study. Our preliminary studies showed that exposure to FCS could cause induction of PTA and c-myc mRNA. Not only did this finding determine the method chosen for lymphocyte subset purification, but it also had serious implications for the interpretation of published data relating to the induction of PTA expression in mitogen stimulated cultures.

We had been unable to find convincing evidence of a regulatory role for c-myc in the induction of PTA expression in $CD34^+$ cells. Therefore, we examined the correlation of these two genes in a common proliferative model that has been used extensively in studies of PTA expression, that of PHA-stimulation of PB MNC. Gene expression of PTA and c-myc, cell viability and proliferation was analysed daily for 4 days in comparison to control cultures lacking mitogen. No published study of PTA expression in mitogen stimulated cells has correlated these four parameters, nor has any study

analysed comparative data in unstimulated, control cultures for the duration of the culture period.

RESULTS

PTA gene expression in lymphocytes and monocytes enriched by two techniques

Assessment of baseline gene expression in subsets of MNC requires purification steps and cell manipulations that might themselves cause gene regulation. Therefore, in the initial assessment of gene expression in pure populations of monocytes and lymphocytes, PB MNCs were separated by two different techniques to assess whether either method caused modulation of gene expression: adherence to plastic of monocytes with subsequent removal of the non-adherent population; and magnetic (MACS) selection of $CD14^+$ monocytes and $CD14^-$ lymphocytes.

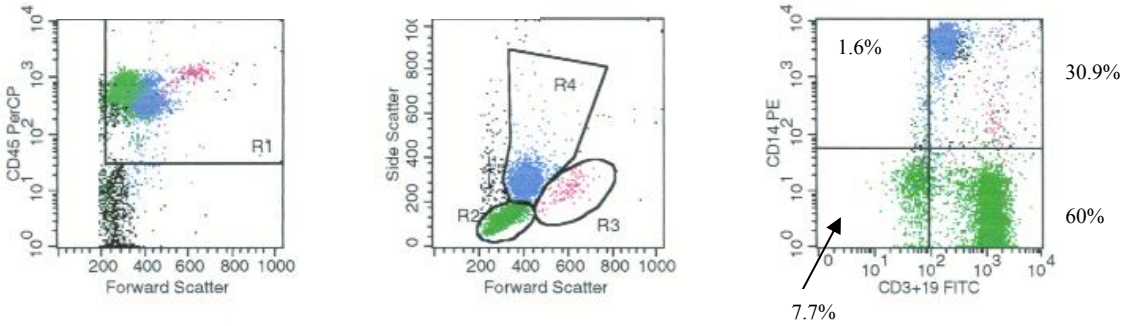
Cell purities were calculated by labelling test aliquots with the triple combination of CD45-PerCP/CD14-PE/CD3+CD19-FITC and control tubes with CD45-PerCP/IgG1-PE/IgG1-FITC. Representative dot-plots of the starting PB MNC fraction, the non-adherent and MACS enriched populations are given in Figure 6.1. The starting population of PB MNCs (expressed as mean ± SEM) was composed of lymphocytes 75% ± 3.6 and monocytes 13% ± 5.9; the non-adherent population was lymphocytes 93% ± 0.6; the purified populations were MACS $CD14^+$ fraction, monocytes 98% ± 0.4 and MACS $CD14^-$ fraction, lymphocytes 94% ± 1.3; n=3 for each analysis.

PTA expression (Fig 6.2A) was significantly up-regulated in the non-adherent (lymphocyte) fraction compared to all other groups (ANOVA $p \leq 0.001$, n=5) indicating that either exposure to monocytes or to FCS, or both, caused induction of gene expression in lymphocytes. PTA gene expression was not significantly induced by the magnetic separation technique. Similarly with c-myc (Fig 6.2B), the non-adherent cell fraction (Non-adh) showed significant mRNA induction (ANOVA $p \leq 0.003$) while the adherent (monocyte) fraction (Adh) exhibited partial gene induction with expression levels between that of the unseparated MNCs and the non-adherent population. The gene modulation in these populations was either in response to factor/s in the FCS and/or to the process of adherence itself, as gene induction was not evident in the cells separated with the MACS technique (compare MNC with MACS CD14+ and MACS CD14-).

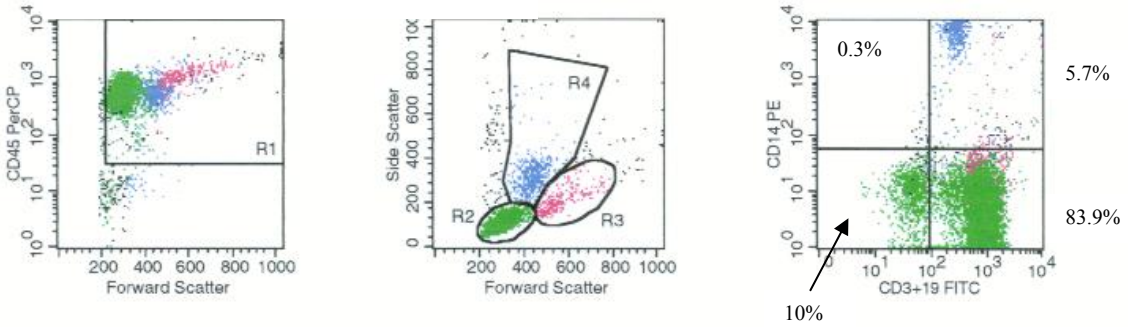
Figure 6.1 (next page) Representative dotplots of (A) PB MNC fraction, (B) non-adherent fraction, (C) MACS CD14$^+$ fraction and (D) MACS CD14$^-$ fraction.

PB MNCs were separated by two different techniques, either adherence to plastic of monocytes with subsequent removal of the non-adherent population, or magnetic separation of CD14$^+$ monocytes and CD14$^-$ lymphocytes. The composition and purity of each population was assessed by flow cytometry using a combination of surface antigen and light scatter to identify distinct cell populations. In the first column, R1 encompasses the CD45$^+$ white blood cells which are displayed in the middle and last columns. The middle column displays cells according to their size (forward scatter) and granularity (side scatter). R2 (green) defines the lymphocyte area, R3 (red) defines the large granular lymphocyte area, and R4 (blue) defines the monocyte area. In the last column, cells are displayed relative to their labelling with CD14 or a combination of CD3 and CD19. The upper left and right quadrants display CD14$^+$ (monocyte) events. The bottom left quadrant contains CD3^{dull}CD19^{dull} lymphocytes; the bottom right quadrant displays the CD3^{bright}CD19^{bright} lymphocytes. Percentages of cells in each quadrant are indicated.

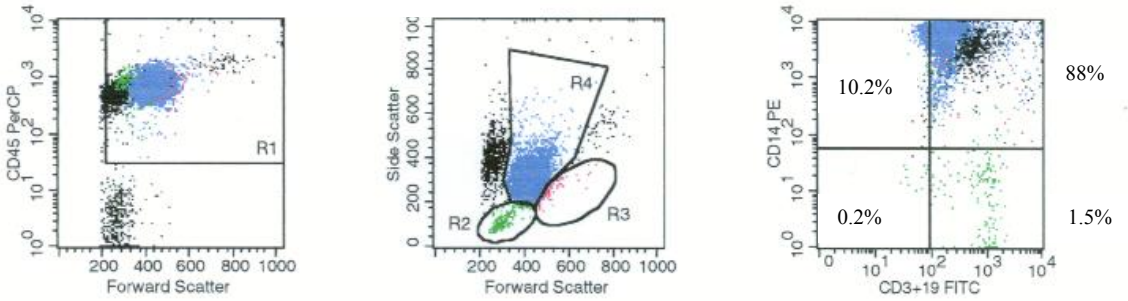
A. PB MNC starting population



B. Non-adherent fraction



C. MACS 14^{+} fraction



D. MACS 14^{-} fraction

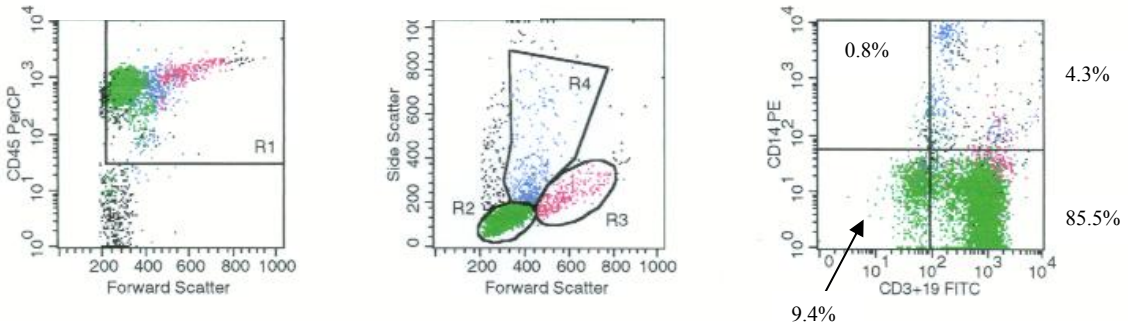
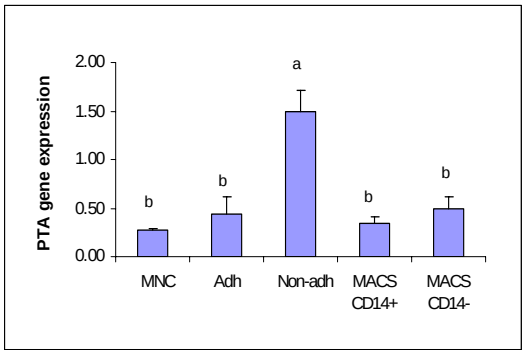


Figure 6.2 Baseline expression of PTA and c-myc in lymphocytes and monocytes separated by either plastic adherence or MACS enrichment.

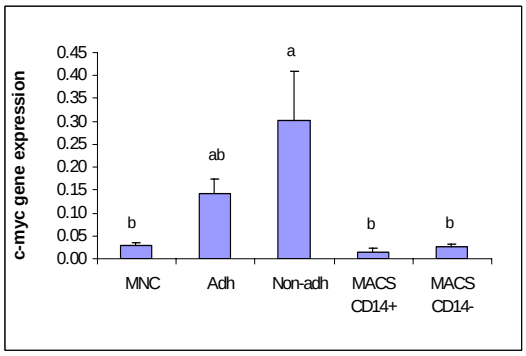
For plastic adherence PB MNC (MNC) were re-suspended in RPMI + 20% FCS, and incubated for 2 hours at 37^{o}C, then non-adherent (Non-adh) cells removed. Adherent (Adh) cells were washed thoroughly and homogenised in TRIzol. In the alternative procedure, PB MNC were labelled with CD14^{+} microbeads and passed through a magnetic field. Flow-through cells (MACS CD14^{-}) were collected and the retained fraction recovered from the column as the MACS CD14^{+} fraction. Gene expression levels are shown in arbitrary units relative to β-actin. For each graph, groups are "lettered" to indicate similarity in gene expression.

A. PTA (n=5)



ANOVA p≤ 0.001

B. C-myc (n=5)



ANOVA p≤ 0.003

Effect of FCS on gene expression in MACS enriched $CD14^+$ and $CD14^-$ subsets

To determine whether gene induction in lymphocytes was due to exposure to FCS or monocytes, a series of experiments was conducted in which PB MNCs were MACS enriched for $CD14^+$ and $CD14^-$ cells and an aliquot of the enriched fractions incubated for 2 hours at 37^{o}C in RPMI + 20% FCS to mimic the culture conditions used for plastic adherence. Gene expression was evaluated in the unseparated PB MNC population and the MACS enriched subsets ($CD14^+$ and $CD14^-$), before and after incubation with FCS.

Flow cytometry was used, as before, to estimate the composition of the starting PB MNC and the purity of the isolated populations. The starting population was composed of (expressed as mean ± SEM) lymphocytes 77% ± 2.0 and monocytes 21% ± 1.7; the purified populations were MACS 14^+ fraction, monocytes 94% ± 1.9 and MACS 14^- fraction, lymphocytes 95% ± 1.6; n=7 for each analysis.

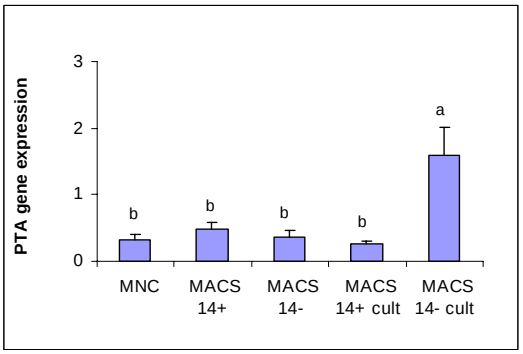
Under these conditions expression of PTA was significantly enhanced in the lymphocyte population after exposure to FCS (MACS CD14- cult) indicating that factor/s in the serum were sufficient to induce gene expression (Fig 6.3A).

The expression pattern of c-myc indicated that significant gene induction occurred in the lymphocytes exposed to FCS compared to all other groups (Fig 6.3B). The monocytes exposed to FCS (Fig 6.3B MACS 14+ cult) showed a trend towards higher c-myc expression compared to the freshly isolated monocytes (MACS 14+), which is consistent with the previous experiment where adherent cells also showed partial induction of c-myc expression (refer Fig 6.2B, compare Adh group to MNC and Non-adh groups)

Figure 6.3 Expression of PTA and c-myc in $CD14^+$ monocytes and $CD14^-$ lymphocytes before and after culture.

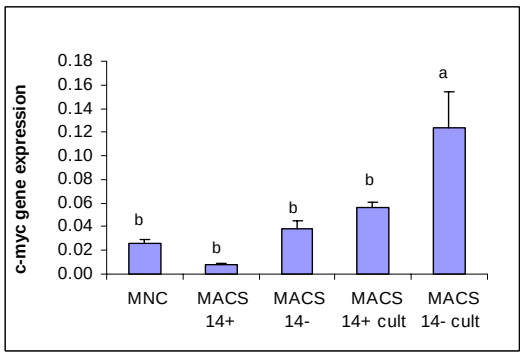
PB MNC were MACS separated into $CD14^+$ monocytes and $CD14^-$ lymphocytes. An aliquot of each purified fraction was incubated for 2 hours at 37°C in RPMI + 20% FCS. Abbreviations are: **MNC**, total mononuclear cells; **MACS 14+**, magnetic separated $CD14^+$ monocytes; **MACS 14-**, magnetic separated $CD14^-$ lymphocytes; **MACS 14+ cult,** magnetic separated $CD14^+$ monocytes cultured for 2 hours; **MACS 14- cult**, magnetic separated $CD14^-$ lymphocytes cultured for 2 hours. Gene expression levels are shown in arbitrary units relative to β-actin. For each graph, groups are "lettered" to indicate similarity in gene expression.

A. PTA (n=7)



ANOVA p≤ 0.003

B. C-myc (n=7)



ANOVA p≤ 0.001

PTA gene expression in lymphoid subsets

Having determined that exposure to FCS could induce expression of PTA and c-myc, magnetic separation of all populations was performed to obtain purified lymphocyte subsets for analysis of baseline gene expression in relatively mature haemopoietic cells. PB MNC underwent 3 sequential rounds of labelling and magnetic separation to enrich T cells ($CD3^+$), B cells ($CD19^+$) and natural killer (NK) cells ($CD56^+$). Real time analysis of gene expression was performed on these samples and on the corresponding depleted MNC ($CD3^-/CD19^-/CD56^-$).

The purities of the final populations were (expressed as mean ± SEM): $CD3^+$, 96% ± 0.9; $CD19^+$, 83% ± 5.0; $CD56^+$, 84% ± 3.4; and the corresponding depleted MNC ($CD3^-/CD19^-/CD56^-$ MNC) was composed of monocytes 42% ± 7.3; granulocytes 45% ± 7.4; and lymphocytes 13% ± 6.1; n=6 for each analysis.

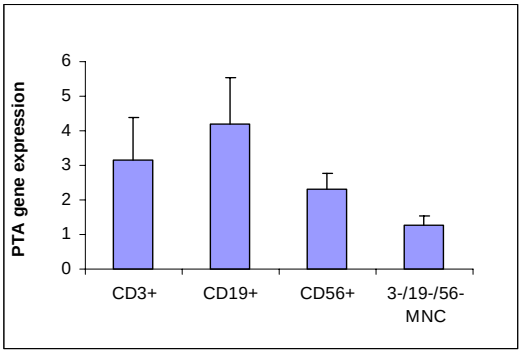
Figure 6.4 illustrates the gene expression of PTA and c-myc in the purified lymphocyte populations and corresponding depleted MNC. Both genes showed a similar pattern of expression with the highest average levels of expression in the T and B cells and lower means in the NK and depleted populations. However, with regard to PTA, there was no significant difference in the baseline expression of PTA amongst the three sub-fractions of lymphocytes nor between the expression of PTA in any of the lymphocyte subsets and the corresponding depleted mononuclear population.

In contrast, c-myc was significantly up-regulated in the T and B cell populations compared to the NK and the depleted MNC. Although PTA and c-myc show the same pattern of expression in the subsets, we were unable to demonstrate statistically significant evidence of regulation of PTA by c-myc.

Figure 6.4 Expression of PTA and c-myc in lymphocyte subsets.

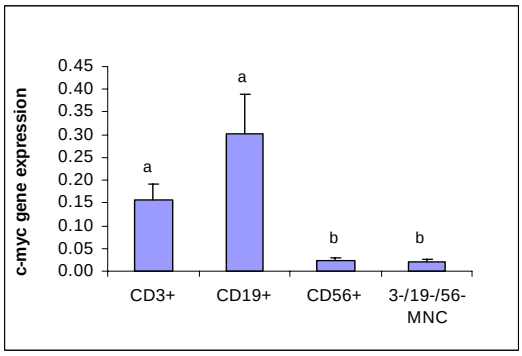
PB MNCs (MNC) underwent 3 sequential MACS purification procedures to isolate $CD3^+$, $CD19^+$, and $CD56^+$ lymphocytes. An aliquot of each purified fraction and the remaining depleted MNC was assessed for gene expression. Gene expression levels are shown in arbitrary units relative to β-actin. Where necessary, groups are "lettered" to indicate similarity in gene expression.

A. PTA (n=6)



ANOVA p≤ 0.198

B. C-myc (n=6)



ANOVA p≤ 0.001

Correlation of PTA and c-myc gene expression in PHA-stimulated PB MNCs

Mitogen stimulation of PB MNC has been used extensively to assess the effect of proliferative stimuli on PTA gene expression. Reports of the induction of PTA vary from 2-fold (Bustelo, *et al* 1991, Szabo, *et al* 1992) to >15-fold (Eschenfeldt and Berger 1986), however, no data exist regarding the response of c-myc in these conditions. Furthermore, only one (Bustelo, *et al* 1991) of the published data relating to mitogen stimulation has controls consisting of cells cultured without mitogen. As we have shown, gene expression can be induced by serum alone and it is possible that the induction of gene expression that has been attributed to mitogen stimulation may in fact be due to the presence of serum in the culture conditions. Therefore, using a common model of cellular proliferation, PHA stimulation of PB MNC, we assessed the correlation of c-myc and PTA expression, cell viability and proliferation in unstimulated cultured cells compared to stimulated cells.

The analyses were conducted using a general linear model (GLM) of ANOVA with pairwise comparisons and a family error rate (α=0.05) to determine significance. As Figure 6.5A demonstrates, cell viability decreased significantly in the cultures stimulated with PHA (ANOVA $p \leq 0.001$, n=10) over the course of the 4 day culture period from an initial mean (±SEM) of 94% (±0.8%) to 81% (± 2.4%). Cell proliferation (Figure 6.5B), measured by S-phase determination, increased significantly in the same cultures by day 2 and remained elevated for the duration of the culture period (ANOVA $p \leq 0.001$). The unstimulated cultures remained viable and quiescent over the same time period (ANOVA NSD).

The expression of PTA and c-myc are depicted in Figure 6.5C and 6.5D, respectively. Surprisingly, the change in PTA expression over the 4 day culture period did not achieve statistical significance (ANOVA $p \leq 0.425$) despite the pronounced cellular proliferation, indicated by the significant and sustained increase in the number of cells in S-phase. It is likely that the large variation in gene expression on day 2, coupled with the mild induction in the unstimulated cultures, has resulted in an apparent loss of statistical significance which may reflect a Type II error (concluding a difference does not exist when it does). Furthermore, we cannot exclude the possibility that significant induction occurred at less than 24 hours as our previous data showed significant regulation of PTA gene expression at 2 hours exposure to FCS. However, the data do

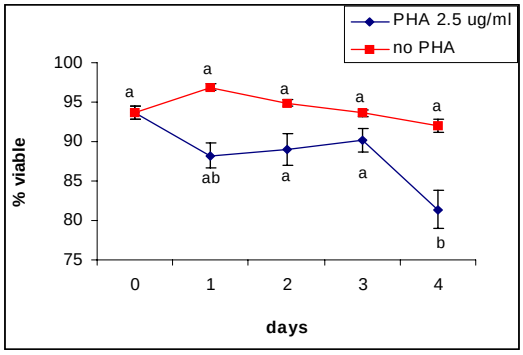
demonstrate that the induction of PTA is not entirely attributable to the proliferative stimuli of exposure to PHA.

In contrast, c-myc expression was significantly enhanced in the PHA-stimulated cultures at day 1 returning to baseline levels by day 3 (ANOVA $p \leq 0.001$). However, the unstimulated cultures also showed a gradual increase in c-myc expression which gained significance by days 3 and 4 of culture. Thus, consistent with our previous data showing induction of c-myc expression by FCS, over a 4 day period in unstimulated cultures c-myc expression is induced in cells that are otherwise quiescent and viable.

Figure 6.5 Effect of culture with PHA on viability, proliferation, PTA and c-myc expression, n=10.

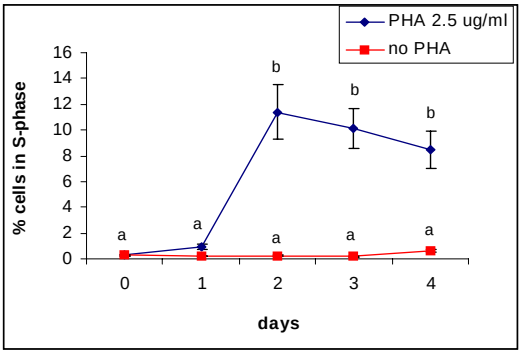
PB MNC were cultured for 4 days (37°C, 5% CO_2) in α-MEM, 10% FCS, L-glutamine, P/S and 2.5 µg/ml PHA. At time-points day 0, 1, 2, 3 and 4 samples were assessed for: **(A)** Viability; **(B)** percentage of cells in S-phase; **(C)** PTA gene expression; and **(D)** C-myc gene expression. Gene expression levels are shown in arbitrary units relative to β-actin. Where necessary, groups are "lettered" to indicate similarity in viability, S-phase or gene expression.

A. Viability



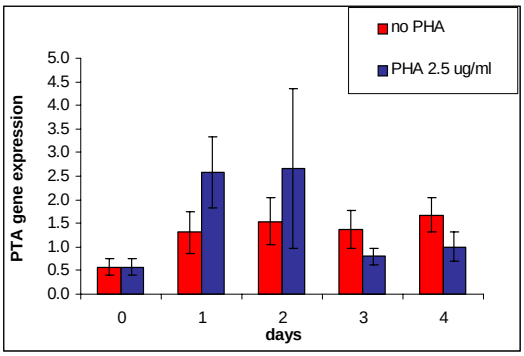
ANOVA p≤ 0.001

B. S-phase determination



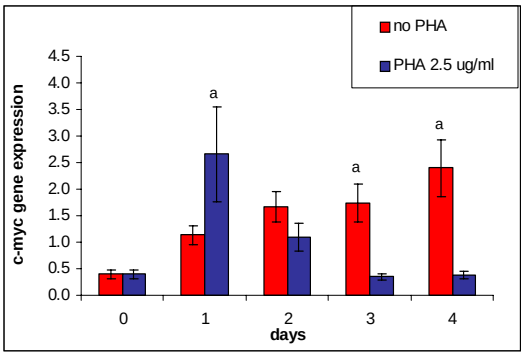
ANOVA p≤ 0.001

C. PTA expression



ANOVA p≤ 0.425

D. C-myc expression



ANOVA p≤ 0.001

DISCUSSION

The experiments detailed in this chapter were designed to investigate two themes: the baseline expression of PTA and c-myc in subsets of mononuclear cells; and the correlation between PTA and c-myc expression in cultured proliferating cells compared to non-proliferating cells.

Effect of cell separation technique on gene expression

Prior to commencing the main investigations it was necessary to ascertain whether common cell purification techniques themselves cause gene induction. In order to obtain homogeneous populations of monocytes and lymphocytes two purification procedures were compared: adherence of monocytes to plastic (with subsequent removal of lymphocytes) and magnetic separation of $CD14^+$ monocytes and $CD14^-$ lymphocytes. The process of culturing PB MNC in plastic tissue culture flasks caused significant up-regulation of both PTA and c-myc in the lymphoid population. Magnetic separation of lymphocytes and exposure to FCS, mimicking the method of plastic adherence (but without the prolonged contact with activated monocytes) showed that factor/s in the serum alone were sufficient to induce gene expression. Where possible, all subsequent experiments were conducted on freshly isolated MACS enriched (or depleted) populations. The induction of gene expression with serum becomes problematic when attempting to analyse the effect of specific treatments on regulation of gene expression and highlights the critical importance of suitable experimental controls. Furthermore, extrapolating from the S-phase data of unstimulated control cells in the PHA study which remained quiescent and viable over 4 days in culture, it is likely that the lymphocytes exposed to FCS were also essentially quiescent. If so, this again represents induction of PTA (and c-myc) in quiescent, viable cells.

Gene expression in lymphoid subsets

Although several studies have assessed gene expression in a variety of normal (Eschenfeldt and Berger 1986) and malignant (Gomez-Marquez*, et al* 1989, Sasaki*, et al* 2001) human tissues there are no studies describing baseline gene expression in subsets of mature haemopoietic cells. The mononuclear cells of peripheral blood are a composite of monocytes, T cells, B cells and NK cells. We purified the lymphoid

subsets from the total MNC population by MACS technique and assessed gene expression with real time PCR. Both PTA and c-myc showed a similar pattern of expression with highest mean mRNA levels in T and B cells and lowest in NK and the depleted MNC. The variations in PTA expression between these subsets were not statistically significant, although it is interesting to note that the pattern of expression correlates with the relative proliferative potentials of the subsets. Lymphoid cells particularly retain a significant proliferative potential and, in response to infectious agents, T and B cells undergo massive clonal proliferation. For example, T cells can expand 1000-fold within one week (Sad and Krishnan 2003). Within the NK population the $CD56^{bright}$ cells have greater proliferative potential than the $CD56^{dull}$ population but comprise only ~10% of the NK total, thus up-regulation of PTA in this population might be difficult to detect without further purification of the cells into $CD56^{bright/dull}$ subsets (Robertson, *et al* 1993). The $CD3^-/CD19^-/CD56^-$ depleted population was comprised predominantly of monocytes and granulocytes. Monocytes have a more limited proliferative capacity (up to 40-fold) (Antonov, *et al* 1997, Cheung and Hamilton 1992), whereas granulocytes which are terminally differentiated cells have virtually no proliferative capacity with a lifespan of 7-10 days.

There were significant differences in c-myc expression with the highest levels occurring in the T and B cell subsets and very low levels in the NK and depleted MNC. This may reflect the relative and on-going importance of c-myc in the maturation of these cells types during haemopoiesis and also the relative maturity of the subsets, as repression of c-myc is required for terminal differentiation (Hoffman, *et al* 2002). Although both PTA and c-myc display the same pattern of expression there is a lack of statistical significance in the variation of PTA expression in contrast to c-myc and this again may indicate a lack of direct regulation of PTA by c-myc in this setting.

Gene expression and PHA stimulation

Experiments with $CD34^+$ cells subjected to proliferative stimuli by cytokines failed to detect any significant increase in PTA expression (Ch. 5, this thesis). However, published literature of several different models of cell proliferation, such as mitogen stimulation, malignancy and hepatic regeneration, has indicated a role for PTA in proliferation. Mitogen stimulation of lymphoid cells has been studied extensively with

regard to modulation of PTA gene expression with the data showing great variability in the degree to which mRNA induction occurs. Furthermore, no data describe the pattern of c-myc expression in these circumstances. Eschenfeldt and Berger (Eschenfeldt and Berger 1986) reported a drastic increase in PTA mRNA of >15-fold in human lymphocytes cultured with staphylococcal enterotoxin A, and significant gene induction with other mitogens including staphylococcal enterotoxin B, PHA, Con A and exfoliatin. Increases in gene expression were noticeable as early as 2 hours. In contrast, rat thymocytes stimulated with Con A and IL-2 demonstrated a more modest 2.5 to 3-fold increase in gene expression, while rat splenic lymphocytes showed a 2.5 to 4-fold induction of mRNA in response to Con A or the calcium ionophore A23187, but not PMA, even though each of these mitogens enhanced proliferation as evident from the marked induction (up to 35-fold) of the S-phase regulated gene H3 (Bustelo, *et al* 1991). Rat splenic B cells stimulated with LPS also showed a 2 to 3-fold induction of PTA gene expression (Szabo, *et al* 1992).

Each of these studies used Northern blots and densitometric scanning to analyse and estimate changes in gene expression. In the time course studies conducted by Eschenfeldt and Berger, changes in PTA mRNA levels were compared to normal resting lymphocytes at time zero (Eschenfeldt and Berger 1986). Bustelo *et al* adopted a different approach to the use of controls. In a preliminary experiment they compared the expression of PTA in freshly isolated, spontaneously proliferating rat thymocytes to that of thymocytes cultured for 24 hours in the absence of mitogen (using a Hepes-buffered RPMI containing 10% heat inactivated FCS, P/S and L-glutamine). They found that after 24 hours PTA levels decreased 4-fold, as did the expression of two cell cycle related genes PCNA (~27-fold decrease) and H3 (~3-fold decrease) and DNA synthesis ceased, as measured by tritiated thymidine uptake. Thereafter, comparisons of PTA expression were made to resting lymphocytes (presumably, cells cultured without mitogen for 24 hours, although this is not explicitly stated), except in time courses where comparisons were relative to cells at time zero. Szabo *et al* also performed a time course of PTA expression and expressed PTA levels relative to an 18S rRNA (used as an internal standard to control for errors in RNA loading and for changes in the amount of RNA per cell through the cell cycle.) Changes in PTA mRNA were measured relative to the unstimulated cells at time zero.

We studied the changes in gene expression over a 4 day period of PB MNC cultured with and without PHA stimulation. Our earlier results had indicated that serum alone could induce expression of PTA and c-myc and thus highlighted the importance of appropriate controls in studies of mRNA induction in treated cells. Of the published studies examining the effect of mitogen on PTA expression (noted above), only one used unstimulated controls cultured in medium containing 10% heat-inactivated FCS, although these were not always cultured for the same length of time as the mitogen stimulated cells (Bustelo, *et al* 1991).

Serum contains a range of proteins (albumin, immunoglobulins, cytokines, complements, etc) that can have inhibitory and stimulatory effects on cellular function. To overcome the variability inherent in using sera, several strategies have been developed, the most common of which are: batch testing of sera in commonly used assays and stockpiling of suitable sera to minimise inter-batch variability; the use of heat-inactivated serum (to destroy heat labile complement and minimise cell death through activated complement cell lysis); using serum-free basic media formulations that require addition of appropriate nutrients (essential to the metabolism and viability of cells and presumed to be inert with respect to other parameters); and commercially prepared liquid and semi-solid complete medias which require the addition of cells only. These latter products have enhanced the reproducibility of clonal assays used commonly in haemopoietic stem cell culture and introduced a level of standardisation of procedure that was hitherto lacking in clinical and research studies.

In our study of PHA stimulation of PB MNC we opted to use FCS (not heat inactivated) as we had shown that it was capable of modulating gene expression and because, with the exception of Bustelo's study, other studies of lectin-induced proliferation had not used heat-inactivated serum, or not stated the details. Additionally, the inclusion of control cells cultured in identical media and conditions, but without PHA, is an important methodological control and a critical parameter in data analysis and interpretation, whether or not FCS, heat-inactivated FCS or serum-free medias are used.

We found that optimal stimulation with PHA did not cause a significant up-regulation of PTA expression at any of the time-points tested, even though proliferation of cells was confirmed by a significant increase in the percent of cells in S-phase from day 2 onwards. The statistical analysis was confounded to some extent by the mild induction of gene expression evident in the unstimulated control cultures (consistent with our

previous studies that had shown induction of PTA following exposure to FCS) and the considerable variability of induction in the treated cultures, especially at day 2. In spite of the statistical result, the data are consistent with previous findings that PTA expression is up-regulated in mitogen stimulated lymphocytes, as careful analysis of the results showed a large degree of individual variability in PTA modulation. However, the results also demonstrate that some of the published data relating to the effect of mitogens on gene induction may have wrongly attributed induction of gene expression solely to proliferative stimuli. Our data show that serum alone in otherwise non-proliferating, viable cells can affect gene expression. The analysis also reflects a more rigorous statistical approach than has previously been demonstrated with Northern blots to assess gene expression, which in turn reflects one of the benefits of real time PCR: namely, the ability to accommodate repetitive experimental models that generate multiple data sets requiring analysis of two or more parameters (the housekeeping gene/s and gene/s of interest, with and without treatments).

Finally, as we had not been able to find evidence of c-myc regulation of PTA expression in $CD34^+$ cells, we wished to examine more closely the correlation of these genes in the PHA stimulation of PB MNC. The mitogen stimulation of MNC showed an unequivocal up-regulation of c-myc at day 1 that returned to baseline levels by day 3. The gene induction reflected an early event in the transduction of proliferation signals, occurring 24 hours before a significant increase in the percent of actively proliferating (S-phase) cells. As noted previously, PTA exhibited suggestive increases in gene expression at day 1 and 2 although these were not statistically significant. More puzzling however was the steady rise in c-myc expression in the unstimulated cells. The induction of gene expression in cells that are quiescent and viable is a phenomenon that we have noted with several genes in our laboratory, but cannot explain. It demonstrates the cautious approach that is required in experimental design and data interpretation. In addition, the steady increase in c-myc expression in the unstimulated cultures does not produce a similar steady increase in PTA expression, possibly because the induction of c-myc in these cultures is aberrant and not reflective of the normal mechanisms controlling c-myc expression. It does however support the thesis that there are some situations in which c-myc alone is not sufficient to induce significant expression of PTA. If a gene were a direct target of c-myc its time course of induction would closely follow that of myc (Dang 1999). Although overall the two genes exhibit

remarkably similar patterns of gene expression there are exceptions to this, as we have shown. Thus, as with the culture of $CD34^+$ cells with various combinations of cytokines to induce expansion and differentiation, it is apparent that induction of c-myc does not always induce expression of PTA. These results suggest several possibilities, including: that PTA is not directly activated by c-myc; in some situations, activation of c-myc is transduced via pathways that bypass induction of PTA; or that inhibitors exist that can block induction of PTA by c-myc.

CHAPTER 7

PTA RECEPTORS IN HAEMOPOIETIC CELLS AND EFFECT OF PTA PROTEIN ON OSTEOCLASTOGENESIS

INTRODUCTION

In the last 10 years receptors for PTA have been described on human PB MNC (Cordero*, et al* 1994) and PHA-stimulated lymphocytes (Cordero*, et al* 1996, Pineiro*, et al* 2001). Recent studies using cross linking of biotinylated or iodinated PTA to the surface of mitogen stimulated lymphocytes with subsequent solubilization of the complexes and analysis by polyacrylamide gel electrophoresis revealed bands of size 75, 57, 45, 43 and 36 kDa (Pineiro*, et al* 2001). Of these the smallest band (36 kDa) was the most intense and showed the highest affinity requiring an excess of native PTA in the range of 500 nM to inhibit labelling. When cross-linking experiments were performed with ^{125}I-PTA as little as 2nM of protein was required for visualisation of the 36 kDa band, which corresponds with concentrations of native protein that have been effective *in vitro*, however, when biotinylated PTA was used for cross-linking a minimum concentration of 15μM was required. The two largest bands identified by cross-linking assays (75 and 57 kDa) were the most easily dislodged in competitive binding assays and the authors surmised that they might represent accessory or non-specific proteins (Pineiro*, et al* 2001).

Affinity chromatography of membrane lysates to a PTA-resin matrix identified three bound proteins of mass 31, 29 and 19 kDa (Pineiro*, et al* 2001). Taken together and assuming a binding ration of 1:1 (protein:receptor), these data indicate the receptor is composed of three molecules of ~31, 29 and 22/19 kDa. Pineiro *et al* hypothesised from their data that the smallest molecule represented the best "fit" of the three candidate receptor bands because it was visualised at concentrations of PTA protein that were biologically active and binding could be abrogated by excess unlabeled PTA. They also suggested that the 31 and 29 kDa bands might comprise a two-chain low affinity receptor, or that one of them might function as an accessory protein.

The identification of receptors goes some way towards explaining the apparent immuno-modulatory effects of PTA both *in vivo* and *in vitro;* which is nonetheless puzzling in a protein that is ubiquitously expressed, located in the nucleus, lacks a secretory signal and has no obvious means of egress from within the cell. As already discussed, the *in vitro* immuno-potentiating activities of PTA are most effective in situations of defective cellular immunity, such as enhancing the anti-tumour responses of cancer patients (Baxevanis, *et al* 1993, Garbin, *et al* 1997) or the MLR of individuals with active SLE (Baxevanis, *et al* 1987). In normal donors, PTA augmented the response of PB MNC (Cordero, *et al* 1991, Cordero, *et al* 1990, Czarnecki 1995) or neutrophils (Wykretowicz 1994) to sub-optimal stimulation by mitogen or phorbol dibutyrate, respectively and NK cell cytotoxicity (Cordero, *et al* 1992)

In addition to the effects on immune responses another extracellular role for PTA has been suggested following the finding that, in apoptotic Hep2 and HeLa cells, truncated PTA protein (its nuclear localisation signal cleaved by caspase-3) was re-located to the cell surface (Evstafieva, *et al* 2003). This group hypothesised that, like phosphatidylserine, surface exposure of a truncated PTA might enhance recognition and removal of apoptotic cells by macrophages.

The aims of the experiments detailed in this chapter were twofold: to examine the receptor profile of unstimulated PB and CB with particular attention to $CD34^+$ cells; and, if receptors were identified on novel populations, to evaluate the effect of PTA protein in functional *in vitro* assays. To achieve these outcomes we adopted the method of Pineiro *et al* (Pineiro, *et al* 2001) and biotinylated calf PTA protein to investigate the presence of receptors for PTA on $CD34^+$ cells. Initially, we repeated the analysis of PB MNC to confirm the utility of the methodology. We also confirmed surface labelling on activated lymphocytes and compared this to unstimulated lymphocytes. We then assessed the PTA receptor status of CB $CD34^+$ cells, but were unable to find convincing evidence of receptors on this cell population. However, our results, like those of Cordero (Cordero, *et al* 1994) indicated the presence of surface receptors on monocytes. Recent publications indicate that bone re-sorbing osteoclasts (OC) may be derived from $CD14^+$ monocytes (Nicholson, *et al* 2000) and that the generation of OC is enhanced if colonies of CB derived CFU-GM are seeded onto dentine slices and cultured with appropriate cytokines (Hodge, *et al* 2004). As several studies indicate that the *in vitro*

activity of monocytes is enhanced by PTA and as they appear to express receptors for PTA, we evaluated the effect of PTA protein on the generation of OC.

RESULTS

PTA receptors in PB

As published data identified PTA receptors on mitogen-stimulated PB MNC we undertook an investigation of receptor profiles on other cell types using a methodology similar to that of Pineiro *et al* (Pineiro*, et al* 2001). Analyses of lysed whole blood or ficoll separated MNC indicated the presence of receptors on monocytes, moderate labelling of neutrophils and an equivocal shift in fluorescence on unstimulated lymphocytes (n=4). Figure 7.1 depicts a typical analysis of lysed adult whole blood.

PTA receptors in CB

Whole CB and MACS enriched and depleted populations were analysed by flow cytometry for the presence of receptors. Fig 7.2 shows a typical analysis of MACS separated $CD14^-$ lymphocytes and granulocytes, $CD3^+$ T lymphocytes and $CD14^+$ monocytes. The MACS $CD14^-$ population (top panel) was analysed for receptor expression on granulocytes and unstimulated lymphocytes, both populations defined by expression of CD45 and their signature forward scatter (size), side scatter (granularity) profiles. Like adult PB, CB neutrophils are positive for PTA receptors (n=3) and unstimulated lymphocytes exhibit a slight shift in fluorescence, but no clear evidence of receptors (n=3). MACS purified CB $CD3^+$ T cells (n=3) showed a shift in fluorescence typical of low density receptor expression and consistent with the data of Pineiro *et al* showing receptor expression on PHA stimulated lymphoblasts. Both anti-CD3 antibodies and PHA activate lymphocytes through the T cell receptor in the presence of monocytes. MACS purified CB $CD14^+$ monocytes (n=5) showed bright staining similar to that seen in the adult PB samples.

PTA receptors in $CD34^+$ cells

$CD34^+$ cells were MACS enriched from CB MNC and assessed for the presence of PTA receptors (n=5). Fig 7.3 shows a typical profile of negligible labelling on $CD34^+$ CB

cells. Additionally, to exclude the possibility of non-receptor based protein up-take, fixed and permeabilised $CD34^+$ cells showed no evidence of intracellular labelling with biotinylated PTA.

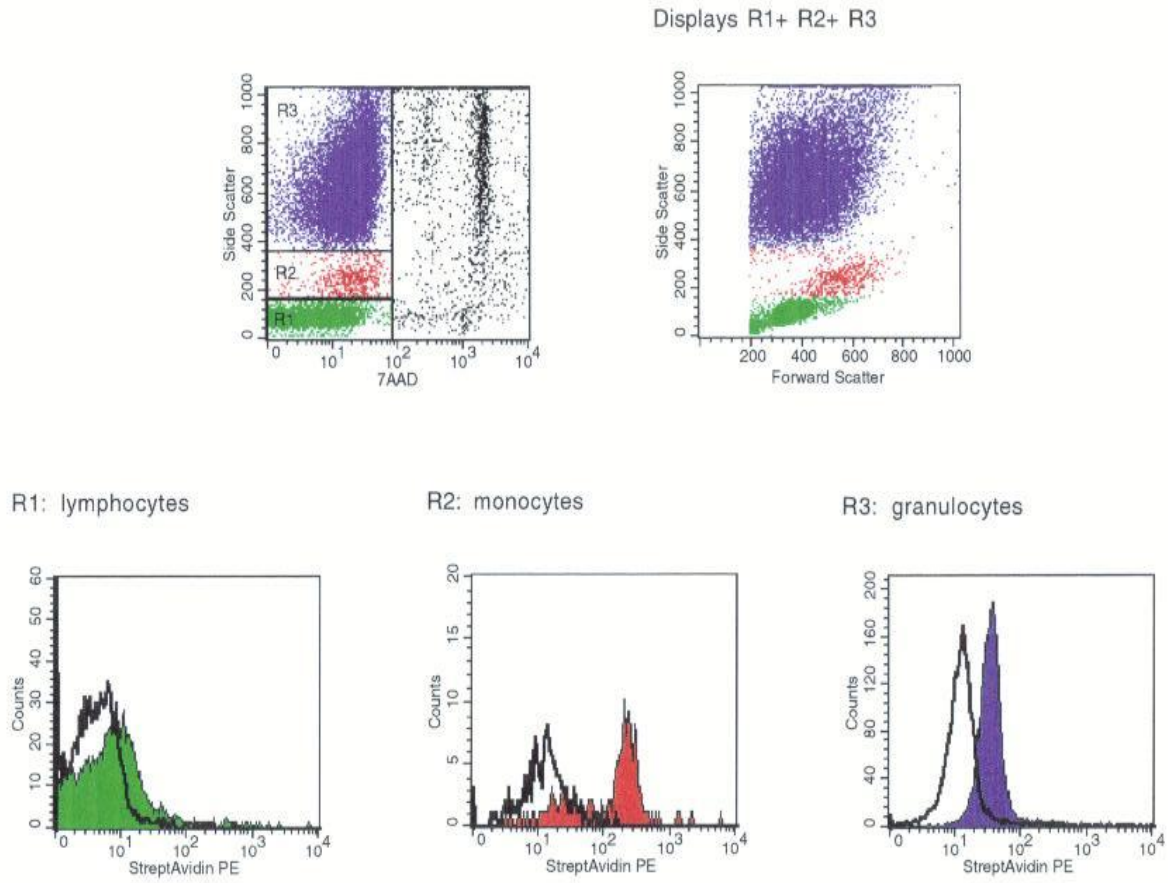


Figure 7.1 Receptors for PTA in lysed adult PB.

Lysed adult whole blood was incubated with biotinylated PTA or carrier (PBS) for control analyses. After thorough washing, samples were incubated with streptavidin-PE for secondary signal detection, washed, resuspended in PBS containing 7-AAD to detect non-viable cells. Samples were analysed by flow cytometry. In the top (L) dotplot viable (7-AAD$^-$) lymphocytes (R1, green), monocytes (R2, red) and granulocytes (R3, blue) are selected and the size (forward scatter) and granularity (side scatter) of each population displayed top (R). In the bottom row the PTA-biotin-Streptavidin-PE labelling of each population is displayed: lymphocytes (left, green), monocytes (middle, red) granulocytes (right, blue). Background labelling of the negative control is overlaid in black.

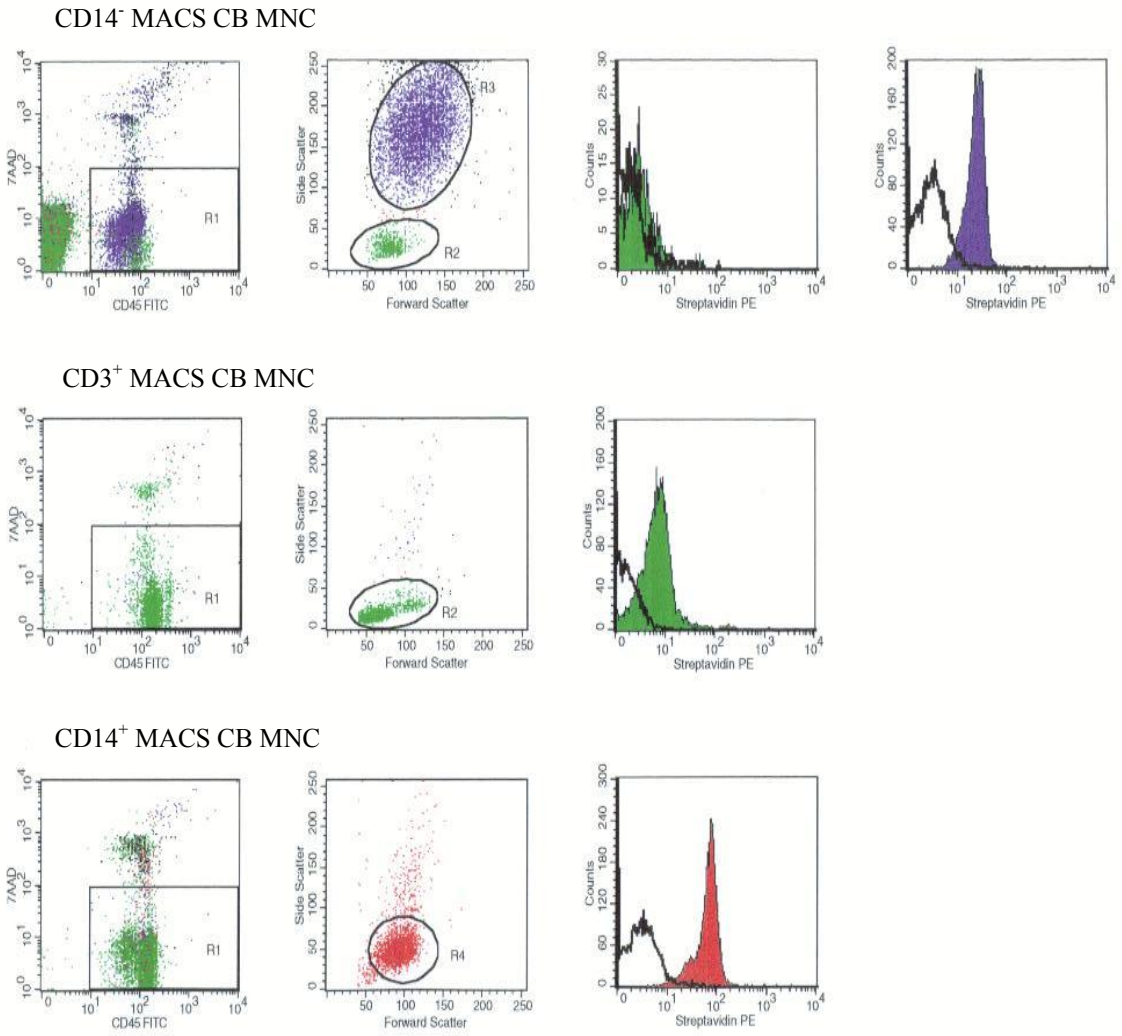
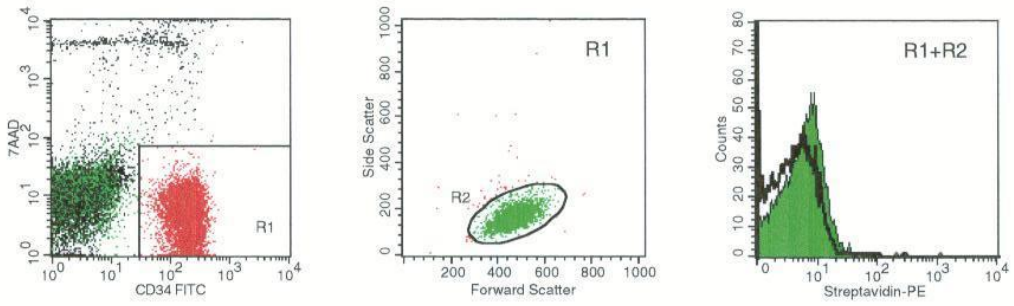


Figure 7.2 PTA receptors in MACS separated CB populations.

MACS depleted and enriched populations were labelled with biotinylated PTA-streptavidin-PE as described and with CD45-FITC (to identify white blood cells) and 7-AAD prior to analysis by flow cytometry. For each of the MACS populations region 1 (R1) (left column) defines viable white blood cells (7-AAD$^-$, CD45$^+$) which are displayed in the second column according to size (forward scatter) and granularity (side scatter). In the top row (CD14$^-$ MACS) lymphocytes (R2, green) and granulocytes (R3, blue) are selected and the specific receptor labelling of each is displayed, respectively, in the histograms at right. In the middle row, CD3$^+$ lymphocytes (R2, green) are selected and the profile of their receptor labelling displayed in the histogram at right. Likewise, in the bottom row CD14$^+$monocytes (R4, red) are selected and the profile of their receptor labelling displayed in the histogram at right. In all histograms background labelling of control cells is overlaid in black.

Figure 7.3 PTA receptor labelling on CB CD34$^+$ cells.

CD34$^+$ MACS enriched cells were incubated with biotinylated PTA as previously detailed. Cells were also labelled with CD34-FITC and 7-AAD as a marker of viability and analysed by flow cytometry. In the dotplot at left, region 1 (R1) defines viable CD34$^+$ cells (7-AAD$^-$, CD34$^+$) which are displayed in the middle dotplot according to size (forward scatter) and granularity (side scatter). Region 2 (R2) defines lymphoid cells, the specific receptor labelling of which is displayed in the histogram at right (solid, green profile). Background labelling of the negative control is overlaid in black.



Effect of PTA protein on osteoclastogenesis

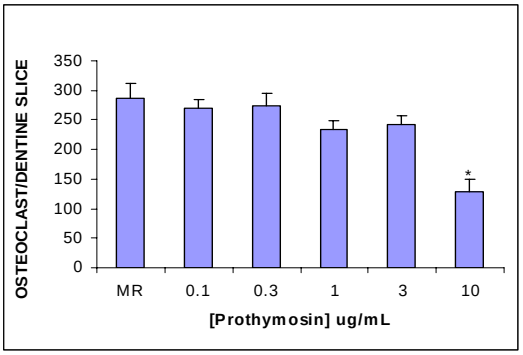
The effect of PTA on the generation of osteoclasts from CB CFU-GM and on their ability to resorb bone was assessed using the modified osteoclastogenesis model of Hodge *et al* (Hodge, *et al* 2004). Day 10 CFU-GM colonies were pooled, disaggregated and cultured with M-CSF, sRANKL and increasing amounts of PTA for 14 days. Control cultures contained M-CSF and RANKL only.

The results of the assays are shown in Fig 7.4. The highest dose of PTA (10μg/ml) inhibited OC formation by ~50% (ANOVA $p \leq 0.001$; n=8), however, the lower doses had no discernible effect. The resorption data show much greater variability of effect, reflecting the inherent variability of the assay which is sensitive to factors such as changes in cell number and different dentine slices. PTA at 10 μg/ml inhibited resorption in comparison with the control cells and in accord with the reduced osteoclast numbers at this protein dose (ANOVA $p \leq 0.027$).

Figure 7.4 Effect of PTA on osteoclast generation and bone resorption (n=8).

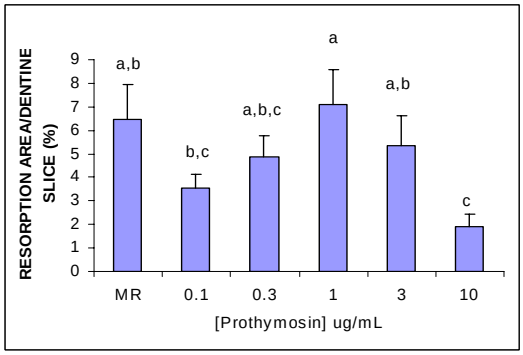
Pooled CB day 10 CFU-GM were cultured on slices of dentine with 25 ng/ml M-CSF (M) and 125 ng/ml sRANKL (R) and increasing concentrations of PTA. Control cultures contained MR only. Cells were assessed at day 14 for (A) osteoclast formation and (B) bone resorption.

A. Osteoclast formation

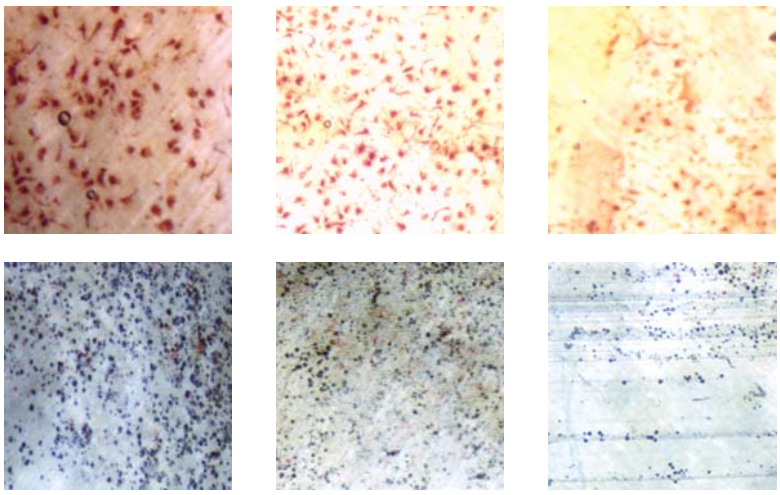


ANOVA p≤ 0.001

B. Bone Resorption



ANOVA p≤ 0.027



Osteoclast formation

Bone resorption

MR

PTA
3 µg/ml

PTA
10 µg/ml

DISCUSSION

In recent years Pineiro *et al* (Pineiro, *et al* 2001) have published a technique they used to identify surface expression of receptors for PTA. The method involved the crosslinking of biotinylated protein to receptors on the cell surface and enabled that group to partially characterise three candidate receptor proteins or accessory proteins. We reproduced (and extended) their published data in MNC from PB and CB with the aim of investigating receptor expression on $CD34^+$ cells. Our studies then prompted the investigation of the effects of PTA in a model of osteoclastogenesis.

PTA receptors in PB and CB

We have demonstrated the presence of receptors for PTA on the surface of granulocytes and monocytes from adult PB and CB. Unstimulated lymphocytes from both sources do not display appreciable binding of PTA-biotin. Pineiro *et al* have previously shown that PHA stimulated PB MNC have surface receptors for PTA. Consistent with their findings, we were able to demonstrate the up-regulation of PTA receptors on magnetically selected $CD3^+$ T-cells. Binding of the T cell receptor (TCR)-CD3 complex by either antigen or CD3 antibody, particularly in the presence of monocytes as was the case in these experiments, activates a cascade of intracellular enzymatic pathways culminating in an increase in intracellular calcium and protein phosphorylation and represents the classic pathway of T cell activation. Plant lectins, such as ConA, pokeweed mitogen, and PHA are mitogenic to peripheral blood lymphocytes through binding to specific carbohydrate residues. In the case of PHA stimulation of PB MNC, activation is triggered through binding to carbohydrate residues present in the TCR.

The kinetics of PTA binding to human PB MNC (Cordero, *et al* 1994) and PHA stimulated lymphocytes (Pineiro, *et al* 2001) have been studied under equilibrium and competitive conditions. Although the dissociation constants (K_D) for each population differ somewhat, both cell populations appear to express high affinity and low affinity receptors. In PHA-stimulated lymphocytes affinity binding and affinity chromatography have permitted further characterisation of the putative receptors, identified as proteins of 31, 29 and 22/19 kDa (Pineiro, *et al* 2001). Whether these proteins represent three different receptors, or one high affinity receptor and another

low affinity receptor composed of two chains, or a receptor and an accessory protein, is yet to be determined. Obviously, cloning of the receptor and identification of its nucleotide and amino acid sequence would enable confirmation of an authentic and specific receptor for PTA.

No evidence of receptors for PTA could be found on the surface of CB $CD34^+$ cells. In addition to surface labelling, and to exclude the possibility of non-receptor based uptake of PTA protein, purified populations of CB $CD34^+$ cells were fixed and permeabilised and assessed for intracellular PTA labelling. As these were also negative for PTA labelling we conclude that CB $CD34^+$ cells do not have receptors for PTA, nor are they able to internalise the protein by any non-receptor based method.

The significance of receptors for PTA is unclear as the protein is almost exclusively nuclear and has no recognisable secretory signal. To date the only convincing evidence of a biologically plausible mode of egress for the protein is the finding that early in apoptosis PTA is cleaved, its NLS destroyed, and the truncated protein re-located to the cell surface (Evstafieva*, et al* 2003). Evstafieva hypothesised that as the only fate of apoptotic cells is degradation and removal, a truncated, externalised PTA may promote immune recognition by phagocytic cells (macrophages and neutrophils). This, however, does not exclude the possibility that a truncated, externalised PTA may also trigger the immune-modulating activities *in vivo* that have been attributed to monocytes and neutrophils exposed to PTA *in vitro*.

Another study has found that incubation of monocytes with PTA (2 μg/ml or ~164 pmol/ml) causes the release of thymosin α_1 ($T\alpha_1$) into the supernatant at a two-fold greater molar basis than the added protein (Frillingos*, et al* 1992). The release of $T\alpha_1$ could be abrogated by the addition of actinomycin D, to inhibit transcription, or cycloheximide, to inhibit and protein synthesis. Incubation of monocytes alone, or with $T\alpha_1$, or incubation of T cells with PTA, did not cause release of $T\alpha_1$ into the supernatant. Furthermore, the RNA content of monocytes increased after incubation with PTA and this was not due to an increase in PTA mRNA. The authors hypothesised that the production of $T\alpha_1$ was due either to the *de novo* production of, and subsequent cleavage of, PTA stimulated by incubation of monocytes with PTA; or the cleavage of $T\alpha_1$ from pre-existing intracellular PTA stocks. Although this study raises the intriguing possibility of an alternative form of the truncated protein being released from the cell, its significance is difficult to interpret as (1) it is PTA, not $T\alpha_1$ that is the

biologically active molecule, and (2) PTA at 2 μg/ml is approximately 70-fold higher than the concentration of PTA in human plasma, a point that will discussed at length below. The biological relevance of this data remains unclear.

Effect of PTA on osteoclastogenesis

The *in vitro* effect of PTA protein was assessed in the generation of osteoclasts and their bone resorbing activity from CB CFU-GM. $CD14^+$ cells cultured for three weeks on bone slices with M-CSF and sRANKL form osteoclasts that demonstrate extensive bone resorption, whereas $CD14^-$ cells do not (Nicholson, *et al* 2000). Hodge *et al* (Hodge, *et al* 2004) increased the efficiency of OC formation in this assay by using cells from day 7-10 CFU-GM colonies derived from CB. They found that CB MNC, cultured for 7-10 days in methylcellulose supplemented with SCF, GM-CSF and IL-3 (a standard haemopoietic progenitor assay) and subsequently cultured in the osteoclastogenic assay for 14 days, were able to produce more OC with greater re-sorbing activity than either unmanipulated CB MNC or PB $CD14^+$ monocytes cultured for 21 days. As the immuno-modulatory effects of PTA appear to target $CD14^+$ cells we were interested to assess the effect of the addition of PTA to the osteoclastogenic assay.

The highest dose (10 μg/ml) was the only one to show any significant effect, causing approximately 50% inhibition of OC formation and a similar reduction in bone resorption. As the precise lineage of the OC and its relationship to other haemopoietic cells is unclear the lack of an effect at lower doses may indicate that OC are derived from haemopoietic progenitors that lack the $CD14^+$ antigen, or that PTA is ineffective in modulating the response of $CD14^+$ cells in this setting. The apparent inhibitory effects at 10 μg/ml most likely reflect high-dose toxicity. Similar high-dose inhibition (at 5 μg/ml) has been noted in cultures of PHA stimulated PB MNC (Czarnecki 1995).

The most comprehensive analysis of the concentrations of PTA in human plasma and blood cells indicates that intracellular levels in leucocytes are the highest (9-13 pmol/ml) (Panneerselvam, *et al* 1987) and comparable to that determined for rat thymus and spleen (Haritos, *et al* 1984b). In human plasma, however, the concentration of PTA is estimated to be 0.75 – 2.3 pmol/ml of whole blood (Panneerselvam, *et al* 1987). Thus, the dose of 10 μg/ml (~820 pmol/ml) is in huge excess to that found in plasma or in cells. In light of the physiological levels of PTA it is difficult to interpret the

significance of its reported immuno-regulatory roles. The effective dose at which PTA augments the proliferation of PHA stimulated PB MNC, the production of IL-2 and the expression of IL-2R is 1 to 5 μg/ml (82-410 pmol/ml) (Baxevanis, *et al* 1990, Cordero, *et al* 1991, Cordero, *et al* 1990, Czarnecki 1995). The effective dose for increasing NK cytotoxicity in normal PB MNC and those of cancer patients is 1 to 2 μg/ml (82-164 pmol/ml) (Baxevanis, *et al* 1993, Cordero, *et al* 1992). In a variety of studies examining the *in vitro* effects of PTA (including its ability to induce expression of HLA-Dr and mRNA production, or IL-2 production and IL-2R expression in normal human PB MNC, and its ability to augment the MLR of SLE patients, or the NK activity of cancer patients) the protein was titrated for every donor and the results reported reflected the optimal dose for each donor (Baxevanis, *et al* 1990, Baxevanis, *et al* 1993, Baxevanis, *et al* 1987, Baxevanis, *et al* 1992). Overall this indicates that the *in vitro* effects of PTA show considerable individual variability and often an effect is significant at doses >35-fold higher than the upper estimate of human plasma concentrations. In the assays of human cells this may reflect cross species differences in protein activity as the most common sources of protein are derived from cow and rat thymus. Furthermore, although the amounts added to culture are non-physiological, the protein concentration available at the cellular level may be much closer to physiological levels as it is not known whether specific carrier molecules are required to permit docking of protein with receptors and/or transport across the plasma membrane.

Several studies have noted significant effects of PTA at doses nearer the upper plasma concentration (2.3 pmol/ml). PTA at 500 ng/ml (42 pmol/ml) augmented the anti-tumour activity of PB MNC from colorectal tumour patients (Garbin, *et al* 1997) and at 100 ng/ml (8.2 pmol/ml) PTA increased the oxidative burst of sub-optimally stimulated neutrophils and the adhesiveness of optimally stimulated neutrophils (Wykretowicz 1994). In any case, although PTA may indeed have a *bona fide* role in immune regulation the ubiquity of its expression and evolutionary conservation imply a general role in cellular metabolism not restricted to cells of the immune system. Rather it is probable that its immuno-modulatory effects flow from its broader housekeeping role which appears to be associated with proliferation, differentiation and possibly, apoptosis.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

INTRODUCTION

The basic methodological strategy adopted in this project was to use differential display to identify genes with differential expression between $CD34^+$ and $CD34^-$ cells from human CB and BM. To broaden the scope of the gene discovery program, samples were incubated for 24 hours with either pooled adult or cord sera. Ten bands were excised that appeared to be differentially expressed, of which two (centromere protein C1 and PTA) were confirmed by real time PCR. The high false positive rate is consistent with the results of others, (Graf 1995) and is an acknowledged shortcoming of the technique (Liang 1995, Liang 1993). The expression pattern of centromere C1 in $CD34^+$ cells appeared to be dependent on the source of sera used in the 24 hour culture. Centromere C1 was not differentially expressed in CB $CD34^+$ cells, but in BM cells was more highly expressed in $CD34^+$ cells cultured with adult sera compared to cord sera. As the gene functions in mitosis, its expression pattern was assumed to reflect the higher proliferation rate of BM $CD34^+$ cells and no further work was pursued with the gene.

The other gene identified, PTA, was more highly expressed in $CD34^+$ haemopoietic cells compared to $CD34^-$ cells from CB and BM. Expression of the gene was ~2.9-fold higher in $CD34^+$ cells compared to $CD34^-$ cells in CB ($p \leq 0.001$, n=25) and ~4.5-fold higher in BM $CD34^+$ cells compared to $CD34^-$ cells ($p \leq 0.001$, n=8). Three other groups have identified differential expression of PTA in haemopoietic progenitors from human and mouse, however there are no published data pertaining to the regulation of the gene in this important population of cells. The $CD34^+$ population is relatively quiescent, so the up-regulation of PTA, a gene closely associated in the literature with active proliferation, is quite intriguing. One of the primary aims of this dissertation was to undertake a comprehensive assessment of the expression and regulation of this gene in subsets of haemopoietic cells and in response to a variety of cytokines well characterised with respect to their ability to induce proliferation and/or differentiation. The results of these studies prompted a comprehensive assessment of PTA and c-myc

gene expression in mature subsets of mononuclear cells and a closer analysis of the induction of gene expression in a well-researched model of cell proliferation, namely the PHA stimulation of PB MNC.

After completing the gene expression and regulation studies we used published techniques to extend the receptor expression data. Finally, we undertook investigations of the effect of the addition of PTA to the culture of OC. This was a particularly pertinent assay system as it melded aspects of the biology of the protein and its receptor (present on $CD14^+$ cells) with that of osteoclastogenesis (possibly derived from $CD14^+$ cells) and the clonal expansion of haemopoietic progenitors (which demonstrably improves the efficiency of OC formation).

CONCLUSIONS

Gene regulation

We compared the levels of gene expression between $CD34^-$ cells, $CD34^+$ cells and a more primitive subset of haemopoietic progenitor cells with the phenotype $CD34^+CD38^-$. There was a significant difference on gene expression between the groups (ANOVA $p \leq 0.002$) with PTA more highly expressed in both the $CD34^+$ and $CD34^+CD38^-$ subset compared to $CD34^-$ cells. Although the level of PTA expression was lower in the more primitive subset compared to the total $CD34^+$ population, the change was not significant. With respect to the expression of PTA in these populations, it is relevant to note that the key features of the $CD34^+$ phenotype and the $CD34^+CD38^-$ subset are immaturity (or primitiveness) and quiescence.

Down regulation of PTA expression in cultures of $CD34^+$ cells purified from BM and CB coincided with differentiation of the cells and significant loss of CD34 positivity over a period of 7 days. Our result is consistent with studies of other cell types where down-regulation of PTA has been associated with terminal differentiation, coincident with a decline in proliferation (Dosil*, et al* 1993, Smith*, et al* 1993). However, we have also shown that combinations of cytokines known to stimulate proliferation and differentiation had no appreciable effect on the expression of PTA over a period of 24 hours. The lack of induction of expression in these circumstances may well reflect that the cells are already maximally expressing the gene, however, the contradiction remains

that an initially quiescent population of cells express a gene reported in other systems to be induced by proliferative stimuli.

To investigate this phenomenon more closely we undertook a detailed study of the induction of PTA expression in PHA stimulated PB MNC. We found that induction of PTA expression occurred within 24 hours following exposure to proliferative stimuli even though the number of actively proliferating cells was unchanged at this time-point. However, the degree and duration of induction was highly variable among individuals, especially in comparison to a gene such as c-myc, the expression of which strongly and reliably correlates with mitogenic activity (reviewed in (Dang 1999)). Our own studies showed significant increase in c-myc expression in response to PHA stimulation at 24 hours (ANOVA $p \leq 0.001$), returning to baseline levels by day4 of culture. Although PTA expression followed a similar pattern the changes did not become statistically significant.

Taken together these results suggest that PTA expression may be less tied to proliferation and more closely associated with differentiation. Although this aspect of PTA expression has been debated extensively in the literature it is worth reviewing the data that support this thesis. Two studies (both of which used the HL60 promyelocytic cell line as a model) specifically addressed the issue of the association of PTA gene expression with proliferation or differentiation and came to slightly different conclusions. Smith *et al* (Smith*, et al* 1993) induced differentiation of HL60 cells with tetradecanoylphorbol acetate (TPA) or bryostatin to induce macrophagic differentiation, DMSO for neutrophilic differentiation and IFN-γ to induce monocytic differentiation while maintaining proliferation. Whereas, with the first 3 differentiation agents (TPA, bryostatin and DMSO), differentiation was clearly associated with a reduction in cycling cells and down regulation of PTA expression to almost undetectable levels by day 1-2, exposure to IFN-γ was associated with differentiation, maintenance of cycling cells and a minor reduction in PTA expression. These authors concluded PTA expression was associated with proliferation. Dosil *et al* (Dosil*, et al* 1993) used TPA and retinoic acid to differentiate HL60 cells along the pathways of macrophage and granulocyte differentiation, respectively, and like Smith *et al* found that PTA expression declined over time (with minor differences in the degree and time-course). To assess the relative contributions of proliferation and differentiation to PTA gene expression they inhibited proliferation in undifferentiated HL60 cells by serum starvation or

treatment with hydroxyurea. Both treatments caused proliferation arrest but only slight decreases in PTA mRNA expression. These results supported the notion that PTA down regulation was associated more strongly with differentiation.

Over-expression of PTA accelerates proliferation by shortening the duration of G1 (Rodriguez, *et al* 1998, Wu, *et al* 1997) and retards or prevents cell differentiation (Rodriguez, *et al* 1998). Down regulation of PTA by antisense oligomers causes significant inhibition of cell proliferation and induces apoptosis (Rodriguez, *et al* 1999, Sburlati, *et al* 1991). In HL60 cells Rodriguez *et al* (Rodriguez, *et al* 1999) found that concentrations of antisense oligomers that inhibited proliferation by up to 50% did not provoke differentiation possibly because the concentrations used may not have produced strong depletion of intracellular levels of PTA mRNA. However, doubling the concentration of antisense oligomers caused a significant increase in apoptosis.

The data supporting a role for PTA in cell proliferation is convincing but this aspect of its biology has overshadowed the other important role in which it appears to have significant influence, differentiation. The data supporting this aspect of its action is strengthened by the finding that PTA is highly expressed in quiescent progenitor populations ($CD34^+$ cells and $CD34^+CD38^-$ subset). The role of haemopoietic progenitor cells is to provide balance to fluctuations in cell number in response to the complex web of signals generated in the haemopoietic microenvironment. It is intellectually satisfying to hypothesise that a broader role for PTA in immature cells is to permit proliferation while helping to keep in check the strong differentiative influences that are provoked by cytokine stimulation. Prolonging the course of differentiation not only increases the output from a single cell (greater clonal expansion) but is a more efficient use of stem and progenitor populations and thus protective of a precious resource.

Receptor Expression

Our data indicates that receptors for PTA are expressed on monocytes and neutrophils, activated T cells but are absent or expressed at negligible levels on unstimulated lymphocytes. We could not find any evidence of receptors on $CD34^+$ cells. The interpretation of the significance of receptors is difficult given that PTA is a ubiquitously expressed nuclear protein, lacking a secretory signal and found at very low

levels in human plasma. To date, the most satisfactory hypothesis for the existence of receptors has been proposed by a group who found a truncated form of the protein on the surface of apoptotic cells and suggested that its presence might elicit recognition by and activation of phagocytic cells (Evstafieva, *et al* 2003). How the truncated form of the protein, cleaved of its NLS, but still lacking a secretory signal, translocates to the outer plasma membrane is yet to be elucidated.

Effect of PTA protein on osteoclastogenesis

The definitive precursor of the OC is not known although published data indicate that it may be either $CD14^+$ (Nicholson, *et al* 2000) or an earlier progenitor of the granulocytic/macrophage line (Hodge, *et al* 2004). As the receptor studies indicated the expression of PTA receptors on monocytes and granulocytes we assessed the effect of PTA on osteoclastogenesis. We were unable to detect any effect of PTA in the generation of OC from CB-derived CFU-GM. This may indicate that $CD14^+$ cells are not the precursors of OC and/or that PTA does not exert an influence on osteoclastogenesis and bone resorption under the conditions used.

The inhibition noted at the highest dose most likely is due to toxicity as it represented a concentration more than 350-fold that of the upper physiological concentration in human plasma.

FUTURE DIRECTIONS

Knock down PTA gene expression

Although a great deal of work has been done regarding the regulation of PTA expression much of it has concentrated on the effects on cell proliferation. As we (and others) have suggested the expression of PTA in non-proliferating cells indicates that its role is not restricted to proliferation alone. An emerging technique that appears useful in the study of gene regulation is RNA interference (RNAi) (Elbashir, *et al* 2002, Fire, *et al* 1998, Harborth, *et al* 2001). In this technique small RNA specific nucleotides, 19-22 bases long, are introduced into the cell which then bind complementary RNA inducing degradation and gene silencing (Elbashir, *et al* 2001a, Elbashir, *et al* 2001b). This phenomenon, first characterized in plants where it is known as “post translational

gene silencing", is thought to be an evolutionarily conserved mechanism for protection against mobile genetic elements (viruses and transposons) reviewed in (Hannon 2002, Maine 2000). At least one study has used RNAi directed at PTA and found that down-regulation of PTA increased the sensitivity of cells to UV-induced apoptosis (Jiang, *et al* 2003). The same study showed that in a reconstituted system using purified enzymes and substrates, recombinant PTA blocked apoptosome formation. However, under normal physiological conditions, PTA is located in the nucleus, rather than the cytoplasm where apoptosome formation occurs. Cleavage of the NLS by caspases is an early event in apoptosis, causing relocation of the truncated PTA to the cytosol, but it is not known whether the truncated protein has the same anti-apoptosome formation effect as the intact protein.

Clearly, further work is required on the role of PTA in differentiation and apoptosis. Given the redundancy evident in metabolic pathways it is unlikely that down regulation of PTA alone would be sufficient to promote "spontaneous" differentiation, which may explain the finding of Rodriguez *et al* (Rodriguez, *et al* 1999) that inhibition of proliferation in HL60 cells by antisense did not coincide with increased differentiation. To extend the work that has been performed in the HL60 model, it may be informative to assess the effect of differentiating agents (RA, TPA, IFN-γ, bryostatin) on cells with down regulated PTA (either through the use of antisense or RNAi).

Another avenue of investigation worthy of consideration is the construction of a knock-out mouse model. Although PTA has been under investigation for over 15 years, no studies using such a model have been conducted, which possibly reflects the numerous difficulties in down regulating a gene that is ubiquitously expressed, not only in the developing embryo but also in tissues of the adult mouse. A conventional, complete inactivation would almost certainly be lethal to the embryo or produce such devastating phenotypic changes as to compromise a detailed functional analysis. However, a conditional knock-out using the Cre/loxP system is feasible. The Cre/loxP system manipulates the activity of the P1 bacteriophage enzyme, Cre recombinase. The enzyme mediates recombination events in a specific 34bp sequence, called the **lo**cus of crossover (**x**) in **P**1, or **loxP**, consisting of two 13bp inverted repeats separated by an 8bp non-palindromic sequence {Torres, *et al* 1997, Nagy, *et al* 2003}. The versatility of this system permits deletion/integration events, translocations or inversions. Furthermore, genetic engineering has produced strains of mice with either constitutive

or inducible tissue-specific Cre recombinase activity. Conditional gene targeting limiting the gene modification both temporally and in its cellular distribution would be particularly useful enabling the study of the role of PTA in specific cell lineages and/or at certain developmentally sensitive times.

PTA regulation by c-myc

The expression of PTA and both c-myc and N-myc are very closely associated in numerous systems studied. Eilers *et al* (Eilers, *et al* 1991) showed that PTA expression was induced in RAT 1A cells engineered to produce a chimeric protein consisting of the hormone-binding domain of the oestrogen receptor (ER) and c-myc, the activity of which was conferred by binding of oestrogen to the ER moiety of the chimera. The PTA gene contains myc binding E-box elements in its promoter and first intron, but whether these cause activation of PTA expression is contested (Desbarats, *et al* 1996, Mol, *et al* 1995). We have suggested an alternative interpretation of the data of Eilers *et al* based on the published evidence that the PTA promoter region contains multiple binding sites for Sp1 (a ubiquitous transcription factor) and that the unliganded ER associates with the DNA-binding domain of Sp1 which becomes transcriptionally active on addition of oestrogen. Our alternative theory (that addition of oestrogen to the engineered RAT 1A cells caused transcription of PTA mediated by Sp1, rather than c-myc) could be tested in the system constructed by Eilers, or in co-transfection studies in other systems, perhaps by down regulating Sp1 expression or blocking/deleting Sp1 and c-myc sites in PTA constructs.

Receptors for PTA

There is much work still to be done on defining and incontrovertibly establishing the existence of specific PTA receptors. The obvious experimental strategy is to clone the receptor so that studies of its expression could be conducted at the molecular level. Definitively identifying the gene involved would facilitate studies of the regulation of receptor expression and possibly illuminate metabolic pathways dependent on either a membrane-bound or soluble form of PTA protein, perhaps even elucidating the means by which PTA is extruded from the cell.

FINAL COMMENT

PTA, first isolated from rat thymus in 1984, has been studied extensively in the intervening years but unfortunately remains a biological enigma. Several putative roles have been suggested including involvement in chromatin remodelling, transcription, RNA splicing or nuclear/cytoplasmic trafficking. It has also been suggested PTA may act as a shuttle protein transporting a variety of molecules from the cytoplasm to their nuclear site of action, chaperoning molecules through highly charged nuclear niches. Of these, the shuttle protein/chaperone role best explains the perplexing and conflicting assortment of proteins to which PTA binds, however definitive evidence is lacking. Recent studies also indicate a role in apoptosis, possibly through inhibition of apoptosome formation.

Although clearly associated with cellular proliferation its regulation and expression are also intrinsically bound to differentiation. Indeed we have demonstrated the differential expression of PTA in $CD34^+$ cells that are inherently quiescent and immature. Further exploration of the role of PTA in differentiation is of particular relevance in the haemopoietic progenitor cell and may be of clinical utility in elucidating the mechanisms by which the twin aims of *in vitro* expansion of progenitor cells while maintaining "stemness" can be obtained.

The challenge of definitively characterising the role and regulation of PTA remains. However, the existence of homologs in non-mammalian cells combined with emerging techniques that provide robust and efficient mechanisms of analysing gene function presents new opportunities for manipulating PTA expression in an effort to determine its function/s.

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