A Study of the Interactions Between Macrophage Inflammatory Protein 1 alpha (MIP-1α) and Transforming Growth Factor Beta (TGF-β) in the Control of Haemopoietic Stem Cell Proliferation

By

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Submission for the Degree of Doctor of Philosophy

in

The Faculty of Medicine

at the

University of Glasgow

June 1995

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Dedication

This thesis is dedicated to my family and Carole

Acknowledgments

Firstly, I would like to thank my supervisor Dr. Gerry Graham for his first class guidance, supervision, unending enthusiasm for my project and consistently feeble attempts at humour. Also, I would like to thank him for teaching me the English language intricacies, no, sorry, the intricacies of the English language. Thanks must also go to both Dr. Ian Pragnell and Dr. Ken Parkinson for their constructive comments and criticisms during my PhD. I would also like to thank all the other members of R2 for both technical and intellectual assistance, including Dr. Jane MacKenzie, Sharon Lowe, Mary Freshney, Alison Sim, and Dr. Robert Nibbs for his incredible ability of repeating things I said that I specifically told him not to repeat. I am extremely grateful to Lynn McGarry for helping with the densitometric analysis.

Thanks must go to all my friends who have made life in Glasgow an interesting and enjoyable experience. Special thanks must go to Dr. Carole Robertson for putting up with me for the past few years. Finally, I would like to thank my parents for their continued support and financial "grants" throughout my three years as a student and beyond.

I acknowledge that this work was supported by a grant from the Medical Research Council.

Declaration

I declare that all work in this thesis was performed by me personally unless otherwise acknowledged

ABSTRACT

TGF-β1 and MIP-1α have recently been identified as potent inhibitors of haemopoietic stem cell proliferation. From previous studies, these molecules appear to have similar functions in the control of stem cell proliferation. This study was designed to investigate the relationship, if any, of these two negative regulators in an attempt to elucidate possible distinctive roles for each within the haemopoietic system. The results presented in this thesis demonstrate that both MIP-1α and TGF-β are capable of potently inhibiting the same stem cell population (colony forming unit [CFU]-A/CFU-S). It is further shown that all three mammalian isoforms of TGF-β are capable of potently inhibiting MIP-1α gene expression in bone marrow-derived macrophages, a likely source of MIP-1α in the bone marrow. These molecules differ slightly in their potency in this regard, and more diverse members of the TGF-β superfamily such as activin and BMP-2 do not exhibit this effect within the same spectrum of concentrations as TGF-β1,-β2 or -β3. This inhibition is not specific to MIP-1α in that expression of MIP-1β, a related molecule that does not exhibit potent stem cell inhibitory properties, is inhibited in a similar manner. However, expression of RANTES, which is a more diverse member of the MIP-1α superfamily, appears to be unaffected by TGF-β. The inhibition of MIP-1α gene expression is also seen as a reduction in MIP-1α protein production by the murine macrophage cell line RAW 264.7. These *in vitro* results suggest that in the presence of active TGF-β *in vivo*, and in the absence of upregulators of MIP-1α expression, very little MIP-1α will be produced. To address how the target cells of MIP-1α, the stem cells, would respond to TGF-β, and the consequently low levels of MIP-1α produced, the effect of TGF-β on MIP-1α receptor levels on FDCP-MIX cells, a murine stem cell line was analysed. TGF-β (100pM) reversibly downregulates MIP-1α receptor levels on these cells to a maximum of around 50-70% after 24hrs. This level of downregulation does not change upon increasing the concentration of TGF-β or the length of time of

exposure of the cells to TGF-β. Scatchard analysis shows that TGF-β downregulates MIP-1α receptor number numbers with no change in affinity of the remaining receptors for ligand. Further, this reduction in MIP-1α receptor numbers by TGF-β is reflected in reduced ability of FDCP-MIX cells to mobilise calcium in response to MIP-1α. These results suggest that TGF-β may be capable of interfering with the role of MIP-1α as a stem cell inhibitor. Indeed, they suggest that in the presence of active TGF-β *in vivo*, and in the absence of upregulators of MIP-1a expression, MIP-1α may only be a weak contributor to the overall physiological inhibition of stem cells. To fully understand the interactions between these two molecules, and to address the possibility that MIP-1α functions either wholly or in part through induction of TGF-β, the effect of MIP-1α on TGF-β1 gene expression and protein production was investigated. The results demonstrate that MIP-1α acts as an inducer of both TGF-β gene expression and protein production in bone marrow macrophages. This suggests that MIP-1α may act through upregulation of TGF-β in these and possibly other secondary cell types. However, inactivation of endogenous TGF-β in the *in vitro* CFU-A assay does not reduce the ability of MIP-1α to inhibit CFU-A colony formation. This suggests that although MIP-1α can induce TGF-β1 in bone marrow macrophages, it does not function as a stem cell inhibitor through this action. Indeed, the results presented in this thesis suggest that while MIP-1α and TGF-β appear to have overlapping roles with respect to inhibition of stem cell proliferation, they both act via independent mechanisms. In summary, the results from my PhD suggest that in cells expressing both MIP-1α and TGF-β, TGF-β will be dominantly expressed and may therefore be the major contributor to physiological inhibition of stem cells.

CONTENTS

APPENDIX - publication

Maltman, J., Pragnell, I.B., and Graham, G.J. Macrophage inflammatory protein 1a: Is it a downregulator of stem cell inhibition by transforming growth factor-b. *J. Exp. Med.* **178**:925-932, 1993.

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Abbreviations

^{32}P	^{32}Phosphorous
5-FU	5-fluorouracil
A	adenine
Ara-C	cytosine arabinoside
ATP	adenosine triphosphate
BM	bone marrow
BMDM	bone marrow derived macrophage
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary deoxyribonucleic acid
CFC	colony forming cell
CFU-A	colony forming unit arbitrary
CFU-G	colony forming unit granulocyte-macrophage
CFU-S	colony forming unit spleen
cpm	counts per minute
CTP	cytidine triphosphate
d8	day 8
d12	day 12
DAG	diacyl glycerol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DHS	donor horse serum
DNA	deoxyribonucleic acid
Dpp	decapentaplegic

ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid,disodium salt
FCS	foetal calf serum
g	gram
G	guanine
G-CSF	granulocyte colony-stimulating factor
GAPDH	glyceraldehyde phosphate dehydrogenase
Gi	inhibitory G protein
GM-CSF	granulocyte-macrophage colony stimulating factor
Gs	stimulatory G protein
GTP	guanosine triphosphate
HCl	hydrochloric acid
HPP-CFC	high proliferative potential-colony forming cell
hr	hour
IL	interleukin
IP3	inositol triphosphate
kDa	kilodalton
l	litre
LPS	lipopolysaccharide
LTBMC	long term bone marrow culture
LTCIC	long term culture initiating cell
LTRC	long term repopulating cell
m	micro
m	milli
M	molar
M-CSF	macrophage colony stimulating factor

mA	milliamps
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
MIS	mullerian inhibiting substance
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	messenger RNA
nM	nanomolar
^{o}C	degrees celsius
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PF-4	platelet factor four
PKC	protein kinase C
PLC	phospholipase C
pM	picomolar
PMA	phorbal myristal acetate
RANTES	regulated on activation normal T expressed and secreted
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SCF	stem cell factor
SCI	stem cell inhibitor
SDS	sodium dodecyl sulphate
SIS	small inducible secretable
Sl	steel
SSC	sodium chloride and sodium citrate
SV40	simian virus 40

T	thymidine
TEMED	tetramethylenediamine
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
TPA	12-O-tetradecanoyl 13-phorbal acetate
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol
U	units
UTP	uridine triphosphate
UV	ultraviolet
V	volts
v/v	volume for volume
w/v	weight for volume
wt	wild type

CHAPTER 1

INTRODUCTION

THE HAEMOPOIETIC SYSTEM

CHAPTER 1: THE HAEMOPOIETIC SYSTEM

1.1 Structure of the haemopoietic system.

Adult mammalian haemopoiesis occurs primarily in the bone marrow and is a highly complex developmental system in which self-renewing pluripotent stem cells give rise, through processes of cellular differentiation and proliferation, to lineage restricted progenitor cells and then to mature functional end cells. This system has classically been viewed as being pyramidal in shape with three overlapping compartments comprising around 0.1-0.4% stem cells, approximately 3% progenitor cells and greater than 90% maturing end cells (Metcalf 1984, Testa and Gale 1988). Haemopoiesis gives rise to at least eight functionally distinct mature populations (see figure 1.1) which have remarkably diverse functions ranging from specific host defence by B and T lymphocytes to oxygen distribution by erythrocytes. In a steady state situation, vast numbers of mature cells need to be manufactured daily due to their relatively short life span. For example, neutrophils expire after around eight hours and erythrocytes after approximately 120 days (Golde 1991). It has been estimated that humans produce around $3.7x10^{11}$ haemopoietic cells per day simply to maintain homeostasis, and that this number can increase up to tenfold when the body is stressed due to infection or disease (Golde, 1991). Cellular disorders in haemopoiesis can lead to a variety of diseases such as leukaemias, anemias and immunodeficiencies. However, the rarity of these disorders in such a vast pool of proliferating cells demonstrates haemopoiesis to be under elaborate and highly efficient mechanisms of control.

1.2 Stem cells

Production of mature blood cells is ultimately controlled at the level of the stem cell and these stem cells constitute a minute population in overall haemopoiesis. The very small numbers of stem cells implies that they must be under tightly regulated control to avoid possible exhaustion. In fact, only about 10% of CFU-S stem cells (see below) are in cell cycle in a steady state situation,

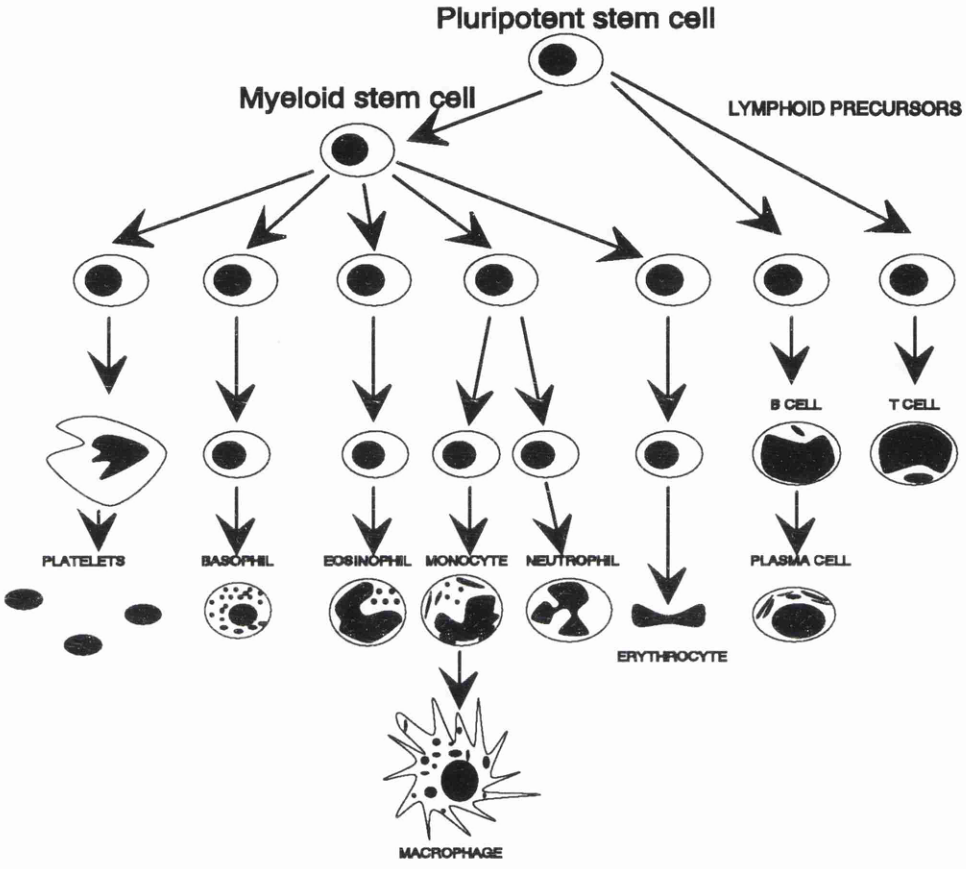


Figure 1.1 A schematic representation of the haemopoietic system

the remainder being quiescent or in the Go stage of the cell cycle (Hodgson *et al* 1982). When the system is stressed, for example by chemotherapy or irradiation, stem cells are triggered into active cell cycle in order to replenish progenitor and mature cell compartments. When this has been accomplished, the stem cells re-enter quiescence. The overall numbers of stem cells do not decrease over the lifetime of an individual however (Zipori 1990).

A stem cell can be defined as that cell which has the capacity to self-renew, or to differentiate. The stem cell is pluripotent i.e. has the potential to differentiate into all haemopoietic cell lineages. Further, stem cells have the property to repopulate a tissue after injury (Lajtha 1979), and indeed to repopulate the haemopoietic system of an animal after otherwise lethal irradiation (Spangrude *et al* 1988, 1991). Stem cells are very difficult to distinguish morphologically, and thus our knowledge of them originates from a series of *in vivo* and *in vitro* assays. These shall be discussed below. It has become apparent from the results of the assays described below that stem cells comprise of a highly heterogeneous population, that can be arranged into distinct but overlapping compartments. Cell types can be arranged into a hierarchical order defined by their "primitiveness". That is, those cell types which possess high self renewal potential and low differentiation tendency are more primitive than those which have limited self renewal potential and high differentiation tendency.

Some insight into the abilities of stem cells to self renew has been gained from repopulation experiments using retrovirally marked stem cells. Capel *et al* (1990) have demonstrated that very few long term repopulating cells (LTRC) are required to maintain adult haemopoiesis, and that they are long lived. Further, other groups have demonstrated that haemopoiesis is maintained by a number of different clones, which can repopulate the system and then return to quiescence allowing other clones to proliferate and differentiate thus continuing the mature cell repopulation (Snodgrass and Keller 1987, Abkowitz *et al* 1990, Harrison *et al* 1988).

1.2.1 The *in vivo* CFU-S assay

The existence of multipotent and self renewing haemopoietic cells can be demonstrated using the murine colony forming unit-spleen assay (CFU-S) (Till and McCulloch 1961). This assay involves lethally irradiating a mouse such that its haemopoietic system is severely compromised, and then intravenously injecting bone marrow from a syngeneic animal. Injection of donor bone marrow results in stem cell mediated rescue of the animal and formation of macroscopic colonies on the spleen which are derived from the inoculated haemopoietic stem cells. The time at which such colonies appear on the spleen indicates their "primitiveness". Put simply, this means that colonies appearing early after transplantation are derived from more mature cells while colonies appearing later are derived from more primitive cells. For example, colonies appearing at day 7/8 after irradiation have been shown to be clonogenic (Williams *et al* 1984) and contain mature haemopoietic lineages. When cells from these colonies are injected into a similarly irradiated animal however, little CFU-S colony formation occurs indicating CFU-S day 7/8 to be relatively lineage restricted and to have limited self renewal capacity (Wolfe and Priestley 1986). Colonies appearing at day 12 however are derived from stem cells capable of generating multilineage colonies. Further, serial transplantation of cells from day 12 colonies results in further CFU-S colony formation indicating both their pluripotency and their extensive self-renewal capacity. Thus, day 12 CFU-S are more primitive than day 7/8 CFU-S as assessed by both their ability to differentiate into a wider range of mature cell types and self renew (Wolfe and Priestley 1986). It was first assumed that day 12 colonies were derived from day 7/8 colonies through differentiation and self renewal. Magli *et al* (1982) have shown however that this may not be the case and that around 50% of the day 7/8 colonies, which are largely erythroid in nature, subsequently regress and are replaced by multilineage day 12 colonies. Two possibilities for the origin of the day 12 colonies not directly derived from day 7/8 colonies have been proposed. The first is that they arise from stem cells which originally seeded in the bone marrow, but were then consequently attracted to the spleen later in the

repopulation process (Van Zant 1984). The second possibility is that they are derived from stem cells which have migrated out from the day 7/8 colonies (Lord *et al* 1989). This remains to be confirmed.

Studies using phase specific cytotoxic drugs have further demonstrated that day 7/8 CFU-S cells are extremely sensitive to killing by these drugs indicating them to be largely in cycle whereas day 12 CFU-S cells are much more refractory to their cytotoxic properties indicating them to be largely quiescent (Hodgson *et al* 1982). Thus, this assay system demonstrates that the portion of the stem cell compartment detectable by its ability to form spleen colonies consists of a heterogeneous population of cells with differing abilities to differentiate and self renew. It was initially believed that day 12 CFU-S might represent the true totipotent haemopoietic stem cell *in vivo*. However, a number of observations suggested that this was not the case. Firstly, CFU-S are incapable of giving rise to lymphoid precursors. Secondly, although donor CFU-S are crucial in the rescue of an otherwise lethally irradiated mouse in the short term (Jones *et al* 1990), they are incapable of setting up long term haemopoiesis *in vivo* or *in vitro*. Thirdly, there is little correlation between CFU-S numbers in the donor marrow and its ability to initiate long term haemopoiesis in an irradiated animal (Chertkov *et al* 1985). This implies that there is a more primitive stem cell type, pre-CFU-S, which can give rise to both lymphoid progenitors and myeloid progenitors such as CFU-S. Indeed, such cells have been identified and separated from the CFU-S compartment by virtue of the fact that they retain the dye Rhodamine 123 (Ploemacher and Brons 1988). These cells have been termed long term repopulating cells (LTRC) and, as their name suggests, are capable of maintaining long term haemopoiesis. They are not in themselves sufficient however to initiate repopulation in an irradiated animal, and require the presence of less primitive CFU-S type cells to initially and quickly repopulate the mature haemopoietic compartments (Jones *et al* 1990). Thus, CFU-S represent a part of the stem cell compartment more primitive than committed progenitors such as GM-CFCs, but less primitive than the long term repopulating cell which shall be discussed below.

1.2.2 *In vitro* stem cell assays

As with the *in vivo* CFU-S assay, *in vitro* stem cell assays also demonstrate the heterogeneity which exists within the stem cell compartment. These assays rely on the fact that clonogenic bone marrow cells treated with the appropriate growth factors give rise to colonies in semi solid culture conditions, and the size and cellular composition of these colonies indicate the level of immaturity of the cell from which they were derived. Although not an exhaustive list, some of these assays are described below.

1.2.3 High proliferative potential colony forming cells (HPP-CFC)

The HPP-CFC assay has been used to identify three distinct populations of stem cells. These have been termed HPP-CFC 1, 2 and 3 whereby HPP-CFC 1 represent the more primitive cells while HPP-CFC 3 are the more mature (reviewed in Bertoncello 1992). Cell cycle toxicity studies have shown that HPP-CFC 1 are highly refractory to the effects of 5-FU treatment (Bradley and Hodgson 1979) whereas HPP-CFC 2 and 3 are more sensitive (Bradley *et al* 1991, McNiece *et al* 1987). Since 5-FU is cytotoxic to cycling cells, this demonstrates that HPP-CFC 2 and 3 are cycling more rapidly than HPP-CFC 1, and are thus more mature. Further, McNiece *et al* (1987) have also demonstrated that HPP-CFC 1 can generate CFU-S day 12 cells suggesting that HPP-CFC 1 in fact represent a pre-CFU-S population, and are probably the most primitive colony forming haemopoietic cell type detectable *in vitro*.

1.2.4 The CFU-A assay

This assay detects a primitive haemopoietic progenitor similar if not identical to day 12 CFU-S (Pragnell *et al* 1988, Lorimore *et al* 1990). The development of this assay has been successfully used to examine a number of regulators of stem cell proliferation, and was used as a screening assay during purification to homogeneity of the stem cell inhibitor macrophage inflammatory protein-1α (Graham *et al* 1990). The assay is set up using two layers of agar

medium, of which the underlayer consists of a 0.6% agar/medium mixture containing the growth factors and additives. Conditioned medium from the cell line L929 is used primarily as a source of the growth factor M-CSF while conditioned medium from the cell line AF-1 is used primarily as a source of GM-CSF. These molecules are believed to act in synergy to promote colony formation. However, Holyoake *et al* (1993a) have recently shown that stem cell factor (SCF) is also required in the human equivalent of the CFU-A assay. The top layer consists of 0.3% agar and normal bone marrow cells. When incubated at 37°C/5% O_2 for 11 days, these conditions give rise to the formation of macroscopic colonies (>2mm) comprised of several haemopoietic lineages including macrophages, granulocytes and megakaryocytes.

1.2.5 Long term bone marrow cultures (LTBMC)

Long term bone marrow cultures represent a novel *in vitro* culture system which involves adding fresh normal bone marrow cells into a tissue culture flask. This results, under the appropriate conditions, in the formation of an adherent stromal layer on the surface of the dish which is capable of maintaining haemopoiesis. The resultant cultures displayed haemopoietic characteristics, and indeed, the presence of all stages of CFU-S cells persisted at readily detectable levels for many weeks, even in the absence of added growth factors (Dexter and Spooncer 1987, Schofield and Dexter 1985). Further, Frazer *et al* (1991) have demonstrated that cells with long term repopulating potential *in vivo*, and with both lymphoid and myeloid repopulating ability are also maintained for many weeks. Thus, LTBMC appear to successfully recreate the bone marrow microenvironment *in vitro*. This assay system has since proved invaluable for assessing the potential *in vivo* roles of many positive and negative regulators of haemopoietic stem cell proliferation and differentiation (Kittler *et al* 1992, Eaves *et al* 1991). Another feature of LTBMC worth noting is that studies with subpopulations of both human and murine bone marrow cells have shown that clonogenic committed progenitors rapidly undergo terminal differentiation in these

conditions. Those which were initially present have disappeared after approximately four weeks, and the clonogenic progenitors detected after this time period are derived from more primitive cell types termed long term culture initiating cells (LTC-IC) (Van der Sluijs *et al* 1990, Winton and Colenda 1987, Sutherland *et al* 1988). LTC-IC can therefore be quantitated by the number of clonogenic progenitors present in the cultures after five weeks, and have also been more accurately estimated using limiting dilution techniques. Indeed, LTC-IC have been shown to be present at 1 in $2x10^4$ nucleated cells in normal bone marrow aspirates (Udomsakdi *et al* 1992), but the proliferative potential of each LTC-IC has also been shown to be highly variable suggesting that the LTC-IC population detected using these techniques may be, and almost certainly is, heterogeneous. It should be noted that LTC-IC and pre-CFU-S are likely to be identical.

1.2.6 Is there a need for long term repopulating cells?

Whilst the *in vitro* assays described above clearly demonstrate the heterogeneity which exists within the stem cell compartment, it is still unclear what contribution cells from each sub-population of the overall compartment make to steady state haemopoiesis. It is conceivable for example that long term repopulating cells are never actually required throughout normal adult life in order to maintain a healthy haemopoietic state, and that mature blood cell production is predominantly controlled at the level of CFU-S type cells, or even at the level of more mature progenitor cells.

1.3 Progenitor cells

Stem cell division can yield, other than identical stem cells, committed progenitors of the megakaryocytic, lymphoid, erythrocytic and myeloid lineages. These cells differ from stem cells in that they have a much reduced proliferative capacity, and can only further differentiate into one or at most two mature cell types. As described above, our knowledge of both stem and progenitor cell types is derived from a series of *in vitro* assays. Progenitor cells are recognised by their

ability to form colonies containing one or more blood cell types in semi-solid tissue culture medium containing agar or methylcellulose and an appropriate "cocktail" of growth factors. Originally, cells from blood forming tissues were cultured with "feeder layers" containing other cell types such as normal embryo fibroblasts (Sachs 1992). These were chosen as possible candidates for the cells which can produce the regulatory molecules required for blood cell formation. This work led to the discovery of clonogenic cells capable of giving rise to colonies containing macrophages, granulocytes or both in various stages of differentiation (reviewed in Sachs 1992). Colony assays, similar in principle to this, have now been developed for many of the other identified haemopoietic lineages (Metcalf 1984). The mature cell types present in the colonies formed in each of these assay systems were used to name each of the corresponding progenitor cell types from which they were derived. For example, cells giving rise to colonies containing granulocytes and macrophages are called CFU-GM or colony forming unit granulocyte-macrophage. Concomitantly, these assays also led to the discovery and ultimate cloning of soluble factors which may be required for the proliferation, differentiation and survival of the progenitor cells under *in vitro* conditions. These shall be discussed in more detail in the following sections.

1.4 Mature End Cells

The ultimate progeny of the stem and progenitor cell compartments are the mature end cells. These cells exhibit a remarkable range of functions ranging from non specific and specific host defence to oxygen distribution throughout the body. At least 8 major cell types are currently recognised (See figure 1.1), although others such as Langerhans cells in the skin are also known to be of haemopoietic origin. The functions of each of these cell types will not be discussed in detail in the text unless relevant.

1.5 Haemopoietic growth factors

A vast array of soluble factors capable of affecting haemopoietic cell growth and differentiation have been identified in the past few years. For myeloid colony formation, four different proteins were identified initially which induced colony formation. These were macrophage colony stimulating factor (M-CSF), granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF) and interleukin 3 (IL-3) (reviewed in Sachs 1992). IL-3 can stimulate formation of colonies containing granulocytes, macrophages, eosinophils, mast cells, erythroid cells or megakaryocytes. Recently, a number of other growth factors have been identified which also appear to play a role in haemopoietic development including stem cell factor (Williams *et al* 1990a, Copeland *et al* 1990), thrombopoietin or megakaryocyte colony stimulating factor (Lok *et al* 1994, De Sauvage *et al* 1994, Wendling *et al* 1994), erythropoietin (reviewed in Ridley *et al* 1994), and a host of molecules termed interleukins. It has become clear from studies analysing the actions of the haemopoietic growth factors that, although some of them e.g. IL-3 can support limited survival of stem cells (Kittler *et al* 1992), combinations of various factors are required for optimum proliferation and differentiation induction (Quesenberry 1992a,b).

It is still unclear what overall role growth factors and cytokines play in regulation of haemopoiesis, and a number of hypotheses have been proposed. It is possible for example that they may direct primitive cells to differentiate along one or another lineage e.g. erythropoietin would stimulate production of erythrocytes and G-CSF production of neutrophils. It is also possible however that they may just be "happiness" factors required to prevent the cells from apoptotic cell death (Koury 1992, Williams *et al* 1990b), and they are not in fact required to instruct stem and progenitor cells to differentiate into multiple lineages. The stem cells may simply follow a "preprogrammed" pattern of proliferation and differentiation as long as the necessary "happiness" factors are present. In agreement with this possibility, Fairbairn *et al* (1993) transfected murine FDCP-MIX cells, an IL-3 dependent, multipotent haemopoietic cell line with the human *bcl-2* gene. The bcl-

2 protein has been demonstrated to be involved in suppressing apoptosis. Indeed, FDCP-MIX cells transfected with this gene become less dependent on IL-3 for survival, and further, are capable of multilineage differentiation in the absence of added growth factors. This data suggests that stem cell differentiation may be intrinsically determined, and that the presence of growth factors is enabling rather than inductive. It should be noted however that horse serum used in these experiments was capable of affecting the differentiation outcome of the cells suggesting that some factors present in the serum are capable of instructive interference.

Although this intrinsically determined stem cell differentiation pathway may be operating, studies using mice defective for certain growth factors have suggested that the presence of these factors may still be required *in vivo* for certain differentiation steps. For example, mice deficient for G-CSF suffered severe neutropenia which was fully reversible upon addition of exogenous G-CSF (Lieschke *et al* 1994a). Low levels of mature neutrophils were still present in the peripheral blood of these mice however, indicating that other factors are capable of compensating for the lack of G-CSF to some extent. Presumably however, absence of these other factors also would result in absolute neutrophil deficiency, stressing the importance of growth factors in normal haemopoietic development. Indeed, mice deficient for both M-CSF and GM-CSF exhibit symptoms common to both the M-CSF and the GM-CSF deficient mice (Stanley *et al* 1994) alone such as defects in osteoclast and macrophage formation (op/op M-CSF deficient mice), and alveolar-proteinosis-like lung pathology (GM-CSF knockout) (Lieschke *et al* 1994b). Further, both Steel (Sl) and white spotting (W) anaemic mice, now known to be deficient in stem cell factor and c-kit, its receptor, respectively, have some similar phenotypic abnormalities including severe anaemia and deficient activity of very primitive haemopoietic stem cells (Russell 1979). Gurney *et al* (1994) have generated c-mpl-deficient mice. C-mpl is the receptor for thrombopoietin, and these mice suffered from an 85% decrease in platelets and megakaryocytes but had

normal amounts of other haemopoietic cell types, demonstrating the importance of c-mpl, and indeed thrombopoietin in megakaryocytopoiesis.

1.5.1 Functional redundancy between positive growth factors?

As mentioned above, very few growth factors are capable of stimulating proliferation of primitive haemopoietic cells on their own. A combination of two or more growth factors is required. It has become apparent from the many studies analysing growth factor actions on haemopoietic cells that there is considerable overlap in their activities. For example, IL-6, leukaemia inhibitory factor (LIF) and IL-11 all share pleiotropic actions on haemopoietic stem cells and hepatic cells. This is not fully understood but may arise from the fact that the receptors for these molecules all share a common β chain called gp130 (Miyajima *et al* 1993). In fact, receptor subunit sharing is a common feature of the haemopoietic growth factor receptor family, and may indeed explain the apparent functional redundancy between certain members (e.g. GM-CSF, IL-3 and IL-5 (Miyajima *et al* 1992)). It should be noted however that the term redundancy used by many workers may be misleading, and that more detailed analysis of the precise signal transduction pathway of each of the haemopoietic growth factors may reveal subtle but important differences in their functions.

Studies of growth factor receptor expression on primitive haemopoietic cells has suggested that there may be some hierarchical response structure to the synergistically acting haemopoietic growth factors. That is, cells may predominantly respond to one factor initially, and this results in upregulation of specific receptors for a different molecule and so on. Bone marrow progenitor cells express relatively low levels of growth factor receptors, and thus any change in number or affinity of these receptors could be manifest as an altered biological response. Jacobsen *et al* (1992) have shown that the synergistic effect of growth factors on colony formation by lin- cells is preceded by an increase in growth factor receptor expression. For example, the synergistic stimulatory effect apparent between G-CSF and GM-CSF in their system was preceded by an upregulation of

GM-CSF receptors of 300%. This effect was apparent at the single cell level and interestingly, combinations of growth factors which did not synergise failed to upregulate receptor expression. They further showed that preincubating lin- cells with IL-1, IL-6, and IL-3 was also capable of increasing GM-CSF induced colony formation. As will be discussed in the following sections, modulation of growth factor receptors has also been proposed as a potential mechanism through which negative regulators of stem cell proliferation may act.

When discussing the roles of growth factors in haemopoiesis, one also has to consider the origin of such factors, and indeed if they are actually present in sites of haemopoiesis.

1.6 The role of stroma in haemopoiesis

Long term bone marrow cultures, mentioned above, clearly indicate the importance of other cell types in maintaining haemopoiesis in the absence of added growth factors. These cultures suggest that an intimate relationship between primitive haemopoietic cells and stromal cells occurs *in vivo*. This relationship does not require direct cell-cell contact however, and Verfaille *et al* (1992) have shown that stromal cells separated from haemopoietic cells by a thin porous mesh can still support haemopoiesis in LTBMC. This suggests that the stromal cells are producing soluble factors required for maintenance of haemopoiesis. As shall be discussed below, detection of these factors *in vivo* and in LTBMCs has proved, in many cases, difficult.

The bone marrow stroma is composed of extracellular matrix and multiple cell types including fibroblasts, macrophages, reticular adventitial cells, endothelial and epithelial cells (reviewed in Quesenberry & Lowry 1992). Using LTBMC as a model, attempts to detect mRNA expression and protein production of various candidate growth factors thought to play a role in maintenance of haemopoiesis have largely produced negative results. For example, several groups have attempted to detect both mRNA and protein of a wide range of growth factors, but could only detect constitutive expression of M-CSF and SCF (Piersma *et al* 1984,

Fibbe *et al* 1988, Sieff *et al* 1988, Schaafsma *et al* 1989, Kittler *et al* 1992). Expression of other cytokines such as IL-6, IL-7, G-CSF, GM-CSF is detectable, but only after stimulation implying that the presence of these molecules in the steady state situation will be very low (Gualtiera *et al* 1984, Quesenberry *et al* 1990). Interestingly, IL-3 has only been detected in LTBMC using sensitive polymerase chain reaction (PCR) techniques (Kittler *et al* 1992). Addition of antibodies directed against IL-3 and GM-CSF to haemopoietic cell depleted stromal cells however results in abolition of the ability of these cells to support the growth of factor dependent haemopoietic cells (Kittler *et al* 1992). Thus, it appears that although many of the positive growth factors may be undetectable in LTBMC and normal bone marrow by conventional techniques, their actual presence there cannot be discounted. It has been suggested that in the bone marrow microenvironment, the stem cell resides in a niche in close association with stromal cells (Schofield 1979). Production of growth factors by these stromal cells may be very low overall in the bone marrow (thus avoiding detection), but may be relatively high in the close proximity of the stem cells (thus fulfilling their assumed role).

Another interesting observation is that many growth factors exist in biologically active membrane or ECM bound forms. For example, IL-1, M-CSF and SCF exist in both soluble and membrane anchored forms (Kurt-Jones *et al* 1985, Stein *et al* 1990). Some growth factors are also capable of binding to ECM components e.g. GM-CSF, IL-3 and LIF where they are then presented to their specific cell surface receptors in an active form (Gordon *et al* 1987, Roberts *et al* 1988). These observations again suggest that growth factors are important in maintenance of haemopoiesis, and yet remain undetectable at the protein level by virtue of the fact that they may be bound rather than exist in soluble forms.

CHAPTER 2

INTRODUCTION

INHIBITION OF HAEMOPOIETIC STEM CELL PROLIFERATION

CHAPTER 2 - INHIBITION OF HAEMOPOIETIC STEM CELL PROLIFERATION

2.1 Inhibition of haemopoietic stem cell proliferation

The need for negative regulation of stem cell proliferation has been questioned. For example, one can postulate that only either positive or negative regulators are required to fully control the proliferation kinetics of a cellular population. Simply put, this hypothesis argues that upregulation of positive regulators will result in increased proliferation whereas downregulation of positive regulators will result in decreased proliferation and vice versa for negative regulators. However, this argument does not adequately explain the situation which occurs in regenerating bone marrow situations. In regenerating bone marrow, even the most primitive stem cells are actively cycling (Harrison & Lerner 1991) presumably in the presence of high levels of growth factor required to stimulate the stem cell. The fact that these primitive stem cells can rapidly return to quiescence suggests the presence of molecules which actively inhibit the cell cycle, rather than the gradual reduction in positive regulators which would result in a much slower return to quiescence. Further, it has been demonstrated that primitive haemopoietic cells undergo apoptosis when deprived of positive growth factors again suggesting a role for active inhibitors of proliferation (Bendall *et al* 1994, Cowling and Dexter 1994, Fairbairn *et al* 1993, Muta and Krantz 1993).

As well as the evidence suggesting that stimulation of stem cell proliferation is important in haemopoiesis, a number of molecules have been identified recently which are capable of inhibiting proliferation of stem cells. As shall be discussed below, these molecules include the tetrapeptide (AcSDKP), the haemoregulatory peptide (pEEDCK), transforming growth factor beta (TGF-β) and macrophage inflammatory protein 1 alpha (MIP-1α). Like the positive growth factors, some of these molecules are capable of affecting proliferation and function

of cells from the stem, progenitor and mature cell compartments, and indeed appear to have considerable overlap in some of their actions.

2.2 Tetrapeptide inhibitor

Frindel and Guigon (1977) first described a low molecular weight activity present in foetal calf bone marrow extracts which has the ability to inhibit the entry of murine bone marrow CFU-S into DNA synthesis following irradiation. They subsequently went on to show that this activity could also increase the long term survival of mice which had received lethal doses of cytosine arabinoside (AraC), a phase specific cytotoxic drug and that this was due to a specific protective effect on bone marrow CFU-S (Guigon *et al* 1981). The active molecule was later purified and identified as a tetrapeptide, with the amino acid sequence acetyl-ser-asp-lys-pro (AcSDKP) (Lenfant *et al* 1989).

Presently, it is unclear as to the origin of this small peptide *in vivo* but current evidence supports proteolytic cleavage of a larger precursor molecule which contains the SDKP sequence. Several candidate molecules have been identified which contain this sequence including rat phenylalanine hydroxylase, human and murine tumour necrosis factor α (TNF-α), human retinal S antigen, human amphiregulin and rat, porcine, human and murine thymosin β4 (Tβ4). Only Tβ4 however contains the sequence at its N-terminus and not internal to its protein chain suggesting Tβ4 to be a possible candidate for the precursor molecule. Also, the SDKP sequence is acetylated in the thymosin β4 chain. Lenfant *et al* (1991) have demonstrated that AcSDKP can be formed in the bone marrow by one-step enzymatic processing of Tβ4, and have further demonstrated that this can be achieved using the endopeptidase Asp-N.

Due to the potential role of this peptide as a protector of the stem cell compartment during chemotherapy using phase specific drugs, much research has concentrated on elucidating its mode of action in inhibiting stem cell proliferation. The tetrapeptide appears only to exhibit an inhibitory effect on the CFU-S stage of the stem cell compartment (Monpezat and Frindel 1989, Bonnet *et al* 1993).

AcSDKP had no effect on the proliferation of isolated adult rat hepatocytes, 3T3, FDC-P2 or K562 cells (Lauret *et al* 1989). Further, no effect of AcSDKP can be detected on committed GM-CFC progenitors, with respect to either colony formation or the proportion of cells in DNA synthesis. The cell cycle specificity of AcSDKP has been investigated, and it has been shown that the peptide is only capable of inhibiting CFU-S when they are in the early part of G1 or at the Go-G1 boundary, not the G1-S transition or once the cells have entered S phase. This suggests that AcSDKP is only capable of preventing quiescent cells from entering the cell cycle and is not able to actively inhibit cycling cells. Indeed, work by Robinson *et al* (1993) demonstrated that AcSDKP had no effect on cycling populations of murine HPP-CFC, but could prevent quiescent HPP-CFC entry into cell cycle when treated with a stimulator of haemopoietic stem cell proliferation (Lord *et al* 1977). These observations may also explain the inability of the tetrapeptide to inhibit proliferation of haemopoietic cell lines and committed progenitors which are in active cell cycle.

AcSDKP is detectable in both serum and bone marrow of mice and is also produced by bone marrow cells in long-term bone marrow cultures (Wdzieczak-Bakala *et al* 1990). AcSDKP like activity has also been detected in both human placenta (Lopez *et al* 1991) and in human white blood cells (Pradelles *et al* 1990) demonstrating a potential physiological role in man. To further investigate the potential physiological role of this peptide, Frindel and Monpezat (1989) injected polyclonal antiserum raised against AcSDKP into untreated normal mice to neutralise endogenous levels of the inhibitor. They observed a dramatic increase in the number of CFU-S cycling in the anti-AcSDKP treated animals suggesting AcSDKP to be an important physiological regulator of CFU-S proliferation in the bone marrow. Further, it appears that levels of AcSDKP in plasma decrease after AraC treatment, and that this precedes CFU-S entry into cell cycle suggesting a correlation between endogenous AcSDKP levels and CFU-S kinetics (Frindel *et al* 1992). Recently, Cashman *et al* (1994) have shown that AcSDKP reversibly inhibits high proliferative potential erythroid and granulocytic progenitors in the

adherent layer of long term bone marrow cultures (LTBMC) without changing their numbers. The inhibitory activity of AcSDKP could be abrogated by addition of macrophage inflammatory protein 1β, an antagonist of the stem cell inhibitor MIP-1α suggesting an indirect mechanism of inhibition via MIP-1α. However, no increase in either MIP-1α mRNA or protein could be detected after AcSDKP treatment of cultures. It is still possible that AcSDKP acts indirectly by upregulating agonists of MIP-1α, or MIP-1α related chemokines, but this awaits further study.

It appears that AcSDKP has a role to play in maintaining steady state haemopoiesis, but it is unlikely that it acts alone in this regard since many other negative regulators of CFU-S proliferation have been described (see below). The exact role of each however in the overall control of haemopoiesis remains uncertain. Interestingly, AcSDKP was inactive on both cycling activity and numbers of neoplastic cells in CML LTBMCs perhaps suggesting that lack of responsiveness to AcSDKP could contribute to the CML phenotype (Cashman *et al* 1994).

2.3 Haemoregulatory peptide

In 1984, Laerum and Paukovits described the structure of a pentapeptide which reversibly inhibited myelopoiesis *in vivo* and *in vitro*. This peptide was isolated from human leukocytes and has the amino acid sequence pyro-Glu-Glu-Asp-Cys-Lys (pEEDCK). Subsequently, a similar peptide (pGlu-Glu-Asp-Ser-Gly) was isolated from mouse epidermis which had the ability to reversibly inhibit epidermal cell proliferation *in vivo* and *in vitro* (Elgjo and Reichelt 1984).

Characterisation of the haemoregulatory peptide has shown that it can exist in two forms, a monomer and a dimer. The dimer arises through oxidation of the cysteine thiol groups of pEEDCK leading to formation of a disulphide bridged homodimer (pEEDCK)2. It is now apparent that the pEEDCK monomer is active as an inhibitor of haemopoietic cells both *in vivo* and *in vitro*. Interestingly, the dimer is active as a stimulator of these cells suggesting that a balance between

monomer and dimer is involved in controlling aspects of haemopoiesis. Synthetic and stable forms of both the pEEDCK monomer and dimer have been manufactured and are functionally identical to their native counterparts.

In vitro, the monomer is active in inhibiting both murine and human CFU-GM in a dose dependent manner (Laerum and Paukovits 1984, Kreja *et al* 1986, Laerum *et al* 1987, Laerum *et al* 1990), with inhibition being seen at concentrations as low as 10^{-13}M. *In vivo*, CFU-GM numbers are decreased following either injection or continuous administration of the monomer, as are CFU-S numbers but to a lesser extent. Prolonged monomer application leads to reduced numbers of CFU-S and CFU-GM in the femora of mice which is later reflected in the mature compartments of haemopoiesis in the periphery (Laerum and Paukovits 1984). Further, Paukovits *et al* (1990) have shown that pEEDCK can inhibit CFU-S recruitment into cell cycle following repeated high doses of the phase specific drug AraC. A physiological role for pEEDCK has been suggested by Paukovits *et al* since ablation of endogenous peptide levels by immunisation leads to an increase in the numbers of CFU-GM progenitors.

The $(pEEDCK)_2$ dimer stimulates the formation of both murine and human CFU-GM colonies in a dose dependent manner *in vitro*. This effect is dependent on the presence of the colony stimulating factor GM-CSF however, since no stimulation of colonies occurs with the dimer alone. The dimer has also been tested for biological effects on a range of normal and malignant cell types (Frostad *et al* 1993). This study concluded that the dimer is an *in vitro* stimulator of CFU-GM with little or no effect apparent on other cell types. *In vivo*, the dimer stimulates an increase in CFU-GM and CFU-S numbers in the femurs and spleens of mice, but has little effect on mature granulocytic numbers in the peripheral blood (Laerum and Paukovits 1989). As mentioned above, the marked opposing effects of the monomer and dimer of pEEDCK have led workers in this field to suggest that a fine balance between these forms is involved in controlling stem and progenitor cell proliferation. This implies that the haemoregulatory peptide may be a valuable myeloprotective agent for use during cancer chemotherapy whereby the

inhibitor (monomer) protects the stem and progenitor cell compartments during drug administration, and then the stimulator (dimer) can enhance recovery of mature cells and reduce neutropenia. Indeed, Paukovits *et al* (1991) have shown that administration of pEEDCK with repeated (twice) clinically relevant doses of Ara-C results in a sustained number of CFU-S in the bone marrow of mice. Mice which did not receive pEEDCK suffered CFU-S losses of approximately 75%. $(pEEDCK)_2$ administration during the second injection of Ara-C delayed the onset of neutropenia by 2-3 days and improved recovery after depression. It should be mentioned however, that cell cycle specific cytotoxic drugs are not widely used in cancer chemotherapy and thus the usefulness of the pentapeptide, and indeed the other inhibitors discussed here, in protection of normal cycling cells from chemotherapeutic damage may be somewhat limited.

Available data indicate that, unlike the tetrapeptide inhibitor discussed above, the pentapeptide is capable of exerting its inhibitory effects on cells regardless of their cell cycle stage, including S phase. The mechanism by which the pentapeptide exerts these effects is still unclear. Paukovits and Paukovits (1975) have shown that cell surface proteins are necessary for its inhibitory action and, by using a ^3H labelled pentapeptide, it appears that murine bone marrow cells but not thymic or spleen cells are capable of taking up the molecule (Eriksen *et al* 1987). pEEDCK has been shown to be a sequence motif present in the effector domain of Gi alpha proteins. However, the significance of this observation and indeed the origin of the pentapeptide *in vivo* is unclear.

2.4 Transforming Growth Factor beta (TGF-β)

The TGF-β superfamily encompasses a large and increasing number of structurally related cytokines with highly diverse activities (Massague 1990, Kingsley 1994). Members of this family are related by sequence homology to the prototype TGF-β1, the most conserved feature being the spacing of nine cysteine residues within the mature carboxy terminus. The members of the TGF-β superfamily have been isolated from several different species ranging from insect to

man. It is remarkable however that sequence identity between homologues from different species is very high (usually greater than 95% from mouse to human) suggesting TGF-β to have a fundamental role in the normal physiology of several species. It is now apparent that this family of molecules contains several subfamilies whose members are more related to each other than to other members of the superfamily (Figure 1.2). These shall be outlined below.

2.4.1 Transforming growth factors beta

This subfamily consist of at least five members, termed TGF-β1, -β2, -β3, -β4 and -β5 which have been isolated from several different species. The prototype TGF-β1 was originally isolated from human platelets (Assoian *et al* 1983), and subsequently cloned from a human cDNA library (Derynck *et al* 1985) and analysed (Derynck *et al* 1986). TGF-β2 and -β3 have also been identified in humans and other mammals at both the cDNA and protein level (reviewed in Massague 1990). TGF-β4 cDNA has only been detected in chicks (Jakowlew *et al* 1988) although recent studies have suggested that it is probably the chick homologue of mammalian TGF-β1 (Burt and Paton 1992, Burt and Law 1994). TGF-β5 was identified as a cDNA from *Xenopus Laevis* (Kondaiah *et al* 1990) and as yet, no mammalian homologue has been found. The TGF-βs have highly diverse activities involving both positive and negative effects (see below) on various developmental and mature cell systems. For example, TGF-β has been found to induce angiogenesis (Roberts *et al* 1986), chondrogenesis (Seyedin *et al* 1985) and osteogenesis (Seyedin *et al* 1987). It can also regulate cellular differentiation and proliferation positively or negatively depending on the cell type and growth conditions. TGF-β has been implicated in wound repair and fibrosis, since it specifically attracts immune cells into the wound site and acts to upregulate extracellular matrix (ECM) components and downregulate degraders of ECM (reviewed in Massague 1990). TGF-β also has a role in haemopoiesis (Ruscetti *et al* 1991) which shall be discussed below.

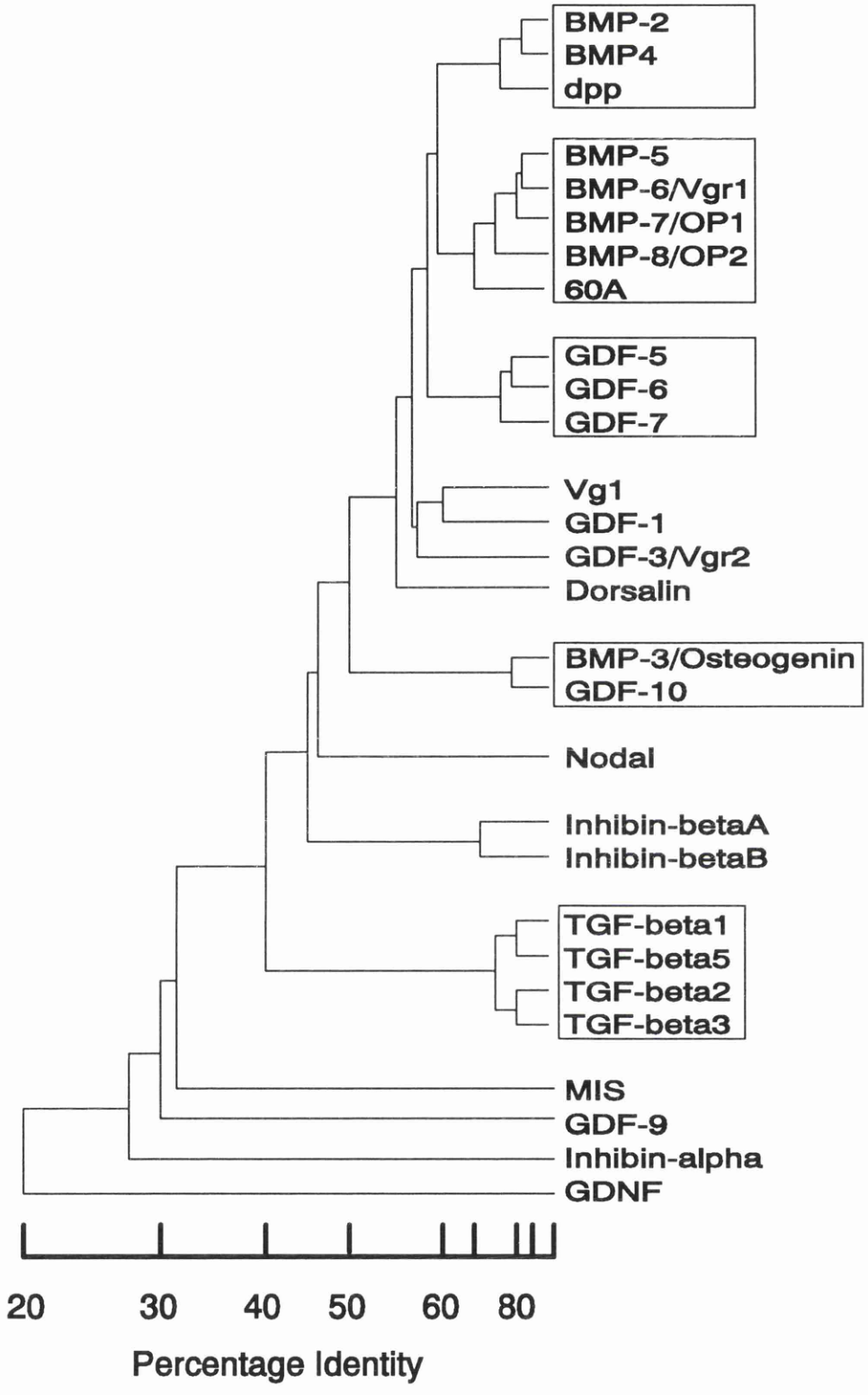


Figure 1.2 Structure of the TGF-β superfamily and percentage identity between members

2.4.2 Inhibins and Activins

This family consists of three genes coding for peptides named inhibin βA subunit, inhibin βB subunit and inhibin α. The products of these genes can dimerise via disulphide bridging and form a number of active polypeptides. Inhibins are formed through dimerisation of either the βA or the βB subunit with the inhibin α subunit yielding inhibin A and inhibin B respectively. Activins are formed through dimerisation of βA-βA subunits generating activin A, βB-βB subunits generating activin B, or βA-βB subunits generating activin AB. Activins and inhibins were initially characterised as gonadal proteins having the ability to stimulate or inhibit respectively the release of follicular stimulating hormone from pituitary cells (Vale *et al* 1986, Ling *et al* 1986). Homodimeric activin A was also independently isolated as a factor produced by human leukaemia cells with the capacity to induce erythroleukaemic cell differentiation (Eto *et al* 1987). Interestingly, this action is also antagonised by inhibins, as are other activin functions, suggesting that a fine balance between them controls their effects. It has been suggested that this antagonistic activity by inhibin is due to competition for the same cell surface binding sites. However, a study by Kondo *et al* (1989) has shown that although inhibin can compete with ^{125}I labelled activin for binding to specific sites on the cell surface, the affinity with which it binds does not explain its highly potent antagonistic activities. It is possible that activin and inhibin may bind to distinct cell surface receptors.

It is now apparent that these molecules have a much wider range of biological activities, with their gene expression being detected in various tissues (Meunier *et al* 1988). Activin in particular has been shown to be involved in cell differentiation. It is capable of inducing human erythroid differentiation and stimulating erythroid colony formation in *in vitro* assays, and also of stimulating multipotential progenitor cells *in vitro* (Yu *et al* 1987). Inhibin acts antagonistically in this context also. Activin has also been suggested to play a major role in embryonic development. It has been shown that activin A can promote mesoderm formation in the frog *Xenopus Laevis* (Rupp and Weintraub

1991). Further, activin A can induce various blood cell types, muscle and notochordal tissue from animal caps of *Xenopus Laevis* in a dose dependent manner (Green and Smith 1990). It is unclear what role activin plays in development in higher vertebrates, but Albano *et al* (1993) have demonstrated mRNA expression of all three inhibin subunits in the murine oocyte, and have also shown that activin protein, but not inhibin, can be detected after the fertilised egg stage. High levels of βA and βB mRNA were detected in embryonal stem (ES) cell lines, but these levels diminished upon induction of differentiation which is consistent with activins proposed role as a regulator of mesoderm induction and early developmental processes. Recently, transgenic mice have been generated carrying a disrupted βB subunit gene. The phenotype of these animals is remarkably mild considering the proposed role of activin in development, with the only noticeable defects being that the animals are infertile and have precocious eyelid opening (Vassalli *et al* 1994). Further, Matzuk *et al* (1995a) have demonstrated mice deficient for the βA subunit develop to term but die within 24hrs of birth. These mice lack whiskers and lower incisors and have secondary palate defects indicating that activin-βA has a role in craniofacial development. Mice deficient for both activin βA and activin βB have a phenotype representative of both individual knockouts, but with no additional abnormalities. To further investigate the potential role of activin in development, Matzuk *et al* (1995c) generated mice deficient for follistatin, an activin binding protein and antagonist. Interestingly, these mice developed many more defects than the activin deficient mice suggesting that follistatin may modulate the actions of several other members of the TGF-β superfamily.

Specific receptors for activin have recently been isolated which belong to a novel family of transmembrane receptors which have a highly characteristic serine/threonine kinase domain on their cytoplasmic surface (Massague *et al* 1992, Lin and Lodish 1993). These receptors shall be discussed in section 2.4.8.1. Matzuk *et al* (1995b) have generated mice deficient for the type II activin receptor (ActRcII) and demonstrated a phenotype quite different from mice deficient in

activin ligand. These mice suffered reduced follicular stimulating hormone production and their reproductive performance was defective suggesting a role for ActRcII in activin signalling in pituitary gonadotrophs.

2.4.3 The DVR group

This subgroup derives its name from the fact that its members are related to the decapentaplegic (DPP) and Vg1 products (hence DPP and Vg1 related). DPP is involved in embryonic and larval pattern formation in drosophila (Spencer *et al* 1982, Ferguson *et al* 1992), and two mammalian homologues of DPP have been found; bone morphogenetic protein 2 (BMP-2) and BMP-4. BMPs (BMP-2 through to BMP-8) have the capacity to induce cartilage and bone formation (Wang *et al* 1990). Interestingly, purified samples of DPP protein are also capable of exhibiting this activity (Sampath *et al* 1990) indicating functional as well as structural conservation. The BMPs have also been implicated in tissue modelling during development (Hogan *et al* 1994). Interestingly, BMPs may have potential clinical roles, particularly in bone healing and repair. Vg1 is involved in embryonic development in *Xenopus Laevis*. Other more distant members of this group, including growth differentiation factor 1 (GDF-1), GDF-3, dorsalin and nodal have all been shown to be involved in embryonic development but shall not be discussed.

2.4.4 Mullerian Inhibiting Substance (MIS)

MIS is distantly related to other members of the TGF-β superfamily. It is produced in the testes and was identified and purified based on its ability to induce regression of the female mullerian ducts in mammalian male embryos (Munsterburg and Lovell-Badge 1991). It may also play in a role in development of female reproductive organs in the adult female ovary.

2.4.5 Structure and Biology of Transforming Growth Factor beta

2.4.5.1 Structure of TGF-β proteins - TGF-β is secreted as a latent complex

Structure of TGF-β related proteins is based on the prototype TGF-β1. Mature active TGF-β1 is a disulphide linked dimer of two identical chains each composed of 112 amino acids (2 x 12.5 kD). However, recombinant TGF-β1 is expressed in mammalian cells as an inactive 105kD precursor known as the small latent complex or pro-TGF-β (Gentry *et al* 1988, Wakefield *et al* 1988). The precursor consists of an N-terminal signal sequence, a pro-region and the C-terminal bioactive domain. The pro-region (residues 30-278) is also known as the latency associated peptide (LAP 75-80kD), and this too consists of two identical disulphide linked chains. The hydrophobic signal sequence is required for transport across the endoplasmic reticulum, but is cleaved prior to secretion. LAP is proteolytically cleaved from the complex at a basic site immediately preceding the bioactive C-terminal domain in a post-golgi compartment. However, it remains associated with the bioactive domain after secretion via non-covalent interactions yielding an inactive complex (Gentry *et al* 1988). The small latent complex of TGF-β1 has been detected in the conditioned media of a number of cell types including bone cultures (Jennings and Mohan 1990, Bonewald *et al* 1991), a human erythroleukaemic cell line (Lioubin *et al* 1991) and a human glioblastoma cell line (Olofsson *et al* 1992), and it is likely that it is ubiquitously expressed.

Another form of latent TGF-β has been detected in human platelets (Wakefield *et al* 1988 and Pircher *et al* 1986), and certain cell lines. This complex consists of the small latent TGF-β complex bound via disulphide bonds from a LAP monomer to a 125-190 kD glycoprotein called latent TGF-β binding protein (LTBP). The function of LTBP is not clear at present, however it may be involved in assisting the assembly and secretion of the large latent TGF-β complex (Miyazono *et al* 1991), and may also stabilise the latent complex. A role for LTBP in assisting TGF-β binding to the cell surface via mannose-6-phosphate residues has also been proposed (Taipale *et al* 1994).

2.4.5.2 Activation of Latent TGF-β

As mentioned above, TGF-β is secreted as a latent complex. Studies by Lawrence *et al* (1985) and Pircher *et al* (1986) have demonstrated that latent TGF-β is incapable of binding to cell surface receptors. This suggests that a complex control mechanism is involved in activation of TGF-β *in vivo* in order to avoid uncontrolled levels of active TGF-β. The exact mechanism of activation *in vivo* remains largely unknown. *In vitro*, activation can be achieved by exposure to extreme pH (<4 or >9), temperature, by exposure to chaotropic agents such as sodium dodecyl sulphate (SDS) or by enzymatic and proteolytic cleavage (Lawrence *et al* 1985, Lyons *et al* 1988). Although, extreme pH conditions can be generated *in vivo* in some circumstances e.g. bone resorption (Silver *et al* 1988), it appears likely that some form of enzymatic or proteolytic cleavage is involved in *in vivo* activation. A number of enzymes have been shown to activate TGF-β including sialidases, endoglycosidase F, neuraminidase and *N*-glycanase (Brown *et al* 1990, Miyanozo and Heldin 1989). However, the very high concentrations of enzyme required for activation make this an unlikely mechanism *in vivo*.

The best *in vitro* model for a potentially relevant physiological activation mechanism involves proteolysis. Both plasmin and cathepsin D are capable of activating TGF-β present in cell conditioned media. The levels of active TGF-β generated via plasmin activation are not as high as those which are found after acid activation (only around 30%) suggesting differential susceptibilities to proteolysis within the latent TGF-β pool. However, as with the other enzymes mentioned above, potentially unphysiologically high levels of protease are required for activation prompting workers to suggest that some form of biological surface mediated activation may occur. It has been shown by various groups that plasminogen can be converted to plasmin by plasminogen activator on a number of biological surfaces including extracellular matrix (ECM), platelets and endothelial cells (reviewed by Harpel *et al* 1992). Sato *et al* (1993) have demonstrated that TGF-β is activated during co-culture of endothelial cells and smooth muscle cells. They further show that plasmin located on the surface of endothelial cells, and the

targeting of latent TGF-β via the LAP to the surface of the smooth muscle cells are required for activation to occur. This represents an attractive model for *in vivo* activation of TGF-β such that its functions will be localised to the site of activation, either autocrinely on the activating cells or paracrinely on nearby cells. In fact, very little active TGF-β can be detected in plasma, lending further credence to this argument. Nearly all active TGF-β which is secreted is quickly bound by α2-macroglobulin, an abundant serum glycoprotein (O`Connor-McCourt and Wakefield 1987). The function of this interaction is unclear, but may serve as a means to remove excess active TGF-β from the periphery. This is important since overproduction of active TGF-β has been implicated in a number of disorders which shall be discussed below. Grainger *et al* (1994) have further implicated plasmin as a potentially important activator of TGF-β *in vivo*. They generated transgenic mice expressing the human apolipoprotein(a) gene, the product of which inhibits the activation of plasminogen to plasmin, and demonstrated that the activation of TGF-β was inhibited as a consequence of the inability of these mice to generate active plasmin. Schultz-Cherry and Murphy-Ullrich (1993) have described a novel mechanism whereby thrombospondin (TSP), an ECM protein, activates latent TGF-β secreted by bovine aortic endothelial cells. They have further shown that both small and large latent TGF-β molecules form specific complexes with TSP. This interaction occurs in the soluble phase, that is, no cell surface interactions are necessary, and does not require proteolytic activity. More recently, this group have also elucidated which region of TSP is responsible for activating latent TGF-β and have shown that the type 1 properdin-like repeats localised in the chymotrypsin resistant core of TSP are necessary for this activity (Schultz-Cherry *et al* 1994).

Another interesting observation is that TGF-β itself potentially controls its own activation. That is, TGF-β downregulates plasminogen activator expression, and strongly upregulates expression of its antagonist plasminogen activator inhibitor (Laiho *et al* 1986). If plasmin is involved in *in vivo* activation of TGF-β, then this suggests that TGF-β downregulates its own activation. Paradoxically,

TGF-β is a potent upregulator of its own mRNA expression in monocytes (McCartney-Francis *et al* 1990), and this autoinduction is mediated by the AP-1 complex (Kim *et al* 1990).

2.4.6 Expression patterns of TGF-β

TGF-β is expressed by an astonishingly wide variety of normal and transformed cells in culture (Derynck *et al* 1985 and reviewed in Sporn *et al* 1987). Its expression is also found in early mouse embryos suggesting a possible role in various aspects of development (reviewed by Mummery and Van Den Eijnden-Van Raaij 1993). In the adult mouse, most tissues and organs have been shown to express mRNA for TGF-β1, β-2 and/or -β3 (Reviewed by Massague 1990) demonstrating TGF-β to be a highly ubiquitous molecule. In particular, mature haemopoietic cells are capable of expressing TGF-β, and it is here that I shall concentrate on the role of TGF-β in haemopoiesis.

2.4.7 The Role of TGF-β in Adult Haemopoiesis

A number of mature haemopoietic cells have been shown to synthesise TGF-β including activated macrophages (Assoian *et al* 1987), B lymphocytes (Kehrl *et al* 1986a) and T lymphocytes (Kehrl *et al* 1986b). Platelets in particular are a very rich source of TGF-β (Assoian *et al* 1983), and indeed TGF-β was originally isolated from platelets. The high level of production of TGF-β by immune cells suggests that TGF-β may be an important immunological mediator, and functional studies have demonstrated that this is almost certainly the case.

2.4.7.1 Immunological effects of TGF-β

The summation of TGF-βs functions on mature blood cells suggest it to be an anti-inflammatory molecule. However in contrast, TGF-β is chemotactic for a number of mature cell types, and is in fact the most potent neutrophil chemoattractant yet identified (Reibman *et al* 1991, Gold *et al* 1990, Parekh *et al* 1994). TGF-β can also inhibit certain functions of a number of mature

immunologically active cell types. *In vitro*, this includes ablation of the ability of macrophages to carry out the respiratory burst (via suppression of H_2O_2 release) (Tsunawaki *et al* 1988), impairment of antibody production by B cells (Kehrl *et al* 1991) and impairment of cytolytic activity of cytotoxic T cells and NK cells (Inge *et al* 1992, Ranges *et al* 1987, Rook *et al* 1986). When administered *in vivo*, the effects apparent are also indicative of TGF-βs potential function as an endogenous immunosuppressant. For example, in rodents, prior injection of TGF-β1 protects animals from the development of arthritis induced by injection of collagen or streptococcal cell wall extracts (Brandes *et al* 1991 and Kuruvilla *et al* 1991). Also, administration of TGF-β delays rejection of ectopic cardiac transplants (Palladino *et al* 1990). A number of immunosuppressive human diseases have been associated with overproduction of TGF-β including glioblastomas, where patients often suffer from impaired T cell function. Purification of glioblastoma cell conditioned medium led to the isolation of TGF-β2 as the property responsible for this impairment (Wrann *et al* 1987). Overproduction of TGF-β is also apparent in individuals with acquired immune deficiency syndrome (AIDS). Peripheral blood mononuclear cells isolated from these patients produce excessive levels of TGF-β, and these levels correlated with those required to cause T and B cell functional impairment (Kekow *et al* 1990, Kekow *et al* 1991). Zauli *et al* (1992) and Lotz *et al* (1994) have further shown that HIV *tat* protein stimulates production of TGF-β1 by bone marrow macrophages and human articular chondrocytes.

Perhaps the best and most direct evidence that TGF-β acts as an endogenous immunoregulatory molecule comes from the TGF-β1 gene disruption experiments. Schull *et al* (1992) and Kulkarni *et al* (1993) disrupted the TGF-β1 gene by homologous recombination in murine ES cells and generated mice which carried the disrupted allele. Surprisingly, these TGF-β1 null mice were born with no gross developmental abnormalities perhaps indicating some functional redundancy between TGF-β1 and its related family members with respect to its developmental roles. Alternatively, maternal transfer of TGF-β1 across the placenta could account for this observation (see below). Approximately three

weeks after birth, mice homozygous for the disrupted allele experienced severe wasting and died. Histological analysis revealed a multifocal, mixed inflammatory cell infiltration, often with necrosis. Analysis of cytokine mRNA in liver and lungs of both control and TGF-β1 deficient mice revealed an upregulation of a number of pro-inflammatory cytokines in TGF-β1 null mice which could contribute to the wasting and inflammatory phenotype observed. These included TNF-α, interferon-γ and macrophage inflammatory protein 1α. It is unclear however whether these observations are causative of the phenotype or emerge as a secondary effect of it. As mentioned above, TGF-β1 has been implicated in controlling T cell function. Lack of functional TGF-β1 may result in aberrant T cell suppression ultimately leading to autoreactivity in the animals. Further, since TGF-β1 downregulates interferon-γ induced MHC class II expression in both lymphoid and non-lymphoid cells, absence of TGF-β1 may augment increased MHC class II expression and presentation of self antigens to immune competent cells, again leading to autoimmunity. Thus, these mice are likely to be invaluable as models of autoimmune disease.

Letterio *et al* (1994) have shown in an elegant series of experiments that maternal transfer of TGF-β1 does indeed contribute to the apparently normal development of the TGF-β1 null mice. TGF-β1 protein can be detected in TGF-β1 null pups born from a heterozygous female, but none can be detected in null pups born from a homozygous TGF-β1 negative female. Further, they have shown that ^{125}I labelled TGF-β1 crossed the placenta from mother to foetus and that it is taken up by cells and bound by extracellular matrices. Null pups born from a null female died perinatally as a result of severe cardiac problems, implicating TGF-β1 to be important in cardiac development. Indeed, this is in agreement with the observations that TGF-β expression is detectable in developing cardiac tissue (Massague 1990).

2.4.7.2 Effects of TGF-β on cell proliferation

Perhaps the most documented property of TGF-β is its ability to affect proliferation, either positively or negatively, of a number of cell types. However, as well as being a reversible inhibitor of proliferation of some cell types, TGF-β has also been implicated in inducing rapid growth arrest followed by apoptosis (programmed cell death.). For example, TGF-β induces apoptosis in cultured primary hepatocytes (Bursch *et al* 1993) and in M1 myeloid leukaemia cells (Selvakumaran *et al* 1994a,b). Thus, one has to be cautious when describing TGF-β as a reversible inhibitor of cell proliferation because, in many of the published cases, this may not necessarily be the case. For the purposes of this work, I shall describe below cell types whose proliferation is inhibited by TGF-β.

In vitro, TGF-β can inhibit the growth of epithelial cells (Moses *et al* 1985), endothelial cells (Roberts *et al* 1985), fibroblasts (Sorrentino *et al* 1989), B and T lymphoid cells (Kerhl *et al* 1986a,b) and haemopoietic progenitor cells (Ohta *et al* 1987, Hampson *et al* 1989). *In vivo*, intravenous injection of TGF-β1 or TGF-β2 had an inhibitory effect on the proliferation of regenerating rat liver (Russell *et al* 1988), and TGF-β1 can inhibit growth of epithelial end buds of immature mammary glands (Silberstein and Daniel 1987). Further, administration of both TGF-β1 and TGF-β2 can protect mice from acutely lethal doses of 5-FU and doxorubicin (Grzegorzewski *et al* 1994). TGF-β can also stimulate cell growth, and was originally isolated by virtue of the fact that it stimulates anchorage-independent growth of certain fibroblasts (Roberts *et al* 1981). TGF-β can also stimulate growth of haemopoietic progenitors as shall be discussed below.

2.4.7.3 Effects on the haemopoietic stem and progenitor cell compartments

All three mammalian forms of TGF-β are active in the haemopoietic system, with differences being generally quantitative rather than qualitative. In general, TGF-β appears to act as a selective inhibitor of primitive multipotent progenitors, with little inhibitory effect on more committed haemopoietic cells. Indeed, in some cases, TGF-β is in fact stimulatory for more mature cells.

In 1990, Keller *et al* demonstrated that TGF-β selectively inhibits colony stimulating factor driven haemopoietic progenitor cell growth in mice. Using highly enriched populations of progenitor cells, they showed that TGF-β could prevent IL-3 induced Thy-1 expression in Thy-1$^-$ cells and that this was a direct effect. TGF-β is extremely potent in this regard with an ED50 of around 5 to 10pM. This data suggests that TGF-β may be active as an inhibitor of differentiation as well as proliferation, although in constrast, Krystal *et al* (1994) have recently shown that TGF-β1 is active as an inducer of erythroid differentiation. Further, Sillaber *et al* (1992) have demonstrated that TGF-β promotes IL-3 dependent differentiation of human basophils, but inhibits IL-3 driven eosinophil differentiation.

Keller *et al* (1990) have demonstrated that TGF-β inhibited the proliferation of all subpopulations of the primitive HPP-CFC compartment suggesting it to play a role in controlling stem cell proliferation throughout the stem cell compartment. Hampson *et al* (1989) have shown that TGF-β suppresses the growth of the IL-3 dependent cell line FDCP-MIX. This cell line is non-transformed, and is capable of generating multilineage colonies *in vitro* akin to the properties ascribed to stem cells. Interestingly, TGF-β had no effect on FDC-P1 or FDC-P2 cells which are unable to undergo further differentiation suggesting that responsiveness to TGF-β is differentiation linked. Sargiacomo *et al* (1991) have reported a direct inhibitory effect by TGF-β on BFU-E and CFU-GM colony formation. Both TGF-β1 and TGF-β2 were active, although TGF-β1 approximately 10 fold more so. Ohta *et al* (1987) found that TGF-β1 was 100 fold more potent that TGF-β2 in inhibiting B6SUtA cell multilineage colony formation, and that this was probably due to the 20 fold lower affinity of TGF-β2 for binding to the specific TGF-β type I receptor.

An obvious problem when interpreting *in vitro* experiments like these is that they are, more often than not, very artificial and may bear little resemblance to what actually happens *in vivo*. For example, the concentrations of exogenous TGF-β required to inhibit cells in colony forming assays may be misleading, in that

it is very difficult to predict *in vivo* localised concentrations around the actual producing and responding cell types. This is especially true with respect to *in vitro* stem cell assays, whereby primitive cells are isolated and then grown in culture with a plethora of positive growth factors. It is now widely accepted that a balance between positive and negative regulators is involved in controlling haemopoietic stem cell proliferation. Indeed, it is apparent that some of the suppressive effects of TGF-β on primitive haemopoietic cells can be abrogated by increasing the concentrations of certain growth factors. For example, Kishi *et al* (1989) have demonstrated that IL-6 and G-CSF can abrogate TGF-βs inhibitory effects on IL-3 and Epo dependent murine progenitors from both normal and 5FU treated mice. Bradley *et al* (1991) have also shown that TGF-β inhibits murine HPP-CFC colony formation and that this effect is antagonised by increasing concentrations of the relevant growth factors.

TGF-β is readily detectable in long term bone marrow cultures (LTBMC). Cashman *et al* (1990) have shown that TGF-β can prevent primitive progenitor cell entry into cycle in LTBMCs in response to a variety of growth stimulators. Eaves *et al* (1991) have further shown that adding anti-TGF-β antibodies to LTMC could prolong the period of proliferation of previously cycling cells and activate the proliferation of quiescent primitive progenitors suggesting TGF-β to have an important role in controlling the normal quiescent state of the stem cell compartment, both in this assay system and possibly in normal bone marrow.

As well as being a potent inhibitor of primitive haemopoietic cell proliferation, TGF-β can also act as a stimulator of more mature lineage restricted progenitors (Moses *et al* 1990). Jacobsen *et al* (1991a) have shown that TGF-β1 and -β2 can exert bimodal dose dependent stimulation of GM-CSF and G-CSF induced day 7 granulocyte-macrophage colony forming units. This effect was due to an increase in granulopoiesis, and was restricted to low doses (0.01 to 1.0 ng/mL) of TGF-β. Thus, TGF-β, like both the haemoregulatory pentapeptide and MIP-1α (see below) appears to be capable of both inhibiting and stimulating proliferation of haemopoietic stem and lineage restricted progenitor cells

respectively. Further, Fan *et al* (1992) have demonstrated that TGF-β can either inhibit or stimulate proliferation of peritoneal exudate macrophages depending on the type of CSF present. That is, TGF-β enhanced proliferation of these cells when they were stimulated with GM-CSF, but inhibited them in the presence of M-CSF.

2.4.8 Mode of action of TGF-β in inhibiting primitive haemopoietic cells

How TGF-β actually achieves inhibition of primitive haemopoietic cells remains largely unknown. While actual cellular mechanisms remain elusive, it has been postulated that TGF-β may inhibit proliferation by somehow preventing the stem cells from responding to positive growth signals. One such mechanism could be by downregulating growth factor receptors on the stem cells themselves. Indeed, Jacobsen *et al* (1991b) have shown that TGF-β specifically inhibits the expression of GM-CSF, IL-3 and G-CSF receptors on both factor dependent and independent murine haemopoietic progenitor cell lines. This was due to a reduction in receptor number with no change in affinity of the remaining receptors for ligand. Interestingly, this receptor downregulation preceded TGF-βs growth inhibitory action, and TGF-βs ED50 for both receptor modulation and inhibition were very alike (1 to 10pM). Similarly, Dubois *et al* (1994) have demonstrated that TGF-β downregulates *c-kit* message stability and cell surface protein expression in haemopoietic progenitors. *c-kit* is the receptor for stem cell factor, a molecule important for the maintenance of haemopoiesis, and TGF-β has previously been shown to inhibit the action of this molecule on both murine and human progenitors (McNiece *et al* 1992). Taken together, these studies suggest that TGF-β is capable of altering, and indeed lessening the response of primitive haemopoietic cells to the actions of positive growth factors and this may be a contributory mechanism to inhibition of proliferation.

In order to fully understand the inhibitory mechanisms of TGF-β, it is necessary to elucidate the molecular events initiated by binding of TGF-β to the cell surface, and the resultant changes in gene expression in the nucleus.

2.4.8.1 Receptors for the TGF-β family

Virtually all cell types examined display cell surface receptors for TGF-β. So far, at least nine distinct proteins have been shown to bind TGF-β (reviewed by Massague 1992 and Massague *et al* 1994). Cross linking to radiolabelled TGF-β has revealed two most widely expressed proteins of 43 kDa and 70 kDa termed TGF-β type I and type II receptors respectively. A third ubiquitous proteoglycan molecule, betaglycan (300 kDa), has been named TGF-β receptor type III. An insight into how TGF-β exerts its highly pleiotropic actions may now be revealed with the recent cloning of these TGF-β receptors.

A number of related type II receptors have been identified including Caenorhabditus Elegans daf-4 for bone morphogenetic proteins (Estevez *et al* 1993), a human TGF-β type II receptor named TβR-II (Lin *et al* 1992) and two mouse activin receptors named ActR-II (Matthews & Vale 1991) and ActR-IIB (Attisano *et al* 1992). These receptors have a small ectodomain rich in cysteine, with the cytoplasmic region largely comprising of a serine/threonine kinase domain. This kinase domain is approximately 40% conserved throughout the type II receptors whereas the extracellular domain similarity is only around 10% (reviewed by Lin & Lodish 1993). However, TβRII can mimic the action of the activin receptor in early Xenopus differentiation in that expression of TβRII in Xenopus embryos allows TGF-β1 to induce mesoderm formation suggesting similar signal transduction pathways are utilised. The kinase domains of the type II receptors are highly characteristic in that they phosphorylate serine/threonine residues on their substrates differing from the classical tyrosine kinase receptors (Dijke *et al* 1994).

Expression cloning and characterisation of the TGF-β type III receptor (Wang *et al* 1991, Lopez-Casillas *et al* 1991, 1994) has revealed it to be an 853 amino acid protein. This protein has a large N-terminal region with at least one site for glycosaminoglycan addition and six glycosylation domains, a hydrophobic transmembrane region and a short 49 amino acid cytoplasmic tail. Interestingly, this tail appears to have no obvious signalling motif, and functional studies of the receptor suggest it is involved in binding of ligand and presentation to the type II

receptor which is capable of further signal transduction. Indeed, Lopez-Casillas *et al* (1993) have demonstrated that betaglycan presents TGF-β directly to the type II receptor forming a ternary complex. Thus, betaglycan may serve as a means to capture TGF-β from the periphery and concentrate it on the cell surface for presentation to the signalling receptors. This possibility is strengthened by the fact that the type II receptors have a very low affinity for TGF-β in cells that do not express functional betaglycan (Lopez-Casillas *et al* 1993).

Several TGF-β type I receptors have recently been cloned and characterised (Ebner *et al* 1993, Franzen *et al* 1993, Attisano *et al* 1993). Although these receptors are also transmembrane serine/threonine kinases, their kinase domains are quite divergent from those of the type II receptors.

2.4.8.2 Signalling through the TGF-β receptors

Much work has concentrated on trying to elucidate the signal transduction pathways of TGF-β. This work is still at a very early stage, but studies so far indicate that both TGF-β type I and type II receptors are required for TGF-β to exert its physiological effects.

Wrana *et al* (1992) have shown that in Mv1Lu cells defective for the type I receptor, the type II receptor is capable of binding ligand but does not signal TGF-β responses. TGF-β responsiveness is restored however when the type I receptor is introduced via transfection into these cells (Franzen *et al* 1993, Attisano *et al* 1993). Cells expressing defective type II receptors which have no functional kinase domain are able to bind TGF-β but unable to signal in response (Wieser *et al* 1993). These experiments demonstrate that both types of receptor are essential for the biological actions of TGF-β to be realised. Recent work by Wrana *et al* (1994) has elucidated the initial mechanics involved in this interaction. TGF-β is bound by the type II receptor which then forms a complex with the type I receptor. Data by Yamashita *et al* (1994) further suggests that the hetero-oligomeric receptor complex formed consists of two molecules each of TβR-I and TβR-II. The kinase domain of the type II receptor phosphorylates serine/threonine residues

on both itself and the cytoplasmic domain of the type I receptor, but has no role in phosphorylating downstream substrates. "Activation" of the type I receptor by this phosphorylation leads to downstream substrate phosphorylation and subsequent changes in gene expression and/or cellular phenotype. Interestingly, inactivation of the type II receptor in Mv1Lu cells reveals two receptor pathways suggesting that the antiproliferative actions of TGF-β are mediated through the type II receptor possibly in conjunction with the type I receptor, while the type I receptors are responsible for TGF-βs actions on extracellular matrix, such as increased fibronectin and plasminogen activator inhibitor expression (Chen *et al* 1993).

The actual details of the TGF-β signal transduction pathway remains a mystery. It has been postulated however that TGF-βs anti-proliferative properties are ultimately mediated at the level of tumour suppresser genes and proto-oncogenes in the nucleus which are involved in cell cycle progression such as the product of the retinoblastoma gene and c-myc.

2.4.8.3 TGF-β effects on gene expression and cell cycle progression

It is likely that TGF-β modulates cellular proliferation in such a vast variety of cells by affecting common genes involved in cell cycle progression. Due to the difficulty of obtaining pure populations of primitive pluripotent stem cells, most of the work in this area has focused on other more homogenous cellular types which are responsive to inhibition by TGF-β. The general consensus regarding TGF-β inhibition is that it prevents cell entry into DNA synthesis at a stage late in G1 or at the G1/S phase boundary.

In both rapidly growing murine keratinocytes and EGF stimulated quiescent keratinocytes, TGF-β inhibits c-myc and KC gene expression, both of which are implicated in growth stimulation (Coffey *et al* 1988). Inhibition of c-myc is post-transcriptional and requires protein synthesis. In agreement with these findings, Pietenpol *et al* (1990a,b, 1991) have also demonstrated TGF-β mediated inhibition of c-myc in keratinocytes. They further show that treatment of murine BALB/MK keratinocytes with c-myc antisense oligonucleotides inhibits proliferation of these

cells in a manner analogous to TGF-β. They demonstrated that this inhibition occurred at the level of transcriptional initiation implying that TGF-β inhibits proliferation in these cells indirectly by inducing synthesis or modification of a protein which can then specifically interact with the c-myc promoter.

A controversial candidate for one possible intermediary protein is the product of the retinoblastoma gene (pRB) (Laiho *et al* 1990). This gene encodes a protein with presumptive growth suppresser activity (reviewed in Zacksenhaus *et al* 1993). Indeed, loss or inactivation of the RB gene has been found in a variety of malignant cell types including retinoblastomas, osteosarcomas, small cell lung carcinoma, breast carcinoma and bladder carcinoma (reviewed in Pietenpol *et al* 1990). Mice deficient for Rb die before birth with defects in neurogenesis and haemopoiesis (Lee *et al* 1992). In normal cells, pRB is expressed throughout the cell cycle in various states of phosphorylation. Significantly, pRB acts as a growth suppresser when in it is underphosphorylated and this form is in abundance in G1 and in growth arrested cells (De Caprio *et al* 1989, Buchkovich *et al* 1989, Chen *et al* 1989). This growth suppressive form of pRB is also selectively bound by the transforming gene products of several DNA tumour viruses including SV40 large T antigen, adenovirus type 5 E1A and human papilloma virus type 16 E7 (Whyte *et al* 1988, DeCaprio *et al* 1989, Dyson *et al* 1989) implying that inactivation of the growth suppressive pRB is essential for cellular transformation in these contexts. Indeed, transient expression of these proteins in human foreskin keratinocytes blocks TGF-βs inhibitory action, and its inhibitory effect on c-myc, implicating pRB to be crucial to TGF-βs function (Pietenpol *et al* 1990b). Interestingly however, a report by Ong *et al* (1991) demonstrates that inactivation of the retinoblastoma gene does not lead to loss of TGF-β receptors or response to TGF-β in breast cancer cell lines. Chen *et al* (1993) have recently shown that TGF-β selectively inhibits FDC-P1 cells, a factor dependent murine myeloid progenitor cell line, possibly through inhibition of M-CSF induced c-myc expression.

Recently, it has become clear that progression through the cell cycle is regulated by a number of cyclin dependent protein kinases (CDKs). TGF-β has

been shown to inhibit Cdk4 synthesis, and that this is related to growth arrest (Ewen *et al* 1993). Mv1Lu cells growth inhibited by TGF-β failed to stably assemble cyclin E-Cdk2 complexes or accumulate cyclin E-associated kinase activity (Koff *et al* 1993). Also, G1 phase extracts from TGF-β treated cells did not support activation of endogenous cyclin-dependent protein kinases by exogenous cyclins. Interestingly, these effects were concomitant with inhibition of phosphorylation of the RB gene product suggesting that TGF-β acts to keep RB in its underphosphorylated and growth suppressive form by inactivating the kinases responsible for this phosphorylation. This led to the identification of kip1, a 27 KDa protein present in extracts from cells made quiescent by TGF-β. This protein binds to cyclin E-Cdk2 and cyclin D-Cdk4 complexes and inhibits their activity *in vitro* in a stoichiometric manner (Polyak *et al* 1994, Toyoshima and Hunter 1994). Another study by Longstreet *et al* (1992) has demonstrated that TGF-β can prevent activation of $p34^{cdc2}$ kinase. This kinase has been associated with phosphorylation of RB, and TGF-βs inhibitory effects on this kinase may be responsible for keeping RB in its unphosphorylated form.

2.4.9 Summary

As has been described above, TGF-β is produced by a bewildering array of cells, and is capable of acting on an equally broad spectrum of cellular types. The high degree of conservation of TGF-βs between species suggests that they are likely to be fundamentally important in normal physiology. With respect to haemopoiesis, TGF-β acts as both an inhibitor of primitive pluripotent cell types, but as a stimulator of less primitive progenitors. This action is also apparent with the haemoregulatory pentapeptide, and also with macrophage inflammatory protein 1α as shall be described below. Further, TGF-β is active in inhibiting epidermal keratinocytes in a manner analogous to that of both the epidermal version of the pentapeptide and of MIP-1α. This suggests that some functional redundancy may occur between the inhibitors of stem cell proliferation in both haemopoiesis and keratopoiesis and possibly in other cellular systems.

2.5 Macrophage Inflammatory Protein 1 alpha (MIP-1α)

In 1990, Graham *et al* reported the identification and characterisation of an inhibitor of haemopoietic stem cell (CFU-S/CFU-A) proliferation. Using the *in vitro* CFU-A assay, they purified to homogeneity an activity from the murine J774.2 macrophage cell line which was capable of reversibly inhibiting CFU-A and CFU-S stem cell proliferation. They named this activity stem cell inhibitor, and sequence analysis revealed that this molecule was identical to a previously described protein, macrophage inflammatory protein-1α (MIP-1α). During purification of MIP-1α, they also isolated a related molecule which was identical to the previously described MIP-1β. However, this molecule did not have potent stem cell inhibitory properties. The biological functions of these chemokines are quite diverse ranging from inhibition of proliferation to potentiating inflammatory responses and will be discussed below.

2.5.1 Structure of the MIP-1α superfamily

MIP-1α belongs to a relatively new family of cytokines, related by limited sequence homology, termed the chemokine family (Schall 1991). Members of this family are also related by the characteristic spacing of four cysteine residues, and it is on the basis of this conserved motif that the family can be further subdivided into two classes. Molecules in which the first two conserved cysteine residues are adjacent have been designated the "C-C" or β branch of the superfamily, whilst molecules whose first two cysteines are separated by an intervening amino acid belong to the "C-X-C" or α branch. All members of this superfamily are small 8-18 kDa heparin binding proteins. The members of each of these subfamilies will be described in the following sections.

2.5.1.1 C-X-C chemokines

This subfamily consists of the prototype platelet factor 4 (PF-4), β thromboglobulin, γIP-10, gro/MGSA, NAP-1/IL-8, *migc* and ENA-78. These names refer to the human homologues, except *migc* which is murine and has no

known human counterpart. A number of them do however have murine homologues, but for the purposes of this brief overview and to avoid complications, only the human homologues will be referred to in the text. The genes for all of these molecules are located on chromosome 11 in the mouse and 4 in humans.

2.5.1.2 Platelet Factor 4 (PF-4)

PF-4 is stored in the alpha granules of platelets, and was first discovered in 1961 by Deutsch *et al* by virtue of its ability to bind to heparin and thus inhibit heparins anticoagulating properties. Subsequent work has revealed that PF-4 is a potent chemoattractant for neutrophils, monocytes and fibroblasts suggesting it to be involved in inflammation and wound repair (Deuel *et al* 1981). Further, Maione *et al* (1990) have also demonstrated that PF-4 is active as an inhibitor of angiogenesis in the chicken chorioallantoic membrane and a specific inhibitor of growth factor stimulated endothelial cell proliferation *in vitro*. Further, Gupta and Singh (1994) have demonstrated that PF-4 inhibits endothelial cells by blocking entry of cells into S-phase. PF-4 is also active as an inhibitor of human erythroleukaemia cells (Han *et al* 1992).

2.5.1.3 β thromboglobulin

β thromboglobulin is also stored in the α granules of platelets, and it too is a potent fibroblast chemoattractant (Senior *et al* 1983). Proteolysis of β thromboglobulin leads to formation of NAP-2, which is a potent chemoattractant and activating factor for neutrophils (Walz *et al* 1989).

2.5.1.4 The gro proteins

Three distinct human gro genes have been identified, namely gro α, gro β and gro γ (Tekamp-Olsen *et al* 1990, Iida and Grotendorst 1990, Haskill *et al* 1990). The nucleotide sequences of gro β and gro γ are 90% and 86% identical to that of gro α respectively. It has been reported by several groups that gro α has

neutrophil chemotactic and activating properties (reviewed in Miller *et al* 1992), and gro α also stimulates autocrine growth of a human melanoma cell line and as such is also known as melanoma growth stimulating activity (MGSA) (Richmond *et al* 1988).

2.5.1.5 Interleukin 8 (IL-8)

Interleukin 8 (IL-8), also known as neutrophil activating protein 1 (NAP-1), is by far the most extensively studied member of the C-X-C branch of the chemokine family. Human IL-8 (there is no known murine homologue) is produced by a wide variety of haemopoietic cell types including monocytes, macrophages, T cells and neutrophils, as well as non-haemopoietic cell types including, amongst others, fibroblasts, keratinocytes and hepatocytes. In general, IL-8 appears to play a role in potentiating inflammatory responses. Indeed, its expression, as is the expression of other members of the superfamily, is regulated by a number of proinflammatory cytokines such as IL-1α, IL-1β and TNF as well as bacterial endotoxin indicating IL-8 to be involved in potentiating inflammatory cytokine cascades. IL-8 seems to act predominantly on neutrophils, both in that it is a potent chemoattractant for these cells (Yoshimura *et al* 1987) and it induces degranulation and the neutrophil respiratory burst (Walz *et al* 1987). Webb *et al* (1993) have further demonstrated that co-treatment of neutrophils with IL-8 plus heparan sulphate enhances the ability of IL-8 to increase cytosolic free calcium levels and neutrophil chemotaxis up to 4-fold. A putative receptor for the as yet unidentified murine homologue of IL-8 has been "knocked out" in mice generating animals which suffered from an increase in B cells and neutrophils (Cacalano *et al* 1994). Further, this receptor was a major mediator of neutrophil migration to sites of inflammation.

2.5.1.6 Other C-X-C chemokines

Monokine induced by IFN-γ (*migc*), has only been identified in mice (Farber 1990) and although sequence analysis places it within the C-X-C

chemokine family, nothing is known to date regarding its possible functions. Also, little is known about ENA-78 other than it too is chemotactic and activatory for neutrophils (Walz *et al* 1991). γ IP-10 was identified by Luster *et al* (1985), and has been suggested to play a role in delayed type hypersensitivity inflammatory situations (Kaplan *et al* 1987).

In summary, members of the C-X-C branch of the chemokine superfamily appear to be proinflammatory molecules which act predominantly, though not entirely, through neutrophils.

2.5.2 C-C chemokines

This subfamily consists of murine MIP-1α (human homologue LD78/GOS19-1), murine MIP-1β (human homologue ACT-2), TCA-3 (human homologue I-309), JE (human homologue MCP-1), RANTES, human HC14 and murine C10. In addition to these, an alternatively spliced form of TCA-3, designated p500 has been cloned (Brown *et al* 1989). Recently, the product of a novel growth factor-activated gene, *fic*, has also been identified and characterised as a C-C chemokine (Heinrich *et al* 1993). The genes for these molecules are located on chromosome 11 in the mouse and chromosome 17 in humans.

2.5.2.1 MIP-1α and MIP-1β

MIP-1α and MIP-1β exert a wide range of biological activities including chemotaxis for monocytes and T cell subsets. MIP-1α has also been shown to be a potent inhibitor of both primitive haemopoietic progenitor cell proliferation *in vivo* and *in vitro*, and of clonogenic epidermal cell proliferation. The biology of these molecules shall be discussed in detail in the following sections.

2.5.2.2 JE and MCP-1

JE was first characterised by Rollins *et al* in 1988 as a gene massively inducible in murine fibroblasts by platelet derived growth factor. This "immediate early gene" property is also shared by other members of the chemokine

superfamily. Subsequently, a human homologue was identified and named monocyte chemoattractant protein-1 (MCP-1) by virtue of its potent monocyte chemoattractant properties (Yoshimura *et al* 1989, Ernst *et al* 1994).

2.5.2.3 RANTES

RANTES (regulated on activation normal T-cell expressed, and secreted) was originally identified and characterised by Schall *et al* in 1988 as a T cell expressed gene. This gene directs synthesis of an 8kD protein, and is expressed by T cells, peripheral blood mononuclear cells and human rheumatoid synovial fibroblasts (Miller *et al* 1989, Rathanawami *et al* 1993). Recently, it has become apparent that a number of other cell types express RANTES in response to TNF-α stimulation including fibroblasts, renal epithelial and mesangial cells (Nelson *et al* 1993). RANTES functions as a potent chemoattractant for monocytes, eosinophils (Kameyoshi *et al* 1992, Ebisawa *et al* 1994) and specific T cell subsets *in vitro* (Schall *et al* 1990).

2.5.2.4 Other C-C chemokines

T cell activation gene-3 (TCA-3) was first cloned in 1987 by Burd *et al*, and has been shown to be expressed by antigen activated T cells and murine mast cells. TCA-3 is unique among the chemokine family in that it contains an extra pair of cysteine residues. Interestingly, it is active on both neutrophils and monocytes. The functions of the product of this gene and its human homologue I-309 remain largely unknown. A role for TCA-3 in inflammation, and in particular neutrophil chemotaxis, has been proposed since injection of partially purified recombinant TCA-3 into the footpads of mice elicited an inflammatory response involving neutrophils (Wilson *et al* 1988).

HC14 is a relatively new member of the C-C family expressed in human macrophages following IFN-γ stimulation (Chang *et al* 1989), and to date nothing is known regarding its function. Murine C10 was identified by virtue of its ability to be induced in GM-CSF stimulated bone marrow cells i.e. myeloid cells (Orlofsky

et al 1991). In addition to those chemokines mentioned above, Kelner *et al* (1994) discovered a novel molecule, related to both C-X-C and C-C chemokines which they have named lymphotactin by virtue of its chemotactic activity for lymphocytes. Interestingly, this molecule lacks two of the four cysteine residues that are characteristic of the other chemokines. It is expressed in activated CD8+ T cells and in CD4-/CD8- αβ T cell receptor+ thymocytes.

Thus, both the C-X-C and the C-C subfamilies of the chemokine superfamily appear to have potentially important roles to play in immune regulation and inflammation, with such effects by the C-X-C members being generally apparent on neutrophils while C-C members generally act on monocytes. However, as mentioned above, MIP-1α also acts as a potent inhibitor of both haemopoietic stem cells and of epidermal keratinocytes demonstrating this molecule to possess both similar and unique properties to its related family members. Below, I shall discuss these diverse properties of MIP-1α in detail.

2.5.3 Structure of the MIP-1 proteins

MIP-1α and MIP-1β were originally co-isolated by Wolpe *et al* in 1988 and named MIP-1. MIP-1 resolves as a doublet on denaturing SDS-PAGE gels, and is composed of two distinct 8kD peptides designated MIP-1α and MIP-1β. Although MIP-1β contains a potential N-terminal glycosylation domain, both of these proteins appear to be non-glycosylated. MIP-1α and MIP-1β are coded for by distinct genes, present on mouse chromosome 11 and share 60% amino acid identity across their entire lengths and 69% identity in the mature peptide sequences. One feature of the both the murine and human MIP proteins which has hampered the progress of investigators searching for specific cell surface receptors is the propensity of them to form high molecular weight self aggregates in physiological buffers *in vitro*. These aggregates are noncovalent and electrostatic in nature and can be disrupted to varying degrees by high ionic strength solutions (Graham *et al* 1992). These aggregates tend to be approximately 100kDa at a concentration of 0.1mg/ml (Graham *et al* 1992), and controversy has reigned over

whether the aggregated form is biologically active, or if disruption of the aggregate to produce monomeric MIP-1α is required. The functional significance of the self aggregation of this molecule is unclear, but may be to stabilise the protein or protect it from proteolysis. Recently, Mantel *et al* (1993) have demonstrated that the aggregated form is biologically inactive with respect to the myelosuppressive effects of MIP-1α, and that the active form is in fact the 8kD monomer. Further, a recent study by Graham *et al* (1994) has shown, by systematically neutralising carboxyl-terminal acidic residues, that these mutants of MIP-1α display native molecular weights representative of tetramers, dimers and monomers of MIP-1α. However, and in contrast to the results of Mantel *et al* (1993), these mutants of MIP-1α appear to be biologically equipotent to each other and to wild type MIP-1α in both stem cell inhibition assays and monocyte shape change assays, suggesting that the wild type multi-aggregate, tetramers and dimers spontaneously dissaggregate and function as monomers in these assay conditions. Thus, the aggregation of MIP-1α appears to have little effect on biological activity *in vitro*. As shall be discussed below, MIP-1α is currently being assessed as a potential myeloprotective drug for use during cancer chemotherapy, and initial results from our laboratory are very encouraging (Dunlop *et al* 1992). Injection of a large molecular weight molecule such as aggregated MIP-1α is unappealing since quantitation of the exact levels of active molecule circulating in the bloodstream are difficult to assess. Thus, these mutants are likely to be prove invaluable when using MIP-1α in such a clinical context.

2.5.4 Expression Patterns of MIP-1α and MIP-1β

2.5.4.1 Expression in normal conditions

MIP-1α and MIP-1β are predominantly expressed by cells of haemopoietic origin. Further, since it appears that the expression of both of these genes is co-regulated, I will only refer to MIP-1α below. Macrophages (Davatelis *et al* 1988, Wolpe *et al* 1988), activated T cells (Yamamura *et al* 1989), stimulated B cells

(Lipes *et al* 1988), mast cells (Burd *et al* 1989), eosinophils (Costa *et al* 1993), neutrophils (Kasama *et al* 1993, 1994) and epidermal Langerhans cells (Heufler *et al* 1992) express MIP-1α mRNA. Thus, it is likely that all haemopoietic cell types are capable of expressing MIP-1α mRNA. However, and like other haemopoietic growth factors, although MIP-1α mRNA expression is detectable in normal bone marrow using sensitive PCR based techniques (Cluitmans *et al* 1995), quantitation and indeed detection of protein remains difficult. It is possible that although MIP-1α is produced in very low and therefore undetectable levels overall in the bone marrow, local concentrations around the target stem cells, perhaps aided by the proteoglycan binding abilities of MIP, may well be high enough to exert physiological effects. In addition to expression of MIP-1α by haemopoietic cells, a report by Nakao *et al* (1990) demonstrates detection of MIP-1α gene transcripts in phorbol ester treated fibroblasts and U105MG cells, a human glioma cell line. Interestingly, both astrocytes and spermatogonia have also been shown to express MIP-1α transcripts (Kim *et al* 1995, Hakovirta *et al* 1994).

An interesting feature of MIP-1α is that its expression can be rapidly super-induced in macrophages by treatment with LPS (Davatelis *et al* 1988), and in T cells by phorbol esters (PMA) and or PHA and cyclohexamide (Obaru *et al* 1986, Yamamura *et al* 1989, Blum *et al* 1990). These inducibility features of MIP-1α suggest that it falls within the class of the rapid-response or immediate early genes. Analysis of the MIP-1α gene reveals five major nuclear protein binding sites in the proximal promoter which bind C/EBP, NF-κB and or c/Ets family members. Changes in promoter binding by members of the C/EBP and NF-κB families correlates with the transcriptional up-regulation seen in serum or endotoxin-stimulated macrophages (Grove and Plumb 1993).

2.5.5 Expression in disease

Aberrant expression of MIP-1α has also been observed in a number of haematological and inflammatory disorders. For example, Maciejewski *et al* (1992) have shown that human MIP-1α mRNA levels are significantly increased in

normal human bone marrow nucleated cells from patients with aplastic anaemia and myelodysplastic syndrome when compared to normal bone marrow. It is unclear however whether or not this upregulation is a secondary effect of dysregulated cytokine production in such patients or if it is in fact partly causative of the diseased state. Similarly, and as mentioned in the previous section describing TGF-β, MIP-1α expression is also upregulated in inflammatory lesions present in TGF-β1 null mice although again, it is unclear if this is primary or secondary to the diseased phenotype of the mice. MIP-1α is also over expressed in alveolar macrophages of patients with interstitial lung disease, again possibly contributing to the inflammatory phenotype observed (Standiford *et al* 1993). Further, Smith *et al* (1994) have demonstrated that passive immunisation of bleomycin-challenged mice with anti-MIP-1α antibodies significantly reduced pulmonary mononuclear phagocyte accumulation and reduced fibrosis.

2.5.6 The role of MIP-1α in adult haemopoiesis

2.5.6.1 Inflammatory functions of MIP-1α and MIP-1β

As mentioned above, both of these proteins were originally co-isolated from the conditioned medium of the murine macrophage cell line RAW 264.7 by Wolpe *et al* in 1988 and were known collectively at that time as MIP-1. They found that injection of MIP-1 subcutaneously into the footpads of mice elicited an inflammatory response consisting largely of a neutrophil infiltrate. Further, MIP-1 was a mild chemoattractant for neutrophils *in vitro* and could potentiate the neutrophil respiratory burst. However, the concentration of MIP-1 protein required to induce these effects was relatively high (>1μg/ml), perhaps indicating that this was not the primary physiological role of MIP-1 *in vivo*.

Recently, it has become clear that MIP-1α and MIP-1β are involved in potentiating the immune response through effects on a wider variety of cell types than was first thought. For example, Fahey *et al* (1992) have shown that MIP-1 protein can enhance the ability of macrophages to kill tumour target cells. Also,

MIP-1 stimulated the proliferation of mature tissue macrophages and this effect was increased in the presence of M-CSF or GM-CSF. They also demonstrated that MIP-1α, but not MIP-1β could increase macrophage secretion of the pro-inflammatory cytokines TNF, IL-6 and IL-1. In fact, an eightfold excess of MIP-1β could abrogate the ability of MIP-1α to exert some of these effects (i.e. increased TNF expression) suggesting that MIP-1α and MIP-1β are in fact antagonistic. However, and unlike TGF-β described in the previous section, MIP-1 could not induce the macrophage respiratory burst or increase MHC expression. A recent study by Taub *et al* (1993) has shown that MIP-1α and MIP-1β can actually exhibit independent functions. They showed that MIP-1α and MIP-1β were potent chemoattractants for CD3 stimulated $CD8^+$ and $CD4^+$ T cells respectively, and that they enhanced the ability of T cells to bind to an endothelial monolayer implicating these cytokines in the initiation of specific immune responses. In support of this, Tanaka *et al* (1993) have demonstrated that proteoglycan immobilised MIP-1β can enhance $CD8^+$ T cell adhesion to the vascular cell adhesion molecule VCAM-1. These experiments suggest that the proteoglycan binding abilities of the chemokines may be important to some of their inflammatory functions such that immune cells can be firstly recruited from the periphery, and then attracted to sites of inflammation or infection.

In addition to having effects on neutrophils and lymphocytic cells, MIP-1α has also been found to activate basophils and mast cells (Alam *et al* 1992). Further, Alam *et al* (1994) injected recombinant MIP-1α into the footpads of mice and observed a significant swelling which was due to a severe inflammatory reaction consisting of neutrophils, monocytes and degranulated mast cells. MIP-1α, but not MIP-1β, can induce the migration and activation of normal human eosinophils (Rot *et al* 1992). Davatelis *et al* (1989) have reported that MIP-1 acts as an endogenous pyrogen i.e. is capable of rapidly inducing fever. This function is analogous to that of other inflammatory cytokines such as tumour necrosis factor (TNF) and interferon γ (IFN-γ). However, and unlike these other pyrogens, the fever induced by MIP-1 could not be abrogated by administration of the

cyclooxygenase inhibitor ibuprofen indicating that MIP-1 induces the febrile response via a prostaglandin independent pathway. This role of MIP-1 in fever induction must therefore be considered when administering antipyretics in fever treatment. Further to this work, Minnano *et al* (1990) have shown that MIP-1 exerts these effects by acting directly on the hypothalamus and at very low concentrations (10^{-15} M). Saukkonen *et al* (1990) have demonstrated that MIP-1α is also capable of inducing inflammatory responses in the central nervous system, in that it induces inflammatory cell infiltration into the subarachnoid space located behind the blood-brain barrier.

In summary, these data indicate that MIP-1α and MIP-1β have important roles to play in inflammation. These cytokines act to recruit specific and non-specific immune cells from the circulation and then attract them to the site of inflammation. Upon arrival, the MIP proteins then contribute to activation of these cells and potentiate many of their cytotoxic functions. MIP-1α,β may also contribute to the cytokine cascade by inducing production of other inflammatory cytokines in fibroblasts and macrophages such as IL-1, IL-6 and TNF (Fahey *et al* 1992).

2.5.7 Effects of MIP-1α on cellular proliferation

Like TGF-β, as well as being an inflammatory mediator, MIP-1α is also capable of controlling cellular proliferation, both positively and negatively in a number of different cell types. The most documented abilities of MIP-1α with respect to proliferative control has come from studies examining its effects on the haemopoietic stem and progenitor cell compartments and these shall be discussed below.

It was not realised until 1990, when Graham *et al* isolated a potent inhibitor of stem cell proliferation identical to MIP-1α, that MIP-1α and its related family members may be involved in control of cellular proliferation and/or differentiation. Subsequent work has revealed that this is indeed the case although conflicting

reports have emerged regarding exactly what members of this family are capable of exhibiting these properties.

MIP-1α is capable of potently (pM concentrations) inhibiting both colony formation by CFU-A type stem cells and the proportion of these same cells in DNA synthesis. It also inhibits their *in vivo* correlate day 12 CFU-S, but is unable to inhibit the proliferation of more committed GM-CFC progenitors (Graham *et al* 1990). This appeared to be a specific effect by MIP-1α since administration of polyclonal antiserum directed against MIP-1 abrogated its inhibitory effects. Further, similar concentrations of MIP-1β were unable to inhibit these same cellular populations. These results indicate that MIP-1α, but not MIP-1β, is a potent inhibitor of primitive stem cell proliferation, and the concentrations at which it is active strongly suggest that this may be an important physiological property of this molecule.

Subsequently, a number of other groups have also reported on the inhibitory properties of MIP-1α. Broxmeyer *et al* demonstrated in 1990 that recombinant murine MIP-1α was active in inhibiting *in vitro* colony formation by a number of primitive haemopoietic progenitor cells. These included suppression of murine CFU-GM stimulated with pokeweed mitogen spleen-conditioned medium (PWMSCM), hu CFU-GM stimulated with optimal recombinant human GM-CSF (rhGM-CSF) + recombinant human IL-3 (rhIL-3), and both murine and human BFU-E and CFU-GEMM stimulated with PWMSCM + erythropoietin or erythropoietin + rhIL-3 or rhGM-CSF respectively. In agreement with the findings of Graham *et al* (1990), MIP-1β was incapable of these effects, and a more recent report by Broxmeyer *et al* (1991) has shown that a 4:1 molar ratio of MIP-1β to MIP-1α can block these inhibitory actions of MIP-1α. Interestingly, they also reported that both recombinant MIP-1α and -1β were capable of enhancing murine CFU-GM colony formation when stimulated by sub-optimal doses of rhM-CSF and rhGM-CSF indicating that, like TGF-β, MIP-1α is active as an inhibitor of primitive haemopoietic stem cells but as a stimulator of more mature lineage restricted progenitors. The apparent contradiction that MIP-1α can both stimulate

CFU-GMs and yet also inhibit them could be representative of differing stages of "maturity" within this progenitor cell compartment. That is, MIP-1α is active as an inhibitor when CFU-GM are more immature and open to stimulation by a plethora of early acting growth factors, and as a stimulator when the presumably more mature CFU-GM are stimulated by a single CSF, as is the case for TGF-β. Broxmeyer *et al* also demonstrated that MIP-1α inhibited highly enriched human progenitor cell populations suggesting that this was a direct effect. However, there remains the possibility that MIP-1α may inhibit stem cell proliferation indirectly. This could be achieved by inducing other inhibitors from secondary contaminating cells or perhaps even autocrinely from the target stem cells. Recently, Cooper *et al* (1994) have performed *in vivo* experiments using monomeric rmMIP-1α rather than the previously used aggregated form (see earlier discussion) and demonstrated that the monomer was around 1000 fold more active than previously described. However, in light of the recent data reported by Graham *et al* (1994), this issue remains unresolved.

Like the other stem cell inhibitors mentioned previously, MIP-1α is currently being evaluated as a possible myeloprotective agent for use during chemotherapeutic regimes (Graham and Pragnell 1991). Dunlop *et al* (1992) have demonstrated protection by MIP-1α of the CFU-S/CFU-A compartment from double injections of cytosine arabinoside (ara-C). Clements *et al* (1992) have also demonstrated that *in vivo* injection of MIP-1α into mice can protect CFU-S from subsequent *in vitro* killing by tritiated thymidine. Similarly, Lord *et al* (1992) have demonstrated that MIP-1α can protect the CFU-S population in mice from the cytotoxic effects of the cell cycle specific drug hydroxyurea (HU). In a dose dependent manner, MIP-1α either reduced HU mediated kill of CFU-S cells and accelerated recovery or completely protected the CFU-S compartment. Maze *et al* (1992) have also shown that MIP-1α (2-10 μg/mouse i.v.) but not MIP-1β rapidly decreased cycling rates and absolute numbers of myeloid progenitor cells in the bone marrow and spleen. These studies indicate that MIP-1α may well be useful in protecting stem cells from cycle specific cytotoxic drugs, but it should be stressed

that these drugs are rarely used alone in cancer chemotherapy in favour of combination regimes using other drugs which do not distinguish between cells according to their cell cycle status. Thus, the actual potential usefulness of MIP-1α in this context is debatable.

In addition to the inhibitory effects of MIP-1α on normal stem and progenitor cells, it has been shown by Ferrajoli *et al* (1994) that it can also inhibit proliferation of progenitors from subsets of acute myelogenous leukaemia in a dose dependent manner. Further, MIP-1α gene expression is detectable in peripheral blood cells from both AML and acute lymphocytic leukaemia patients, and yet appears to have little or no function in endogenous inhibition of these neoplastic cells. This perhaps suggests that although MIP-1α mRNA is expressed, this is not translated into protein in these cells. However, this possibility awaits further study. It has been shown by various groups that MIP-1α is, unlike TGF-β, inactive in inhibiting chronic myeloid leukaemia cell proliferation (Holyoake *et al* 1993b, Eaves *et al* 1993, Chasty *et al* 1993). This suggests that MIP-1α may have clinical potential as a protective agent during chemotherapy or for chemotherapeutic purging of CML bone marrow grafts *in vitro* such that MIP-1α will inhibit and protect the normal stem cell population, whereas the malignant cells will be more susceptible to drug toxicity.

The role of MIP-1α in long term bone marrow cultures (LTBMC) has also been investigated. Eaves *et al* (1993) have demonstrated that exogenous addition of MIP-1α to LTBMCs reversibly and specifically blocks the entry into cycle of primitive high proliferative potential progenitors present in the adherent layer, but has no effect on more mature progenitors. Further, MIP-1α had no such effect when the LTBMCs were supporting growth of CML cells. An interesting report by Verfaille *et al* (1993) also investigated the role of MIP-1α in LTBMCs, and in particular its effect on the recovery of LTBMC-initiating cells (LTBMC-IC). These cells are capable of setting up secondary LTBMC. This group had previously described a novel stroma-noncontact culture in which haemopoietic progenitors are grown at a distance from the stromal layer via separation by a

0.4mm filter membrane. This membrane is however permeable to soluble factors, and these factors alone are capable of maintaining progenitor viability, and indeed production of committed progenitors. However, in both the traditional LTBMC and the noncontact version, it was apparent that after approximately five weeks, up to 50% of the initial LTBMC-IC inoculated into the cultures were lost. As mentioned above, a number of growth factors are capable of increasing total cell expansion and committed progenitor cell numbers, but none are capable of increasing recovery of LTBMC-ICs. The prolonged addition of IL-3 alone to these cultures results in eventual depletion of primitive LTBMC-ICs, likely to be as a result of their terminal differentiation. Co-addition of MIP-1α and IL-3 however results in greatly increased maintenance of LTBMC-IC for up to eight weeks. Verfaille *et al* suggest that this observation may not be due to growth inhibition by MIP-1α but rather as a result of this cytokine blocking terminal differentiation since administration of the known anti-proliferative cytokine TGF-β with IL-3 results in a decreased recovery of LTBMC-IC. In a more recent publication, Verfaille and Miller (1994) show that up to 30% of CD34+/CD33- cells present in MIP-1α + IL-3 supplemented cultures are capable of initiating and sustaining long term *in vitro* haemopoiesis compared with only around 6% in cultures not receiving the MIP-1α supplement. Thus, treatment of such cultures with both MIP-1α and IL-3 may prove an excellent method of obtaining large numbers of very primitive stem cells for both phenotypic analysis, and for clinical stem cell expansion.

Although MIP-1α inhibits subpopulations of progenitors that are activated in myelodepressed animals, a study by Quesniaux *et al* (1993) has shown that MIP-1α has no inhibitory effect on the induction of long term repopulating cells (LTRC) into the cell cycle by treatment with two doses of 5-FU *in vivo*. However, the concentrations of MIP-1α used in this experiment were significantly lower than those required to inhibit CFU-S/CFU-A type progenitor cells. Further, a recent report by Keller *et al* (1994) also demonstrated that MIP-1α was incapable of inhibiting LTRC at concentrations analogous to those required to inhibit CFU-

S/CFU-A. Thus, it appears that the inhibitory properties of MIP-1α are apparent on a very small window of the stem cell compartment, namely CFU-S/CFU-A. LTRC represent the most primitive stem cells detectable, and are capable of sustaining long-term haemopoiesis *in vivo*. Thus, the definition of MIP-1α as a stem cell inhibitor may not be generally accurate since the most primitive stem cells appear to be refractory to its inhibitory properties.

As well as being able to inhibit and enhance sub-populations of haemopoiesis, MIP-1α, like TGF-β, is also active as an inhibitor of both murine and human clonogenic epidermal keratinocyte proliferation (Parkinson *et al* 1993). Further, MIP-1α mRNA is detectable in epidermal Langherans cells, but not in keratinocytes or other epidermal cellular populations (Heufler *et al* 1992, Parkinson *et al* 1993). These cells have been shown to be intimately associated with the epidermal keratinocyte (Potten and Allen 1976), suggesting local production of MIP-1α to play an important role in the regulation of this constantly regenerating tissue. A recent paper by Hakovirta *et al* (1994) also suggests that MIP-1α may function as a regulator of germ cell development, by both increasing DNA synthesis in primitive spermatogonia and inhibiting DNA synthesis of more mature & differentiated spermatogonia. They also demonstrated using immunohistochemical analysis that MIP-1α protein was localised in these cell types and in areas where cells were undergoing DNA synthesis.

As mentioned above, controversy exists over exactly which members of the chemokine superfamily are capable of stem cell inhibitory effects. Work from our laboratory by Graham *et al* (1993) has shown that MIP-1α, its human homologue LD78 and ACT-2 (human MIP-1β) are active in this context, while JE, TCA-3, MCAF (human JE) and all of the C-X-C family members are inactive. In contrast to these findings, Broxmeyer *et al* (1993) has demonstrated dose dependent inhibition of progenitors by a different but wider range of chemokines. The differences are difficult to explain, but may result from differing growth factor combinations present in the different types of assay system utilised by each group.

2.5.8 The mode of action of MIP-1α in inhibiting stem cell proliferation

To date, very little is known regarding the mechanisms by which MIP-1α inhibits these cell types. Studies on the actual kinetics of inhibition by MIP-1α have been hampered by lack of a suitable homogeneous cell system which is responsive to these inhibitory effects. Aronica *et al* (1994) have recently described a human cell line MO7e, whose proliferative status could be downregulated by a number of chemokines, so it may now be possible to investigate the antiproliferative signal transduction pathway utilised by MIP-1α. It is tempting to speculate that due to the many similarities between MIP-1α and TGF-β in this regard, MIP-1α too may ultimately mediate its effects via genes and proteins implicated in cell cycle control which are affected by TGF-β. This possibility however awaits further study.

With the recent cloning and characterisation of a receptor for the C-C chemokine family (Neote *et al* 1993), some of the initial mechanisms of the mode of action of MIP-1α may now be studied.

2.5.9 Receptors for MIP-1α and the chemokine family

Two receptors for human IL-8 (designated A and B) were the first receptors to be cloned for the chemokine family (Holmes *et al* 1991, Murphy and Tiffany 1991). Recently, Neote *et al* (1993) isolated a cDNA from phorbol myristate acetate treated HL60 cells encoding a protein acting as a receptor for C-C chemokine family members. This receptor has been named C-C CKR1 and has a predicted molecular mass of around 41 kD. Functional expression of this receptor in human embryonic kidney cells confers both binding ability for MIP-1α and a consequent mobilisation of calcium. Subsequently, another chemokine binding receptor has been identified on red blood cells and shown to be identical to the cloned Duffy antigen, which also acts as a receptor for the malarial parasite Plasmodium vivax (Neote *et al* 1994). Interestingly, this receptor is capable of binding all members of the C-C and C-X-C branches of the superfamily with the notable exception of MIP-1α. The significance of this, and indeed the role of the

Duffy antigen is unclear, but as this receptor is present on cell types incapable of further signal transduction such as erythrocytes, it may act as a "sink" for excess chemokine levels present in the periphery. Charo *et al* (1994) have recently cloned two MCP-1 receptors, and functional expression of these receptors in *Xenopus* oocytes reveals them to be alternatively spliced at the carboxy tail. Four potential viral counterparts of the chemokine receptors have also been identified (reviewed in Horuk 1994). Interestingly, CC-CKRI shows approximately 33% homology to the US28 open reading frame encoded by human cytomegalovirus (Neote *et al* 1993). US28 can act as a functional receptor when transfected into 293 cells, and elicit calcium fluxes in response to MIP-1α, MIP-1β, RANTES and MCP-1, but not C-X-C chemokines (Neote *et al* 1993, Gao and Murphy, 1994). The biological significance of this observation is unclear.

Functional and structural analysis of these receptors has revealed them to belong to the seven-membrane spanning G-protein linked receptor superfamily. This family also includes receptors for the chemoattractant molecules f-Met-Leu-Phe and complement molecule C5a. Classically, signalling through such G-protein linked receptors results in a dissociation of the heteromeric G proteins into Gα and $\beta\gamma$ subunits. The α subunits then activate phospholipase C, an enzyme which then generates diacylglycerol (DAG) and inositol triphosphate (IP3). DAG then activates protein kinase C, while IP3 diffuses into the cytosol and releases the secondary messenger calcium from intracellular stores. Both IL-8 RA and IL-8 RB have been shown to elicit these effects in response to IL-8 and MGSA, although two different pertussis toxin sensitive and insensitive pathways appear to be involved in activation of phospholipase C (Wu *et al* 1993). It has been demonstrated by Sozzani *et al* (1993) that MIP-1α, MCP-1 and RANTES stimulate calcium influx in human monocytes, and by Rot *et al* (1992) that MIP-1α and RANTES induce calcium influx in normal human eosinophils suggesting that many of these chemokines may utilise similar initial signal transduction pathways.

Receptors for MIP-1α and related C-C chemokines have been found on a variety of haemopoietic cell types including monocytes (Napolitano *et al* 1990),

murine T cells and macrophages (Oh *et al* 1991) and primitive haemopoietic cell lines. Graham *et al* (1993) have demonstrated that the primitive human myeloerythroleukaemic cell line K562 expresses around 15,000 receptors of a single class (Kd of 600pM) which can recognise both MIP-1α and the rest of the C-C chemokines (with the exception of TCA-3) perhaps indicating that this is a general receptor for this branch of the superfamily. Further, the murine stem cell like line FDCP-MIX displays around 18,000 receptors of a single class (Kd of 330pM), but MIP-1α binding to this receptor is less prone to competition by more diverse MIP family peptides. It should be noted at this point that the receptors present on FDCP-MIX cells are most likely distinct from the cloned C-C CKRI receptor based on both the apparent "affinity for ligand" differences between them, and on their different binding and competition patterns using other chemokines. That is, the FDCP-MIX receptor recognises only MIP-1α, MIP-1β and their human homologues LD78 and ACT-2, while C-C CKRI binds MIP-1α, MIP-1β and RANTES and MCP-1. Thus, it is possible that MIP-1α exerts its many diverse effects on different cell types by signalling through a number of different receptor types. Further, it has been suggested by Graham *et al* (1993) that the high affinity receptor present on FDCP-MIX cells may be responsible for mediating the inhibitory function of MIP-1α since it can only bind members of the chemokine family with inhibitory properties. However, confirmation of this awaits cloning and functional characterisation of this receptor type.

A recent report by Avalos *et al* (1994) has demonstrated the presence of both high and low affinity receptors for monomeric MIP-1α on human leukaemic $CD34^+$ blast cells, promyelocytic cells, monocytes, peripheral blood neutrophils and T cells. They show that THP-1 monocytes express a single class of high affinity receptor for MIP-1α, that also binds MIP-1β, LD78 and ACT-2, with no detectable competition for binding by RANTES. Further, they detected both this receptor, and a low affinity receptor present on neutrophils which binds MIP-1α, MIP-1β, LD78, RANTES but not ACT-2. Compilation of the data discussed above suggests that the high affinity receptor detected on a number of cell types by

Avalos *et al* may be identical to that observed on FDCP-MIX cells by Graham *et al*, and the low affinity receptor may be identical to the low affinity C-C CKRI cloned by Neote *et al* (1993) and Gao *et al* (1993).

Understanding of the diverse functions of MIP-1α are now only beginning to surface. Cloning of receptors for this molecule will provide a valuable tool for elucidating many of these mechanisms, perhaps by artificial expression in cell types which are not normally responsive to either the inflammatory effects or inhibitory effects of MIP-1α, or by disruption of the receptor gene in cells which are responsive (e.g. IL-8 receptor knockouts). Such experiments should allow us to distinguish between the different receptor types with respect to their functional roles. However, as yet, little is understood regarding the inhibitory activities of MIP-1α. Like TGF-β, MIP-1α may ultimately regulate cellular proliferation at the level of nuclear proto-oncogenes, tumour suppresser genes and molecules involved in cell cycle progression such as the cyclins and cyclin inhibitors. This represents an exciting area for future study.

Aims of Thesis

In order to fully understand the complex process of haemopoiesis, it is necessary to understand how the proliferation, differentiation and self renewal of the stem cell is controlled. It has become apparent in the last few years that the proliferation of the haemopoietic stem cell compartment is open to control by a number of both positive and negative regulators. Many of these factors appear to be capable of exerting similar if not identical effects on the same stem cell type suggesting that there is "functional redundancy" between them. Our group has recently identified and characterised a potent inhibitor of haemopoietic stem cell proliferation, namely macrophage inflammatory protein 1 alpha (MIP-1α). It has struck us throughout our studies on this molecule that it bears a number of functional similarities to other inhibitors of stem cell proliferation, namely the tetrapeptide, the haemoregulatory peptide and in particular, transforming growth factor beta (TGF-β). The aim of this project therefore, was to investigate the extent and possible mechanisms of functional similarity between these molecules. It is possible for example that one, or all, may act through induction of another in secondary cell types present within the bone marrow microenvironment such that a heirarchy between the inhibitors may exist. Also, it is possible that one may prime the target stem cells to respond to another by upregulating specific receptors for that molecule. Investigation of these possibilities could potentially explain the functional similarities between them, and also elucidate possible distinctive roles for each in the overall control of haemopoietic stem cell proliferation.

CHAPTER 3

MATERIALS

CHAPTER 3 - Materials

3.1 Tissue Culture Supplies

Beatson Institute Central Services	Sterile PBS Sterile glassware and pipettes
Amicon (USA)	Centriprep 10 spin columns
Gibco Life Technologies (Paisley, UK)	Special Liquid Medium Foetal calf serum MEM alpha stock 200mM glutamine DH5α competent cells
A/S Nunc (Roskilde, Denmark)	Tissue culture flasks Nunc cryotubes
Beta Labs (UK)	Yeast extract
Biological Industries (Israel)	RPMI 1640 media
Costar (USA)	96 well cell culture plates Cell scrapers
DIFCO Laboratories (Michigan, USA)	Lipopolysaccharide W Bactoagar Bactotryptone
Gelman Sciences (UK)	Sterile acrodiscs syringe filters (0.2μm and 0.45μm)
Becton Dickinson, U.K. Ltd. (Plymouth, UK)	Tissue culture plates (35mm, 60mm) Falcon tubes (15ml and 50ml)
SIGMA Chemical Co. (Poole, UK)	Ampicillin Horse serum (donor herd)

3.2 Cytokines and Antibodies

R&D Systems Europe (Abingdon, UK)	Recombinant human TGF-β1 Natural porcine TGF-β2 Recombinant human TGF-β3 Recombinant human LAP (TGF-β1) Recombinant anti-murine MIP-1α antibody

	Recombinant murine MIP-1α
Genetics Institute (Boston, USA)	Recombinant murine activin Recombinant murine BMP-2
Sigma Immunochemicals (UK)	Anti-goat IgG (peroxidase conjugate)

3.3 Kits

Amersham International (Amersham UK)	ECL Western blotting detection reagents
Boehringer-Mannheim UK (Lewes, UK)	SP6/T7 transcription kit
Qiagen Inc. (Chatsworth, CA, U.S.A)	Qiagen plasmid preparation kits
R&D Systems (Abingdon, UK)	Quantikine transforming growth factor-β1 immunoassay
Pharmacia Biotech (St Albans, UK)	Ready-To-Go DNA labelling kit

3.4 Membranes, paper and X-ray film

Amersham International (Amersham, UK)	Hybond N nylon membranes Hybond C nitrocellulose membranes
Dow Chemical Company (UK)	Saranwrap
Dupont NEN (UK)	Genescreen membrane
Schleicher & Schuell (Dassel, Germany)	Nitrocellulose membranes
Vernon-Carus, Ltd. (Preston, UK)	Gauze swabs
Whatmann International Ltd. (Maidstone, UK)	3MM filter paper
Eastman Kodak Co. (Rochester, USA)	X-OMAT AR X-ray film DUP-1 duplicating film X-ray cassettes
Technical Photo Systems (Cumbernauld, UK)	Fuji Medical RX X-ray film

3.5 Nucleotides, polynucleotides, DNA and restriction enzymes

Amersham International, plc. (Amersham, UK)	[α-^{32}P]-dCTP: 3000 Ci/mmol [α-^{32}P]-UTP: 800 Ci/mmol

	[γ-^{32}P]-ATP: 5000 Ci/mmol
Boehringer-Mannheim (Lewes, UK)	GTP, ATP, CTP (100mM lithium salts)
Dupont NEN (UK)	^{125}Iodine
SIGMA Chemical Co. (Poole, UK)	Salmon sperm DNA Polyadenylic acid (5')
Gibco Brl	Restriction enzymes

3.6 Gels and columns

GIBCO, Ltd (Paisley, UK)	Agarose and Low Melting Point Agarose
Severn Biotech Ltd. (Kidderminster, UK)	Design-a-Gel 40% (w/v) acrylamide, 2% (w/v) bis-acrylamide solution
BDH Chemicals Ltd. (Poole, UK)	Acrylamide bis-acrylamide
Schleicher and Schuell (Dassel, Germany)	Elutip-D-columns
Pierce & Warriner (UK)	GF5 excellulose column
Pharmacia Biotech (St Albans, UK)	NICK columns

3.7 Chemicals

BDH Chemicals (Poole, UK)	All chemicals other than those listed below
Amersham International, plc. (Amersham, UK)	Rainbow molecular weight markers
SIGMA chemical Co. (Poole, UK)	Bromophenol blue Dithiothreitol (DTT) MOPS TEMED Triton-X 100 FURA-2 AM
James Burroughs Ltd. (Witham, UK)	Ethanol
Cinna/ Biotecx Laboratories Inc. (Houston, USA)	RNazol B reagent

GIBCO BRL (Paisley, UK)	TRIzol reagent
Northumbria Biologicals Ltd. (Cramlington, UK)	Bovine serum albumin (20% w/v)
Fisons Scientific Equipment (Loughborough, UK)	Formaldehyde (38% w/v) Propan-2-ol Glycerol
Fluka Chemika-Biochemica AG (Switzerland)	Formamide
Pierce & Warriner (UK)	Iodogen reagent

3.8 Solutions

3.8.1 Tissue Culture

MEM Alpha Stock (Gibco)	Dissolve 5 litre pack in water, add 50ml MEMx100 vitamins (Gibco) and 100mg gentamycin sulphate. Make up to 1500ml with water and filter sterilise.
MEM Alpha x 2 (for 100ml)	MEM alpha stock 21ml L-Glutamine (200mM) 1ml Sodium Bicarbonate (7.5%) 3ml Donor Horse Serum 25ml (For MEM Alpha x 1, add equal volume of distilled water or agar)
RPMI 1640	RPMI 10x stock 100ml Distilled water 860ml L-Glutamine (200mM) 10ml Sodium Bicarbonate (7.5%) 1.5ml
INT Stain	50mg of 2-(-4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) (BDH, UK) was added to 50ml of PBS, and the mixture heated to dissolve the solid. This solution was allowed to cool, and was filter sterilised through a 0.22μm filter.
L-βroth (L-Amp broth)	Yeast extract 5g

	Bactotryptone	10g
	NaCl	10g
	Ampicillin (50μg/ml)	

3.8.2 Agarose Gel Electrophoresis, Northern Blotting and Hybridisation

MOPS Buffer 10x pH 7.0 (for 1l)	MOPS (Sigma)	41.80g
	Sodium Acetate	6.80g
	EDTA	3.72g
RNA Sample Loading Buffer	Formamide	55%
	10x MOPS	11%
	Formaldehyde	17.9%
	Water	7.5%
	Glycerol	7.5%
	Bromophenol blue to colour	
10 x TE (for 1 litre)	Tris/HCl pH8	12.1g
	EDTA	3.7g
	Water to 1 litre	
20 x SSC pH 7.0 (for 1 litre)	Sodium Chloride	175.3g
	Sodium Citrate	88.4g
20 x SSPE pH 7.4 (for 1 litre)	Sodium Chloride	175.3g
	Sodium Citrate	88.2g
	EDTA	7.4g
50 x Denhardts Solution	Ficoll-400	1% (w/v)
	Polyvinylpyrrolidine	1% (w/v)
	Bovine Serum Albumin (Pentax Fraction V)	1% (w/v)
(Pre-) Hybridisation Buffer (50mls)	Formamide	25ml
	50x Denhardts	2.5ml
	20 x SSPE	12.5ml
	Poly C (1mg/ml)	100ml
	Salmon Sperm DNA (10mg/ml)	0.5ml
	10% SDS	0.5ml
	Complete to 50mls with water	
(Pre-) Hybridisation Buffer pH 7	Na_2HPO_4	0.25M
	SDS	7%

3.8.3 SDS-PAGE Polyacrylamide Gel Electrophoresis

15% Polyacrylamide gel	Water	7.5ml
	Acrylamide mix (30%)	12.5ml
	1.5M Tris (pH 8.8)	6.3ml

	10% SDS	0.25ml
	10% ammonium persulphate	0.25ml
	TEMED	0.01ml
5% Stacking gel	Water	6.8ml
	Acrylamide mix (30%)	1.7ml
	1.0M Tris (pH 6.8)	1.25ml
	10% SDS	0.1ml
	10% ammonium persulphate	0.1ml
	TEMED	0.01ml
SDS/DTT	20% SDS	0.1ml
	1M DTT	0.1ml
	1M Tris (pH 6.8)	30µl
	Complete to 1ml with water	
Protein Sample Buffer	SDS/DTT	5µl
	Bromophenol Blue (0.2%)	5µl
	Glycerol	5µl
SDS/PAGE buffer	Glycine	28.8g
	Tris.HCl	6.05g
	SDS	2.0g
	up to 2l with dH_2O	
Western blot solution 1	0.3M Tris/20% methanol pH 10.4	
Western blot solution 2	25mM Tris/20% methanol pH 10.4	
Cathode solution	40mM 6-amino-n-hexanoic acid	
	20% methanol	
	pH 7.2	

3.8.4 Calcium flux studies

HACM buffer	NaCl	125mM
	KCl	5mM
	BSA	0.025% (w/v)
	$CaCl_2$ (optional)	1mM
	$MgCl_2$	1mM
	HEPES	20mM
	Water to appropriate volume	

CHAPTER 4

METHODS

CHAPTER 4 - Methods.

4.1 Mice

All mice were housed within the animal facility of the Beatson Institute. Female mice, aged between 8-12 weeks were used throughout all experimental procedures. For the *in vitro* CFU-A assay, femora bone marrow from strain B6D2F1 mice was used, whereas for the derivation of bone marrow macrophages, femur marrow from strain CD1 mice was used.

4.2 Cell Lines

All cell lines used in this study were obtained from the frozen stocks of the Beatson Institute. All cell lines were maintained at 37°C in a dry atmosphere of 5% CO_2 in air.

4.2.1 RAW 264.7

The murine monocyte-macrophage cell line RAW 264.7 (Ralph and Nakoinz 1977), derived from an Abelson murine leukaemia virus induced tumour was maintained in Special Liquid Medium (SLM) (Gibco-BRL, UK) containing 4mM glutamine and 10% foetal calf serum (FCS) in NUNC 75cm^2 tissue culture flasks. RAW cells are an adherent macrophage cell line. Upon reaching near confluence, the growth medium was removed using a pipette and the cells were rinsed in warm PBS. Approximately 90% of the PBS was discarded and the adherent cells were removed by gently scraping using a disposable sterile cell scraper (Costar). The cells were then thoroughly resuspended using an automatic pipette aid, and suspended in fresh medium at a concentration of 2x10^5/ml.

4.2.2 WEHI-3B

WEHI-3B cells, a partially adherent cell line derived from a monocytic leukaemia in a BALB/c mouse (Ralph and Nakoinz 1977), was maintained in SLM / 10% FCS / 4mM L-glutamine in NUNC 75cm^2 tissue culture flasks. Cells were sub-

cultured every 3 days, and suspended in fresh medium at a concentration of $2x10^5$/ml. These cells were used to generate WEHI conditioned medium for use during culture of FDCP-MIX cells (see section 4.2.3).

4.2.3 FDCP-MIX cells

The factor dependent murine stem cell line FDCP-MIX, derived from src-infected long term bone marrow cultures (Dexter *et al* 1977) was maintained in SLM + 4mM glutamine / 10%DHS / 10%WEHI conditioned medium as a source of interleukin 3 in NUNC $75cm^2$ tissue culture flasks. Such a source of IL-3 is essential to maintain these cells in a healthy undifferentiated state. FDCP-MIX cells grow in suspension and were subcultured every 2-3 days and resuspended in fresh medium at a concentration of $5x10^5$/ml.

4.2.4 U937 cells

The human monocytic cell line U937, derived from a histiocytic lymphoma, was maintained in RPMI 1640 + 4mM glutamine / 10%FCS in NUNC $75cm^2$ tissue culture flasks. U937 cells grow in suspension and were subcultured every 2-3 days and resuspended in fresh medium at a concentration of $2x10^5$/ml.

4.2.5 L929 cells

The murine fibroblast cell line L929 was maintained in SLM/10% FCS in NUNC $75cm^2$ tissue culture flasks. L929 cells are an adherent cell line, and were subcultured every 7 days. Firstly, growth medium was discarded, and cells washed in 10mls of PBS. Cells were then detached from tissue culture flask by incubating in 10 mls of 0.25% trypsin in PBS for 2-3 minutes at room temperature. Cells were then resuspended in fresh medium containing serum and quantitated using a Coulter counter, after which they were spun down at 1000 rpm for 10 minutes and resuspended in fresh medium at a concentration of $3x10^4$ cells/ml.

4.2.6 AF1-19T cells

Murine AF-1 cells were maintained in SLM/10% FCS in NUNC 75cm^2 tissue culture flasks. AF-1 cells are an adherent cell line, and were subcultured every 7 days. Firstly, growth medium was discarded, and cells washed in 10mls of PBS. Cells were then detached from tissue culture flask by incubating in 10 mls of 0.25% trypsin in PBS for 2-3 minutes at room temperature. Cells were then resuspended in PBS and quantitated using a Coulter counter, after which they were spun down at 1000 rpm for 10 minutes and resuspended in fresh medium at a concentration of $3x10^4$ cells/ml.

4.2.7 HT-2 cells

The murine helper T cell line was maintained in RPMI 1640 + 2mM glutamine / 10% FCS / 0.05mM mercaptoethanol / 200 U/ml IL-2 in NUNC 75cm^2 tissue culture flasks. These cells were subcultured every 2-3 days and resuspended in fresh medium at a concentration of $2x10^5$/ml.

4.3 Production of conditioned media

As a source of M-CSF for use in the CFU-A assay and for derivation of bone marrow macrophages, conditioned medium (CM) from the murine L929 cell line was prepared. As a source of GM-CSF for use in the CFU-A assay, CM from AF1-19T cells was prepared. As a source of Interleukin 3 for maintenance of FDCP-MIX cells, CM from WEHI-3B cells was prepared. All CM samples were prepared by growing each cell line in roller bottles in SLM/10%FCS to around 50% confluence and then removing spent medium. The medium was renewed and the cells grown for a further 3 days to generate conditioned medium. This medium was then removed, filter sterilised through a 0.42μm filter and then through a 0.2μm filter, aliquoted and stored at -20^{o}C.

4.4 Harvesting of bone marrow

Bone marrow was obtained from femora of mice. Mice were killed either by CO_2 asphyxiation or cervical dislocation. The femurs were then carefully removed using surgical scissors, and stripped of excess muscle using a tissue soaked in ethanol. Both ends of the femur were removed with scissors and the bone marrow plug expelled by flushing with 2mls of SLM through a 21 gauge needle. The cells were re-suspended thoroughly by vigorous pipetting using an automatic pipette aid. The cells were then washed in PBS and nucleated cells quantitated using a haemocytometer. Approximately $1.5x10^7$ cells were routinely obtained from each femur. Cells were then resuspended at the appropriate concentration and in the appropriate medium for their application (see below).

4.4.1 Derivation of bone marrow derived macrophages

Total bone marrow cells obtained as above were suspended at $5x10^4$/ml in α-modified MEM supplemented with 25% DHS and 20% L929 CM as a source of M-CSF. 15ml aliquots of this suspension were incubated in Nunc T75 flasks for 7 days in a dry atmosphere of 5% CO_2 in air. These gave rise to highly homogeneous (>95%) populations of macrophages as verified by staining for the macrophage specific enzyme alpha-napthyl acetate esterase (see table 4.4.1). Cells were used at 7 days, after which they quickly lose their adherence and die, as assessed by visual inspection.

4.4.2 Growth factor starvation of bone marrow derived macrophages

Quiescent bone marrow derived macrophages express MIP-1α at very low levels. In order to obtain a readily detectable level of MIP-1α expression, bone marrow macrophages were starved of L929 CM and thus M-CSF by removing medium from cells after 7 days and adding fresh α-modified MEM supplemented with 25% DHS. Cells were maintained for 16 hrs as such and then re-fed with fresh α-modified MEM supplemented with 25% DHS and 20% L929. This has previously been shown to rapidly induce MIP-1α mRNA expression to a peak at around 4 hrs

Time (days)	% positive for non specific (alpha napthyl acetate) esterase
4	82
5	96
6	98.5
7	99
8	100

Table 4.4.1 Staining of bone marrow derived macrophages with the macrophage specific stain non specific esterase (NSE). This service was carried out by the Haematology Laboratory at the Dept. of Veterinary Pathology, University of Glasgow. Results are shown as the percentage of cells positively stained with NSE, at days 4 to 8 after initiation of cultures.

post re-feeding (A. Reid. PhD thesis 1993). Expression remains at an elevated level for around 24 hours after which it returns to normal levels.

4.5 The *In vitro* CFU-A Assay

Bone marrow cells were obtained as described above. For the detection of primitive progenitor cells, CFU-A assays were carried out. $2x10^4$ total bone marrow cells in 4mls of supplemented α-modified MEM containing 25% DHS and 0.3% Bacto agar (Difco, USA) were seeded on top of an underlayer of the same medium containing 0.6% Bacto agar / 10% L929 CM / 10% AF1-19T CM in 45mm Petri dishes (Sterilin). Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO_2, 5% O_2 and 85% N_2 for 11 days. This semi solid agar culture gave rise to the formation of macroscopic colonies (>2mm in size), which histological analysis has previously revealed to consist of mature haemopoietic lineages (Pragnell *et al* 1988, Lorimore *et al* 1990). Although colonies smaller than 2mm in size do appear in the CFU-A assay, only colonies greater than 2mm were scored since it has been previously demonstrated that these larger colonies are derived from primitive haemopoietic cells. For all experimental procedures involving this assay, a "direct addition" protocol was adopted whereby putative inhibitory cytokines are assessed for their potential effects on CFU-A colony formation by directly adding various concentrations to the bottom layer of agar.

4.6 RNA and DNA Methodology

4.6.1 DNA cloning

The murine MIP-1α and MIP-1β cDNAs were amplified using EcoRI and XhoI tailed primers in a PCR reaction. The PCR products were then ligated into the pBluescript II^- plasmid (pSK^-). Both plasmids were constructed in the Beatson Institute.

The pcDNA-3 murine RANTES construct and the pcDNA-3 human C-C CKRI receptor construct were both kind gifts from Dr. Thomas Schall, DNAX Research Institute, USA.

The murine pSP65 TGF-β1 construct was a kind gift from Dr. R. Derynck, USA.

The murine pCRII glyceraldehyde phosphate dehydrogenase (GAPDH) construct was obtained from Max Walker at the Beatson Institute.

The 7S RNA specific probe is a 0.4kb Hinf I fragment of the plasmid pAT153 containing the 7S cDNA, and was obtained from Frances Fee at the Beatson Institute.

4.6.2 Propagation of plasmid DNA in bacterial host cells

Commercially available competent cells, DH5α, were transformed with the appropriate plasmid construct as follows. DH5α cells were removed from the -70°C freezer and thawed on ice. 20μl aliquots were dispensed into pre-chilled sterile 1.5ml Eppendorf tubes, then 1μl of plasmid construct (10ng/μl) was added and incubated on ice for 30 minutes. Cells were then heat shocked at 42°C for 40 seconds, returned to ice for approximately 2 minutes, and 80μl of S.O.C. medium (2% w/v bactotryptone, 0.5% w/v yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was added. Mixture was shaken at 225 rpm for 1 hour at 37°C to allow expression of the ampicillin resistance gene. The transformed cell mixture was spread evenly using a glass spreader sterilised by alcohol flaming onto L-amp plates (5mls of 1% w/v bactotryptone, 0.5% w/v yeast extract, 1% NaCl, 1.5% bactoagar) supplemented with 60μg/ml ampicillin. Plates were then inverted and incubated at 37°C overnight.

4.6.3 Growth of plasmids

5ml of L-βroth containing ampicillin (50μg/ml) was inoculated with cells from a single colony (from plates prepared in section 4.6.2) using a sterile loop. This was incubated at 37°C with shaking at 225 rpm overnight in a 20ml Universal container. 1ml of this was then added to 100mls of L-amp broth and incubated

under the above conditions overnight in a 1 litre conical flask. Suspensions were then transferred to 500ml centrifuge bottles (Sorvall instruments) and spun at 3000rpm for 30 minutes in a Beckmann J-6B centrifuge containing a GS-3 rotor. Plasmid preparation was begun immediately or pellets were frozen at -20°C until use.

4.6.4 Plasmid preparation

Plasmids were prepared using the commercially available QIAGEN plasmid kit (Qiagen Inc, USA) as follows. Bacterial pellets were resuspended in 4ml of resuspension buffer (100mg/ml RNase A, 50 mM Tris/HCl, 10mM EDTA, pH 8.0). The suspension was transferred to 50ml centrifuge tubes (Sorvall Instruments) and 4ml of lysis buffer (200mM NaOH, 1% SDS) was added, the solutions mixed by gentle inversion and incubated at room temperature for 5 minutes. 4ml of chilled neutralisation buffer (3M KAc, pH 5.5) was added, mixed and incubated on ice for 15 minutes. Tubes were then spun at 4°C for 30 minutes at 10000 rpm in a Sorvall RC-5B superspeed centrifuge containing SS-34 rotors. The supernatant was then poured through a double layered gauze swab to remove particulate material. To purify the DNA, a QUIAGEN-tip 100 column was equilibrated with 4ml of equilibration buffer (750mM NaCl, 50mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7). The filtered supernatant was then applied to the column, and the column washed with 2 x 10ml of wash buffer (1M NaCl, 50mM MOPS, 15% ethanol, pH 7). DNA was then eluted using 5ml of elution buffer (1.25M NaCl, 50mM Tris/HCl, 15% ethanol, pH 8.5). DNA was precipitated with 0.7 volumes of isopropanol and centrifuged at 10000 rpm at 4°C for 30 minutes as described above. The DNA pellet was washed with 5ml of cold 70% ethanol, air dried for 5 minutes, and redissolved in a suitable volume of sterile distilled water. The DNA concentration was then quantitated using spectrophotometry as follows. 5µl of DNA solution was added to 495µl of water and the optical density determined at wavelengths of 260nm and 280nm. An OD_{260} of 1 is equal to 50µg of plasmid or genomic DNA, thus allowing sample

concentrations to be estimated absolutely. Samples were stored in water at -20°C until use.

4.6.5 Restriction digests of plasmid DNA

For large scale digests, plasmid DNA was digested in a solution containing 50μg of DNA, 50μl of appropriate 10x React™ buffer, 10μl of appropriate enzyme and made up to 500μl using distilled water. Choice of React™ buffer was dependent on optimum efficiency conditions of each enzyme. Where two different enzymes were used, a compromise was reached where both enzymes would work in the same buffer at their highest possible efficiency. Digestion was then carried out for 2 hours at 37°C in 1.5ml Eppendorf tubes, after which DNA was precipitated by adding 0.1 volumes of 3M sodium acetate and 2.5 volumes of absolute ethanol. Tubes were left on dry ice for 10 minutes at then spun at 13000rpm for 10 minutes in a benchtop micro centaur centrifuge (MSE). Supernatant was carefully removed using a 1ml Gilson pipetman, the tube respun and residual supernatant removed leaving the DNA pellet intact. The pellet was washed x 2 in 70% ethanol and resuspended in 100μl of distilled water. The optical density of the DNA was measured using a spectrophotometer as described in the previous section. The DNA was then reprecipitated as described above and the DNA pellet dissolved in a volume of distilled water to give a stock solution of 1μg/μl.

4.6.6 Isolation of Total RNA

Total RNA was isolated using a commercially available phenol based extraction solution, RNAzol™. For RNA isolation from adherent cells, growth medium was removed and cells washed twice in PBS. For 75cm^2 flasks, 10mls of RNAzol was added and left for 5 minutes at room temperature. The flasks were then shaken and the solution pipetted several times to ensure complete lysis of cells. The solution was transferred to a 15ml polypropylene tube (Falcon 2059) and 1ml of chloroform added. Tubes were shaken vigorously for 15 seconds, placed on ice for 15 minutes to allow separation of aqueous and non-aqueous phases and spun at

10000 rpm for 15 minutes in a Sorvall centrifuge using HB4 or HB6 swing-out rotors. The top aqueous layer was removed to a fresh tube and an equal volume of isopropanol added. Tubes were then incubated at -20°C for 30 minutes to facilitate precipitation of RNA, after which they were spun at 10000 rpm for 15 minutes. Isopropanol was discarded and the RNA pellet was resuspended in 2mls of 75% ethanol and agitated to wash the RNA. Tubes were spun at 10000 rpm for 15 minutes and ethanol was removed. RNA pellets were then air dried to ensure complete absence of ethanol and RNA was resuspended in 105µl of sterile distilled H_20. RNA was quantitated by adding 5µl to 495µl of distilled H_20 and optical density assessed using a Beckman spectrophotometer. Readings were taken at wavelengths of 260nm and 280nm. The ratio between readings at 260nm and 280nm gives an indication of the purity of the RNA obtained with a ratio value of 1.8-2.0 being best. With the knowledge that an OD_{260} of 1 corresponds to approximately 40µg/ml in the case of RNA, then concentrations can be calculated for each sample. Samples were then stored, dissolved in water, at -20°C until use. The similar TRIzol RNA extraction reagent was sometimes used in place of RNazol. The methodology for extraction of RNA with TRIzol is very similar to that described above with the only difference being that none of the stages have to be incubated on ice or at -20°C. They were left at room temperature.

4.6.7 Agarose gel electrophoresis and Northern Blotting

For further analysis of RNA species, total RNA was visualised and separated in an agarose gel as follows. Approximately 20µg of total RNA was freeze-dried in a speedivac, and resuspended in 16µl of RNA sample loading buffer (see section 3.8.2) containing 1µl of ethidium bromide(10µg/ml stock). Samples were heated at 65°C for 5 minutes and placed immediately on ice to prevent renaturation of RNA strands. Samples were then resolved on a denaturing gel (1.4% (w/v) agarose, 1 x MOPS buffer, 2.2M formaldehyde and made up to a final volume of 200mls with distilled H_2O). Gels were run at a constant 80 volts for approximately 2-3 hours, after which RNA integrity was checked by visualisation of ethidium

bromide stained RNA bands on a UV transilluminator (312nm). The gel was then photographed using Polaroid Type 57 high speed film alongside a fluorescent ruler to enable subsequent sizing of RNA species. Excess agarose was trimmed from the gel, and the RNA transferred to Genescreen or Hybond-N^+ membrane overnight by capillary transfer in 10x SSC. Briefly, a rectangular piece of Whatman 3MM blotting paper was cut and placed on a raised platform in a glass tank. 10xSSC was then poured over the paper to partially fill the tank such that either end of the paper dipped into the SSC thus forming a paper wick. The agarose gel was then placed on top of the wet paper and layered with a prewetted Hybond-N+ membrane cut to the size of the gel and then 6 pieces of pre-cut Whatmann 3MM paper. The surrounding area of the paper wick was then covered using plastic sheets and 3 layers of paper towelling (Scott), each 6 paper towels thick, layered over the gel. RNA was fixed onto the membrane following transfer either by baking at 80°C for 2 hours or by UV linking on a UV Stratalinker 1800 (Stratagene). Efficient transfer was confirmed by viewing the agarose gel under UV light to ensure no RNA was visibly left on the gel. Membranes were wrapped in Saranwrap and stored at room temperature until use.

4.7 Preparation of radiolabelled probes

4.7.1 Uniform labelling of anti-sense RNA riboprobes

The plasmid, either pBluescript II SK (pSK$^-$ or pSK$^+$), containing the gene of interest was linearised using the appropriate restriction enzyme as described above. This was to prevent vector sequences being used as template by the enzyme (either T3 or SP6 polymerase) and thus increase the efficiency and specificity of the reaction. Probes were then generated as *in vitro* transcription products of the relevant gene coding sequence. Labelling was carried out using a commercially available SP6/T7 transcription kit (Boehringer Mannheim) according to manufacturers instructions yielding an [α-^{32}P]-UTP labelled antisense RNA transcript with a specific activity of approximately 10^8 cpm/μg.

4.7.2 Oligolabelling of DNA fragments

Denatured DNA restriction fragments (25-50ng/ml) were labelled for 15 minutes at 37°C with [α-^{32}P]-dCTP (3000 Ci/mmol) using a commercially available random priming kit, Ready-to-Go (Pharmacia) according to manufacturers instructions. This method involves mixing random oligodeoxyribonucleotides with the DNA to be labelled which anneal to random sites on the DNA. These then serve as primers for DNA synthesis by a DNA polymerase resulting in a labelled DNA fragment of high specific activity.

4.7.3 Purification of radiolabelled RNA and DNA probes

All radiolabelled probes were subsequently separated from unincorporated ^{32}P-labelled nucleotides by passing through a NICK column (Pharmacia), pre-equilibrated with 1 x TE, according to manufacturers instructions. DNA probes were denatured by boiling for 2-3 minutes, and placing on ice prior to use.

4.7.4 Hybridisation conditions for northern blots

Two methods of prehybridisation and hybridisation were utilised in this study.

1. RNA Riboprobe hybridisation: Membranes were incubated in approximately 10mls of 50% formamide prehybridisation solution (50% formamide, 5 x SSPE, 5 x Denhardts, 0.1% SDS, 0.2% Poly(A), 12.8% distilled H_20, and 0.1% salmon sperm DNA of a 10mg/ml stock solution) for 3 hours at 42°C or 65°C in a Hybaid Mini 10 hybridisation oven. After this time, prehybridisation buffer was removed and replaced with 10mls of fresh buffer containing the appropriate radiolabelled probe (see below). Membranes were then hybridised at 42°C or 65°C overnight after which the radioactive buffer was discarded and the membrane washed as follows. The membrane was washed initially in 2 x SSPE/0.1% SDS for 30 minutes at room temperature, after which the radioactivity still bound to the membrane was assessed using a series 900 radiation mini-monitor (Mini-Instruments Ltd., UK). If the background radiation was still high, then membrane

was washed for a further 30 minutes using 2 x SSPE/0.1% SDS at 65°C. If high background radiation was still detectable then membrane was washed more stringently in 0.2 x SSPE/0.1% SDS for 30 minutes at 65°C, or until background radiation was negligible. After washing, the membranes were wrapped in Saranwrap and transferred to an exposure cassette containing an intensifying screen. Membranes were exposed to Kodak X-Ray film (X OMAT-AR) at -70°C for approximately 4 hours in the first instance and then overnight if appropriate.

2. Pharmacia Ready to Go labelling kit hybridisation Prehybridisation and hybridisation was carried out essentially as above except pre and hybridisation fluid was 0.25M Na_2HPO_4 / 7% SDS, pH7 and pre and hybridisation was carried out at 65°C. Membranes were then washed x 2 in 20mM Na_2HPO_4 / 5% SDS, pH 7.2 for 30 minutes at room temperature and/or 65°C, and then x 2 in 20mM Na_2HPO_4 / 1% SDS, pH 7.2 for 30 minutes at 65°C.

4.7.5 Stripping of Northern blots

Blots were stripped of bound radiolabelled probe by boiling for 10 minutes in several hundred millilitres of 0.1% SDS. The efficiency of stripping was verified by exposing the membrane to X-ray film for 18hrs. Membranes were then used for further hybridisation protocols as described above.

4.7.6 Image analysis of Northern blots

Northern blots were scanned using a Molecular Dynamics laser densitometer, and data analysed on a Sun Workstation using Protein Databases Inc. Quantity One software. The assistance of Lynn McGarry is gratefully acknowledged.

4.8 Protein Detection

4.8.1 Preparation of RAW cell conditioned medium

RAW cells were grown in 15mls of SLM/10% FCS as described in section 2.1, and then several flasks treated with TGF-β at a concentration of 250pM. Medium was collected from flasks after 24, 48, 72 and 96hrs, and partially concentrated using a Centriprep 10 protein purification column by spinning 10mls of each sample at 10000rpm for 30 minutes in a benchtop centrifuge.

4.8.2 SDS Polyacrylamide Gel Electrophoresis

15% polyacrylamide gels were prepared using a commercially available acrylamide / bisacrylamide stock solution (30% w/v acrylamide / 0.8% w/v bisacrylamide) (Severn Biotech Ltd.) as follows:

Water	7.5ml
Acrylamide mix	12.5ml
1.5M Tris (pH 8.8)	6.3ml
10% SDS	0.25ml
10% ammonium persulphate	0.25ml
TEMED	0.01ml

This solution was mixed and poured between two minigel glass plates. Approximately 500µl of water saturated iso-butanol was layered on top of the solution to enable a smooth interface between stacking and resolving gels to be formed, and the gel left to set at room temperature. A 5% stacking gel was prepared essentially as above but with differing amounts of additives (6.8ml water, 1.7ml acrylamide mix, 1.25ml 1M Tris (pH 6.8), 0.1ml SDS (10% stock), 0.1ml ammonium persulphate (10% stock), 0.01ml TEMED). The water saturated butanol was poured off, and the top of the gel thoroughly washed using distilled water. Approximately 3-4mls of stacking gel solution was then poured between

the glass plates overlaying the solid gel and an appropriate comb was inserted to construct wells. This was then left at room temperature to set and the comb carefully removed. Samples were prepared for electrophoresis by adding 20µl of each sample to 15µl of gel loading buffer in 1.5ml Eppendorf tubes (5µl SDS/DTT (see section 3.8.3), 5µl bromophenol blue solution/ 5µl glycerol) and heated at 100°C for 5 minutes to denature and reduce the proteins. Samples were loaded onto the gel along with low molecular weight rainbow markers (Amersham) to enable sizing of discrete bands and to check transfer had occurred during Western blotting. Electrophoresis was carried out at room temperature in SDS-PAGE electrophoresis buffer (28.8g glycine, 6.05g tris.HCl, 2g of SDS and made up to 2 litres with dH2O) at 150V until the blue dye front almost reached the bottom of the gel.

4.8.3 Western blotting

MIP-1α content of cell conditioned media was then analysed by Western blotting. The contents of the gel were transferred to nitrocellulose membranes (Schleicher & Schuell) by semi-dry blotting using minigel electroblotting equipment (Biotech Instruments Ltd.) at a constant current of 8mA/cm^2 gel area for 30 minutes as follows. Briefly, many pieces of Whatman 3MM blotting paper were cut to match the dimensions of the polyacrylamide gel (usually 6cm x 9cm), as was a piece of nitrocellulose. Six pieces of blotting paper were soaked in western blot solution 1 (see section 3.8.3) and placed onto the electroblotter, followed by 3 pieces soaked in western blot solution 2 (see section 3.8.3). Any air bubbles were removed using a plastic pipette. The piece of nitrocellulose was soaked in western blot solution 2, and layered on top of the blotting papers, followed by the polyacrylamide gel, again removing away any air bubbles. Six pieces of blotting paper soaked in cathode solution (see section 3.8.3) were placed on top of the gel and the apparatus assembled. The electroblotter was then turned on and run at the above mentioned conditions. After blotting, the membrane was incubated in BLOTTO solution (5% Marvel dried milk in PBS, 0.1% NP40) overnight to block non-

specific binding sites, after which washing was continued for 30 minutes in several changes of BLOTTO. The membrane was then exposed to the primary polyclonal goat anti-MIP-1α antibody at the appropriate dilution (usually 1:1000) in 10mls of BLOTTO for 1 hour. This was discarded and the membrane washed in several changes of BLOTTO for 45 minutes. The membrane was then incubated with the secondary anti-goat IgG antibody conjugated to horse radish peroxidase in 10mls of BLOTTO for 1 hour after which this was discarded and the membrane washed in several changes of BLOTTO for 30 minutes and then in several changes of PBS/0.1% tween for 30 minutes. Antibody binding was then visualised using an enhanced chemiluminescence (ECL) kit (Amersham), which involved agitating the membrane in ECL solutions for 60 seconds. The membrane was then wrapped in Saranwrap clingfilm and exposed to X-OMAT AR X-ray film for the appropriate time to enable visualisation of protein bands.

4.8.4 TGF-β1 protein quantitation using ELISA

TGF-β1 protein content of bone marrow derived macrophage conditioned media was determined using a commercially available "sandwich technique" ELISA kit (R&D systems, UK) which detects both murine and human TGF-β1. This kit adopts an alternative protocol to traditional ELISA based methodology in that initial TGF-β binding to the plate is accomplished via TGF-β type II receptor adherent to the well (rather than an antibody) which recognises all TGF-β isoforms. The kit then specifically detects TGF-β1 via a polyclonal secondary antibody conjugated to horse radish peroxidase. As only active TGF-β is capable of binding to the type II receptor, this method necessitates acid activation of TGF-β present in conditioned media samples. 1ml of each BMDM conditioned media sample, previously concentrated using Centriprep 10 spin columns according to manufacturers instructions, was acid treated by adding 200µl of 1N HCl for 10 minutes and then neutralised by adding 200µl of 1.2N NaOH / 0.5M HEPES. As a control, a standard curve of pure TGF-β1 was prepared by diluting 500µl of a 2000pg/ml stock into 500µl of RD5I diluent (see manufacturers instructions).

Serial dilutions from 1000pg/ml to 31.25pg/ml were then carried out. Although human TGF-β1 was used as a control, the assay kit detects murine TGF-β1 with equivalent accuracy. 200µl of standards and samples were then loaded in duplicate into a 96 well plate, covered with a plastic film and incubated at room temperature for 3 hours. Plate contents were discarded and each well washed x 3 with 400µl of wash buffer. 200µl of anti-TGFβ1 conjugate was then added to each well and incubated at room temperature for a further 90 minutes. Each well was then washed x 3 with 400µl of wash buffer after which 200µl of substrate solution was added to each well. The plate was incubated at room temperature for 20 minutes to allow colour development and 50µl of stop solution was then added to each well. The optical density of each well was then determined using a Beckman DU 650 spectrophotometer set to 450nm. Readings at 570nm were also taken and subtracted from the 450nm readings to correct for optical imperfections in the polystyrene microtiter plate.

4.9 Receptor Binding Methodology

4.9.1 ^{125}I labelling of MIP-1α protein

MIP-1α protein was labelled using Iodo-gen (Pierce, U.S.A.), a commercially available iodination reagent which resembles a four-fold chloramine T molecule. 10µg of MIP-1α in PBS was incubated with 10µg of immobilised iodogen and 1mCi Na^{125}I (New England Nuclear) in Eppendorf tubes for 15 minutes on ice. All work involving ^{125}I was carried out behind a protective lead impregnated screen. Unincorporated iodine was then separated from labelled protein by applying the reaction mixture to a disposable desalt column (GF5 excellulose columns; Pierce) and eluting with PBS. 500µl fractions were collected and counts per minute assessed in a gamma counter to detect the peak of protein associated radioactivity. The three most active fractions were then pooled yielding a solution of MIP-1α protein labelled to high specific activity (2.5x10^7 cpm/mg). The integrity of the labelled protein was then checked by SDS-PAGE, and the proteins biological

activity checked in the previously described CFU-A assay. Iodination did not affect the biological potency of the protein in the CFU-A assay (Graham *et al* 1993). Labelled MIP-1α was then stored in polypropylene tubes at 4^{o}C and used within 1 month.

4.9.2 MIP-1α receptor binding studies on FDCP-MIX cells

FDCP-MIX cells were counted in a haemocytometer as previously described, spun down at 1000rpm and washed twice in PBS. Cells were then resuspended at 1x10^{6}/ml and 5x10^{5} cells per point were aliquoted into individual Eppendorf tubes. These were spun in a microcentrifuge at 13000rpm for 1 minute and PBS was carefully removed using a pipette. To each aliquot, varying concentrations of ^{125}I labelled MIP-α were added in binding buffer (SLM / 10% DHS / 0.2% azide) and either PBS or 100-fold excess unlabelled MIP-1α competitor up to a final volume of 250μl. Cells were then incubated in the above conditions at 37^{o}C for 90 minutes after which they were washed x 3 in PBS, and the incorporated radiation in the cell pellets assessed in a gamma counter (Beckman Gamma 5500B). To detect possible TGF-β effects on MIP-1α receptor levels on FDCP-MIX cells, cells were incubated at a concentration of 1x10^{6} cells/ml for 16 hours in the presence of varying concentrations of TGF-β (for details see figure legends) prior to receptor binding assay. Analysis of binding isotherms was then performed using the LIGAND computer program (Munson and Robard 1980).

4.9.3 Calcium flux studies in FDCP-MIX cells

FDCP-MIX cells were counted in a haemocytometer, spun down at 1000rpm and washed twice in PBS. Cells were then resuspended in calcium free HACM buffer (see materials) at a concentration of 1x10^{6}/ml. The fluorescent dye FURA-2 AM was added to the cell suspension at a concentration of 0.5μM, and the cells incubated at 37^{o}C for 45 minutes to allow the FURA-2 to permeate the cells. This phase was carried out in the dark since FURA-2 is light sensitive. The cells were then spun, washed twice in warm PBS and resuspended at a concentration of

$1x10^6$/ml in HACM buffer containing calcium. Cells were then incubated in the dark at 37°C for a further 30 minutes prior to use. 2mls of each cell suspension was aliquoted directly into a spectrophotometer cuvette, and the appropriate stimulants added directly to the cell suspension. Overall changes in the intensity of the FURA-2 were assessed using a Perkin Elmer spectrophotometer.

CHAPTER 5

RESULTS

CHAPTER 5 - Results

5.1 Overview

In order to investigate any potential interactions between the negative regulators of stem cell proliferation, it was necessary to firstly find a suitable *in vitro* assay system which was responsive to each individual molecule. Using such a system, any potential additive, synergistic or antagonistic effects between them should be easily determined. The *in vitro* CFU-A assay, which as described in section 1.2.4 detects a primitive multipotent haemopoietic cell, is in routine use in our laboratory and thus represented an attractive possibility.

5.1.1 Effect of MIP-1α on CFU-A colony formation

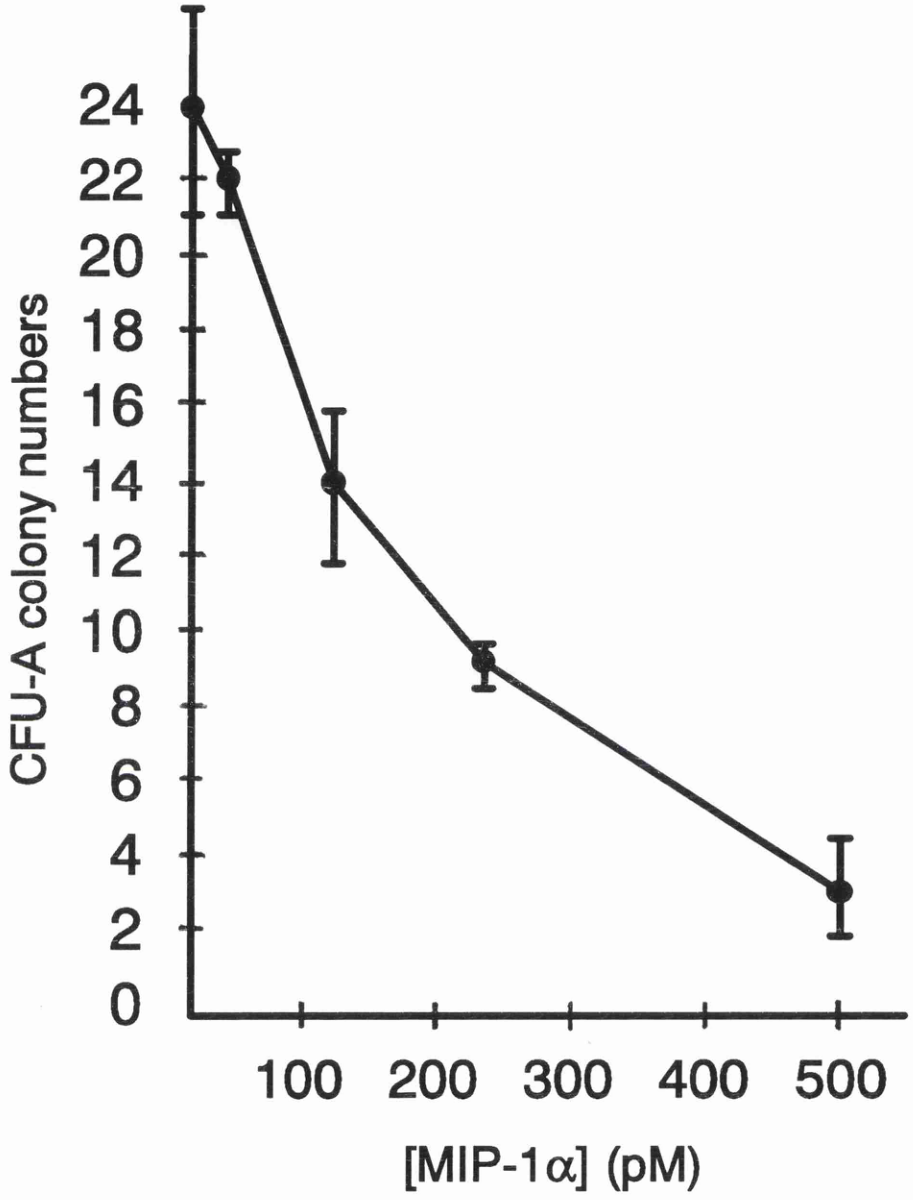
The inhibitory effects of MIP-1α on CFU-A colony formation have been previously reported (Graham *et al* 1990). However, in order to standardise the methodology for analysis of the other potential inhibitors, the effect and potency of MIP-1α in this assay was investigated. This assay was performed using a titration of MIP-1α concentrations up to 500pM. The results, representative of 3 separate experiments are shown in figure 5.1. Control plates contained an average of 24 colonies in this assay. These results show that MIP-1α is a potent inhibitor of CFU-A colony formation, with an ED50 of approximately 100pM. This is in agreement with the previous findings of Graham *et al* (1990).

5.1.2 Effect of TGF-β1 on CFU-A colony formation

The inhibitory effects of TGF-β1 on haemopoietic cells have been previously reported (see introduction). As TGF-β1 has been shown to inhibit CFU-S type stem cells, it was expected to exhibit an effect in the CFU-A assay, an *in vitro* correlate of the CFU-S assay. Control plates contained an average of 21 colonies in this assay. Figure 5.1.2 shows that direct addition of a titration of TGF-β1 concentrations (up to 500pM) resulted in dose dependent inhibition of CFU-A colony formation analogous to that shown by MIP-1α. Both of these

Figure 5.1.1 Effect of Macrophage Inflammatory Protein 1 alpha (MIP-1α) on CFU-A colony formation.

MIP-1α was tested for inhibitory activity in the direct addition CFU-A assay as described in section 4.5. MIP-1α was added directly to the feeder layers (0.6% agar in α-MEM with sources of M-CSF and GM-CSF) of the CFU-A assays, and the target cells were normal bone marrow cells plated at $5x10^3$/ml in the upper layer (0.3% agar in α-MEM). These assays were carried out in 45mm diameter Petri dishes. The assay plates were incubated at 37°C in a fully humidified atmosphere of 10% CO_2, 5% O_2, and 85% N_2 for 11 days. Colonies >2mm were scored as being CFU-A type. The results represent the effect of increasing cytokine concentrations on CFU-A colony formation and are depicted as mean values +/- standard deviation. The results depicted are representative of at least 3 separate experiments.


CFU-A colony numbers
24
22
20
18
16
14
12
10
8
6
4
2
0
100
200
300
400
500
[MIP-1α] (pM)

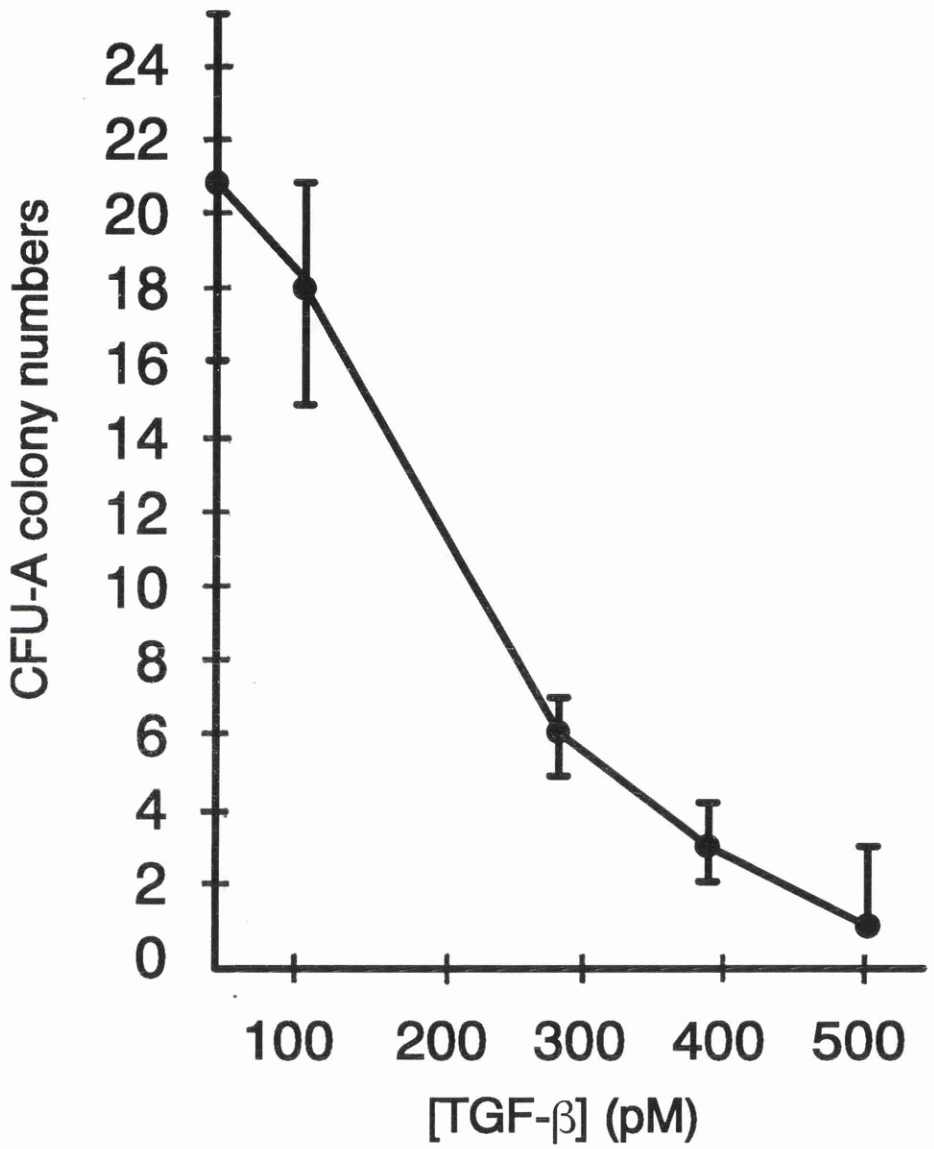


Figure 5.1.2 Effect of Transforming Growth Factor beta (TGF-β) on CFU-A colony formation

CFU-A assays were performed as described in section 4.5. The results represent the effect of increasing cytokine concentrations on CFU-A colony formation and are shown as mean values +/- standard deviation. The results depicted are representative of at least 3 separate experiments.

molecules are active in the picomolar range, again emphasising their apparent functional similarities on haemopoietic stem cells.

5.1.3 Effect of tetrapeptide on CFU-A colony formation

As is described in the introduction, the inhibitory effects of the tetrapeptide on haemopoietic stem cells appear to be limited to cells falling within the CFU-S region of the stem cell compartment. Furthermore, the tetrapeptide appears to be unable to actively render cycling cells quiescent, and appears to act by holding already quiescent cells in that state. It was anticipated however that the tetrapeptide may still exhibit an effect in the CFU-A assay since the cells detected by this *in vitro* assay are very similar phenotypically to CFU-S cells, and also, a high proportion (approximately 90%) are quiescent at the onset of the assay. However, no inhibitory effect could be routinely obtained using the tetrapeptide in a direct addition CFU-A assay. Control plates contained an average of 18 colonies in this assay. Figure 5.1.3 represents the mean of three separate assays, and shows that no inhibition could be obtained.

5.1.4 Effect of haemoregulatory pentapeptide (pEEDCK) on CFU-A colony formation

Like the other inhibitors mentioned above, pEEDCK has previously been shown to exert an inhibitory effect of primitive haemopoietic cells including CFU-S (Paukovits *et al* 1990). However, as figure 5.1.4 shows, no inhibition of CFU-A colony formation was observed using pEEDCK over a wide range of concentrations. Control plates contained an average of 14 colonies in this assay

5.1.5 Summary

These results demonstrate that of the four inhibitors of haemopoietic stem cell proliferation analysed in this study, only MIP-1α and TGF-β1 appear to be potently active in inhibiting CFU-A colony formation. Indeed, the similar results using these two molecules in this regard confirms the apparent overlap in actions of

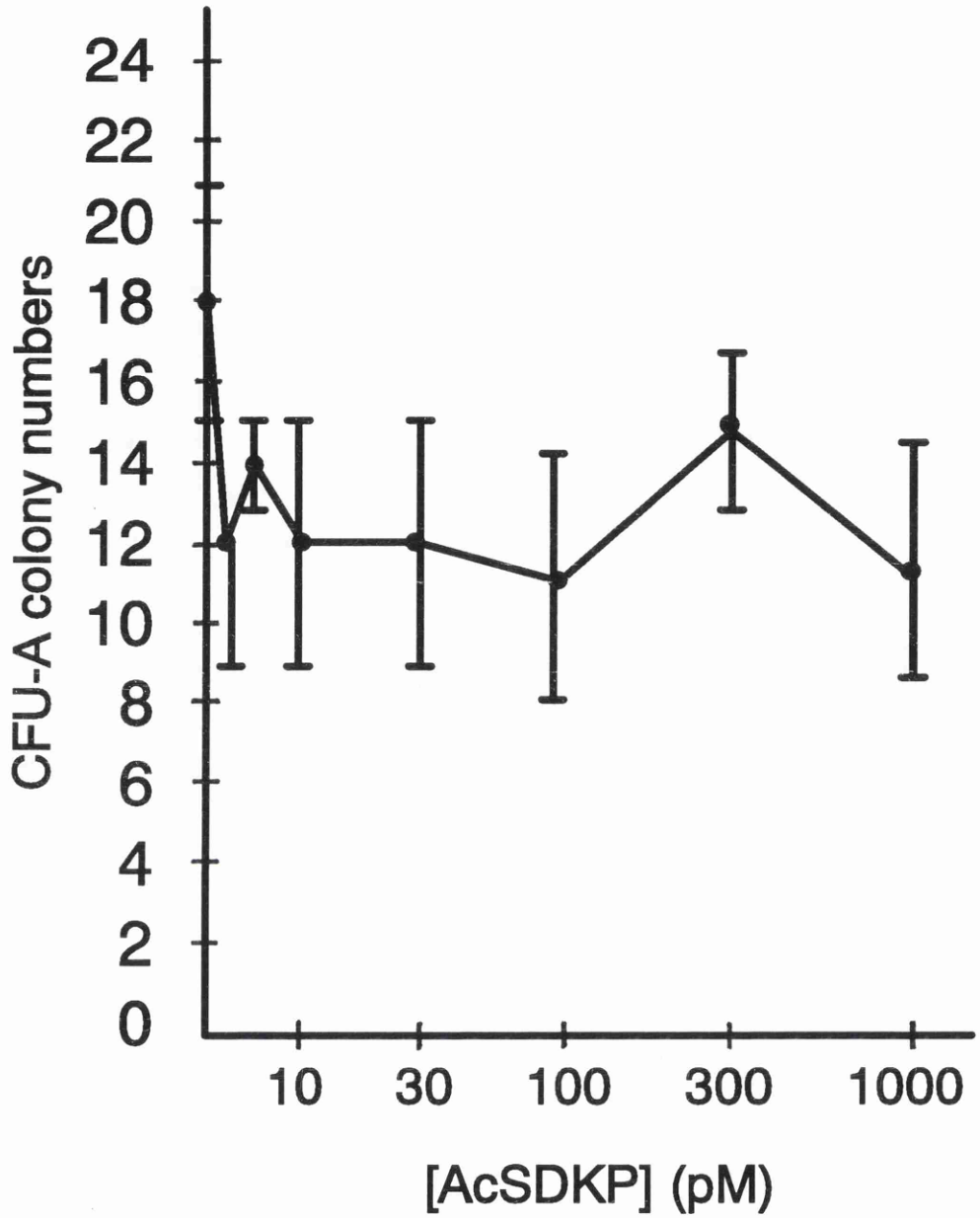


Figure 5.1.3 Effect of the Tetrapeptide (AcSDKP) on CFU-A colony formation

The tetrapeptide (AcSDKP) was tested for inhibitory activity in the direct addition CFU-A assay as described in section 4.5. The results represent the effect of increasing tetrapeptide concentrations on CFU-A colony formation and are shown as mean values +/- standard deviation The results depicted are representative of at least 3 separate experiments.

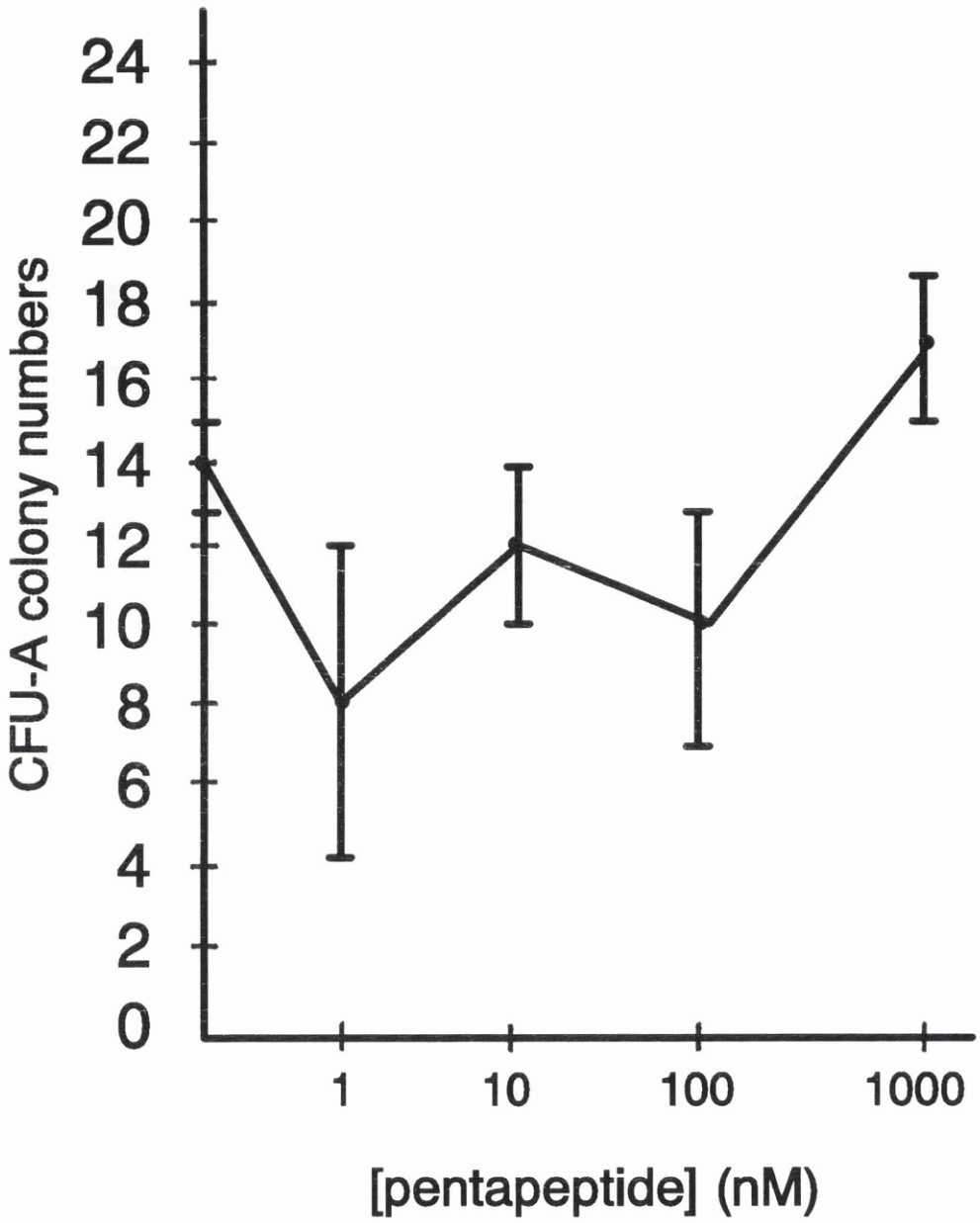


Figure 5.1.4 Effect of the pentapeptide (pEEDCK) on CFU-A colony formation

The pentapeptide (pEEDCK) was tested for inhibitory activity in the direct addition CFU-A assay as described in section 4.5. The results represent the effect of increasing pentapeptide concentrations on CFU-A colony formation and are shown as mean values +/- standard deviation. The results depicted are representative of at least 3 separate experiments.

these cytokines on this cellular population. One complicating feature of these studies is that the *in vitro* CFU-A assay, like normal bone marrow, consists of a multicellular environment, and it is possible that one or other of these two molecules may be acting indirectly by inducing expression of the other in non-target cells such as macrophages which are known to produce both cytokines. To investigate this possibility in more detail, it was decided to firstly address the possibility that TGF-β1 may act through possible inductive effects on MIP-1α expression in normal bone marrow derived macrophages, a potential source of MIP-1α in the bone marrow.

5.2 Effect of TGF-β1 on MIP-1α expression in murine bone marrow derived macrophages

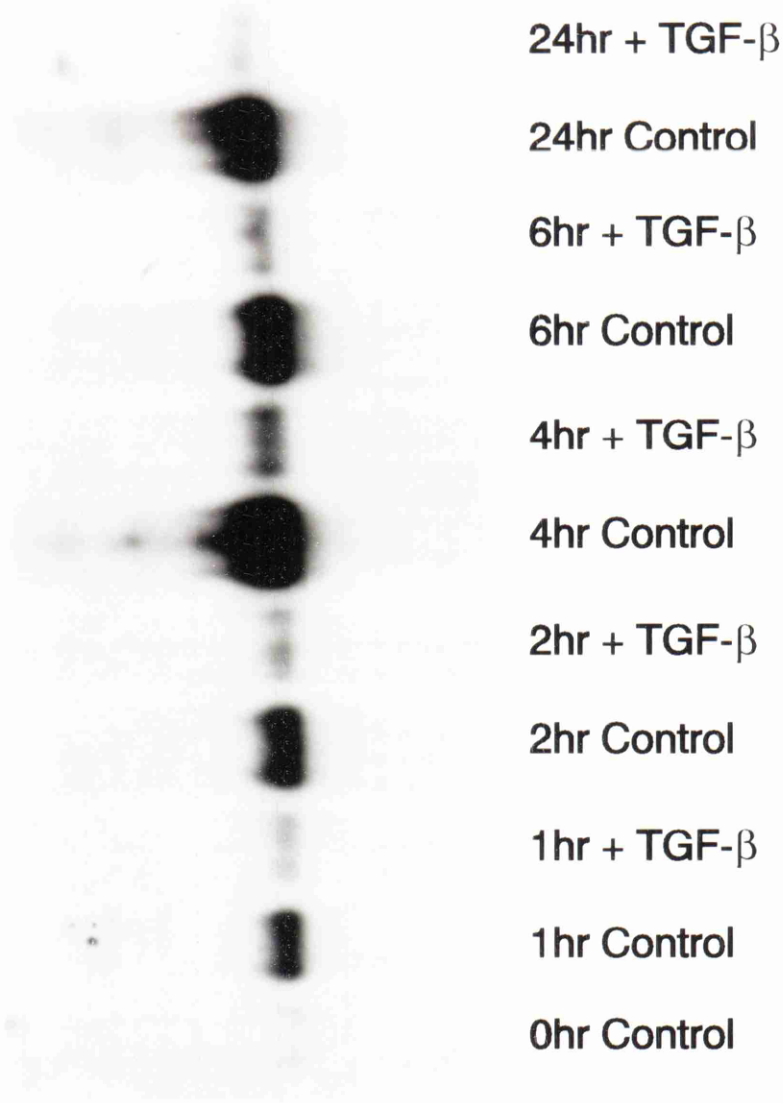
5.2.1 Inhibition of MIP-1α gene expression induction by TGF-β1 in murine bone marrow derived macrophages

It has been shown previously that murine bone marrow derived macrophages starved of the growth factor M-CSF for 16hrs enter growth arrest (Tushinski and Stanley 1985). Upon refeeding with M-CSF, MIP-1α mRNA expression, as detected by Northern blot analysis, is rapidly induced to an optimum at around 4 hrs. Given the very low level of MIP-1α mRNA expression observed in cultured macrophages, it was decided to use this technique of MIP-1α mRNA expression induction to obtain a readily detectable level of expression. Any possible effect, either inhibitory or stimulatory, of TGF-β1 on MIP-1α mRNA expression could then be more easily determined.

The effect of TGF-β1 on the induction of MIP-1α mRNA expression was investigated. Figure 5.2.1 shows a time course of MIP-1α mRNA expression in cells after refeeding with M-CSF, with or without TGF-β1 (250pM) as measured by Northern blot analysis. As previously demonstrated, in the absence of TGF-β1, MIP-1α expression peaks approximately 4 hrs after feeding, and remains elevated. In the presence of TGF-β1 however, the induction of MIP-1α expression is

Figure 5.2.1 Northern blot analysis of the effect of TGF-β1 on MIP-1α expression in bone marrow derived macrophages induced by M-CSF.

Bone marrow derived macrophages were starved for 16hrs of the growth factor M-CSF, and refed with fresh medium containing M-CSF at time 0 plus or minus TGF-β1 at a concentration of 250pM. Total RNA was prepared 0, 1, 2, 4, 6 and 24hrs following addition of M-CSF plus or minus TGF-β1, and 20μg of total RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred by Northern blotting, and the membrane probed using a radiolabelled MIP-1α specific riboprobe. The autoradiograph was exposed for 18hrs. Details of each individual lane are shown in the figure. This blot is representative of at least three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining and reprobing for GAPDH.


24hr + TGF-β
24hr Control
6hr + TGF-β
6hr Control
4hr + TGF-β
4hr Control
2hr + TGF-β
2hr Control
1hr + TGF-β
1hr Control
0hr Control

markedly inhibited throughout all the time-points investigated. Even after 24 hrs, densitometric analysis shows that expression of MIP-1α mRNA in TGF-β1 treated cells remains inhibited by approximately 90% compared with the control cells. Ethidium bromide staining and reprobing of these blots for the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) revealed similar loading of RNA in all tracks (data not shown). This demonstrates that the TGF-β1 mediated inhibition of MIP-1α mRNA expression is not simply due to TGF-β1 induced cell death since expression of house-keeping genes is unaffected. Further, since ethidium bromide staining and GAPDH probing revealed similar loading of RNA, ethidium bromide staining was routinely used to standardise RNA loading in subsequent experiments.

5.2.2 Titration of the inhibitory effect of TGF-β1 on MIP-1α mRNA expression

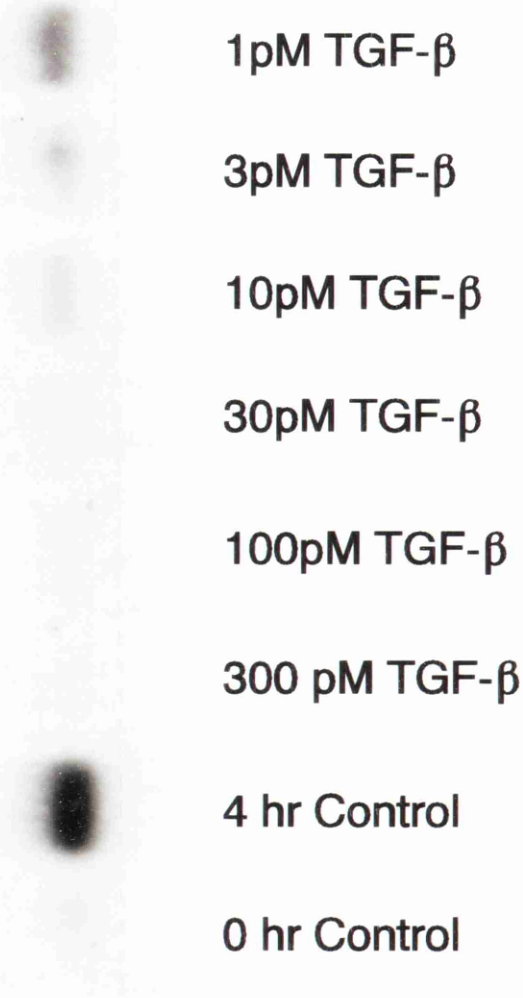
To assess the potency of the inhibitory effect of TGF-β1 on MIP-1α mRNA expression in bone marrow macrophages, a titration of TGF-β1 (1pM to 300pM) was used in the same experimental protocol. Cells were studied at 4 hrs after feeding as this leads to the optimum induction of MIP-1α expression, therefore allowing the extent of inhibition by TGF-β1 to be easily measured. Figure 5.2.2 shows MIP-1α mRNA expression to be inhibited in a dose dependent manner, and even using concentrations of TGF-β1 as low as 1pM, densitometric analysis shows that MIP-1α mRNA expression is reduced by approximately 70% compared with control values. This data indicates TGF-β1 to be remarkably potent with respect to inhibition of MIP-1α mRNA expression in macrophages, and indeed suggests that TGF-β1 is capable of exerting this effect at femtomolar concentrations.

5.2.3 Inhibition of MIP-1α gene expression by TGF-β1 in RAW 264.7 cells

Although it has been demonstrated that TGF-β1 is a potent inhibitor of MIP-1α expression in bone marrow derived macrophages, it is of more relevance

Figure 5.2.2 Northern blot analysis of the effect of a titration of TGF-β1 on MIP-1α expression in BMDM induced by M-CSF.

BMDM were starved overnight of the growth factor M-CSF, and refed with fresh medium containing M-CSF at time 0 plus or minus TGF-β1 at the concentrations shown. Total RNA was prepared 0 and 4hrs after cytokine addition, and 20μg of total RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred by Northern blotting, and the membrane probed using a radiolabelled MIP-1α specific riboprobe. The autoradiograph was exposed for 18 hrs. The blot is representative of at least three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining.


1pM TGF-β
3pM TGF-β
10pM TGF-β
30pM TGF-β
100pM TGF-β
300 pM TGF-β
4 hr Control
0 hr Control

when considering potential functional consequences to ascertain if this inhibition is reflected at the protein level also. It was discovered throughout the course of this work that bone marrow derived macrophages express very low and often undetectable levels of MIP-1α protein as assessed by Western blot analysis. It would therefore be very difficult to investigate if TGF-β1 inhibited MIP-1α protein production in these cells. Thus, it was decided to look for similar inhibitory effects of TGF-β1 on MIP-1α in a murine macrophage cell line which is capable of producing readily detectable levels of MIP-1α protein. RAW 264.7 cells are such a cell line and as with the BMDM, it was desirable to obtain an increased and easily detectable level of MIP-1α expression in these cells also. Since they are a transformed cell line however, they are growth factor independent, and it was decided to induce MIP-1α expression by starving the cells of FCS for 16 hrs and then refeeding with fresh FCS. As shown in figure 5.2.3, TGF-β1 is also active as an inhibitor of MIP-1α mRNA expression in these cells. This Northern blot also demonstrates that the TGF-β1 diluent (HCl/BSA) does not potently inhibit MIP-1α mRNA expression in these cells. Although it appears that MIP-1α expression is slightly inhibited by HCl-BSA in this experiment, this was not the case routinely and represents an aberrant point. Addition of equal volumes (equivalent to the highest volume of TGF-β used) of HCl/BSA was routinely added to all control cells.

5.2.4 Inhibitory effect of TGF-β1 on MIP-1α protein production by RAW 264.7 cells

Figure 5.2.4 shows an analysis of conditioned medium from control and TGF-β1 treated RAW cells for presence of MIP-1α protein using Western blotting. Conditioned medium samples were collected later than total RNA due to the potentially longer time required for changes in mRNA to be reflected at the protein level. It was anticipated that any inhibitory effect of TGF-β1 on MIP-1α protein synthesis would be apparent around 24hrs after treatment with TGF-β1. The results indicate that in the presence of TGF-β1, MIP-1α protein levels are

Figure 5.2.3 Northern blot analysis of the effect of TGF-β1 on MIP-1α expression in RAW 264.7 cells

RAW 264.7 cells were starved of foetal calf serum (FCS) overnight, and refed with fresh medium containing FCS at time 0 plus or minus TGF-β at a concentration of 250pM. HCl/BSA, the diluent for TGF-β was included as a control. Total RNA was prepared 0 and 4hrs after cytokine addition, and 20μg of total RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred by Northern blotting, and the membrane probed using a radiolabelled MIP-1α specific riboprobe. The autoradiograph was exposed for 18 hrs. The blot is representative of at least three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining.

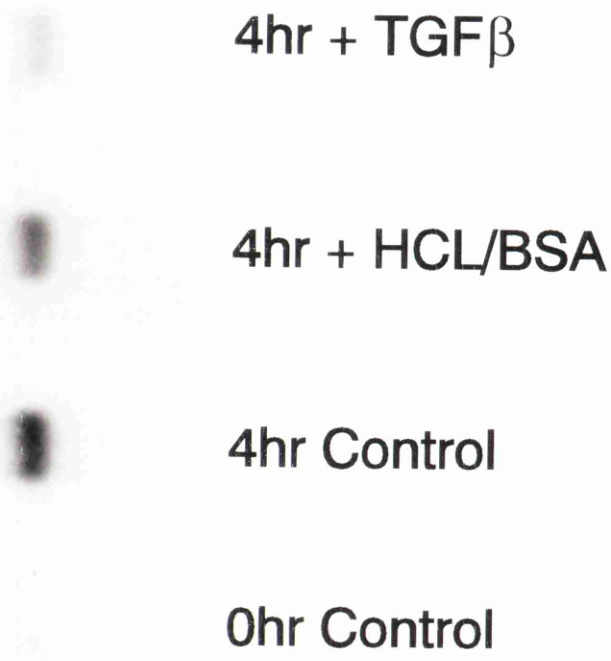

4hr + TGFβ
4hr + HCL/BSA
4hr Control
0hr Control

Figure 5.2.4 Western blot analysis of the effect of TGF-β1 on MIP-1α protein production by RAW264.7 cells

RAW 264.7 cells were starved overnight of foetal calf serum (FCS), and refed with fresh medium containing FCS at time 0 plus or minus TGF-β1 at a concentration of 250pM. Medium was harvested after 24, 48, 72 and 96hrs. MIP-1α protein presence in this harvested medium was determined by Western blotting using a commercially available polyclonal antibody to MIP-1α (R&D Systems) at a concentration of 1:1000. Lane details are shown opposite.

96hr + TGF-β

96hr Control

72hr + TGF-β

72hr Control

48hr + TGF-β

48hr Control

24hr + TGF-β

24hr Control

substantially reduced, and that this effect is evident after 24 hrs. Further, this inhibition was maintained over the 96 hr time course of this experiment. These results suggest that the inhibition of MIP-1α mRNA expression by TGF-β1 is also observed as an inhibition of protein synthesis and that the potent blocking of MIP-1α production by TGF-β1 may have important physiological and functional implications.

5.3 Effect of TGF-β1 on mRNA expression of MIP-1α family members

5.3.1 Inhibitory effect of TGF-β1 on MIP-1β mRNA expression in bone marrow derived macrophages

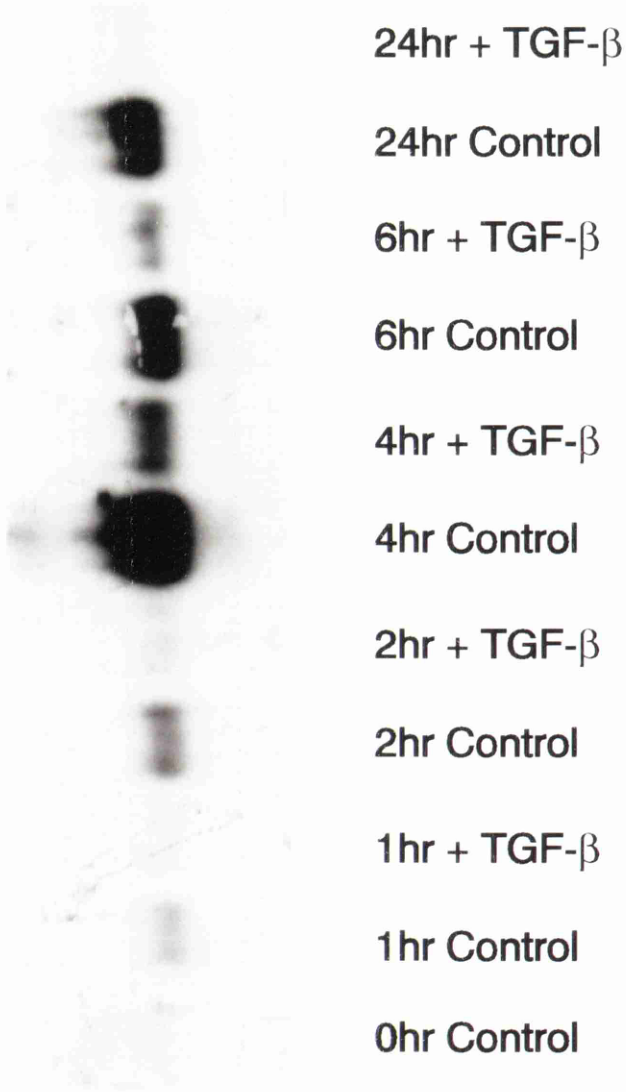
As was mentioned in the introduction, MIP-1α belongs to a large family of related molecules, some of which are capable of exerting stem cell inhibitory properties, and some which are not. To determine whether the TGF-β1 inhibition of MIP-1α expression was a specific phenomenon, the effect of TGF-β1 on MIP-1β mRNA expression in bone marrow derived macrophages was investigated. MIP-1β is a related molecule to MIP-1α which exhibits weak stem cell inhibitory properties. The blot shown in figure 5.2.1 was stripped of MIP-1α specific probe as described in section 4.7.5. The results (figure 5.3.1) show firstly that MIP-1β mRNA expression mirrors MIP-1α expression upon refeeding of the cells with M-CSF. Further, TGF-β1 can also inhibit MIP-1β mRNA expression, and therefore it appears that TGF-β1 is not specific in inhibiting MIP-1α gene expression in bone marrow derived macrophages.

5.3.2 TGF-β1 inhibits LPS stimulated MIP-1α expression induction but does not affect LPS stimulated RANTES mRNA expression

To further investigate the specificity of TGF-β1 in inhibiting MIP-1α and MIP-1β mRNA expression, the effect of TGF-β1 on more diverse members of the chemokine family which display no stem cell inhibitory activity was investigated. RANTES, like MIP-1α and MIP-1β, is a member of the C-C branch of the

Figure 5.3.1 Northern blot analysis of the effect of TGF-β1 on MIP-1β expression in BMDM induced by M-CSF

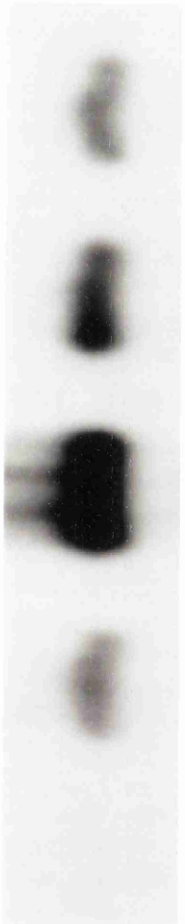
The membrane shown in figure 5.2.1 was stripped of MIP-1α specific riboprobe by boiling for 10 minutes in several hundred millilitres of 0.1% SDS, and the membrane probed using a radiolabelled MIP-1β specific riboprobe. The autoradiograph was exposed for 18hrs. Details of each individual lane are shown in the figure. This blot is representative of at least three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining.


24hr + TGF-β
24hr Control
6hr + TGF-β
6hr Control
4hr + TGF-β
4hr Control
2hr + TGF-β
2hr Control
1hr + TGF-β
1hr Control
0hr Control

chemokine family whereas IL-8 is a member of the C-X-C branch. Both of these molecules appear to have important roles in the immune system, and inflammation in particular, with no detectable stem cell inhibitory properties in the CFU-A assay. It is possible, and indeed likely, that the downregulation of MIP-1α and MIP-1β by TGF-β1 is a result of the ability of TGF-β1 to act as a general anti-inflammatory molecule. Indeed, TGF-β1 is a potent de-activator of macrophages, and it is possible that the observed downregulation falls into this category of cellular control. It is interesting to note in this context that TGF-β1 is also active in inhibiting induction of MIP-1α mRNA after treatment of bone marrow derived macrophages with LPS, a bacterial mitogen. Figure 5.3.2 shows that MIP-1α induction by LPS in these cells is inhibited by TGF-β1 in a dose dependent manner. This lends further credence to the argument that the TGF-β1 mediated inhibition of MIP-1α expression is part of the inflammatory cytokine network (see discussion). Such a conclusion would predict that other pro-inflammatory molecules belonging to the chemokine family might also be downregulated by TGF-β1. To this end, the effect of TGF-β1 on RANTES expression in the murine T cell line HT-2 was investigated. Figure 5.3.3 shows however, that TGF-β1 does not affect, either positively or negatively, the expression of RANTES in these cells. Interestingly, expression of RANTES does not appear to be induced by LPS in these cells, and this is in agreement with results of Xing *et al* (1994) who demonstrate that RANTES is not induced by endotoxin in neutrophils and macrophages *in vivo*. HT-2 cells have previously been shown to be able to respond to TGF-β and thus they possess functional TGF-β receptors (Taipale *et al* 1994, Chao *et al* 1992). Therefore, the fact that TGF-β1 does not affect expression of RANTES is not simply due to the cells being unable to respond to TGF-β1. Interestingly, Mauviel *et al* (1993) have also demonstrated that TGF-β1 and TGF-β2 do not alter cytokine induced IL-8 expression in human adult skin fibroblasts. IL-8 is another member of the pro-inflammatory chemokine superfamily, and it thus appears that TGF-β1 appears to specifically inhibit expression of the stem cell inhibitory molecules MIP-1α and MIP-1β, but not other more diverse members of the

Figure 5.3.2 Northern blot analysis of the effect of TGF-β1 on MIP-1α expression in BMDM induced by LPS

BMDM were treated with LPS (20ng/ml) plus or minus TGF-β at the concentrations shown opposite. Total RNA was prepared 0 and 4 hrs after additions, and 20mg of RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred by Northern blotting, and the membrane probed using a radiolabelled MIP-1α specific riboprobe. The autoradiograph was exposed for 18hrs. The blot is representative of three separate experiments. Equal loading of RNA was confirmed by ethidium bromide staining.



4 hrs + LPS + 1nM TGF-β

4 hr + LPS + 250pM TGF-β

4 hr + LPS

4 hr Control

0 hr Control

Figure 5.3.3 Northern blot analysis of the effect of TGF-β1 on RANTES expression in HT-2 cells

HT-2 cells were sub-cultured and then grown for 2 days, after which TGF-β1 was added at a concentration of 250pM. Total RNA was prepared 0 and 4hrs after cytokine addition and, 20μg of total RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred by Northern blotting, and the membrane probed using a radiolabelled RANTES specific DNA probe. The autoradiograph was exposed for 24hrs. Details of each individual lane are shown in the figure. This blot is representative of at least three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining.

4 hrs + LPS + TGF-β

4 hrs + LPS

4 hr Control

0 hr Control

chemokine family. It should be noted here that since TGF-β1 is also capable of potently inhibiting MIP-1α expression induced by LPS, this method of MIP-1α induction was used henceforth rather than starving the cells of M-CSF for 16 hrs. This method represented an easier and less time consuming alternative.

5.4 Effect of TGF-β family members on MIP-1α expression in BMDM: TGF-β2 and TGF-β3 inhibit MIP-1α mRNA expression in bone marrow derived macrophages, but activin and BMP-2 do not

As was discussed in the introduction, TGF-β1 belongs to a large family of related molecules, some of which, its isoforms in particular, are capable of exerting similar if not identical effects to the prototype TGF-β1, where the differences may be quantitative rather than qualitative. To more fully understand the interactions between MIP-1α and TGF-β, it was therefore important to investigate whether or not other TGF-β isoforms could also inhibit MIP-1α mRNA expression in bone marrow derived macrophages. Figures 5.4.1 and 5.4.2 show total RNA made from control BMDM and BMDM treated with LPS (20ng/ml) with or without a titration of TGF-β2 and TGF-β3 respectively. The results demonstrate that both TGF-β2 and TGF-β3 are also capable of inhibiting MIP-1α mRNA expression induction in a manner analogous to that of TGF-β1. It appears from the data however, that TGF-β2 is slightly weaker in this regard than either TGF-β1 or TGF-β3. Although the Northern shown indicates that TGF-β3 appears more potent than TGF-β1, other similar experiments have suggested that TGF-β1 and TGF-β3 appear equipotent. This observation of differing potencies is of interest, and may be due to the differing affinities with which each of these isoforms binds the TGF-β receptors (see discussion).

As with MIP-1α, the TGF-β family contains many other members which have no reported role in inhibition of haemopoietic stem cells such as activin and bone morphogenetic protein 2 (BMP-2). To investigate the specificity of TGF-β1, -β2 and -β3 isoforms in inhibition of MIP-1α mRNA expression, similar experiments to those described above were performed using activin and BMP-2.

Figure 5.4.1 Northern blot analysis of the effect of TGF-β2 on MIP-1α expression in BMDM induced by LPS

Bone marrow derived macrophages were treated with LPS at a concentration of 20μg/ml plus or minus TGF-β2 at the concentrations shown in each lane. Total RNA was prepared 0 and 4hrs after cytokine addition, and 20μg of total RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred via Northern blotting, and the membrane probed using a radiolabelled MIP-1α specific riboprobe. The autoradiograph was exposed for 18hrs. Details of each individual lane are shown in the figure. This blot is representative of at least three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining.

4 hrs + LPS + 100pM TGF-β1

4 hrs + LPS + 1nM TGF-β2

4 hrs + LPS + 300pM TGF-β2

4 hrs + LPS + 100pM TGF-β2

4 hrs + LPS + 30pM TGF-β2

4 hrs + LPS + 10pM TGF-β2

4 hrs + LPS + 3pM TGF-β2

4 hr + LPS

4 hr Control

0 hr Control

Figure 5.4.2 Northern blot analysis of the effect of TGF-β3 on MIP-1α expression in BMDM induced by LPS

Bone marrow derived macrophages were treated with LPS at a concentration of 20μg/ml plus or minus TGF-β3 at the concentrations shown in each lane. Total RNA was prepared 4hrs after cytokine addition, and 20μg of total RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred by Northern blotting, and the membrane probed using a radiolabelled MIP-1α specific riboprobe. The autoradiograph was exposed for 18hrs. Details of each individual lane are shown in the figure. This blot is representative of at least three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining.

4 hrs + LPS + 300pM TGF-β1

4 hrs + LPS + 1nM TGF-β3

4 hrs + LPS + 300pM TGF-β3

4 hrs + LPS + 100pM TGF-β3

4 hrs + LPS + 30pM TGF-β3

4 hrs + LPS + 10pM TGF-β3

4 hrs + LPS + 3pM TGF-β3

4 hr + LPS

0 hr Control

Figure 5.4.3 shows MIP-1α expression in total RNA made from BMDM stimulated with LPS plus or minus a titration of activin ranging from 10pM to 3nM. The last track shows the inhibitory effect of TGF-β3 on MIP-1α mRNA expression, and was included as a positive control. This result demonstrates that, at low concentrations where the other TGF-β isoforms have been shown to be active, activin appears to have little if any inhibitory effect on MIP-1α mRNA expression in BMDM. It does appear however, that at a concentration of 3nM, activin is capable of inhibiting MIP-1α expression. Ethidium bromide staining of RNA indicated equal loading in each track.

Figure 5.4.4 shows total MIP-1α expression in total RNA made from BMDM stimulated with LPS plus or minus a titration of BMP-2 ranging from 3pM to 1nM. The last track shows the inhibitory effect of TGF-β3 on MIP-1α mRNA expression, and was included as a positive control. This figure shows that BMP-2 appears to have no effect on the induction of expression of MIP-1α mRNA by LPS using concentrations up to 1nM.

In summary, it appears that only the TGF-β isoforms are capable of potently inhibiting the expression of MIP-1α mRNA in BMDM, with more diverse members of the TGF-β superfamily, i.e. activin and BMP-2, being incapable of potently inhibiting its expression at equivalent concentrations.

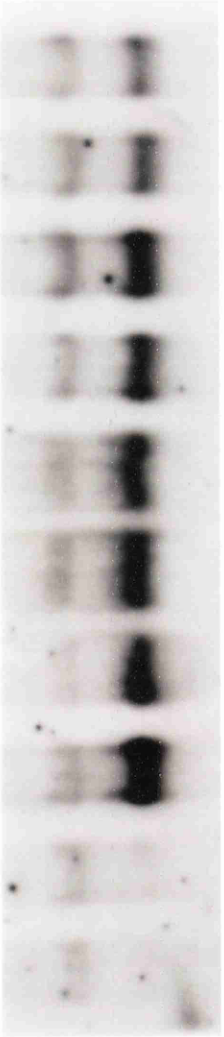
5.5 Effect of TGF-β1 on MIP-1α receptor levels on FDCP-MIX cells

5.5.1 TGF-β1 downregulates MIP-1α specific receptors on FDCP-MIX cells

Given the observed response of macrophages (a likely *in vivo* source of MIP-1α), it was also investigated what, if any, response is observed in the MIP-1α target cells i.e. stem cells. It is possible for example that, *in vivo*, in the presence of active TGF-β1 and therefore the presumed downregulation of MIP-1α, the stem cells may compensate for this downregulation of MIP-1α by upregulating specific receptors for that molecule. To address this question, any possible effects of TGF-β1 on the levels of receptor for MIP-1α on a haemopoietic stem cell like line were

Figure 5.4.3 Northern blot analysis of the effect of activin on MIP-1α expression in BMDM induced by LPS

Bone marrow derived macrophages were treated with LPS at a concentration of 20μg/ml plus or minus activin at the concentrations shown in each lane. Total RNA was prepared 0 and 4hrs following cytokine treatment, and 20μg of total RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred by Northern blotting, and the membrane probed using a radiolabelled MIP-1α specific riboprobe. The autoradiograph was exposed for 18hrs. Details of each individual lane are shown in the figure. This blot is representative of at least three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining.



4hr + LPS + 100pM TGF-β3

4hr + LPS + 3nM Activin

4hr + LPS + 1nM Activin

4hr + LPS + 300pM Activin

4hr + LPS + 100pM Activin

4hr + LPS + 30pM Activin

4hr + LPS + 10pM Activin

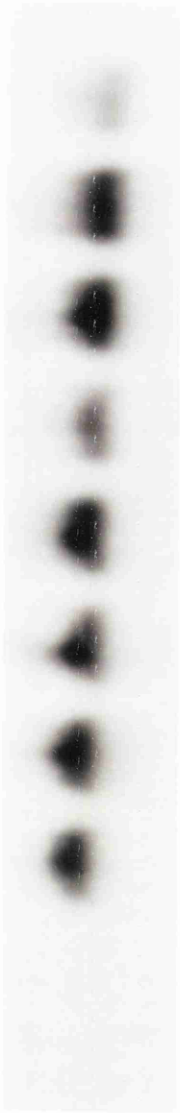
4hr + LPS

4hr Control

0hr Control

Figure 5.4.4 Northern blot analysis of the effect of BMP-2 on MIP-1α expression in BMDM induced by LPS

Bone marrow derived macrophages were treated with LPS at a concentration of 20μg/ml plus or minus BMP-2 at the concentrations shown in each lane. Total RNA was prepared 0 and 4hrs after cytokine addition, and 20μg of total RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred by Northern blotting, and the membrane probed using a radiolabelled MIP-1α specific riboprobe. The autoradiograph was exposed for 18hrs. Details of each individual lane are shown in the figure. This blot is representative of at least three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining.



4 hrs + 300pM TGF-β3

4 hrs + 1nM BMP-2

4 hrs + 300pM BMP-2

4 hrs + 100pM BMP-2

4 hrs + 30pM BMP-2

4 hrs + 10pM BMP-2

4 hrs + 3pM BMP-2

4 hrs + LPS

4 hr Control

0 hr Control

investigated. FDCP-MIX cells, as initially isolated, display considerable similarity to the CFU-S stem cell (Spooncer *et al* 1984). It has been previously demonstrated by Graham *et al* (1993) that these cells have numerous cell surface receptors for MIP-1α, and further, that these receptors are specific in recognising only those members of the MIP-1α peptide family that are capable of stem cell inhibition. It is therefore a good candidate for a MIP-1α responsive cell line. To investigate the effect of TGF-β1 on MIP-1α receptor levels on these cells, cells were incubated with TGF-β1 for 16hrs and MIP-1α receptor binding assays were performed as described in materials and methods section 4.9.

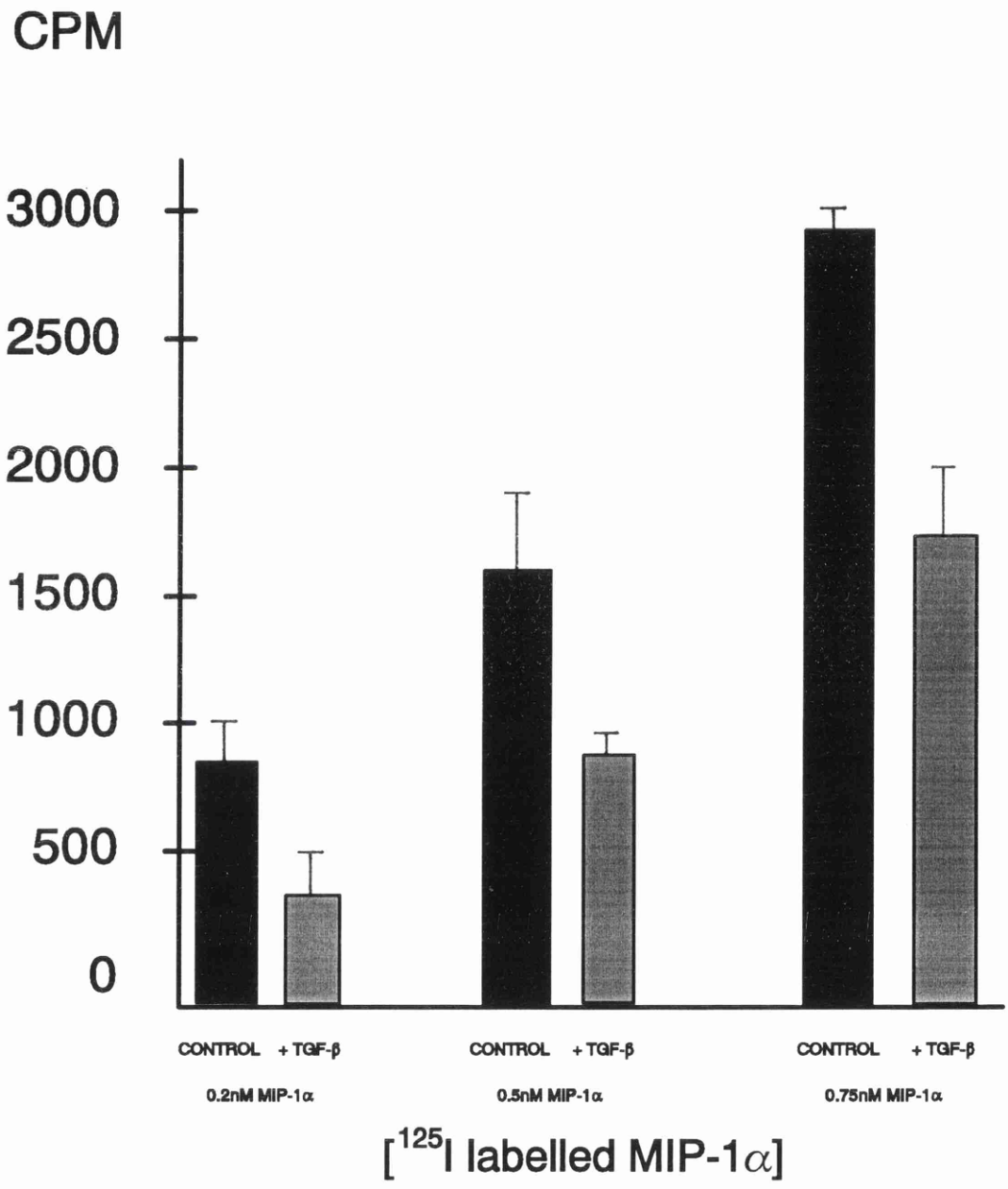
Initially, binding was performed using a titration of radiolabelled MIP-1α to investigate the effects of TGF-β1 on MIP-1α receptor levels. MIP-1α receptor levels are typically saturated at around 1nM. Figure 5.5.1 shows however, that in the presence of TGF-β1, specific MIP-1α binding to the cell surface appears to be inhibited by around 50% at each of the three radiolabelled MIP-1α concentrations. Binding of radiolabelled MIP-1α to the cell surface is substantially competable using a 100 fold excess of unlabelled MIP-1α, and importantly, is not competable by high concentrations of unlabelled TGF-β1 (data not shown). This demonstrates that the reduced binding of radiolabelled MIP-1α to these cells is not simply due to TGF-β1 directly blocking binding of MIP-1α to its receptor.

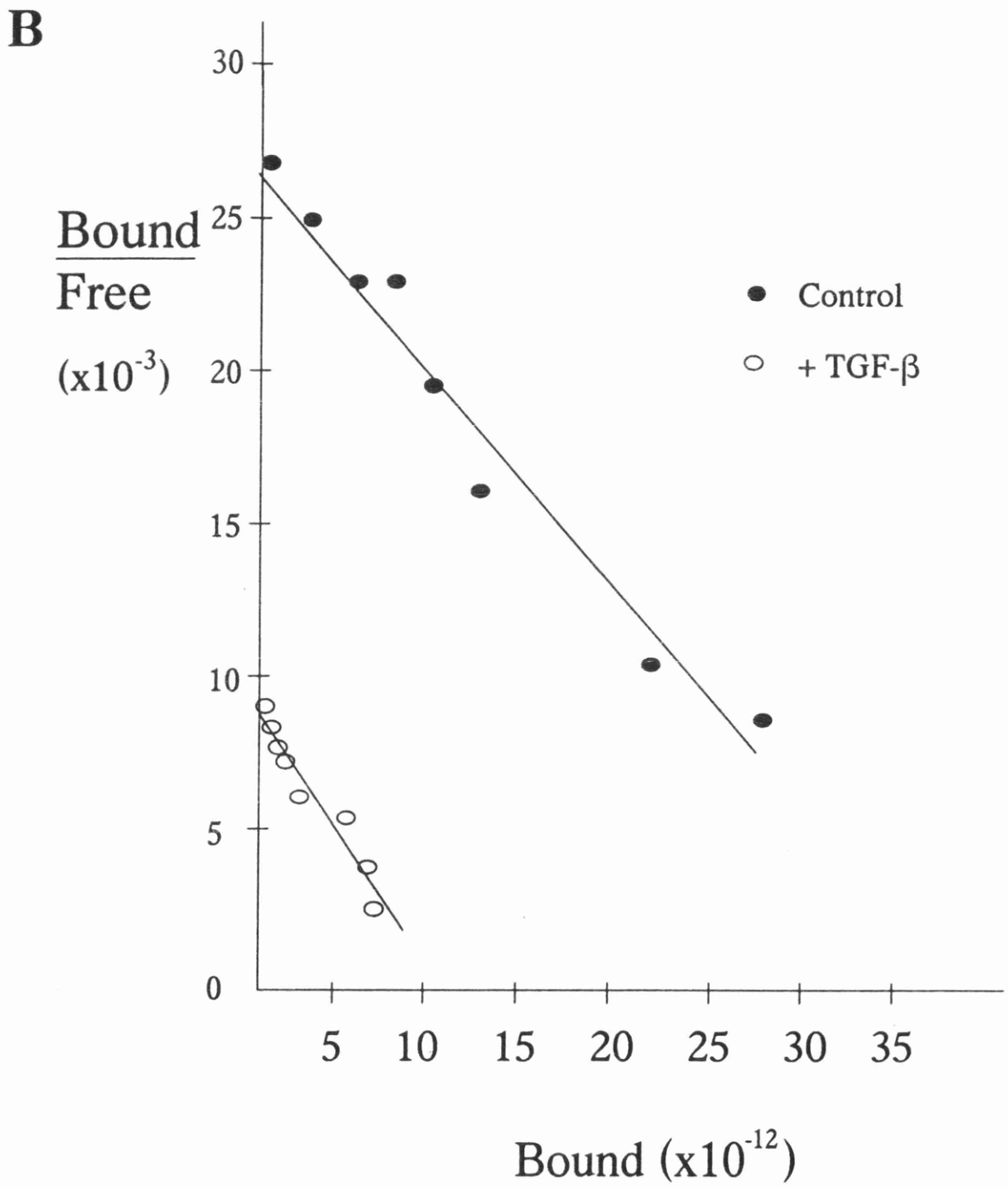
5.5.2 TGF-β1 downregulates MIP-1α receptor numbers with no change in affinity of the remaining receptors for ligand

A reduction in binding of a ligand to the cell surface can be the result of either a reduction in overall receptor numbers for that molecule on the cell surface, or of a reduction in affinity of those receptors for ligand. More comprehensive binding curves were carried out (see figure 5.5.2), and Scatchard analysis of the data performed to investigate whether the observed downregulation results from reduction in receptor numbers or receptor affinity. The results indicate that whereas the control cells expressed approximately 11,000 receptors per cell, the TGF-β-treated cells expressed only around 3,400 receptors per cell, indicating a

Figure 5.5.1 Effect of TGF-β1 on MIP-1α receptor levels on FDCP-MIX cells

FDCP-MIX cells were incubated for 16hrs at a concentration of 10^6 cells/ml plus or minus TGF-β1 at a concentration of 100pM. This concentration was chosen because it results in almost complete inhibition of MIP-1α mRNA expression in macrophages. Cells were then washed twice in PBS and $5x10^5$ cells per point aliquoted into eppendorf test tubes and pelleted. To each aliquot, various concentrations of ^{125}I labelled MIP-1α were added in binding buffer (Special Liquid Medium / 10% DHS / 0.2% azide), and either PBS or 100-fold excess unlabelled MIP-1α competitor. Cells were then incubated at 37oC for 90 minutes after which they were washed 3 times in PBS and the incorporated radiation assessed in a gamma counter. Data are representative of of 3 experiments and are plotted as the mean of duplicate determinations. Background radiation has been subtracted and thus these plots represent specific counts.


CPM
3000
2500
2000
1500
1000
500
0
CONTROL
+ TGF-β
CONTROL
+ TGF-β
CONTROL
+ TGF-β
0.2nM MIP-1α
0.5nM MIP-1α
0.75nM MIP-1α
[125I labelled MIP-1α]


B
Bound
Free
(x10-3)
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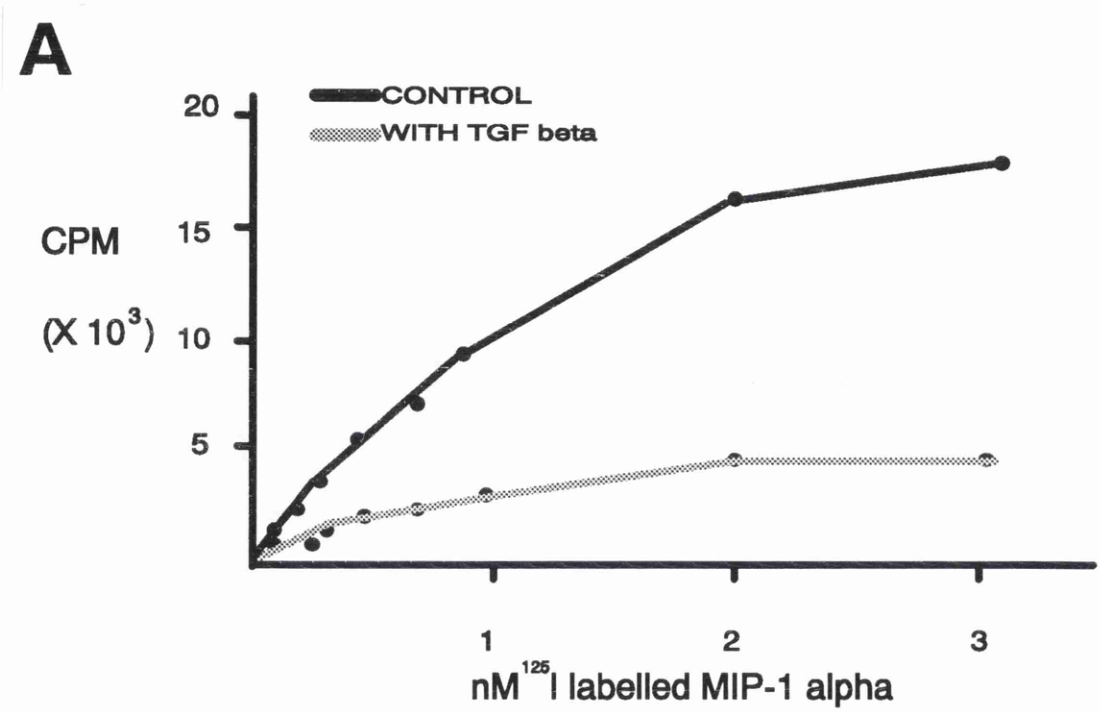


Figure 5.5.2 Comprehensive binding curves of the effect of TGF-β on MIP-1α receptor levels on FDCP-MIX cells

A Equilibrium binding of ^{125}I labelled MIP-1α to FDCP-MIX cells. Cells were pre-incubated in medium with or without TGF-β at a concentration of 100pM for 18 hrs, and binding of radiolabelled MIP-1α assessed as outlined in section 4.9.2.

B Scatchard analysis of the data shown in figure 5.5.2 part A. Analysis of binding isotherms was performed using the LIGAND computer program (Munson and Robard 1980).

70% reduction in receptor numbers. MIP-1α receptors on control cells exhibited a Kd of $1.3x10^{-9}$M compared with $1.21x10^{-9}$M for the TGF-β1-treated cells. Thus, TGF-β1 mediated inhibition of MIP-1α receptors is due to a decrease in receptor number and not to a decrease in receptor affinity.

5.5.3 TGF-β1 mediated inhibition of MIP-1α receptors on FDCP-MIX cells is potent and fully reversible

To fully characterise the kinetics of this receptor downregulation, and to ascertain the maximum downregulation that could be obtained, a titration from 50pM to 1nM TGF-β1 was performed. Figure 5.5.3 shows that after 16hrs, the maximum downregulation appears to be approximately 70%, and that this is achieved using TGF-β1 at a concentration of 100pM. The maximum downregulation achievable varied from between 50-70% in other experiments. Increasing the concentration of TGF-β1 does not however cause a further decrease in MIP-1α receptor numbers. The possible functional significance of this level of downregulation is addressed in the discussion.

In vivo, it is possible that stem cells are continually exposed to active TGF-β1, and figure 5.5.4 shows the effects on MIP-1α receptors of long term exposure of FDCP-MIX cells to active TGF-β1. These results show that continued daily addition of TGF-β1 results in prolonged MIP-1α receptor downregulation over the 96hr time course of this experiment. However, a single treatment of TGF-β1 results in an initial reduction of MIP-1α receptors which then return to normal levels by 72hrs and indeed overshoot at 96hrs. This suggests that TGF-β1 mediated inhibition of MIP-1α receptor levels is fully reversible, but that on prolonged exposure, such as may be encountered *in vivo*, MIP-1α receptor levels will be downregulated on cells for the duration of their exposure to TGF-β1.

The MIP-1α specific receptor present on FDCP-MIX cells remains to be cloned and fully characterised, thus analysis of the effects of TGF-β1 on the actual mRNA expression of this receptor are not possible. However, as mentioned in the introduction, a C-C chemokine receptor has recently been cloned from HL-60 cells

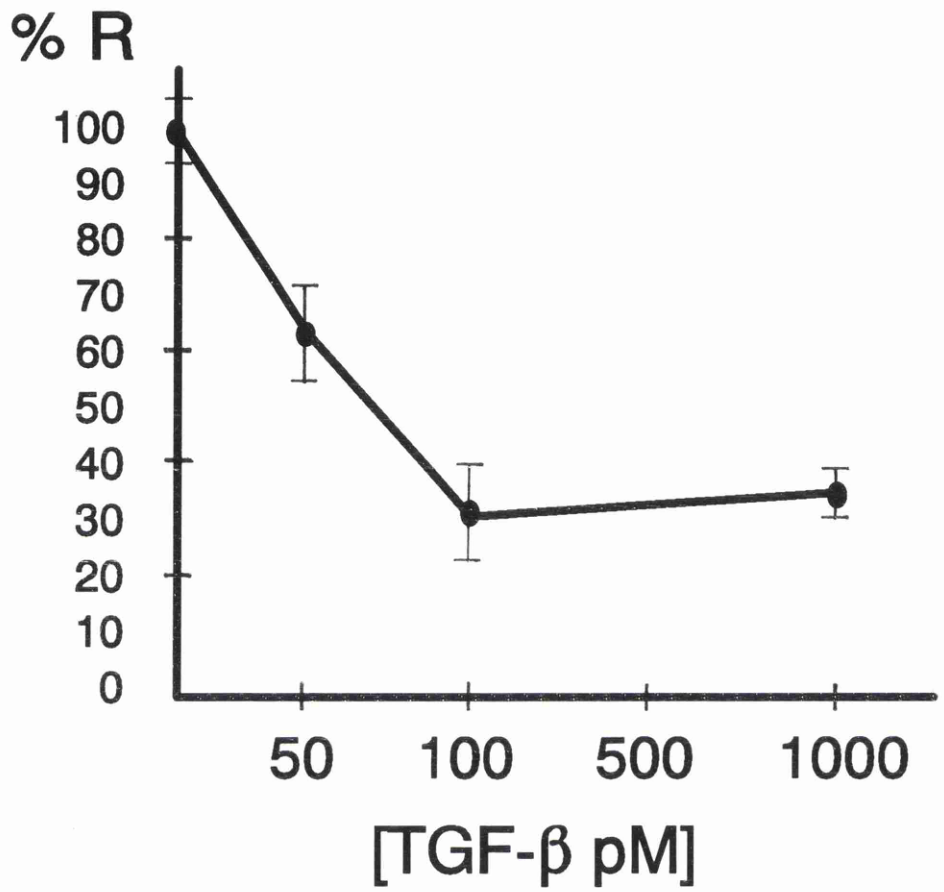


Figure 5.5.3 Titration of TGF-β mediated inhibition of MIP-1α receptors on FDCP-MIX cells

FDCP-MIX cells exposed to the concentrations of TGF-β detailed above were treated as outlined in figure legend 5.5.1, and specific binding of ^{125}I labelled MIP-1α (0.5nM) measured. The results are expressed as receptor percentage and are representative of at least 3 separate experiments.

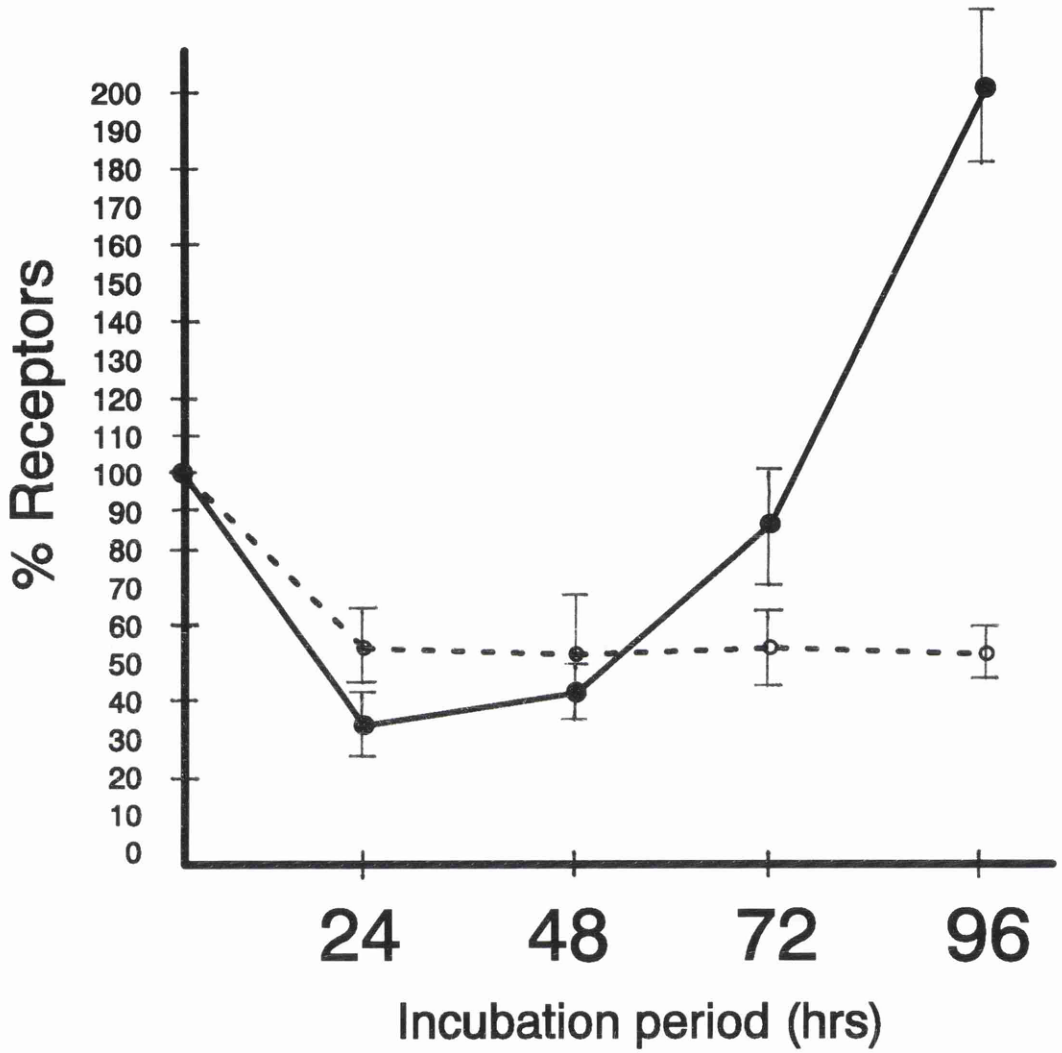


Figure 5.5.4 Time course of TGF-β mediated downregulation of MIP-1α receptors on FDCP-MIX cells

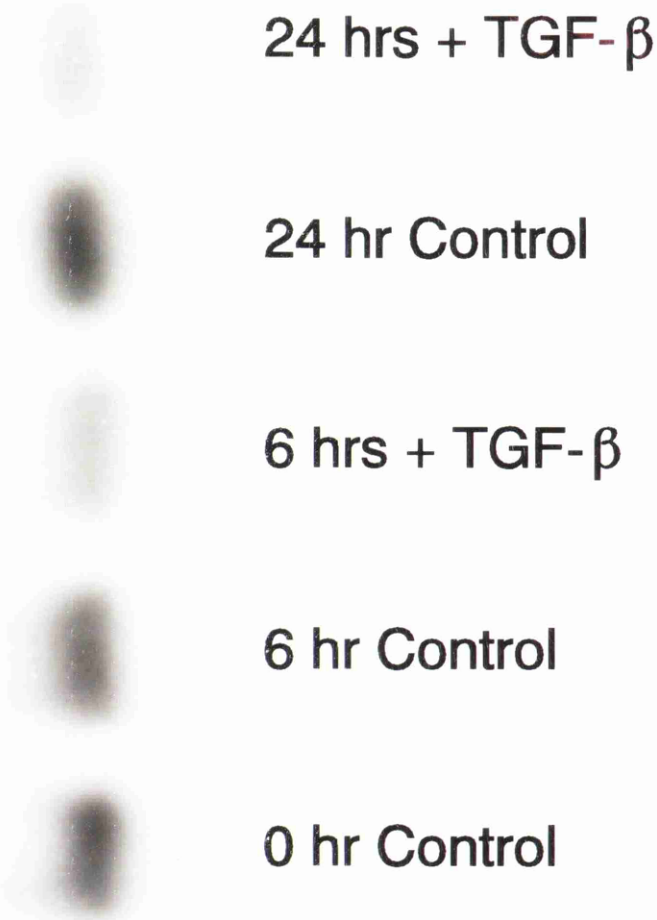
FDCP-MIX cells exposed to TGF-β at a concentration of 100pM were treated as outlined in figure 5.5.1, and specific binding of ^{125}I labelled MIP-1α (0.5nM) measured. Cells were treated with either one dose of TGF-β at time 0 (full line), or continual daily doses of TGF-β (broken line). The results are expressed as percent receptors and are representative of at least 3 separate experiments.

(Neote *et al* 1993), and it was of interest to investigate any possible effects TGF-β1 may have on this receptor type also. Interestingly, figure 5.5.5. shows that TGF-β1 is capable of downregulating mRNA expression of CC-CKRI in U937 cells suggesting that TGF-β1 may be capable of inhibiting surface expression of this MIP-1α receptor also. This data further suggests that the inhibitory effect of TGF-β1 on MIP-1α receptors on FDCP-MIX cells may also be regulated at the mRNA level.

At present, there are conflicting reports regarding the percentage of cytokine receptors which need to be ligand occupied in order to elicit a biological response. That is, although a downregulation of MIP-1α receptor numbers of up to 70% is observed, it is still possible that the remaining 30% are fully capable of causing a biological response. As described in the introductory section, the receptor for MIP-1α present on FDCP-MIX cells is likely to be a member of the G-protein linked seven membrane spanning family. A feature of all G-protein linked receptors is that upon ligand binding and receptor activation, a rise in intracellular calcium levels is observed as part of the signal transduction process. This rise in intracellular calcium results from release of calcium from intracellular stores, or influx of calcium from the extracellular periphery. Both mechanisms can also work together. A number of workers have previously shown that MIP-1α is capable of causing intracellular calcium mobilisation in both monocytes and eosinophils (Sozzani *et al* 1993, Rot *et al* 1992), and it was reasoned that MIP-1α may also signal via calcium in FDCP-MIX cells. Thus, a reduction in receptor numbers on the surface of these cells may result in a decrease in the ability of MIP-1α to elicit a calcium flux in these cells, which in turn may result in functional compromise. This possibility was therefore investigated with a view to determining whether a 50-70% reduction in MIP-1α receptors by TGF-β1 could have functionally relevant consequences.

Figure 5.5.5 Effect of TGF-β on CC-CKRI MIP-1α receptor expression in U937 cells

U937 cells were treated with TGF-β at a concentration of 250pM. Total RNA was prepared after 0, 6 and 24hrs and 20μg of RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred by Northern blotting, and the membrane probed using a radiolabelled CC-CKRI specific riboprobe. The autoradiograph was exposed for 24hrs. Details of each individual lane are shown in the figure. This blot is representative of three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining.


24 hrs + TGF-β
24 hr Control
6 hrs + TGF-β
6 hr Control
0 hr Control

5.6 Downregulation of MIP-1α receptors on FDCP-MIX cells by TGF-β1 is reflected in reduced ability of these cells to mobilise calcium in response to MIP-1α

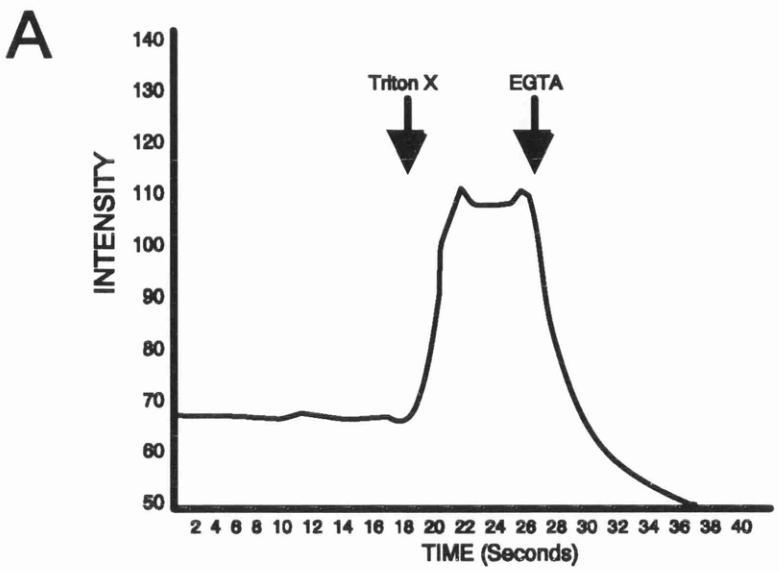
FDCP-MIX cells were treated overnight with TGF-β1 (100pM), and then examined for their ability to mobilise calcium in response to MIP-1α. To check that the cells had been efficiently loaded with the fluorescent dye FURA-2, total calcium release was induced with the detergent triton-X. As seen in figure 5.6 A, addition of triton X to the cells causes a large increase in intensity. This is due to lysing of the cells, and release of all intracellular FURA-2 into the extracellular medium where it binds calcium, and thus an increase in fluorescence intensity is observed. Addition of the calcium chelator EGTA however, results in removal of all available calcium ions, and thus a decrease in fluorescence intensity is observed. Figure 5.6 B shows a calcium flux in control FDCP-MIX cells in response to addition of 60μl of a 1mg/ml solution of MIP-1α. Figure 5.6 C shows a calcium flux in FDCP-MIX cells pre-treated for 16 hrs with TGF-β1 (100pM) in response to addition of 60μl of a 1mg/ml solution of MIP-1α. These figures demonstrate that the calcium flux elicited in TGF-β1 treated FDCP-MIX cells by MIP-1α is approximately 86% lower than that observed in control FDCP-MIX cells. This demonstrates that pre-treatment of FDCP-MIX cells with TGF-β1 results in not only a significant decrease in MIP-1α receptor numbers on these cells, but also a reduction in the ability of MIP-1α to signal into these cells. Thus, the TGF-β1 mediated inhibition of MIP-1α receptor levels on stem cells may have important implications with respect to the suggested role of MIP-1α as an endogenous physiological stem cell inhibitor.

5.7 Overnight incubation of normal bone marrow cells with TGF-β1 reduces the ability of MIP-1α to inhibit the CFU-A population

If the TGF-β1 mediated inhibition of MIP-1α receptors on stem cells does reduce their ability to respond to MIP-1α, then it is possible that this may be reflected in a functional biological assay. To investigate this possibility in more

Figure 5.6 Effect of preincubation of FDCP-MIX cells with TGF-β on the ability of MIP-1α to induce calcium mobilisation

FDCP-MIX cells were incubated overnight with or without TGF-β at a concentration of 100pM. Cells were then washed twice in PBS and resuspended at a concentration of $5x10^5$/ml in calcium free HACM buffer (see Materials and Methods). FURA-2 was added to the cell suspension at a concentration of 0.5μM, and the cells incubated at 37°C for 45 minutes in the dark to allow the FURA-2 fluorescent dye to permeate the cells. The cells were then washed twice in warm PBS, and resuspended in calcium containing HACM buffer at a concentration of $1x10^6$/ml. Cells were incubated at 37°C for 30 minutes, and then used immediately. 2mls of each cell suspension was aliquoted into a spectrophotometer cuvette, and the appropriate stimulants added directly to the suspension. Changes in fluorescence intensity of FURA-2 were assessed in a Perkin-Elmer spectrophotometer. Part A shows the effect of addition of 100μl of a 10% solution (v/v) of triton-X, followed by addition of 100μl of a XM solution of EGTA. Part B shows the effect of addition of 60μl of MIP-1α at a concentration of 1mg/ml to control FDCP-MIX cells. Part C shows the effect of addition of 60μl of MIP-1α at a concentration of 1mg/ml to TGF-β pre-treated FDCP-MIX cells. The Y axis on each graph represents the overall intensity of fluoresence of the dye FURA-2 upon calcium binding.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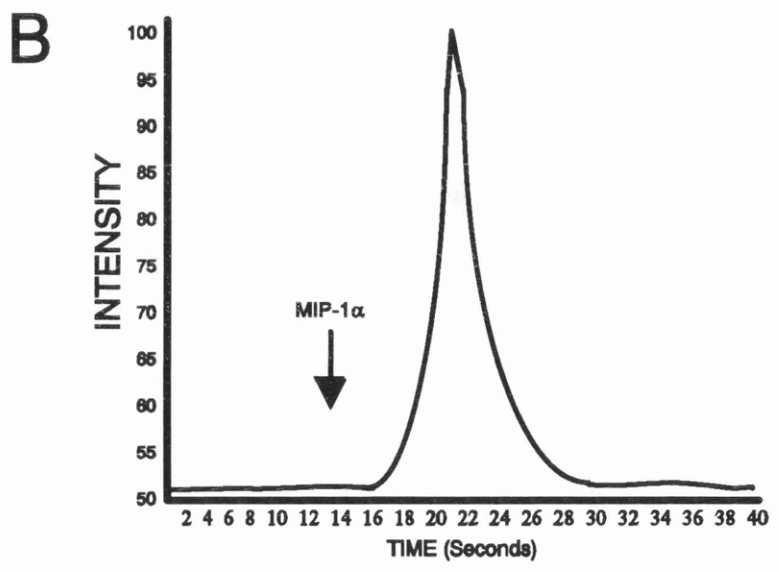
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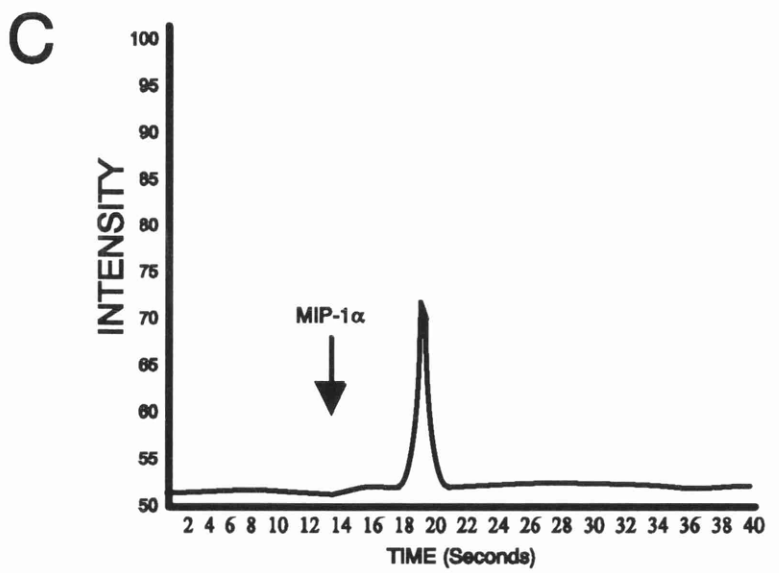
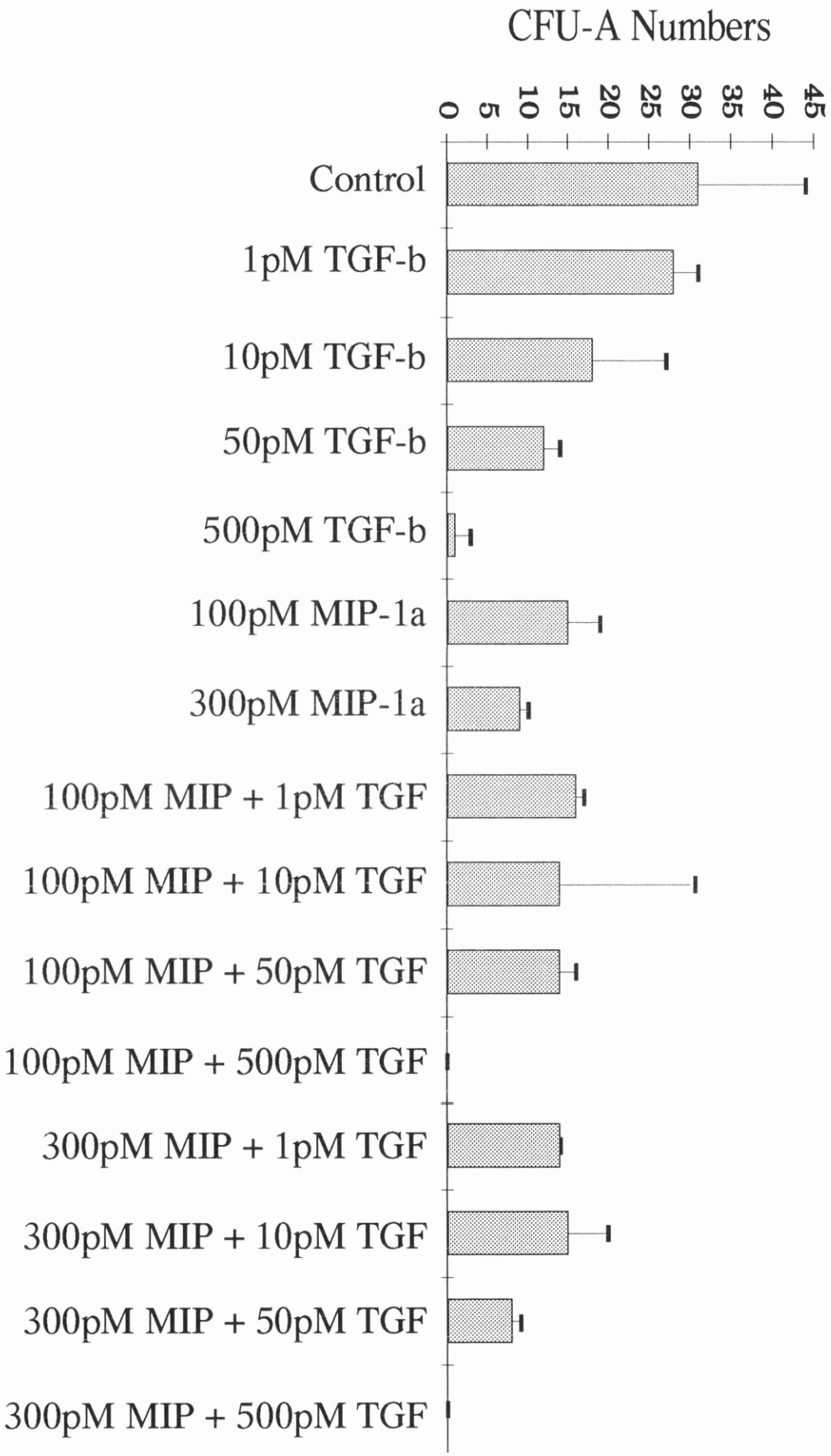

C
INTENSITY
100
95
90
85
80
75
70
65
60
55
50
MIP-1α
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
TIME (Seconds)

detail, normal bone marrow cells were incubated with a titration of TGF-β1 (1pM to 500pM) for 16 hours. This should result in a maximal downregulation of MIP-1α receptors on CFU-A cells. The cells were then washed 3 times in PBS, to remove any excess TGF-β1. If the resultant downregulation of MIP-1α receptors had any functional significance, then this may be manifest in the reduced potency of MIP-1α in the CFU-A assay. It was anticipated that overnight (16 hrs) incubation of normal bone marrow cells with TGF-β1 would result in a reduction in the ability of MIP-1α to inhibit CFU-A colony formation. The results shown in figure 5.7 demonstrate that this may be the case. For example, it appears that overnight incubation of bone marrow cells with TGF-β is capable of inhibiting CFU-A colony formation in a dose dependent manner. When using sub-optimal concentrations of TGF-β such as 50pM, colony formation is inhibited by approximately 60%. MIP-1α when used at a concentration of 100pM inhibits CFU-A colony formation by around 50%. Both of these molecules are ultimately capable of causing 100% inhibition in this assay. However, exposure of CFU-A cells to both TGF-β and MIP-1α does not result in increased inhibition of colony formation, as might be expected from earlier results demonstrating that both are highly potent in this assay system. It appears that after TGF-β treatment, MIP-1α is incapable of inhibiting CFU-A colony formation beyond that achieved by TGF-β suggesting that the cells may indeed be less responsive to the inhibitory properties of MIP-1α. It should be noted that the potencies of both MIP-1α and TGF-β1 in this particular assay appear different from those shown in figures 5.1.1 and 5.1.2. This is most likely due to inter assay variation, which is an unavoidable feature of *in vitro* colony forming assays.

The results of this assay are quite intriguing. It appears that overnight incubation of normal bone marrow cells with a titration of TGF-β1 results in dose dependent inhibition of colony formation after 11 days without the requirement for subsequent TGF-β addition. This surprising result suggests at least two possibilities. Either this short exposure of the cells to TGF-β1 is sufficient to keep them out of cell cycle and thus prevent them from forming colonies over the 11 day

Figure 5.7 Effect of preincubation of total bone marrow cells with TGF-β1 on the ability of MIP-1α to inhibit CFU-A colony formation

Normal bone marrow cells were incubated for 16hrs in SLM/10% DHS at 37°C with or without a titration of TGF-β1. Following this, the cells were washed three times in PBS. MIP-1α was tested for inhibitory activity in the direct addition CFU-A assay as described in section 4.5. Varying concentrations of MIP-1α were added directly to the feeder layers (0.6% agar in α-MEM with sources of M-CSF and GM-CSF) of the CFU-A assays, and the TGF-β1 pretreated bone marrow target cells plated at $5x10^3$/ml in the upper layer (0.3% agar in α-MEM). The assay plates were incubated at 37°C in a fully humidified atmosphere of 10% CO_2, 5% O_2 and 85% N_2 for 11 days. Colonies >2mm were scored as being CFU-A type. The results depicted are representative of 3 separate experiments.


CFU-A Numbers
0
5
10
15
20
25
30
35
40
45
Control
1pM TGF-b
10pM TGF-b
50pM TGF-b
500pM TGF-b
100pM MIP-1a
300pM MIP-1a
100pM MIP + 1pM TGF
100pM MIP + 10pM TGF
100pM MIP + 50pM TGF
100pM MIP + 500pM TGF
300pM MIP + 1pM TGF
300pM MIP + 10pM TGF
300pM MIP + 50pM TGF
300pM MIP + 500pM TGF

time period of this assay, or the washing steps carried out prior to plating the cells into the assay dishes was insufficient, and the cells were actually exposed to active TGF-β1 for considerably longer than 16 hrs. Also, as will be discussed in the next chapter, it is possible that TGF-β1 is not actually inhibiting these cells from cycling, but is in fact inducing apoptotic cell death.

In summary, the results from these experiments suggest that TGF-β1 downregulates MIP-1α receptor numbers on stem cells, and this downregulation is reflected in decreased secondary messenger release. Further, overnight incubation of normal bone marrow with TGF-β1 reduces the ability of MIP-1α to inhibit CFU-A colony formation.

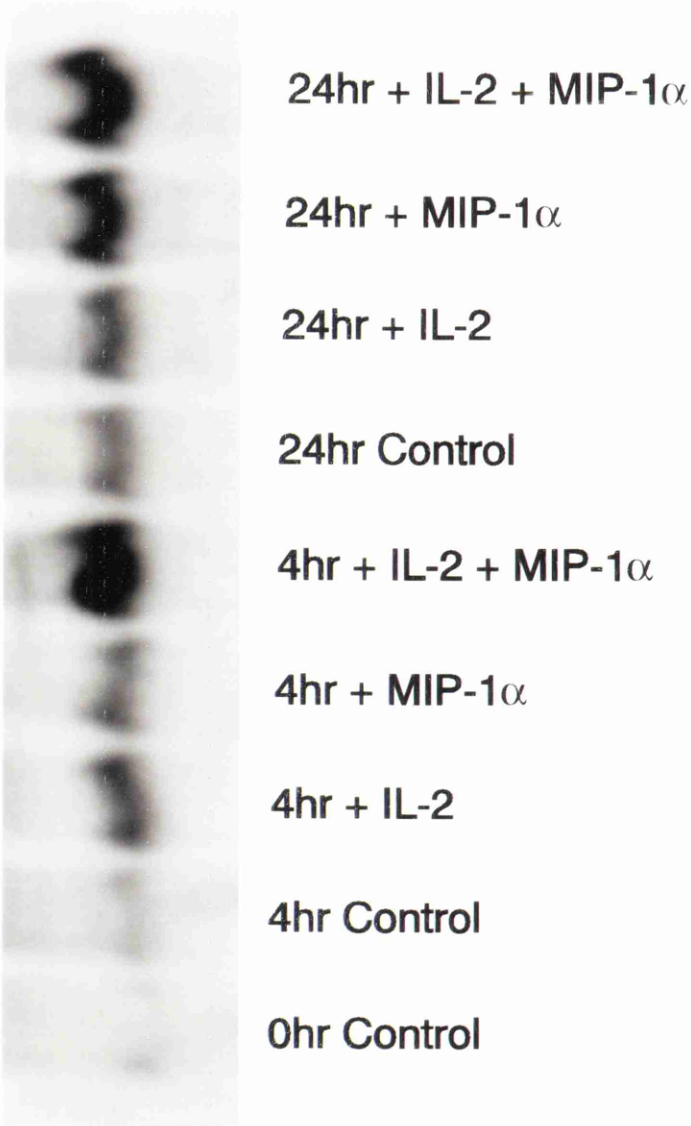
5.8 Effect of MIP-1α on TGF-β1 expression in murine BMDM

5.8.1 MIP-1α and IL-2 increase TGF-β1 mRNA expression in murine bone marrow derived macrophages

In order to fully understand the interactions between these two molecules in the overall control of haemopoietic stem cell proliferation, it was next decided to investigate any potential reciprocal effects of MIP-1α on TGF-β1. Although it is quite clear that TGF-β1 functions as an inhibitor independently of MIP-1α, it is still possible that MIP-1α mediates common functions, either wholly or in part, via upregulation of TGF-β1. To investigate this possibility, the effect of MIP-1α on TGF-β1 mRNA expression in BMDM was examined. Addition of interleukin 2 (IL-2) to macrophages has previously been shown to upregulate TGF-β1 mRNA expression, and was thus included as a positive control. BMDM were grown to confluence over seven days as described previously, after which MIP-1α (2.5nM), IL-2 (100 U/ml) or a combination of the two were added to the cells. Total RNA was prepared after 4 hrs and 24 hrs, and analysed for TGF-β1 expression using a TGF-β1 specific radiolabelled probe. Figure 5.8.1 shows that very little TGF-β1 expression is detectable in resting macrophages at time 0, and 4 hrs after commencing the experiment. Addition of IL-2 alone results in an increase in TGF-

Figure 5.8.1 Northern blot analysis of the effect of MIP-1α and IL-2 on TGF-β1 expression in BMDM

BMDM were grown for 7 days, after which they were treated with either IL-2 at a concentration of 100 units/ml, MIP-1α at a concentration of 2.5nM or a combination of both IL-2 and MIP-1α. Total RNA was prepared 0, 4 and 24hrs following cytokine addition, and 20μg of RNA was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred via Northern blotting, and the membrane probed using a radiolabelled TGF-β1 specific DNA probe. The autoradiograph was exposed for 24hrs. Details of each individual lane are shown in the figure. This blot is representative of three separate experiments. Equal loading of total RNA was confirmed by ethidium bromide staining.


24hr + IL-2 + MIP-1α
24hr + MIP-1α
24hr + IL-2
24hr Control
4hr + IL-2 + MIP-1α
4hr + MIP-1α
4hr + IL-2
4hr Control
0hr Control

β1 expression evident after 4hrs and 24hrs. Addition of MIP-1α alone also causes an increase in TGF-β1 expression after 4 hrs and 24 hrs, and addition of both MIP-1α and IL-2 causes an increase in TGF-β1 expression considerably greater than that caused by either cytokine alone. Thus, it appears that MIP-1α is indeed capable of inducing TGF-β1 mRNA expression in bone marrow macrophages, and that this property is augmented in the presence of other TGF-β1 inducing molecules such as IL-2.

5.8.2 MIP-1α upregulates TGF-β1 protein production by BMDM

As with the inhibitory effect of TGF-β1 on MIP-1α expression, it is of more physiological importance and significance to confirm that the observations apparent at the mRNA level are reflected at the secreted protein level. Detection of TGF-β1 protein by Western blot analysis is notoriously difficult due to very poor transfer of TGF-β1 protein to nitrocellulose membranes, so it was decided to examine any potential increase in TGF-β1 protein using a commercially available enzyme linked immunosorbent assay (ELISA) system. This assay utilises soluble TGF-β type II receptor bound to 96 well plates, which binds any of the three mammalian TGF-β isoforms present in cell conditioned medium. The assay only detects TGF-β1 however, since the second polyclonal antibody is TGF-β1 specific. Figure 5.8.2 shows that, firstly, resting bone marrow macrophages produce around 230 pg/ml of TGF-β1. Since all the conditioned media sample is acidified prior to analysis in the assay, this result represents the total pool of TGF-β1 molecules present, and does not distinguish between active and non-active forms. The overall levels of TGF-β1 present has increased to around 350 pg/ml after 24 hrs, presumably due to accumulation of TGF-β1 normally produced by the cells, but no significant differences are detectable between control cells and those treated with either 40ng/ml or 80ng/ml of MIP-1α. After 48 hrs however, the levels of TGF-β1 present in the CM of those cells treated with MIP-1α is considerably higher than control cells. This increase appears to be related to the dose of MIP-1α added to the cells 48 hrs previously. Control cell CM contains around 610 pg/ml after 48

Figure 5.8.2 ELISA analysis of the effect of MIP-1α on TGF-β protein production by BMDM

BMDM were grown for 6 days, after which MIP-1α was added at concentrations of 40ng/ml (5nM) and 80ng/ml (10nM). Conditioned medium was harvested at times 0hrs, 24hrs and 48 hrs after addition of MIP-1α, and then concentrated using Centriprep 10 spin columns. Presence of TGF-β1 protein in each conditioned medium sample was then quantitated using a TGF-β1 specific ELISA system (R&D Systems). This assay relies on active TGF-β1 binding to the type II TGF-β receptor, and as such, each conditioned media sample was acidified to yield active TGF-β1. Acidification of samples was carried out by adding 0.2ml of 1N HCl to 1ml of conditioned media for 10 minutes, and then adding 0.2ml of 1.2N NaOH/0.5M HEPES. TGF-β1 is also present in the BMDM growth medium, and so the results represent the amount of TGF-β1 present in the BMDM conditioned media after subtraction of these levels of TGF-β1. These results are representative of at least three separate experiments.

TGF-β (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
0 hr Control
24 hr Control
24 hrs + 40ng/ml MIP-1α
24 hrs + 80ng/ml MIP-1α
48 hr Control
48 hrs + 40ng/ml MIP-1α
48 hrs + 80ng/ml MIP-1α

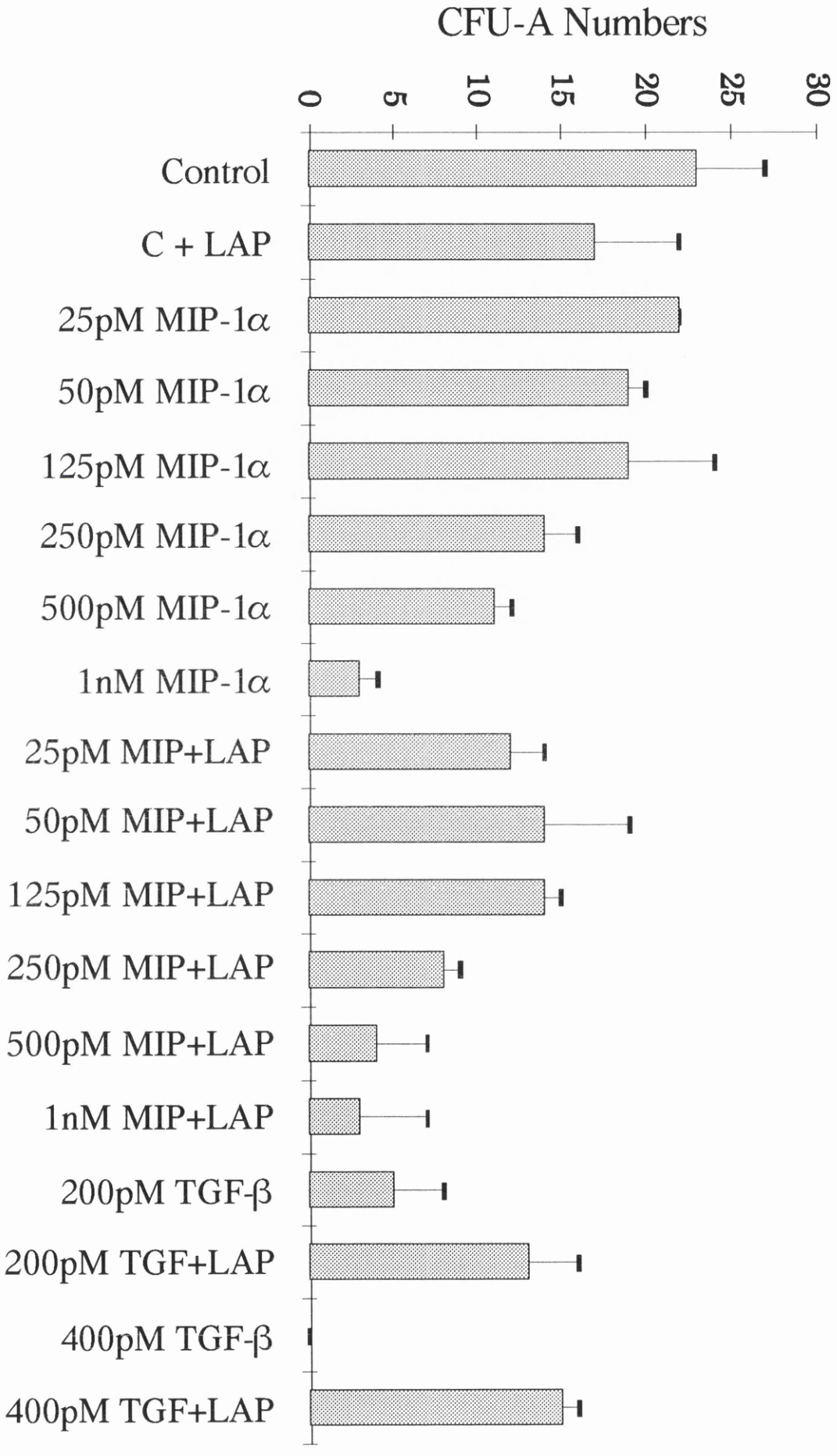
hrs whereas CM from cells treated with 40ng/ml contains approximately 740 pg/ml and CM from cells treated with 80 ng/ml contains 1020 pg/ml. Each point was assayed in duplicate, with almost identical values in each demonstrating this to be a significant difference. This data suggests that the MIP-1α mediated increase in TGF-β1 mRNA is reflected at the protein level, and further suggests that MIP-1α may function in part as a stem cell inhibitor by upregulation of TGF-β1 in either target or non-target cells present within the bone marrow environment.

5.9 Inactivation of endogenous TGF-β levels in the CFU-A assay does not reduce the ability of MIP-1α to inhibit CFU-A colony formation

In order to test if MIP-1α may act as an inhibitor either wholly or in part through upregulation of TGF-β, it was reasoned that inhibition of TGF-β function may abrogate the ability of MIP-1α to inhibit CFU-A colony formation. To this end, CFU-A assays were performed using MIP-1α in the presence of the TGF-β latency associated peptide (LAP), which is a natural and potent antagonist of TGF-β function. Figure 5.9 shows that firstly, LAP (80ng/ml) is successfully preventing TGF-β (200pM and 400pM) from inhibiting CFU-A colony formation. The results further show that MIP-1α is potently inhibiting, and that even in the presence of LAP, MIP-1α is still a potent inhibitor of CFU-A colony formation. This suggests that MIP-1α does not function as an inhibitor via upregulation of TGF-β, and that the two molecules inhibit CFU-A type stem cells entirely independently of each other.

Figure 5.9 Effect of LAP on the ability of MIP-1α and TGF-β to inhibit CFU-A colony formation

MIP-1α and TGF-β were tested for inhibitory activity in the direct addition CFU-A assay as described in section 4.5. Varying concentrations of MIP-1α and TGF-β were added directly to the feeder layers of the CFU-A assay (0.6% agar in α-MEM with sources of M-CSF and GM-CSF) plus or minus LAP at a concentration of 80ng/ml. Normal bone marrow cells were plated at $5x10^3$/ml in the upper layer and the assay plates were incubated 37°C in a fully humidified atmosphere of 10% CO_2, 5% O_2 and 85% N_2 for 11 days. Colonies >2mm were scored as being CFU-A type. The results depicted are representative of 3 separate experiments.


CFU-A Numbers
0
5
10
15
20
25
30
Control
C + LAP
25pM MIP-1α
50pM MIP-1α
125pM MIP-1α
250pM MIP-1α
500pM MIP-1α
1nM MIP-1α
25pM MIP+LAP
50pM MIP+LAP
125pM MIP+LAP
250pM MIP+LAP
500pM MIP+LAP
1nM MIP+LAP
200pM TGF-β
200pM TGF+LAP
400pM TGF-β
400pM TGF+LAP

CHAPTER 6

DISCUSSION

Chapter 6 - DISCUSSION

6.1 Introduction

It has become clear over the last few years that the proliferation of the haemopoietic stem cell is open to both positive and negative regulation by a wide array of cytokines and peptide molecules. It has also become clear that, among both the positive and negative regulators, considerable overlap in actions, or redundancy, is apparent between several members of each group. Our laboratory has been particularly interested in the inhibitors of haemopoietic stem cell proliferation, and the work presented in this thesis has attempted to investigate the extent and possible mechanisms of functional redundancy between these molecules.

To investigate any possible interactions between the inhibitors MIP-1α, TGF-β, tetrapeptide and pentapeptide, these molecules were first assayed for activity in the *in vitro* CFU-A assay. As can be seen in the results section, only MIP-1α and TGF-β appear to be capable of inhibiting CFU-A colony formation, with no notable inhibition by the tetrapeptide or pentapeptide. The reasons for lack of inhibition by both the tetrapeptide and the pentapeptide are unclear but it is possible that they are not able to overcome the positive stimuli conferred by the high levels of growth factors present in the assay. However, given the potency of both MIP-1a and TGF-β in this assay, it is possible that CFU-A cells are refractory to the inhibitory properties of both the tetrapeptide and pentapeptide. The results further demonstrate that both MIP-1α and TGF-β are active in this assay system at picomolar concentrations, suggesting functional similarities between them with respect to inhibition of CFU-A haemopoietic stem cell proliferation. Indeed, the functional similarities between MIP-1α and TGF-β do not end there. Both MIP-1α and TGF-β are also capable of stimulating the proliferation of more mature GM-CFC progenitors (Broxmeyer *et al* 1991, Jacobsen *et al* 1991a), and both are also capable of inhibiting proliferation of human clonogenic keratinocytes (Parkinson *et al* 1993, Moses *et al* 1985) suggesting that the functional similarities

between MIP-1α and TGF-β in haemopoiesis may also be apparent in other regenerating tissues.

6.2 Inhibition of MIP-1α by TGF-β1 and related family members

Given the lack of inhibition by the tetrapeptide and the pentapeptide, it was decided to concentrate on possible interactions between MIP-1α and TGF-β. As has been mentioned previously, the *in vitro* CFU-A assay consists of a multicellular environment, and it is possible that either MIP-1α or TGF-β could be acting indirectly by inducing synthesis of the other in either secondary non-target cells present in assay, or indeed in the actual target stem cells. Bone marrow macrophages are a potential source of both cytokines in the CFU-A assay and in the bone marrow, so any possible inductive effects of these molecules on each other were examined using these cell types. The results shown in figures 5.2.1 to 5.2.4 demonstrate that, rather than acting as an inducer of MIP-1α mRNA expression and protein production in bone marrow derived macrophages, TGF-β potently inhibits both of these functions in macrophages. This data clearly shows that TGF-β does not act as an inhibitor of haemopoietic stem cell proliferation by induction of MIP-1α in bone marrow macrophages. Further, the remarkable potency with which TGF-β exerts this inhibition suggests that in the presence of active TGF-β *in vivo*, very little MIP-1α will be expressed and synthesised. However, as will be discussed below, this may be dependent on the presence of other growth factors and cytokines which can potentially upregulate expression of MIP-1a.

It is important to remember when interpreting such experiments that both MIP-1α and TGF-β are actively involved in the inflammatory response and, from their properties described in the introduction, may have antagonistic roles. That is, TGF-β generally plays an anti-inflammatory role while MIP-1α plays a pro-inflammatory role. Thus, the inhibition of MIP-1α expression by TGF-β observed in macrophages may have little to do with stem cell inhibition and may simply be another of the anti-inflammatory functions of TGF-β. Such a scenario would

predict that TGF-β may also be able to downregulate other members of the pro-inflammatory chemokine superfamily such as MIP-1β, and RANTES. As shown in figure 5.3.1, TGF-β is indeed capable of downregulating expression of MIP-1β, a molecule highly related to MIP-1α and which displays weak stem cell inhibitory properties being capable of inhibiting CFU-A type cells at approximately 20 fold higher concentrations than those required for MIP-1α. This result is unsurprising since the expression of both MIP-1α and MIP-1β appears to be co-regulated in response to positive or mitogenic stimulation e.g. LPS or serum. The structure of the MIP-1β promoter has recently been elucidated by Proffitt *et al* (1995), and interestingly, the activities of the MIP-1α and MIP-1β promoters in transfected cells are very similar. The ability of TGF-β to suppress both MIP-1α and MIP-1β expression may therefore be due to their very similar promoter sequences which are capable of binding identical transcription factors. Thus, treatment of macrophages with TGF-β (or indeed positive stimuli) may result in cognate changes in expression of both MIP-1α and MIP-1β. Interestingly, mRNA expression of RANTES, a more diverse member of the MIP-1α superfamily which does not share such a high degree of nucleic acid conservation with MIP-1α appears to be unaffected by TGF-β. Similarly, Mauviel *et al* (1993) have demonstrated that TGF-β1 and TGF-β2 are incapable of affecting cytokine induced IL-8 expression in human adult skin fibroblasts. If the inhibition of MIP-1α by TGF-β is purely an anti-inflammatory function of TGF-β, then these results are quite surprising. Indeed, the apparent ability of TGF-β to only inhibit those members of the chemokine family which are capable of inhibiting stem cell proliferation suggests that this may not simply be an anti-inflammatory function and may therefore be more specific to stem cell inhibition.

When attempting to relate these results to possible physiological consequences *in vivo*, it is necessary to take into account other molecules which could potentially affect expression of MIP-1α. Examples of such molecules include other members of the TGF-β superfamily, some of which are capable of exerting similar effects to TGF-β1 in certain biological systems. Recently, mice

carrying a disrupted TGF-β1 allele have been generated to try to unravel some of the diverse functions of TGF-β both in development and in the adult. As described in the introduction, these mice develop normally to birth, but die some weeks later due to uncontrolled inflammatory reactions. As part of their analysis, Schull *et al* (1993) examined inflammatory cytokine expression in the inflamed organs of these animals and, interestingly, observed a dramatic increase in the expression of MIP-1α. There are two possibilities which could account for this observation. The first is that MIP-1α is upregulated in response to other inflammatory mediators and general macrophage activation, and is part of the inflammatory cytokine cascade. The second is that, in light of the data presented here, MIP-1α is upregulated due to the removal of a specific block on MIP-1α expression by TGF-β1. MIP-1α could then potentiate the inflammatory response via induction of other pro-inflammatory molecules such as IL-1, IL-6 and TNF-α. Such a conclusion would predict that other members of the TGF-β superfamily such as TGF-β2 and TGF-β3 would be incapable of potently inhibiting MIP-1α expression in macrophages, or indeed other MIP-1α producing cells. The Northern blots shown in figures 5.4.1 and 5.4.2 demonstrate that this is not the case however, and that both TGF-β2 and TGF-β3 are capable of inhibiting MIP-1α expression in bone marrow derived macrophages. Further, TGF-β3 appears as potent as TGF-β1 in this regard, with TGF-β2 being slightly less potent.

The reason for this difference in potency is unclear, but similar differences in potency between the TGF-β isoforms have been reported previously (Ohta *et al* 1987). It is possible that the differences in potency between the isoforms results from differing abilities to bind to the specific TGF-β receptors. Indeed, Ohta *et al* (1987) observed that the binding affinity of TGF-β2 to the type I receptor present on B6SUtA cells was 20 fold lower than that of TGF-β1. This difference in affinities could perhaps explain the 100 fold higher potency with which TGF-β1 inhibits proliferation of B6SUtA cells. Further, Chiefetz *et al* (1990) have demonstrated that distinct TGF-β receptor subsets determine responsivensess of cells to each of the TGF-β isoforms. Lopez-Casillas *et al* (1993) have also

demonstrated that TGF-β2 binds to the TGF-β type II receptor with a lower affinity than TGF-β1. It would be of interest to perform TGF-β binding studies on bone marrow derived macrophages to determine if the degree of inhibition of MIP-1α expression in macrophages by TGF-β1,-β2, -β3 is related to the affinity with which each isoform binds to the cells.

Given that all of the TGF-β isoforms are capable of inhibiting MIP-1α expression, one might predict that the increased MIP-1α expression in TGF-β1 deficient mice is secondary to the absence of TGF-β1. However, it is still possible that this represents a specific effect, and that the other TGF-β isoforms in these animals are not expressed at high enough levels, or perhaps are expressed in different locations to TGF-β1, such that they are unable to actively inhibit MIP-1α expression.

Interestingly, more diverse members of the TGF-β superfamily such as activin and BMP-2 appear to be incapable of inhibiting MIP-1α gene expression in macrophages at concentrations equivalent to TGF-β1. Although activin has been shown to have a role in erythroid differentiation, and is capable of stimulating multipotential progenitor cells *in vitro* (Yu *et al* 1987, Broxmeyer *et al* 1988), it has not been reported as having stem cell inhibitory properties. Likewise for BMP-2. The fact that these molecules appear incapable of inhibiting MIP-1α expression again suggests that the relationship between TGF-β and MIP-1α may be specific and related to stem cell inhibition.

More recent experiments performed subsequent to and following on from the data presented in this thesis have investigated the possibility of an endogenous relationship between MIP-1α and TGF-β expression levels in bone marrow derived macrophages. To this end, endogenous TGF-β1 protein in bone marrow derived macrophage cultures was neutralised by adding an excess of TGF-β1 latency associated peptide (LAP), and the effect of LAP treatment on MIP-1α mRNA expression analysed by Northern blot. The results of this experiment demonstrate that MIP-1α expression is elevated (approximately 2 to 3 fold) in macrophage cultures treated with LAP suggesting that MIP-1α expression, at the mRNA level

at least, is endogenously suppressed by TGF-β1 (J.Maltman, G.Graham, unpublished observations). It remains to be seen whether this is effect is reflected at the protein level, but given the very low and often undetectable levels of MIP-1α produced by these cells, it is unlikely that such a small change would be detectable using conventional techniques. However, the results of this experiment suggest that the inhibitory activity of TGF-β on MIP-1α expression is not dependent on exogenous TGF-β addition and may also be at play *in vivo* resulting in undetectable MIP-1α protein secretion.

6.3 Relative expression of MIP-1α and TGF-β

These results raise questions over the actual role MIP-1α plays as an inhibitor of stem cell proliferation *in vivo*. Its effects on stem cells have been extensively studied *in vitro*, and it quite clearly functions as a potent inhibitor in certain *in vitro* and *in vivo* assay systems (Graham and Pragnell 1992). The potency with which TGF-β is capable of inhibiting MIP-1α expression suggests however that in normal bone marrow, MIP-1α expression will be permanently "turned off" or at least substantially downregulated in the presence of active TGF-β and in the absence of upregulators of MIP-1α expression. Interestingly, a recent study by Cluitmans *et al* (1995) has demonstrated both MIP-1α and TGF-β mRNA expression in human peripheral blood and normal bone marrow biopsy samples, suggesting that these molecules at least have the capacity to be co-expressed in normal bone marrow and that MIP-1α is not completely suppressed by endogenous TGF-β. Whether or not this is reflected at the protein level *in vivo* remains to be elucidated. From our own studies it is clear that although very low levels of MIP-1α mRNA expression can be seen in bone marrow macrophages, no MIP-1α protein can be detected in bone marrow extracts using sensitive assays. TGF-β1 protein on the other hand can be readily detected in bone marrow extracts using both sensitive ELISA based methods and the HT-2 bioassay (A.Sim personal communication). However, although TGF-β protein can be detected in both normal bone marrow and in long term bone marrow cultures (LTBM) (Cashman *et*

al 1990), it is difficult to assess how much is actually active, and therefore capable of inhibiting expression of MIP-1α. Evidence for the possible existence of biologically active endogenous TGF-β in LTBM cultures comes from the studies of Eaves *et al* (1991) whereby addition of anti-TGF-β antibodies to normal LTBM cultures resulted in activation of proliferation of quiescent primitive progenitors. Interestingly, and perhaps unexpectedly in the light of the data presented in this thesis, addition of anti-MIP-1α antibodies also results in stimulation of primitive progenitor cell proliferation also suggesting that MIP-1α protein is expressed and does play an endogenous inhibitor role. Thus it is possible that, although no MIP-1α protein can be detected in the LTBM cultures or real bone marrow, localised production of the molecule in the vicinity of the stem cell can produce high enough concentrations to elicit a biological response. The potent proteoglycan binding abilities of MIP-1α, and indeed its propensity to self-aggregate, may help in this context to build up high localised concentrations. If this is the case, proteoglycan immobilised MIP-1α would be undetectable in the supernatant of LTBM, and indeed in bone marrow extracts. It is thus very difficult to draw conclusions regarding both the abundance and the relative roles of each cytokine in *in vivo* haemopoiesis. It is clear however that although TGF-β is capable of potently inhibiting MIP-1α expression *in vitro*, MIP-1α expression is not completely suppressed in LTBMCs or in human peripheral blood or bone marrow.

6.4 Inhibition of MIP-1α specific receptors by TGF-β1

If one assumes that *in vivo*, in the presence of active TGF-β, very little MIP-1α will be produced, then it is of interest to investigate how the target cells of MIP-1α would respond to the presence of TGF-β. If MIP-1α does play an important endogenous physiological role in the control of stem cell proliferation, it is possible that the stem cells would respond to TGF-β and therefore the corresponding decrease in MIP-1α protein by upregulation of specific receptors for that molecule. This possible "yin-yang" relationship may act to compensate for the reduced levels of MIP-1α and maintain the stem cells in a quiescent state. To this

end, the effects of TGF-β on MIP-1α receptor levels on the actual target stem cells were investigated. As mentioned previously, FDCP-MIX cells are a CFU-A/CFU-S stem cell like line which are capable of self renewal and multilineage differentiation, and of forming spleen colonies in early passage (Dexter *et al* 1977, Schofield and Dexter 1985). They also express high levels of high affinity receptors for MIP-1α and have previously been shown to respond, though inconsistently, to its inhibitory properties (Graham *et al* 1993, Clements *et al* 1992). Thus these cells appeared ideal candidates for investigating the effect of TGF-β on MIP-1α receptors. Figures 5.5.1. to 5.5.3 in the results section clearly demonstrate that TGF-β is in fact a potent inhibitor of MIP-1α binding to the surface of FDCP-MIX cells. Scatchard analysis revealed that this was due to a reduction in receptor numbers with no change in affinity of the remaining receptors for ligand. Further, this effect was fully reversible upon removal of TGF-β from the medium. The possible significance of this receptor downregulation will be discussed in more detail below.

The MIP-1α receptor present on FDCP-MIX cells remains to be cloned and characterised. However, a receptor (CC-CKRI) specific for MIP-1α and other C-C chemokines has been cloned from HL60 cells (Neote *et al* 1993). This receptor is also expressed on U937 cells and THP-1 monocytes, and possibly on other cell types also (Avalos *et al* 1994). As mentioned in the introduction, the FDCP-MIX receptor is likely to be different from CC-CKRI, and thus it was of interest to investigate the specificity with which TGF-β downregulates the receptor type present on FDCP-MIX cells. Also, it was possible to investigate the potential mechanism of TGF-β mediated MIP-1α receptor downregulation in more detail. A cDNA probe was obtained for CC-CKRI, and the effect of TGF-β on mRNA expression of CC-CKRI in U937 cells was investigated. Interestingly, TGF-β appeared capable of downregulating CC-CKRI mRNA in this cell type suggesting that TGF-β may be capable of downregulating all receptors capable of binding MIP-1α, and probably achieves this through altering mRNA expression.

Again, this raises the question as to whether or not this is an inflammatory or a stem cell related phenomenon. It appears that TGF-β may be capable of downregulating receptors for MIP-1α on a number of cell types, including those cell types unresponsive to the inhibitory properties of MIP-1α, and suggests that this is a general function of TGF-β. Further evidence suggesting receptor downregulation by TGF-β to be a general phenomenon comes from observations that TGF-β is also capable of downregulating receptors on primitive haemopoietic cells for a number of other cytokines including GM-CSF, G-CSF and IL-3 (Jacobsen *et al* 1991). Indeed it has been suggested that this is one possible mechanism by which TGF-β acts to inhibit proliferation of these cell types. The observed downregulation of CC-CKRI may result in decreased sensitivity to MIP-1α (see below) and decreased inflammatory response of exposed cells. This may be part of the natural progression of an inflammatory reaction. For example, high concentrations of TGF-β are likely to be present in inflammatory foci due to platelet degranulation. TGF-β is capable of attracting monocytes and inducing expression of other pro-inflammatory cytokines such as TNF-α and IL-1 in these cell types (McCartney-Francis *et al* 1990). Conversely, upon monocyte differentiation into macrophages, TGF-β deactivates macrophages and reduces their ability to kill via the respiratory burst (Tsunawaki *et al* 1988). Thus, the downregulation of CC-CKRI may be part of the process by which TGF-β deactivates macrophages and reduces their ability to respond to inflammatory mediators such as the chemokines and in particular MIP-1α. This hypothesis suggests that the expression of CC-CKRI and MIP-1α is regulated by the presence of both TGF-β and other stimulatory cytokines. Interestingly, as will be discussed later, MIP-1α is active as an inducer of both TGF-β mRNA expression and protein production. It is likely that production of pro-inflammatory molecules such as MIP-1α need to be controlled such that excessive production and consequently unwanted tissue damage would not occur. In light of the presented data, it is possible that MIP-1α acts both to inhibit its own expression and to help dampen

down the inflammatory situation via induction of TGF-β in a negative feedback loop.

In summation, it appears that TGF-β is capable of downregulating both arms of the MIP-1α inhibitory machinery. That is, TGF-β downregulates MIP-1α mRNA and protein expression in the potential MIP-1α producing cells i.e. macrophages, and at the same time downregulates MIP-1α specific receptors on the target stem cells.

6.5 Significance of MIP-1α receptor downregulation

Although TGF-β is capable of downregulating MIP-1α specific receptors on FDCP-MIX cells to a maximum of between 50-70%, the functional significance of this downregulation required further investigation. Numerous other studies reporting on the physiological significance of decreased growth factor receptor expression have presented widely different results with respect to the levels or numbers of receptors required to elicit a full biological response. For example, it has been demonstrated that only 1% of IL-1 receptors need to be occupied for IL-1 to elicit a biological response whereas other groups have suggested that as much as 50-70% of cell surface receptors need to be occupied (Dinarello et al 1994). Thus, it is important to determine whether a 50-70% decrease in MIP-1α receptor numbers on FDCP-MIX cells (or indeed normal stem cells) will reduce the ability of MIP-1α to inhibit that cell type.

To address this possibility, it was decided to investigate the effect of TGF-β on MIP-1α signal transduction processes. As was mentioned in the introduction, the receptor for MIP-1α present on FDCP-MIX cells is likely to belong to the seven membrane spanning family of G-protein linked receptors. Signalling through such receptors classically results in an increase in intracellular calcium which then acts as a second messenger for further downstream events. Indeed, MIP-1α has previously been shown to elicit a calcium flux in a number of different cell types including eosinophils and monocytes (Rot *et al* 1993, Sozzani *et al* 1993). It was

of interest therefore to investigate the possible effects of TGF-β on the ability of FDCP-MIX cells to release calcium upon MIP-1α binding to its receptor. A reduction or indeed abrogation of this response by TGF-β would suggest that the receptor downregulation may indeed have physiological and functional consequences. Figure 5.6 in the results section shows that this appears to be the case. Overnight incubation of FDCP-MIX cells results in receptor downregulation and a substantial reduction in the ability of these cells to mobilise calcium in response to MIP-1α. It appears therefore that the TGF-β mediated downregulation of MIP-1α receptors on these cells will affect the MIP-1α signal transduction pathway and, presumably, further downstream events.

Given the reduction of MIP-1α receptors, and reduction in MIP-1α induced calcium mobilisation by TGF-β, it was anticipated that pre-treating normal bone marrow with TGF-β would result in downregulation of MIP-1α receptors on the CFU-A population, and thus these cells would be less responsive to inhibition by MIP-1α. Figure 5.7 shows that pre-incubating bone marrow cells with TGF-β does indeed reduce the potency of MIP-1α in the CFU-A assay and therefore clearly has functional consequences. It must be stressed here that the bone marrow cells were only exposed to TGF-β overnight, and were then thoroughly washed in PBS to remove any excess TGF-β. However, the number of CFU-A colonies formed using TGF-β pre-treated bone marrow only was still markedly reduced after the eleven day time course of this assay. This suggests two possibilities. The first is that washing the cells three times in PBS does not adequately wash off TGF-β bound to the cell surface, and therefore, the cells are still exposed to active TGF-β after initiation of the assay. Given that the assay is carried out over a period of eleven days however, it is unlikely that sufficient TGF-β will remain to cause such marked inhibition. It is probable that the exogenously added TGF-β will have been internalised and degraded early during the assay time course. The other, and more likely, possibility is that TGF-β is inducing programmed cell death, or apoptosis, in the majority of the CFU-A cells present in the bone marrow population during overnight incubation. However, when using lower

concentrations of TGF-β, some colony formation does still occur, and presumably these cells should still be open to inhibition by MIP-1α. The fact that MIP-1α appears incapable of adding to the "inhibitory" effect of TGF-β suggests that the TGF-β mediated downregulation of MIP-1α receptor numbers on the clonogenic cells does affect the ability of MIP-1α to inhibit CFU-A colony formation.

There is considerable precedent for suggesting that TGF-β may induce apoptosis in CFU-A cells since TGF-β1 has been shown to induce apoptosis in a number of both haemopoietic and non-haemopoietic cell types. For example, Bursch *et al* (1993) have demonstrated the involvement of TGF-β1 in the induction of apoptosis in certain epithelia *in vivo*. Chuang *et al* (1994) have further characterised the apoptotic signals TGF-β1 confers on human hepatoma cells and have shown that TGF-β1 induced cell death is independent of cytosilic calcium and protein kinase C. With respect to haemopoiesis, TGF-β1 can prevent the stem cell factor mediated rescue of mast cells from apoptosis after IL-3 deprivation (Mekori and Metcalfe 1994). Further, TGF-β2 induces apoptosis of murine T cell clones (Weller *et al* 1994), and TGF-β1 is capable of abrogating the effects of haematopoietins (IL-3, IL-5 and GM-CSF) on eosinophils and induces their apoptosis (Alam *et al* 1994). Interestingly, TGF-β1 also induces apoptosis in M1 myeloid leukaemia cells, and this effect is mediated by the primary response gene MyD1118, and the proto-oncogenes myb, myc and bcl-2 (Selvakuramin *et al* 1994).

Thus, there is considerable evidence that TGF-β can induce apoptosis in haemopoietic cell types, and it remains possible that this is occurring on the CFU-A stem cells. It would therefore be of interest to investigate if the CFU-A type cells incubated overnight with TGF-β exhibit any classical apoptotic morphology such as condensation of chromatin, blebbing of the cell surface and fragmentation of chromatin. However, it would be very difficult obtaining sufficient numbers of these cells for such analysis. Interestingly, data shown in figure 5.5.4 demonstrates that the inhibition of MIP-1α receptors on FDCP-MIX cells is reversible, implying

that TGF-β1 does not induce apoptosis in these cell types. If these cells were undergoing apoptosis then one would predict that, as time progressed, the ability of MIP-1α to bind to the cells would be markedly lower due to the decreasing number of viable cells present. However, MIP-1α receptor levels on those cells which received continuous addition of TGF-β over a 96hr period remained constantly downregulated by around 50% and this level of downregulation did not increase as time progressed. It appears therefore that TGF-β does not induce apoptosis in FDCP-MIX cells. This may however be a consequence of the fact that the FDCP-MIX cells used in the above mentioned experiment were always grown in the presence of the growth factor IL-3. In contrast, in the experiments involving TGF-β overnight treatment of bone marrow CFU-A cells, bone marrow cells were incubated overnight in medium containing only serum and no added growth factors. Normally, overnight incubation of bone marrow cells without growth factors has no great detrimental effect on the CFU-A compartment because colony formation by CFU-A cells is normal. However, the lack of growth factors and therefore positive growth stimuli may actually predispose these cells to the active induction of apoptosis by TGF-β. Therefore, it is possible that TGF-β, in contrast to MIP-1α, is actually an inducer of apoptosis in haemopoiesis rather than an inhibitor of proliferation.

6.6 Induction of TGF-β by MIP-1α

In order to fully understand the possible relationship between MIP-1α and TGF-β, both in the control of stem cell proliferation and also in potential inflammatory reactions, it was decided to examine any possible effects of MIP-1α on TGF-β mRNA and protein expression. Although it appears quite clear that TGF-β does not function as a stem cell inhibitor via induction of MIP-1α, it is still possible that MIP-1α functions, either wholly or in part, through induction of TGF-β.

To address this possibility, the effect of MIP-1α on TGF-β expression was examined in bone marrow derived macrophages, which are capable of expressing

both TGF-β mRNA and protein. Indeed, MIP-1α and IL-2 appear to be capable of inducing both TGF-β mRNA and protein production by these cell types. This effect is enhanced to a level greater than that achieved using either cytokine alone when the cells are treated with both MIP-1α and IL-2, a known inducer of TGF-β expression. These results suggest that MIP-1α may indeed function as a stem cell inhibitor via induction of TGF-β expression in secondary cell types such as macrophages present within the bone marrow microenvironment or indeed in target cells. Such a conclusion would predict that MIP-1α will be unable (or less able) to inhibit stem cell proliferation in the presence of molecules which act to inhibit the function of TGF-β. A number of such molecules are available for neutralising TGF-β bioactivity including anti-TGF-β antibodies, the TGF-β type II receptor in soluble form and the TGF-β1 latency associated peptide (LAP), a natural and potent TGF-β1 antagonist. Since LAP appeared to be the most potent neutraliser of TGF-β1 activity, it was decided to use this molecule to investigate if MIP-1α was capable of inhibiting CFU-A colony formation in the presence of blockers of TGF-β function. The results shown in figure 5.9 demonstrate that this is clearly the case, with LAP having no effect on the ability of MIP-1α to inhibit CFU-A cells. It is capable however of reversing the ability of exogenously added TGF-β to inhibit CFU-A cells indicating that LAP is efficiently neutralising the activity of TGF-β in this assay system. Thus, although MIP-1α is capable of inducing TGF-β expression in macrophages, it does not appear to function as a stem cell inhibitor through this mechanism. Indeed, the results presented in this thesis suggest that although MIP-1α and TGF-β appear to have overlapping functions in the control of stem cell proliferation, they function in this context independently of each other.

6.7 The role of MIP-1α and TGF-β in haemopoiesis?

When attempting to relate this data to possible *in vivo* consequences, it is necessary to take into account all of the other molecules which are at play during steady state and aberrant haemopoiesis. As was mentioned above, these

experiments were performed *in vitro*, and thus cannot adequately recreate the complexity of the bone marrow. However, the results suggest firstly that in the presence of active TGF-β *in vivo*, very little MIP-1α will be produced, and secondly, due to receptor downregulation, the target stem cells may not be able to respond to any MIP-1α which is present. However, steady state haemopoiesis is likely to be controlled by an intricate interplay between various different molecules, many of which are potentially capable of stimulating proliferation of stem cells. It is possible that many of these molecules are capable of positively regulating the expression of MIP-1α and that the expression of MIP-1α is regulated by a balance between TGF-β and stimulators of MIP-1α expression. This remains to be clarified.

The results presented in this thesis raise the question as to what role each of these molecules plays in the overall control of haemopoietic stem cell proliferation. Clearly they are able to inhibit the same population of stem cells through apparently independent mechanisms. It is interesting to note however that TGF-β is capable of inhibiting a much wider spectrum of cell types than MIP-1α. It may be that the endogenous role of TGF-β in haemopoiesis is to inhibit subpopulations of stem cells not open to control by MIP-1α such as pre-CFU-S. It is also possible that MIP-1α may only be required as a stem cell inhibitor in times of extreme stress e.g. a systemic infection or excessive inflammation. Such a scenario would require generation of large numbers of mature immune cells such as macrophages and neutrophils. These cells are likely to be generated by the induction of CFU-S type cells into cell cycle. The role of MIP-1α as a stem cell inhibitor may be to act as a negative feedback molecule to control the production of mature myeloid cells, and at the same time upregulate TGF-β expression such that TGF-β can act to dampen down the inflammatory response through its wide range of anti-inflammatory functions. Thus, when enough mature myeloid cells have been produced to deal with the inflammation, presumably activation of such large numbers of immune cells would result in an increase in expression of MIP-1α. The increased concentration of this molecule in the bloodstream would feed back to the bone

marrow, where it can then act to inhibit the cycling CFU-S compartment thus controlling the overall levels of mature myeloid cells produced. While TGF-β expression may also be increased at the site of inflammation, the levels of active TGF-β are likely to be very localised. Active TGF-β has a very short half-life and is bound very quickly by the serum component β2-macroglobulin (O`Connor-McCourt and Wakefield 1987), and thus it is unlikely that sufficient amounts of active TGF-β will feed back to the bone marrow and inhibit the bone marrow resident CFU-S cells. Thus, TGF-β may be involved in steady state inhibition of stem cells in the locality of the bone marrow microenvironment whereas MIP-1α may be involved in stem cell inhibition in times of stress. In addition, MIP-1α may play a role in controlling proliferation of peripheral CFU-S stem cells present in the blood. By the same criteria above, it is unlikely that TGF-β would play a major role in this context due to its very short half life in the circulation.

6.8 Summary, conclusions and future prospects

The aim of this thesis was to try to elucidate the functional roles of the inhibitors of haemopoietic stem cell proliferation. In particular, MIP-1α and TGF-β appeared to have many similar functions in this regard perhaps suggesting that there may be some form of productive interaction between them. That is, one may work indirectly via induction of the other. As has been described above, this appears not to be the case. TGF-β acts as a potent inhibitor of both MIP-1α mRNA expression and protein production in macrophages, and also downregulates MIP-1α specific receptors on the stem cell like line FDCP-MIX. This demonstrates that TGF-β is unlikely to function via induction of MIP-1α. On the other hand, MIP-1α appears to act as an inducer of both TGF-β mRNA and protein expression by macrophages suggesting that MIP-1α may work via induction of TGF-β. However, inactivation of endogenous TGF-β does not appear to affect either the ability or the potency of MIP-1α to inhibit CFU-A colony. In

conclusion, the data presented in this thesis suggests that MIP-1α and TGF-β inhibit the proliferation of stem cells through, initially at least, independent mechanisms. It is still unclear whether the reciprocal effects between MIP-1α and TGF-β are a consequence of inflammatory regulation, but the fact that TGF-β cannot inhibit expression of other pro-inflammatory chemokines such as IL-8 or RANTES suggests these interactions may be more specific to control of stem cell proliferation. Both of these molecules have the capacity to be expressed in the bone marrow where they can presumably interact in the vicinity of the stem cells. This work presents evidence for a heirarchy of stem cell inhibitors such that TGF-β will be dominantly expressed over MIP-1α. However, it is still unclear what might happen in an *in vivo* situation, where upregulators of MIP-1α expression are likely to be present. Addition of either anti-MIP-1α antibodies or anti-TGF-β antibodies to LTBMCs results in entry of primitive cells into cycle suggesting that although TGF-β may be dominantly expressed in bone marrow macrophage cultures, both are endogenously active in LTBMCs (Eaves *et al* 1991). In conclusion, although TGF-β can downregulate both arms of the MIP-1α inhibitory machinery, this is not sufficient to wipe out endogenous activity of MIP-1α as a stem cell inhibitor.

It would be interesting to examine this scenario in more detail, perhaps using a murine model undergoing extensive immune reactions, such that the relative expressions of MIP-1α and TGF-β can be more easily determined. When the putative inhibitory receptor for MIP-1α has been cloned and characterised, signal transduction analysis will be possible. It will be of interest to compare and contrast the resultant changes in gene expression by both MIP-1α and TGF-β. Although the initial signal transduction systems are likely to be distinct due to the different receptor types for these molecules, it is possible that the similar functions of these molecules may be due to similar changes in the expression of genes regulating cell cycle progression and growth arrest.

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