The effects of fibroblast growth factor-2 on human bone marrow cells.

by
Melanie-Jane Hannocks

Supervisor: Prof. E. Lynette Wilson

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Declaration.

PhD THESIS TITLE: THE EFFECTS OF FIBROBLAST GROWTH FACTOR-2 ON HUMAN BONE MARROW CELLS.

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Index.

Abstract.

Fibroblast growth factor-2 (FGF-2), a potent angiogenic growth factor, induces the generation of plasmin which, in turn, regulates cell migration and growth factor activity. FGF-2 acts on both primitive and committed haematopoietic progenitor cells, directly and in concert with other haematopoietic growth factors, to induce their proliferation and/or differentiation. FGF-2 is expressed by bone marow stromal cells and in the bone marrow stromal microenvironment, is found bound to heparan sulphate proteoglycans, forming a reservoir of biologically active growth factor protected from proteolytic degradation.

To functionally characterise FGF-2 as a haematopoietic growth factor, I investigated the role of FGF-2 in haematopoiesis *in vitro* in human stromal cell and long-term bone marrow cultures. I focused my analysis on three aspects:

1. the effects of FGF-2 on the stromal compartment,
2. the effects of FGF-2 on haematopoietic cell production, and
3. the potential mechanism of FGF-2 action via the regulation of the plasminogen activator/plasmin system.

I observed that FGF-2 had dramatic effects on the morphology, proliferation and senescence of bone marrow stromal cells. Nanogram concentrations of FGF-2 greatly accelerated the formation of the stromal layer and caused the cells to become spindle shaped, to loose their contact inhibition, and to grow to high cell densities in multilayered sheets. I also noted that bone marrow stromal cell senescence was considerably delayed when the cells were cultured continuously in the presence of this growth factor.

In human long-term bone marrow cultures, I found that FGF-2 significantly increased the number of haematopoietic progenitor cells in both the adherent layer and supernatant. This was due to an increase in the numbers of granulocyte-macrophage-colony stimulating factor- and granulocyte-colony stimulating factor-responsive progenitor cells.

In addition, I found that FGF-2, and two other growth factors relevant to haematopoiesis, interleukin-1 and transforming growth factor-ß, regulated the production of plasminogen activators and, in some cases, their specific inhibitors, plasminogen activator inhibitor-1 and -2, by bone marrow stromal fibroblasts. FGF-2 increased predominantly tissue-type plasminogen activator and, interleukin-1 and transforming growth factor, urokinase-type plasminogen activator. Therefore, in the vicinity of haematopoietic cells, plasmin can be generated which can modulate growth

factor activity via activation of latent or inactive growth factors, mobilisation of growth factors from the cell surface/extracellular matrix, or the shedding of growth factor receptors from cell surfaces. Thus, the plasminogen activator/plasmin system can potentially regulate progenitor cell development.

In summary, my work indicates that FGF-2 has potent effects on both the stromal and haematopoietic compartments. It regulates stromal cell morphology and significantly stimulates stromal cell proliferation and myelopoiesis, the latter possibly indirectly by regulating the plasmin- or t-PA-catalysed processing of growth factors and/or receptors. I therefore conclude that FGF-2, as well as the proteolytic cascade of plasminogen activation, have the potential to be used clinically to enhance haematopoiesis *in vivo* and deserves further investigation.

Abbreviations.

αMEM: alpha minimal essential medium
FGF-2: fibroblast growth factor-2
BM: bone marrow
BFU-E: burst forming unit-erythroid
CAMs: cell adhesion molecules
Cdks: cyclin dependent kinases
CFU-Bas: basophil colony forming unit
CFU-E: erythroid colony forming unit
CFU-Eos: eosinophil colony forming unit
CFU-F: fibroblast colony forming unit
CFU-GEMM: granulocyte erythroid macrophage megakaryocyte colony forming unit
CFU-GM: granulocyte macrophage colony forming unit
CFU-Meg: megakaryocyte colony forming unit
CFU-s: colony forming unit spleen
CSFs: colony stimulating factors
DNA: deoxyribose nucleic acid
EBV: Epstein Barr virus
ECM: extracellular matrix
EGF: epidermal growth factor
Epo: erythropoietin
FCS: fetal calf serum
GAGs: glycosaminoglycans
G-CSF: granulocyte-CSF
GM-CSF: granulocyte-macrophage-CSF
GTP: guanosine-5' triphosphate
HEMCAM: haematopoietic cell adhesion molecule
HGF: hepatocyte growth factor
HLA-DR: histocompatibility class II antigen,
HP-CAM: haematopoietic progenitor cell adhesion molecule
HPP-CFC: high proliferating potential-colony forming cell
HSPG: heparan sulphate proteoglycan
ICAM: intercellular cell adhesion molecule
IFN: interferon
IGF: insulin-like growth factor
IL: interleukin
LFA-1: lymphocyte function-related antigen-1
LIF: leukaemia inhibitory factor
LTBM: long-term bone marrow
LTC-ICs: long-term culture-initiating cells
MAPCs: multipotent adult progenitor cells
M-CSF: macrophage-CSF
Meg-CSF: megakaryocyte-CSF
MIPs: macrophage inflammatory proteins
MSCs: mesenchymal stem cells
NOD: non-obese diabetic
PA: plasminogen activator
PAI: plasminogen activator inhibitor
PARs: protease activated receptors
PBS: phosphate buffered saline
PDGF: platelet derived growth factor
mRNA: messenger ribonucleic acid
si RNA: small interfering RNA
RT-PCR: reverse transcriptase-polymerase chain reaction
RS: recycling stem cells
SCF: stem cell factor
SCID: servere combined immune-deficient
SD: standard deviation
SDF-1α: stromal derived factor-1α
SDS: sodium dodecyl sulphate
SRC: SCID repopulating cell
SV40: simian virus 40
TCA: trichloroacetic acid
t-PA: tissue type plasminogen activator
TGF-α: transforming growth factor-alpha
TGF-ß: transforming growth factor-beta
TNF: tumour necrosis factor
Tpo: thrombopoietin
u-PA: urokinase type plasminogen activator
VCAM-1: vascular cell adhesion molecule-1
VLA: very late antigen

Chapter 1.

Introduction.

The regulation of haematopoiesis is complex and involves cellular interactions as well as a variety of growth factors. For my thesis I chose to investigate the role of FGF-2 in haematopoiesis *in vitro* in human stromal cell and long-term bone marrow cultures. I analysed on the effects of FGF-2 on the stromal compartment, haematopoietic cell production in LTBM cultures, and the potential mechanism of FGF-2 action via regulation of the plasminogen activator/plasmin system.

1. FGF-2 and the FGF family.

FGFs make up a large family of polypeptide growth factors, of which twenty-three members in vertebrates have so far been described, ranging in molecular mass from 17 to 34 kDa and sharing 13 - 71% amino acid identity (Ornitz and Itoh, 2001; Galzie et al., 1997). FGF-2 and FGF-1 were the first to be isolated and were originally named basic and acidic FGF, respectively, based on their isoelectric points (Ornitz and Itoh, 2001; Galzie et al., 1997). Other members of the FGF family are described as oncogene products (FGF-3 as *int-2*, FGF-4 as *hst-1*, FGF-5, and FGF-6 as *hst-2*) or tissue-specific growth factors (e.g. FGF-7 as keratinocyte growth factor, FGF-8 as androgen-induced growth factor, and FGF-9 as glia-activating factor)(Ornitz and Itoh, 2001; Galzie et al., 1997).

FGF-2, as well as FGF-1, were characterised over a decade ago as angiogenic and pleiotropic factors involved in the regulation of the proliferation and differentiation of numerous cell types (Schofield and Gallagher, 1994; Rifkin and Moscatelli, 1989). *In vivo*, FGF-2 has been shown to initiate neo-vascularisation, wound repair, and mesoderm formation during embryonic development (Rifkin and Moscatelli, 1989). *In vitro* studies show FGF-2 to be a potent autocrine growth factor for endothelial cells inducing cell migration, cell proliferation, PA and metalloproteinase production (Sato and Rifkin, 1988). However, the recent generation and characterisation of mice lacking FGF-1, FGF-2, or FGF-1 and FGF-2, which are viable and fertile, has put question to the relevance of these two growth factors in development and homeostasis (Miller et al., 2000; Ortega et al., 1998; Dono et al., 1998; Ozaki et al., 1998). It has been suggested that FGF-2 and FGF-1 only play a role in specific situations, such as following stress or injury (Pintucci et al., 2002; Pellieux et al., 2001; Yoshimura et al., 2001; Miller et al., 2000), and a limited role in

physiological processes (Miller et al., 2000). However, it is also possible that there is a high degree of redundancy and that other FGFs or growth factors are compensating for the lack of FGF-1 and FGF-2 in these mice. Other members of the FGF family can bind to the same 4 high affinity FGF-receptors identified so far compensating for the lack of FGF-2 and/orFGF-1. For example, FGF-3 as well as FGF-2 can induce the differentiation of epithelial cells (Venesio et al., 1992).

FGF-2 has been isolated from a number of organs and is synthesised by normal and tumour cell lines (Gospodarowicz et al., 1987b; Moscatelli et al., 1986). Multiple molecular weight forms of human FGF-2 have been identified (of approximately 18, 22, 22.5, and 24 kDa) resulting from alternative translation initiation of a single mRNA (Florkiewicz and Sommer, 1989; Prats et al., 1989). The higher molecular weight forms are found located in the cell nucleus, whereas the 18 kDa isoform is found preferentially in the cytoplasm of FGF-2-expressing cells (Florkiewicz et al., 1991; Renko et al., 1990). FGF-2 does not contain the classical signal sequence necessary for secretion and, so far, no mechanism of release from cells has been characterised besides release from dead or injured cells (Nickel, 2003). Nevertheless, FGF-2 is found bound to HSPGs in the ECM and on cell surfaces where it forms a reservoir of biologically active growth factor (Brunner et al., 1991 and 1993; Saksela et al., 1988; Vlodavsky et al., 1987) which is protected from degradation by extracellular proteinases (Saksela et al., 1988). Biologically active FGF-2-HSPG complexes can be proteolytically released from the ECM or cell surfaces (Whitelock et al., 1996; Rich et al., 1996; Brunner et al., 1994 and 1991) enabling the complex to mediate its biological activity by interacting with high-affinity FGF-receptors (FGFR) on cells at adjacent sites.

The biological activity of FGF-2 is mediated through interaction with high-affinity FGF-receptors (FGFRs) as well as low-affinity heparan sulphate proteoglycan (HSPG)-receptors (Yayon et al., 1991; Rapraeger et al., 1991; Rhogani et al., 1994). Four high affinity FGFRs have been identified to date: FGFR-1 (Dionne et al., 1990), FGFR-2 (Housssaint et al., 1990), FGFR-3 (Keegan et al., 1991), and FGFR-4 (Partanen et al., 1991), coded for by separate genes. For FGFR-1, FGFR-2, and FGFR-3, alternative splicing gives rise to multiple isoforms (Galzie et al., 1997; Schofield and Gallagher, 1994). Several mechanisms have thus evolved to regulate signalling including cell type-specific alternate splicing of the receptors, selective recognition of growth factor ligands, and the heparan sulphate cofactor requirement for ligand binding receptors (Schofield and Gallagher, 1994). For example, the isoforms of FGFR-1 and FGFR-2 that vary in the third immunoglobulin loop region include soluble

receptors (Schofield and Gallagher, 1994) as well as two isoforms with different ligand-binding specificities. A single cell will express one of the membrane-bound splice variants, FGFR-2-IIIb or FGFR-2-IIIc, resulting in specific binding of FGF-7 or FGF-2 respectively (Galzie et al., 1997; Schofield and Gallagher, 1994). Furthermore, the secreted form of FGFR-1 binds FGF-2 but not FGF-1 with high affinity (Galzie et al., 1997; Schofield and Gallagher, 1994). Nevertheless there is still considerable overlap between the binding sites of the FGFs and the different FGFRs, and the relevance therefore of the various FGFR isoforms is still unclear.

2. FGF-2 and haematopoiesis.

Although FGF-2 is expressed mostly in tissues of mesodermal and neuroectodermal origin, the role of FGF-2 in regulating the haematopoietic system, which is of mesodermal origin, is not fully understood (Moroni et al., 2002). FGF-2 is produced by human primary BM stromal cells as well as haematopoietic cells (Yoon et al., 2001; Brunner et al., 1993; Blotnick et al., 1994).

FGF-2 can increase the number of primitive progenitor cells by acting as a survival factor. Recently, haematopoietic progenitor cell lines derived from embryonic stem cells (e.g. the multipotent haematopoietic progenitor cell line, A6) have been isolated that are dependent on FGF-2 for their self-renewal (Faloon et al., 2000; Anzai et al., 1999). In addition, FGF-2 increases the number of murine haematopoietic cells in the spleen colony forming assays after 9 and 12 days of culture (Gallicchio et al., 1991). FGF-2, although ineffective on its own, also enhances the colony stimulating activity of GM-CSF and IL-3 on primitive haematopoietic progenitor cells with a $CD34^{+}CD33^{-}$ phenotype (Gabbianelli et al., 1990). Furthermore, FGF-2 synergises with SCF to augment GM-CSF-mediated progenitor cell growth (Gabrilove et al., 1994) and can also partially counteract the suppressive effects of TGF-ß on these cells (Gabrilove et al., 1993). In addition, FGF-2 can enhance the differentiation of primitive progenitor cells by acting in concert with other growth factors. For example, FGF-2 synergises with SCF, IL-3, IL-6, IL-11, GM-CSF, and Epo to support the megakaryocyte differentiation of embryonic stem cells (Berthier et al., 1997). However, FGF-2 can also inhibit differentiation of progenitor cells. This growth factor can antagonize transforming growth factor-beta mediated erythroid differentiation in K562 cells (Burger et al., 1994).

FGF-2 can also influence specific lineage-derived haematopoietic progenitors. For example, FGF-2 is a potent mitogen for the primitive erythroid cell line, EryP (Yuen et al., 1998). FGF-2 acts synergistically with GM-CSF, Epo and Meg-CSF to

induce committed precursors giving rise to GM-colonies, erythroid bursts and megakaryocyte colonies, respectively (Gallicchio et al., 1991). Further research has shown that FGF-2 directly promotes megakaryocyte progenitor cell growth and, in addition, stimulates BM accessory cells to release growth factors such as IL-3 and GM-CSF which have megakaryocyte colony stimulating activity (Han et al., 1992; Bikfalvi et al., 1992; Bruno et al., 1993). The enhancing effects of FGF-2 on megakaryocytopoiesis may also involve the stimulation of IL-6 secretion by megakaryocytes as well as the adhesion of these cells to the stromal fibroblasts (Bikfalvi et al., 1992, Avraham et al., 1994).

Taken together, these findings show that FGF-2 *in vitro* acts on both primitive and committed progenitor cells directly or in concert with other haematopoietic growth factors to induce their proliferation and/or differentiation and, therefore, may play a role in haematopoiesis.

Nevertheless, in vivo experiments suggest otherwise, since mice lacking FGF-1, FGF-2, or FGF-1 and FGF-2, exhibit no significant haematopoietic defects (Miller et al., 2000). However, mice lacking FGF-2 display impaired proliferation and differentiation of their haematopoietic progenitor cells in culture which cannot be compensated for by FGF-1 (Miller et al., 2000). This impairment appears to be due to a defect in the stromal cell layer rather than in the haematopoietic cells (Miller et al., 2000). In such mice, other FGFs may be compensating for the lack of FGF-1 and FGF-2, or possibly another signal that is lost upon culture, since, *in vitro* at least, FGF-4 stimulates haematopoiesis (Quito et al., 1996).

3. Haematopoiesis

The process of haematopoiesis generates the various types of cells found in peripheral blood and in some tissues such as thymus, liver, spleen and lymph nodes. These cells comprise macrophages, basophils, eosinophils, neutrophils, megakaryocytes/platelets, erythrocytes and B- and T-lymphocytes, all of which arise from a common, primitive, pluripotent stem cell (Fig. 1). In adult mammals, haematopoiesis occurs almost exclusively in the intersinusoidal spaces of the marrow cavity known as haematopoietic cords (Dorshkind, 1990). Mature blood cells have a finite life span and, thus, must be constantly replaced throughout the lifespan of the organism.

Pluripotent stem cells can either undergo self-renewal or differentiate into myeloid or lymphoid stem cells (Fig. 1) (Markus et al., 2002; Weissman et al., 2001). These stem cells differentiate further to give progeny that are lineage- restricted,

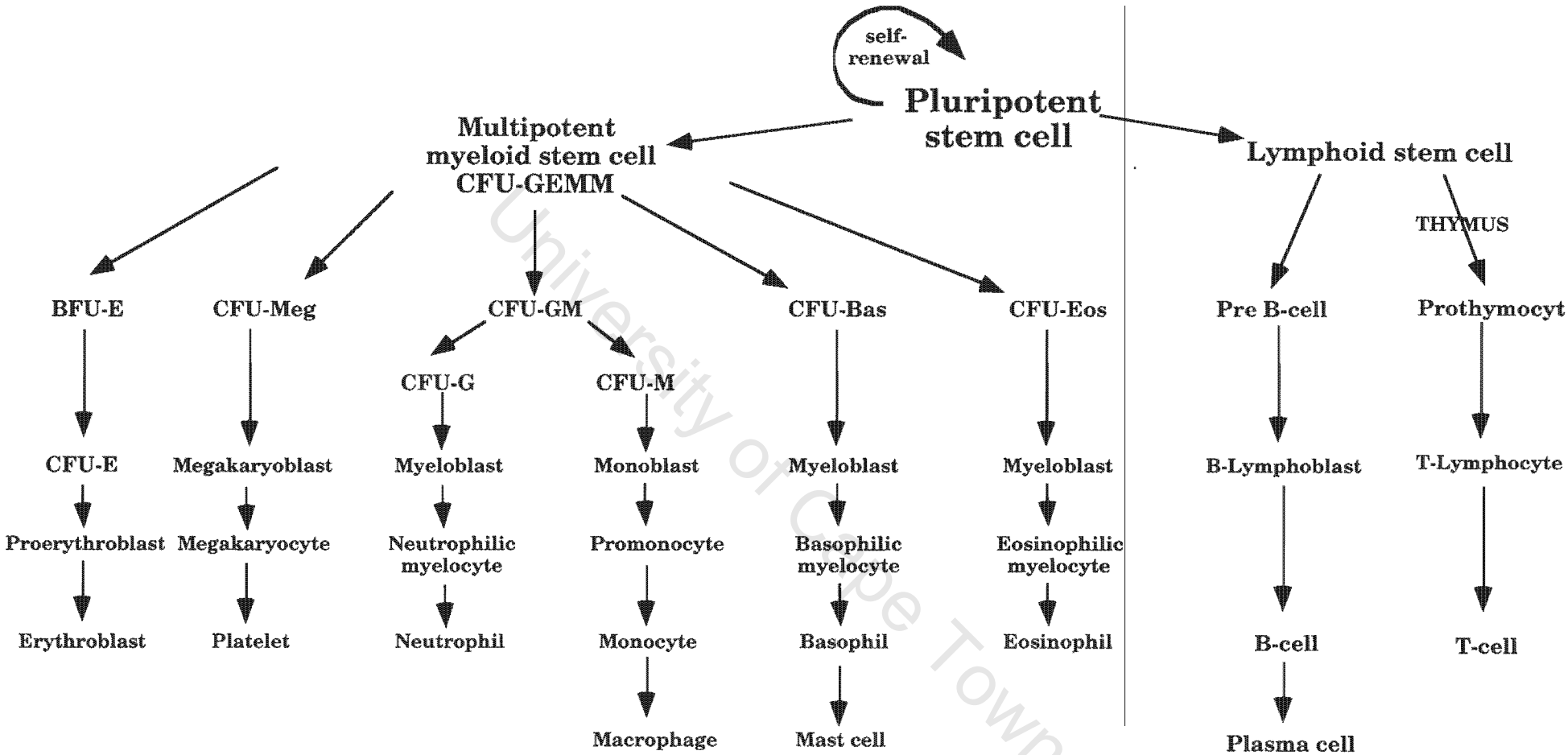

Fig. 1. The haematopoietic lineages. Primitive pluripotent stem cells can either undergo self-renewal or differentiate into multipotent myeloid or lymphoid stem cells. These multipotent stem cells become committed to development, differentiating further to form lineage-restricted progenitor cells: erythroid-burst forming unit (BFU-E), erythroid-colony forming unit (CFU-E), megakaryocyte-CFU (CFU-Meg), granulocyte macrophage-CFU (CFU-GM), granulocyte-CFU (CFU-G), macrophage-CFU (CFU-M), basophil-CFU (CFU-Bas), eosinophil-CFU (CFU-Eos), pre-B-cells and prothymocytes.

committed progenitor cells (Weissman et al., 2001). Myeloid progenitor cells include the erythroid burst forming unit (BFU-E), granulocyte macrophage colony forming unit (CFU-GM), eosinophil colony forming unit (CFU-Eos), megakaryocyte colony forming unit (CFU-Meg), and basophil colony forming unit (CFU-Bas) (Fig. 1). Lymphoid stem cells give rise to pre-B-cells and prothymocyte progenitor cells (pre-T-cells) (Fig. 1) (Kondo et al., 1997).

Myeloid and lymphoid progenitor cells continue to divide and differentiate and, therefore, one pluripotent stem cell can give rise to thousands of mature blood cells. However, the pluripotency of BM stem cells also allows these cells to give rise to mature cells of multiple mesenchymal cell types. BM stem cells can also differentiate into marrow stromal cells, endothelial cells, as well as osteoblasts, chondrocytes, adipocytes, heart and skeletal myoblasts, and epithelial cells of the liver, lung, kidney, skin and GI tract (Herzog et al., 2003; Jiang et al., 2002a and b; Reyes et al., 2002 and 2001; Krause, 2002; Stocum, 2001). Therefore, there may be a common stem cell within the BM for stromal cells (see section 3.1), haematopoietic cells, as well as epithelial cells, which has the potential to form functional tissue when engrafted into other tissues such as the brain or cardiac muscle (Zhao et al., 2002; Stocum, 2001; Goodell et al., 2001).

3.1. *Stromal components involved in haematopoiesis.*

A highly organised stromal component in BM supports the proliferation and differentiation of the haematopoietic cells. The stromal component is comprised of two major cell types: reticular cells (adventitial and fibroblastic) which are of mesenchymal origin and macrophages which are of haematopoietic origin. Adipocytes are also found in the marrow cavity and are thought to function as a 'flexible cushion', increasing when haematopoietic activity is low to prevent the empty cord space from collapsing, and decreasing when haematopoiesis is stimulated (Tavassoli, 1989). The reticular cells provide both nutrients as well as a scaffolding for developing haematopoietic cells (Wilson and Tavassoli, 1994; Shaklai, 1989). Macrophages take up and digest the extruded nuclei of developing erythroid cells as well as the remains of megakaryocytes after the megakaryocytes have completely expelled their platelet-rich cytoplasm into the circulation (Wilson and Tavassoli, 1994). Macrophages also play a nutritive role in erythropoiesis, supplying iron and growth factors to the erythroid progenitors (Wilson and Tavassoli, 1994).

4. *In vitro* culture models of haematopoiesis.

The establishment of *in vitro* long-term BM (LTBM) cultures supporting myelopoiesis (Dexter cultures) (Dexter et al., 1977a) and cultures supporting B-lymphocyte production (Whitlock cultures) (Whitlock and Witte, 1982) have created model systems that closely mimic haematopoiesis in its natural environment (see Chapter 2, section 4 and Table 1 for the culture conditions). After culturing freshly isolated BM mononuclear cells for ten to fourteen days, an adherent stromal cell layer develops, initially with the appearance of long, spindle shaped cells forming fibroblastic colonies (Colter et al., 2000 and 2001; Prockop et al., 2001). The fibroblastic cells spread out over the tissue culture dish forming a "blanket" layer comprising of fibroblasts with characteristics of vascular smooth muscle cells, macrophages, endothelial cells and adipocytes, (Dennis and Charbord, 2002). Primitive and committed progenitor cells become lodge in this layer to form "cobblestone" colonies (Moore et al., 1997). Both mature cells and progenitor cells at various stages of development are released into the supernatant.

4.1. *Types of haematopoiesis supported in LTBM cultures.*

Dexter LTBM cultures primarily support the growth and differentiation of progenitor cells of the granulocyte-macrophage lineages (Quesenberry, 1991). Erythropoiesis and megakaryocytopoiesis also occur in Dexter LTBM cultures but to a limited extent. The growth and differentiation of these lineages require the addition of growth factors. Epo, in combination with SCF and IL-3, SCF and IL-11, FGF-4, or HGF supports the growth and differentiation of primitive erythroid cells (Hassan et al., 1996; Quito et al., 1996; Galimi et al., 1994). The addition of Tpo, the principal regulator of megakaryocytopoiesis and platelet formation, augments megakaryocyte production which is further enhanced in the presence of GM-CSF, IL-1, or SCF and IL-3 (Ziegler et al., 1994; Banu et al., 1995; Angchaisuksiri et al., 1996; Dolzhanskiy et al., 1997). Furthermore, human LTBM cultures treated with a neutralising anti-TGF-ß antibody exhibit significant numbers of megakaryocytes suggesting a role for TGF-ß in down-regulating CFU-Meg differentiation (Waegell et al., 1994).

Whitlock-Witte LTBM cultures support the growth and differentiation of B-lymphocytes which is significantly enhanced by the addition of IL-7 (Winkler et al., 1995; Moreau et al., 1993).

Thus, Dexter and Whitlock-Witte LTBM cultures permit studies on the role of the stromal cells, extracellular matrix (ECM) and growth factors, which make up the

haematopoietic microenvironment, in the growth and differentiation of primitive and committed progenitor cells.

5. Regulation of haematopoiesis by the adherent layer.

The specific role of each stromal cell type in haematopoiesis is poorly understood. It is known, however, that the stromal compartment produces growth factors, cytokines, adhesion molecules, ECM molecules, and enzymes that support and regulate the proliferation and differentiation of primitive and committed progenitor cells (Sensebe et al., 1997*a* and *b*; Witte et al., 1993; Kittler et al., 1992; Eaves et al., 1991). In some cases these factors are critical for haematopoiesis to occur. For example, stromal cells that do not express growth arrest-specific gene-6 are unable to support the growth of haematopoietic stem cells (Dormady et al., 2000). The mechanism by which this occurs is unknown but contact between stromal cells and haematopoietic cells producing the receptor Axl (Heide et al., 1998), is important (Dormady et al., 2000).

In vivo, stem cells and maturing haematopoietic cells exhibit distinct and consistent lineage-specific spatial locations (Nilsson et al., 2001; Jacobsoen and Osmond, 1990; Shaklai, 1989). It is hypothesised that the heterogeneous nature of the stromal cells allows "niches" to be created in which haematopoietic stem cells are maintained and, in which progenitor cells can differentiate along a certain lineage defined by the microenvironment of that niche (Whetton and Graham, 1999; Craddock et al., 1997; Wang and Sullivan, 1993). The microenvironment of a niche would be created by the stromal production of specific molecular signals that mediate haematopoiesis (Nilsson et al., 1998; Gupta et al., 1998; Simmons et al., 1994; Crittenden et al., 1992). Thus, while most stromal cell lines support mutilineage progenitor cells (Wineman et al., 1996; Li et al., 1997; Kameoka et al., 1995), this appears to be less efficient than heterogenous cultures as niches supporting stem cells are not provided (Koller et al., 1997). Indeed, stromal cell lines that maintain stem cells *in vitro* for prolonged periods of time are rare (Wineman et al., 1996; Müller-Sieburg and Deryugina, 1995) indicating that stem cells interact selectively with distinct stromal cell types.

5.1. *Regulation of haematopoiesis through cell-cell, cell-matrix, and growth factor-matrix interactions.*

Multiple types of ECM macromolecules (including collagen I, III, IV and V, fibronectin, laminin, haemonectin, thrombospondin, vitronectin, tenascin, and

CD164), CAMs, (including intercellular-CAM-1, vascular-CAM-1, haematopoietic-CAM) as well as proteoglycans, and integrins such as the very late antigens (VLA)-4 and -5 (or CD49d/CD29 and CD49e/CD29, respectively), and lymphocyte function-related antigen-1 (LFA-1 or CD11a/CD18) have been identified in *in vitro* culture systems (Simmons et al., 1994 and 1997; Ohta et al., 1998; Nilsson et al., 1998; Campbell et al., 1987; Zannettino et al., 1998; Gu et al., 2003; Dittel et al., 1993; Vainio et al., 1996; Siczkowski et al., 1992; Liesveld et al., 1993; Simmons et al., 1994) Some ECM molecules play a dual role in haematopoiesis by acting as a stimulator/inhibitor in addition to providing anchorage. For example, fibronectin stimulates erythroid progenitor proliferaton (Weinstein et al., 1989), thrombospondin inhibits megakaryocytopoiesis (Chen et al., 1997), and CD164 (sialomucin) suppresses progenitor cell proliferation (Zannettino et al., 1998).

Similarly, proteoglycans also play an important role in the presentation of growth factors and cytokines that regulate stem cell and progenitor cell proliferation and differentiation apart from regulating cell adhesion (Table 1). Studies have identified multiple chondroitin/dermatan sulphates, heparan sulphates, and hyaluronic acid glycosaminoglycans involved in this process (Gupta et al., 1998; Nilsson et al., 1998; Zuckerman et al., 1989). Growth factors or cytokines bound to proteoglycans in the ECM or on the cell surface form reservoirs of biologically activity and, are thus presented to target cells at an effectively higher local concentration than soluble growth factors (Ruoslahti and Yamaguchi, 1991; Gordon et al., 1987a; Roberts et al., 1988), and are also protected from proteolytic degradation as has been shown for FGF-2 (Saksela et al., 1988).

Furthermore, membrane-bound or ECM-associated growth factors and cytokines (Table 1) are thought to act as "anchor factors", i.e. to mediate cell adhesion between stromal and primitive progenitor cells in addition to being biologically active growth factors for the latter (Bruno et al., 1995; Anklesaria et al., 1990; Lowry et al., 1992; Cui et al., 1997; Richard et al., 1995; Avraham et al., 1992 and 1994; Uemura et al., 1993).

The expression of certain CAMs by haematopoietic cells is dependent on their stage of maturation suggesting that these molecules facilitate the attachment/release of primitive/mature cells to/from the marrow stroma (Craddock et al., 1997; Mohle et al., 1997; Arroyo et al., 1996; Liesveld et al., 1993). For example, the binding of erythrocytes to fibronectin is mediated by VLA-5. When erythrocytes differentiate into reticulocytes, VLA-5 is lost accompanied by the parallel loss of their adhesion to fibronectin (Patel and Lodish, 1987; Virtanen et al., 1987). Similarly, myeloid stem

cells adhere to BM stroma via specific haematopoietic progenitor-CAMs expressed on their cell surface which are absent on lineage-restricted progenitor cells (Gordon et al., 1990; Campbell et al., 1987).

Table 1. Membrane-bound, cell surface- and ECM-associated forms of growth factors and cytokines.

Growth factors or cytokines that are produced in a membrane bound form:	TNF-α *(1)* pro-TGF-α *(2)*
Growth factors or cytokines that are produced in a secreted and membrane bound form:	M-CSF *(3)* SCF *(4)* flk-2 ligand *(5)* EGF *(6)*
Secreted growth factors or cytokines that can associate with cell surfaces or the ECM:	FGF-2 *(7)* LIF *(8)* IL-3 *(9)* GM-CSF *(9)* TGF-ß *(10)* TGF-α *(11)* MIP-1ß *(12)* IL-1 α *(13)* M-CSF *(14)* IGF *(15)* HGF *(16)* Tpo *(17)* IL-6 *(18)*

(1) Kriegler et al., 1988; *(2)* Bringman et al., 1987; Massagué, 1990; *(3)* Heard et al., 1987; Cerretti et al., 1988; *(4)* Anderson et al., 1990; Huang et al., 1992; *(5)* Lisovsky et al., 1996; *(6)* Mroczkowski et al., 1988: Higashiyama et al 1993; *(7)* Vlodavsky et al., 1987; Saksela et al., 1988; Brunner et al., 1991; *(8)* Rathjen et al., 1990b; *(9)* Gordan et al., 1987a; Roberts et al., 1988; *(10)* Ruoslathi and Yamaguchi, 1991; McCaffrey et al., 1992; *(11)* Massagué, 1991; *(12)* Tanaka et al., 1993; *(13)* Kurt-Jones et al., 1985; Conlon et al., 1987; *(14)* Price et al., 1992; *(15)* Remacle-Bonnet 1997; *(16)* Lyon et al., 1994 and 1998; Schuppan et al., 1998; *(17)* Cui et al., 1997; *(18)* Mummery and Rider, 2000.

5.2. *Regulation of haematopoiesis by growth factors and cytokines.*

The regulation of haematopoiesis by growth factors and cytokines is a complex process involving the effects of both stimulatory and inhibitory factors on proliferation, differentiation and survival of stem cells and progenitor cells. Stromal cells and mature haematopoietic cells are thought to form part of an intercellular network in which mature haematopoietic cells produce and secrete the growth factors or cytokines that regulate haematopoiesis either directly or indirectly by inducing/inhibiting the

production of growth factors or cytokines by BM stromal cells. In this way, the body can meet the changing requirements for cells of a particular lineage of haematopoietic cells.

The stromal cells constitutively produce haematopoietic growth factors and cytokines such as granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF), M-CSF (or CSF-1), FGF-2, stem cell factor (SCF), interleukin (IL)-1, -2, -3, -4, -6, and -7, leukaemic inhibitory factor (LIF), transforming growth factor (TGF)-ß, nerve growth factor (NGF), insulin growth factor (IGF), flk-2 ligand, hepatocyte growth factor (HGF), erythropoietin (Epo), and thrombopoietin (Tpo) (Fibbe et al., 1988; Sensebe et al., 1997a and b; Brunner et al., 1993; Witte et al., 1993; Wetzler et al., 1991, 1994; Kittler et al., 1992; Eaves et al., 1991; Gutierrez-Ramos et al., 1992; Heinrich et al, 1993; Lisovsky et al., 1996; Takai et al., 1997; Auffray et al., 1996; Weimar et al., 1998; Sungaran et al., 1997).

Of the CSFs, IL-3 and GM-CSF act on the broadest range of cell types (Heyworth et al., 1990). Both promote proliferation and differentiation of pluripotent and myeloid stem cells as well as committed progenitor cells. The other CSFs are more specific in their function. M-CSF stimulates macrophage formation (Stanley et al., 1983) and G-CSF neutrophil formation from CFU-GM (Heyworth et al., 1990). In addition, G-CSF and M-CSF potentiate the biological function of their respective mature target cells.

Other cytokines and growth factors that are specific in their function are IL-7, Tpo and Epo. Tpo is expressed in very immature haematopoietic cells and promotes the differentiation and maturation of stem cells into megakaryocytes and pro-platelets (Ziegler et al., 1994; Banu et al., 1995). Indeed, mice deficient in Tpo or c-Mpl (the receptor for Tpo) display thrombocytopenia (Kaushansky et al., 2002; Alexander, 1999). Similarly, Epo is required for erythrocyte formation. Epo acts on the committed progenitor cells, BFU-E and erythroid colony forming units (CFU-E) and promotes their proliferation and differentiation (Broxmeyer, 1990). IL-7 stimulates B- and T-progenitor cell proliferation but does not promote differentiation (Winkler et al., 1995; Gibson et al., 1993; Dorshkind, 1990).

Most growth factors or cytokines (permissive as well as inhibitory) act directly in synergy with CSFs and other haematopoietic growth factors or cytokines to promote/inhibit the proliferation of progenitor cells. SCF on its own has a negligible capacity to stimulate the proliferation of stem and progenitor cells but is a potent stimulator when used in combination with normal or subliminal levels of M-CSF, G-CSF, GM-CSF, IL-1, IL-3, IL-4 or IL-7 (Tsuji et al., 1992; Metcalf and Nicola, 1991;

Lowry et al., 1992; Briddell et al., 1991; Keller et al., 1994b; Fahlman et al., 1994). Likewise, Flk-2 ligand in combination with IL-3, IL-6, or G-CSF, and IL-1 in synergy with GM-CSF, M-CSF, or IL-3 are potent stimulators of primitive progenitor cell proliferation (Namikawa et al., 1996; Shapiro et al., 1996).

Inhibitory growth factors or cytokines include TGF-ß, IFNs, TNF, and MIPs and mostly inhibit haematopoiesis by diminishing the proliferation and differentiation of stem cells and primitive (non-differentiated) progenitor cells (Waegell et al., 1994; Eaves et al., 1991; Graham et al., 1990; Broxmeyer et al., 1990; Khoury et al., 1994; Eliason and Vassalli, 1988). IFNs inhibit myeloid progenitor growth and differentiation (Galvani and Cawley, 1990; Coutinho et al., 1986). TNF, at subliminal levels, can also synergise with low levels of IFN to suppress myeloid progenitor cell growth (Broxmeyer et al., 1986). However, some inhibitory growth factors are stimulatory for differentiated progenitors. For example, TGF-ß in the presence of GM-CSF, or MIP-1ß as well as MIP-2, enhances the differentiation of granulocyte and/or macrophage progenitor cells. (Keller et al., 1991; Broxmeyer et al., 1990).

6. Regulation of growth factor activity.

Haematopoiesis is thus a highly complex process that is regulated by a variety of both stimulatory and inhibitory growth factors and cytokines. The activity of these factors in the BM microenvironment will determine the net effect on stem cell proliferation and differentiation. Apart from transcriptional regulation, the activity of growth factors and cytokines can be regulated by various posttranscriptional local mechanisms involving proteolytic processing mediated by serine proteases, metalloproteinases and phospholipases. (Whitelock et al., 1996; Falcone et al., 1993a; Taipale et al., 1992; Rich et al., 1996; Brunner et al., 1994 and 1991; Metz et al., 1994). Although not well studied, it is possible that proteases, such as the serine protease plasmin, whose generation can be increased by FGF-2-stimulation of plasminogen activators (Mignatti et al., 1990), may have a role in regulating growth factor/cytokine activity in the BM microenvironment.

6.1. *Activation of latent growth factors.*

Certain factors including IL-1ß, TGF-ß, and HGF, are synthesised as inactive precursor molecules which require activation to be biologically active. For example, plasmin converts inactive IL-1ß precursor and latent TGF-ß into active molecules (Hazuda et al., 1991; Harpel et al., 1992), and pro-HGF is converted by urokinase, HGF activator, or kallikrein into active HGF (Naldini et al., 1992; Kataoka et al.,

2001; Peek et al., 2002). Activation of latent or inactive growth factors effectively increases the concentration of these molecules in the cell's vicinity.

In a similar manner, protease-activated receptors (PARs) induce signalling events after activation by proteolytic cleavage of the extracellular domain of the receptor (Mackie et al., 2002). PAR-1, -3, and -4, are activated by thrombin, and PAR-2, by plasmin or elastase (Mackie et al., 2002). Blood cells (platelets, monocytes, macrophages) differentially express PARs which mediate platelet activation and inflammatory responses (Ofosu, 2003; Colognato et al., 2003).

6.2. *Mobilisation of cell surface / ECM bound growth factors.*

The activity of other growth factors or cytokines can be regulated through their enzymatic mobilisation from cell surfaces or ECM, e.g. by proteases or phospholipases (Ehlers and Riordan, 1991). The release of membrane-bound or membrane/ECM-associated growth factors or cytokines (see Table 1) not only reduces the concentration of these factors at specific sites in the cell's microenvironment, but also allows them to interact with their receptors on cells at adjacent sites. Furthermore, the release of "anchor factors" from the cell surface may leads to a decrease in cell-cell interactions.

Active FGF-2-HSPG complexes are released from the cell surface by plasmin or phospholipase D (Saksela and Rifkin, 1990; Brunner et al., 1991; Falcone et al., 1993a and b) and may represent one of the mechanisms regulating the local availability of this growth factor in the BM microenvironment (Falcone et al., 1993b) since TGF-ß-induced u-PA in macrophages results in the increased release of FGF-2 from the ECM (Falcone et al., 1993b). Active TGF-ß itself binds to the ECM via interactions with the core proteins of decorin and biglycan, and to cell surfaces via endoglin or betagylcan (Fortunel et al., 2000; Robledo et al., 1996; Ruoslahti and Yamaguchi, 1991; Massagué, 1991). The binding of TGF-ß to decorin and biglycan neutralises its activity which can be restored after its release from decorin (Yamaguchi et al., 1990; Ruoslathi and Yamaguchi, 1991), thus representing an additional mechanism by which TGF-ß activity can be regulated via proteolytic processing events. Latent TGF-ß is also released from the ECM by serine proteases including plasmin (Falcone et al., 1993a; Taipale et al., 1992).

Other examples include the cleavage of transmembrane pro-TGF-α by elastase-like enzymes to form soluble TGF-α (Massagué, 1990) and the release of membrane-bound IL-1α by plasmin (Matsushima et al., 1986; Conlon et al., 1987).

6.3. *Shedding of membrane bound growth factor receptors.*

The response of a cell to a growth factor is also influenced by the number of receptors on its surface, the higher the number of a particular receptor the greater the respons. Certain membrane-bound receptors including FGFR, TNFR, M-CSFR, IL-1R, IL-4R, IL-5R, IL-6R, EGF-R isoforms, CD117 (c-kit), and nerve growth factor (NGF)-R, can be proteolytically cleaved generating soluble receptors (Levi et al., 1996; Porteu et al., 1991, Downing et al., 1989, Penton-Rol et al., 1999; Jung et al., 1999; Liu et al., 2002; Montero-Julian, 2001; Chang et al., 2003; Lévesque et al., 2003; Kanning et al., 2003). Soluble receptors can prolong the half-life of the factors they bind by protecting them from degradation but, at the same time, soluble receptors can reduce ligand activity by competing with membrane bound receptors for the ligand (Jones and Rose-John, 2002). Similarly, soluble co-receptors (e.g. HSPG for FGF-2, betaglycan for TGF-ß, or IGF-binding protein-4) can regulate growth factor activity.

7. Aims

The first aim of this thesis was to explore the effects of FGF-2 on haematopoiesis, which were poorly understood at the time that this project was started. To approach this, I studied the effects of FGF-2 on BM stromal cells (Chapter 2). I added FGF-2 to BM stromal cells and noted its effects on proliferation, senescence, and morphology of the BM stromal cells. I also investigated the role of FGF-2 in stimulating haematopoiesis *in vitro* in LTBM Dexter cultures (Chapter 3). FGF-2 was added to LTBM cultures and I determined the number of progenitor cells in the adherent stromal layer as well as the supernatant of these cultures.

The second aim of this thesis was to determine if the proteolytic cascade of plasminogen activation in the BM stromal microenvironment could be modulated by FGF-2. Plasminogen activators (PAs) directly control the level of plasmin which may indirectly regulate haematopoietic growth factor activity. This can occur, for example through the proteolytic activation of latent forms of growth factors, the mobilisation of active growth factors from cell surfaces and/or extracellular matrix reservoirs, or through the shedding of growth factor receptors from the cell surface. In Chapter 4, I therefore investigated whether urokinase-type PA or tissue-type PA activity, expressed by BM stromal cells, could be modulated by haematopoietic growth factors such as FGF-2 as well as IL-1 or TGF-ß. In addition, the effects of these growth factors on the inhibitors, PA inhibitor-1 (PAI-1) and -2 (PAI-2), were also determined.

In summary, my work indicates that FGF-2 has potent effects on both the stromal and haematopoietic compartments. It regulates stromal cell morphology and

is a potent mitogen for BM stromal cell proliferation, significantly delaying their senescence. Furthermore, FGF-2 significantly stimulates myelopoiesis, possibly indirectly by regulating the plasmin-catalysed processing of growth factors and/or receptors. Thus, FGF-2 has the potential to be used clinically to enhance haematopoiesis *in vivo* and therefore deserves further investigation.

Chapter 2.

FGF-2 induces morphological changes, delays senescence, and is mitogenic for human BM stromal cells.

Introduction.

In this chapter I have analysed the effects of FGF-2 on the stromal cell compartment of *in vitro* BM cultures. I have also investigated the contribution of the culture medium supplements for Dexter LTBM cultures on the growth of primary and passaged human BM stromal cells in the presence or absence of FGF-2. Little is known about the growth factors and cytokines that regulate the growth and differentiation of the non-haematopoietic cells in LTBM cultures or *in vivo*. Since FGF-2 is a potent mitogen for a variety of cells of mesenchymal origin (Lindner and Reidy, 1993; Gitter and Koehneke, 1991), it may be involved in the regulation of BM stromal cells.

1. The stromal cell layer.

Both myelopoietic and lymphopoietic LTBM cultures are dependent on the establishment of an adherent layer of stromal cells for the growth and differentiation of stem cells and progenitor cells (Dexter et al., 1977a and b; Whitlock and Witte, 1982). These cells are believed to arise from mesenchymal stem cells (MSCs)(Dennis and Charbord, 2002; Simmons et al., 2001). A subgroup of MSCs, referred to as recycling stem cells (RS) since they are rapidly self-renewing, appear to represent the most earliest progenitor cells (as suggested by the expression of cell surface receptors and proteins) isolated from colonies of BM stromal cells (Colter et al., 2001 and 2000; Prokop et al., 2001). Like MSCs, RS cells are negative for CD34, CD45, and c-kit (Colter et al., 2001 and 2000; Majumdar et al., 2000). Unlike MSCs, RS cells are negative for STRO-1, PDGF and EGF and are positive for Flk-1, TRK (a NGF receptor), transferrin receptor, and annexin II (Colter et al., 2001; Gronthos and Simmons, 1995). RS cells also have a greater capacity for multilineage differentiation than MSCs (Colter et al., 2001; Prokop et al., 2001).

2. Growth factor regulation of BM stromal cell growth and differentiation.

There are a number of reports in the literature showing several growth factors and cytokines that regulate haematopoiesis may also be involved in the regulation of

BM stromal cell proliferation.

Oliver et al. (1990) have shown that low concentrations of FGF-2 stimulate the growth of BM stromal cells, that this effect is reversible, that significant stimulation of growth is also obtained when the BM cells were exposed to FGF-2 for brief periods of time, and that the addition of heparin substantially potentiates the growth observed with low concentrations of FGF-2. Brunner et al. (1993), subsequently, showed that BM stromal cells produce FGF-2. Therefore, it appears that FGF-2, an endogenous stromal growth factor, may function in the autocrine growth regulation of BM stromal cells. Further evidence for the involvement of FGF-2 in BM stromal cell growth comes from the murine preadipocyte cell line, PA6, which requires either FGF-2 or EGF for its maintenance when grown in defined media (i.e. medium without serum)(Nishikawa et al., 1994). Sensebe et al. (1995) have established a method for developing non-transformed human BM stromal cell "lines" with high proliferative potential by stimulation with FGF-2 (Sensebe et al., 1995) suggesting a role for this growth factor in the growth of stromal precursor cells.

Other growth factors and cytokines involved in the proliferation of BM stromal precursor cells include platelet derived growth factor (PDGF), EGF, IL-1, IL-2, IL-3, IL-6, TNF-α, TGF-ß, M-CSF, SCF, and IFN-α (Sensebe et al., 1995; Gronthos and Simmons, 1995; Andrades et al., 1999; Satomura et al., 1998; Kuznetsov et al., 1997; MacDonald et al., 1990; Carron and Cawley, 1991; Galvani and Cawley, 1990; Wang et al., 1990, 1992). Which of these factors play a role in stromal cell differentiation in addition to growth is not yet known. VSMC differentiation is modulated by TGF-ß and PDGF (Dennis and Charbord, 2002; Yamashita et al., 2000) and may involve ECM molecules (Dennis and Charbord, 2002). Thrombospondin-1 stimulates the expression of α-smooth muscle actin in fibroblasts which might be mediated via TGF-ß as thrombospondin is an activator of latent TGF-ß (Dennis and Charbord, 2002). Since TGF-ß is produced by stromal cells (Sensebe et al., 1997b) it might act in an autocrine manner to regulate stromal cell growth and differentiation. Other cytokines involved in stromal differentiation include IL-1ß, IL-11, IL-6, and IFN-α which inhibit adipocyte formation (Delikat et al., 1993; Kawashima and Takiguchi, 1992; Gimble et al., 1994).

The ability to isolate and expand MSCs and RS cells *ex vivo* (Colter et al., 2001 and 2000; Prokop et al., 2001) should now make it possible to characterise the factors that control the growth and differentiation of BM stromal cells.

3. Medium requirements for LTBM cultures.

The culture conditions for the two types of LTBM cultures, Dexter and Whitlock-

Table 1. Culture conditions for LTBM cultures supporting myelopoiesis or lymphopoiesis.

	Myelopoiesis		Lymphopoiesis	
	Murine[a]	Human[b]	Murine[c]	Human[d]
Medium	DMEM	McCoy's 5A	RPMI	Opti-MEM
Horse Serum	25 %	12.5 %	—	—
Fetal Calf Serum	—	12.5 %	25 %	10 %
Hydrocortisone	10^{-6}-10^{-7} M	10^{-6}-10^{-7} M	—	—
ß-Mercaptoethanol	10^{-4} M	—	10^{-4} M	5×10^{-5} M
Glutathione	—	5×10^{-4} M	—	—
Temperature	33°C	33°C	37°C	37°C

a: Greenberger et al., 1978 *b*: Gartner and Kaplan, 1980
c: Whitlock and Witte, 1982 *d*: Moreau et al., 1993

Witte cultures, are depicted in Table 1 and are important for the successful maintenance of the progenitor cells. However, the effect of the supplements on the growth of the stromal cells is not known.

3.1. *Hydrocortisone.*

The survival of haematopoietic cells in murine LTBM cultures, originally described by Dexter, appeared to be dependent on the batch of horse serum used (Greenberger et al., 1978). Supplementing the culture medium with hydrocortisone, however, allowed the successful long-term maintenance (over one year) of murine LTBM cultures even with the use of "poor" batches of horse serum (Greenberger et al., 1978). It was concluded that horse serum contains a corticoid(s) which is important for the generation and proliferation of myelopoietic cells. Hydrocortisone is also added to human myelopoietic cultures. Nevertheless, the culture conditions for human LTBM cultures support myelopoiesis for only 2 to 3 months.

Hydrocortisone which inhibits the differentiation of lymphocytes by altering the calmodulin pathway (Hyden-Martinez et al., 2000; Balakumaran et al., 1996; Evans-Storms and Cidlowski, 1995), as well as horse serum are therefore omitted from the culture medium of lymphopoietic LTBM cultures (Whitlock and Witte, 1982). With some strains of mice, lymphopoietic LTBM cultures can be successfully maintained for over one year, but attempts to mimic such culture conditions with human BM have been less successful (LeBien, 1989; Wolf et al., 1991). Human lymphopoietic cultures that support the proliferation and differentiation of B-cell precursor cells for approximately 3 weeks can be established by seeding human fetal BM (which is highly

enriched for lymphoid precursors compared to adult BM) onto preformed human stromal cell layers (Moreau et al., 1993).

3.2. *Anti-Oxidants.*

An anti-oxidant is also added to the culture medium of LTBM cultures to prevent oxidative damage to the haematopoietic cells. Human myelopoietic cultures are normally cultured in "Gartners" medium (Gartner and Kaplan, 1980) which uses McCoy's 5A medium that contains glutathione as an anti-oxidant. The culture medium of lymphopoietic cultures and murine myelopoietic cultures are supplemented with ß-mercaptoethanol to prevent oxidative damage.

4. Aims and findings.

The effects of FGF-2 on BM stromal cell growth had not, prior to the studies in our laboratory (Oliver et al., 1990), been described. To further characterise the role of FGF-2 on the stromal compartment of BM cultures, I studied the effect of this growth factor, as well as the contribution of the culture medium supplements, on the growth of primary and passaged human BM stromal cells, and my findings are described in this chapter. I noted that FGF-2 greatly accelerated the formation of the adherent layer following inoculation of primary BM buffy coat cells into tissue culture dishes, that in the presence of FGF-2, the stromal cells lost their contact inhibition and grew to high cell densities in multilayered sheets, and that the senescence of the stromal cells was delayed considerably when cultured continuously in the presence of FGF-2.

In addition, I noted that horse serum was a supplement important for the optimum growth of primary BM stromal cells and that the stimulation of primary, but not passaged, BM stromal cell growth by FGF-2 was dependent on hydrocortisone.

These findings suggest that FGF-2 may play a functional role in the growth of BM stromal cells, which may be important for supporting the proliferation and differentiation of BM progenitor cells. Indeed, Miller at al. (2000) have demonstrated that BM stromal cells deficient in FGF-2 display diminished support for haematopoiesis *in vitro* due to a defect in the stromal layer, although *in vivo* normal haematopoiesis was observed. Nevertheless, understanding the factors and mechanisms that regulate the BM stromal cell compartment may provide additional insight into the role of the BM microenvironment in haematopoiesis and, in addition, the use of MSCs or RS cells could potentially be used to treat BM stromal cell disorders such as myelofibrosis in patients with chronic myeloid leukaemia, B-cell lymphocytic leukaemia, and aplastic anaemia (Van Damme et al., 2002; Aman et al., 1993; Lagneaux et al., 1993).

Methods and Results.

1. BM collection and establishment of cultures.

In order to establish cultures of adherent BM stromal cells, BM cells were aspirated from healthy volunteers after they had given informed consent and were collected in sterile syringes containing preservative-free heparin. Twenty to forty ml of BM were obtained from ten to twenty separate "first-pull" aspirates. Two to three ml aliquots of BM were added to 5-ml polystyrene tubes and then centrifuged at 600 x *g* for 5 minutes. The BM buffy coat cells (BM cells at the interface of the plasma and red blood cell layer) were collected and seeded into 35-mm dishes at 3 x 10^6 cells/dish in 2 ml of "stromal medium" (αMEM containing 12.5% horse serum, 12.5% fetal calf serum, 10^{-6} M hydrocortisone, 10^{-4} M ß-mercaptoethanol, 2 mM glutamine, 500 U/ml penicillin and 200 μg/ml streptomycin) or into 75-cm^2 flasks at 20 x 10^6 cells/flask in 20 ml of this medium. Parallel cultures were treated with FGF-2 at concentrations indicated in each section.

2. The effect of FGF-2 on the establishment of the adherent BM stromal layer.

To determine the effect of FGF-2 on the morphology and growth of BM stromal cells in developing cultures, the growth factor was added to BM buffy coat cells seeded into 35-mm dishes.

2.1. *Morphology*

BM buffy coat cells (3 x 10^6) were seeded into 35-mm dishes as described above, in the absence or presence of 0.2, 2, or 20 ng/ml FGF-2, and the developing adherent cell layer was observed microscopically. Three to five days after inoculation, clusters of fibroblast-like cells with a spindle shaped morphology appeared in all culture dishes. Eight days after inoculation, the colonies of fibroblastic cells in cultures that had not been treated with FGF-2 consisted of large, well-spread cells (Fig. 1*A*). In contrast, the cultures to which 20 ng/ml FGF-2 had been added continued to exhibit spindle-shaped cells at a higher cell density than the control cultures (Fig. 1*D*). Lower concentrations of FGF-2, 0.2 ng/ml (Fig. 1*B*) and 2 ng/ml (Fig. 1*C*), also induced a spindle-shaped morphology of the fibroblastic cells and increased cell their density. The effect was not as marked as noted with 20 ng/ml FGF-2. Sparser cultures of BM stromal cells treated with low concentrations of FGF-2 also displayed morphological changes.

Thus, FGF-2 was observed to induce morphological changes in primary human BM stromal cells and to increase the density of the cultures.

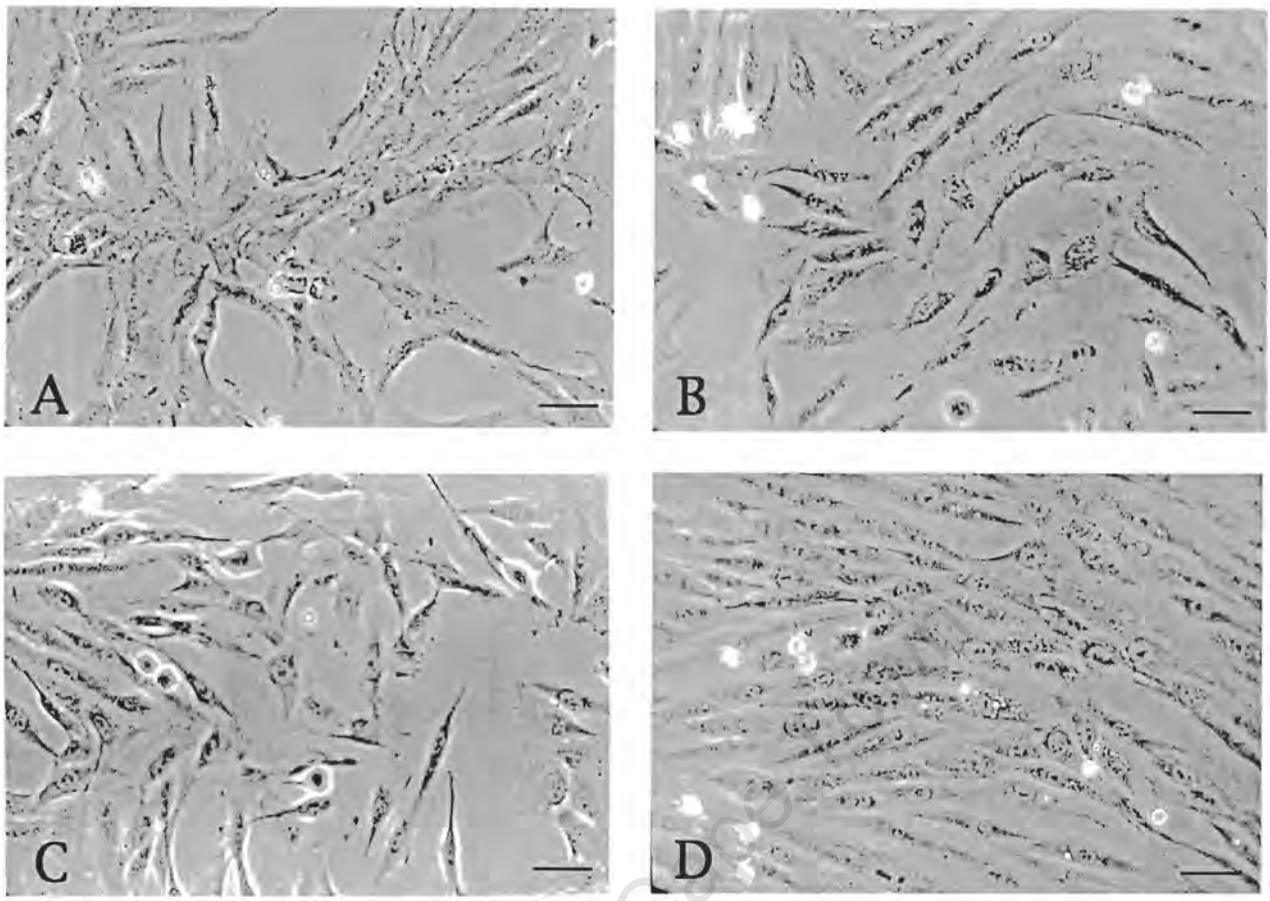

Fig. 1. The effect of FGF-2 on the morphology of primary BM stromal cells. BM buffy coat cells (3 x 10^6) were seeded into 35-mm dishes in stromal medium in the absence (*A*) or presence of 0.2 (*B*), 2 (*C*) or 20 (*D*) ng/ml of FGF-2. The cultures were photographed 8 days after inoculation using a phase contrast microscope.
Bar = 50 μm (applies to all four photographs).

2.2. *Growth*

In order to quantitate the effects on growth, 3 x 10^6 BM buffy coat cells were seeded into 35-mm dishes, as described above, in the absence or presence of 0.02, 0.2, 2, or 20 ng/ml FGF-2. After one week, the supernatant was replaced, at three day intervals with fresh medium with and without FGF-2 and the number of adherent cells was determined on days 11 and 15 (Fig. 2). The *t*-test (Quito et al., 1996) was used to assess the significance of the results. On day 15, cultures to which no FGF-2 had been added contained 3.3 ± 0.9 x 10^5 cells/dish. The cell number was 5-fold increased in the presence of 20 ng/ml FGF-2 (15.7 ± 0.6 x 10^5 cells/dish; $p < 0.001$) and 2-fold with 0.2 20 ng/ml FGF-2 (6.4 ± 0.8 x 10^5 cells/dish; $p < 0.02$)(Fig. 2).

Thus, FGF-2 significantly increased the proliferative activity of primary human BM stromal cells even at very low concentrations.

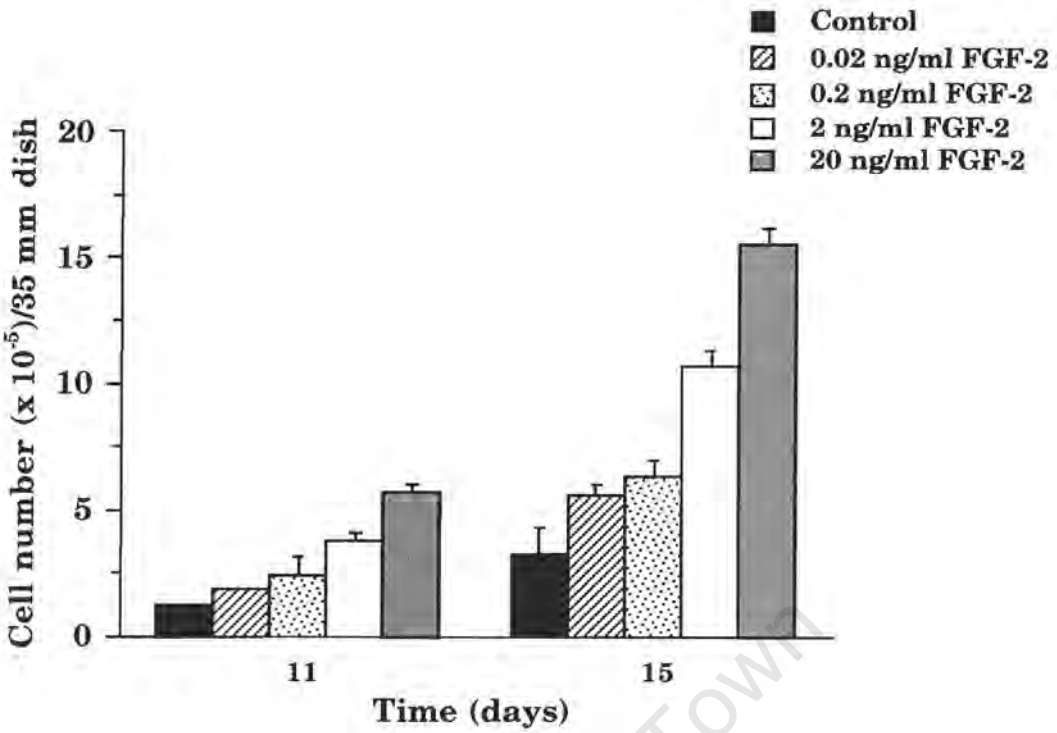

Fig. 2. The effect of FGF-2 on the growth of primary BM stromal cells. BM buffy coat cells (3 x 10^6) were seeded into 35-mm dishes in stromal medium in the absence or presence of 0.02, 0.2, 2 or 20 ng/ml FGF-2. After one week the supernatant was replaced with fresh medium with or without FGF-2 at three day intervals. The number of adherent cells on duplicate dishes was determined 11 and 15 days after inoculation. The results are expressed as the mean ± standard deviation (SD).

2.3. *Senescence*

In contrast to primary cultures of embryonic or skin fibroblasts or aortic endothelial cells which proliferate readily and can be propagated, human BM stromal cells senesce after approximately 2 generations and therefore have limited expansion potential. As FGF-2 has been shown to delay the senescence of granulosa cells and corneal endothelial cells (Gospodarowicz et al., 1986) I determined its effect on the senescence of BM stromal cells.

For these experiments, BM buffy coat cells were seeded into 75-cm^2 flasks at 2 x10^7 cells/flask in 20 ml of stromal medium in the absence or continuous presence of 20 ng/ml FGF-2. After one week, the supernatant was replaced every three to four days with fresh medium with or without FGF-2, and cell numbers in duplicate flasks were determined after 10 to 13 days of culture. The cells were then reseeded into new 75-cm^2 flasks at 1.3 x 10^6 cells/flask in 20 ml of stromal medium in the absence or continuous presence of FGF-2. This process was repeated until cell growth ceased. The results are presented in Fig. 3.

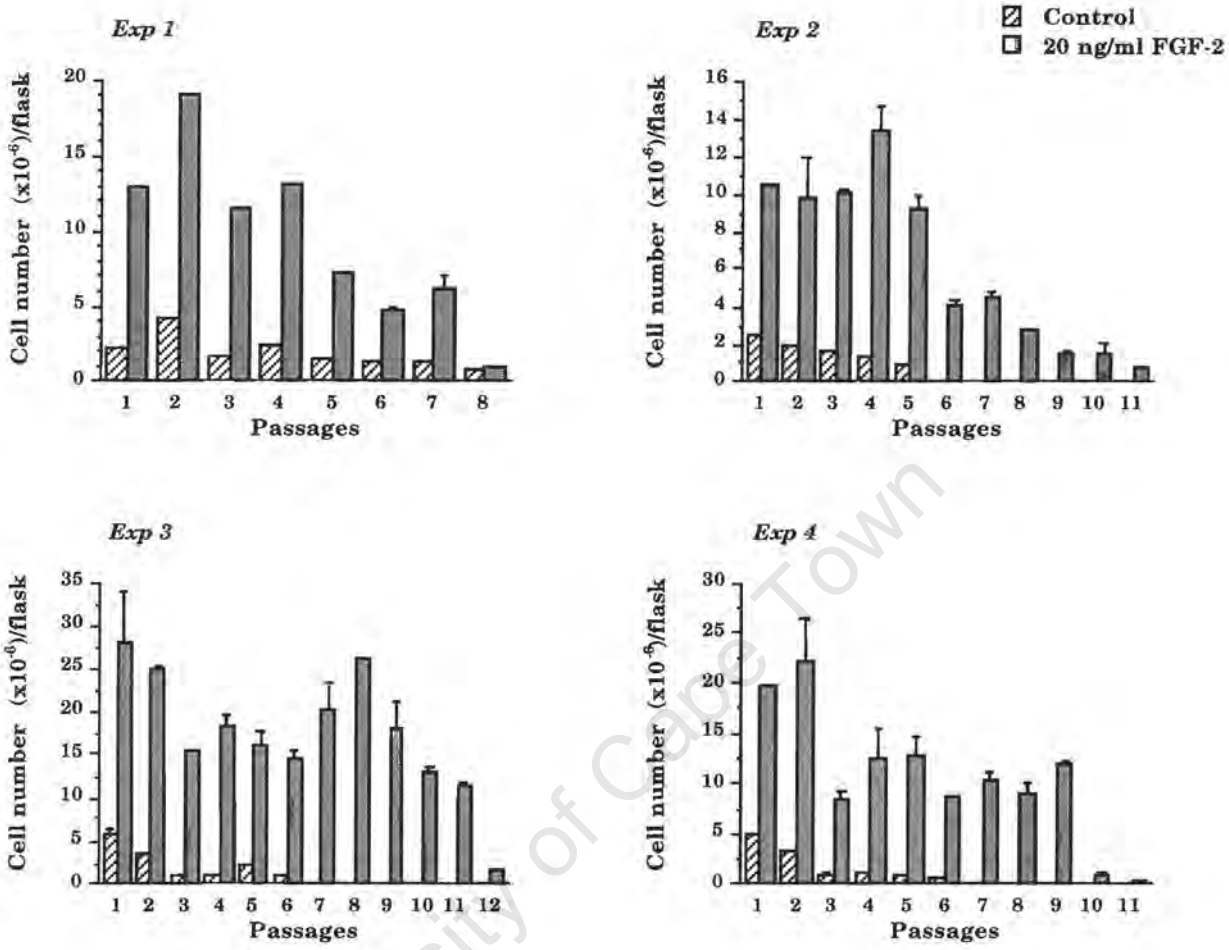

Fig. 3. The effect of FGF-2 on the senescence of BM stromal cells. BM buffy coat cells (2×10^7) were seeded into 75-cm^2 flasks in stromal medium in the absence or continuous presence of 20 ng/ml FGF-2. At 10 to 13 day intervals the number of adherent cells in duplicate flasks was determined and 1.3×10^6 cells were reseeded into new flasks in the absence or continuous presence of FGF-2. The process was repeated until cell growth ceased. This experiment was repeated four times with BM removed from four different donors. Results are given as the mean of the total number of cells ± SD recovered at each passage for each experiment.

In each experiment, untreated BM stromal cells stopped growing after approximately two to three passages. In contrast, BM stromal cells treated with 20 ng/ml FGF-2 only ceased growing after eight to twelve passages (Fig. 3). At each 10 to 13 day interval the number of cell generations that had arisen was calculated. A generation was defined as a doubling in the cell population that arose due to each cell dividing and giving rise to two daughter cells. The total number of generations calculated for untreated and FGF-2 treated cultures is presented in Table 2. In the absence of FGF-2, stromal cells gave rise to approximately 2 generations before they senesced. However, in the presence of FGF-2 cells could be passaged for approximately 26 generations before senescing.

The addition of FGF-2 to BM stromal cells therefore resulted in considerable cell expansion and greatly delayed their senescence.

Table 2. The Effect of FGF-2 on the senescence of BM stromal cells.

Exp. No.	Generations	
	Control	20 ng/ml FGF-2
1	3.1	21.8
2	0.8	16.8
3	1.4	39.5
4	1.8	24.7
Average	1.8 ± 0.9	25.7 ± 9.8*

BM buffy coat cells (2×10^7) were seeded into 75-cm^2 flasks in stromal medium in the absence or continuous presence of 20 ng/ml FGF-2. At 10 to 13 day intervals the number of adherent cells in duplicate flasks was determined and 1.3×10^6 cells were reseeded into new flasks in the absence or continuous presence of FGF-2. The process was repeated until cell growth ceased. The number of generations were calculated from the total number of population doublings that occurred prior to senescence.

* the average number of generations was significantly different to the control value, $p < 0.005$.

3. The contribution of stromal medium supplements to the growth of BM stromal cells in the absence or presence of FGF-2.

The medium used to culture human BM in Dexter LTBM cultures contains fetal calf serum, horse serum, hydrocortisone, and the anti-oxidant, ß-mercaptoethanol, as medium supplements. I was interested in determining:

(1) which culture medium supplements were important for the growth of the stromal cells, and

(2) if the mitogenic effect of FGF-2 on stromal cell growth was dependent on the presence of one or more of these supplements.

I therefore investigated the effect of FGF-2 on the growth of both primary and passaged stromal cells in complete medium or medium lacking one or more of the supplements.

3.1. *Primary BM stromal cultures*

To determine which culture medium supplements were important for the growth of primary stromal cells, BM buffy coat cells were seeded into 35-mm dishes at 3 x10^6 cells/dish in 2 ml of stromal medium, or in stromal medium that lacked either horse serum, hydrocortisone, ß-mercaptoethanol, or all three supplements. Cultures were also grown in the absence or continuous presence of 20 ng/ml FGF-2 in order to determine if the mitogenic effect of this growth factor on stromal cell growth was dependent on the presence of one or more of these supplements. After one week, the supernatant was replaced at three to four day intervals with fresh medium with or without FGF-2. Cell numbers in triplicate dishes were determined after 18 days of culture to ensure adequate development of the stromal layer. The results are presented in Table 3. Control cultures are defined as BM stromal cell cultures grown in complete stromal medium in the absence of FGF-2. The *t*-test was used to test the significance of the results. As an example for further illustration, the results of *Exp. 3* are presented in Fig. 4 as the average cell number/35 mm dish.

Table 3 and Fig. 4 show that in the absence of FGF-2, the number of cells in the developing primary cultures was significantly decreased by the omission of horse serum from the medium in two out of three experiments ($p < 0.001$ in each experiment) whereas, the omission of ß-mercaptoethanol had either no effect ($p > 0.05$) or stimulated cell growth ($p < 0.001$ in this experiment). The omission of hydrocortisone from the medium did not significantly alter the number of cells in three out of four experiments ($p > 0.05$, Table 3 and Fig. 4). When all three medium supplements, horse serum, ß-mercaptoethanol and hydrocortisone, were omitted from the medium of control cultures,

the number of stromal cells was significantly reduced ($p < 0.01$ in all four experiments) (Table 3 and Fig. 4).

As noted previously (Fig. 2), FGF-2 is mitogenic for primary BM stromal cells. Therefore, as expected, the addition of 20 ng/ml FGF-2 to stromal cells grown in stromal medium significantly increased the number of cells in the cultures. Table 3 and Fig. 4 show that this concentration of FGF-2 increased cell number by 5 to 14-fold ($p < 0.01$ in all four experiments).

The addition of FGF-2 to BM stromal cells grown in stromal medium lacking horse serum increased the number of cells by approximately 2 to 8-fold above control ($p < 0.02$ in each of the three experiments). It should be noted, however, that in the presence of FGF-2 and absence of horse serum, the cell number was still lower than that determined in cultures grown in complete medium and FGF-2 (Table 3 and Fig. 4). This indicated that horse serum was required for the optimal growth of BM stromal cells and that FGF-2 could only partially compensate for its absence.

Table 3. The contribution of stromal medium supplements to the growth of primary BM stromal cells in the absence or presence of FGF-2.

	Stromal Medium		Stromal Medium without Horse Serum		Stromal Medium without ß-Mercaptoethanol		Stromal Medium without Hydrocortisone		Stromal Medium without Horse Serum, Hydrocortisone and ß-Mercaptoethanol	
Exp.	-FGF-2	+FGF-2	-FGF-2	+FGF-2	-FGF-2	+FGF-2	-FGF-2	+FGF-2	-FGF-2	+FGF-2
1	100%	1427%	142%	805%	ND	ND	82%	36%	25%	31%
2	100%	462%	54%	351%	ND	ND	54%	55%	18%	14%
3	100%	735%	31%	183%	117%	655%	104%	52%	58%	61%
4	100%	455%	ND	ND	257%	1177%	122%	64%	19%	7%

BM buffy coat cells (3 x 10^6 cells/dish) were seeded into 35-mm dishes in stromal medium or stromal medium lacking either horse serum, hydrocortisone, ß-mercaptoethanol, or all three supplements, in the absence or presence of 20 ng/ml FGF-2. After one week the supernatant was replaced with fresh medium with or without FGF-2 at three to four day intervals. The number of cells in triplicate dishes was determined after 18 days of culture. This experiment was repeated four times with BM taken from four different donors. The cell numbers obtained in each experiment are expressed as a percentage of the cell number noted in control dishes. Control cultures were defined as those cultures containing BM stromal cells grown in complete stromal medium in the absence of FGF-2. The mean number of cells determined in control cultures on day 18 in each experiment was: *Exp. 1* = $1.4 \pm 0.4 \times 10^4$ cells/dish; *Exp. 2* = $40.7 \pm 3.2 \times 10^4$ cells/dish; *Exp. 3* = $16.9 \pm 3.5 \times 10^4$ cells/dish; and *Exp. 4* = $4.7 \pm 0.8 \times 10^4$ cells/dish. ND = not determined.

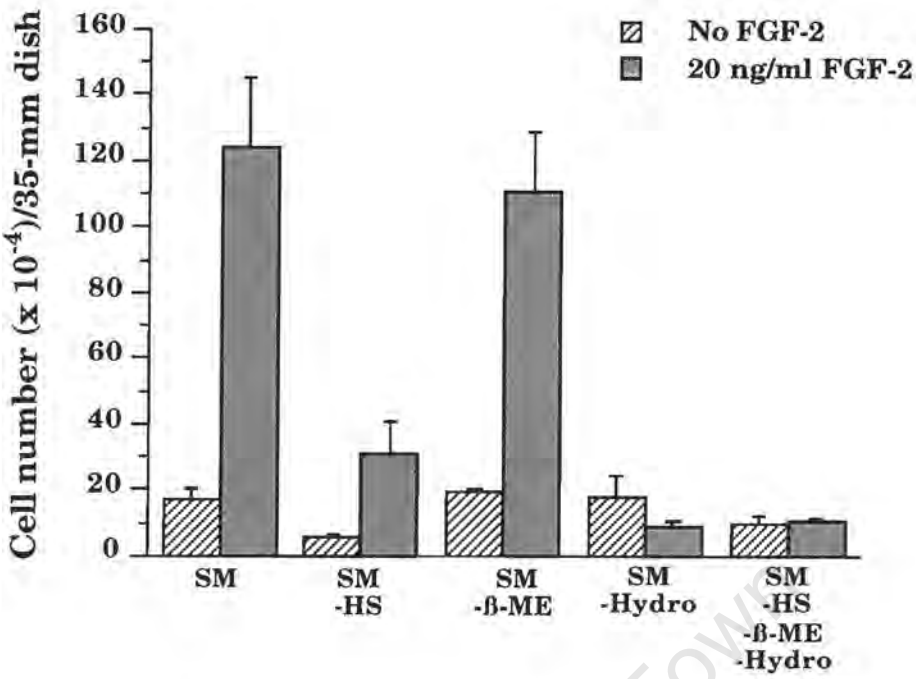

Fig. 4. The contribution of stromal medium supplements to the growth of primary BM stromal cells in the absence or presence of FGF-2. Absolute cell numbers from *Exp. 3* of Table 3 are presented as the mean number of cells/dish ± SD.
SM: stromal medium; HS: horse serum; ß-ME: ß-mercaptoethanol; Hydro: hydrocortisone.

FGF-2 stimulated the growth of stromal cells grown in stromal medium without ß-mercaptoethanol by 7 to 12-fold above control ($p < 0.001$ in both experiments). Thus, FGF-2 stimulation in these cultures was comparable to that in cultures grown in complete medium.

The most striking observation was that the omission of hydrocortisone from the culture medium completely abrogated the stimulation of cell growth by FGF-2 and in three experiments cell growth was actually inhibited (Table 3 and Fig. 4, $p < 0.02$ in the three experiments). In keeping with this observation, FGF-2 did not stimulate cell growth following the omission of all three medium supplements, namely, horse serum, ß-mercaptoethanol, and hydrocortisone (Table 3 and Fig. 4, $p > 0.05$ in each experiment).

These results indicate that horse serum is an essential medium supplement for the growth of primary BM stromal cells, whereas ß-mercaptoethanol and hydrocortisone are not required. Indeed, the results suggest that ß-mercaptoethanol may be growth inhibitory for these cells. LTBM culture require however, the presence of an anti-oxidant to prevent oxidative damage to the haematopoietic cells

In addition, the results show that FGF-2 stimulates the growth of stromal cells grown in medium lacking ß-mercaptoethanol or horse serum and can partially replace

the requirement for the latter. Most intriguingly FGF-2 is not mitogenic for primary BM stromal cells in the absence of hydrocortisone suggesting that hydrocortisone is required for the biological action of FGF-2 in these cultures.

3.2. *Passaged BM stromal cultures*

In order to determine whether passaged BM stromal cells differed from primary stromal cells in their requirement for culture medium supplements, cultures of such cells were established by removing the non-adherent progenitor and mature haematopoietic cells from two to three week old primary BM cultures by extensive washing with phosphate buffered saline (PBS). The adherent stromal cells were removed from the flasks by adding 8 ml of a 0.5% trypsin/0.02% EDTA solution for 5 minutes at 37°C. An equal volume of αMEM containing 10% FCS was added to each flask to neutralise the effect of the trypsin. The cells were then washed twice by centrifugation with stromal medium and reseeded at 5 x 10^4 cells/35-mm dish in 2 ml of stromal medium. The following day the supernatant was replaced with 2 ml of complete stromal medium, or stromal medium lacking either horse serum, hydrocortisone, ß-mercaptoethanol, or all three supplements, in the absence and continuous presence of 20 ng/ml FGF-2. After four days the culture supernatant was replaced with fresh medium with or without FGF-2, and cells were counted in duplicate dishes on day 7.

The results presented in Table 4 show the cell numbers obtained in each experiment expressed as a percentage of control. Control cultures are defined as BM stromal cells grown in complete stromal medium in the absence of FGF-2. The *t*-test was used to perform statistical analysis on the data. Absolute cell numbers from *Exp. 2* are presented in Fig. 5 for further illustration.

As limited stromal growth was noted in the absence of FGF-2 (Table 4 and Fig. 5), it was not possible to determine the effects of the supplements (horse serum, hydrocortisone, or ß-mercaptoethanol) on the growth of passaged BM stromal cells.

As noted previously in Fig. 3 and shown by Oliver et al. (1990), FGF-2 is mitogenic for passaged BM stromal cells. As expected, Table 4 and Fig. 5 show that the addition of 20 ng/ml FGF-2 to stromal cultures significantly increased the number of cells by 3 to 17-fold ($p < 0.01$ in all four experiments).

Table 4. The contribution of stromal medium supplements to the growth of passaged BM stromal cells in the absence or presence of FGF-2.

	Stromal Medium		Stromal Medium without Horse Serum		Stromal Medium without ß-Mercaptoethanol		Stromal Medium without Hydrocortisone		Stromal Medium without Horse Serum and Hydrocortisone		Stromal Medium without Horse Serum, Hydrocortisone and ß-Mercaptoethanol	
Exp.	-FGF-2	+FGF-2	-FGF-2	+FGF-2	-FGF-2	+FGF-2	-FGF-2	+FGF-2	-FGF-2	+FGF-2	-FGF-2	+FGF-2
1	100%	1741%	89%	603%	116%	1058%	149%	691%	107%	662%	69%	324%
2	100%	924%	105%	591%	119%	1307%	137%	1108%	154%	833%	133%	792%
3	100%	288%	86%	218%	92%	268%	95%	408%	81%	362%	91%	220%
4	100%	341%	72%	236%	47%	173%	123%	414%	109%	275%	76%	108%

Primary BM cultures that had been washed extensively with PBS to remove non-adherent haematopoietic cells, were trypsinised and seeded onto 35-mm dishes at 5 x 10^4 cells/dish in 2 ml of stromal medium. The following day the number of adherent cells was determined by counting the cells in duplicate dishes. These values were as follows: *Exp. 1* = 3.6 ± 0.1 x 10^4 cells/dish; *Exp. 2* = 3.9 ± 0.3 x 10^4 cells/dish; *Exp. 3* = 2.9 ± 0.2 x 10^4 cells/dish; *Exp. 4* = 3.9 ± 0.3 x 10^4 cells/dish. The supernatant in the remaining dishes was replaced with complete stromal medium or stromal medium lacking either hydrocortisone, horse serum, ß-mercaptoethanol; horse serum and hydrocortisone, or all three supplements, with or without 20 ng/ml FGF-2. After 4 days, the culture supernatant was replaced with fresh medium with or without FGF-2. Cell numbers on duplicate dishes were determined on day 7. This experiment was repeated four times with BM from four different donors. The cell numbers obtained in each experiment are expressed as a percentage of the cell number noted in control dishes. Control cultures were defined as those cultures containing BM stromal cells grown in complete stromal medium in the absence of FGF-2. The number of cells determined on day 7 in control cultures was: *Exp. 1* = 5.9 ± 0.5 x 10^4 cells/dish; *Exp. 2* = 6.9 ± 1.1 x 10^4 cells/dish; *Exp. 3* = 4.9 ± 0.4 x 10^4 cells/dish; *Exp. 4* = 6.1 ± 0.8 x 10^4 cells/dish.

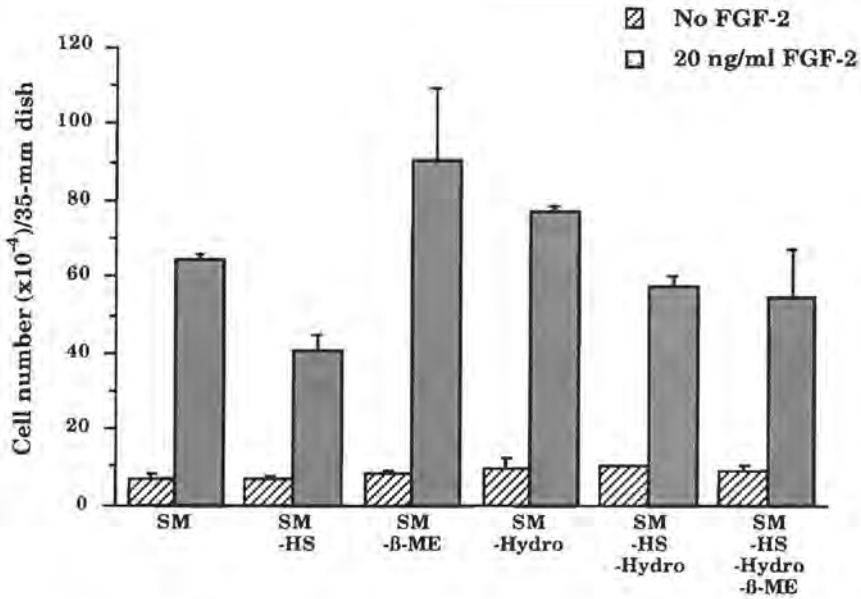

Fig. 5. The contribution of stromal medium supplements to the growth of passaged BM stromal cells in the absence or presence of FGF-2. Cell numbers from *Exp. 2* of Table 4 are presented as the mean number of cells/dish ± SD.
SM: stromal medium; HS: horse serum; ß-ME: ß-mercaptoethanol; Hydro: hydrocortisone.
* $p < 0.01$, ** $p < 0.02$ with respect to the control.

The addition of FGF-2 to stromal cells grown in stromal medium lacking horse serum increased the number of cells by 2 to 6-fold above control cultures ($p < 0.02$ in all four experiments). Similarly, the addition of FGF-2 stimulated cell growth by approximately 2 to 13-fold in cultures grown without ß-mercaptoethanol ($p < 0.01$ in all four experiments). Thus, the stimulation of cell proliferation by FGF-2 was not affected by the omission of horse serum or ß-mercaptoethanol from the medium.

In contrast to primary cultures, the omission of hydrocortisone from the stromal medium did not affect the growth-stimulatory effect of FGF-2 (4 to 11-fold above control cultures; $p < 0.02$ in all four experiments). Similarly, when both horse serum and hydrocortisone were omitted from the medium, FGF-2 increased cell growth by approximately 3 to 8-fold ($p < 0.01$ in all four experiments). When all three medium supplements, horse serum, hydrocortisone, and ß-mercaptoethanol, were omitted from the medium, FGF-2 stimulated cell growth in three out of four experiments by 2 to 3-fold ($p < 0.02$ in each of the three experiments).

FGF-2 was therefore noted to stimulate the growth of passaged BM stromal cells equivalently in control medium and medium lacking hydrocortisone, horse serum and ß-mercaptoethanol.

Discussion.

1. Summary of results.

The establishment of an adherent layer of BM stromal cells is important for the proliferation and differentiation of haematopoietic cells in LTBM cultures. Considerable effort has been directed towards elucidating the manner by which the stromal layer supports myelopoiesis and lymphopoesis. A large number of cytokines produced by stromal cells and acting on haematopoietic cells have been described (Quesenberry et al., 1991; Kittler et al., 1992; Eaves et al., 1991). In contrast, not much is known about the growth factors that regulate the proliferation and differentiation of the stromal cells. A previous student in Dr Wilson's laboratory demonstrated that FGF-2, is mitogenic for BM stromal cells (Oliver et al., 1990). I therefore chose to extend these studies, especially as it was noted that FGF-2 is produced by BM stromal cells (Brunner et al., 1993) and could therefore act in an autocrine manner to stimulate stromal proliferation.

I added FGF-2 to BM buffy coat cells seeded in culture and observed that this growth factor:

(1) accelerated the formation of the stromal layer by acting as a mitogen for primary human BM stromal cells;

(2) significantly delayed the senescence of BM stromal cells which normally cease growing after approximately two to three passages. This resulted in considerable expansion of the stromal cells in culture.

(3) induced morphological changes in the developing adherent stromal layer;

I also observed that:

(4) horse serum is an important medium supplement for the establishment of the stromal layer in primary BM cultures whereas ß-mercaptoethanol may be inhibitory for the growth of these cells;

(5) the mitogenic effect of FGF-2 on primary BM stromal cells was significantly inhibited by the omission of hydrocortisone from the culture medium. In contrast, this inhibition was not noted with passaged BM stromal cells.

2. Morphological changes induced by FGF-2.

The addition of FGF-2 to primary human BM cultures altered the morphology of the adherent stromal layer. The cells lost their contact inhibition and formed densely packed layers of spindle shaped cells. Similar morphological changes have also been observed in other cell types such as 3T3 fibroblasts, skin fibroblasts, vascular endothelial cells, and smooth muscle cells, to which FGF-2 has been added

(Gospodarowicz and Moran, 1974; Gospodarowicz et al., 1987a and b). These morphological features, elongation, criss-cross patterns, and high cell density, are typical of transformed cells. The similarity in the phenotype of transformed and FGF-2-treated cells suggests that FGF-2 may induce cellular transformation. Indeed, several groups have demonstrated that FGF-2 promotes the growth of normal cells in soft agar, a characteristic of transformed cells (Jaye et al., 1988; Rogelj et al., 1989; Sasada et al., 1988). In addition, FGF-2-transfected 3T3 fibroblasts exhibit transformed morphology and are tumorigenic (Quarto et al, 1991). Furthermore, a number of tumour cell lines produce FGF-2 and their clonogenic growth is dependent upon its expression (Morrison et al., 1993). Transformation of rat kidney fibroblasts by the simian sarcoma virus and the spontaneous immortalisation of rabbit smooth muscle cells are also associated with increased expression of FGF-2 (Milner, 1991; Winkles et al., 1993). Thus, in addition to its mitogenic activity, FGF-2 may also be a transforming factor.

3. FGF-2 is a mitogen for primary BM stromal cells.

My work shows that FGF-2 is a mitogen for BM stromal cells and significantly promoted the development of the primary stromal cell layer.

3.1. *BM stromal cell types that respond to FGF-2.*

The stromal layer consists of a number of cell types arising from a common stromal precursor cell (MSC). Since FGF-2 is mitogenic for numerous cells of mesodermal origin (Gospodarowicz et al., 1987b), as well as passaged BM stromal cells (Oliver et al., 1990), it is likely that a number of cell types in the primary BM stromal cultures respond to FGF-2.

FGF-2 could therefore enhance the formation of the adherent layer in LTBM cultures by stimulating the growth of differentiated stromal cells. In addition, FGF-2 could increase stromal cell numbers by initially acting on RS cells and MSCs to potentiate their survival and/or proliferation since some, but not all, of these cells express FGFRs (Colter et al., 2001). This could be tested by determining the number as well as the size of CFU-Fs in four to seven day old primary cultures treated with or without FGF-2. An increase in the number of CFU-Fs in FGF-treated cultures would indicate that FGF-2 enhances the survival of stromal precursor cells. An increase in the size of the CFU-F colonies size would indicate that this growth factor stimulates the proliferation of stromal precursor cells.

In keeping with this it has been observed that RS cells plated at low cell density (1.5 - 3 cells/cm^2) exhibit a log phase of rapid growth whereas cell plated at high cell

densities (6 cells/cm^2) do not (Colter et al., 2000). Colter et al. (2000) suggest that the slow growth of RS cells at high cell densities might be due to either cell-cell contact or factors that the cells secrete into the medium. Presumably therefore, RS cells either grow slowly or even cease to grow in LTBM cultures. Since I have shown that FGF-2 delays the senescence of BM stromal cells (see section 4), it could be speculated that FGF-2 could overcome this slow rate of growth at high cell densities thus allowing RS cells to continue contributing to the development of the stromal cell layer. RS cells could therefore be isolated as described by Colter et al. (2000) and seeded in culture at different cell densities in the presence or absence of FGF-2 to compare growth rates.

3.2. *BM stromal cell types that produce FGF-2.*

The vast majority of cells in stromal cell cultures have been shown to express FGF-2 (Brunner et al., 1993). FGF-2 is therefore likely to act in an autocrine manner to promote the proliferation of MSCs and their progeny.

However, FGF-rich niches may be created by the selective expression of FGFR isoforms. For example, one cell type within the adherent layer might express the FGFR-2-IIIc splice variant, while another might express FGFR-2-IIIb, which selectively bind FGF-2 and FGF-7 respectively (Galzie et al., 1997; Schofield and Gallagher, 1994). Selective expression of FGFR-isoforms by the different cell types within the BM stromal cell layer could be determined by sorting the stromal cells according to type by FACS. The mRNA synthesis of the various FGFR-isoforms as well as different FGFs could then be analysed on the different stromal cell fractions.

3.3. *Comparison of FGF-2 with other BM stromal cell mitogens.*

Other growth factors described in the literature as mitogens for BM stromal cells are PDGF, EGF, and TNF (Rosenfield et al., 1985; Rogalsky et al., 1992; Kimura et al., 1988). PDGF is the most effective of these three growth factors and is also produced by a BM stromal cell line, MBA-2 (Abboud, 1993). PDGF stimulates the growth of CFU-Fs as well as passaged human marrow fibroblasts and murine marrow endothelial cells (Abboud, 1993; Fontenay et al., 1992; Bryckaert et al., 1988; Hirata et al., 1985; McIntyre and Bjornson, 1986; Rosenfeld et al., 1985). The relative mitogenicity of FGF-2 and PDGF is unknown, as I and the other authors have used different assays. I have studied the stimulatory effects of FGF-2 on stromal cell growth by determining cell numbers. The other authors have investigated the effect of PDGF on the proliferation and survival of MSCs by determining the number and size of stromal colonies formed after seeding BM buffy coat cells in the absence or presence of PDGF (Hirata et al.,

1985; McIntyre and Bjornson, 1986). These results showed that 3 ng/ml PDGF increased the size of the colonies and increased the number of colonies by 8-fold (Hirata et al., 1985; McIntyre and Bjornson, 1986). The effect of PDGF on the proliferation of passaged BM stromal cells was determined by measuring the amount of radioactive thymidine incorporated into DNA. These studies showed that with concentrations between 6 - 100 ng/ml PDGF the amount of ^{3}H-thymidine incorporated into the DNA of human marrow fibroblasts was increased by 3 to 6-fold (Rosenfeld et al., 1985; Kimura et al., 1988; Bryckaert et al., 1988), and into the DNA of a murine marrow endothelial cell line the increase was 5 to 8-fold (Abboud, 1993). I have observed that 2 - 20 ng/ml FGF-2 induced a 3 to 17-fold increase in the number of stromal cells in primary human BM cultures. Therefore, both PDGF and FGF-2 are potent mitogens for BM stromal cells but their relative potencies are still unknown. It is also not known whether BM stromal cells stimulated by PDGF are functionally the same as those stimulated by FGF-2. However, studies indicate that PDGF stimulates haematopoiesis indirectly by inducing BM stromal cells to produce GM-CSF, IL-3, and IL-6 (Yang et al., 2001).

4. Senescence of BM stromal cells.

My observation that the number of cell generations obtained from human BM stromal cells cultured in the presence of 20 ng/ml FGF-2 (26 generations) greatly exceeded that of control cultures (2 generations) indicated that FGF-2 significantly delayed the senescence of these cells. The mechanism for this is not clear. Senescence of normal somatic cells is characterised by cessation of cell proliferation after a finite number of population doublings known as 'limited replicative life-span' (Wynford-Thomas, 1999; Goldstein, 1990). One underlying clock driving the arrest of cell growth involves the progressive erosion of chromosome telomeres which occurs with each round of DNA replication (Wynford-Thomas, 1999; Reddel, 1998a and b). Reduction beyond a critical length is a signal for cellular senescence. Other mechanisms inducing senescence involve the repression of genes promoting proliferation with the reciprocal expression of anti-proliferative genes (Wynford-Thomas, 1999; Reddel, 1998a and b; Garkavtsev et al., 1998; Goldstein, 1990; Phillips et al., 1992).

4.1. Mechanisms by which FGF-2 might delay senescence.

4.1.1. *Inhibition of telomere shortening.*

One of the cellular mechanisms used to overcome proliferative restriction, as in many tumour cells, is the activation of the enzyme telomerase (Allsopp et al., 2003). Telomerase is a ribonucleoprotein enzyme that stably maintains telomere length by the

addition of the sequence of TTAGGG repeats to telomeres. Activation of telomerase appears to involve the suppression of a telomerase repressor gene (Tanaka et al., 1998) and thus, might represent a mechanism by which FGF-2 delays the senescence of BM stromal cells. However, some immortalised human cells avoid telomeric shortening by non-telomerase mechanisms which may include the replacement of telomeric DNA by recombination and copy switching, or retrotransposition (Reddel, 1998a).

4.1.2. *Expression of genes promoting proliferation.*

One of the genes that has been implicated in the promotion of cell proliferation is the proto-oncogene, c-*fos*, and its expression has been shown to be an early and essential prerequisite for the initiation of DNA synthesis in dividing fibroblasts (Holt et al., 1986; Nishikura and Murray, 1987). In senescent fibroblasts, the c-*fos* gene appears to be under specific transcriptional repression (Seshadri and Campisi, 1990; Phillips et al, 1992). Proteins such as insulin-like growth factor binding protein-3, statin, PAI-1, W53-10, and terminin, which are highly abundant in senescent but not in proliferating cells, have been implicated in the inhibition of transcription of growth-promoting genes (Goldstein et al., 1993; Wang et al., 1989 and 1996; Mu et al., 1998; Grigoriev et al., 1996; Wang and Tomaszewski, 1991). Thus, FGF-2 may delay the senescence of BM stromal cells by inhibiting the expression of proteins that block the transcription of growth promoting genes or by inducing the expression of growth promoting genes. Indeed, FGF-2 upregulates c-*fos* mRNA and protein levels in adrenal cells (Lotfi and Armelin, 2001; Viard et al., 1993; Puchacz et al., 1993). While it is unknown whether this is due to a direct stimulation of c-*fos* transcription or to a decreased expression of inhibitors of c-*fos* transcription, it indicates that FGF-2, at least in certain cell types, may shift the transcriptional balance of these factors towards growth promotion. Therefore, the effect of FGF-2 on the mRNA or protein levels of c-*fos* or possible inhibitors of the transcription of growth-promoting genes such as insulin-like growth factor binding protein-3, statin, PAI-1, W53-10, and terminin could be determined on senescent BM stromal cells.

4.1.3. *Repression of anti-proliferative genes.*

Genes such as tumour suppressor genes (e.g. the retinoblastoma (Rb) gene and the *p53* gene), the GADD45 gene (Kastan et al., 1992) and the WAF1/CIP1 gene (El-Deiry et al., 1993) encode proteins that inhibit cell growth. The protein products of tumour suppressor genes arrest cell proliferation in response to DNA damage (Hinds and Weinberg, 1994). However, their role in the regulation of normal cell growth is not

well understood although the Rb and *p53* gene are constitutively activated during senescence (Garkavtsev et al., 1998; Gire et al., 1998). Phosphorylation of the Rb protein product (pRb), which is carried out by complexes of cyclins and cyclin dependent kinases (Cdks)(Dulic et al., 1992; Hinds et al., 1992; Ewen et al., 1993a; Kato et al., 1993), is important for regulating its function. In its active form (i.e. when the cell is dormant), pRb is hypophosphorylated and becomes hyperphosphorylated when the cell begins to cycle (Hinds and Weinberg, 1994). Indeed, FGF-2 has been shown to increase the expression of the hyperphosphorylated form of pRb in a number of non-haematopoietic cells (Liu et al., 2001; Nath et al., 1999; Takuwa et al., 1993). Thus, FGF-2 may delay the senescence of BM stromal cells by inhibiting the expression of anti-proliferative genes such as *p53*, and WAF1/CIP1 which could be determined by measuring the mRNA synthesis of these gene products in stromal cells treated with or without FGF-2. The expression of hyper- and hypo-phosphorylated forms of pRb could also be assessed by Western blot analysis.

There is also evidence suggesting a link between the inhibitory effects of TGF-ß and a block in hyperphosphorylation of pRb. TGF-ß has been shown to block hyperphosphorylation of pRb by inhibiting the complex formation between cyclin E and its partner kinase, Cdk 2 (Koff et al., 1993), as well as the expression of Cdk4 (Ewens et al., 1993b). Thus, one possible mechanism by which FGF-2 may delay the senescence of BM stromal cells is through the inhibition of TGF-ß action. Indeed, FGF-2 has been shown to overcome the suppressive effect of TGF-ß on myeloid progenitor cell growth (Gabrilove et al., 1993), although the mechanism by which TGF-ß inhibits myeloid growth is unknown.

4.1.4. *Inhibition of differentiation inducing factors.*

Another mechanism by which FGF-2 might delay senescence is by inhibiting the activity of growth factors that induce cell differentiation, e.g. TGF-ß. Indeed, FGF-2 has been shown to inhibit the TGF-ß induced differentiation of the leukaemic cell line, K562 (Burger et al., 1994), and of muscle cells (Rosenthal et al., 1991; Olwin and Rapraeger, 1992). In synergism with PDGF, FGF-2 also inhibits the differentiation of oligodendrocyte-type 2 astrocyte progenitor cells (Bogler et al., 1990; Wolswijk and Noble, 1992), and together with SCF and LIF, FGF-2 promotes the proliferation and survival of primordial germ cells (Matsui et al., 1992; Resnick et al., 1992).

The inhibition of cell differentiation could be achieved by a variety of mechanisms such as inhibition of growth factor synthesis, activation and/or presentation, signalling in the downstream pathway, or inhibition of growth factor receptor synthesis and/or

signalling. For example, FGF-2 inhibits insulin-like growth factor (IGF)-II expression in BC3H-1 muscle cells. This results in increased IGF-I receptor expression by these cells and consequently, increased IGF-I signalling which inhibits muscle cell differentiation (Rosenthal et al., 1991).

FGF-2 might also delay senescence by inhibiting the expression of receptors for growth factors that induce differentiation or by stimulating receptor expression of growth factors that mediate inhibition of differentiation. For example, FGF-2 has been shown to decrease the expression of TGF-ß receptors on endothelial cells making the cells less responsive to the inhibitory effects of TGF-ß (Fafeur et al., 1990). FGF-2 has also been shown to stimulate the expression of IL-1 receptors on chondrocytes resulting in increased IL-1-induced metalloproteinase levels by these cells (Chandrasekhar and Harvey, 1989).

4.1.5. *Growth factor presentation and mobilisation.*

FGF-2 might delay the senescence of BM stromal cells by inducing changes in the composition of the ECM of these cells since this growth factor affects the synthesis and deposition of ECM proteins such as collagen, fibronectin and proteoglycans (Kinsella et al., 1997; Sternberg et al., 1996) and induces metalloproteinases and plasmin levels (Edwards et al., 1987; Pepper et al., 1993). This may result in an altered presentation of growth factors and cytokines to the cells (Sternberg et al., 1996). The ECM composition of proliferating fibroblasts differs from that of non-proliferating fibroblasts. For example, senescent fibroblasts express lower levels of collagen mRNA (Furth, 1991) and secrete increased levels of fibronectin, stromelysin, and MMP-1 compared to proliferating fibroblasts (Millis et al., 1992; Girard et al., 1993; Tresini et al., 1998). Thus, FGF-2 treatment of BM stromal cells may lead to remodelling of the ECM which may, in turn, affect the activity and availability of growth factors and cytokines which either promote or inhibit differentiation resulting in a delay of stromal senescence.

Alternatively, FGF-2 could delay senescence by affecting the availability of active extracellular growth factors by preventing the activation of latent growth factors that induce differentiation. In fact, FGF-2 has been shown to modulate latent TGF-ß activation in endothelial cells by regulating PA levels (Flaumenhaft et al., 1992). In this system, however, TGF-ß activation is induced by FGF-2 due to the increase in plasmin levels as a result of FGF-2 stimulation of PA which converts plasminogen to plasmin.

5. Contribution of medium supplements to the growth of BM stromal cells.

As described before, human Dexter LTBM cultures are grown in medium containing FCS supplemented with horse serum, hydrocortisone and an anti-oxidant. In order to determine which supplements were important for stromal cell growth, I determined their effects on the growth of passaged and primary stromal cells. The results showed that horse serum is an essential medium supplement for the establishment of the stromal layer in primary BM cultures. This suggests that horse serum contains a factor(s) that may act on stromal progenitor cells to stimulate their proliferation which can only be partially compensated for by the addition of FGF-2. In contrast to primary cultures of stromal cells, passaged stromal cells displayed relatively little to no growth over a one week period even when grown in stromal medium with all three supplements added which was most likely due to these cells senescing. Thus, it was not possible to determine which of the medium supplements were important for the growth of passaged stromal cells.

I also attempted to determine if FGF-2 was mitogenic for stromal cells cultured in stromal medium lacking one or more of the medium supplements. The most intriguing observation from these studies was that the omission of hydrocortisone from the culture medium, although not affecting stromal growth in the absence of FGF-2, completely abolished the mitogenic effect of FGF-2 on primary BM stromal cells but not on passaged stromal cells. The reason for this difference is unknown. It may be explained if a subpopulation of stromal or haematopoietic cells in primary BM cultures produce a factor(s) that specifically inhibits FGF-2 mitogenicity and whose own activity is inhibited by glucocorticoids such as hyrocortisone. This subpopulation of cells may no longer be present in the adherent layer of passaged BM cultures. This inhibitory factor could antagonise FGF-2 action by interfering with FGF-2 mobilisation or receptor presentation, or with FGF-2 receptor signalling (Grazul-Bilska et al., 2002; Sheffield, 1998; Chaidarun et al., 1994; Grieb and Burgess, 2000; Blanquaert et al., 2000).

Such a regulatory system may well exist because glucocorticoids decrease the expression of TGF-ß (Wen et al., 2002; Danielpour et al., 1991; Ayanlar-Batuman et al., 1991), as well as diminishes the activation of latent to active TGF-ß (Rowley, 1992). TGF-ß itself, inhibits the mitogenic effects of PDGF on BM fibroblasts (Bryckaert et al., 1988; Fontenay et al., 1992). Since TGF-ß has been shown to antagonise the effects of FGF-2 on myelopoiesis and K562 differentiation (Gabrilove et al., 1993; Burger et al., 1994), it is a likely candidate factor to counteract FGF-2 effects on stromal growth and to be inhibited by hydrocortisone. Indeed, in Chapter 4 I show that hydrocortisone inhibits TGF-ß induced urokinase plasminogen activator synthesis. Interestingly, the

catalytic domain of urokinase is required for the mitogenic activity of FGF-2 (Padró et al., 2002). Nevertheless, factors which block the mitogenic effect of FGF-2 on BM stromal cells and that are inhibited by hydrocortisone still need to be identified.

6. Conclusions.

The results presented in this Chapter indicate that FGF-2 may be an important cytokine for promoting the growth of the adherent stromal layer in LTBM cultures and thus may play a role in the growth of BM stromal cells as well as their progenitor cells *in vivo*. Indeed, FGF-2 protects mice from the toxic effects of irradiation by rapidly restoring the architecture of the BM (Okunieff et al., 1996; Ding et al., 1996 and 1997). Furthermore, the optimal concentration of FGF-2 required for radioprotection does not increase the rates of tumour growth or metastasis, nor does it decrease the radiosensitivity of the tumours (Ding et al., 1996). Thus, FGF-2 may prove useful in the treatment of cancer patients with radiotherapy by offering them protection against the high doses of radiation needed to ablate their tumours without promoting further tumour growth and dissemination.

My results also provide further insight into the mechanisms of regulation of FGF-2 and stromal cell growth. The requirement of horse serum for successful establishment of LTBM cultures appears to be due to the presence of factors in the serum which are essential for stromal cell growth and which can only be partially replaced by FGF-2. In addition, heterogeneous primary BM cultures, in contrast to the more homogeneous passaged stromal cell cultures, appear to produce an activity that specifically interferes with FGF-2 action without affecting stromal cell growth and that is down-regulated by hydrocortisone. This activity is possibly TGF-ß.

Chapter 3.

FGF-2 stimulates myelopoiesis in human LTBM cultures.

Introduction.

In this chapter I have focused my analysis on the effects of FGF-2 on the haematopoietic compartment. I have analysed the number of progenitor cells in the supernatant and adherent layer of LTBM cultures treated with and without FGF-2.

1. FGF-2 action on haematopoietic cells.

Although *in vivo* studies indicate otherwise (Ortega et al., 1998; Dono et al., 1998; Ozaki et al., 1998; Miller et al., 2000), a large body of evidence from *in vitro* studies suggests that FGF-2, although not essential, may be relevant in regulating haematopoiesis. *In vitro*, FGF-2 acts on both primitive and committed progenitor cells directly or in concert with other haematopoietic growth factors to induce their proliferation and/or differentiation and, therefore, may function in the survival and differentiation of haematopoietic cells. FGF-2 is a survival factor for embryonic stem cells (Faloon et al., 2000; Anzai et al., 1999; Yuen et al., 1998) and acts in concert with SCF, GM-CSF, and IL-3 to stimulate the growth of primitive, multipotent haematopoietic progenitor cells with a $CD34^+CD33^-$ phenotype (Gabbianelli et al., 1990; Gabrilove et al., 1994). FGF-2 can also influence specific lineage-derived haematopoietic progenitors. FGF-2, by synergising with GM-CSF, Epo and Meg-CSF, can induce committed precursors to give rise to GM-colonies, erythroid bursts and megakaryocyte colonies, respectively (Gallicchio et al., 1991; Han et al., 1992; Bikfalvi et al., 1992; Bruno et al., 1993).

2. *In vitro* assays to study haematopoiesis.

2.1. Soft-agar colony forming assays.

Before long-term liquid BM culture systems were developed, *in vitro* studies on stem cell differentiation were performed with freshly isolated BM cells seeded in soft-agar colony assays in the presence of an exogenous supply of factors stimulating colony growth (Bradley and Metcalf, 1966; Pluznik and Sachs, 1966). Colony forming assays are still used extensively in haematopoietic research and can also be utilised to identify primitive BM cells. Some of the BM cells seeded in soft agar give rise to very

large colonies (the colonies contain an average of 50 000 cells and have a diameter greater than 5 mm). These cells are called high proliferating potential-CFCs (HPP-CFCs) and are capable of differentiation into multiple haematopoietic lineages in response to growth factors (McNiece et al., 1990; Stewart et al., 1993). The limitations of the CFC-assay are: (1) the cultures do not allow the study of stem cell and progenitor cell interactions with the stromal cell microenvironment in which they exist *in vivo*, and (2) the differentiation and proliferation of the haematopoietic cells are dependent on the exogenous supply of CSFs which are expensive to buy.

2.2. LTBM cultures.

The development by Dexter and colleagues of an *in vitro* long-term liquid culture system that supports the renewal and differentiation of murine myeloid stem cells was a major advancement for studying the regulation of haematopoiesis (Dexter et al., 1977a).

A significant advantage of Dexter LTBM cultures and Whitlock-Witte LTBM cultures (developed subsequently to study the differentiation of B-lymphocytes) is that the differentiation and proliferation of the progenitor cells is driven by the stromal cells thus obviating the need for exogenously added growth factors (Dexter et al., 1977a). A second advantage is that primitive progenitor cell self-renewal is also supported and, therefore, cultures can be maintained for extended periods of time. A third advantage is that since the differentiation and proliferation of myeloid and lymphoid stem cells is mediated by an adherent layer of stromal cells, studying the interactions of these primitive haematopoietic cells and their progenitors with the stromal cells is possible.

Dexter LTBM cultures can also be utilised to identify human primitive BM cells. A subpopulation of low density (< 1.068 g/ml) BM progenitor cells is capable of initiating long-term haematopoiesis (more than five weeks) on pre-established, irradiated stromal cell layers (Sutherland et al., 1990). These cells, referred to as long-term culture-initiating cells (LTC-ICs), have similar characteristics to HPP-CFCs (are predominantly non-cycling, give rise to multiple haematopoietic lineages, $CD34^+$, c-kit^+) and may represent one and the same cell type (Sutherland et al., 1993, Zandstra et al., 1997).

In comparison to murine LTBM cultures, human LTBM cultures tend to be relatively short lived and produce few multipotential progenitor cells (Quesenberry et al., 1991). A number of investigators have tried to improve the output of primitive progenitor cells and, thereby the longevity of human LTBM cultures, by adding

growth factors important for haematopoiesis. SCF, IL-3, IL-4, GM-CSF, G-CSF, and FGF-4 added to human LTBM cultures enhance the expansion of haematopoietic progenitor cells (Quito et al., 1996; Keller et al., 1994b; Firkin et al., 1993; Countinho et al., 1990; Platzer et al., 1988). However, only FGF-4 was found to increase the longevity of human LTBM cultures suggesting that this growth factor may act on primitive progenitor cells within the culture and that SCF, IL-3, IL-4, GM-CSF, and G-CSF are acting on progenitor cells at a more differentiated stage of development.

3. Human BM stem cells.

A major challenge in haematopoiesis has been the identification and purification of human BM stem cells. The hemangioblast, derived from an embryonic stem cell, is a pluripotent stem cell that can give rise to haematopoietic cells as well as endothelial cells (Robb and Elfanty, 1998). However, the use of human embryonic cells in research and for clinical applications is limited because of ethical considerations. It is also unknown whether the hemangioblast exists in postnatal life. Therefore, the challenge has remained to isolate BM stem cells from postnatal sources. The ability to identify and purify human BM stem cells is not only useful for studying the mechanisms that regulate their growth and differentiation, but is also important for clinical applications such as BM transplants, gene therapy, or diseases.

Murine stem cells have been successfully identified and purified due to the availability of an *in vivo* assay system. In the 1960's murine stem cells were identified and named CFU-spleen (CFU-s) because of their ability to repopulate the haematopoietic and lymphoid organs of lethally irradiated mice after intraveneous injection of BM nucleated cells (Till and McCulloch, 1961). The frequency of stem cells in this cell fraction (about 0.05% of murine BM cells) was determined by quantitating the number of cells capable of forming colonies in the spleen of irradiated mice (Till and McCulloch, 1961). Murine stem cells are Thy-1$^+$, Sca-1$^+$, c-kit$^+$ and CD34$^+$ and negative for haematopoietic lineage markers [CD45R (B-cells), TER119 (erythroid cells), CD11b/CD18 (macrophages), or Ly-6G (granulocytes)] (Krause et al., 1994; Orlic et al., 1993).

A major obstacle in isolating and characterising human stem cells has been the lack of an *in vivo* assay, equivalent to the CFU-s, to identify them. Only in the last decade has an *in vivo* method been developed that identifies primitive human haematopoietic cells. Following intravenous injection, human haematopoietic cells are able to repopulate the BM of non-obese diabetic (NOD)/severe combined immuno-deficient (SCID) mice with both myeloid and lymphoid human cells (Larochelle et al.,

1996; Cashman et al., 1997). The engrafting cells have thus been called SCID repopulating cells (SRCs) and represent approximately 1 in 3 x 10^6 human BM cells (Larochelle et al., 1996; Wang et al., 1997). SRCs are more primitive than HPP-CFCs and LTC-ICs since, in contrast to HPP-CFCs and LTC-ICs, they are rarely transduced with retroviruses (an indication of their quiesent state)(Larochelle et al., 1996), their maintenance is not supported by BM stromal cells in LTBM cultures (Gan et al., 1997), and they have a higher repopulating capacity than HPP-CFCs and LTC-ICs (Gan et al., 1997).

Multipotent progenitor cells (MAPCs) have now been isolated from human and murine BM and represent approximately 1 in 10^6 BM cells, (Reyes et al., 2000 and 2001; Jiang et al., 2002a and b). MAPCs are CD34, CD44, CD45, ckit, HLA-DR, and HLA-class I negative; express low levels of Flk-1, AC133, and Flt-1; and higher levels of CD13 (Reyes et al., 2002; Jiang et al., 2002b). MAPCs can give rise to haematopoietic cells *in vivo* but no culture conditions for their differentiation into blood cells have yet been found (Jiang et al., 2002b). Human stem cells that generate haematopoietic cells in culture, are characterised as being $CD34^+$, $HLA\text{-}DR^-$, $c\text{-}kit^+$, $CD38^-$, $CD33^-$, $Thy\text{-}1^{low}$, $Flk\text{-}1^+$, and $AC133^+$; lack lineage-specific markers [e.g. CD19 (B-cells), CD41a (megakaryocytes), and CD11b (granulocytes and monocytes)] (Briddell et al., 1992a; Andrews et al., 1986; Graig et al., 1993; Miraglia et al., 1997); give rise to long-term cultures in LTC-IC assays; and have multilineage potential (Berardi et al., 1995a).

4. Aims and findings.

In Chapter 2 I showed that FGF-2 has significant effects on the stromal compartment of BM cultures. A small number of $CD34^+$ haematopoietic progenitor cells express FGFRs (Berardi et al., 1995; Testa et al., 1996; Le Bousse-Kerdilès et al., 1996; Burger et al., 1998) and because FGF-2 has both synergistic and direct effects on progenitor cell proliferation, and promotes the self-renewal of primitive haematopoietic cell lines (Gabbianelli et al., 1990; Gabrilove et al., 1994; Gallicchio et al., 1991; Bruno et al., 1993; Anzai et al., 1999; Yuen et al., 1998), it may be a relevant growth factor in early blood cell development.

Therefore, in this chapter I have investigated the effect of this growth factor on the proliferation and differentiation of haematopoietic cells in both the supernatant and the adherent layer of LTBM cultures. My results show that:

(1) low concentrations of FGF-2 (0.2 to 2 ng/ml) increase the total number of haematopoietic cells in the supernatant of LTBM cultures, in particular the

number of GM- and G-CSF-responsive progenitor cells;

(2) low concentrations of FGF-2 increase the number as well as the size of cobblestone foci of haematopoietic cells in the adherent layer by increasing the number of GM-CSF responsive progenitor cells in this layer.

Since FGF-2 is produced by BM stromal cells and is present in haematopoietic cells *in vivo* (Brunner et al., 1993), my results suggest that FGF-2, *in vivo*, may form part of the complex cytokine network that regulates haematopoiesis.

Methods.

LTBM cultures were established and cultured in the absence and presence of FGF-2 and the effect of this growth factor on the proliferation and differentiation of haematopoietic cells in the supernatant and the adherent layer was determined using soft agar colony forming assays.

1. Establishment of LTBM cultures.

LTBM cultures were established as previously described in Chapter 2. The BM buffy coat cells were seeded at 2 x 10^7 cells/75-cm^2 tissue culture flask in 20 ml Gartner's medium (McCoy's 5A medium supplemented with 10 % heat-inactivated FCS, 10% heat-inactivated horse serum, 0.8 mM sodium pyruvate, 0.6% (vol/vol) of a 50x stock solution of essential amino acids, 0.3% (vol/vol) of a 100x stock solution of nonessential amino acids, 1.5 mM L-glutamine, 10^{-7} M hydrocortisone, 77 U/ml penicillin and 31 µg/ml streptomycin) and incubated in 5% CO_2 incubators at either 33°C or 37°C as indicated in the text (Gartner and Kaplan, 1980). The total number of BM buffy coat cells collected from each donor ranged between 3 - 7 x 10^8 cells, thus allowing between 15 and 35 LTBM flasks to be established for each experiment. After 4 days, all non-adherent cells were removed and layered over Ficoll-Hypaque (1.077 g/cm^3) to remove red blood cells (Greenberg et al., 1981) and mature granulocytes (this procedure was done on day 4 rather than at the start of the experiment so as to allow the stromal cell to adhere to the flask). The tubes were centrifuged for 30 minutes at 1, 000 x *g*. The low density cells were collected and washed twice with McCoy's 5A medium containing 1% FCS and returned to their original flasks in 20 ml of Gartner's medium. The number of cells recovered corresponded to 45% ± 9% of the 20 x 10^6 mononuclear cells originally seeded. Cultures were then supplemented with different concentrations of FGF-2 as indicated in the text.

2. Feeding of LTBM cultures.

The schedule for feeding the LTBM cultures is shown in Table 1. At week 1, the protocol differed slightly between LTBM cultures established to determine the effect of FGF-2 on the number of haematopoietic progenitor cells in the supernatant and those established to determine the effect of FGF-2 on the adherent layer. At this time point, the stromal layer in the cultures was not well formed and therefore, the number of adherent progenitor cells was not determined. At week 1, the cultures established to determine the numbers of progenitor cells in the adherent layer were left undisturbed

(indicated in italics in Table 1). Beginning at week 3, two thirds of the supernatant of each LTBM culture was removed once a week (day 1). Fresh Gartner's medium and the respective concentration of FGF-2 were added to the cultures twice a week (day 1 and day 4).

Table 1. Schedule for feeding the LTBM cultures.

Week	Day	Supernatant Removed (ml)	Medium Added (ml)	Total Volume per Flask (ml)
0	1[a]	-	20	20
	4[b]	20	20	20
1	1	10 *(0)**	10 *(0)**	20
	4	0	10	30
2	1	15	15	30
	4	0	15	45
3-11	1	30	15	30
	4	0	15	45

a: Initiation of cultures
b: Separation of low density cells from red blood cells on Ficoll-Hypaque
*LTBM cultures established to determine the number of haematopoietic cells in the adherent layer (indicated in italics) differed in the protocol from LTBM cultures established to determine the number of progenitor cells in the supernatant.

3. Determining progenitor cell number in the supernatant.

The number of progenitor cells recovered each week from the supernatant of each LTBM culture was determined using soft agar colony forming assays. An aliquot of the cells in supernatant was diluted in 3% glacial acetic acid in PBS (to lyse any contaminating red blood cells), and the total number of haematopoietic cells was determined using a Neubauer haemocytometer. The supernatant cells were then seeded in colony forming assays which were done in collaboration with Dr Janice Gabrilove, an expert haematologist, at Memorial Sloan Kettering Cancer Center, New York. The cells were plated at 10^5 haematopoietic cells/dish in 1 ml of 0.3% agar in McCoys 5A medium containing 10% FCS, in the absence and presence of 10 ng/ml GM-CSF or 100 ng/ml G-CSF. The number of clusters (3 to 40 cells) and colonies (>40 cells) was determined after 7 and 14 days of incubation at 37°C and 5% CO_2. After 7

days of culture, clusters develop in colony forming assays and reflect a mixture of both primitive and more differentiated progenitor cells in the assay sample. The more primitive progenitor cells have greater proliferative capacities than the more differentiated progenitor cells. Thus, the latter stop dividing because of their limited proliferative capacity, whereas the primitive progenitor cells continue to divide giving rise to colonies which are larger after 14 days of culture. The colony forming assay, therefore, provides a measure of the number of GM- and G-CSF-responsive progenitor cells in the haematopoietic cell population collected each week.

4. Identification of progenitor cell types.

To determine the lineage composition of the non-adherent haematopoietic cells, differential morphological counts were performed by Dr Gabrilove on cytospin preparations stained with Diff Quik stain. Differential counts were made based on the morphology of the nucleus and the cytoplasm.

5. Determining progenitor cell number in the adherent layer.

The number of haematopoietic progenitor cells recovered from the adherent stromal layer of each LTBM culture was also determined using soft agar colony-forming assays. Since the stromal layers required approximately 10-14 days to become established, the adherent layers were usually harvested from week 3 onwards, although in some experiments the adherent layers were removed at week 2. Sets of cultures were removed at weekly intervals, and the cells in the supernatant were collected. The remaining stromal cell layers were washed once with serum-free medium. Ten ml of 0.25% trypsin in a versene-containing buffer (Appendix 1.8) was added to the cultures. After 5 minutes at 37°C all the adherent cells were detached and an equal volume of medium containing 10% FCS was added to the flasks. (The FCS provided a competing substrate for the trypsin thereby quenching the effects of this enzyme on the cells). The cells were washed twice by centrifugation with McCoy's 5A medium containing 10% FCS. An aliquot of the cells in medium was diluted in 3% glacial acetic acid in PBS (to lyse any contaminating red blood cells), and the total number of cells was determined using a Neubauer haemocytometer.

Two types of cells were observed in the adherent layer. One cell type was small, round, with smooth edges and was assumed to represent haematopoietic cells. The other type of cell was larger, round, with rough edges. These cells were assumed to represent the stromal cells. The total number of haematopoietic cells present in the stromal layer was calculated based on these criteria so that each condition in the

colony-forming assay would contain the same number of "haematopoietic" cells, namely 10^5/35-mm dish. To prevent stromal cells from adhering to the culture dish, a feeder layer of 1 ml of 0.3% agar in McCoys 5A medium containing 10% FCS was first added to the culture dishes. The number of clusters (3 to 40 cells) and colonies (> 40 cells) was determined 7 and 14 days after plating the cells in agar in the presence of 10 ng/ml GM-CSF.

6. Determining the number of foci in the adherent layer.

The establishment and appearance of the adherent stromal layers was monitored weekly by examination under an inverted phase microscope. The number of "cobblestone" foci of haematopoietic cells in the adherent layer of LTBM cultures (Moore et al., 1997) was determined at weekly intervals. The foci were counted by placing the flask on a 75 x 38 mm glass slide etched with a grid. The results obtained from 4 flasks for each condition were then adjusted to represent the mean number of foci ± the SD/flask.

7. Statistical analysis.

The statistical significance of differences between the FGF-2-treated and control cultures was determined using the *t* test for paired samples (Quito et al., 1996). Statistical values were considered significant with $p < 0.05$ value.

Results.

1. The effect of FGF-2 on the generation of haematopoietic cells in the supernatant of LTBM cultures.

LTBM cultures were established by inoculating freshly isolated BM cells into tissue culture flasks, and the effect of FGF-2 on the production of haematopoietic progenitor cells in the supernatant was determined. At weekly intervals the total number and lineage composition of the haematopoietic cells, as well as the number of GM-CSF and G-CSF responsive progenitors, were determined in the supernatant.

1.1. *The effect of FGF-2 on the total number of non-adherent cells.*

To determine the effect of FGF-2 on the total number of haematopoietic cells in the supernatant of LTBM cultures, LTBM cultures were established as described in the Methods. Cultures were incubated at 37°C (*Exp. 1*) or 33°C (*Exp. 2 - 5*) [In the latter experiments I was primarily interested in investigating the effect of FGF-2 on the number of haematopoietic cells in the adherent stromal layer, and the literature (Platzer et al., 1988; Cashman et al., 1990; Eaves et al., 1991) indicates that 33°C, as opposed to 37°C, is the optimal temperature for studying this]. On day 4, after the red blood cells and mature granulocytes had been removed, different concentrations of FGF-2 (0.2, 1, 2, or 20 ng/ml of FGF-2 in *Exp. 1* and *2*, and 1 ng/ml of FGF-2 in *Exp. 3, 4,* and *5*) were added to quadruplicate flasks. The number of haematopoietic cells in the supernatant of these cultures was determined at various time points and compared to control cultures.

In Fig. 1, *Exp. 1*, it can be noted that after 1 week of culture the total number of cells in the supernatant of all cultures had decreased and continued to do so until week 3. The decrease in the number of cells in the supernatant may be explained by death of the mature cells and attachment of the haematopoietic cells to the developing adherent stromal cell layer. The cell number in the supernatant of LTBM cultures treated with 1 and 0.2 ng/ml FGF-2 decreased more rapidly than those in control cultures or cultures treated with higher concentrations of FGF-2 (Fig. 1, *Exp. 1*). The reason for this effect is not understood, but may possibly occur if FGF-2 at 0.2 and 1 ng/ml stimulates the attachment of the progenitor cells to the stromal cells and/or if the death of mature progenitor cells is faster in these cultures.

Furthermore, it can be seen in Fig. 1, *Exp. 1* that at week 4, the number of cells in the supernatant of control LTBM cultures and those to which 20 ng/ml of FGF-2 had been added continued to decrease reaching approximately 2 x 10^5 haematopoietic

cells/flask at week 6. From weeks 7 through 11 these two cultures yielded approximately 1.5 x 10^5 cells/ flask/ week (Fig. 1, *Exp. 1*). In contrast, the cell number in the supernatants of LTBM cultures, to which 0.2, 1, or 2 ng/ml of FGF-2 had been added, increased by approximately 9-fold compared to control cultures between weeks 6 and 7, reaching a maximum of 1 x 10^6 haematopoietic cells/flask ($p < 0.001$ at week 6, Fig. 1, *Exp. 1*). After week 7, the number of cells in the supernatant of these LTBM cultures also decreased. At weeks 9 and 10, however, these cultures still contained 2-fold ($p < 0.005$ at week 9) and 3-fold ($p < 0.05$ at week 10) more cells in the supernatant than control cultures or cultures treated with 20 ng/ml of FGF-2 (Fig. 1, *Exp. 1*).

Thus, from weeks 4 through 10, low concentrations of FGF-2 (0.2, 1 or 2 ng/ml) increased the total number of haematopoietic cells in the supernatant of LTBM cultures compared to control cultures incubated at 37°C.

In contrast to *Exp. 1*, the total number of non-adherent cells in both control and FGF-2-treated cultures incubated at 33°C decreased at approximately the same rate during the first 3 weeks (see Fig. 1, *Exp. 2 - 5*). In addition there was no significant difference at weeks 4 through 6 in the total number of non-adherent cells between control cultures and those treated with 1 ng/ml of FGF-2 in *Exp. 3 - 5* (Fig. 1).

In summary, the experiments demonstrate that low concentrations of FGF-2 increase the total number of haematopoietic cells in the supernatant of LTBM cultures incubated at 37°C but not in those incubated at 33°C. The reason for the latter might be due to a decreased rate in the establishment and development of the cultures as a result of the lower incubation temperatures of the flasks. Due to the limited amount of material and the nature of the experiments, i.e. the adherent layers were removed to determine progenitor number in them, I did not investigate the effects of FGF-2 on the number of haematopoietic cells in the supernatant of the LTBM cultures incubated at 33°C (see *Exp. 2 – 5)* beyond week 6. Therefore, I could not determine whether similar increases in the total number of haematopoietic cells in the supernatant such as those observed in *Exp. 1* would also have been noted at later time points in cultures incubated at 33°C.

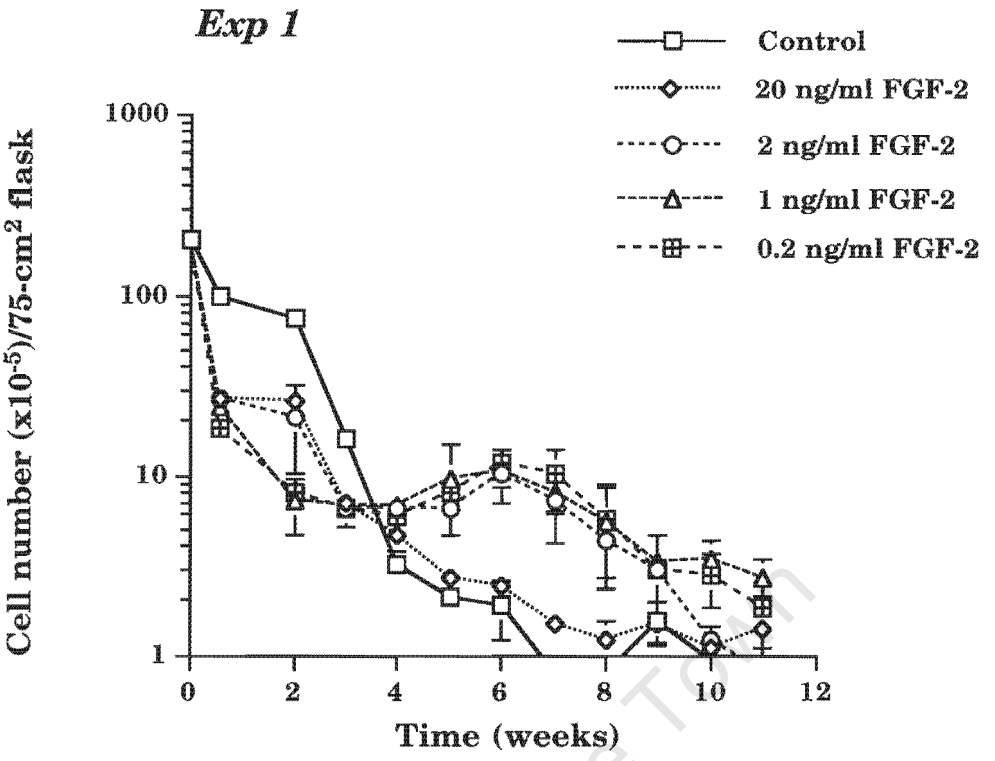

Fig. 1. The effect of FGF-2 on the number of haematopoietic cells in the supernatant of LTBM cultures. BM buffy coat cells (2 x 10^7) were inoculated into 75-cm^2 flasks. After 4 days, the non-adherent cells were removed and layered onto Ficoll-Hypaque to remove red blood cells. The low density cells were collected, washed and returned to their original flasks in 20 ml of Gartner's medium to which different concentrations of FGF-2 were added as indicated. The cultures in *Exp. 1* were incubated at 37°C, whereas the cultures in *Exp. 2 - 5* (see over the page) were cultured at 33°C. The total number of haematopoietic cells in the supernatant of each LTBM culture was determined at weekly intervals. Results shown represent the mean number of cells ± SD of four flasks for each condition. At some time points the error bars are too small to be observed.

Fig. 1 continued.

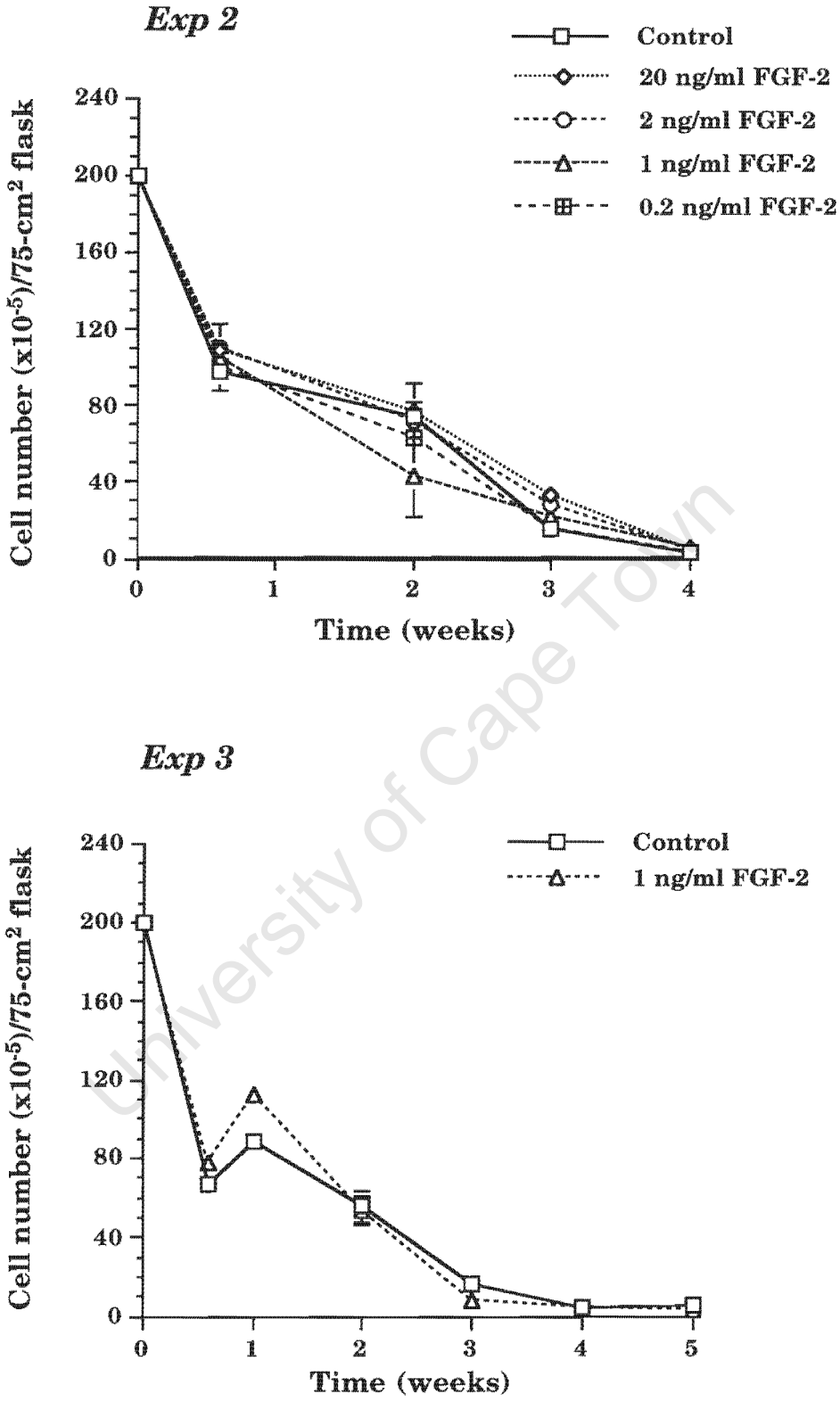

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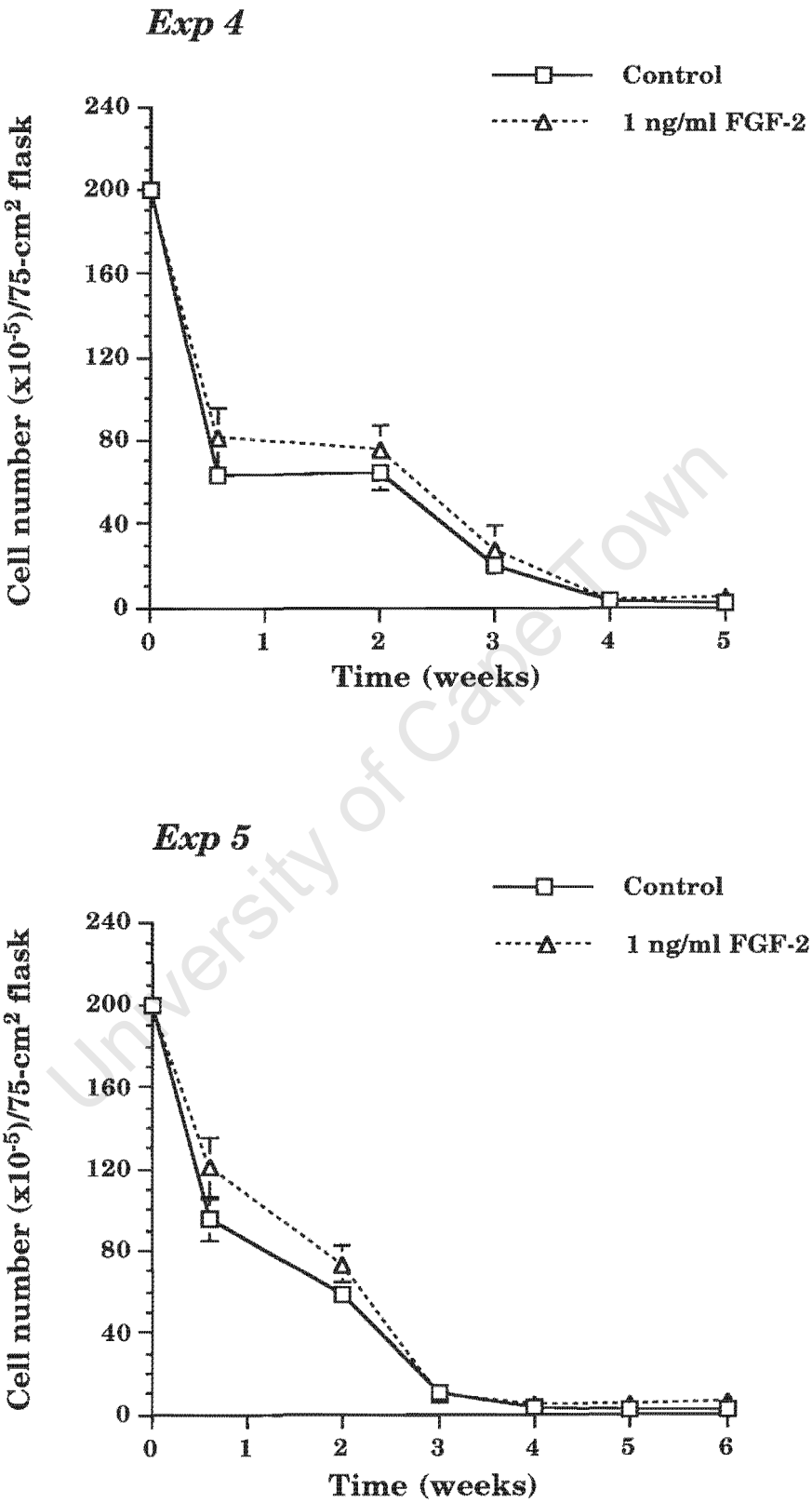
Fig. 1 continued.



1.2. *The effect of FGF-2 on the lineage composition of the non-adherent haematopoietic cells.*

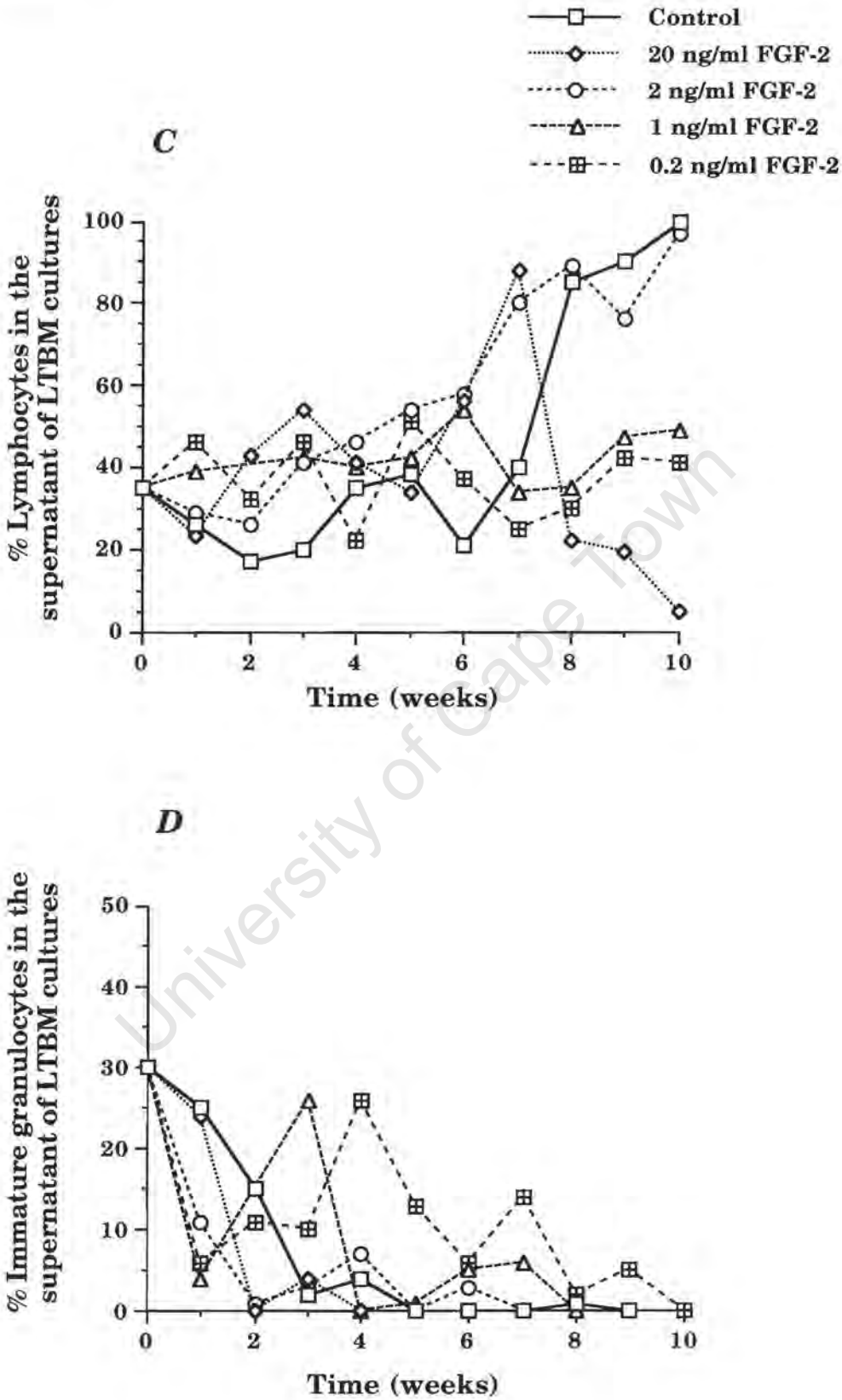
The lineage composition of the haematopoietic cells in the supernatant of control and FGF-2 treated LTBM cultures in *Exp. 1* were determined on cytospin preparations stained with Diff Quik as described in the Methods. There were significant differences in the cell types of the non-adherent cells between control cultures and those to which 0.2, 1, 2, or 20 ng/ml of FGF-2 had been added.

During the first 2 weeks of culture, 50 to 70% of the supernatant cells of all cultures were progenitors and mature cells of the granulocyte/neutrophil lineage (consisting mainly of bands and segmented neutrophils) (Fig. 2*A*). This decreased to 40 to 50% during weeks 3 to 5 in both control and FGF-2 treated cultures and remained so through weeks 6 to 10 in LTBM cultures treated with either 0.2 or 1 ng/ml of FGF-2. At weeks 8 through 10, LTBM cultures treated with 20 ng/ml of FGF-2 had mainly monocytes in the supernatant (Fig. 2*B*).

Control cultures and those treated with 2 ng/ml of FGF-2 showed a predominance of lymphocytes in the supernatant at weeks 8 through 10 (Fig. 2*C*). Other investigators have also reported a change in the composition of the supernatant cells in control cultures from granulocytic to either lymphocytic or monocytic (Gartner and Kaplan, 1980: Hocking and Golde, 1980). The reasons for this are unknown since Dexter LTBM cultures do not support the development of mature B- and T-cell lymphocytes (Shibita and Inoue, 1986; Touw and Löwenberg, 1984). It is most likely that the presence of lymphocytes in these cultures is due to spontaneous Epstein Barr virus (EBV) transformation of pre-B-cells since it has been shown that B-cell lines can be established in LTBM cultures when BM from EBV-positive donors are used (Pavlova et al., 1995; Novotny et al., 1990). Only 10 to 15% of the non-adherent cells in control cultures and those treated with 2 ng/ml of FGF-2 were myeloid.

In addition, it was noted that in LTBM cultures treated with 0.2 or 1 ng/ml of FGF-2, a greater percentage of the non-adherent cells were early progenitor cells (promyelocytes, myelocytes, and metamyelocytes) compared to control cultures (Fig. 2*D*).

Fig. 2 continued.



The lineage composition of the non-adherent cells was also determined in *Exp. 5* in which control or FGF-2 (1 ng/ml) treated LTBM cultures were incubated at 33°C (Fig. 3). Since I did not detect any significant difference in the total number of non-adherent cells between control and FGF-2 treated cultures (see Fig. 1, *Exp. 5*), I did not expect to observe any differences in the composition of the cells in these cultures. It was however noted, as in *Exp. 1*, that FGF-2 treated cultures in *Exp. 5* contained a higher percentage of early progenitor cells (11% at week 4) than control cultures (2% at week 4) which were of the granulocyte/neutrophil lineage ($p < 0.05$, Fig. 3). Control LTBM cultures in *Exp. 5* also became predominantly lymphocytic after week 4, which, may have been due to EBV transformation of the pre-B-cells.

Thus, based on the morphology of the cells, the results suggest that LTBM cultures treated with FGF-2 at either 33°C or 37°C contain a higher number of primitive progenitor cells of the granulocyte/neutrophil lineage compared to control cultures.

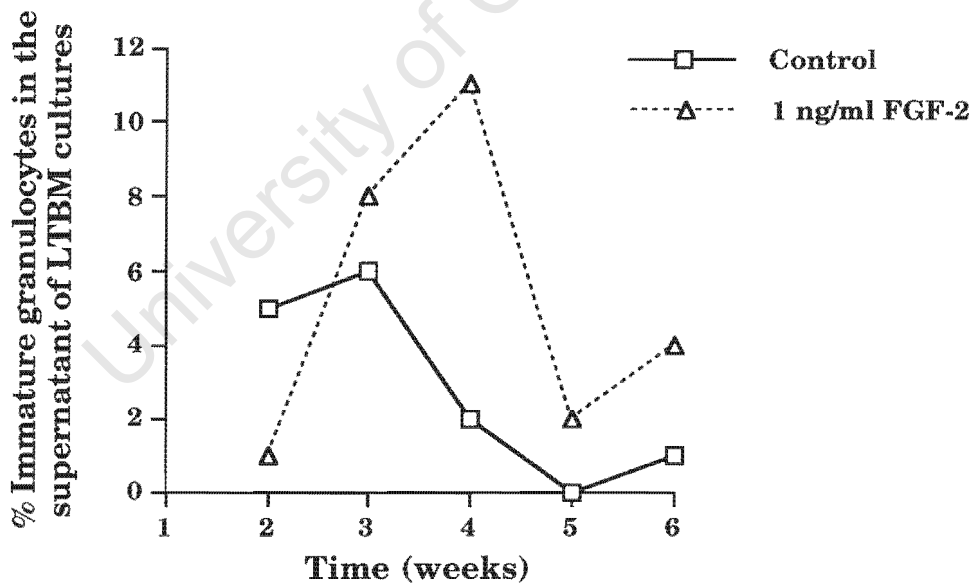


Fig. 3. The effect of FGF-2 on the lineage composition of the haematopoietic cells in the supernatant. BM buffy coat cells (2 x 10^7) were inoculated into 75-cm^2 flasks. After 4 days, the non-adherent cells were removed and layered onto Ficoll-Hypaque to remove red blood cells. The low density cells were collected, washed and returned to their original flasks in 20 ml of Gartner's medium and then cultured in the absence or presence of 1 ng/ml FGF-2. The cultures were incubated at 33°C. To determine the lineage composition of the cells in the supernatant, the cells were removed at weekly intervals and cytospin preparations, stained with Diff Quik, were prepared. One flask/point was used to prepare the cytospin preparations. Differential morphological counts were performed on the stained cells and the results expressed as a percentage of the total number of cells.

1.3. *The effect of FGF-2 on the number of non-adherent primitive progenitor cells.*

In addition to determining the effect of FGF-2 on the total number and composition of the haematopoietic cells in the supernatant of LTBM cultures, I also investigated the effect of this growth factor on the number of progenitor cells in this fraction. Haematopoietic cells from the supernatant of control and FGF-2 treated cultures were seeded at weekly intervals in colony-forming assays in the presence of GM-CSF or G-CSF. The number of colonies of the granulocyte-macrophage or granulocyte lineage, respectively, were scored 14 days after seeding the cells in the agar.

The results presented in Fig. 4 show the effect of FGF-2 on the number of primitive progenitor cells in the supernatant of control and FGF-2 treated cultures incubated at 37°C (*Exp. 1*). During the first 3 weeks of culture the number of GM-CSF responsive progenitor cells in the supernatant of control LTBM cultures and those treated with 0.2, 1, 2, or 20 ng/ml of FGF-2 decreased (Fig. 4*A*). At the time of initiation of the LTBM cultures, there were 2.1 x 10^4 colonies/flask which decreased to 200 - 400 colonies/flask by week 3. The number of GM-CSF responsive progenitors continued to decrease in control LTBM cultures and those treated with 20 ng/ml of FGF-2, such that, by week 5, there were no GM-CSF responsive progenitor cells in the supernatant of these cultures (Fig. 4*A*). LTBM cultures treated with 0.2, 1, or 2 ng/ml of FGF-2, however, contained significant numbers of GM-CSF responsive progenitors at weeks 4 through 8 (Fig. 4*A*). At weeks 4 through 7, these cultures contained 350 - 550 colonies/flask ($p < 0.001$ at week 6), which, decreased to approximately 50 colonies/flask by week 8. Thus, these results show that low concentrations of FGF-2 dramatically increased the number of GM-CSF responsive progenitor cells in the supernatant of LTBM cultures compared to control cultures or LTBM cultures treated with higher concentrations of FGF-2 (20 ng/ml).

The number of G-CSF responsive progenitors in the supernatant of control LTBM cultures and those to which 0.2, 1, 2, or 20 ng/ml of FGF-2 had been added (*Exp. 1*) decreased during the first 3 weeks of culture in a similar manner to that noted for GM-CSF responsive progenitors. At the time of initiation, the cultures contained 9 x 10^3 colonies/flask (Fig. 4*B*). By week 3, control LTBM cultures and those to which 20 ng/ml of FGF-2 had been added, had approximately 80 colonies/flask, whereas LTBM cultures to which 0.2, 1, or 2 ng/ml of FGF-2 had been added, contained 200 - 350 colonies/flask ($p < 0.02$). The number of G-CSF responsive progenitors in the supernatant continued to decrease in control LTBM cultures and those treated with 20 ng/ml of FGF-2, such that by week 5 there were no G-CSF

responsive progenitor cells in the supernatant of these culture. The number of G-CSF responsive progenitors in the supernatant of LTBM cultures treated with 0.2, 1, or 2 ng/ml of FGF-2 was also increased through weeks 4 to 6 compared to control LTBM cultures (Fig. 4*B*). At weeks 4 through 6, these cultures had 100 - 250 G-CSF ($p < 0.001$) colonies/flask which decreased to less than 50 colonies/flask by week 8.

This experiment shows that in cultures incubated at 37°C, low concentrations of FGF-2 increased the number of GM-CSF as well as G-CSF responsive progenitor cells in the supernatant of LTBM cultures compared to control LTBM cultures or those treated with a higher concentration of FGF-2 (20 ng/ml). This suggests that low concentrations of FGF-2 increase the total number of haematopoietic cells in the supernatant of LTBM cultures by increasing the number of primitive progenitor cells. In *Exp. 1*, the total number of haematopoietic cells in the supernatant of control LTBM cultures and those treated with 20 ng/ml of FGF-2 was low at week 6 (see Fig. 1, *Exp. 1*) and contained no detectable GM-CSF or G-CSF progenitors (see Fig. 4). In contrast, LTBM cultures treated with low concentrations of FGF-2 contained up to 1 x 10^6 haematopoietic cells/flask (see Fig. 1, *Exp. 1*) with 350 - 550 GM-CSF responsive progenitor cells/flask as well as 100 - 250 G-CSF responsive progenitors/flask. The reason for the higher proportion of GM-CSF responsive progenitor cells as compared to G-CSF responsive progenitor cells is most likely that GM-CSF acts on non-differentiated progenitors as well as lineage committed progenitors, whereas, G-CSF acts predominantly on the latter type (Heyworth et al., 1990).

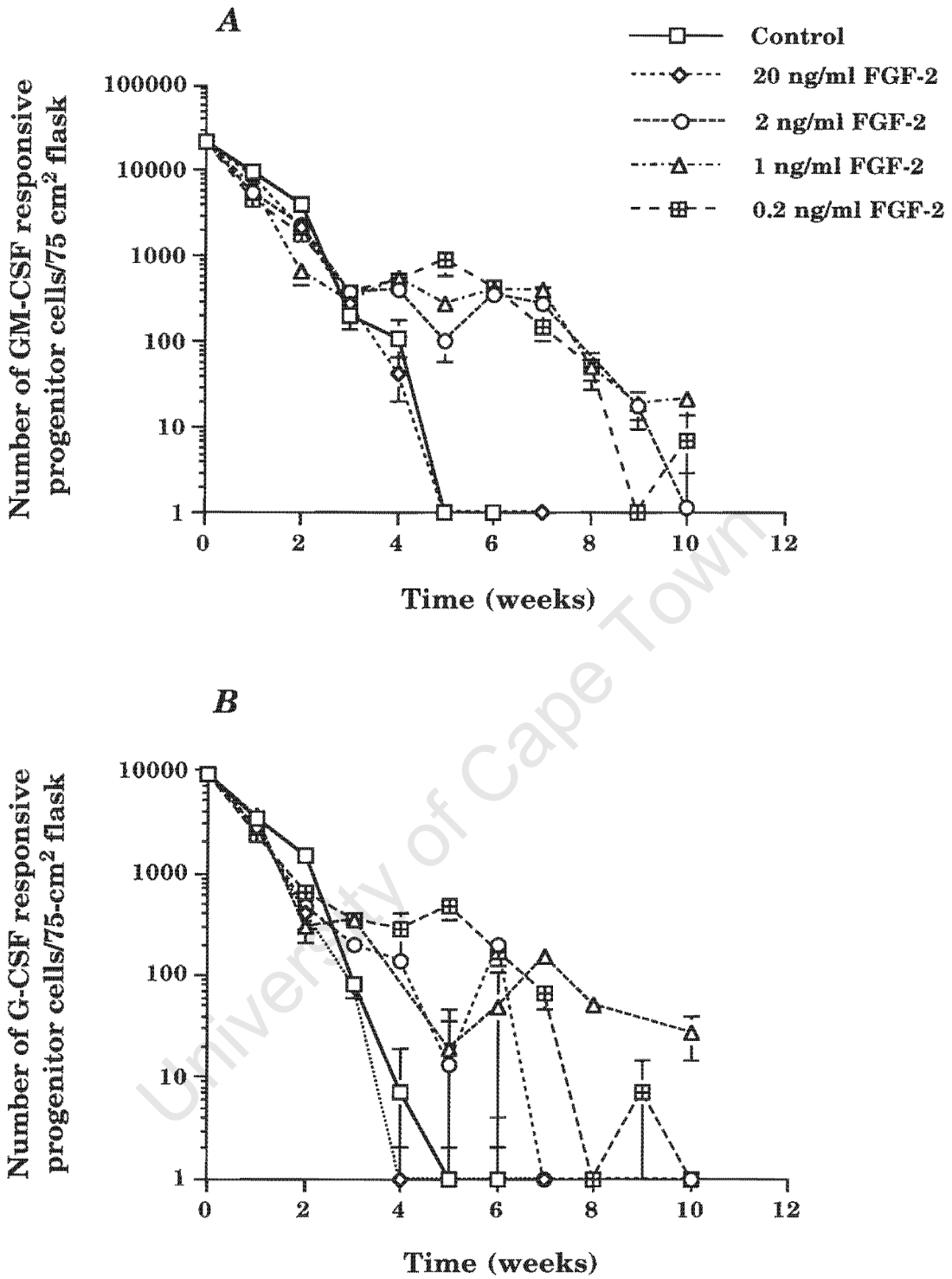


Fig. 4. The effect of FGF-2 on the number of primitive progenitor cells in the supernatant of LTBM cultures. BM buffy coat cells (2 x 10^7) were inoculated into 75-cm^2 flasks. After 4 days, the non-adherent cells were removed and layered onto Ficoll-Hypaque to remove red blood cells. The low density cells were collected, washed and returned to their original flasks in 20 ml of Gartner's medium to which 0, 0.2, 1, 2 or 20 ng/ml of FGF-2 was added. The cultures were maintained at 37°C. Haematopoietic cells were removed from the supernatant at weekly intervals and cultured in soft-agar at 10^5 haematopoietic cells/ml in the presence of (*A*) 10 ng/ml of GM-CSF or (*B*) 100 ng/ml of G-CSF. Results shown are the mean number of colonies ± SD from 4 dishes scored on day 14. At some time points the error bars are too small to be observed.

I also determined the number of GM-CSF responsive progenitor cells in the supernatant of five LTBM experiments that were incubated at 33°C (*Exp. 2 - 6*). In *Exp. 2*, LTBM cultures were treated with 0, 0.2, 1, 2 or 20 ng/ml FGF-2. From Fig. 5 it can be noted that, at week 3, the number of GM-CSF responsive progenitor cells was increased by 2.5-fold ($p < 0.02$) and 3.2-fold ($p < 0.001$) in the supernatant of LTBM cultures treated with 1 or 20 ng/ml of FGF-2, respectively. Cultures treated with 0.2 or 2 ng/ml of FGF-2 did not exhibit significantly more progenitor cells ($p > 0.1$) in the supernatant than control cultures.

In the other 4 experiments, LTBM cultures were treated with or without 1 ng/ml of FGF-2. However, this concentration of FGF-2 had little to no effect on the number of GM-CSF responsive progenitor cells in the supernatant of LTBM cultures ($p > 0.05$, data not shown). It was noted earlier that there was no significant difference in the total number of haematopoietic cells in the supernatant of control and FGF-2 treated LTBM cultures incubated at 33°C (see Fig. 1, *Exp. 2 - 5*). Therefore, it was not likely that control and FGF-2-treated cultures would show significant differences in the numbers of primitive progenitors.

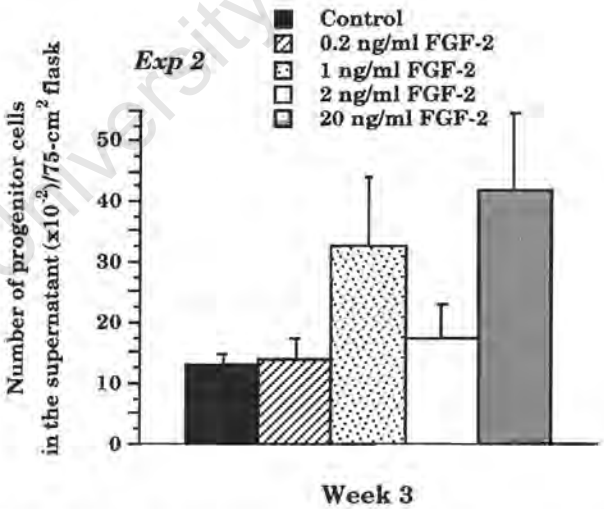


Fig. 5. The effect of FGF-2 on the number of GM-CSF responsive progenitor cells in the supernatant of LTBM cultures. BM buffy coat cells (2 x 10^7) were inoculated into 75-cm^2 flasks. After 4 days, the non-adherent cells were removed and layered onto Ficoll-Hypaque to remove red blood cells. The low density cells were collected, washed and returned to their original flasks in 20 ml of Gartner's medium to which different concentrations of FGF-2 were added as indicated. The cultures were maintained at 33°C. Haematopoietic cells were removed from the supernatant at weekly intervals and cultured in soft-agar at 10^5 haematopoietic cells/ml in the presence of 10 ng/ml GM-CSF. Results shown are the mean number of colonies ± SD from 4 dishes scored on day 14.

2. The effect of FGF-2 on the adherent layer of LTBM cultures.

2.1. *The effect of FGF-2 on the establishment and appearance of the adherent layer.*

I had previously observed (in Chapter 2) that the addition of FGF-2 to BM buffy coat cells seeded in culture affected the morphology and growth of the developing adherent stromal layer. Similarly, the adherent stromal layers in LTBM cultures (maintained at 37°C or 33°C) to which FGF-2 had been added lost their contact inhibition and formed a densely packed sheet of spindle-shaped cells compared to untreated cultures. In some cases when LTBM cultures were treated with 20 ng/ml of FGF-2, the adherent stromal cells formed multilayered sheets of spindle shaped cells.

Round, refractile, haematopoietic cells adhered to the stromal layer (Fig. 6*A*) and later became embedded in this layer forming typical cobblestone foci of haematopoietic cells (Fig. 6*B*). Adipocytes also developed in the stromal layer of both control (Fig. 6*C*) and FGF-2-treated cultures.

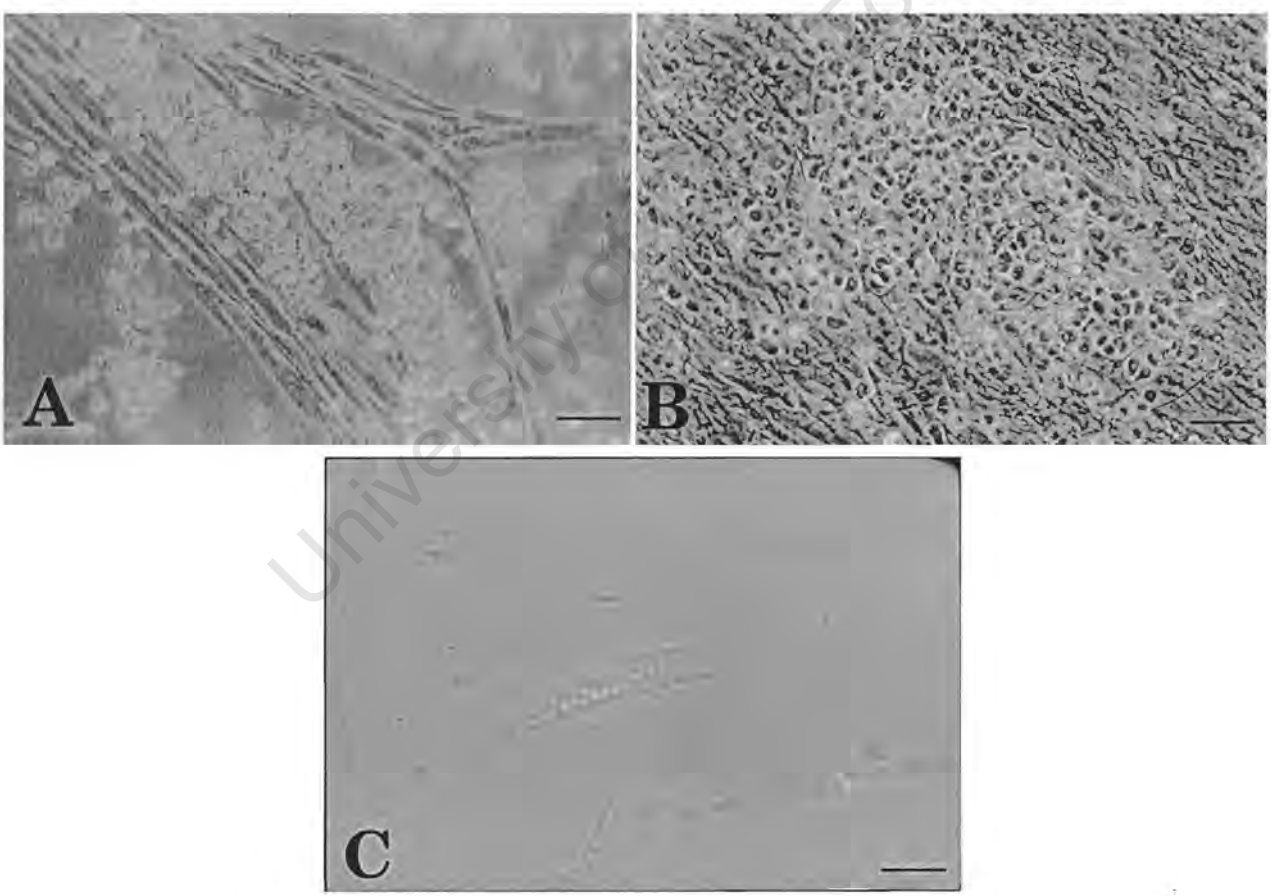


Fig. 6. The effect of FGF-2 on the morphology of the stromal cells in the adherent layer of LTBM cultures.

A. A micrograph showing haematopoietic cells adhering to the stromal layer of LTBM cultures. Cultures were treated with 20 ng/ml of FGF-2 for 9 days.

B. A micrograph of a typical cobblestoned colony of haematopoietic cells in the stromal layer of LTBM cultures. Cultures were treated with 2 ng/ml of FGF-2 for 5 weeks.

C. A micrograph showing an adipocyte in the stromal layer of an 8-day old control LTBM culture.

Bar = 50 μm (applies to all three photographs).

2.2. *The effect of FGF-2 on the number of cobblestone foci of haematopoietic cells in the adherent layer.*

I observed that typical cobblestone foci of haematopoietic cells formed in the adherent layer of control and FGF-2 treated LTBM cultures. Since I had noted that LTBM cultures cultured at 37°C contained more progenitor cells in the supernatant of FGF-2 treated cultures compared to control cultures and that these haematopoietic cells presumably were shed into the supernatant from the adherent layer, I determined if there were differences in the number of foci of haematopoietic cells in the adherent layers of these cultures. The number of cobblestone foci of haematopoietic cells in the adherent layer was determined at weekly intervals as described in the Methods.

In *Exp. 1*, LTBM cultures (maintained at 37°C) were treated with 0, 0.2, 2, 1, or 20 ng/ml of FGF-2. The foci in the stromal layers of these flasks were counted from weeks 2 through 11. LTBM cultures treated with FGF-2 had significantly more foci in the stromal layer during this time period than control cultures (Fig. 7, *Exp. 1*). LTBM cultures treated with 1 or 2 ng/ml of FGF-2 had approximately 10-fold more foci of haematopoietic cells at week 6 (420 foci/flask) than untreated cultures (40 foci/flask; $p < 0.001$ at week 6) (Fig. 7, *Exp. 1*). In addition, the size of the foci in FGF-2-treated cultures was larger than those noted in control dishes.

The number of foci of haematopoietic cells in the stromal layer was also determined in LTBM cultures maintained at 33°C. As shown in Fig. 7, LTBM cultures treated with 1 ng/ml of FGF-2 in *Exp. 3* had 4-fold more foci of haematopoietic cells than control cultures at week 5 ($p < 0.001$). This increase was lower than that noted for cultures treated with 1 ng/ml of FGF-2 and maintained at 37°C, which, may be due to the slower rate of establishment and development of the adherent layer in cultures incubated at lower temperatures. As noted before, the size of the foci in FGF-2-treated cultures was larger than those noted in control dishes.

These results show that FGF-2 increased the number as well as the size of the cobblestone foci of haematopoietic cells in the stromal cell layers of LTBM cultures. This finding suggests that FGF-2 increases the number of progenitor cells in the adherent layer by enhancing their adherence to the stromal cell layer (leading to an increase in the number of foci) as well as stimulating progenitor cell proliferation (leading to an increase in the size of the foci).

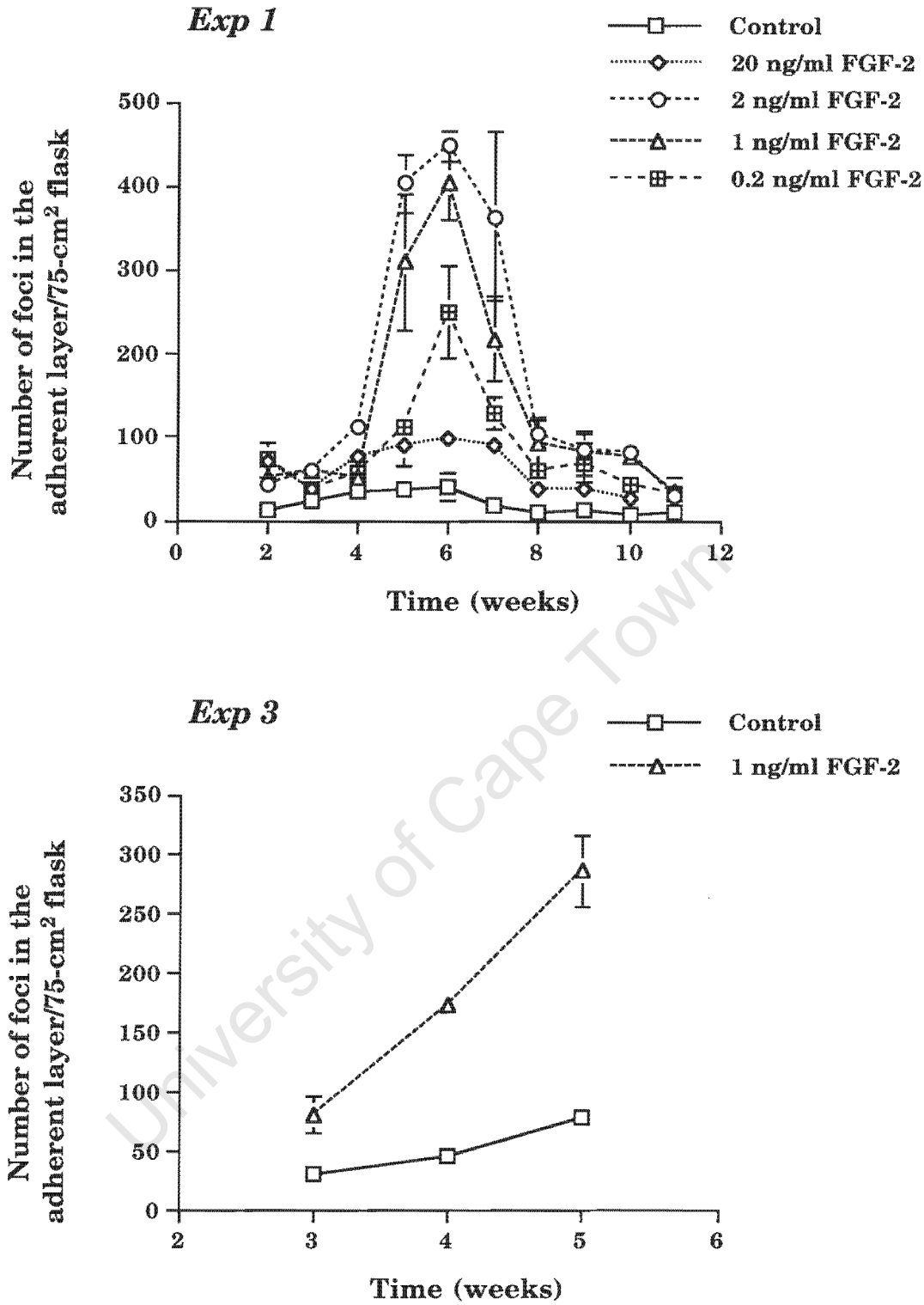


Fig. 7. The effect of FGF-2 on the number of foci of haematopoietic cells in the adherent layer of LTBM cultures. BM buffy coat cells (2×10^7) were inoculated into 75-cm^2 flasks. After 4 days, the non-adherent cells were removed and layered onto Ficoll-Hypaque to remove red blood cells. The low density cells were collected, washed and returned to their original flasks in 20 ml of Gartner's medium to which 0, 0.2, 1, 2, or 20 ng/ml (*Exp. 1*), or 0 and 1 ng/ml of FGF-2 (*Exp. 3*) were added. The cultures in *Exp. 1* were incubated at 37°C, whereas the cultures in *Exp. 3* at 33°C. At weekly intervals, the number of foci of round, refractile haematopoietic cells present in the stromal layer was counted. Results shown represent the mean of foci ± SD of three flasks (*Exp. 1*) and five flasks (*Exp. 3*) for each condition. At some time points the error bars are too small to be observed.

2.3. *The effect of FGF-2 on the total number of cells in the adherent layer of LTBM cultures.*

The total number of cells (stromal plus haematopoietic) in the adherent layers of LTBM cultures treated with or without FGF-2 was determined. This was done because colony-forming soft-agar assays were used to determine the effect of FGF-2 on the number of primitive progenitor cells in the adherent layers and I wanted to access whether colony growth might be affected if different numbers of stromal cells were co-seeded with the "haematopoietic" cells in the assays.

Therefore, the total number of adherent cells as well as the number of haematopoietic cells in the adherent layer (identified morphologically, by arbitrarily defining these cells as small, round cells with smooth edges), were determined using a haemocytometer (the results for *Exp. 2 - 6* are shown in Table 2). It is assumed that any error in the morphological identification of the haematopoietic cells remained constant between experiments.

In Chapter 2 I showed that FGF-2 is a mitogen for the adherent layer of primary LTBM cultures. Therefore, I expected to see significant differences in the total number of adherent cells (stromal plus haematopoietic) between control and FGF-2 treated cultures. Concentrations of 1, 2, or 20 ng/ml FGF-2 were most effective in stimulating the total number of adherent cells. The increase, however, was not dependent on the amount of FGF-2 added as previously noted in Chapter 2. The number of adherent cells was increased by approximately 2.5-fold at week 3 (an average of 12.1 x 10^6 cells/flask for the three FGF-2 concentrations, $p < 0.001$), and, by approximately 2.9-fold at week 4 (an average of 16.8 x 10^6 cells/flask for the three FGF-2 concentrations, $p < 0.001$) compared to control cultures (Table 2, *Exp. 2*). [The reason that the increase in adherent cell numbers appears to be independent of FGF-2 concentration is most likely due to cell numbers being determined in LTBM cultures with confluent adherent layers. In Chapter 2 I determined the rate of growth of sparsely plated cells after 15 days.] The average mediated increase in cell number by 1 ng/ml FGF-2 in *Exp. 3, 4,* and *5* was 3.7 ± 0.8 at week 2 ($p < 0.05$), 3.2 ± 1.4 at week 3 ($p < 0.02$), 4.5 ± 1.2 at week 4 ($p < 0.01$), and 4.9 ± 0.8 at week 5 ($p < 0.01$)(Table 2).

Because FGF-2 stimulates the proliferation of stromal cells, colony-forming assays established with haematopoietic cells from FGF-2-treated LTBM cultures may have contained a different number of stromal cells than colony forming assays established with cells from control LTBM cultures. However, this would only occur if the increase in stromal cell number by FGF-2 is independent of the increase in haematopoietic cell number by this growth factor or, in other words, if the ratio of

Table 2. The ratio of stromal to "haematopoietic" cells in the adherent layers of LTBM cultures.

Exp. No	Week	Condition	Total number of cells/ 75-cm^2 flask (x 10^{-6})	Number of "haematopoietic" cells/75-cm^2 flask (x 10^{-6})	Number of stromal cells/ 75-cm^2 flask (x 10^{-6})	Ratio of stromal to haematopoietic cells
2	3	Control	4.9 ± 0.7	0.9	4.0	4.6:1
		0.2 ng/ml FGF-2	8.1 ± 1.0§	1.5	6.6	4.3:1
		1 ng/ml FGF-2	12.6 ± 0.1*	2.8	9.8	3.6:1
		2 ng/ml FGF-2	11.6 ± 0.3*	2.3	8.3	3.6:1
		20 ng/ml FGF-2	12.0 ± 1.0*	2.8	9.2	3.3:1
	4	Control	5.8 ± 0.5	1.4	4.3	3.0:1
		0.2 ng/ml FGF-2	9.8 ± 1.8§	2.0	7.8	3.9:1
		1 ng/ml FGF-2	16.3 ± 1.0*	3.7	12.6	3.4:1
		2 ng/ml FGF-2	18.8 ± 1.2*	3.3	10.6	3.2:1
		20 ng/ml FGF-2	15.2 ± 2.2**	4.1	11.2	2.7:1
3	3	Control	4.8 ± 0.0	1.2	3.5	2.9:1
		1ng/ml FGF-2	9.2 ± 0.7*	1.4	7.8	5.6:1
	4	Control	6.0 ± 0.2	1.4	4.6	3.3:1
		1ng/ml FGF-2	20.7 ± 0.3*	3.2	17.5	5.4:1
	5	Control	8.2 ± 1.3	1.5	6.7	4.5:1
		1ng/ml FGF-2	34.5 ± 4.8**	6.9	27.5	4.0:1
4	3	Control	3.8 ± 0.2	1.1	2.7	2.5:1
		1ng/ml FGF-2	18.6 ± 1.0*	4.1	14.5	3.5:1
	4	Control	2.8 ± 0.3	0.7	2.1	2.9:1
		1ng/ml FGF-2	16.1 ± 1.5*	4.6	11.5	2.5:1
	5	Control	3.5 ± 0.4	0.7	2.8	4.2:1
		1ng/ml FGF-2	20.2 ± 1.4*	5.2	14.9	2.9:1
5	3	Control	6.2 ± 0.5	1.3		3.8:1
		1ng/ml FGF-2	23.1 ± 1.8*	5.3	17.8	3.4:1
	4	Control	6.2 ± 0.4	0.9	5.3	5.8:1
		1ng/ml FGF-2	26.3 ± 2.2*	6.7	19.6	2.9:1
	5	Control	5.2 ± 0.9	0.9	4.3	5.0:1
		1ng/ml FGF-2	24.2 ± 0.2*	6.8	17.4	2.6:1

BM buffy coat cells (2 x 10^7) were inoculated into 75-cm^2 flasks. After 4 days, the non-adherent cells were removed and layered onto Ficoll-Hypaque to remove red blood cells. The low density cells were collected, washed and returned to their original flasks in 20 ml of Gartner's medium and cultured in the absence or presence of FGF-2. At weekly intervals the adherent layers were removed and the numbers of stromal cells and "haematopoietic" cells in the layer were determined morphologically using a haemocytometer. Haematopoietic cells were defined as small, round cells with smooth edges and stromal cells were defined as large, round cells with rough edges. Results represent the mean number of cells ± SD of duplicate flasks for each condition.

* $p < 0.001$, ** $p < 0.005$, § $p < 0.02$

stromal to haematopoietic cells in FGF-2-treated cultures was different from that in control cultures. The effect of the stromal cells on the haematopoietic cells in the colony forming assays is unknown. Stromal cells could have no effect, or, they could alter the number and/or size of the colonies by either (a) producing growth factors that stimulate/inhibit progenitor cell growth, or by (b) depleting the medium of nutrients, or by (c) releasing toxic substances after cell death.

It is possible that a certain number of stromal cells are required to support the growth and development of a particular number of haematopoietic cells. Therefore, in order to determine whether there was a statistically significant change in the ratio of stromal to "haematopoietic" cells in FGF-2-treated LTBM cultures compared to control cultures, these ratios were tabulated as shown in Table 2. Statistical analysis of the data (Table 3) indicated that there was no significant difference in the ratio of stromal to "haematopoietic" cells between control and FGF-2 treated LTBM cultures. This suggests that the number of stromal cells co-seeded with the haematopoietic cells in the colony forming assays did not vary significantly from one experimental condition to another. Thus, the effects of the stromal cells on the growth and development of the colonies would have been similar for each experimental condition.

These findings also imply that a specific number of stromal cells is required to support the growth and development of a specific number of "haematopoietic" cells suggesting that the effects of FGF-2 on haematopoietic cell numbers in LTBM cultures might be indirect, i.e. stromal-cell mediated. From Table 3 it can be noted that approximately 4 stromal cells are required to generate 1 "haematopoietic" progenitor cell at any given time point. While, this suggests that the density of the stromal cells alone may account for an increase in progenitor cell numbers, my results (see Tables 4 and 5) clearly indicate that FGF-2 acts on haematopoietic cells, most likely by maintaining them in a more primitive state which can lead to their expansion.

Table 3. Statistical significance of the stromal to haematopoietic cell ratios (from Table 2) between 1 ng/ml FGF-2-treated and control LTBM cultures.

Week 3	*Exp. 2*	*Exp. 3*	*Exp. 4*	*Exp. 5*	Average
Control	4.6	2.9	2.5	3.8	3.5 ± 0.9
FGF-2	3.6	5.6	3.5	3.4	4.0 ± 1.1*

Week 4	*Exp. 2*	*Exp. 3*	*Exp. 4*	*Exp. 5*	Average
Control	3.0	3.3	2.9	5.8	3.8 ± 1.4
FGF-2	3.4	5.4	2.5	2.9	3.6 ± 1.3*

Week 5	*Exp 2*	*Exp. 3*	*Exp. 4*	*Exp. 5*	Average
Control	ND	4.5	4.2	5.0	4.6 ± 0.4
FGF-2	ND	4.0	2.9	2.6	3.2 ± 0.7*

ND: not determined
* $p > 0.05$, i.e. no significant difference to control LTBM cultures.

2.4. *The effect of FGF-2 on the number of haematopoietic progenitor cells in the adherent layer.*

Haematopoietic progenitor cells in the adherent layer of LTBM cultures are continuously shed into the supernatant as they mature. I observed that the addition of FGF-2 to LTBM cultures (maintained at 37°C) increased the number of progenitor cells in the supernatant. This finding, combined with the observation that FGF-2 stimulated the number of foci of haematopoietic cells in the stromal layer of these LTBM cultures, suggested that FGF-2 increased the number of haematopoietic progenitor cells in the adherent layer of LTBM cultures. I therefore investigated the effect of FGF-2 on the number of haematopoietic progenitor cells in the adherent layers of LTBM cultures. Since the literature indicated that incubating LTBM cultures at 33°C was more effective than 37°C for studying haematopoietic progenitor cells in the stromal cell layer (Platzer et al., 1988; Cashman et al., 1990; Eaves et al., 1991), the LTBM cultures in these experiments were maintained at the lower temperature.

The adherent layers were removed from LTBM cultures and the number of "haematopoietic" cells was determined by identifying them morphologically as described in the Methods. The number of GM-CSF responsive progenitor cells in the adherent layers of control and FGF-2 treated LTBM cultures were then determined using colony-forming assays as described in the Methods.

In *Exp. 2*, sufficient BM cells were collected to establish 25 flasks of cells. This allowed me to determine the number of progenitor cells in the adherent layer at two time points from duplicate (and in some cases from triplicate) LTBM flasks treated with different concentrations of FGF-2 (0.2, 1, 2, or 20 ng/ml FGF-2).

Table 4 shows the number of GM-CSF responsive progenitor cells/flask determined at weeks 3 and 4 for *Exp. 2*, in which LTBM cultures were treated with 0, 0.2, 1, 2, or 20 ng/ml of FGF-2. Results are expressed as the mean ± the standard deviation of four dishes for each condition. Table 4*A* shows that the number of progenitor cells in the stromal layer calculated from cluster counts made on day 7 was increased by all concentrations of FGF-2 used. Control LTBM cultures had 299 ± 106 progenitors/flask at week 3, and, 187 ± 86 progenitors/flask at week 4 (Table 4*A*). Concentrations of 1 or 2 ng/ml FGF-2 stimulated the number of progenitor cells in the adherent layer at both weeks 3 and 4 by approximately 14-fold above control cultures (Table 4*A*, $p < 0.001$ in each case).

Similarly, Table 4*B* shows that all concentrations of FGF-2 used stimulated the number of progenitors in the adherent layer calculated from colony counts made on day 14. Control LTBM cultures had 484 ± 70 progenitors/flask at week 3 and 230 ± 72 progenitors/flask at week 4 (Table 4*B*). At week 3, LTBM cultures treated with 1, 2, or 20 ng/ml of FGF-2 contained on average 6.3-fold more GM-CSF responsive progenitors than control cultures (Table 4*B*, $p < 0.001$). At week 4, concentrations of 1 or 2 ng/ml FGF-2 were the most effective at stimulating the number of progenitor cells in the adherent layer. LTBM cultures treated with these concentrations of FGF-2 contained on average 14 times more progenitor cells than control cultures (Table 4*B*, $p < 0.001$).

Table 4. Total number of GM-CSF responsive progenitor cells in the adherent layer or supernatant of LTBM cultures cultured in the absence or presence of FGF-2.

	Week	FGF-2 (ng/ml)	Number of progenitor cells in the adherent layer/75-cm^2 flask	FGF-2-mediated increase in the number of progenitor cells in the adherent layer/75-cm^2 flask	Number of progenitor cells in the supernatant / 75-cm^2 flask	FGF-2-mediated increase in the number of progenitor cells in the supernatant / 75-cm^2 flask
A	3	0	299 ± 106	-	4576 ± 1888	-
		0.2	1920 ± 240	6.4*	11792 ± 414	2.6*
		1	5713 ± 1187	19.0*	23496 ± 3234	5.1*
		2	4509 ± 456	15.0*	14388 ± 2046	3.1*
		20	2296 ± 420	7.7*	22836 ± 4752	4.9*
	4	0	187 ± 86	-	ND	
		0.2	1160 ± 150	6.2*	ND	
		1	2686 ± 699	14.3*	ND	
		2	3280 ± 689	17.5*	ND	
		20	1102 ± 408	5.9**	ND	
B	3	0	484 ± 70	-	1296 ± 176	-
		0.2	1560 ± 270	3.2*	1360 ± 350	1.1 NS
		1	3091 ± 331	6.4*	3278 ± 1122	2.5*
		2	3146 ± 342	6.5*	1716 ± 594	1.3 NS
		20	2940 ± 252	6.1*	4191 ± 1254	3.2*
	4	0	230 ± 72	-	ND	
		0.2	1320 ± 340	5.7*	ND	
		1	3422 ± 552	14.9*	ND	
		2	2854 ±164	12.4*	ND	
		20	1632 ± 530	7.1*	ND	

BM buffy coat cells (2 x 10^7) were inoculated into 75-cm^2 flasks. After 4 days, the non-adherent cells were removed and layered onto Ficoll-Hypaque to remove red blood cells. The low density cells were collected, washed and returned to their original flasks in 20 ml of Gartner's medium to which 0, 0.2, 1, 2, or 20 ng/ml of FGF-2 was added. At weeks 3 and 4, the supernatant and the adherent layers were removed and the cells seeded in colony forming assays in the presence or absence of 10 ng/ml GM-CSF. Clusters (3-40 cells) and colonies (> 40 cells) were scored 7 (*A*) and 14 (*B*) days after seeding the cells in agar. Results shown are the total number of progenitors/75-cm^2 flask and are the mean ± the SD of four dishes for each condition.

*$p < 0.001$, **$p < 0.005$ with respect to the control.

NS: not significant with respect to the control.

ND = not determined

In addition, the results show that at weeks 3 and 4 the number of colonies/flask determined on day 14 in control LTBM cultures and those treated with 0.2 or 20 ng/ml of FGF-2 was either greater than, or approximately equal to, the number of clusters/flask determined on day 7 for these cultures. This suggests that the clusters were formed from primitive progenitor cells which continued to grow to form colonies. In contrast, the number of colonies/flask was lower than the number of clusters/flask in LTBM cultures treated with 1 or 2 ng/ml of FGF-2 at week 3. This suggests that the clusters were formed from a mixture of primitive and differentiated progenitor cells and that it was the former cell type, with the greater proliferative potential, that continued to grow and form colonies. Therefore, the adherent layers of LTBM cultures treated with 1 or 2 ng/ml of FGF-2 appear to contain a mixture of primitive and more differentiated progenitor cells, whereas control LTBM cultures and those treated with 0.2 or 20 ng/ml of FGF-2 contained mainly primitive progenitor cells.

Furthermore, it can be noted from Table 4*A* and *B*, that the FGF-2-mediated increase in the number of progenitor cells was greater in the adherent layer than in the supernatant (the number of progenitor cells determined in the supernatant of LTBM cultures at week 3 were discussed earlier in section 1.3 and Fig. 5). This most likely reflects the primitive nature of the progenitor cells in the adherent layer.

These results therefore demonstrate that FGF-2 increased the number of GM-CSF responsive progenitors in the adherent layer of LTBM cultures with low concentrations of FGF-2 (1 or 2 ng/ml) being the most effective. Higher concentrations of FGF-2 (20 ng/ml) were significantly less effective and, although the reasons for this are not understood, it may reflect the biphasic nature of growth factor action.

This experiment was repeated several times using one concentration of FGF-2 to determine its effect on the number of progenitor cells in the adherent layers of LTBM cultures over a longer period of time rather than at 2 time points as in *Exp. 2*. A concentration of 1 ng/ml FGF-2 was chosen, as it was noted in *Exp. 2* that this concentration of FGF-2 was more effective than higher concentrations at stimulating the number of progenitor cells in the stromal layer (Table 4). The adherent layers of *Exp. 3*, *4*, *5*, and *6* were removed at weeks 2 through 6 and seeded into colony forming assays as described for *Exp.* 2. The results are shown in Table 5. For easier visualisation, the results of *Exp. 3, 4,* and *5* are shown in graph form in Fig. 8.

As noted before, 1 ng/ml of FGF-2 significantly stimulated the number of GM-CSF responsive progenitor cells in the adherent layers compared to control LTBM cultures, particularly at weeks 4 and 5 (Table 5*A* and *B* and Fig. 8*A* and *B*). Table 5*A* shows that at week 4 the FGF-2-mediated increase in the number of progenitors/flask,

calculated from cluster counts scored on day 7 of the colony forming assay, ranged from 156-fold to > 1766-fold. Likewise, Table 5*B* shows that the FGF-2-mediated increase in the number of progenitors/flask, calculated from colony counts scored 14 days after seeding the cells in agar, ranged from 95-fold to > 804-fold. The reason why the FGF-2-mediated increases are higher than those noted in Table 4 is most likely due to the lower colony counts obtained with control cultures which reflects the variation in growth from one BM sample to another.

The number of progenitor cells/flask found in the supernatant of these LTBM cultures (discussed earlier in section 1.3 and Fig. 5) are also shown in Table 5*A* and *B* as well as Fig. 8*A* and *B*. As noted before the most striking observation is that FGF-2 was more effective at increasing the number progenitor cells in the adherent layer than in the supernatant.

A further observation noted in Table 5*B* is that the primitive progenitor cells in FGF-2-treated LTBM cultures are more persistent in the adherent layers than in the supernatant. In contrast, in control LTBM cultures, haematopoietic cells appear to be more persistent in the supernatant than in the adherent layer. For example, Table 5*B* shows that in *Exp. 3* the number of progenitor cells in the supernatant of control LTBM cultures was greater than the number of progenitor cells in the adherent layer at week 4 (245 ± 34 progenitor cells/flasks vs. 42 ± 36 progenitor cells/flask, respectively). In contrast, FGF-2 treated LTBM cultures contained 4013 ± 1361 progenitor cells in the adherent layer as compared to 420 ± 175 progenitor cells in the supernatant (see also Fig. 8*B*). Since there is a spatial sequence in which progenitor cells proliferate and mature in the adherent layer and then move into the supernatant of LTBM cultures (mimicking the *in vivo* egress of haematopoietic cells from the BM into the circulation), this observation suggests that low concentrations of FGF-2 may inhibit the differentiation of primitive progenitor cells, thus decreasing the rate at which these cells are shed from the adherent layer into the supernatant compared to control LTBM cultures.

In summary, the addition of low concentrations of FGF-2 to human Dexter LTBM cultures significantly stimulates the number of haematopoietic cells in the adherent layer and to a lesser extent in the supernatant and appears to do so by increasing the percentage of immature progenitor cells of the granulocyte-macrophage lineage.

Table 5A. Number of progenitor cells in the adherent layer and supernatant of control LTBM cultures or cultures treated with 1 ng/ml FGF-2 (calculated from clusters scored on day 7 of the colony forming assay).

Exp No	Week	Number of progenitor cells in the adherent layer/75-cm^2 flask		FGF-2-mediated increase in the number of progenitor cells in the adherent layer/75-cm^2 flask	Number of progenitor cells in the supernatant /75-cm^2 flask		FGF-2-mediated increase in the number of progenitor cells in the supernatant/75-cm^2 flask
		Con	1 ng/ml FGF-2		Con	1 ng/ml FGF-2	
3	2	1832 ± 433	1804 ± 449	1^{NS}	12719 ± 1797	8224 ± 2990	0.6^{NS}
	3	303 ± 48	958 ± 51	3*	2622 ± 291	3266 ± 700	1.2^{NS}
	4	4 ± 7	626 ± 196	156*	427 ± 146	464 ± 98	1^{NS}
	5	0	504 ± 103	> 504*	ND	ND	-
4	2	68 ± 57	460 ± 145	7*	13081 ± 2286	7928 ± 1737	0.6§
	3	76 ± 27	3362 ± 1804	44*	1235 ± 154	2998 ± 578	2.4***
	4	0	1766 ± 134	> 1766*	361 ± 18	713 ± 90	1.97§
	5	0	432 ± 270	> 432*	ND	ND	-
5	2	ND	ND	-	13432 ± 701	20350 ± 1976	1.5**
	3	102 ± 77	6970 ± 792	68*	61 ± 20	1196 ± 193	19.6*
	4	0	1005 ± 335	> 1005*	29 ± 20	448 ± 55	15.5*
	5	0	0	0	0	0	0
	6	0	0	0	0	0	0
6	2	ND	ND	-	24012 ± 3058	16600 ±950	0.7**
	3	5 ± 11	1350 ± 180	270*	1245 ± 85	2099 ± 333	1.7**

BM buffy coat cells (2 x 10^7) were inoculated into 75-cm^2 flasks. After 4 days, the non-adherent cells were removed and layered onto Ficoll-Hypaque to remove red blood cells. The low density cells were collected, washed and returned to their original flasks in 20 ml of Gartner's medium and then cultured in the absence or presence of 1 ng/ml FGF-2. All cultures were maintained at 33°C. At weeks 2 - 6, the medium and the stromal layers were removed and the cells seeded in colony forming assays in the presence of 10 ng/ml GM-CSF. Clusters (3-40 cells) were scored on day 7. Results represent the mean number of progenitors ± the SD calculated from four dishes for each condition.

ND = not determined.

* $p < 0.001$; ** $p < 0.002$; *** $p < 0.005$, § $p < 0.05$

NS: not significantly different

Table 5*B*. Number of progenitor cells in the adherent layer and supernatant of control LTBM cultures or cultures treated with 1 ng/ml FGF-2 (calculated from colonies scored on day 14 of the colony forming assay.)

Exp No	Week	Number of progenitor cells in the adherent layer/75-cm^2 flask		FGF-2-mediated increase in the number of progenitor cells in the adherent layer/75-cm^2 flask	Number of progenitor cells in the supernatant/75-cm^2 flask		FGF-2-mediated increase in the number of progenitor cells in the supernatant/75-cm^2 flask
		Con	1 ng/ml FGF-2		Con	1 ng/ml FGF-2	
3	2	813 ± 365	2904 ± 290	3.6*	5696 ± 1582	13884 ± 1655	2.4**
	3	386 ± 145	1454 ± 209	3.8*	2765 ± 810	1528 ± 218	0.6^{NS}
	4	42 ± 36	4013 ± 1361	95*	245 ± 34	420 ± 175	1.7^{NS}
	5	0	380 ± 131	> 380*	ND	ND	-
4	2	787 ± 137	2856 ± 1056	3.6**	10605 ±1397	9891 ±3322	0.9^{NS}
	3	153 ± 19	3547 ± 1304	23***	151 ± 12	154 ± 12	1.0^{NS}
5	2	ND	ND	-	9110 ± 1869	12590 ± 1244	1.4***
	3	90 ± 13	2429 ± 475	27*	297 ± 85	1224 ± 55	4.2*
	4	0	804 ± 335	> 804*	38 ± 24	252 ± 21	6.6*
	5	9 ± 5	68 ± 34	7.6*	0	22 ± 7	> 22.0**
	6	0	440 ± 110	> 440*	0	0	0
6	2	ND	ND	-	11120 ± 626	8050 ± 1650	0.7**
	3	224 ± 67	3150 ± 810	14*	1519 ± 359	1562 ± 410	1.0^{NS}

The colony forming assays from which the results for Table 5*A* were calculated were incubated for a further 7 days. Colonies (> 40 cells) were scored on day 14 of the colony forming assay. Results represent the mean number of progenitors ± the SD calculated from four dishes for each condition.
ND = not determined
* $p < 0.001$; ** $p < 0.005$; *** $p < 0.005$
NS: not significantly different

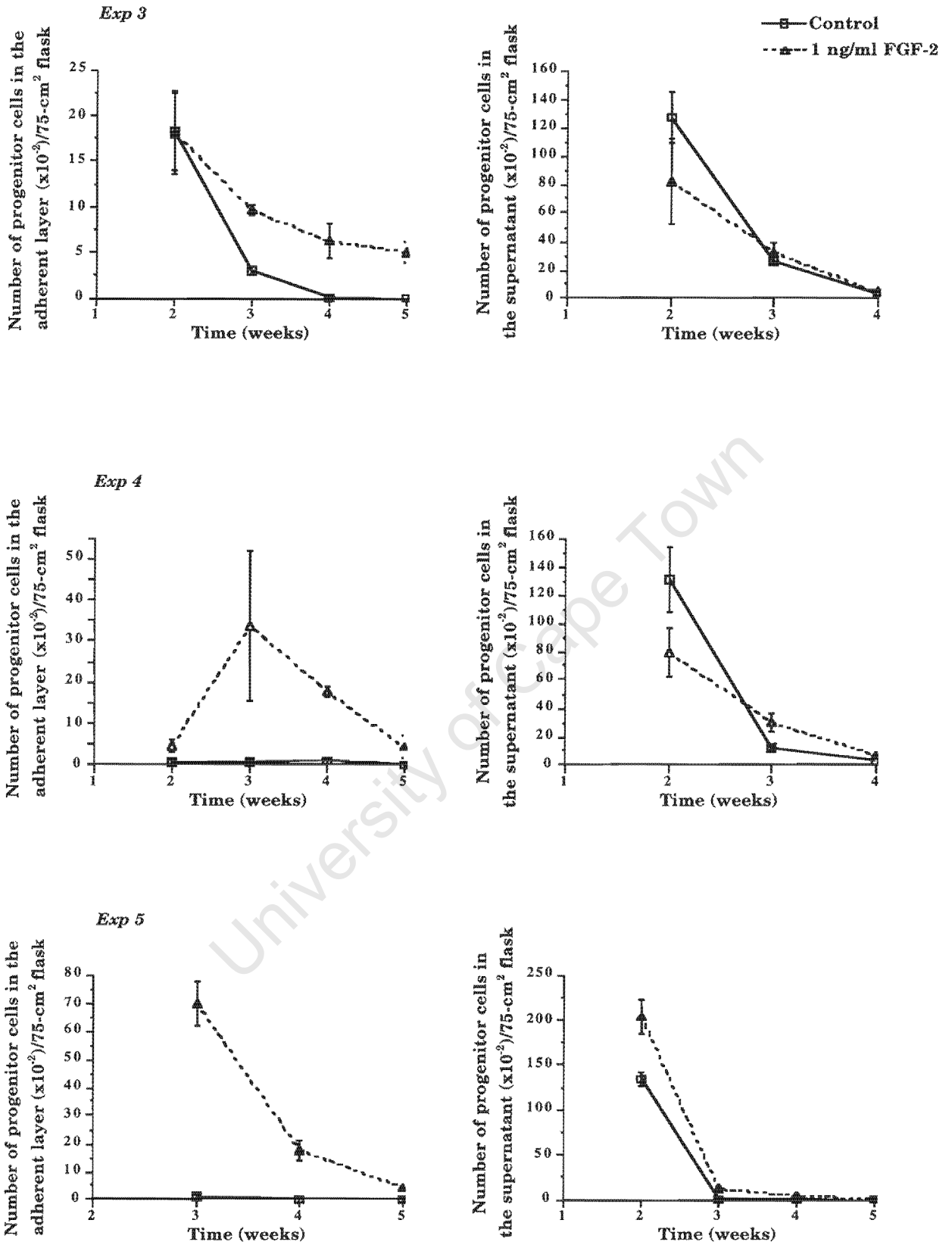


Fig. 8A. The effect of FGF-2 on the number of progenitor cells in the adherent layer and supernatant of LTBM cultures calculated from clusters scored on day 7 of the colony forming assay. The results shown in Table 5*A* are represented in graph form.

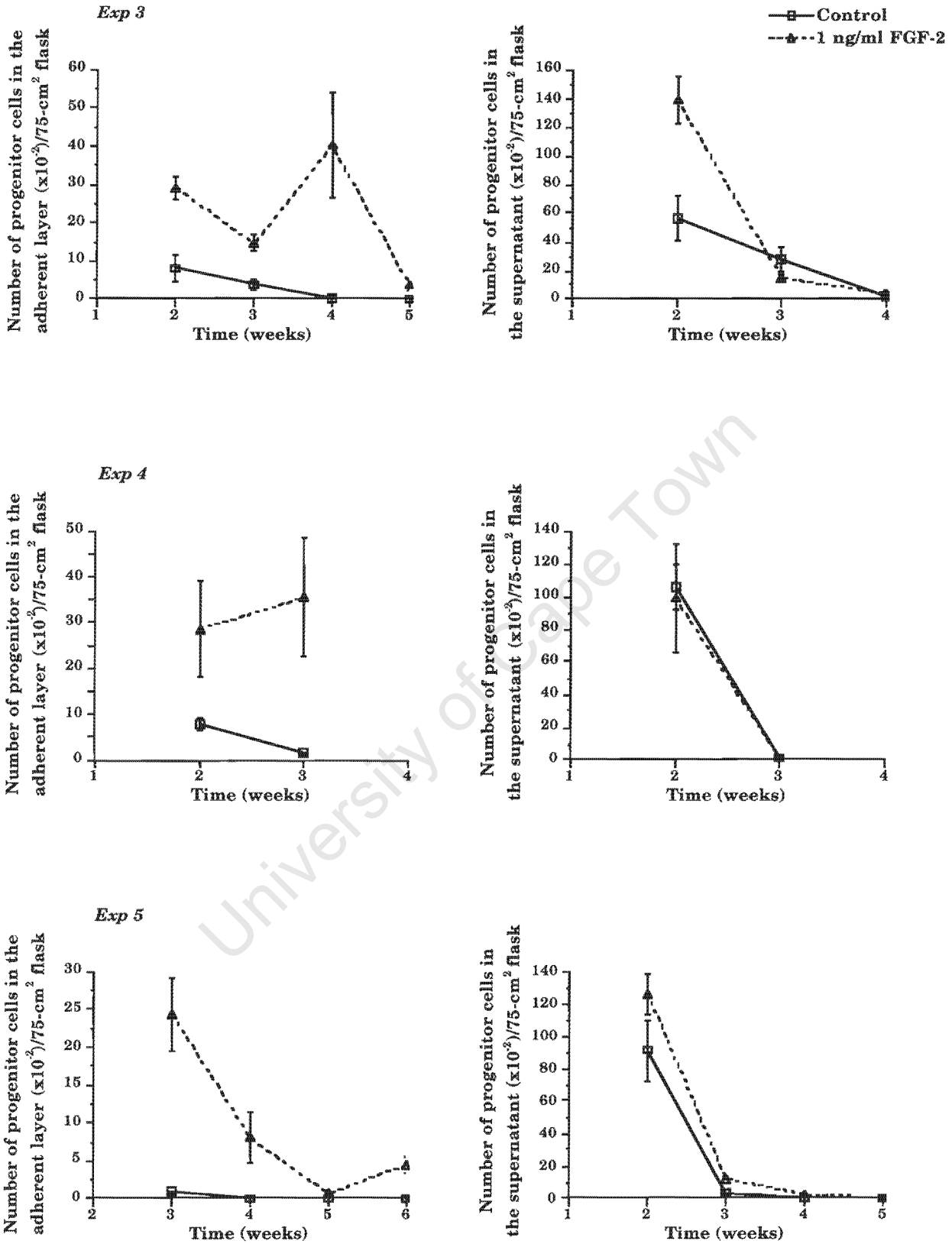


Fig. 8*B*. The effect of FGF-2 on the number of progenitor cells in the adherent layer and supernatant of LTBM cultures calculated from colonies scored on day 14 of the colony forming assay. The results of Table 5*B* are represented here in graph form.

Discussion.

In vivo studies indicate that FGF-2 may be a relevant growth factor for the survival and differentiation of haematopoietic cells (Faloon et al., 2000; Anzai et al., 1999; Yuen et al., 1998; Gabbianelli et al., 1990; Gabrilove et al., 1994; Gallicchio et al., 1991; Han et al., 1992; Bikfalvi et al., 1992; Bruno et al., 1993; Burger et al., 1998). Therefore, I investigated the effect of this growth factor on the proliferation and differentiation of haematopoietic cells in LTBM cultures. LTBM cultures were treated with different concentrations of FGF-2 and the number of progenitor cells was determined in the supernatant and adherent layers. My results show that low concentrations of FGF-2 (0.2 to 2 ng/ml) stimulate haematopoiesis in both the supernatant and adherent layers of LTBM cultures. FGF-2 is produced by BM stromal cells and is present in haematopoietic cells *in vivo* (Brunner et al., 1993). Thus my results, together with other investigators, suggest that FGF-2 may form part of the complex cytokine network that regulates haematopoiesis.

1. FGF-2 increases progenitor cell numbers in LTBM cultures.

The most significant observation was that the increase in the number of progenitor cells by low concentrations of FGF-2 was greater in the adherent layer than in the supernatant. This is most likely because the progenitor cells in the adherent layer have a more primitive phenotype than most of the haematopoietic cells in the supernatant (Keller et al., 1994b; Coulombel et al., 1983), and FGF-2 may have its greatest effect upon this cell group. My observation is consistent with the findings of other investigators who treated LTBM cultures with CSFs and observed that IL-3, GM-CSF, or G-CSF preferentially stimulated progenitor cell growth in the adherent layers (Coutinho et al., 1990).

My findings that FGF-2 stimulates the number of primitive progenitor cells in LTBM cultures are also consistent with the reported actions of this growth factor on primitive cells from other sources. The senescence of primordial germs cells is delayed by FGF-2 (Matsui et al., 1992) and embryonic stem cells (which can be generated from primordial germs cells) are dependent are FGF-2 for their self-renewal (Anzai et al., 1999; Yuen et al., 1998). FGF-2 also inhibits the differentiation of oligodendrocyte-type 2 astrocyte progenitor cells and promotes their proliferation (Bogler et al., 1990).

In addition to the observation that FGF-2 increased the number of progenitor cells in the adherent layer and supernatant of LTBM cultures, I made a number of additional observations:

(1) High concentrations of FGF-2 (20 ng/ml) was less effective than lower concentrations of this growth factor in stimulating the number of progenitor cells in the adherent layer and supernatant of LTBM cultures (Table 4 and Fig. 4A respectively). The reason for this is not clear. A concentration-dependent response to FGF-2 has been reported for hippocampal progenitor neurons (Ray et al., 1993). Low concentrations of FGF-2 (50 pg to 1ng/ml) induced survival of the neurons whereas higher concentrations (10-20 ng/ml FGF-2) induced proliferation of these cells (Ray et al., 1993). This indicates that the effects of FGF-2 on certain cell types may occur in a concentration-dependent manner. If FGF-2 is acting in synergy with other growth factors to stimulate progenitor cell growth, low concentrations may be more effective than higher concentration (Lowry et al., 1992). Alternatively, high concentrations of FGF-2 may stimulate inhibitory cytokines which might be unaffected by low concentrations of FGF-2.

(2) The incubation temperature had an effect on the FGF-2-mediated increase in the total number of haematopoietic cells in the supernatant of LTBM cultures. At 37°C, but not at 33°C (Fig. 1), the total number of haematopoietic cells in the supernatant was increased by low concentrations of FGF-2. The reason for this is most likely that the lower incubation temperature decreased the rate at which the LTBM cultures established and developed thus delaying an increase in the number of progenitor cells in the supernatant of these cultures. Due to limited amounts of material and the nature of the experiments, i.e. the adherent layers were removed to determine progenitor number in them, haematopoietic cells in the supernatant and adherent layers of the LTBM cultures incubated at 33°C were not analysed beyond week 6. Therefore, I could not establish whether similar increases in the total number of haematopoietic cells in the supernatant of LTBM cultures at 37°C would also have been noted at later time points in cultures incubated at 33°C.

2. Comparison of my results with published literature.

2.1. The effects of FGF-2 on haematopoiesis in LTBM cultures in comparison with other growth factors.

I demonstrated a significant increase in the number of progenitor cells in LTBM cultures in the presence of FGF-2. I have compared the effects that I observed with FGF-2 with those noted by other investigators using other growth factors or cytokines (Table 6). The addition of FGF-4, SCF, IL-3, IL-4, GM-CSF, and G-CSF stimulated the number of progenitor cells in the supernatant and the stromal layer of LTBM cultures (Table 6; Quito et al., 1996; Firkin et al., 1993; Coutinho et al., 1990; Keller et al.,

1994b; Platzer et al., 1988) whereas M-CSF (not shown in Table 6) inhibited haematopoiesis (Mayani et al., 1991). It is further evident from Table 6 that FGF-2 is considerably more effective than any of the other growth factors or cytokines and dramatically stimulates the number of primitive progenitor cells in both the adherent stromal layer and the supernatant of LTBM cultures. Surprisingly, Dooley et al. (1995) have reported that the addition of FGF-2 to human LTBM cultures inhibits progenitor cell production. This finding is unexpected given the wealth of evidence indicating a supportive role for FGF-2 in haematopoiesis.

Table 6. Growth-factor mediated increase in the number of progenitor cells in the supernatant and adherent layers of LTBM cultures.

Growth Factor	Supernatant	Adherent layer	Lineages induced
FGF-2 (1ng/ml)	> 395-fold (37°C) > 22-fold (33°C)	Not determined at 37°C 450-fold (33°C)	neutrophils
FGF-4[a] (30 ng/ml)	1.8 - 2.5-fold	5-fold	neutrophils
SCF[b] (50 ng/ml)	2-fold	no increase	neutrophils
IL-3[c] (2 ng/ml)	5-fold	2 - 18-fold	neutrophils, lymphocytes
IL-4[d] (500 U/ml)	2 - 3-fold	3-fold	neutrophils
GM-CSF[e] (10 ng/ml)	3-fold	2 - 18-fold	neutrophils, monocytes, eosinophils, lymphocytes
G-CSF[e] (50 ng/ml)	2-fold	2 - 8-fold	neutrophils

a: Quito et al., 1996 *b:* Firkin et al., 1993 *c:* Coutinho et al., 1990
d: Keller et al., 1994b *e:* Platzer et al., 1988

Of particular interest are the effects of FGF-4 on haematopoiesis (Quito et al., 1996) since it shares 40% homology with FGF-2 (Galzie et al., 1997). Maximal stimulation was noted with 30 ng/ml FGF-4 (Quito et al., 1996) compared to 1 ng/ml FGF-2 in my LTBM cultures (Table 6). In contrast to FGF-2, FGF-4 did not display a biphasic nature in that low concentrations as well as high concentrations (up to 100 ng/ml of FGF-4) stimulated haematopoiesis (Quito et al., 1996). I observed that 20

and 8 that the differences in my protocol for establishing LTBM cultures resulted in a more rapid depletion of the progenitor cells from the supernatant and adherent layer of control LTBM cultures compared to the results published by other investigators. This rapid decline in progenitor numbers in the supernatant occurred because two thirds in place of half of the supernatant was removed at weekly intervals (Wilson et al., 1991). Nevertheless, although the life span of my LTBM cultures was shortened by these changes in protocol, the stimulatory effects of FGF-2 on the number of progenitor cells in LTBM cultures was clearly demonstrated using these culture conditions.

Table 7. Comparison of the number of progenitor cells in the supernatant of my LTBM cultures with those of other investigators.

	Number of CFU -GM/10^7 BM buffy coat cells originally seeded in the culture*									
	Exp 1		*Exp 3*		*Exp 5*		Published results			
Week	Con	FGF-2 1ng/ml	Con	FGF-2 1ng/ml	Con	FGF-2 1ng/ml	Countinho et al., 1990	Mayani et al., 1991	Chauvet et al., 1990	Quito et al., 1996**
1	4941	3345	ND	ND	ND	ND	4666	1277	5100	ND
2	2035	339	2848	6442	4555	6295	1666	456	2500	1180
3	103	149	1383	764	149	612	300	281	500	ND
4	55	286	123	210	19	126	280	144	500	440
5	0	138			0	11	166	118	500	ND
6	0	207			0	0	300	40	500	250
7		200					133	35	400	ND
8		25					133		55	65
9		10					100		65	
10		11					80		10	
11									3	
Temp	37°C		33°C		33°C		33°C	33°C	37°C	37°C

ND = not determined
* In order to be comparable, the results have been expressed as the number of CFU-GM/10^7 BM buffy coat cells originally seeded in the flask.
** Results were published as the number of CFU-c per flask and, in addition to CFU-GM, include BFU-E, CFU-G, and CFU-GEMM colony counts. The data are presented as the number of CFU-c/10^7 whole bone marrow cells because whole bone marrow rather than BM buffy coat cells was used to initiate the LTBM cultures.

ng/ml of FGF-2 was less effective than lower concentration in augmenting haematopoiesis. This suggests that the mechanism of action of FGF-4 in LTBM cultures may be different to that of FGF-2. One possibility might be that FGF-4 primarily mediates its effects directly on haematopoietic cells whereas FGF-2 mediates its effects indirectly via the stromal cells.

The increases in progenitor cell numbers in LTBM cultures treated with 3 ng/ml FGF-4 or FGF-2 observed by Quito et al. (1996) were significantly lower (5-fold in the adherent layer and 1.75-fold in the supernatant at week 6) than those I observed with low concentrations of FGF-2 (Table 6). The reason for this is unclear but may be due to differences in the culture conditions. For example, 80 x 10^6 cells were seeded into 25-cm^2 flasks (Quito et al., 1996) whereas I seeded 20 x 10^6 cells in 75-cm^2 flasks. Thus, the ratio of cells to surface area was higher and their cells may have been deprived of nutrients (see section 2.2).

2.2. *Differences in the protocols for the establishment and maintenance of LTBM cultures.*

My protocol for establishing LTBM cultures differs significantly from those described in the literature (Gartner and Kaplan, 1980; Coulombel et al., 1983; Chauvet et al., 1990; Coutinho et al., 1990; Quito et al., 1996) in the following respects:

(1) Two x 10^7 cells were inoculated into flasks with a larger surface area (75 cm^2) in place of a similar or greater number of cells added to 25-cm^2 flasks or dishes. I found that it was necessary to increase the surface area of the culture vessel to prevent premature detachment of the stromal layer in flasks treated with high concentrations of FGF-2 (20 ng/ml). The stromal cells in these cultures grew to high cell densities in multiple layers and, in some cases, detached from the surface of the flask after extended periods of culture.

(2) Because FGF-2-treated cultures grew to higher cell densities than control cultures, the cells were fed twice a week (Table 1) instead of once a week to prevent nutrient depletion in the FGF-2-treated cultures.

(3) Two thirds of the supernatant (Table 1) in place of half of the supernatant was removed at weekly intervals. Thus, the progenitor cells in the supernatant were depleted more rapidly as compared to removing half the supernatant as it is described in the literature.

Because of these differences, I have compared the life span of my LTBM cultures with that published by other investigators (Tables 7 and 8). It is evident from Tables 7

Table 8. Comparison of the number of progenitor cells in the adherent layers of my LTBM cultures and those published by other investigators.

	Number of CFU-GM/10^7 BM buffy coat cells originally seeded in the culture*						
	Exp. 3		*Exp. 5*		Published results		
Weeks	Control	FGF-2 1 ng/ml	Control	FGF-2 1 ng/ml	Platzer et al., 1988	Coulombel et al., 1983	Quito et al., 1996**
1	ND	ND	ND	ND	230	ND	ND
2	406	1456	ND	ND	350	800	140
3	193	727	77	1774	120	240	ND
4	21	2006	0	402	ND	400	140
5	0	190	4	34		140	ND
6			0	220		88	32
7						40	ND
8							25
Temp	33°C		33°C		33°C	33°C	37°C

ND = not determined
* In order to be comparable, the results have been expressed as the number of CFU-GM/10^7 BM buffy coat cells originally seeded in the flask.
** Results were published as the number of CFU-c/flask and, in addition to CFU-GM, include BFU-E, CFU-G, and CFU-GEMM counts. The data are presented here as the number of CFU-c/10^7 whole bone marrow cells as LTBM cultures were initiated with whole bone marrow rather than BM buffy coat cells.

3. Mechanisms by which FGF-2 may stimulate haematopoiesis.

The mechanisms by which FGF-2 could stimulate the proliferation of haematopoietic cells in LTBM cultures include both indirect (stromal mediated) and direct mechanisms.

3.1. *Direct mechanisms.*

Direct mechanisms by which FGF-2 may increase the number of haematopoietic progenitor cells include:

(1) the alteration in affinity and/or number of receptors for haematopoietic growth factors/cytokines on the surface of these cells. Growth factor/cytokine receptor numbers on primitive haematopoietic cells can be regulated. For example, IL-6 increases IL-3 receptors, IL-3 upregulates receptors for both GM-CSF and Epo, and GM-CSF increases Epo and decreases IL-1 receptor numbers on primitive myeloid cells (Testa et al., 1993; Shieh et al., 1993). In addition, TNF-α decreases c-kit and IL-1 receptors on myeloid cells (Khoury et al., 1994; Shieh et al., 1993). FGF-2 regulates receptor numbers on other cell types: it induces IL-1 receptors on chondrocytes (Chandrasekar and Harvey, 1989; Olashaw et al., 1986) and decreases TGF-ß receptor

expression on endothelial cells (Fafeur et al., 1990). Thus, an alteration in the affinity and/or number of growth factor/cytokine receptors by FGF-2 may be a potential mechanism by which this growth factor stimulates haematopoiesis which does not require an increase in growth factor production.

In addition, FGF-2 could also act as a permissive factor, similar to SCF. Potential mechanisms include the induction of signalling components, adhesion molecules (to facilitate the binding of progenitor cells to the stromal cells), enzymes (to mobilise growth factors), and ECM molecules (as shown for FGF-2, cross talk between integrins and ECM molecules is required for growth factor signalling to take place [Yayon et al., 1991; Rapraeger et al., 1991]).

(2) FGF-2 may also directly increase haematopoiesis by inhibiting the differentiation of haematopoietic cells and thereby maintaining cells with a more primitive phenotype. FGF-2 promotes the survival of a number of cell types including stem cells of various origins. FGF-2 increases the survival of fibroblasts, endothelial cells and neuronal cells (Tamm et al., 1991; Araki et al., 1990; Ray et al., 1993) and inhibits the differentiation of muscle cells (Rosenthal et al., 1991; Olwin et al., 1992). In synergism with PDGF, FGF-2 promotes the survival of oligodendrocyte-type 2 astrocyte progenitor cells (Bogler et al., 1990), and in combination with SCF and LIF, FGF-2 promotes the survival of primordial germ cells (Matsui et al., 1992; Resnick et al., 1992).

Similar effects have also been observed with FGF-2 on BM-derived cell types. FGF-2 inhibits the differentiation of the leukaemic cell line, K562 (Burger et al., 1994), the primitive erythroid cell line, EB-PE (Yuen et al., 1998), the multipotent haematopoietic cell line, A6 (Anzai et al., 1999), and my results in Chapter 2 show that FGF-2 delayed the senescence of BM stromal cells. Thus, in LTBM cultures FGF-2 might act directly on progenitor cells by inhibiting their differentiation leading to increased numbers of progenitor cells in the cultures.

(3) stimulating the self-renewal of haematopoietic cells. Maintaining progenitor cells in a primitive state might also favour conditions that promote self-renewal. In this way FGF-2 could augment haematopoiesis in LTBM cultures by inducing the self-renewal of primitive progenitor resulting in a higher number of these cells in the cultures. For example, the multipotent haematopoietic cell line, A6, derived from embryonic cells is dependent on FGF-2 for its self-renewal (Anzai et al., 1999).

3.2. *Indirect mechanisms.*

It is also possible that the stimulatory effects of FGF-2 on haematopoiesis observed in LTBM cultures resulted from indirect, stroma-mediated effects. The findings of Miller et al. (2000), which demonstrate that impaired haematopoiesis in cultures derived from FGF-2-null mice arises primarily from a defect in the stromal layer and is not a consequence of a defect in the haematopoietic cells, supports an indirect mechanism for FGF-2 action. Furthermore, this impairment is not compensated for by FGF-1, suggesting a specific role for FGF-2 in haematopoiesis (Miller et al., 2000). Indirect mechanisms by which FGF-2 may stimulate the growth of haematopoietic cells include:

(1) modulating the production of haematopoietic growth factors or cytokines produced by the stromal cells. Stromal cells produce both stimulating and inhibiting growth factors/cytokines and it is the net negative or positive proliferative effect on the progenitor cells that regulates haematopoiesis. Thus, FGF-2 may induce myelopoiesis in LTBM cultures by stimulating stromal cells to produce growth-stimulatory haematopoietic growth factors/cytokines, or alternatively, by suppressing the production of inhibitory ones. For example, FGF-2 stimulates both the production of M-CSF mRNA and M-CSF protein in a murine stromal cell line (Abboud et al., 1991; Abboud and Pinzani, 1991), as well as IL-6 and LIF production by human BM stromal cells (Berardi et al., 1995b; Rougier et al., 1998; Rathjen et al., 1990a). Furthermore, FGF-2 antagonises TGF-ß-mediated erythroid differentiation in K562 cells (Burger et al., 1994), and counteracts the suppressive effect of TGF-ß on human myeloid progenitor cells (Gabrilove et al., 1993). The mechanism by which FGF-2 inhibits the negative actions of TGF-ß are not known but may include the down regulation of TGF-ß production.

(2) FGF-2 may also indirectly stimulate haematopoiesis by increasing and/or decreasing the concentration of biologically active growth factors or cytokines available in the BM microenvironment via the increased production of ECM or cell surface molecules, e.g. proteoglycans, which complex growth factors and cytokines. For example, IL-3 or GM-CSF bound to HSPGs in stromal cell matrices (Gordon et al., 1987a; Roberts et al., 1988), are localised and concentrated forming a reservoir of biologically active growth factor. Growth factor/cytokine protection from degradation (Saksela et al., 1988) effectively increases the size of the reservoir and thus the local concentration and biological activity of the bound factors.

Furthermore, FGF-2 might regulate the availability of growth factors by modulating growth factor mobilisation from the reservoirs, or by modulating growth

factor activation (e.g. TGF-ß) by the induction of enzymes, such as u-PA, t-PA (see Chapter 4), heparanase, or phospholipase D, that control these processes.

(3) Alternatively, progenitor cell numbers may be increased in LTBM cultures by FGF-2 acting as an "anchor factor" and promoting stromal cell-haematopoietic cell interactions. FGF-2 can mediate cell attachment by linking receptors expressed on haematopoietic cells with HSPGs on neighbouring stromal cells (Richard et al., 1995). For example, FGF-2 enhances the adhesion of megakaryocytes to BM stromal cells (Avraham et al., 1994). Such attachments may promote the interaction between ECM- or cell surface bound-CSFs and progenitor cells leading to an increase in progenitor cell proliferation and, in turn, a stimulation in myelopoiesis.

4. Experiments to further define the mechanism(s) by which FGF-2 stimulates haematopoiesis.

Do FGFs act on stromal cells to indirectly promote haematopoiesis in LTBM cultures?

FGF-2 deficient BM stromal cells do not efficiently support haematopoiesis *in vitro* which appears to be specifically due to FGF-2 as FGF-1 can not compensate for it (Miller et al., 2000). This finding therefore indicates that FGF-2 can act indirectly to stimulate haematopoiesis.

Potential experiments to further define indirect mechanisms by which members of the FGF-family stimulate haematopoiesis include the analysis of growth factor production by the stromal cells in the presence or absence of FGFs. Although ELISAs are available for many growth factors and cytokines, it is difficult to detect them in the supernatant of LTBM cultures because they are mostly (1) produced by BM stromal cells at subliminal levels, (2) produced in a membrane-bound form or localised to the cell surface/ECM through binding molecules, or (3) rapidly utilised by stem cells and progenitor cells. For example, GM-CSF, G-CSF, IL-1, and IL-3 are not detected in the supernatant of LTBM cultures (I have also not been able to detect, by immunoprecipitation with specific antibodies, the presence of these growth factors in the supernatant or matrix extracts of LTBM cultures, data not shown). We have however, shown the presence of M-CSF by radioimmunoassay (kindly done by Dr Richard Stanley at Albert Einstein College of Medicine, Bronx, New York) and IL-6 by ELISA (kindly done by Dr Gabrilove at Sloan Kettering Institute, New York) in the supernatant of LTBM cultures (data not shown). TGF-ß has also been determined in the supernatant of low-density BM cultures by measuring TGF-ß-mediated suppression of plasminogen activator expression in bovine aortic endothelial cells (Gabrilove et al., 1993) or by using a bioassay (Abe et al., 1994; Eaves et al., 1991).

In a preliminary study I determined the mRNA levels of a number of haematopoietic growth factors and cytokines in the stromal cells of LTBM cultures treated with and without FGF-2. Using standard northern blotting techniques, I noted the constitutive production of M-CSF, G-CSF, IL-1, IL-6, SCF, and TGF-ß by the adherent layers of primary LTBM cultures (data not shown). However, no consistent, significant change in the mRNA levels of these factors by FGF-2 was observed. Although this argues against the possibility that FGF-2 regulates human haematopoiesis by modulating the production of these particular growth factors and cytokines, it does not exclude the possibility that FGF-2 modulates the synthesis of other unknown growth factors or cytokines, or factors such as IL-3 and Flt-2-ligand that I did not investigate.

More sensitive techniques allowing comparative gene expression to study the effect of bFGF on growth factor production by BM adherent cells include real-time RT-PCR and the Bio-chip (Torok-Storb et al., 1999; Marshall and Hodgson, 1998). The Bio-chip, however, is very expensive to purchase due to the research and the state of the art technology required to produce it. The main advantage of this technique is the ability to compare the amounts of many different mRNAs in two cell populations simultaneously with high sensitivity. Since Bio-chips containing DNA sequences for 2400 human genes are available (with the potential for many more), this technique may also be useful for characterising ECM and cell surface proteoglycans synthesis, enzyme synthesis (e.g. u-PA, t-PA, heparanases, phospholipase D, MMP-9 and other metalloproteinases), cell surface adhesion molecule synthesis, as well as growth factor/cytokine receptor synthesis by BM stromal cells in response to FGF-2 and other members of the FGF-family. Alternatively, the production of these molecules by BM stromal cells from FGF-2-deficient mice could be compared to wild type mice.

What is the role of endogenous FGF-2 in LTBM cultures?

My results demonstrate that exogenously added FGF-2 promotes haematopoiesis *in vitro*. This finding together with the findings of Quito et al., (1996) that FGF-4 also stimulates haematopoiesis in LTBM cultures, indicates a possible role for endogenously produced FGFs *in vivo*. Therefore, I attempted to determine whether endogenous FGF-2 has a functional role as a haematopoietic growth factor in LTBM cultures. I did this by adding neutralising antibodies directed against FGF-2 to LTBM cultures and determining the number of GM-CSF responsive progenitor cells in the supernatant and adherent layers. Preliminary results suggested that the number of progenitor cells in the supernatant and adherent layer was decreased in the presence

of anti-FGF-2 antibodies relative to control cultures receiving control antibodies. Relatively high concentrations of immunoglobulin (0.5 mg/ml) were added to the cultures to ensure complete inhibition of FGF-2 since the antibodies were not affinity-purified. In some experiments the control antibodies decreased the number of progenitor cells making the interpretation of the results difficult. The reason for this non-specific effect of the antibodies might be that the F_c portion of immunoglobulins binds to F_c receptors on macrophages and/or neutrophils present in the cultures. This could cause the production of stimulatory and/or inhibitory cytokines which, in turn, act on progenitor cells. Therefore, these experiments could be repeated using $F(ab)_2$ fragments of the antibodies which lack the F_c portion.

Alternatively, RNA interference (RNAi) has emerged as a powerful tool for the post-transcriptional silencing of gene expression (Tuschl and Borkhardt, 2002) and could be used to show the endogenous role of FGF-2 or FGF-4 in haematopoiesis in LTBM cultures. Double-stranded RNA (dsRNA) can inhibit gene expression in a sequence-specific manner by triggering the degradation of mRNA (Tuschl and Borkhardt, 2002). In cells, the mediators of mRNA degradation are small interfering RNA duplexes (siRNAs), which are produced from long dsRNA by the RNase III enzyme Dicer (Tuschl and Borkhardt, 2002). Chemically synthesised siRNAs can be effectively delivered by electroporation or cationic liposome-mediated transfection to transiently suppress the expression of target genes (Tuschl and Borkhardt, 2002). However, methods for the endogenous expression of short hairpin RNA (shRNA) molecules which give rise to siRNAs *in vivo* have been developed (Yu et al., 2002; Paddison et al., 2002). This allows the construction of continuous cell lines in which RNAi enforces stable and heritable gene silencing. Thus, primary BM stromal cells (or RS cells) could be transfected with a plasmid containing a vector into which DNA oligonucleotides encoding shRNAs for FGF-2 or FGF-4 are ligated (Yu et al., 2002; Paddison et al., 2002). In this way, the expression of the chosen FGF by BM stromal cells should be silenced thus allowing one to determine the ability of such cells to support haematopoiesis.

It should be remembered that the neutralisation of one growth factor may not result in the inhibition of haematopoiesis because another growth factor or indeed another member of the FGF-family (FGF-4 for example [Quito et al., 1996]) may perform the same function.

Do FGFs act on progenitor cells to promote haematopoiesis in LTBM cultures?

FGF-2 might act directly on primitive progenitor cells to stimulate haematopoiesis. Thus, further experiments to define the mechanism(s) by which this might occur include defining the target cell populations and determining the effects of FGF-2 or other FGFs such as FGF-4 on these populations. As mentioned before, a small subpopulation of $CD34^+$ cells express FGFRs (Berardi et al., 1995b; Testa et al., 1996; Le Bousse-Kerdilès et al., 1996; Burger et al., 1998) as well as differentiated progenitor cells (Berardi et al., 1995b; Ratajczak et al., 1996). In addition, $CD34^+FGFR\text{-}1^+$ cells express antigens found on haematopoietic stem cells as well as antigens found on endothelial cells (Burger at al., 2002). These cells give rise to endothelial cells in vitro, indicating that the $CD34^+FGFR\text{-}1^+$ population contains endothelial stem/progenitor cells (Burger at al., 2002). Such stem cells are of particular interest because of their potential to differentiate into multiple cell types (Krause , 2002; Zhao et al., 2002; Mertelsmann, 2000; Jiang et al., 2002b). Furthermore, murine long-term repopulating stem cells can be expanded in culture with FGF-1 and as these cell differentiate they loose FGFR expression (de Haan et al., 2003). Therefore, it would be interesting to determine if the survival of human $CD34^+FGFR\text{-}1^+$ cells are supported by FGF-2 (or other FGFs such as FGF-1 or -4) as has been shown for embryonic stem cells (Faloon et al., 2000; Anzai et al., 1999) or whether $CD34^+FGFR\text{-}1^+$ cells require FGF signalling (or loss thereof) for their differentiation. $CD34^+FGFR\text{-}1^+$ cells can be obtained and their maintenance in serum-free medium supplemented with FGFs (FGF-1, -2, or -4) could be assessed (de Haan et al., 2003). Progeny from such cultures could be assessed for their continued expression of CD34 and FGFR as well as the expression of AC133, c-kit and Thy-1, typical antigens of haematopoietic stem cells (Markus et al., 2002; Briddell et al., 1992; Graig et al., 1993; Miraglia et al., 1997) indicating that FGFs can maintain such cells in culture. Alternatively, FGFR expression in BM stem cells ($CD34^+FGFR\text{-}1^+$ cell population) could be silenced using siRNAs as described above (plasmid-based expression of short hairpin loops which give rise to siRNAs *in vivo* is the preferred method for undifferentiated cells, Yu et al., 2002) to determine the involvement of FGF signalling in BM stem cell self-renewal and differentiation.

In addition, FGF-2 can partially overcome the inhibitory effects of TGF-ß on haematopoietic cells including $CD34^+$ cells (Burger et al., 1994; Gabrilove 1994). Disruption of TGF-ß signalling leads to enhanced survival and proliferation of haematopoietic progenitors (Fan et al., 2002; Le Bousse-Kerdiles et al., 1996). Indeed, increased expression of FGF-2 and FGFRs 1 and 2 in $CD34^+$ progenitor cells isolated

form patients with myelofibrosis and myeloid metaplasia are associated with a reduction in TGF-ß type II receptor (Le Bousse-Kerdiles et al., 1996). Thus, determining the levels of TGF-ß type II receptor in the $CD34^+FGFR\text{-}1^+$ cell population treated with and without FGF-2 may provide insight into the mechanism by which this growth factor stimulates haematopoiesis. The levels of TGF-ß type II receptor in $CD34^+FGFR\text{-}1^+$ cells could be determined by RT-PCR (Le Bousse-Kerdiles et al., 1996).

5. Summary and conclusions.

In summary, I have demonstrated that the addition of low concentrations rather than high concentrations of FGF-2 to LTBM cultures augmented the number of primitive progenitor cells in these cultures. I noted that the increase in the number of progenitor cells by FGF-2 was greater in the adherent layer than in the supernatant. These results suggest that FGF-2 may be a relevant growth factor for haematopoiesis.

Chapter 4

Role of growth factors/cyokines in regulating plasminogen activators in the BM stromal microenvironment.

Introduction.

Proteolysis plays an important role in regulating the availability and activity of certain growth factors and cytokines including FGF-2, TGF-ß, IL-1ß, IL-1α, and M-CSF (Brunner et al., 1991; Lyons et al., 1988; Gonias et al., 1989; Matsushima et al., 1986; Mignatti et al., 1989). Many of these growth factors are produced by BM stromal cells and are presented in a biologically active form to developing stem and progenitor cells in the BM microenvironment. Proteolytic regulation of growth factor activity includes the mobilisation of active growth factors from cell surfaces and/or ECM reservoirs, the activation of latent forms of growth factors, the shedding of growth factor receptors from the cell surface, as well as growth factor degradation. Furthermore, proteolysis is required for cell migration, being necessary for the breakdown of ECM molecules (Basbaum and Werb, 1996; Brunner and Priessner, 1994). Although it is known that proteases are produced by BM cells (McWilliam et al., 1998 and 1996; Hamilton et al., 1991a; Wilson and Francis, 1987), their functional role in haematopoiesis is not known but may include the regulation of growth factor/cytokine activity and/or migration of stem cells and progenitor cells from the BM cavity into the circulation.

1. Evidence for possible role of PAs and plasmin in haematopoieisis.

Several of the processes regulating growth factor/cytokine activity and ECM degradation are mediated by the serine protease, plasmin (Lyons et al., 1988; Gonias et al., 1989; Matsushima et al., 1986; Saksela and Rifkin, 1990; Brunner et al., 1991; Taipale et al., 1992; Falcone et al., 1993a; Whitelock et al., 1996). It is possible that plasmin may affect growth factors/cytokine activity in the BM microenvironment, thereby regulating haematopoiesis.

Both tissue-plasminogen activator (t-PA) and urokinase (u)-PA, which convert plasminogen to plasmin (see section 2.2), are secreted by a variety of haematopoietic cells and their production can be regulated by growth factors (Hamilton et al., 1991a; Hart et al., 1991; Wilson and Francis, 1987). In BM derived macrophages u-PA mRNA

levels and u-PA activity increase in response to M-CSF, GM-CSF, or IL-3 (Hamilton et al., 1991a; Hart et al., 1991). GM-CSF, in the presence of IFN-γ, as well as IL-4 also stimulate t-PA production by human monocytes (Hart et al., 1989 and 1991). The presence of t-PA-generated plasmin in the BM has also been demonstrated (McWilliam et al., 1998 and 1996). Furthermore, primitive myeloid progenitor cells and leukaemic cells have been shown to secrete t-PA, while mature blood cells secrete u-PA (Wilson and Francis, 1987; Wilson et al., 1983). This suggests that the expression of u-PA by mature haematopoietic cells might be a prerequisite for their migration and egression from the BM into the circulation. In addition, the conversion from t-PA to u-PA production might be accompanied by the co-expression of the u-PAR on the cell surface. In mature haematopoietic cells (monocytes and T-lymphocytes) as well as in other cell types, a stimulation in u-PA activity is accompanied by the co-expression of the u-PAR (Lund et al., 1991; Mignatti et al., 1991; Pepper et al., 1993; Estreicher et al., 1990; Nykjær et al., 1992a and b) thus facilitating cell migration.

A wide range of proteolytic enzymes including plasmin, thrombin, elastase, cathepsin G, and MMPs (Lapidot and Petit, 2002) are involved the breakdown of ECM molecules resulting in the migration of haematopoietic cells from the marrow cavity into the circulation. Indeed, u-PA and, to a lesser degree, t-PA promote the migration of T-lymphoblasts through an ECM barrier (Reiter et al., 1997). In addition, a number of cytokines/growth factors (e.g. SCF, G-CSF, TGF-ß), chemokines (e.g. stromal cell-derived factor-1α [SDF-1α] and IL-8) and chemokine receptors (e.g. CXCR4, the receptor for SDF-1α, and cb2, a cannabinoid receptor) function in the mobilisation of haematopoietic cells (De La Rosa et al., 2003; Moore, 2002; Lapidot and Petit, 2002; Mohle et al., 2001). Proteolysis appears to have further roles in regulating this process by acting on a number of these molecules leading to the disruption of/decrease in stromal-haematopoietic cell interactions. For example, serine proteases are responsible for the shedding of CXCR4 and c-kit (Levesque et al., 2003a and b), and dipeptidylpeptidase IV cleaves SDF-1α from cell surfaces (Christopherson et al., 2002). Furthermore, TGF-ß, whose own activity is regulated by plasmin, can decrease SDF-1α expression (Wright et al., 2003).

Fig 1 shows the cascade of proteolytic enzyme activation reactions, beginning with the activation of pro-u-PA and ending with the activation of pro-metalloproteinases (Mignatti et al., 1989). Since FGF-2 is a known inducer of this cascade (Pepper et al., 1993; Mignatti et al., 1989), it might indirectly regulate haematopoietic growth factor activity in the BM microenvironment which may well account for the stimulation in haematopoiesis observed in LTBM cultures treated with

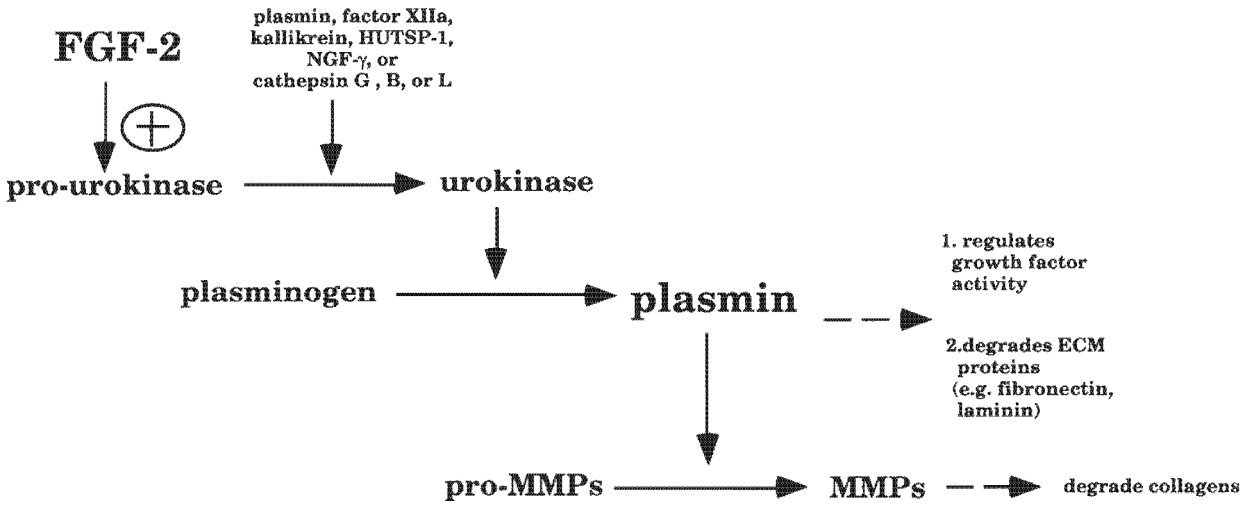


Fig. 1. Schematic representation of the proteolytic cascade leading to the formation of plasmin and MMPs which degrade ECM proteins.
HUTSP-1: human T-cell specific serine proteinase
MMPs: matrix matalloproteinases

this growth factor. The resulting generation of plasmin may cause the release of important haematopoietic growth factors/cytokines that are associated with the cell surfaces or the ECM, or are membrane bound. Alternatively, plasmin may activate latent growth factors, or shed growth factor receptors from the cell's surface and, in doing so, stimulate haematopoiesis. In addition, FGF-2 could potentially regulate progenitor cell migration. For example, TGF-ß, which can be activated by plasmin, downregulates the expression of SDF-1α leading to a decrease in haematopoieic cell migration (Wright et al., 2003). Furthermore, the proteolytic release of FGF-2 from cell surfaces or the ECM may represent one mechanism for regulating its own activity in the BM microenvironment.

The aims in this Chapter were to determine the cellular and secreted levels of u-PA and t-PA activity as well as their specific inhibitors, plasminogen activator inhibitor (PAI)-1 and PAI-2, by passaged BM stromal cells. The effects of FGF-2, IL-1, TGF-ß and hydrocortisone on the production of u-PA, t-PA, PAI-1, and PAI-2 were also investigated. In addition, the regulation of t-PA activity by PAI-1 in the BM microenvironment was studied by following the internalisation of the t-PA/PAI-1 complex by BM stromal cells. I found that all three cytokines increased PA activity, and in some cases PAI-1 and PAI-2 protein was also modulated by these growth factors. PAI-1 was also noted to rapidly bind t-PA in the culture medium, inhibiting its activity and removing it from the culture medium. Furthermore, hydrocortisone

inhibited both basal as well as TGF-ß-stimulated increases in PA activity in a reversible manner.

2. The plasminogen activator/plasmin system.

2.1. *Plasminogen/plasmin.*

The serine protease, plasmin, is generated from the inactive pro-enzyme plasminogen, by the two serine proteases, t-PA and u-PA. Plasminogen is a single-chain, inactive pro-enzyme, which is activated by PAs to form plasmin, a two-chain polypeptide connected by a disulphide bridge (Robbins et al., 1967). Plasminogen is ubiquitous in human body fluids and is found in high concentrations in the circulation. Both plasminogen and plasmin bind to a variety of low-affinity receptors, which are expressed in high numbers on the surface of many cell types including peripheral blood cells, such as, platelets, neutrophils, monocytes, and lymphocytes, but not erythrocytes (Plow et al., 1995; Redlitz and Plow, 1995; Hajjar et al., 1995). Plasminogen and plasmin bind via their lysine binding sites, which are associated with their kringle domains and recognise carboxy-terminal lysine residues of cell surface proteins such as α-enolase or annexin II (Redlitz and Plow, 1995; Hajjar et al., 1994; Miles et al., 1991). Plasminogen and plasmin also bind to ECM components such as fibronectin, laminin, vitronectin, or thrombospondin (Salonen et al., 1984 and 1985; Silverstein et al., 1986; Preissner et al., 1990).

Several functions for plasminogen receptors have been described which contribute to enhancing plasmin activity. Plasmin(ogen) receptors serve to localise these enzymes in the pericellular environment which enables directed proteolysis. Receptor bound plasmin is protected from inactivation by its inhibitor, α_2-antiplasmin, which is present in serum and other body fluids (Gonias, 1992; Sprengers and Kluft, 1987; Knudsen et al., 1986), thus allowing proteolysis to occur on cell surfaces and in the ECM in the presence of this inhibitor. Bound plasminogen is more readily activated to plasmin than free plasminogen and, furthermore, bound plasmin has increased enzymatic activity in comparison to free plasmin (Redlitz and Plow, 1995).

2.2. *PAs*

t-PA, a 70-kDa protein, and u-PA, a 55-kDa protein, are the protein products of two genes (Rajput et al., 1988). t-PA and u-PA are structurally similar, but functionally and immunologically distinct serine proteases (Andreasen et al., 1990; Saksela and Rifkin, 1988). Since both enzymes are able to activate plasminogen to form plasmin, the distinct roles that t-PA and u-PA play are not fully understood. u-

PA expression has been repeatedly associated with processes involving ECM degradation and cell migration, while t-PA, the main PA in plasma, appears to be primarily responsible for plasmin generation during fibrinolysis (Fazioli and Blasi, 1994; Carmeliet et al., 1994; Collen and Lijnen, 1991; Andreasen et al., 1990; Saksela and Rifkin, 1988).

Both t-PA and u-PA are secreted as single-chain proenzymes. While both pro-u-PA and pro-t-PA have some intrinsic activity (van der Werf et al., 1986; Andreasen et al., 1990), they are converted by plasmin to form active two chain u-PA and t-PA, respectively (Andreasen et al., 1990; Saksela and Rifkin, 1988). In addition, kallikrein, trypsin, factor XIIa, the human T-cell specific serine proteinase, HUTSP-1, cathepsins B, L, and G, as well as NGF-γ have also been shown to mediate the conversion of pro-u-PA to active u-PA (Fig. 1)(Ichinose et al., 1986; Koivunen et al., 1989; Brunner et al., 1992; Kobayashi et al., 1991; Goretzki et al., 1992; Learmonth et al., 1992; Wolf et al., 1993).

PA activity is regulated through synthesis (transcriptional regulation) and by specific inhibitors or co-factors (post-transcriptional regulation). PA synthesis may directly modulate extracellular PA activity and is under the control of hormones, growth factors, cyclic nucleotides, and tumour promoters (Saksela and Rifkin, 1988). Post-transcriptional regulation of PA activity also involves ECM components, e.g. sulphated glycosaminoglycans (Brunner et al., 1998), besides PA inhibitors (see section 2. 4).

2.3. *PA receptors.*

2.3.1. *t-PA receptors.*

Receptors for t-PA include those that localise its activity, resulting in focused and restricted extracellular protease activity, and those that mediate its clearance. Annexin II (Kim and Hajjar, 2002; Hajjar et al., 1994; Cesarman et al., 1993), α-enolase (Felez et al., 1991), and amphoterin (Parkkinen and Rauvala, 1991) have been implicated as receptors which serve to localise t-PA activity to the cell surface. The overlapping binding specificities of plasminogen and t-PA with certain receptors (e.g. annexin II and α-enolase) is important for co-localisation, leading to the enhancement of plasminogen activation (Felez et al., 1993a and b; Cesarman et al., 1994).

The liver is the main organ responsible for removing t-PA from the circulation. Carbohydrate-specific receptors, including the mannose receptor (Otter et al., 1991 and 1992) and the αfucose receptor (Hajjar and Reynolds, 1994), function as clearance receptors for t-PA. In addition, low density lipoprotein receptor related protein/α_2-

macroglobulin (LRP/α_2-M) receptor, which binds numerous ligands, is as a clearance receptor for t-PA complexed to PAI-1 (Bu et al., 1992), and possibly free t-PA (Camani et al., 1994; Orth et al., 1994). Gp330, another receptor of the low density lipoprotein receptor family, may also contribute to t-PA removal (Willnow et al., 1992).

2.3.2. *The u-PA receptor.*

Pro-uPA and active u-PA bind to a specific high-affinity urokinase receptor (u-PAR, CD87) which is attached to the membrane via a glycosyl-phosphatidylinositol (GPI) anchor (Ploug et al., 1991). The binding of pro-u-PA to its receptor accelerates its activation and also localises the resulting active u-PA to specific sites on the cell's surface (Nykjær et al., 1992b; Estreicher et al., 1990; Ellis et al., 1989). Two additional roles have been attributed to the u-PAR other than its role in plasminogen activation, namely cell-cell adhesion (by modulating integrin-mediated cell-adhesion; Wei et al., 1996) as well as cell-ECM adhesion (via vitronectin; Kanse et al., 1996), and signal transduction events (also via integrins)(Plesner et al., 1997).

The GPI anchor is cleaved by phosphatidylinositol-specific phospholipase C, GPI-specific phospholipase D, and metalloproteinase-12, thus releasing the u-PAR from cell surfaces (Wilhelm et al., 1999; Ploug et al., 1991; Koolwijk et al., 2001). Interestingly, u-PA also cleaves its own receptor from cell surfaces, releasing the ligand binding domain (Hoyer-Hansen, 1992). These shedding events might regulate the biological function of cell surface u-PAR and it's soluble counterpart by reducing the concentration of u-PA on the cell's surface (Koolwijk et al., 2001). Receptor-bound u-PA is not protected from inhibition by PAI-1, instead it forms a ternary complex with PAI-1 which induces internalisation of the entire complex via the LRP/α_2-M receptor leading to degradation of u-PA and PAI-1 in lysosomes (Nykjær et al., 1992c and 1997; Cubellis et al., 1990). The u-PAR is recycled back to the cell surface thus providing additional mechanisms for regulating pericellular plasmin activity (Nykjær et al., 1997). Recently, soluble u-PAR has been shown to bind to the mannose 6-phosphate receptor, which modulates the subcellular distribution of u-PAR and is capable of directing it to lysosomes, thus possibly functioning in the disposal of u-PAR (Nykjær et al., 1998).

A variety of cell types have been reported to express the u-PAR including monocytes, granulocytes, and activated B- and T-lymphocytes (Plesner et al., 1994 and 1997; Nykjær et al., 1990 and 1992b; Miles and Plow, 1987), thus further supporting a role for plasmin in haematopoiesis.

2.4. *PA inhibitors (PAIs).*

Three inhibitors of PAs, PAI-1, PAI-2, and protease nexin-1 (PN-1), serve to limit plasmin formation and thus its activity.

2.4.1. *PAI-1*

PAI-1 is a single-chain, 45-kDa glycoprotein and is produced by a number of cell types including vascular endothelial cells, hepatocytes, haematopoietic cells such as megakaryocytes, as well as by a number of transformed or tumour cell lines (Saksela and Rifkin, 1988; Andreasen, 1990). PAI-1 inhibits u-PA and t-PA as well as pro-t-PA by rapidly forming a 1:1 molar complex. Secreted PAI-1 is active but is rapidly converted into a latent form that is unable to complex with u-PA or t-PA (Sprenger and Kluft, 1987;). However, PAI-1 remains active when bound to ECM components such as vitronectin (a major binding protein of PAI-1), heparin, or fibronectin (Keijer et al., 1991; Owensby et al., 1991; Edelberg et al., 1991) allowing inhibition of pericellular activation of plasminogen by PAs (Ciambrone and McKeown-Longo, 1990). In addition, the binding of PAI-1 to vitronectin blocks cell migration independently of u-PA inhibition (Stefansson and Lawrence, 1996; Kjøller et al., 1997). PAI-1 can compete with the vitronectin receptor, $\alpha_v\beta_3$ (CD51/CD61), for vitronectin binding, thus, reducing the number of cell-matrix adhesion sites available for vitronectin-mediated migration, thereby inhibiting cell movement (Stefansson and Lawrence, 1996; Kjøller et al., 1997). The u-PAR has also been identified as a receptor for vitronectin (Wei et al., 1994), however, PAI-1 does not appear to inhibit cell migration by blocking the vitronectin-u-PAR interaction (Kjøller et al., 1997).

2.4.2. *PAI-2*

The synthesis of PAI-2 is restricted to the skin, placenta and macrophages/monocytes suggesting that PAI-2 functions in inflammatory/ phagocytic processes and/or morphogenesis (Lyons-Giordano et al., 1994; Chapman and Stone, 1985; Sprenger and Kluft, 1987). PAI-2 is secreted as a 60-kDa glycosylated protein, but inefficiently as it lacks a cleavable signal peptide (Ye et al., 1987). Therefore, PAI-2 accumulates intracellularly as a 47-kDa, non-glycosylated protein (Sprenger and Kluft, 1987; Saksela and Rifkin, 1988). Secreted PAI-2 is stable in soluble form for prolonged periods of time (Saksela and Rifkin, 1988). PAI-2 is the most effective inhibitor of u-PA. It also inhibits t-PA, although less effectively than PAI-1, and is a poor inhibitor of pro-t-PA (Sprenger and Kluft, 1987; Saksela and Rifkin, 1988; Andreasen et al., 1990).

2.4.3. *PN-1*

PN-1, a 45-kDa glycoprotein, is another inhibitor of u-PA and t-PA that is produced by a variety of cell types (Sprenger and Kluft, 1987). PN-1 is less effective than PAI-1 and PAI-2 and is also not specific for PAs, as it inhibits a broad range of serine proteases including trypsin, thrombin, and plasmin (Sprenger and Kluft, 1987; Saksela and Rifkin, 1988). Receptor-bound u-PA is also not protected from inhibition by PN-1 (similar to PAI-1). Instead, the binding of PN-1 to receptor-bound u-PA initiates the internalisation and degradation of the u-PA/ PN-1 complex via the LRP/α_2-M receptor (Conese et al., 1994).

5. The importance of the PA /plasmin system to mammalian physiology.

In vivo studies using gene targeting have emphasised the extensive involvement and importance of the PA/plasmin system as well as their inhibitors in mammalian physiology especially in wound healing (Carmeliet et al., 1997; Romer et al., 1996). However, similar studies have also shown that the PA/plasmin system plays a less essential role in reproduction as was previously thought. Transgenic mice overexpressing PAI-1 or u-PA, and mice with deficiencies in t-PA, u-PA, PAI-1, u-PAR, or plasminogen, survive embryonic development, have a normal life span and are also fertile (Lijnen et al., 1995; Bugge et al., 1995; Carmeliet and Collen, 1995).

Nevertheless, u-PA-deficient mice display an array of unusual phenotypic abnormalities such as dystrophic calcification, rectal prolapse, pleuritis, and the effacement of lymphoid follicles in the region of the lymph nodes and spleen, as well as a reduced immune response making the mice susceptable to infections (Shapiro et al., 1997; Gyetko et al., 2002). Likewise, u-PAR-deficient mice also suffer from diminished immune responses and markedly diminished platelet survival (Gyetko et al., 2000; Piguet et al., 2000). These mice maintain normal platelet numbers by increasing platelet production (Piguet et al., 2000). Furthermore, the healing of skin wounds is defective in mice with a disrupted plasminogen gene. In these mice, keratinocytes fail to proliferate and migrate to cover the injured area (Romer et al., 1996). Plasminogen-deficient mice also display after arterial wall injury, impaired vascular wound healing and neointima formation due to poor smooth muscle cell migration, impaired tissue remodelling, and severe thrombosis (Carmeliet et al., 1997). In a further study, bleomycin-induced pulmonary fibrosis has been noted in mice that either lack or overexpress the PAI-1 gene (Eitzman et al., 1996).

Other than the reports describing defects in platelet survival and reduced immuno-responses (due to a decrease in B-cell production) in u-PAR-deficient mice

(Gyetko et al., 2000; Piguet et al., 2000), there have been no reports of impaired haematopoiesis in mice with deficiencies in t-PA, u-PA, PAI-1, u-PAR, or plasminogen. This would suggest that the plasmin/plasminogen activator system has a limited role in this process. However, many proteolytic processes involving plasmin can also be mediated by other enzymes. For example, plasmin as well as phospholipase D releases active FGF-2-HSPG complexes from the cell surface (Saksela and Rifkin, 1990; Brunner et al., 1991; Falcone et al., 1993a and b), and plasmin as well as thrombin can cleave fibronectin and laminin into multiple fragments (Liotta et al., 1981). Pro-u-PA can be activated to u-PA by HUTSP-1, kallikrein, factor XIIa, cathepsin B, G, and L, as well as NGF-γ in addition to plasmin (Ichinose et al., 1986; Koivunen et al., 1989; Brunner et al., 1992; Kobayashi et al., 1991; Goretzki et al., 1992; Learmonth et al., 1992; Wolf et al., 1993). Furthermore, both t-PA and u-PA can activate plasminogen to plasmin. Therefore, it may be that the overlap in enzyme specificity allows mice lacking t-PA, u-PA, u-PAR, or plasminogen to survive embryonic development and birth. Indeed, mice lacking both t-PA and u-PA suffer from poor health, significant growth retardation, have a shorter life-span, and are less fertile than wild-type mice or mice with a single deficiency of t-PA or u-PA. Therefore, *in vitro* studies are still valuable for characterising and defining the specific roles that the plasmin/PA system may play in haematopoiesis.

6. Conclusions.

Although the proteolytic regulation of growth factor activity and cell migration in the BM have not been well studied, there is evidence supporting a role for plasmin in these processes. My findings that BM stromal cells secrete PAs and that their production can be differentially regulated by FGF-2, IL-1, and TGF-ß support a role for PAs in modulating haematopoiesis. In the BM this could occur via the regulation of plasmin-catalysed processing of growth factors and/or their receptors, and possibly cell migration.

Chapter 4

Role of growth factors/cyokines in regulating plasminogen activators in the BM stromal microenvironment.

Introduction.

Proteolysis plays an important role in regulating the availability and activity of certain growth factors and cytokines including FGF-2, TGF-ß, IL-1ß, IL-1α, and M-CSF (Brunner et al., 1991; Lyons et al., 1988; Gonias et al., 1989; Matsushima et al., 1986; Mignatti et al., 1989). Many of these growth factors are produced by BM stromal cells and are presented in a biologically active form to developing stem and progenitor cells in the BM microenvironment. Proteolytic regulation of growth factor activity includes the mobilisation of active growth factors from cell surfaces and/or ECM reservoirs, the activation of latent forms of growth factors, the shedding of growth factor receptors from the cell surface, as well as growth factor degradation. Furthermore, proteolysis is required for cell migration, being necessary for the breakdown of ECM molecules (Basbaum and Werb, 1996; Brunner and Priessner, 1994). Although it is known that proteases are produced by BM cells (McWilliam et al., 1998 and 1996; Hamilton et al., 1991a; Wilson and Francis, 1987), their functional role in haematopoiesis is not known but may include the regulation of growth factor/cytokine activity and/or migration of stem cells and progenitor cells from the BM cavity into the circulation.

1. Evidence for possible role of PAs and plasmin in haematopoieisis.

Several of the processes regulating growth factor/cytokine activity and ECM degradation are mediated by the serine protease, plasmin (Lyons et al., 1988; Gonias et al., 1989; Matsushima et al., 1986; Saksela and Rifkin, 1990; Brunner et al., 1991; Taipale et al., 1992; Falcone et al., 1993a; Whitelock et al., 1996). It is possible that plasmin may affect growth factors/cytokine activity in the BM microenvironment, thereby regulating haematopoiesis.

Both tissue-plasminogen activator (t-PA) and urokinase (u)-PA, which convert plasminogen to plasmin (see section 2.2), are secreted by a variety of haematopoietic cells and their production can be regulated by growth factors (Hamilton et al., 1991a; Hart et al., 1991; Wilson and Francis, 1987). In BM derived macrophages u-PA mRNA

levels and u-PA activity increase in response to M-CSF, GM-CSF, or IL-3 (Hamilton et al., 1991a; Hart et al., 1991). GM-CSF, in the presence of IFN-γ, as well as IL-4 also stimulate t-PA production by human monocytes (Hart et al., 1989 and 1991). The presence of t-PA-generated plasmin in the BM has also been demonstrated (McWilliam et al., 1998 and 1996). Furthermore, primitive myeloid progenitor cells and leukaemic cells have been shown to secrete t-PA, while mature blood cells secrete u-PA (Wilson and Francis, 1987; Wilson et al., 1983). This suggests that the expression of u-PA by mature haematopoietic cells might be a prerequisite for their migration and egression from the BM into the circulation. In addition, the conversion from t-PA to u-PA production might be accompanied by the co-expression of the u-PAR on the cell surface. In mature haematopoietic cells (monocytes and T-lymphocytes) as well as in other cell types, a stimulation in u-PA activity is accompanied by the co-expression of the u-PAR (Lund et al., 1991; Mignatti et al., 1991; Pepper et al., 1993; Estreicher et al., 1990; Nykjær et al., 1992a and b) thus facilitating cell migration.

A wide range of proteolytic enzymes including plasmin, thrombin, elastase, cathepsin G, and MMPs (Lapidot and Petit, 2002) are involved the breakdown of ECM molecules resulting in the migration of haematopoietic cells from the marrow cavity into the circulation. Indeed, u-PA and, to a lesser degree, t-PA promote the migration of T-lymphoblasts through an ECM barrier (Reiter et al., 1997). In addition, a number of cytokines/growth factors (e.g. SCF, G-CSF, TGF-ß), chemokines (e.g. stromal cell-derived factor-1α [SDF-1α] and IL-8) and chemokine receptors (e.g. CXCR4, the receptor for SDF-1α, and cb2, a cannabinoid receptor) function in the mobilisation of haematopoietic cells (De La Rosa et al., 2003; Moore, 2002; Lapidot and Petit, 2002; Mohle et al., 2001). Proteolysis appears to have further roles in regulating this process by acting on a number of these molecules leading to the disruption of/decrease in stromal-haematopoietic cell interactions. For example, serine proteases are responsible for the shedding of CXCR4 and c-kit (Levesque et al., 2003a and b), and dipeptidylpeptidase IV cleaves SDF-1α from cell surfaces (Christopherson et al., 2002). Furthermore, TGF-ß, whose own activity is regulated by plasmin, can decrease SDF-1α expression (Wright et al., 2003).

Fig 1 shows the cascade of proteolytic enzyme activation reactions, beginning with the activation of pro-u-PA and ending with the activation of pro-metalloproteinases (Mignatti et al., 1989). Since FGF-2 is a known inducer of this cascade (Pepper et al., 1993; Mignatti et al., 1989), it might indirectly regulate haematopoietic growth factor activity in the BM microenvironment which may well account for the stimulation in haematopoiesis observed in LTBM cultures treated with

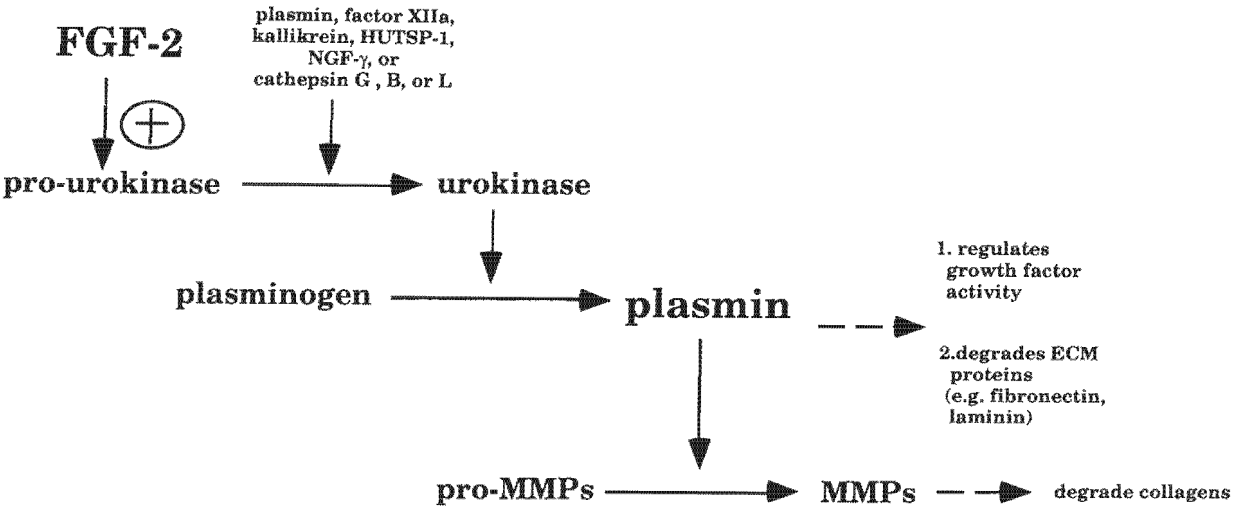


Fig. 1. Schematic representation of the proteolytic cascade leading to the formation of plasmin and MMPs which degrade ECM proteins.
HUTSP-1: human T-cell specific serine proteinase
MMPs: matrix matalloproteinases

this growth factor. The resulting generation of plasmin may cause the release of important haematopoietic growth factors/cytokines that are associated with the cell surfaces or the ECM, or are membrane bound. Alternatively, plasmin may activate latent growth factors, or shed growth factor receptors from the cell's surface and, in doing so, stimulate haematopoiesis. In addition, FGF-2 could potentially regulate progenitor cell migration. For example, TGF-ß, which can be activated by plasmin, downregulates the expression of SDF-1α leading to a decrease in haematopoieic cell migration (Wright et al., 2003). Furthermore, the proteolytic release of FGF-2 from cell surfaces or the ECM may represent one mechanism for regulating its own activity in the BM microenvironment.

The aims in this Chapter were to determine the cellular and secreted levels of u-PA and t-PA activity as well as their specific inhibitors, plasminogen activator inhibitor (PAI)-1 and PAI-2, by passaged BM stromal cells. The effects of FGF-2, IL-1, TGF-ß and hydrocortisone on the production of u-PA, t-PA, PAI-1, and PAI-2 were also investigated. In addition, the regulation of t-PA activity by PAI-1 in the BM microenvironment was studied by following the internalisation of the t-PA/PAI-1 complex by BM stromal cells. I found that all three cytokines increased PA activity, and in some cases PAI-1 and PAI-2 protein was also modulated by these growth factors. PAI-1 was also noted to rapidly bind t-PA in the culture medium, inhibiting its activity and removing it from the culture medium. Furthermore, hydrocortisone

inhibited both basal as well as TGF-ß-stimulated increases in PA activity in a reversible manner.

2. The plasminogen activator/plasmin system.

2.1. *Plasminogen/plasmin.*

The serine protease, plasmin, is generated from the inactive pro-enzyme plasminogen, by the two serine proteases, t-PA and u-PA. Plasminogen is a single-chain, inactive pro-enzyme, which is activated by PAs to form plasmin, a two-chain polypeptide connected by a disulphide bridge (Robbins et al., 1967). Plasminogen is ubiquitous in human body fluids and is found in high concentrations in the circulation. Both plasminogen and plasmin bind to a variety of low-affinity receptors, which are expressed in high numbers on the surface of many cell types including peripheral blood cells, such as, platelets, neutrophils, monocytes, and lymphocytes, but not erythrocytes (Plow et al., 1995; Redlitz and Plow, 1995; Hajjar et al., 1995). Plasminogen and plasmin bind via their lysine binding sites, which are associated with their kringle domains and recognise carboxy-terminal lysine residues of cell surface proteins such as α-enolase or annexin II (Redlitz and Plow, 1995; Hajjar et al., 1994; Miles et al., 1991). Plasminogen and plasmin also bind to ECM components such as fibronectin, laminin, vitronectin, or thrombospondin (Salonen et al., 1984 and 1985; Silverstein et al., 1986; Preissner et al., 1990).

Several functions for plasminogen receptors have been described which contribute to enhancing plasmin activity. Plasmin(ogen) receptors serve to localise these enzymes in the pericellular environment which enables directed proteolysis. Receptor bound plasmin is protected from inactivation by its inhibitor, α_2-antiplasmin, which is present in serum and other body fluids (Gonias, 1992; Sprengers and Kluft, 1987; Knudsen et al., 1986), thus allowing proteolysis to occur on cell surfaces and in the ECM in the presence of this inhibitor. Bound plasminogen is more readily activated to plasmin than free plasminogen and, furthermore, bound plasmin has increased enzymatic activity in comparison to free plasmin (Redlitz and Plow, 1995).

2.2. *PAs*

t-PA, a 70-kDa protein, and u-PA, a 55-kDa protein, are the protein products of two genes (Rajput et al., 1988). t-PA and u-PA are structurally similar, but functionally and immunologically distinct serine proteases (Andreasen et al., 1990; Saksela and Rifkin, 1988). Since both enzymes are able to activate plasminogen to form plasmin, the distinct roles that t-PA and u-PA play are not fully understood. u-

PA expression has been repeatedly associated with processes involving ECM degradation and cell migration, while t-PA, the main PA in plasma, appears to be primarily responsible for plasmin generation during fibrinolysis (Fazioli and Blasi, 1994; Carmeliet et al., 1994; Collen and Lijnen, 1991; Andreasen et al., 1990; Saksela and Rifkin, 1988).

Both t-PA and u-PA are secreted as single-chain proenzymes. While both pro-u-PA and pro-t-PA have some intrinsic activity (van der Werf et al., 1986; Andreasen et al., 1990), they are converted by plasmin to form active two chain u-PA and t-PA, respectively (Andreasen et al., 1990; Saksela and Rifkin, 1988). In addition, kallikrein, trypsin, factor XIIa, the human T-cell specific serine proteinase, HUTSP-1, cathepsins B, L, and G, as well as NGF-γ have also been shown to mediate the conversion of pro-u-PA to active u-PA (Fig. 1)(Ichinose et al., 1986; Koivunen et al., 1989; Brunner et al., 1992; Kobayashi et al., 1991; Goretzki et al., 1992; Learmonth et al., 1992; Wolf et al., 1993).

PA activity is regulated through synthesis (transcriptional regulation) and by specific inhibitors or co-factors (post-transcriptional regulation). PA synthesis may directly modulate extracellular PA activity and is under the control of hormones, growth factors, cyclic nucleotides, and tumour promoters (Saksela and Rifkin, 1988). Post-transcriptional regulation of PA activity also involves ECM components, e.g. sulphated glycosaminoglycans (Brunner et al., 1998), besides PA inhibitors (see section 2. 4).

2.3. *PA receptors.*

2.3.1. *t-PA receptors.*

Receptors for t-PA include those that localise its activity, resulting in focused and restricted extracellular protease activity, and those that mediate its clearance. Annexin II (Kim and Hajjar, 2002; Hajjar et al., 1994; Cesarman et al., 1993), α-enolase (Felez et al., 1991), and amphoterin (Parkkinen and Rauvala, 1991) have been implicated as receptors which serve to localise t-PA activity to the cell surface. The overlapping binding specificities of plasminogen and t-PA with certain receptors (e.g. annexin II and α-enolase) is important for co-localisation, leading to the enhancement of plasminogen activation (Felez et al., 1993a and b; Cesarman et al., 1994).

The liver is the main organ responsible for removing t-PA from the circulation. Carbohydrate-specific receptors, including the mannose receptor (Otter et al., 1991 and 1992) and the αfucose receptor (Hajjar and Reynolds, 1994), function as clearance receptors for t-PA. In addition, low density lipoprotein receptor related protein/α_2-

macroglobulin (LRP/α_2-M) receptor, which binds numerous ligands, is as a clearance receptor for t-PA complexed to PAI-1 (Bu et al., 1992), and possibly free t-PA (Camani et al., 1994; Orth et al., 1994). Gp330, another receptor of the low density lipoprotein receptor family, may also contribute to t-PA removal (Willnow et al., 1992).

2.3.2. *The u-PA receptor.*

Pro-uPA and active u-PA bind to a specific high-affinity urokinase receptor (u-PAR, CD87) which is attached to the membrane via a glycosyl-phosphatidylinositol (GPI) anchor (Ploug et al., 1991). The binding of pro-u-PA to its receptor accelerates its activation and also localises the resulting active u-PA to specific sites on the cell's surface (Nykjær et al., 1992b; Estreicher et al., 1990; Ellis et al., 1989). Two additional roles have been attributed to the u-PAR other than its role in plasminogen activation, namely cell-cell adhesion (by modulating integrin-mediated cell-adhesion; Wei et al., 1996) as well as cell-ECM adhesion (via vitronectin; Kanse et al., 1996), and signal transduction events (also via integrins)(Plesner et al., 1997).

The GPI anchor is cleaved by phosphatidylinositol-specific phospholipase C, GPI-specific phospholipase D, and metalloproteinase-12, thus releasing the u-PAR from cell surfaces (Wilhelm et al., 1999; Ploug et al., 1991; Koolwijk et al., 2001). Interestingly, u-PA also cleaves its own receptor from cell surfaces, releasing the ligand binding domain (Hoyer-Hansen, 1992). These shedding events might regulate the biological function of cell surface u-PAR and it's soluble counterpart by reducing the concentration of u-PA on the cell's surface (Koolwijk et al., 2001). Receptor-bound u-PA is not protected from inhibition by PAI-1, instead it forms a ternary complex with PAI-1 which induces internalisation of the entire complex via the LRP/α_2-M receptor leading to degradation of u-PA and PAI-1 in lysosomes (Nykjær et al., 1992c and 1997; Cubellis et al., 1990). The u-PAR is recycled back to the cell surface thus providing additional mechanisms for regulating pericellular plasmin activity (Nykjær et al., 1997). Recently, soluble u-PAR has been shown to bind to the mannose 6-phosphate receptor, which modulates the subcellular distribution of u-PAR and is capable of directing it to lysosomes, thus possibly functioning in the disposal of u-PAR (Nykjær et al., 1998).

A variety of cell types have been reported to express the u-PAR including monocytes, granulocytes, and activated B- and T-lymphocytes (Plesner et al., 1994 and 1997; Nykjær et al., 1990 and 1992b; Miles and Plow, 1987), thus further supporting a role for plasmin in haematopoiesis.

2.4. *PA inhibitors (PAIs).*

Three inhibitors of PAs, PAI-1, PAI-2, and protease nexin-1 (PN-1), serve to limit plasmin formation and thus its activity.

2.4.1. *PAI-1*

PAI-1 is a single-chain, 45-kDa glycoprotein and is produced by a number of cell types including vascular endothelial cells, hepatocytes, haematopoietic cells such as megakaryocytes, as well as by a number of transformed or tumour cell lines (Saksela and Rifkin, 1988; Andreasen, 1990). PAI-1 inhibits u-PA and t-PA as well as pro-t-PA by rapidly forming a 1:1 molar complex. Secreted PAI-1 is active but is rapidly converted into a latent form that is unable to complex with u-PA or t-PA (Sprenger and Kluft, 1987;). However, PAI-1 remains active when bound to ECM components such as vitronectin (a major binding protein of PAI-1), heparin, or fibronectin (Keijer et al., 1991; Owensby et al., 1991; Edelberg et al., 1991) allowing inhibition of pericellular activation of plasminogen by PAs (Ciambrone and McKeown-Longo, 1990). In addition, the binding of PAI-1 to vitronectin blocks cell migration independently of u-PA inhibition (Stefansson and Lawrence, 1996; Kjøller et al., 1997). PAI-1 can compete with the vitronectin receptor, $\alpha_v\beta_3$ (CD51/CD61), for vitronectin binding, thus, reducing the number of cell-matrix adhesion sites available for vitronectin-mediated migration, thereby inhibiting cell movement (Stefansson and Lawrence, 1996; Kjøller et al., 1997). The u-PAR has also been identified as a receptor for vitronectin (Wei et al., 1994), however, PAI-1 does not appear to inhibit cell migration by blocking the vitronectin-u-PAR interaction (Kjøller et al., 1997).

2.4.2. *PAI-2*

The synthesis of PAI-2 is restricted to the skin, placenta and macrophages/monocytes suggesting that PAI-2 functions in inflammatory/ phagocytic processes and/or morphogenesis (Lyons-Giordano et al., 1994; Chapman and Stone, 1985; Sprenger and Kluft, 1987). PAI-2 is secreted as a 60-kDa glycosylated protein, but inefficiently as it lacks a cleavable signal peptide (Ye et al., 1987). Therefore, PAI-2 accumulates intracellularly as a 47-kDa, non-glycosylated protein (Sprenger and Kluft, 1987; Saksela and Rifkin, 1988). Secreted PAI-2 is stable in soluble form for prolonged periods of time (Saksela and Rifkin, 1988). PAI-2 is the most effective inhibitor of u-PA. It also inhibits t-PA, although less effectively than PAI-1, and is a poor inhibitor of pro-t-PA (Sprenger and Kluft, 1987; Saksela and Rifkin, 1988; Andreasen et al., 1990).

2.4.3. *PN-1*

PN-1, a 45-kDa glycoprotein, is another inhibitor of u-PA and t-PA that is produced by a variety of cell types (Sprenger and Kluft, 1987). PN-1 is less effective than PAI-1 and PAI-2 and is also not specific for PAs, as it inhibits a broad range of serine proteases including trypsin, thrombin, and plasmin (Sprenger and Kluft, 1987; Saksela and Rifkin, 1988). Receptor-bound u-PA is also not protected from inhibition by PN-1 (similar to PAI-1). Instead, the binding of PN-1 to receptor-bound u-PA initiates the internalisation and degradation of the u-PA/ PN-1 complex via the LRP/α_2-M receptor (Conese et al., 1994).

5. The importance of the PA /plasmin system to mammalian physiology.

In vivo studies using gene targeting have emphasised the extensive involvement and importance of the PA/plasmin system as well as their inhibitors in mammalian physiology especially in wound healing (Carmeliet et al., 1997; Romer et al., 1996). However, similar studies have also shown that the PA/plasmin system plays a less essential role in reproduction as was previously thought. Transgenic mice overexpressing PAI-1 or u-PA, and mice with deficiencies in t-PA, u-PA, PAI-1, u-PAR, or plasminogen, survive embryonic development, have a normal life span and are also fertile (Lijnen et al., 1995; Bugge et al., 1995; Carmeliet and Collen, 1995).

Nevertheless, u-PA-deficient mice display an array of unusual phenotypic abnormalities such as dystrophic calcification, rectal prolapse, pleuritis, and the effacement of lymphoid follicles in the region of the lymph nodes and spleen, as well as a reduced immune response making the mice susceptable to infections (Shapiro et al., 1997; Gyetko et al., 2002). Likewise, u-PAR-deficient mice also suffer from diminished immune responses and markedly diminished platelet survival (Gyetko et al., 2000; Piguet et al., 2000). These mice maintain normal platelet numbers by increasing platelet production (Piguet et al., 2000). Furthermore, the healing of skin wounds is defective in mice with a disrupted plasminogen gene. In these mice, keratinocytes fail to proliferate and migrate to cover the injured area (Romer et al., 1996). Plasminogen-deficient mice also display after arterial wall injury, impaired vascular wound healing and neointima formation due to poor smooth muscle cell migration, impaired tissue remodelling, and severe thrombosis (Carmeliet et al., 1997). In a further study, bleomycin-induced pulmonary fibrosis has been noted in mice that either lack or overexpress the PAI-1 gene (Eitzman et al., 1996).

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Methods.

Cultures of passaged BM stromal cells were established and the cellular and secreted levels of u-PA and t-PA under different experimental conditions was investigated. The synthesis of the specific inhibitors, PAI-1 and PAI-2, was determined by enzyme-linked immunosorbent assays.

1. BM collection and establishment of cultures.

LTBM cultures were established in 75-cm^2 flasks in 20 ml of stromal medium as previously described in Chapter 2. Cultures of passaged BM stromal cells were established by removing the non-adherent progenitor and mature haematopoietic cells from two to three week old primary BM cultures by extensive washing with PBS. The adherent stromal cells were removed from the flasks by adding trypsin as described in Chapter 3. The washed cells were then seeded in 24-well Linbro plates (2.5 x 10^4 cells/well) or 35-mm dishes (5 x 10^4 cells/dish) in 2 ml of stromal medium. Before using the cells in an experiment, hydrocortisone (present in the stromal medium) was removed from the cultures by washing four times with serum-free RPMI medium.

2. Preparation of harvest fluids.

To prepare harvest fluids, the cells were washed twice with serum-free RPMI medium to remove the FCS. One ml of serum-free RPMI medium with or without the relevant of growth factor/cytokine was added to each well. Twenty-four hours later the supernatants were collected. Protease- and protease inhibitor-free BSA (Appendix 1.1) was added to the supernatant at a final concentration of 0.4 mg/ml BSA.

3. Preparation of cell lysates.

Adherent cells on 35-mm dishes or 24-well Linbro plates were washed twice with ice-cold PBS and scraped with a rubber policeman into 1 ml of PBS. The cells were centrifuged at 350 x *g* for 5 min at 4°C. To lyse the cells, 100 µl of 0.5% Triton X-100 in 0.1 M Tris HCl, pH 8.1, was added to the pellet. The lysate was incubated on ice for 1 hour and then cleared by centrifugation at 650 x *g* for 5 min. The samples were stored at -20°C.

The protein concentration of each sample was determined in order to express the results as the amount of enzyme production in international milliunits (I mU)/mg protein. Alternatively, cells from additional dishes were removed with trypsin and

counted to express the results as the amount of enzyme or inhibitor production in I mU/10^5 cells.

4. Protein concentration determinations.

The protein concentration in cell lysates was determined using the Bio-Rad protein assay. This assay allowed for the determination of protein in the presence of up to 0.1% Triton X-100. The methodology used was according to the manufacturer's instructions. Briefly, 20 µl of sample (dilutions were necessary) were mixed with 1 ml of Bio-Rad dye and allowed to stand at room temperature for 5 to 60 minutes. The optical density was measured in a spectrophotometer at 595 nm. Serial dilutions of BSA were used as standards against which the samples were compared.

5. Purification of antibodies.

Sheep anti-human u-PA and t-PA antibodies, control IgG from non-immune sheep serum, rabbit anti-human PAI-1 antibodies, and control rabbit IgG from non-immune serum, were purified using a protein-G sepharose column (Appendix 1.6).

6. Plasminogen activator assay.

Samples were assayed for PA activity by measuring the plasminogen-dependent release of soluble, radioactive fibrin degradation peptides from an insoluble substrate of ^{125}I-fibrin prepared and adsorbed to the bottom surface of a plastic well (Appendix 2.1)(Wilson and Dowdle, 1978). Each well contained 2 µg of purified human plasminogen and 80 µg BSA in a final volume of 300 µl of 0.1 M Tris-HCl, pH 8.1. To initiate the reaction, 5 to 10 µl of cell lysate or 40 µl of supernatant were added to duplicate wells. Control wells contained plasminogen alone (no sample). Standards were included in each assay and contained u-PA in doubling decrements starting from 0.25 international units (IU)/well. The covered plates were incubated at 37°C in a humid atmosphere. Fibrinolysis was monitored by measuring the solubilised radioactivity in 50 µl aliquots at various time intervals. Results are expressed as IU PA/10^5 cells/24 h ± SD, unless otherwise stated.

7. Immunochemical identification of PA type.

Plasminogen activators present in the supernatant or cell lysates were identified as u-PA or t-PA by measuring residual enzyme activity after incubation of the sample with specific neutralising antibodies to each enzyme type. Thirty-five µl of supernatant or 10 µl of cell lysate were incubated for 1 hour at 4°C with an equal

volume of a 1.25 mg/ml antibody preparation (sheep anti-human u-PA or t-PA antibodies, Appendix 1.6) and 80 µg BSA made up to a final volume of 80 µl with 0.1 M Tris-HCl, pH 8.1. An aliquot thereof was assayed for residual PA activity as described above.

8. Zymography of plasminogen activators and inhibitors.

Samples (22.5 µl supernatant or 5 µg protein [lysates]) were subjected to 11% polyacrylamide/0.1% SDS gel electrophoresis (Appendix 2.2). Following electrophoresis, the gel was washed for 1 hour with gentle shaking in a solution of 2.5% Triton X-100 in water to remove the SDS. The gel was rinsed in distilled water, drained and layered onto a fibrin-plasminogen agar indicator gel slab (Appendix 2.3) and incubated in a humid chamber at 37°C. Bands of enzyme activity were detected zymographically as plasminogen-dependent zones of fibrinolysis in the fibrin-plasminogen agar gel (Wilson et al., 1980) and their apparent molecular weights calculated by reference to co-electrophoresed molecular weight marker proteins.

Reverse zymography, for demonstrating the presence of PAI-1, was initiated by including 3 mU u-PA in the indicator layer. Complete lysis of the fibrin occurred unless PAI-1 was present to inhibit this reaction. Thus, inhibitor bands were detected as opaque zones in a background of complete lysis of the fibrin indicator gel (Erickson et al., 1984).

The fibrin indicator gels were preserved and stained by soaking in fixative (70% methanol, 10% acetic acid and 20% water) containing 0.1% amido black for 1 hour, and were destained by repeated soaking in fixative containing no dye.

9. Enzyme-linked immunosorbent assay (ELISA).

PAI-1 and PAI-2 were measured with specific ELISA kits (American Diagnostica, USA) which used the double-antibody sandwich principle. Peroxidase-conjugated reagents and an enzyme-linked colourimetric reaction with phenylenediamine-HCl as the substrate yielded a yellow colour upon reaction that could be read at an optical density of 492 nm. It should be noted that the PAI-1 assay kit detected only free PAI-1 and not complexes of t-PA or u-PA with PAI-1. Results are expressed as ng PAI/10^5 cells/24 h (supernatant values) or as ng PAI/10^5 cells (cellular values).

10. PAI-1 inhibition of t-PA activity.

Passaged BM stromal cells (3') were seeded in 35-mm culture dishes and depleted of hydrocortisone as described above. After one week all cultures were washed three times with RPMI medium to remove all traces of FCS. The cells were then incubated at 37°C with or without rabbit anti-PAI-1 antibodies, or with irrelevant rabbit IgG at 1.14 mg/ml in a final volume of 750 µl of RPMI medium containing 0.4 mg/ml BSA. After 10 min, an equal volume of RPMI medium containing 0.4 mg/ml BSA and 100 ng/ml pro-t-PA was added to each culture condition. At time intervals of 1, 2, 4, 6, 8, and 24 hours, 100 µl aliquots of the supernatants were removed and assayed for PA activity using the ^{125}I-fibrin plate assay. After 4 hours of culture, one set of cultures treated with PAI-1 antibodies received a second aliquot of rabbit anti-PAI-1 antibodies such that the final antibody concentration in these cultures was 1.15 mg/ml. An equivalent volume of PBS was added to the other cultures.

To control for non-specific binding of pro-t-PA to the plastic and to verify the stability of pro-t-PA over the assay period of 24 hours, control dishes were prepared by adding pro-t-PA to culture dishes that contained no cells but which had been treated with RPMI medium containing 10% FCS. These culture dishes were subjected to the same experimental protocol as described above. "Supernatants" were collected and PA activity was determined using the ^{125}I-fibrin plate assay.

11. Clearance of t-PA by PAI-1.

Passaged BM stromal cells (5') were seeded into 24-well Linbro plates and depleted of as described above. After one week the cells were washed three times with RPMI medium to remove all traces of FCS. The cultures were then treated with 500 µl of RPMI medium containing 0.4 mg/ml BSA and 10^5 cpm of ^{125}I-t-PA (equivalent to 10 ng t-PA; ^{125}I-t-PA was a gift from Dr. Lillian Ossowski, Mt Sinai School of Medicine, NY). At time intervals of 0, 15 min, 30 min, 1, 2, 4, 6, 8, 10, and 24 hours, the supernatants were collected and cell lysates and matrix extracts were prepared. The supernatants were centrifuged at 1,000 x *g* for 1 minute in an Eppendorf centrifuge and then transferred to a new tube containing 10 µl of 0.25% Tween 80. To prepare cell lysates the cells were washed three times with cold PBS and then incubated for 10 minutes at 37°C with 250 µl of PBS containing 0.5% Triton X-100. The matrix layer was then washed with three changes of cold water. Matrix extracts were prepared by scraping the matrix layer into 250 µl of a 0.1% SDS solution. Samples were stored at -20°C. To identify ^{125}I-t-PA, aliquots of supernatants, cell lysates and matrix extracts

were subjected to 11% SDS-PAGE (Appendix 2.2), and the gel was dried for autoradiography. After exposing the gels, labelled proteins in the high and low molecular weight fractions present in the supernatant was quantitated by excising the relevant areas of the gel and determining radioactitvity using a gamma counter.

12. PAI-1 identification by Western blot.

PAI-1 antigen was identified in the supernatants, cell lysates and matrix extracts by using standard Western blotting procedures (Appendix 2.3). Aliquots of the samples as well as 3.13 ng of a PAI-1 standard were subjected to 11% SDS-PAGE (Appendix 2.2) and the proteins in the resultant gel transferred onto nitrocellulose membranes by semi-wet blotting. The membranes were then incubated with anti-PAI-1 antibodies conjugated to ^{125}I-labelled protein A.

13. Trichloroacetic acid (TCA) precipitation of proteins.

Proteins in the supernatant were precipitated using TCA in order to determine the total amount of TCA precipitable ^{125}I-t-PA in each sample. To do this, 100 µl aliquots of supernatant were mixed with an equal volume of BSA (8 mg/ml) and 200 µl of 10% TCA. The samples were incubated at 4°C for 30 minutes before centrifugation at 1,000 x *g* for 5 minutes. The precipitates were washed twice with 5% TCA.

Results.

The cellular production and secretion of u-PA and t-PA as well as their specific inhibitors, PAI-1 and PAI-2, by passaged BM stromal cells were determined because of the role that they could play in modulating haematopoiesis via the regulation of plasmin-catalysed processing of growth factors and/or their receptors (Hannocks et al., 1992). Furthermore, u-PA-mediated plasmin generation stimulates cell migration and may therefore be important for the egress of haematopoietic cells from the BM into the circulation.

1. Basal PA/PAI levels in passaged BM stromal cells.

Table 1 shows the mean basal PA, PAI-1, and PAI-2 values from the individual values obtained in the various experiments. PA activity, and PAI-1 and -2 protein levels, were determined in harvest fluids and cell lysates of passaged BM stromal cells after removing hydrocortisone from the culture medium for four days (see section 2). The stromal cells were then incubated in serum-containing RPMI medium for 24 hours. Harvest fluids were then prepared by incubating the stromal cells in serum-free RPMI for a further 24 hours. Cell lysates were prepared in 0.5% Triton X-100.

Passaged BM stromal cells produced and secreted very low levels of PA activity (0.39 ± 0.28 mU/10^5 cells and 0.27 ± 0.16 mU/10^5 cells/24 h, respectively). This was due to the relatively high endogenous levels of PAI-1 (Table 1). The type of PA produced by BM stromal cells (using immunochemical techniques) was mainly u-PA.

Table 1. Basal PA and PAI levels in BM stromal cells.

	PA	PAI-1	PAI-2
Supernatant mU/10^5 cells/24 h	0.27 ± 0.16 (n = 10 exp.)	68.4 ± 37.6 (n = 9 exp.)	3.9 ± 3.26 (n = 7 exp.)
Cell Associated mU/10^5 cells	0.39 ± 0.28 (n = 5 exp.)	0.76 ± 0.33 (n = 6 exp.)	0.39 ± 0.14 (n = 3 exp.)

In the majority of the experiments, BM stromal cells used were at passage three. while in some experiments, stromal cells were also used at passage two or four.
Results represent the mean value ± SEM

2. Regulation of BM stromal PA activity by hydrocortisone.

Previous studies have demonstrated that glucocorticoids inhibit the proteolytic activity expressed by a number of different cell types in culture (Saksela and Rifkin, 1988). Since Dexter LTBM cultures require medium containing the glucocorticoid, hydrocortisone (see Chapter 2, Table 1), I was interested in determining the effect of this glucocorticoid on BM stromal cell PA activity. Hydrocortisone was removed from BM stromal cells and PA activity was determined in the supernatant at different time intervals following its removal. Hydrocortisone was also added at various concentrations to BM stromal cells grown in glucocorticoid-free medium to determine its effect on both PA activity and PAI-1 production.

2.1. *The inhibition of PA activity by hydrocortisone.*

Passaged BM stromal cells (3') were seeded in 24-well Linbro plates as described in the methods. The cells (washed free of hydrocortisone) were then incubated with 1 ml of RPMI medium containing 10% FCS in the presence or absence of 10^{-6} M hydrocortisone. After 1, 2, 4, and 5 days, fresh RPMI medium containing 10% FCS was added, and the cells were treated with or without 10^{-6} M hydrocortisone in the presence or absence of 0.2 ng/ml TGF-ß for 24 hours. Because the basal PA secretion of passaged BM stromal cells was low (see Table 1), TGF-ß, a potent inducer of BM stromal cell PA activity (noted in preliminary experiments), was added to the cultures to stimulate the basal levels of PA activity in order to observe the effects of hydrocortisone. Harvest fluids were prepared as described in the methods. The supernatants were then assayed for PA activity using the ^{125}I-fibrin plate assay.

Fig. 2 shows that hydrocortisone inhibited the basal secretion of PA activity as well as the TGF-ß-induced stimulation of PA activity by BM stromal cells. BM stromal cells treated with 10^{-6} M hydrocortisone alone or in the presence of 0.2 ng/ml TGF-ß did not secrete detectable PA activity (Fig. 2). However, the inhibitory effect of hydrocortisone on TGF-ß-stimulated PA production by BM stromal cells was reversed when this glucocorticoid was removed from the culture medium for one day. At this time interval, TGF-ß-treated BM stromal cells secreted 23-times more PA activity than control cells ($p < 0.01$). Maximum stimulation (59-fold) in PA activity by TGF-ß was noted after hydrocortisone was removed from the medium for two days (from 0.8 ± 0.35 to $47 \pm 7.5\%$ of the total trypsin-soluble ^{125}I-fibrin, $p < 0.02$)(Fig. 2).

The basal secretion of PA activity was measurable in the supernatant of BM stromal cells cultured in the absence of hydrocortisone for a minimum of four days (the supernatant contained a maximum of $6 \pm 0.21\%$ of the total trypsin-soluble ^{125}I-fibrin

compared to hydrocortisone treated cells which did not secrete detectable PA activity, $p < 0.001$).

These results show that hydrocortisone in the medium of Dexter LTBM cultures inhibited the secretion of PAs by BM stromal cells. In addition, hydrocortisone completely inhibited the TGF-ß-mediated induction of PAs. These effects were reversed when BM stromal cells were grown in the absence of hydrocortisone. Thus, in all experiments, unless otherwise stated, BM stromal cells were cultured in medium without hydrocortisone (RPMI medium containing 10% FCS) for four days with a medium change after two days to ensure that the inhibitory effect of hydrocortisone on basal PA activity was completely reversed.

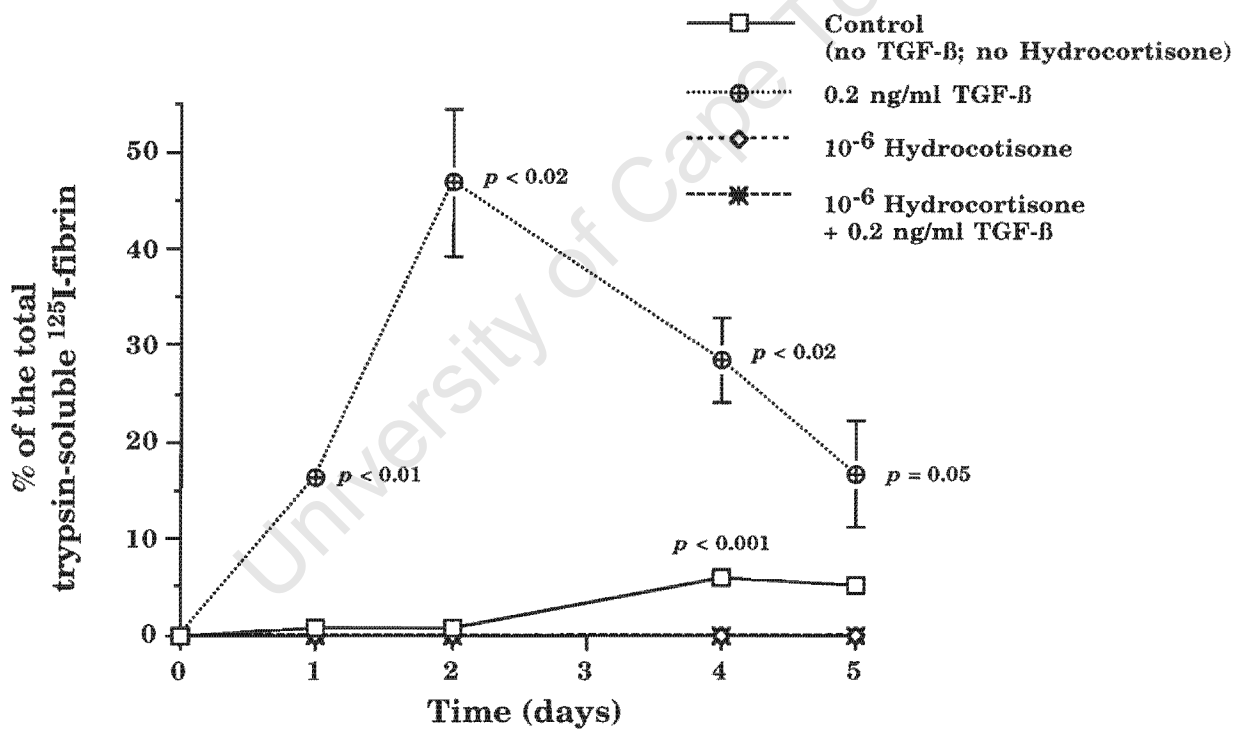


Fig. 2. The regulation of PA activity by hydrocortisone. Passaged BM stromal cells were seeded into 24-well Linbro plates at 2.5 x 10^4 cells/well in stromal medium. This medium, containing 10^{-6} M hyrocortisone, was replaced on day zero with RPMI medium containing 10% FCS in the presence or absence of 10^{-6} M hydrocortisone. After 1, 2, 4, and 5 days, the supernatants were replaced with RPMI medium containing 10% FCS, with or without 10^{-6} M hydrocortisone and in the presence or absence of 0.2 ng/ml TGF-ß. After 24 hours, harvest fluids were prepared by replacing the supernatants with 1 ml of serum-free RPMI medium with or without 10^{-6} M hydrocortisone and in the absence or presence of 0.2 ng/ml TGF-ß. Twenty four hours later PA activity in the supernatants was determined using the ^{125}I-fibrin plate assay. Results are expressed as the mean percentage of the total trypsin-soluble ^{125}I-fibrin ± SD from duplicate wells for each condition. This experiment was performed only once.

2.2. *Dose-dependency of PA regulation by hydrocortisone.*

To further characterise the inhibitory effects of hydrocortisone on PA activity, BM stromal cells were treated with different concentrations of hydrocortisone in the absence or presence of TGF-ß, and the levels of PA and PAI-1 in the supernatant were determined. Passaged BM stromal cells (4') were seeded into 24-well Linbro plates in BM stromal medium and washed free of hydrocortisone as described above. Hydrocortisone (10^{-6} to 10^{-9} M) was added to the cultures for 24 hours in 1 ml RPMI medium containing 10% FCS in the absence or presence of 0.2 ng/ml TGF-ß. Harvest fluids were prepared as described in the methods in serum-free RPMI medium containing hydrocortisone (10^{-6} to 10^{-9} M) in the absence or presence of 0.2 ng/ml TGF-ß and assayed for PA activity and PAI-1 protein.

In Fig. 3*A*, BM stromal cells cultured in the absence of hydrocortisone and TGF-ß secreted little PA activity (0.45 ± 0.2 mU/10^5 cells/24 h). Furthermore, in the absence of TGF-ß, all concentrations of hydrocortisone inhibited the basal PA activity expressed by BM stromal cells. However, PA activity was stimulated approximately 273-fold by 0.2 ng/ml TGF-ß (123 ± 23 mU/10^5 cells/24 h, $p < 0.001$, Fig. 3*A*). Concentrations of 10^{-6} and 10^{-7} M hydrocortisone resulted in the most significant inhibition of TGF-ß-induced PA stimulation with activities being reduced by 99 ($p < 0.005$) and 79% ($p < 0.02$) respectively (Fig. 3*A*). Hydrocortisone concentrations of 10^{-8} and 10^{-9} M did not significantly inhibit TGF-ß-induced PA secretion (Fig. 3*A*).

The levels of PAI-1 in the supernatants were also determined (Fig. 3*B*). BM stromal cells cultured in the absence of hydrocortisone and TGF-ß secreted 6 ± 2 ng PAI-1/10^5 cells/24 h (Fig. 3*B*). PAI-1 levels were induced by approximately 4-fold (to approximately 26 ± 3 ng PAI-1/10^5 cells/24 h, $p < 0.02$) by all concentrations of hydrocortisone tested. TGF-ß alone stimulated PAI-1 levels by approximately 7-fold above control cultures (from 6 ± 2 to 42 ± 5 ng/10^5 cells/24 h, $p < 0.01$). The presence of hydrocortisone (10^{-6} and 10^{-9} M), had no significant effect on the levels of PAI-1 induced by TGF-ß (Fig. 3*B*, $p > 0.05$ for each hydrocortisone concentration).

Thus, both basal and TGF-ß-mediated increases in PA activity were inhibited by hydrocortisone in a concentration dependent manner. No changes in PAI-1 were noted indicating that the inhibition was not due to increases in PAI-1 production.

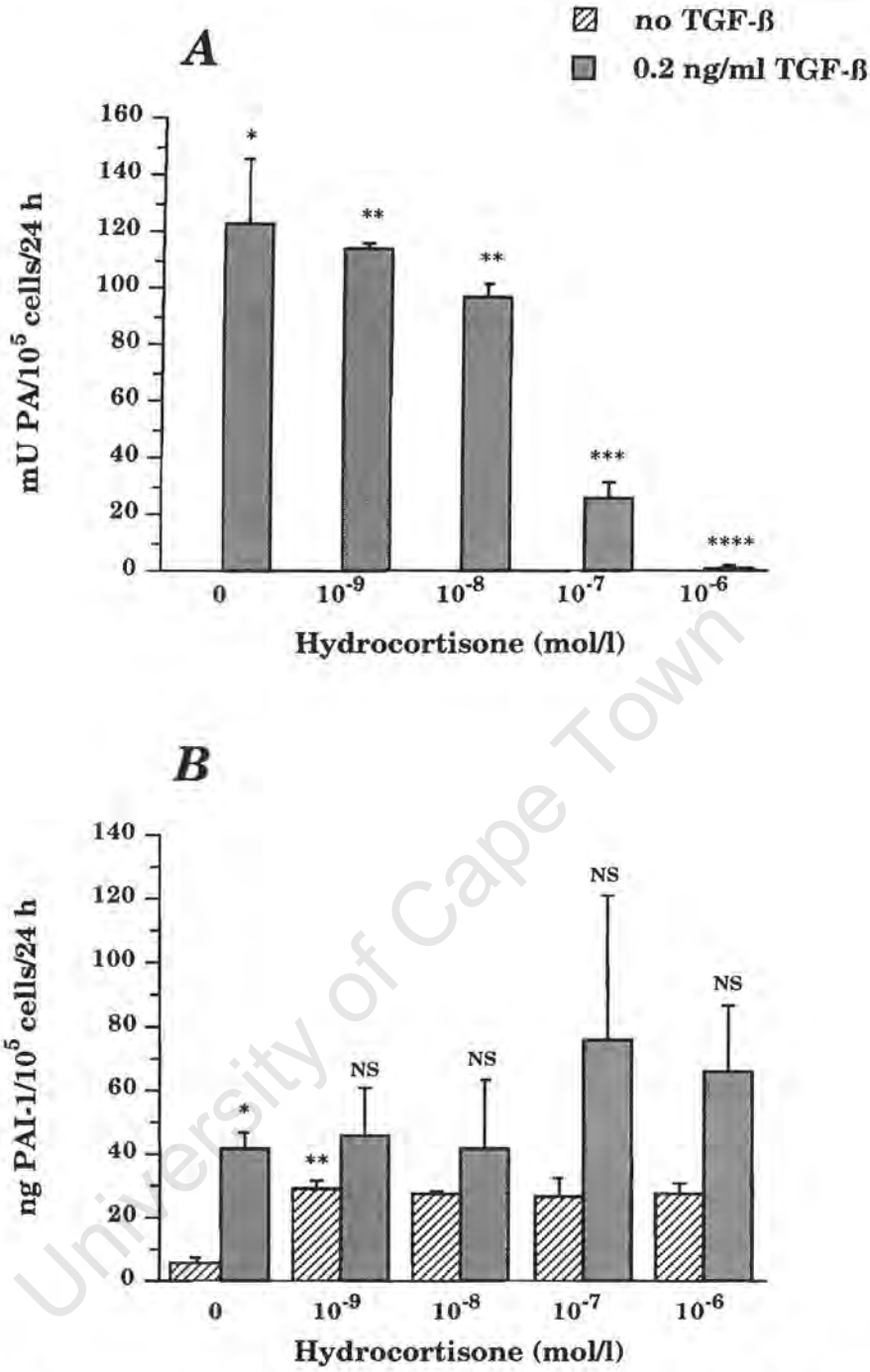


Fig. 3. Regulation of PA secretion by hydrocortisone. Passaged BM stromal cells were seeded into 24-well Linbro plates at 2 x 10^4 cells/well in BM stromal medium. Hydrocortisone (10^{-6} to 10^{-9} M) was added to the cultures for 24 hours in 1 ml RPMI containing 10% FCS in the absence or presence of 0.2 ng/ml TGF-ß. The BM stromal cells were then washed twice with RPMI medium to remove the FCS and incubated for a further 24 hours in serum-free RPMI medium containing hydrocortisone (10^{-6} to 10^{-9} M) in the absence or presence of 0.2 ng/ml TGF-ß. This experiment was performed only once.

A. The supernatants were assayed for PA activity using the ^{125}I-fibrin plate assay. Results are expressed as the mean mU PA/10^5 cells/24 h ± SD from duplicate wells for each condition.
* $p < 0.001$ relative to control (i.e. no TGF-ß and no hydrocortisone).
** $p > 0.05$, *** $p < 0.02$, and **** $p < 0.005$ relative to maximal stimulation with TGF-ß.

B. PAI-1 protein levels in the supernatants were determined by ELISA. Results are expressed as ng PAI-1/10^5 cells/24 h ± SD from duplicate wells for each condition.
* $p < 0.01$ and ** $p < 0.02$ relative to control (i.e. no TGF-ß and no hydrocortisone).
NS: not significant relative to 0.2 ng/ml TGF-ß and no hydrocortisone, $p > 0.05$.

3. Growth factor regulation of PA activity in BM stromal cells.

3.1. *Regulation of PA activity by FGF-2, IL-1ß, and TGF-ß.*

FGF-2 and two other growth factors known to be important in the regulation of haematopoiesis, IL-1ß and TGF-ß, were added to BM stromal cells, and their effects on secreted and intracellular PAs, u-PA and t-PA, as well as their inhibitors, PAI-1 and PAI-2, were investigated.

3.1.1. Secreted PA activity.

To determine the effects of FGF-2, IL-1ß, and TGF-ß on the PA activity secreted by BM stromal cells, passaged BM stromal cells (3') were seeded into 24-well Linbro dishes and depleted of hydrocortisone FGF-2 (0.2 to 20 ng/ml), IL-1 (10 to 10^4 U/ml), or TGF-ß (0.2 to 20 ng/ml) were added to the cells for 24 hours in 1 ml of RPMI medium containing 10% FCS. Harvest fluids were prepared as described in the methods and PA activity and PA species were determined. PAI-1 and PAI-2 protein levels were determined by ELISA. PAs and PAI-1 were also visualised by zymography and reverse zymography, respectively.

PAs

BM stromal cells to which no growth factor had been added secreted little PA activity (mean of 0.27 ± 0.16 mU/10^5 cells/24 h, Table 1). The predominant PA secreted was identified immunochemically as being u-PA.

In Fig. 4*A*, untreated cells secreted 0.43 ± 0.0 mU/10^5 cells/24 h. Addition of FGF-2 (0.2 to 20 ng/ml), IL-1ß (10^2 to 10^4 U/ml), and TGF-ß (0.02 to 20 ng/ml) stimulated secretion PA activity considerably.

The most effective concentration of FGF-2 in stimulating PA activity was 0.2 ng/ml, which increased activity by 10-fold from 0.43 ± 0.0 to 4.3 ± 1.2 mU/10^5 cells/24 h ($p < 0.01$, Fig. 4*A*). Other concentrations of FGF-2 were less stimulatory and yielded a 2-fold (0.02 ng/ml, $p < 0.001$), 3-fold (20 ng/ml, $p < 0.05$), or 4-fold (2 ng/ml, $p < 0.001$) increase in PA activity. It was also noted that FGF-2 stimulated t-PA production significantly more than u-PA (Fig. 4*A*).

IL-1ß at 10^4 U/ml stimulated PA activity approximately 12-fold from 0.43 ± 0.0 to 5.4 ± 0.7 mU/10^5 cells/24 h ($p < 0.01$). Lower concentrations were less stimulatory with only a 7-fold increase from 0.43 ± 0.0 to approximately 3.1 ± 0.2 mU/10^5 cells/24 h ($p < 0.005$) with 10^2 and 10^3 U/ml IL-1ß. In contrast to FGF-2, IL-1ß primarily stimulated u-PA production (Fig. 4*A*).

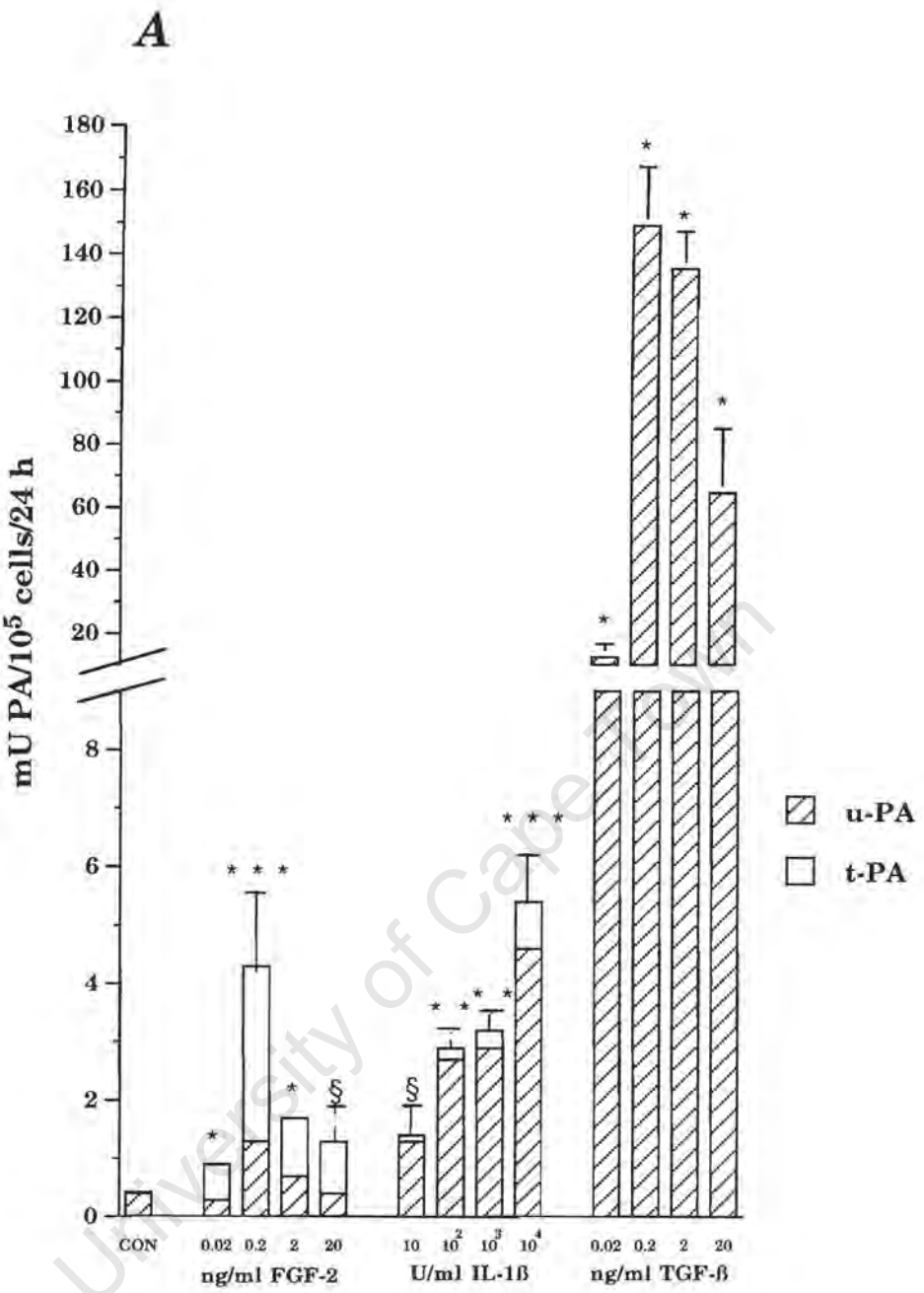


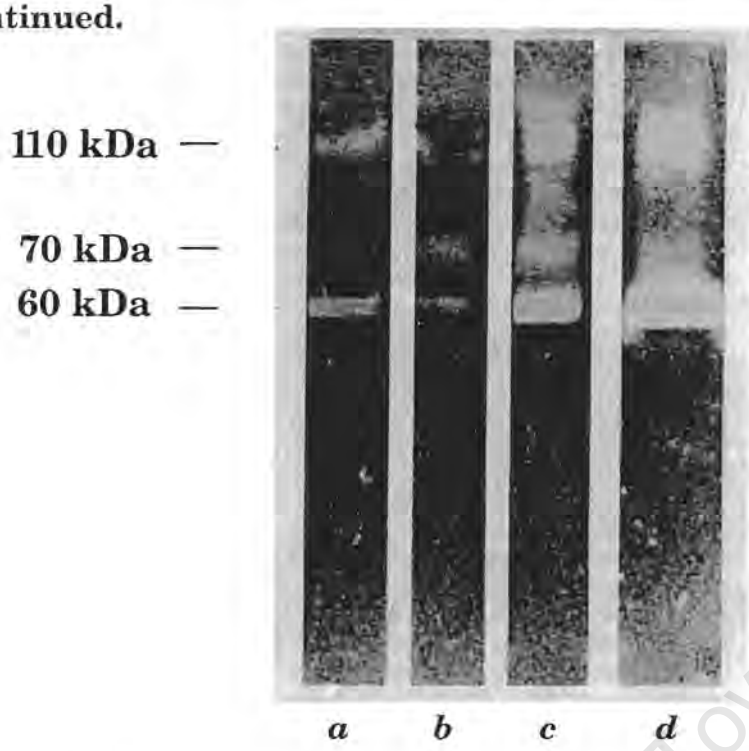
Fig. 4. Regultion of PA secretion by FGF-2, IL-1ß, and TGF-ß. Passaged BM stromal cells were seeded into 24-well Linbro plates at 2 x 10^4 cells/well in BM stromal medium. The medium was replaced with RPMI containing 10% FCS for four days with one medium change after two days to remove the hydrocortisone. FGF-2, IL-1ß, or TGF-ß were added to BM stromal cells for 24 hours at the indicated concentrations in RPMI medium containing 10% FCS. The cells were washed twice with RPMI medium to remove the FCS and then incubated for 24 hours in serum-free RPMI with the appropriate concentrations of growth factors.

A. The supernatants were assayed for PA activity using the ^{125}I-fibrin plate assay. Results are expressed as mU PA/10^5 cells/24 h ± SD from duplicate wells.

Similar results by these three growth factors were noted in a further nine experiments.

* $p < 0.001$, ** $p < 0.005$, *** $p < 0.01$, § $p < 0.05$, relative to the control.

Fig. 4 continued.



B. Zymography of PA activity secreted by control cells (lane *a*) and cells treated with 0.2 ng/ml FGF-2 (lane *b*), 10^4 U/ml IL-1ß (lane *c*), or 0.2 ng/ml TGF-ß (lane *d*). PA activity is visualised as bands of lysis in the opaque indicator gel.

The most marked stimulation of PA activity in BM stromal cell cultures occurred with TGF-ß, which caused up to 350-fold increase in secreted PA activity. The optimal stimulatory concentration was 0.2 ng/ml, which stimulated PA activity from 0.43 ± 0.0 to 149.6 ± 18.7 mU/10^5 cells/24 h ($p < 0.001$, Fig. 4*A*). Even 20 pg/ml TGF-ß still stimulated PA activity approximately 30-fold ($p < 0.001$, Fig. 4*A*). All concentrations exclusively stimulated u-PA production (Fig. 4*A*).

PA activity was also visualised as clear bands of lysis in fibrin-plasminogen agar indicator gels as illustrated in Fig. 4*B*. In supernatant from control cultures (lane *a*), two lysis bands were evident, one at 60 kDa and one at 110 kDa. Supernatant from stromal cells stimulated with 0.2 ng/ml of FGF-2 (lane *b*) generated three lysis bands: the first at 60 kDa, the second at 70 kDa, and the third at 110 kDa. Supernatant from stromal cells stimulated with 10^4 U/ml IL-1ß (lane *c*) produced four lysis bands: the first at 60 kDa, the second at 70 kDa, the third at 110 kDa, and the fourth at 130 kDa. Lane *d* contained supernatant from stromal cells stimulated with 0.2 ng/ml TGF-ß. Two major bands of lysis were noted at 60 kDa and 110 kDa. The band at 60 kDa represents u-PA, while the band at 110 kDa represents a complex between PAI-1 and u-PA (Pepper et al., 1991). The band at 70 kDa represents t-PA, while the band at 130 kDa represents the complex between t-PA and PAI-1 (Pepper et al., 1991). The

u-PA/PAI-1 and t-PA/PAI-1 complexes are normally enzymatically inactive but after SDS gel electrophoresis some of the complex dissociates such that in the gel a zone of PA activity is visable (Andreasen et al., 1990).

The results therefore show that FGF-2, IL-1ß, and TGF-ß stimulated the levels of secreted PA activity in the BM microenvironment with TGF-ß having the most significant effect and increasing exclusively u-PA production (Fig. 4*A*).

PAIs

BM stromal cells to which no growth factor had been added secreted high levels of PAI-1 (mean of 68.4 ± 37.6 ng/10^5 cells/24 h, Table 1), but relatively low levels of PAI-2 (mean of 3.9 ± 3.0 ng/10^5 cells/24 h, Table 1).

Addition of FGF-2 did not significantly affect PAI-1 levels (Fig. 5*A*) over a concentration range of 0.02 - 20 ng/ml ($p > 0.05$). Similarly, FGF-2 had no effect on PAI-2 secretion (data not shown).

IL-1ß (at concentrations between 10 and 10^4 U/ml) significantly decreased the net levels of PAI-1 compared to control cells by approximately 50% (from 111.3 ± 3.6 to between 40 and 60 ng/10^5 cells/24 h, $p < 0.01$, Fig. 5*A*). In Fig. 4B, a significant increase in the level of the u-PA-PAI-1 complex can be noted with this cytokine compared to control cultures. PAI-1 complexed to u-PA is, however, not measured by ELISA. It appears that the increase in PAI-1 by IL-1ß was smaller than the increase in PA activity and that most of the PAI-1 was complexed with u-PA. In contrast, IL-1ß (10^2 - 10^4 U/ml), stimulated PAI-2 levels by approximately 7.5-fold (from 0.8 ± 0.3 to between 6 and 10 ng/10^5 cells/24 h, $p < 0.05$, Fig. 5*C*).

TGF-ß (at concentrations between 0.02 and 20 ng/ml) consistently elevated PAI-1 secretion by approximately 2-fold compared to control cultures. This resulted in PAI-1 levels of approximately 227 ± 12 ng/10^5 cells/24 h ($p < 0.005$, Fig. 5*A*). In contrast, TGFß was found to have no effect on PAI-2 secretion (data not shown).

PAI-1 levels were also analysed by reverse zymography. Fig. 5*B* shows that the PAI-1 inhibitor band, corresponding to 44 kDa, was detectable under all assay conditions. It is evident that the inhibitor band was unaffected by FGF-2 (lane *b*), decreased by IL-1ß (lane *c*), and increased by TGF-ß (lane *d*) confirming above ELISA data.

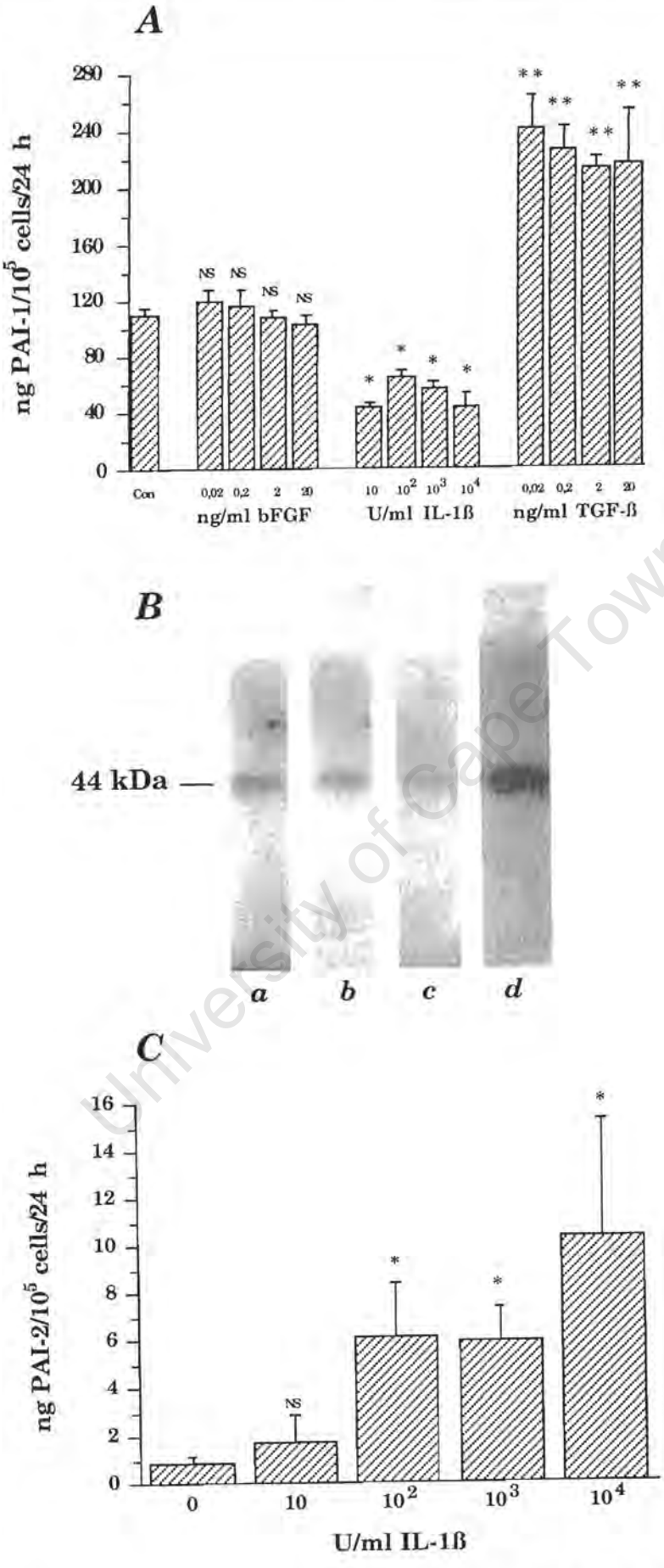


Fig. 5. Regulation of PAI-1 secretion by FGF-2, IL-1ß, and TGF-ß.

Fig. 5. Passaged BM stromal cells were seeded into 24-well Linbro plates at 2 x 10^4 cells/well in BM stromal medium. To remove the hydrocortisone, the medium was replaced with RPMI medium containing 10% FCS for 4 days with one medium change after 2 days. The cells were then treated with FGF-2, IL-1ß, or TGF-ß for 24 h at the indicated concentrations in RPMI medium containing 10% FCS. To prepare harvest fluids, the cells were washed twice with RPMI medium to remove the FCS and incubated for a further 24 hours in serum-free RPMI medium with the appropriate concentration of growth factor.

A. PAI-1 levels in the supernatants were determined by ELISA. Results are expressed as the mean ng PAI-1/10^5 cells/24 h ± SD from duplicate wells for each condition.
Similar results were noted in eight other experiments.
* $p < 0.01$ with respect to the control.
** $p < 0.005$ with respect to the control.
NS: not significant with respect to the control, $p > 0.05$.

B. Reverse zymography of PA inhibitors secreted by control cells (lane *a*) and cells treated with 0.2 ng/ml FGF-2 (lane *b*), 10^4 U/ml IL-1ß (lane *c*), or 0.2 ng/ml TGF-ß (lane *d*). PAI-1 was visualised as an opaque band in a completely lysed indicator gel. Similar results were noted in eight other experiments.

C. PAI-2 levels in the supernatant were determined by ELISA. Results are expressed as the mean ng PAI-2/10^5 cells/24 h ± SD from duplicate wells for each condition. Similar results were noted in a further six experiments.
* $p < 0.05$ with respect to the control.
NS: not significant with respect to the control, $p > 0.05$.

In summary, FGF-2 had little to no effect on PAI-1 or PAI-2 secretion in BM stromal cells, whereas IL-1ß inhibited PAI-1 levels and induced PAI-2, and TGF-ß stimulated PAI-1 levels but had no effect on PAI-2. However, the addition of IL-1ß and TGF-ß to BM stromal cells still favoured plasmin generation as there was an overall increase in PA activity. This is in contrast to what has been previously noted with TGF-ß in other cell systems where this growth factor stimulates PAI-1 production resulting in an overall suppression of PA activity and, therefore, plasmin generation (Saksela et al., 1987; Laiho et al., 1986a and b).

3.1.2. Cell-associated PA activity.

To determine the effect of FGF-2, IL-1ß and TGF-ß on the cell-associated (intracellular plus cell surface) levels of PA activity of BM stromal cells, passaged BM cells (3') seeded into 35-mm dishes were depleted of hydrocortisone as described in the methods. FGF-2 (0.2 to 20 ng/ml), IL-1 (10 to 10^5 U/ml), or TGF-ß (0.2 to 20 ng/ml) were added to the cells for 24 hours in 1 ml of RPMI medium containing 10% FCS. The cells were then washed twice with serum-free RPMI medium to remove the serum and incubated for a further 24 hours with 1 ml of serum-free RPMI medium containing the relevant concentration of growth factor. Cell lysates were prepared as described in the methods, and PA activity, PA species, and PAI-1 and PAI-2 protein levels determined.

PAs

PA activity in cell lysates of BM stromal cells cultured in the absence of growth factors was low (mean of 0.39 ± 0.28 mU/10^5 cells). Control cells contained 0.26 mU PA/10^5 cells (Fig. 6). Addition of FGF-2 (0.02 to 20 ng/ml) did not significantly stimulate cellular PA activity.

All concentrations of IL-1ß tested stimulated PA activity, the most effective being 10^2 U/ml which stimulated PA activity approximately 5-fold ($p < 0.005$, Fig. 6). In addition, it was noted that the stimulation in cellular PA activity by IL-1ß was lower than the stimulation in PA secretion by this growth factor (10^4 U/ml IL-1ß stimulated PA secretion by approximately 12-fold; see Fig. 4*A*).

The addition of TGF-ß to stromal cells also stimulated PA activity in cell lysates compared to control cells but not as dramatically as PA secretion. A maximum stimulation of 4.5-fold (from 0.26 ± 0.2 to 1.18 ± 0.35 mU/10^5 cells, $p = 0.005$) was obtained with 0.2 ng/ml TGF-ß (Fig. 6) compared to a 350-fold increase in PA secretion with the same concentration of this cytokine (Fig. 4*A*). TGF-ß at 20 ng/ml stimulated cell lysate PA activity by approximately 3.3-fold ($p < 0.02$), and no increase was noted with 20 pg/ml TGF-ß.

Using neutralising antibodies, the type of PA activity in the lysates of control cells and cells treated with FGF-2, IL-1ß, or TGF-ß was determined as being only u-PA. In contrast to secreted t-PA levels, no cell-associated t-PA was detected in FGF-2-treated BM stromal cultures. Since FGF-2 did not stimulate cellular PA levels, this may indicate that t-PA was effectively secreted into the supernatant, leaving cellular t-PA levels below the detection limit of the assay.

The results therefore show that FGF-2 had little to no effect on the levels of cell-associated PA activity. In contrast, IL-1ß or TGF-ß stimulated cellular PA activity in BM stromal cells, but the stimulation was significantly lower than the increase noted in PA secretion.

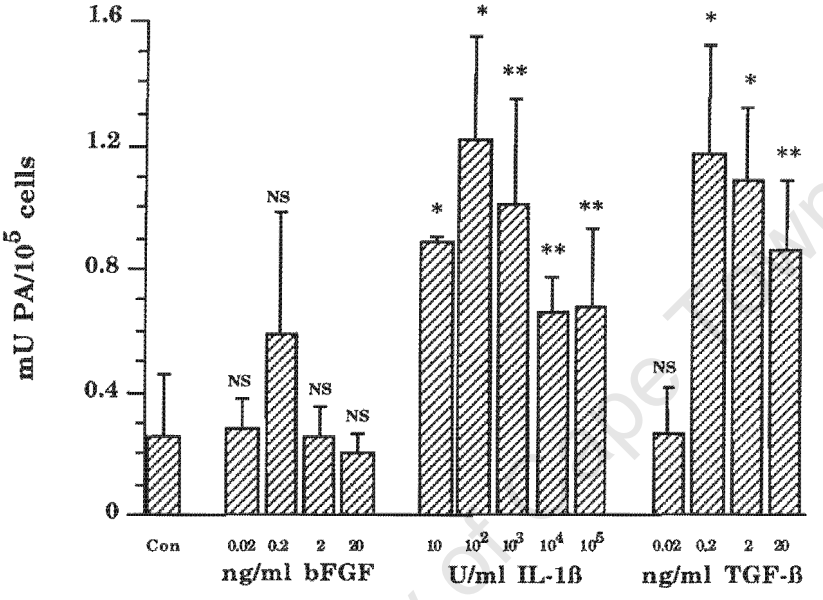


Fig. 6. Regulation of cell-associated PA activity by FGF-2, IL-1ß, and TGF-ß. Passaged BM cells were seeded into 35-mm dishes at 1 x 10^5 cells/dish in BM stromal medium. To remove the hydrocortisone, the medium was replaced with RPMI medium containing 10% FCS for four days with one medium change after two days. The stromal cells were then treated with FGF-2, IL-1ß, or TGF-ß at the concentrations indicated in 1 ml of RPMI medium containing 10% FCS. After 24 hours, the cells were incubated for a further 24 hours with 1 ml serum-free RPMI medium containing the relevant concentration of growth factor. Cell lysates were prepared in 0.5% Triton X-100. PA activity was determined using the ^{125}I-fibrin plate assay. Results are expressed as the mean mU PA/10^5 cells ± SD from duplicate wells. Similar results were noted in four other experiments.
* $p < 0.005$ with respect to the control.
** $p < 0.02$ with respect to the control.
NS: not significant with respect to the control, $p > 0.05$.

PAIs

The cellular basal levels for both PAI-1 and PAI-2 in human BM stromal cells were low (means of 0.76 ± 0.33 ng/10^5 cells for PAI-1 and 0.39 ± 0.14 ng/10^5 cells for PAI-2, Table 1).

In Fig. 7 the cell lysate of control cells contained 0.92 ng PAI-1 and 0.53 ng PAI-2/10^5 cells. In contrast to secreted PAI-1 levels, addition of FGF-2 at 0.2 and 2 ng/ml increased PAI-1 levels by approximately 2-fold to 1.9 ng/10^5 cells. FGF-2, at all concentrations used, did not regulate PAI-2 levels (data not shown).

Addition of IL-1ß (10 to 10^5 U/ml) to BM stromal cells had little to no effect on cellular PAI-1 levels (Fig. 7*A*). This effect was in contrast to that found in the supernatant where similar concentrations of IL-1ß decreased PAI-1 secretion by approximately 50% (Fig. 5*A*). Furthermore, all concentrations of IL-1ß increased cellular PAI-2 levels between 10- and 16-fold (Fig. 7*B*).

TGF-ß (0.02 to 20 ng/ml) increased the cellular PAI-1 levels by approximately 2-fold (Fig. 7*A*) as was found for the secreted PAI-1 levels. Similar to FGF-2, TGF-ß (0.02 to 20 ng/ml) did not regulate cellular PAI-2 levels in BM stromal cells, which is consistent with the effects of these two growth factors on PAI-2 secretion (data not shown).

The results therefore show that low concentrations of both FGF-2 and TGF-ß stimulated cell-associated PAI-1 levels, whereas IL-1ß increased cellular PAI-2 levels.

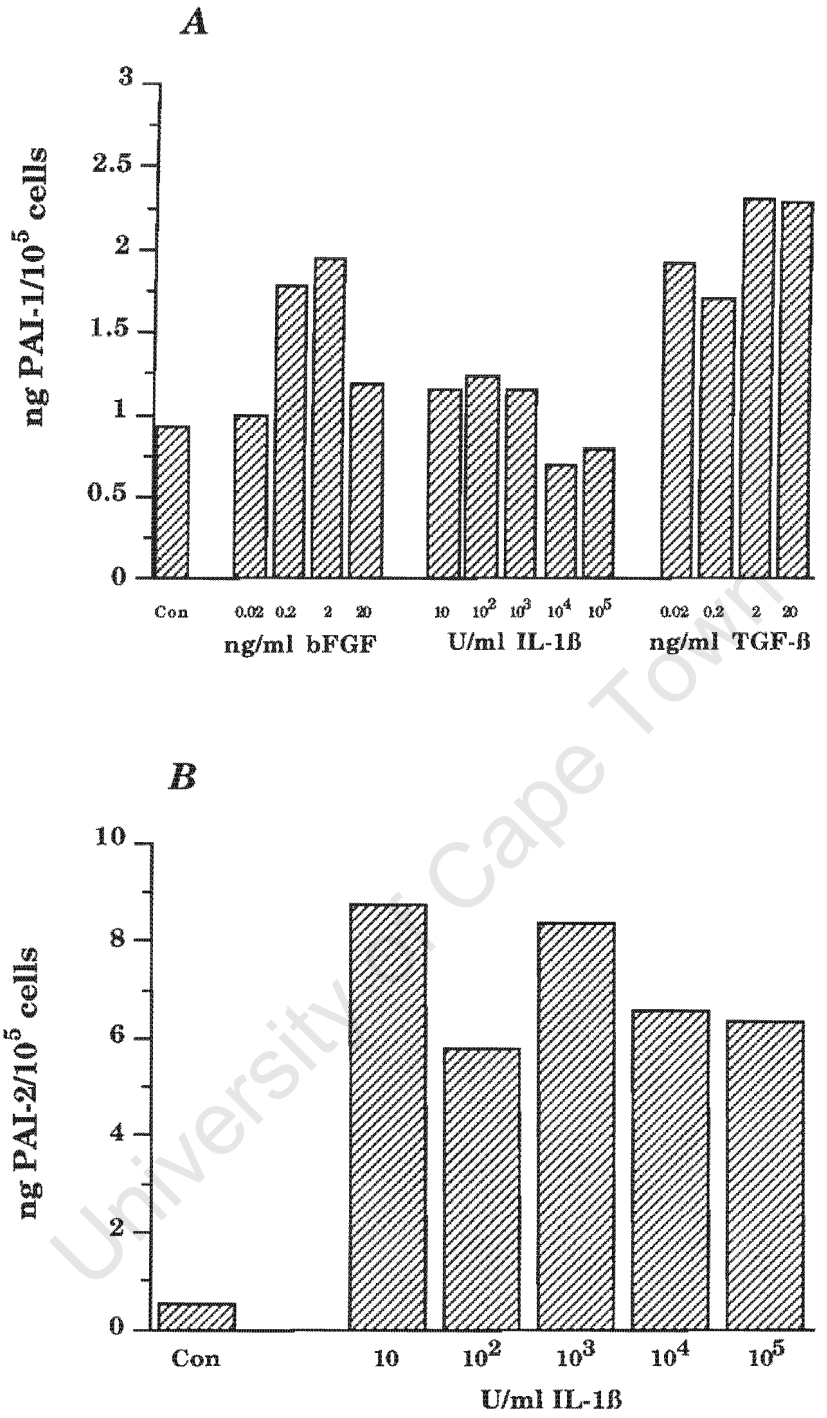


Fig. 7. Regulation of cellular PAI-1 levels by FGF-2, IL-1ß, and TGF-ß. Passaged BM cells were seeded into 35-mm dishes at 1 x 10^5 cells/dish in BM stromal medium. The medium was replaced with RPMI medium containing 10% FCS for four days with one medium change after two days to remove the hydrocortisone. FGF-2, IL-1ß, or TGF-ß were added to the cells at the concentrations indicated for 24 hours in 1 ml of RPMI containing 10% FCS. The cells were then incubated with 1 ml serum-free RPMI medium containing the relevant concentration of growth factor for a further 24 hours. Cell lysates were prepared in 0.5% Triton X-100. PAI-1 (***A***) and PAI-2 (***B***) protein levels were determined by ELISA. Results are expressed as ng PAI-1 or PAI-2/10^5 cells from one well. Similar results for PAI-1 were noted in five experiments and for PAI-2 in three experiments.

These experiments demonstrated that FGF-2, IL-1ß, and TGF-ß differentially modulated production and secretion of PAs and their inhibitors, PAI-1 and PAI-2, by BM stromal cells. It was noted that FGF-2 stimulated PA secretion by inducing mainly t-PA levels but had no effect on cell-associated PA activity. Furthermore, it was observed that FGF-2 increased only cellular PAI-1 levels.

TGF-ß was the most significant inducer of both secreted and cell-associated PA, increasing only u-PA and with the increase in secretion being more profound. It was further noted that TGF-ß increased both PAI-1 production and secretion. Nevertheless, u-PA activity was still permissive.

IL-1ß also increased both cellular and secreted PA activity, increasing primarily u-PA, but to a lesser extent than TGF-ß. Interestingly, it was noted that only IL-1ß increased PAI-2 levels, although plasmin generation was still favoured as there was an overall increase in u-PA activity.

Thus, FGF-2, TGF-ß and IL-1ß differentially regulated the PA/plasmin system in BM stromal cells such that the modulation in PA activity by these growth factors varied according to the type of PA/PAI modulated, compartment in which the regulation took place, and to the extent of the regulation.

3.2. *TGF-ß regulation of PA activity as a function of time.*

The previous experiments indicated that TGF-ß was a potent stimulator of BM stromal cell PA activity (up to 350-fold in secretion and 4.5-fold in cellular levels) as well as PAI-1 levels. Therefore, TGF-ß has the capacity for regulating its own activation and its own activity. Increasing u-PA activity would increase local plasmin generation and convert latent to active TGF-ß (Lyons et al., 1988). Active TGF-ß would also induce PAI-1, which by inhibiting u-PA activity would diminish the conversion of plasminogen to plasmin. Therefore, TGF-ß via its stimulation of both u-PA and PAI-1 in a possible sequential time frame could regulate its own activity. In addition, plasmin can release latent TGF-ß from the ECM (Taipale et al., 1992; Falcone et al., 1993a) therefore providing an additional mechanism for this growth factor regulating its own activity. I therefore decided to determine the increase in PA activity and PAI-1 levels as a function of time.

Passaged BM stromal cells (3') seeded into 24-well Linbro plates were depleted of hydrocortisone as described in the methods. Harvest fluids were prepared with 0.2 ng/ml TGF-ß in 1 ml RPMI medium as described in the methods. (This concentration of TGF-ß was chosen as it was the most effective in stimulating PA secretion, see Fig. 4A). Supernatants were collected from duplicate wells after 8, 12, 24, 36, and 48 hours.

To determine cellular PA levels, the cells were treated with 0.2 ng/ml TGF-ß in 1 ml of RPMI medium containing 10% FCS. Cell lysates were prepared from duplicate wells after 6, 12, 18, 24, and 48 hours as described in the methods. The supernatants and cell lysates were assayed for PA activity using the ^{125}I-fibrin plate assay and PAI-1 protein levels by ELISA. PAI-1 activity was also visualised by reverse zymography.

Figs. 8*A* and *B* shows that both secreted and cell-associated PA activity in BM stromal cells were increased by 7- and 8-fold, respectively, following incubation with TGF-ß for 12 hours (from 0.21 ± 0.1 to 1.48 ± 0.88 mU/10^5 cells/12 h for secreted PA [$p < 0.05$], and from 50 ± 0.0 to 400 ± 44 mU/mg protein for cellular PA [$p < 0.01$]).

Increases in PAI-1 production in BM stromal cells treated with TGF-ß were noted at an earlier time point, namely 8 hours, as shown in Fig. 8*C*. Here, TGF-ß induced PAI-1 secretion from BM stromal cells by approximately 10-fold from 4.8 ± 8.4 to 55 ± 13 ng PAI-1/10^5 cells ($p < 0.05$). After 12 hours, however, PAI-1 levels secreted in the presence of TGF-ß were only approximately 2-fold higher compared to control cells (Fig. 8*C*).

PAI-1 activity in the supernatants was also visualised by reverse zymography. PAI-1 activity was evident as an opaque band of 46 kDa in the completely lysed indicator gel as illustrated in Fig. 8*D*. This figure confirms that an increase in PAI-1 levels by TGF-ß was observed after 8 hours.

These results suggest that TGF-ß stimulated BM stromal cells to produce PAI-1 and u-PA in a time-dependent manner. However, since RNA levels for u-PA and PAI-I were not determined in this experiment, I do not know whether the increase in u-PA activity by TGF-ß was due to an increase in u-PA production or rather the release of u-PA from ECM storage sites or possible intracellular storage sites (the latter has not been demonstrated for u-PA as it has been for t-PA by Pepper et al. (2001)). PAI-1 is a major target gene for TGF-ß activity and is induced early which might explain the presence of PAI-1 in the supernatant at an earlier time than u-PA. Although TGF-ß stimulates PAI-1 production by BM stromal cells, which initially might inhibit u-PA activity, plasmin generation is still favoured by the significant increases in u-PA activity by this growth factor.

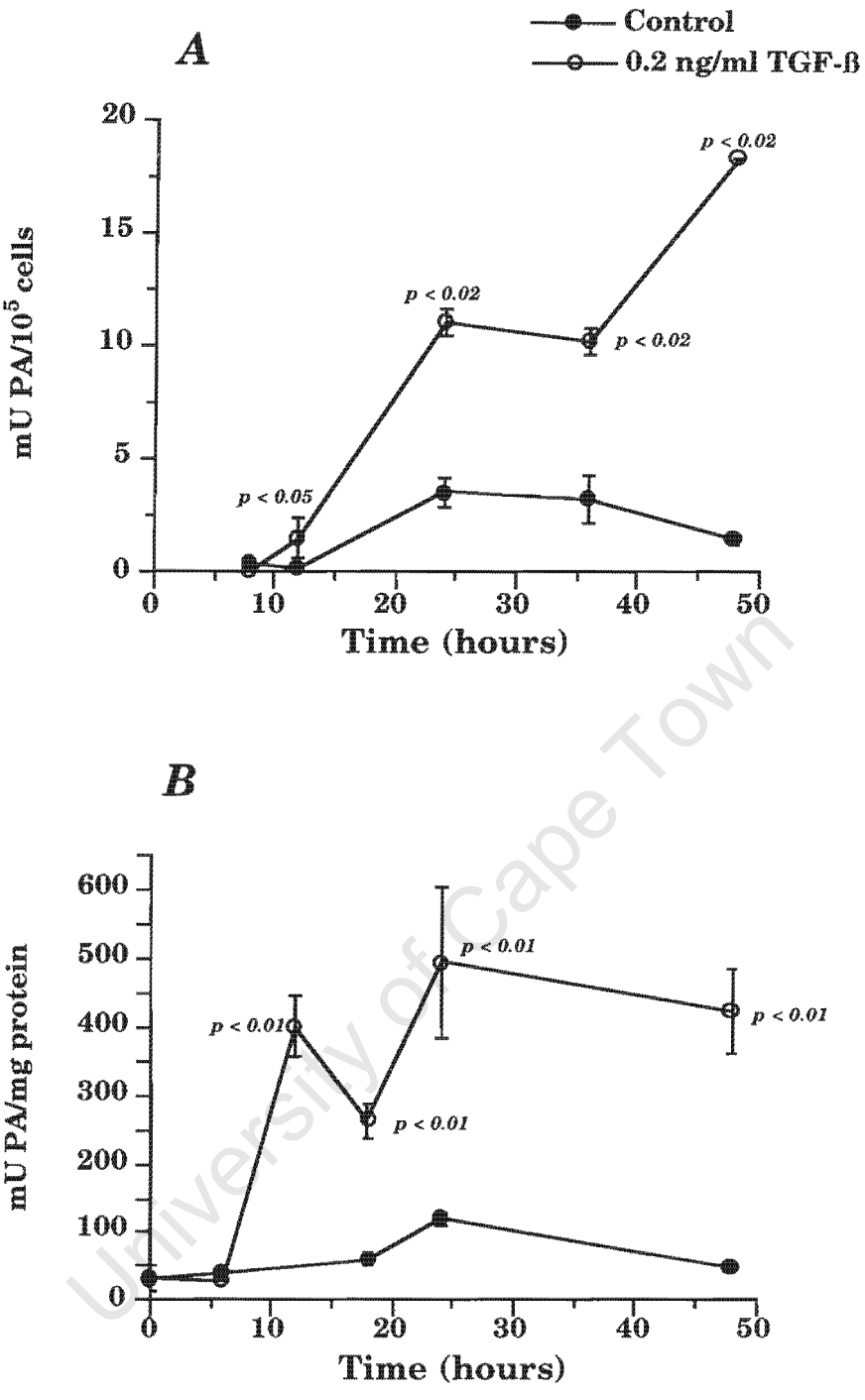
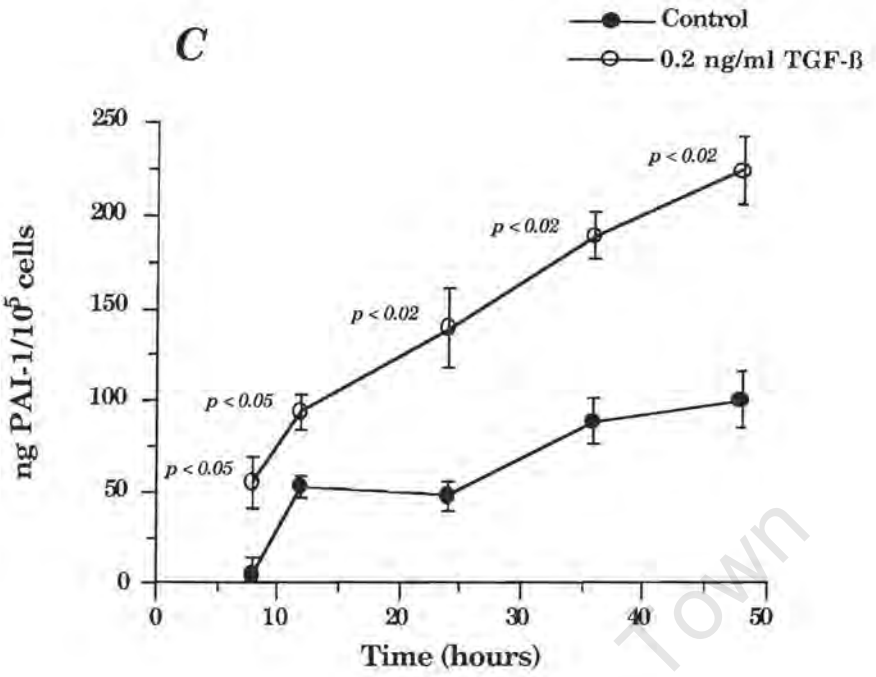


Fig. 8. The effect of TGF-ß on PA activity as a function of time. Passaged BM stromal cells were seeded into 24-well Linbro plates at 2.5 x 10^4 cells/well in BM stromal medium. The medium was replaced with RPMI medium containing 10% FCS for four days with one medium change after two days to remove the hydrocortisone.

A. BM stromal cells were treated with 0.2 ng/ml TGF-ß in 1 ml of serum-free RPMI medium. Supernatants were collected after 8, 12, 24, 36, and 48 hours and were assayed for secreted PA activity using the ^{125}I-fibrin plate assay. Results are expressed as the mean mU PA/10^5 cells ± SD from duplicate wells.
p values were determined with respect to the control value at each time point.

B. The cells were treated with 0.2 ng/ml TGF-ß in 1 ml of RPMI medium containing 10% FCS. Cell lysates were prepared from duplicate wells after 6, 12, 18, 24, and 48 hours in 0.5% Triton X-100 and assayed for intracellular PA activity using the ^{125}I-fibrin plate assay. Results are expressed as the mean mU PA/mg protein ± SD from duplicate wells.
p values were determined with respect to the control value at each time point.



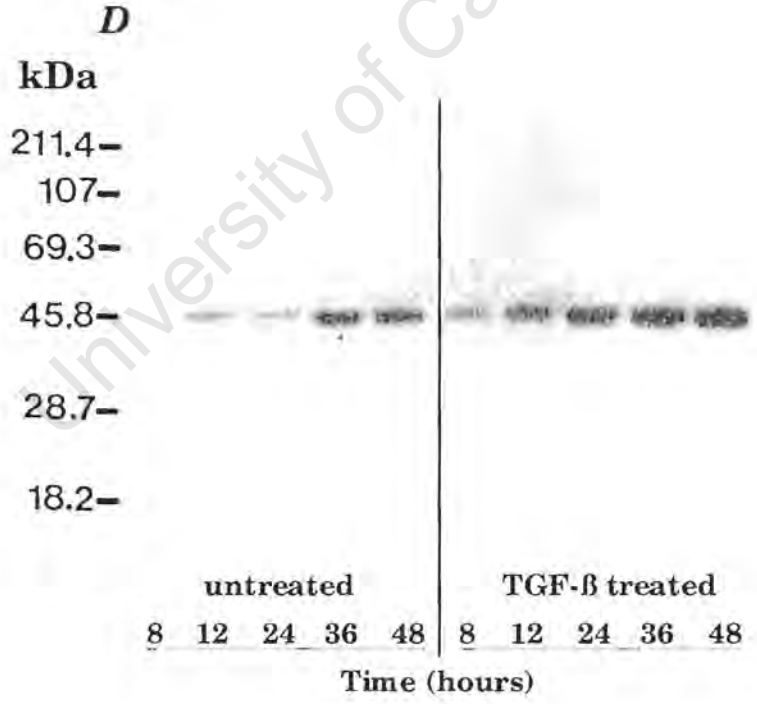


Fig. 8 continued.
C. PAI-1 levels in the supernatants of BM stromal cells used in ***A*** were determined by ELISA. The results are expressed as the mean ng PAI-1/10^5 cells ± SD from duplicate wells. *p* values were determined with respect to control values.

D. PAI-1 activity in the supernatants from *A* were visualised by reverse zymography as opaque bands in a lysed indicator gel.

3.3. *Regulation of PA activity by TGF-ß and PDGF.*

The finding (see Table 2) that TGF-ß is a potent stimulator of BM stromal cell PA activity when these cells are pretreated with this growth factor in the presence of FCS (before harvest fluids were prepared with this growth factor in the absence of FCS), suggests that FCS sensitises the cells for the stimulation of PA activity by TGF-ß. Since serum is a rich source of PDGF (Gronthos and Simmons, 1995), I hypothesised that PDGF might be involved in this process to induce the production of BM stromal cell PA activity by TGF-ß. In contrast, the increased levels of PAI-1 production by TGF-ß is independent of FCS sensitisation since PAI-1 levels were not

Table 2. PA activity and PAI-1 levels in the supernatant of BM stromal cells treated with TGF-ß in the absence or presence of FCS.

Experimental Conditions	PA activity ($mU/10^5$ cells/24 h)	PAI-1 levels ($ng/10^5$ cells/24 h)
24 h + FCS; 24 h – FCS[a]	117 ± 16	132 ± 13
24 h – FCS; 24 h – FCS[b]	4.3 ± 2.0 ($p < 0.01$)	120 ± 13 (NS)
24 h – FCS[c]	12.3 ± 2.5 ($p < 0.01$)	131 ± 19 (NS)
48 h – FCS[d]	9.3 ± 1 ($p < 0.01$)	113 ± 8 (NS)

Passaged BM stromal cells (4') were seeded into 24-well Linbro plates at 2.5 x 10^4 cells/well in BM stromal medium. The medium was replaced with RPMI medium containing 10% FCS for four days with one medium change after two days to remove the hydrocortisone. The cells were then treated with TGF-ß in the absence or presence of FCS for different time periods as described below. Supernatants were then collected and assayed for PA activity using the ^{125}I-fibrin plate assay. Results are expressed as the mean mU PA/10^5 cells/24 h ± SD from duplicate wells for each condition.

a: BM stromal cells were pretreated with 0.2 ng/ml TGF-ß in the presence of FCS for 24 hours. Harvest fluids were then prepared by treating the cells with this growth factor in the absence of FCS for 24 hours.

b: BM stromal cells were pretreated with 0.2 ng/ml TGF-ß in the absence of FCS for 24 hours. Harvest fluids were then prepared by treating the cells with this growth factor in the absence of FCS for 24 hours.

c: Harvest fluids were prepared from BM stromal cells treated with 0.2 ng/ml TGF-ß in the absence of FCS for 24 hours (i.e. there was no pretreatment of the cells with TGF-ß in the presence of FCS).

d: Harvest fluids were prepared from BM stromal cells treated with 0.2 ng/ml TGF-ß in the absence of FCS for 48 hours (i.e. there was no pretreatment of the cells with TGF-ß in the presence of FCS).

p values determined with respect to *a*.

NS: not significant compared to PAI-1 levels in *a*, $p > 0.05$.

affected by the pretreatment of the BM stromal cells with TGF-ß in the presence of FCS (Table 2).

To further investigate this hypothesis, passaged BM stromal cells at 3' (seeded into 24-well Linbro plates and washed free of hydrocortisone as described in the methods) were pretreated with RPMI medium containing either 0.2 ng/ml TGF-ß, 1 ng/ml PDGF, or 0.2 ng/ml TGF-ß and 1 ng/ml PDGF, in the absence or presence of 10% FCS for 24 hours. Harvest fluids were then prepared as previously described and assayed for PA activity and PAI-1.

PAs

BM stromal cells to which no growth factors had been added secreted low amounts of PA activity, both in the presence of FCS (0.49 ± 0.0 mU PA/10^5 cells/24 h, Fig. 9*A*) and in the absence of FCS (1.54 ± 0.44 mU PA/10^5 cells/24 h, Fig. 9*B*). PDGF alone did not significantly stimulate PA production by BM stromal cells in the presence (Fig. 9*A*) or absence (Fig. 9*B*) of FCS. As previously observed, the stimulation in PA activity by TGF-ß was significant when BM stromal cells were preincubated with this growth factor in the presence of serum (approximately 100-fold, $p < 0.001$, Fig. 9*A*). In contrast, BM stromal cells pretreated with TGF-ß in the absence of serum exhibited a smaller increase in PA activity (approximately 6-fold, $p < 0.01$, Fig. 9*B*).

PDGF further enhanced TGF-ß-mediated stimulation of PA activity levels by approximately 4-fold, resulting in an overall increase of 428-fold above control cells ($p < 0.001$, Fig. 9*A*). PDGF also enhanced the TGF-ß-mediated PA activity in BM stromal cells preincubated with these growth factors in the absence of serum by approximately 45-fold above control cells ($p < 0.001$, Fig. 9*B*). Although this increase in PA activity was significantly higher than that noted for TGF-ß alone (pretreated in the absence of FCS), the data suggest that other growth factors or components in FCS apart from PDGF may increase the sensitivity of the BM stromal cells to TGF-ß. It was further noted that increasing the concentration of PDGF to 5 ng/ml did not result in any additional increases in PA activity (data not shown).

PAI-1

Since the stimulation in BM stromal cell PAI-1 levels by TGF-ß was not dependent on FCS (Table 2), PAI-1 levels were only determined in the supernatants of BM stromal cells that had been preincubated with growth factors in the presence of FCS. Fig. 9*C* shows that BM stromal cells to which no growth factors had been added secreted small amounts of PAI-1 (5.3 ng/10^5 cells/24 h). The addition of PDGF or TGF-

ß to BM stromal cells stimulated the production of PAI-1 by approximately 3.5-fold and 9-fold, respectively. Furthermore it was noted that TGF-ß and PDGF had additive effects and stimulated PAI-1 levels by approximately 20-fold above control cells (Fig. 9*C*).

The results show that PDGF sensitises stromal cells for TGF-ß induction of PA. PDGF might increase the number of TGF-ß receptors or co-receptors (betaglycan or endoglin) on stromal cell surfaces such that the effect of TGF-ß on PA induction is enhanced. Furthermore, PDGF and TGF-ß have additive effects on PAI-1 induction. However, the results also suggest that other growth factors and/or components in FCS contributed to the enhancement of TGF-ß-mediated PA induction.

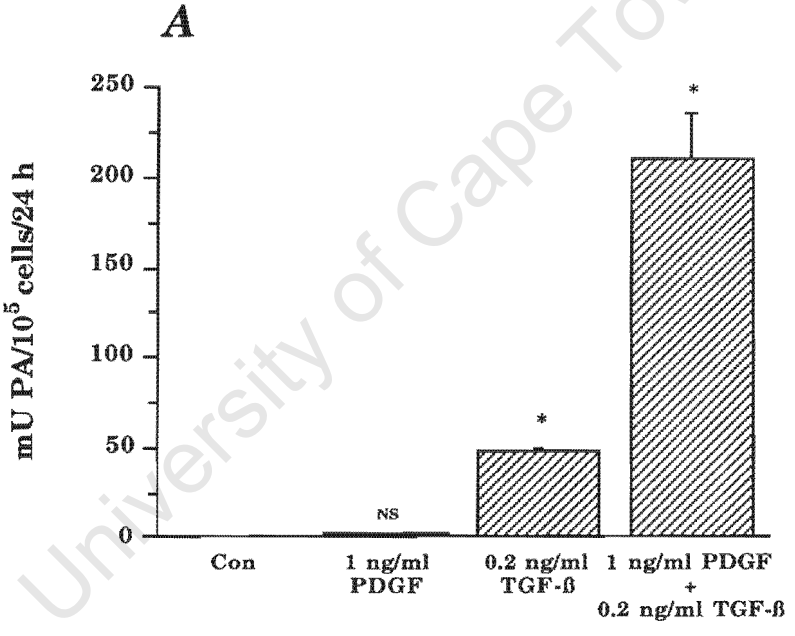


Fig. 9. Regulation of PA secretion and PAI-1 levels in BM stromal cells by TGF-ß and PDGF. Passaged BM stromal cells were seeded into 24-well Linbro plates at 2.5 x 10^4 cells/well in BM stromal medium. The medium was replaced with RPMI containing 10% FCS for four days with one medium change after two days to remove the hydrocortisone. The cells were treated with 0.2 ng/ml TGF-ß, 1 ng/ml PDGF, or 0.2 ng/ml TGF-ß and 1 ng/ml PDGF, in the presence (*A* and *C*) or absence (*B*) of FCS for 24 hours. Supernatants were collected after the cells were incubated with serum-free RPMI medium containing the relevant growth factors for a further 24 hours. PA activity was determined using the ^{125}I-fibrin plate assay, and the results are expressed as the mean mU PA/10^5 cells/24 h ± SD from duplicate wells (*A* and *B*). PAI-1 protein levels were determined by ELISA and the results are expressed as the mean ng PAI-1/10^5 cells/24 h ± SD from duplicate wells (*C*).
Similar results were noted in a second experiment.
* $p < 0.001$, with respect to control.
NS: not significant with respect to the control.

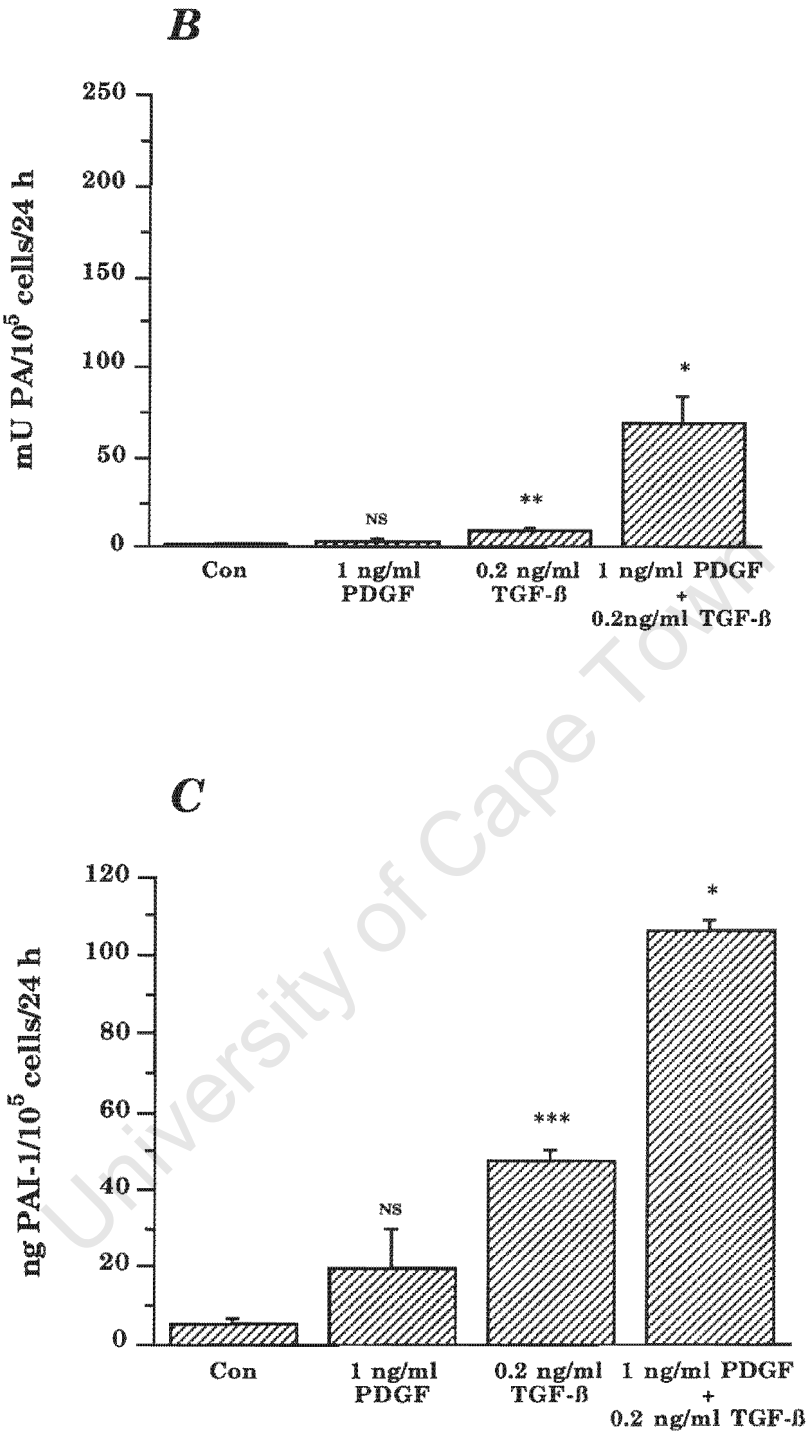


Fig. 9 continued.
* $p < 0.001$, * *$p < 0.01$, * **$p < 0.005$, with respect to control.
NS: not significant with respect to the control.

4. The regulation of t-PA activity by PAI-1 in BM stromal cells.

Of the two specific inhibitors of PA activity, PAI-1 is the most effective inhibitor of t-PA and thus is important for the regulation of t-PA activity (Sprenger and Kluft, 1987; Saksela and Rifkin, 1988). Furthermore, PAI-1 functions in the clearance of t-PA by the liver which is the main organ responsible for removing t-PA from the circulation. Clearance of t-PA by the LRP/α_2-M receptor is dependent on t-PA forming a complex with PAI-1 (Morton et al., 1989; Orth et al., 1992), although the uptake of free t-PA can also be mediated by the LRP/α_2-M receptor (Camani et al., 1994; Orth et al., 1994). Since little is known about the PA system in the BM stromal cell microenvironment and since t-PA induction is potentially instrumental in mediating the stimulatory effects of FGF-2 on haematopoiesis, I decided to further characterise the regulation of t-PA activity by PAI-1. To do this I investigated the inhibition of t-PA activity by BM stromal cell produced PAI-1 as well as the clearance of t-PA from the supernatant by BM stromal cells. The lowering of t-PA activity through inhibition by PAI-1 and/or removal of t-PA from the pericellular environment would effectively decrease plasmin generation in the BM. Diminished levels of plasmin could have both a permissive and an inhibitory effect on haematopoiesis depending on the local environment in which the plasmin level is lowered.

4.1. *PAI-1 inhibits t-PA activity in the stromal cell microenvironment.*

BM stromal cells secrete relatively high levels of PAI-1 (68.4 ± 37.6 mU/10^5 cells/24 h, see Table 1) into the culture medium. In order to determine how effectively endogenous BM stromal cell PAI-1 inhibits t-PA activity, I added t-PA to passaged BM stromal cells in the presence of anti-PAI-1 antibodies and measured the levels of t-PA activity in the supernatant over 24 hours.

The results show (Fig. 10) that BM stromal cells, to which no pro-t-PA and antibody had been added, secreted little PA activity (0.04 U PA/dish). Furthermore, dishes that contained no BM stromal cells but to which pro-t-PA had been added contained approximately 7 U PA /dish throughout the 24 hour experimental period, indicating no significant loss of activity. However, PA activity in the supernatant of BM stromal cells, to which pro-t-PA had been added, decreased in a time-dependent manner (Fig. 10). It was noted that the $^1/_2$-life for pro-t-PA (i.e. the time point at which 3.5 U PA activity remained detectable), was approximately 4.5 hours ($p < 0.001$). After 8 hours the activity had reached near baseline levels (0.4 U/dish).

The addition of anti-PAI-1 antibodies to the culture medium effectively slowed down the loss of t-PA activity in the supernatant of BM stromal cells. In these cultures

the $^1/_2$-life for pro-t-PA was extended almost 3-fold to approximately 12 hours ($p < 0.001$, Fig. 10). This inhibition was further enhanced by the repeated addition of anti-PAI-1 antibodies, such that the $^1/_2$-life for pro-t-PA became was approximately 15 hours ($p < 0.001$). Therefore, endogenous BM stromal cell PAI-1 effectively inhibits t-PA activity.

This effect was not observed when irrelevant IgG was used in place of specific antibody. In cultures containing irrelevant IgG, the $^1/_2$-life for pro-t-PA was approximately 4 hours ($p < 0.001$, Fig. 10). Nevertheless, after 24 hours, BM cultures

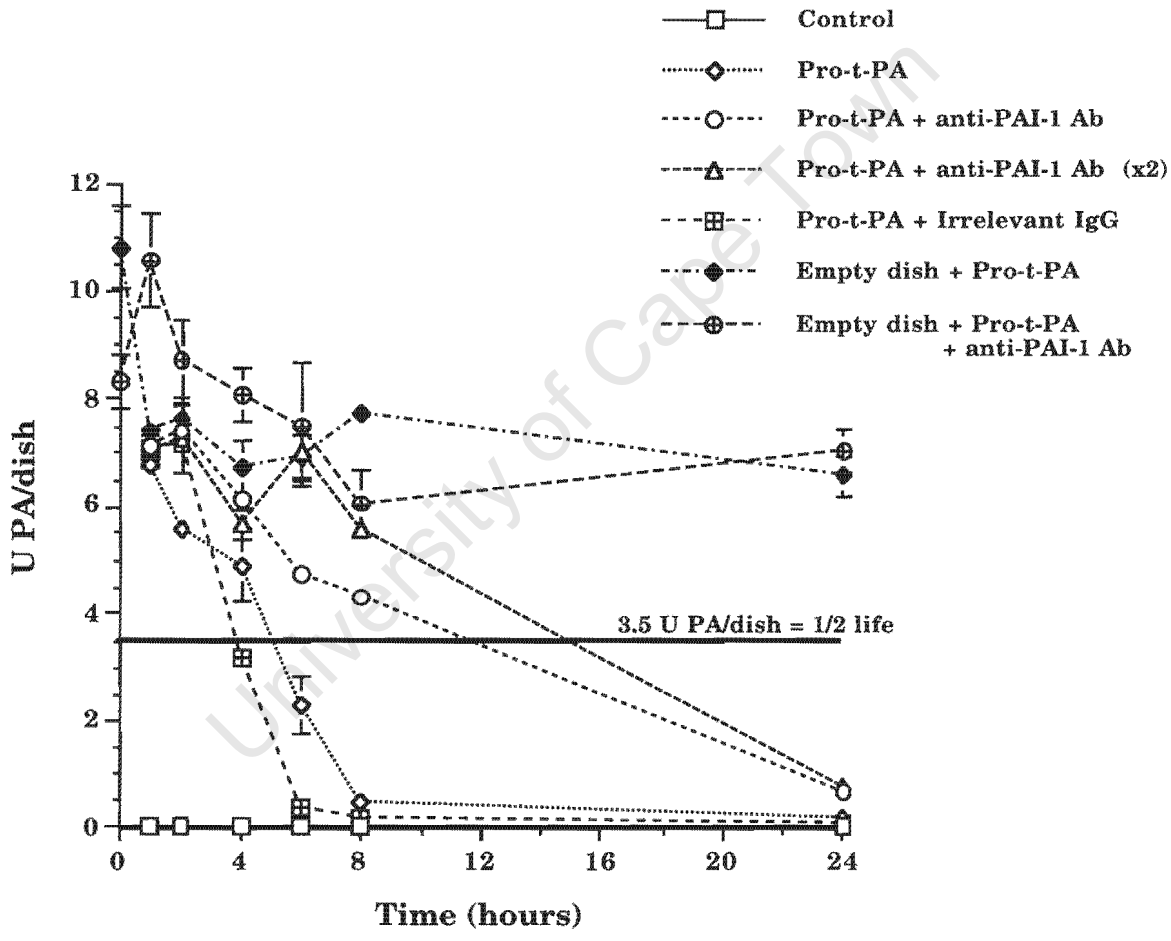


Fig. 10. Regulation of t-PA activity by PAI-1. Passaged BM stromal cells, seeded in 35-mm culture dishes at 5 x 10^4 cells/dish, were incubated at 37°C with or without rabbit anti-PAI-1 antibodies or irrelevant rabbit antibodies. After 10 min, pro-t-PA was added to each culture. To control for non-specific binding of pro-t-PA to the plastic, pro-t-PA was also added to culture dishes that contained no cells but which had been treated with RPMI medium containing 10% FCS. At time intervals of 1, 2, 4, 6, 8, and 24 hours, 100 µl aliquots of supernatant were removed and assayed for residual PA activity using the ^{125}I-fibrin plate assay. After four hours of culture, one set of cultures (-- Δ --) received a second aliquot of rabbit anti-PAI-1 antibodies. Results are expressed as the mean U t-PA/dish ± SD from triplicate dishes. Similar results were noted in a second experiment.

to which anti-PAI-1 antibodies had been added also contained near baseline levels of PA activity (0.9 U PA/dish). Thus, after 24 hours the BM stromal cells in these cultures had produced sufficient PAI-1 to bind all the anti-PAI-1 antibodies present in the supernatant as well as to inhibit the entire PA activity.

These results therefore indicate that PAI-1 produced by BM stromal cells is an effective inhibitor of t-PA activity in the BM stromal cell microenvironment.

4.2. *The role of PAI-1 in the clearance of t-PA from the stromal cell microenvironment.*

In order to determine the fate of t-PA and whether the complexing of t-PA to PAI-1 is necessary for its clearance from the BM stromal cell microenvironment, passaged BM stromal cells were treated with ^{125}I-t-PA and the amount of radioactivity and PAI-1 antigen present in the supernatant, cell lysate, and matrix was determined over 24 hours. Proteins in the supernatant were also precipitated using TCA in order to determine the total amount of TCA precipitable ^{125}I-t-PA in each sample. The radioactivity in each sample was determined using a gamma counter.

The results show that ^{125}I-t-PA, which has a molecular weight of approximately 70 kDa, was completely removed from the BM stromal cell supernatant during the first 8 hours of incubation (Figs. 11*A* and *D*). The results also indicate that, within 15 minutes of incubation, a protein band with a molecular weight of approximately 130 kDa appeared in the supernatant. This complex, which corresponds to ^{125}I-t-PA bound to PAI-1, reached maximum accumulation after 6 hours and was then slowly removed from the supernatant such that by 24 hours only traces of the complex remained (Figs. 11*A* and *D*).

The ^{125}I-t-PA/PAI-1 complex (130 kDa) appeared in the extracellular matrix extract after 15 minutes of incubation with maximum accumulation by 6 hours (Fig. 11*B*). The complex was also rapidly turned over such that by 24 hours no remaining label was detected (Fig. 11*B*). However, there was some accumulation of free ^{125}I-t-PA (70 kDa) in the matrix with a peak at 4 hours indicating that some t-PA was associated with the ECM.

As indicated in Fig. 11*C*, the ^{125}I-t-PA/PAI-1 complex (130 kDa) appeared in the cell lysate at zero time. This suggests that the complex was rapidly internalised by the BM stromal cells. The complex reached maximum accumulation in the cell lysate between 2 and 4 hours but was completely degraded by 24 hours. No free ^{125}I-t-PA was detected in the lysate.

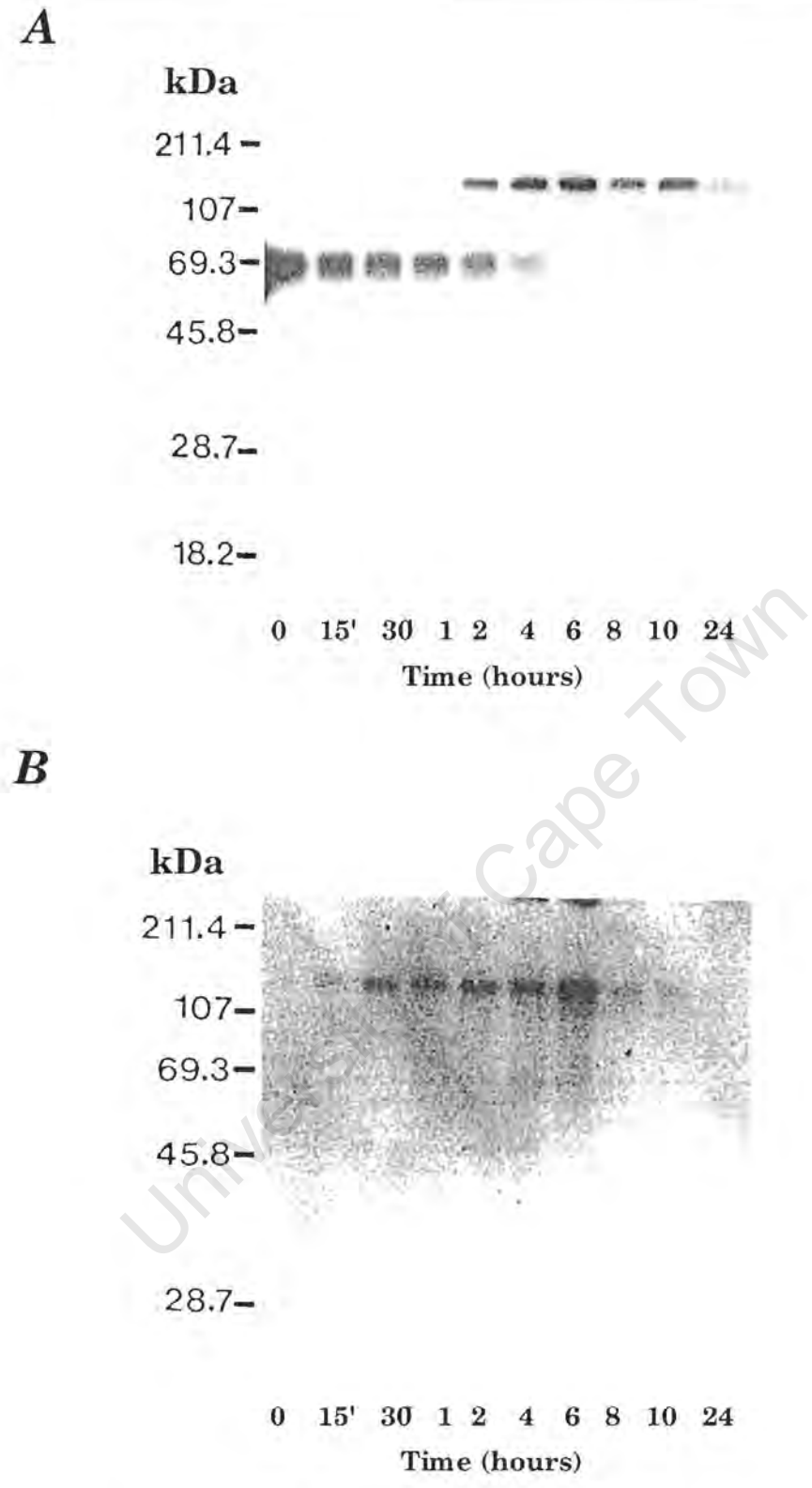


Fig. 11. Regulation of t-PA binding to PAI-1. Passaged BM stromal cells, seeded into 24-well Linbro plates at 2.5 x 10^4 cells/well, were treated with 10^5cpm of ^{125}I-t-PA (equivalent to 10 ng t-PA). At time intervals, 15 min, 30 min, 1, 2, 4, 6, 8, 10, and 24 hours, supernatants, cell lysates, and matrix extracts were collected and aliquots subjected to 11% SDS-PAGE and autoradiography to identify ^{125}I-t-PA. Free, uncomplexed ^{125}I-t-PA has an apparent molecular weight of 70 kDa, and the ^{125}I-t-PA/PAI-1 complex is approximately 130 kDa.
(***A***) a 24-hour exposure of supernatants.
(***B***) a 15-day exposure of matrix extracts.

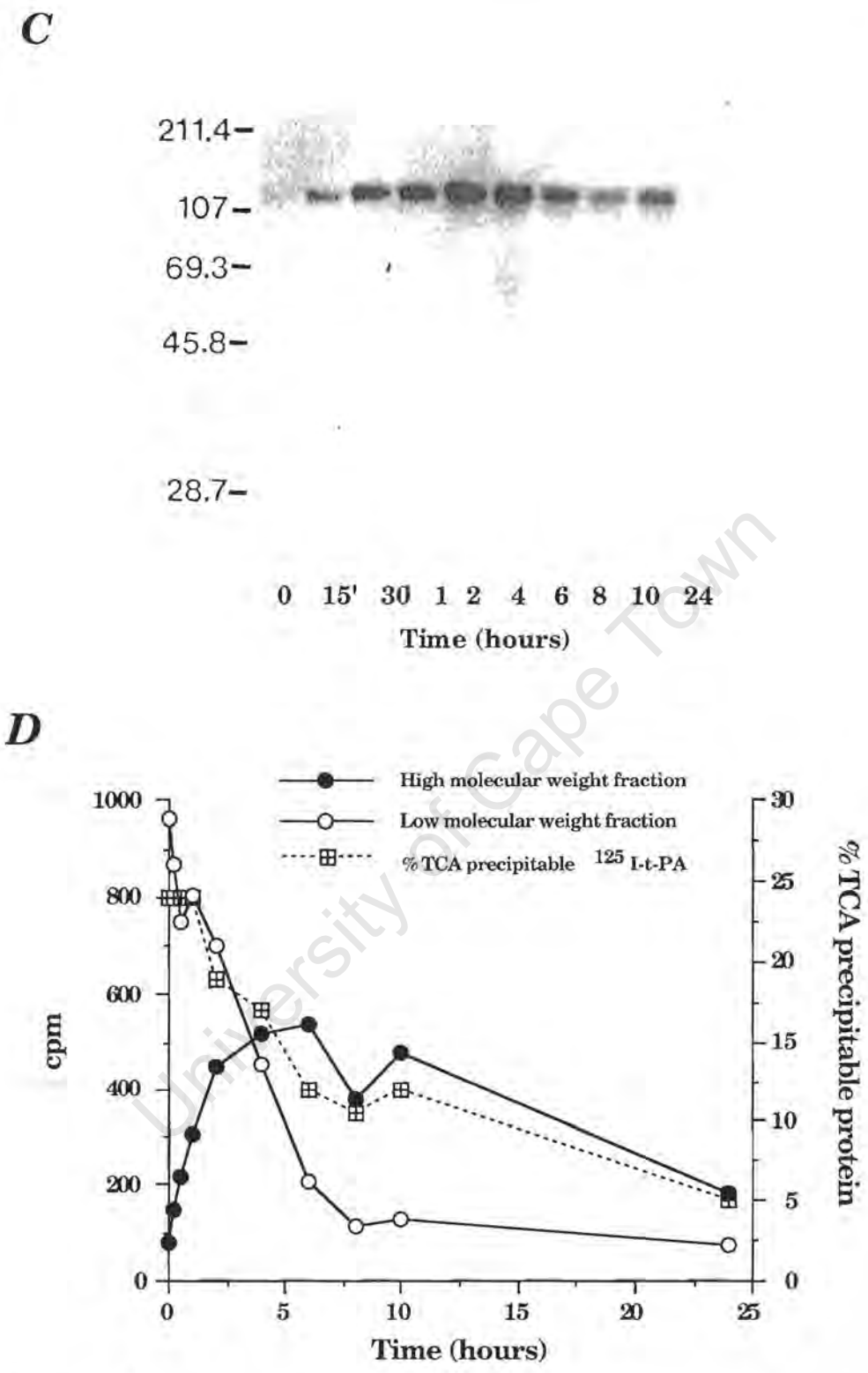


Fig. 11. continued.
(*C*) a 4-day exposure of cell lysates.
(*D*) After exposing the gels for autoradiography, the labelled proteins in the high and low molecular weight fractions present in the supernatant was quantitated by cutting the relevant areas from the gel used in (*A*) and measuring radioactivity using a gamma counter. Proteins in the supernatants were also precipitated with TCA in order to determine precipitable ^{125}I-t-PA in each fraction.

To confirm that ^{125}I-t-PA formed a complex with PAI-1, free and complexed PAI-1 was identified in the samples using Western blotting techniques. Non-complexed PAI-1, which has a molecular weight of approximately 44 kDa, was detected in the supernatant after 8 hours and in matrix and cell lysate over the entire 24-hour time period in approximately equal amounts (Fig. 12, data not shown for supernatant and matrix samples).

A high molecular weight form of PAI-1 was also detected in the cell lysate with maximum accumulation at 2 hours (Fig. 12). This high molecular weight form of PAI-1, of approximately 110 kDa, was taken to represent the internalised complex between PAI-1 and exogenously added t-PA. However, no PAI-1/t-PA complex was detected in the supernatant or matrix extracts. The experiment was repeated in the presence of an excess amount of cold t-PA but this did not result in the formation of a high molecular weight form of PAI-1 in either the supernatant or the matrix extract. The reason for this is not known but it may be that in the supernatant and matrix samples the binding site for the anti-PAI-1 antibody on the t-PA/PAI-1 complex was not recognised by the antibodies. Upon internalisation the complex may have undergone a conformational change such that the binding site was exposed and could bind with the anti-PAI-1 antibodies.

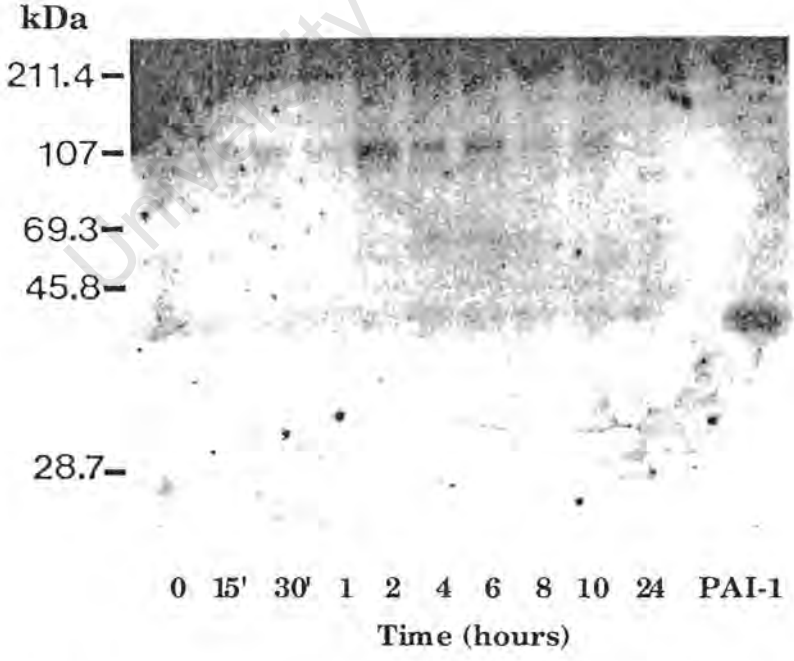


Fig. 12. Identification of PAI-1 in the cell lysates of BM stromal cells. Cell lysates used in Fig 13*B* were subjected to 11% PAGE-SDS. The proteins in the resultant gel were transferred onto nitrocellulose membranes with semi wet blotting. To identifiy PAI-1, the membranes were then incubated with anti-PAI-1 antibodies bound to ^{125}I labelled protein-A using standard Western blotting techniques. Free, uncomplexed PAI-1 had an apparent molecular weight of 44 kDa.

Alternatively, since the PAI-I/t-PA complex is rapidly internalised, the amount of complex remaining in the supernatant and ECM may have been below the detection limit for Western blot. Determining the rate of synthesis for PAI-1 as well as the turnover of protein and mRNA, should help to explain the inability to detect the PAI-1/t-PA complex in these fractions.

These results demonstrate that t-PA complexes with PAI-1 in the BM stromal cell microenvironment and that the t-PA/PAI-1 complex is rapidly internalised. In addition, while trace amounts of free t-PA were detected in the ECM, uncomplexed t-PA did not appear to be internalised by BM stromal cells.

Discussion.

I studied the regulation of PA activity in passaged BM stromal cells by FGF-2 and two other growth factors important for haematopoiesis, TGF-ß and IL-1ß. These growth factors stimulate plasmin generation which is dependent on PAs. Plasmin is a mediator of many proteolytic processes regulating growth factor activity and cell migration (Plow et al., 1995; Brunner and Priessner, 1994). I found that u-PA, t-PA, and their specific inhibitors, PAI-1 and PAI-2, are differentially regulated by FGF-2, TGF-ß, and IL-1ß. Furthermore, PAI-1 not only inhibits t-PA activity, but is required for the binding of t-PA to the cell surface and for its internalisation. I also noted that hydrocortisone modulates u-PA activity in the BM stromal cell microenvironment. Thus, the regulation of plasmin generation in the BM microenvironment maybe a potential mechanism by which growth factors such as FGF-2, TGF-ß, and IL-1 regulate haematopoiesis in LTBM cultures.

1. The inhibition of BM PA activity by hydrocortisone.

My results show that hydrocortisone in BM stromal medium completely and reversibly inhibits TGF-ß-mediated u-PA induction as well as basal PA secretion, which was found to be u-PA, by BM stromal cells. Since hydrocortisone is a medium supplement essential for the generation and proliferation of myelopoietic cells in Dexter LTBM cultures (see Chapter 2), u-PA might be a negative regulator of blood cell development. Since u-PA is primarily associated with ECM degradation and cell migration, its presence in LTBM cultures might alter the formation of the ECM such that myelopoiesis is not favoured. u-PA might also be involved in the activation of additional TGF-ß (positive feedback), or the release of other inhibiting cytokines. Increased levels of active TGF-ß within niches of the BM could lead to a decrease in haematopoiesis as this growth factor is a potent inhibitor of primitive progenitor cell growth (Fan et al., 2002; Fortunel et al., 2000; Waegell et al., 1994; Gabrilove et al., 1993; Cashman et al., 1990; Eaves et al., 1991; Sing et al., 1988). However, in other niches TGF-ß could stimulate haematopoiesis because in the presence of GM-CSF, it enhances the differentiation of granulocyte progenitor cells (Keller et al., 1991).

The inhibition of PA activity by hydrocortisone cannot be entirely explained by the increased levels of PAI-1, as the stimulation of PAI-1 synthesis was independent of hydrocortisone concentration. As has been described for other glucocorticoids (Saksela and Rifkin 1988), this finding suggests that hydrocortisone may directly affect u-PA gene activity, which could occur, for example, through induction of a repressor gene.

In Chapter 2, I showed that the mitogenic effect of FGF-2 on primary BM stromal cells required the presence of hydrocortisone in the culture medium. One possible explanation for this is that hydrocortisone inhibits u-PA. In primary BM cultures, u-PA may antagonise FGF-action by interfering with FGF-2 mobilisation via the generation of plasmin (Whitelock et al., 1996; Falcone et al., 1993b; Brunner et al., 1991). However, the mechanism is not that simple as the FGF-2-mediated stimulation of passaged BM stromal cells did not show the same dependence on hydrocortisone.

Furthermore, I noted that the basal PA activity found in passaged BM stromal cells was u-PA. My findings are in contrast to the findings of McWilliam et al. (1998 and 1996) who show that in normal marrow, t-PA is the primary PA present. Passaged BM stromal cells consist mainly of fibroblast-like cells whereas the BM samples used by McWilliam et al. contained all the stromal cell types as well as haematopoietic progenitor cells. The difference in cell type between the two cell samples may account for the difference in the type of PA detected. Thus, the results of McWilliam et al. may reflect a predominance of primitive progenitor cells in the sample since the primary PA type expressed by these cells is t-PA (Wilson and Francis, 1987). It may be that *in vivo* the basal expression of u-PA by fibroblast-like cells is very low, but that, in a particular niche, both u-PA and t-PA can be induced by growth factors. While u-PA is usually associated with ECM degradation and cell migration, and t-PA with plasmin generation during fibrinolysis (Fazioli and Blasi, 1994; Carmeliet et al., 1994; Collen and Lijnen, 1991), the distinct roles of u-PA and t-PA in haematopoiesis is not known. There is some evidence to suggest that the expression of t-PA or u-PA by haematopoietic cells is linked to their state of differentiation since differentiated haematopoietic cells express u-PA (Wilson and Francis, 1987). However, myeloid leukaemic patients, u-PA is the primary PA present (Scherrer et al., 1999; McWilliam et al., 1998). This might reflect the malignant state of the haematopoietic cells since malignant cell transformation has been associated both *in vitro* and *in vivo* with the increased expression of u-PA, possibly contributing to the invasive nature of such cells (Scherrer et al., 1999; McWilliam et al., 1998).

2. Growth factors regulate PA activity in the BM microenvironment.

I chose to study the effects of three growth factors relevant to haematopoiesis, namely FGF-2, TGF-ß, and IL-1ß, on BM stromal cell PA activity. These growth factors modulate the production of PAs and their inhibitors which, in turn, regulate growth factor activity and cell migration in cellular processes such as wound healing and angiogenesis, and thus, in a similar manner, these growth factors could regulate

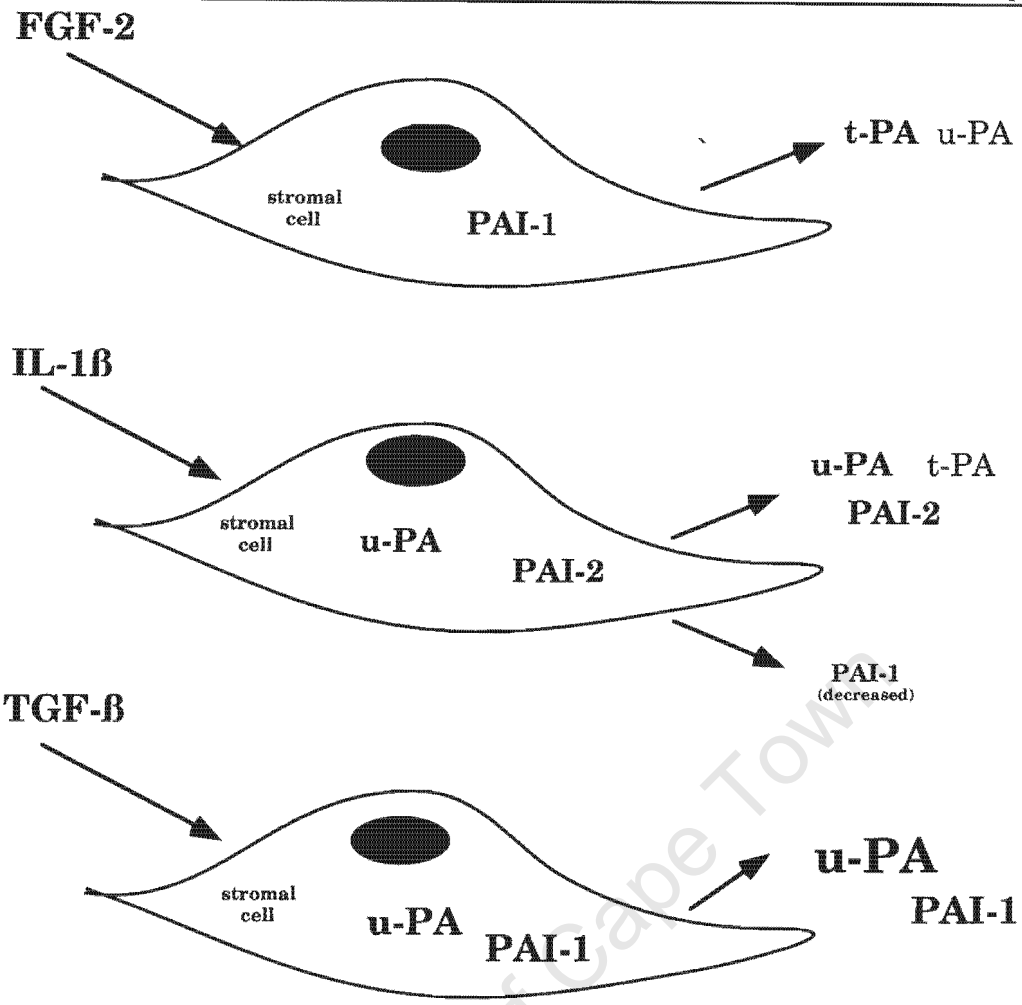


Fig. 14. A schematic representation summarising the regulation of PAs and their inhibitors by FGF-2, IL-1ß, and TGFß. FGF-2, IL-1ß, and TGFß were found to differentially modulate PAs and their inhibitors according to the type of PA or PAI affected, the compartment in which the effect took place (cellular and/or extracellular), as well as the extent of the effect (denoted by script size and boldness).

haematopoiesis. The results are summarised in Fig. 14. FGF-2, IL-1ß, and TGF-ß were found to differentially modulate the production and secretion of PAs and their inhibitors, PAI-1 and PAI-2, by BM stromal cells.

FGF-2 induced mainly t-PA secretion. Low concentrations were the most effective as was similarly found with FGF-2 stimulating haematopoiesis in LTBM cultures. TGF-ß increased exclusively, and IL-1ß predominantly, cellular and secreted u-PA levels. Furthermore, it was noted that IL-1ß stimulated PAI-2 production and secretion, while PAI-1 levels were stimulated by TGF-ß (cellular and secreted) and FGF-2 (only cellular) (Fig. 14).

The most profound stimulatory effects were noted with TGF-ß, which exclusively increased u-PA secretion by up to 350-fold (Fig. 14). Cellular u-PA was also stimulated by TGF-ß, but not to the same magnitude as secreted u-PA. The optimal

concentration of TGF-ß was 0.2 ng/ml but even 20 pg/ml TGF-ß increased PA activity approximately 30-fold. These concentrations of TGF-ß inhibit the proliferation of primitive progenitors *in vitro* (Fortunel et al., 2000) and, in addition, correspond to the concentration of active TGF-ß in plasma which is less than 300 pg/ml (Junker et al., 1996).

I also investigated the effect of GM-CSF, IL-3, and G-CSF on PA activity in BM stromal cells and found that these growth factors had no affect on the basal PA activity (data not shown). However, these growth factors stimulate u-PA activity in BM derived macrophages (Hamilton et al., 1991a). This difference may reflect the specificity of growth factor-action for a particular cell type (human BM stromal cells consist predominantly of fibroblast-like cells with VSMC characteristics) which would contribute to niches forming in the BM microenvironment allowing haematopoiesis to occur along a particular lineage.

The finding that FGF-2, IL-1ß, and TGF-ß modulate the production of u-PA and t-PA is potentially interesting since VSMC proliferate and migrate in response to FGF-2-stimulated u-PA, or PDGF-stimulated t-PA (Herbert et al., 1997; Padró et al., 2002). My findings therefore suggest that PAs might regulate the proliferation of BM stromal cells. Interestingly, the ability of u-PA or t-PA to stimulate VSMC growth and migration was not dependent on the generation of plasmin, but on the binding of the PAs to their respective receptors and subsequent internalisation (Herbert et al., 1997). Indeed, the LRP/α_2-M receptor was found to play a significant role in the internalisation of t-PA and, to a lesser degree, that of u-PA. Antibodies to the LRP/α_2-M receptor completely abrogated the effects of PDGF on VSMC growth and migration by blocking t-PA internalisation (Herbert et al., 1997).

Since human LTBM stromal cells consist mostly of fibroblastic cells expressing phenotypic markers of smooth muscle cells, a similar mechanism may exist (although distinct to that noted with VSMCs), in which the mitogenic activity of FGF-2 on BM stromal cells (see Chapter 2) is mediated by t-PA binding and internalisation, independent of plasmin. Alternatively, FGF-2 mediated t-PA induction could render the cells responsive to growth stimulation by PDGF.

In addition to these observations, I also noted that the TGF-ß-mediated increase in PAI-1 levels occurred much earlier than the increase in PA activity. The reason for the apparent delay in detecting PA secretion may be the fact that the initial amounts of u-PA produced by the stromal cells within the first 12 hours were rapidly inactivated by PAI-1. Another possible explanation is that u-PA is produced as an inactive pro-enzyme which requires proteolytic processing by plasmin for activation

(Andreasen et al., 1990). Since my experiments were performed under serum-free conditions, i.e. plasminogen-free conditions (although it must be assumed that some plasminogen as well as some plasmin remained bound to the cell surface or ECM even after washing the cells several times), the post-transcriptional activation of pro-uPA to active u-PA by plasmin may have been slow during the first 12 hours due to the initially limited amounts of plasmin in the system. A third reason for the delay in detecting PA activity may be that there are differences in the signalling pathway for PA and PAI-1 production. For example, the transcription of PA, but not PAI-1, may depend on the synthesis of a transcription factor which may result in a lag phase in the synthesis of PA compared to PAI-1. PAI-1 is a major target gene for TGFß activity and is induced early.

I also observed that the growth factors were significantly more effective at stimulating PA activity, when the stromal cells were first incubated with the growth factors in the presence of FCS. To try and explain this, I substituted PDGF for FCS (FCS contains significant amounts of this growth factor; Gronthos and Simmons, 1995) and determined the stimulatory effects of TGF-ß on u-PA and PAI-1 production by BM stromal cells. Although u-PA and PAI-1 levels were significantly enhanced by TGF-ß in the presence of PDGF, it was nevertheless noted that the stimulatory effects of PDGF on TGF-ß-mediated PA induction were further enhanced by FCS suggesting that other growth factors and/or components synergise in this process. Indeed, FCS appears to increase the responsiveness of the BM stromal cells to growth factor treatment. However, the mechanisms by which this occurs are not fully understood.

3. The regulation of t-PA activity by PAI-1.

I demonstrated, using anti-PAI-1 antibodies, that PAI-1 effectively inhibits t-PA activity in the BM microenvironment. However, the addition of FGF-2 apparently shifts the proteolytic balance producing a significant net t-PA activity in stromal culture supernatants which favours the generation of plasmin. In addition, PAI-1 mediates t-PA internalisation. My results show that t-PA complexes with PAI-1 in the culture supernatant and that this binding appears to be a requirement for the removal of extracellular t-PA from the BM microenvironment. The t-PA/PAI-1 complex was rapidly internalised by the stromal cells, whereas free t-PA was not as it was not detected in the lysates. Trace amounts of t-PA were, however, associated with the ECM, possibly via LRP/α_2-M receptors (Camani et al., 1994).

It was also noted that the internalisation of the t-PA/PAI-1 complex by the stromal cells was more efficient than binding of the complex to the ECM. This

observation is also supported by the long exposure time required for the visualisation of ECM bound complexes (15 days, [see Fig 13*C*] as compared to 4 days for cell lysates [see Fig 13*B*]).

4. Potential functions for the PA/plasmin system in haematopoiesis and the role of FGF-2.

The haematopoietic process is an exceedingly complex one involving the coordinated expression of many cytokines and growth factors. A number of these factors regulate PAs and their inhibitors and therefore either favour or diminish plasmin generation. Plasmin has a documented role in generating active TGF-ß and IL-1ß and in releasing biologically active FGF-2 and latent TGF-ß from the stromal matrix (Hazuda et al., 1991; Lyons et al., 1988; Saksela and Rifkin, 1990; Brunner et al., 1991; Falcone et al., 1993a and b; Taipale et al., 1992;). Thus, it is possible that PA generation in a specific microenvironment of the BM may be one of the factors orchestrating the complex series of events that results in a regulated haematopoietic process. My findings that BM stromal cells secrete u-PA and t-PA and their inhibitors, PAI-1 and -2, and that their production is differentially regulated by growth factors that are essential to BM physiology as well as by glucocorticoids, suggests that the PA/plasmin system plays a relevant role in haematopoiesis and possibly mediates the stimulating effects of FGF-2. Fig. 15 illustrates the potential functions that I envisage for the PA/plasmin system in haematopoiesis, namely, regulation of growth factor activity, progenitor cell egression, and possibly BM stromal cell proliferation. Primitive haematopoietic progenitor cells also secrete PAs (Wilson and Francis, 1987). Thus, in various niches of the BM, t-PA and u-PA could be produced by different cell types in response to growth factors. The resulting generated plasmin could then regulate haematopoiesis in either an autocrine or paracrine manner.

Plasmin generated in the BM microenvironment could regulate the effective concentration of growth factors in a number of ways. First, plasmin could affect the availability of active growth factors such as L-1ß, TGF-ß, and HGF by activating the latent forms (Hazuda et al., 1991; Lyons et al., 1988; Naldini et al., 1992). Paradoxically, plasmin generation could have both a permissive and an inhibitory effect on haematopoiesis, the net effect being dictated by the local environment in which the plasmin is generated. IL-1ß and HGF are permissive growth factor in haematopoiesis, while TGF-ß is an inhibitor of haematopoiesis.

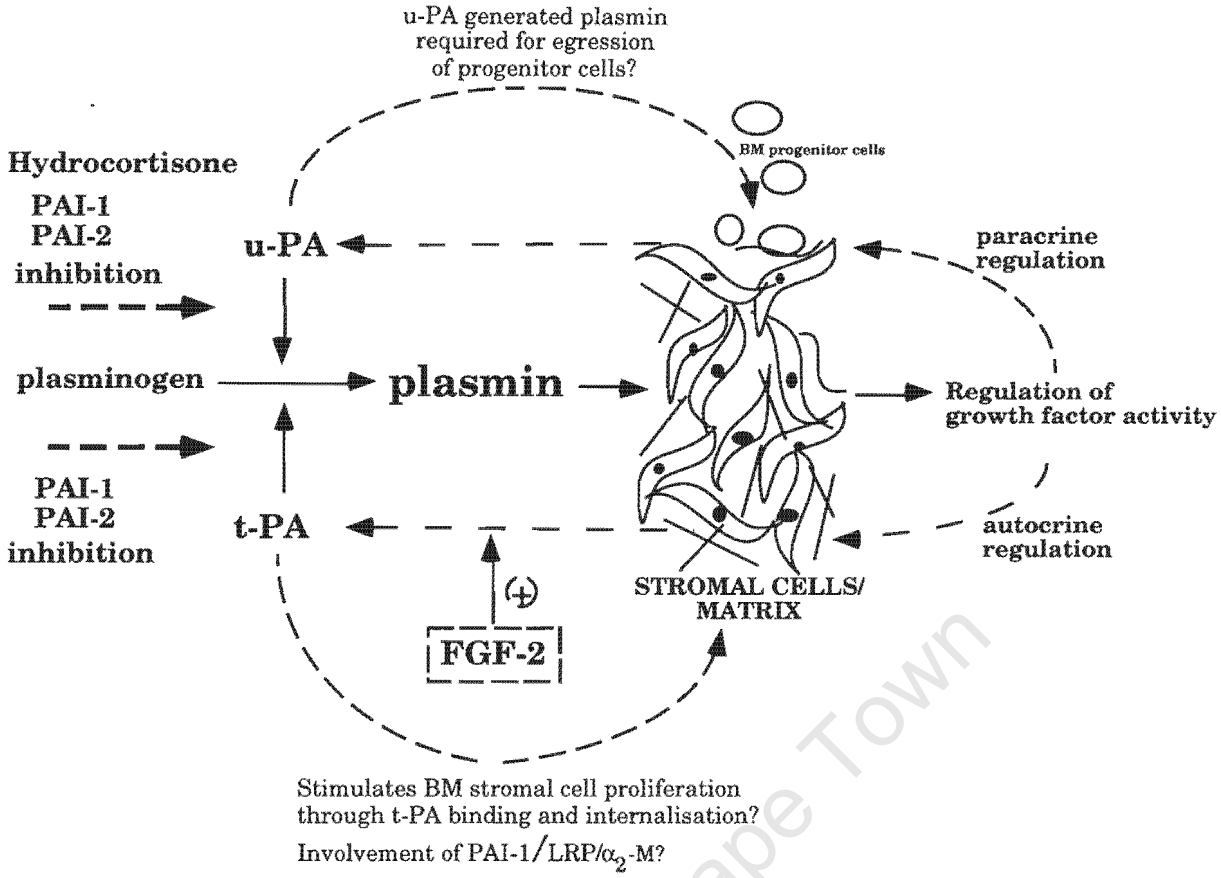


Fig. 15. Schematic representation of the possible roles of the PA/plasmin system in haematopoiesis.

Second, plasmin might directly release active growth factors from the stromal cell matrix, increasing their likelihood of engaging their specific cell surface receptors. Plasmin releases FGF-2-HSPG complexes (Whitelock et al., 1996; Falcone et al., 1993b; Brunner et al., 1991) which are available for binding to the cell surface high-affinity FGFRs.

In Chapter 3 I demonstrated that FGF-2 stimulates myelopoiesis in LTBM cultures. It is possible that the increase in haematopoiesis by FGF-2 might occur via the plasmin-mediated release of FGF-2-HSPG complexes. FGF-2-HSPG complexes could then interact with high-affinity FGFRs to elicit stimulatory effects on haematopoietic cells, overcoming the inhibitory effects of TGF-ß. Other enzymes such as PI-PLC, GPI-PLD, heparanses, and elastase (Ishai-Michaeli, 1990; Whitelock et al., 1996; Brunner et al., 1991 and 1994; Rich et al., 1996) can also mobilise FGF-2-HSPGs from the cell surface or ECM and could also, in a similar manner to plasmin, contribute to the increase in haematopoiesis by FGF-2.

Both GM-CSF and IL-3 bind in a biologically active form HSPGs in the BM stroma (Gordon et al., 1987a; Roberts et al., 1988). As has been demonstrated for FGF-2 and TGF-ß, these factors might also be released by plasmin in a proteoglycan-cytokine complex in which the IL-3 and GM-CSF are available for binding to their high-affinity cell surface receptors.

Third, plasmin generation might regulate growth factor activity by shedding growth factor receptors from the cell surface. Other proteases also participate in the process of growth factor receptor shedding. For example, MMP-2 cleaves FGFR-1 from cell surfaces, releasing an active soluble form (Levi et al., 1996) which might reduce FGF signalling.

Thus, the increase in the pericellular plasmin activity by FGF-2, IL-1ß, or TGF-ß could further increase the activation and/or release of these growth factors as well as others that function in haematopoiesis. In this manner a positive cascade of plasmin generation and cytokine activation could result. In certain instances, such as in the case of TGF-ß, this process might be self-limiting due to release of PAI-1. The plasmin generated could have either a permissive or non-permissive effect on haematopoietic processes. In one particular environment it could result in the release of growth factors such as FGF-2 or latent TGF-ß from the cell surface or ECM, and therefore have a stimulating effect. In another compartment, plasmin generation could increase the local concentration of active TGF-ß, which would negatively affect haematopoiesis.

Plasmin might also function in the egression of progenitor cells from the marrow to the circulation. Plasmin-released FGF-2 from BM stromal cell surfaces or the ECM, allows FGF-2 to act upon haematopoietic cells, possibly affecting their migration. FGF-2 increases the expression of u-PAR (Mignatti et al., 1991) and stimulates the production of metalloproteinases, such as MMP-9 (Liu et al., 2002). Similar increases in u-PAR and/or MMP-9 by FGF-2 in BM stromal cells or haematopoietic cells could promote the migration of haematopoietic cells from the marrow into the circulation. Indeed, circulating haematopoietic stem cells have been identified and purified from peripheral blood (Burger et al., 2002; Asahara et al., 1997; Lin et al., 2000). Circulating haematopoietic stem cells have been implicated in promoting angiogenesis and regenerating organs (Takakura et al., 2000; Goodell et al., 2001; Zhao et al., 2002; Krause, 2002) although whether this occurs under normal physiological conditions is still under debate (Wagers et al., 2002). Alternatively, FGF-2 might down-regulate progenitor cell migration in certain niches of the BM. Active FGF-2 released from the fibroblast matrices decreases elastin transcription leading to a decrease in elastase digestion of the ECM (Rich et al., 1996).

5. Experiments to further characterise the role of the PA/plasmin system in haematopoiesis.

I propose a number of experiments to answer the following questions which try to explain the stimulatory effect of FGF-2 on BM cells and to elucidate possible roles that the PAs/plasmin system may play in haematopoiesis.

Does FGF-2 mediate its stimulatory effects on haematopoiesis via the t- PA regulation of growth factor activity?

I have shown that the addition of FGF-2 to passaged BM stromal cells results primarily in an increase in t-PA secretion. In angiogenesis, FGF-2 and TGF-ß are two closely linked regulatory growth factors. In this system, TGF-ß increases u-PA levels resulting in the release of FGF-2 from the ECM (Falcone et al., 1993b), presumably through elevated levels of plasmin which is known to release ECM associated FGF-2 (Saksela and Rifkin, 1990; Brunner et al., 1991). Furthermore, FGF-2 can increase active TGF-ß levels due to the stimulation in plasmin generation (Flaumenhaft et al., 1992). The observation that FGF-2 partially inhibits the negative effects of TGF-ß on haematopoietic cells (Gabrilove et al., 1993), suggests that these two growth factors may also be linked in their regulation of haematopoiesis although how this occurs is not known. A decrease in TGF-ß-mediated signalling could be achieved through plasmin-mediated release of latent/active TGF-ß from the ECM (Falcone et al., 1993a; Taipale et al., 1992). Therefore, the levels of active TGF-ß in the supernatants of BM stromal cells treated with or without FGF-2 could be determined using a bioassay. This assay measures the TGF-ß-mediated suppression of PA by bovine aortic endothelial cells (Flaumenhaft and Rifkin, 1992).

Does FGF-2-stimulated-t-PA stimulate BM stromal cell proliferation?

VSMC proliferate and migrate in response to FGF-2 or PDGF via the stimulation of PAs, in particular u-PA (Padró et al., 2002; Herbert et al., 1997). However, these effects are not dependent on plasmin generation but rather, in mice, on the binding of the PAs to their respective receptors and subsequent internalisation (Herbert et al., 1997) and, in humans, on the catalytic domain of u-PA. Since human LTBM stromal cells consist mostly of fibroblastic cells expressing phenotypic markers of smooth muscle cells, a similar mechanism may exist, in which the mitogenic activity of FGF-2 on BM stromal cells independent of plasmin generation.

To determine whether the FGF-2-mediated stimulation in BM stromal cell growth is independent of plasmin, the mitogenic response of these cells to FGF-2 could

be determined in the presence of ε-aminocaproic acid or aprotinin (plasmin inhibitors) or neutralising antibodies (α_2-antiplasmin) at concentrations which abrogate the effects of plasmin. An alternative would be to use BM stromal cells from plasminogen-deficient mice and to determine the effect of t-PA or u-PA on BM stromal cell growth. However, since the stromal cell layer in murine *in vitro* BM cultures consists mainly of macrophages (Quesenberry et al., 1991), the desired effects may not be observed using BM stromal cells from plasminogen-deficient mice.

Is the internalisation of the t-PA/PAI-1 binary complex by BM stromal cells dependent on it forming a ternary complex with LRP/ α_2-M receptors?

I have demonstrated that the removal of t-PA from the supernatant of BM stromal cells is dependent on it forming a complex with PAI-1. In human HepG2 hepatoma cells and parenchymal hepatocytes (Morton et al., 1989; Wing et al., 1991; Andreasen et al., 1994; Orth et al., 1992), the clearance of the t-PA/PAI-1 binary complex from the ECM is dependent on it forming a ternary complex with LRP/α_2-M receptors. A similar mechanism may therefore exist in BM stromal cells. To determine whether the t-PA/PAI-1 complex in supernatant of BM stromal cells binds to LRP/α_2-M receptors on BM stromal cell surfaces and is then internalised, the clearance of ^{125}I-t-PA from the supernatant of BM stromal cells can be followed in the presence and absence of antibodies to LRP/α_2-M receptors. The binding of t-PA to LRP/α_2-M receptors might have direct implications for the mitogenic effect of FGF-2 on BM stromal cell growth. Thus, antibodies to LRP/α_2-M receptors should also block the proliferation of BM stromal cells in the presence of FGF-2 if the internalisation of t-PA (stimulated by FGF-2) promotes BM stromal cell growth.

Does the PA/plasmin system play a role in the migration of progenitor cells into the circulation?

There is some evidence indicating that the migration of progenitor cells into the circulation may involve the PA/plasmin system. Reiter et al. (1997) have shown that the transmigration of T-leukaemic cells through an ECM barrier requires PA-dependent proteolysis, which can be provided by u-PA or t-PA, but is significantly faster in T-cells expressing u-PA. Thus, although the PA/plasmin system is not essential for haematopoiesis since mice with deficiencies in t- PA, u-PA, PAI-1, u-PAR, or plasminogen do not display impaired haematopoiesis, this system may still play a relevant role in regulating haematopoiesis. Human stem cells that do not express t-PA or u-PA (using siRNA techniques) could be tested for their ability to migrate through

Chapter 5.

Summary and relevance of FGF-2 for clinical applications.

Haematopoiesis is the process in which the various cell types found in peripheral blood are generated. This process occurs primarily in the BM and is dependent on a stromal cell component. The stromal cells produce stimuli which regulate the proliferation, differentiation as well as the retention and migration of the haematopoietic cells.

FGF-2 is a well described angiogenic factor and collected evidence indicated that this growth factor may also regulate blood cell development. FGF-2 acts on both primitive and committed haematopoietic progenitor cells, directly and in concert with other haematopoietic growth factors, to induce their proliferation and/or differentiation. FGF-2 is expressed by BM stromal cells and is found bound to heparan sulphate proteoglycans in the BM stromal microenvironment forming a reservoir of biologically active growth factor.

The aim of this thesis was to explore the effects of FGF-2 on the stromal compartment in stromal cell cultures, and on haematopoietic cell production in human LTBM Dexter cultures. In addition, I investigated the effects of FGF-2 on PAs production by BM stromal cells as a potential mechanism for FGF-2 action. Plasmin, generated from plasminogen by PAs, could regulate growth factor activity and stimulate cell migration in the BM.

1. Summary of the results.

The addition of low concentrations of FGF-2 to human BM buffy coat cells accelerated the formation of the layer by acting as a mitogen for primary human BM stromal cells. In addition, FGF-2 significantly delayed the senescence of BM stromal cells which normally cease to grow after approximately 2 to 3 passages. This resulted in considerable expansion of the stromal cells in culture. FGF-2 also induced morphological changes in the developing adherent stromal layer. In the presence of this growth factor, stromal cells lost their contact inhibition and formed densely packed layers of spindle shaped cells. These results suggest that FGF-2 may be a relevant growth factor for promoting the growth of the adherent stromal layer in LTBM cultures and may be required for the growth of these cells *in vivo*.

The addition of low concentrations of FGF-2 to human LTBM cultures significantly increased the number of haematopoietic progenitor cells in both the adherent layer and supernatant. In particular, FGF-2 stimulated the number of GM-CSF and G-CSF-responsive progenitor cells in the supernatant of LTBM cultures, and furthermore, increased the number as well as the size of cobblestone foci of haematopoietic cells in the adherent layer by increasing the number of GM-CSF responsive progenitor cells in this layer.

I also demonstrated that BM stromal cells produce u-PA and t-PA as well as PAI-1 and PAI-2 and that their synthesis can be regulated by hydrocortisone and growth factors relevant to haematopoiesis, namely FGF-2, TGF-ß, and IL-1. I also demonstrated that endogenous PAI-1 effectively inhibits t-PA activity as well as mediates its internalisation by BM stromal cells. These results indicate that PA levels can be regulated in the BM microenvironment thereby regulating plasmin generation. Thus, the PA/plasmin system may serve in modulating BM development.

In Fig. 1 I have illustrated my findings with the known actions of FGF-2. In the BM, stromal cells secrete FGF-2 where it binds to HSPGs in the ECM forming a reservoir of active growth factor (Brunner et al., 1993; Blotnick et al., 1994). FGF-2 is proteolytically released from the ECM making it available to both haematopoietic and non-haematopoietic cells in the neighbouring vicinity (Saksela and Rifkin, 1990; Ishai-Michaeli, 1990; Whitelock et al., 1996; Brunner et al., 1991 and 1994; Rich et al., 1996). Here, depending on the needs of the body, FGF-2 could directly stimulate proliferation or promote/delay differentiation of haematopoietic cells at different stages of development (Faloon et al., 2000; Anzai et al., 1999; Gallicchio et al., 1991; Gabbianelli et al., 1990; Gabrilove et al., 1993 and 1994; Berthier et al., 1997; Burger et al., 1994). Alternatively, FGF-2 could modulate growth factor activity by stimulating the generation of plasmin via the increase in t-PA production. Increased plasmin levels could also result in the migration of a particular mature haematopoietic cell type(s) into the blood if required by the body.

In vivo studies in which mice lacking FGF-1 and/or FGF-2 are viable, fertile and display no significant haematopoietic defects, has questioned the relevance of FGF-2 in blood cell development as well as other physiological processes. It is possible that in these mice other FGFs and/or other growth factors are compensating for the lack of FGF-1 and FGF-2. For example, FGF-4 effectively stimulates haematopoiesis in LTBM cultures (Quito et al., 1996). The results that I have presented in this thesis show that *in vitro* FGF-2 is a positive regulator of haematopoiesis, acting on both stromal cells and early progenitor cells. My findings together with those of other researchers suggest

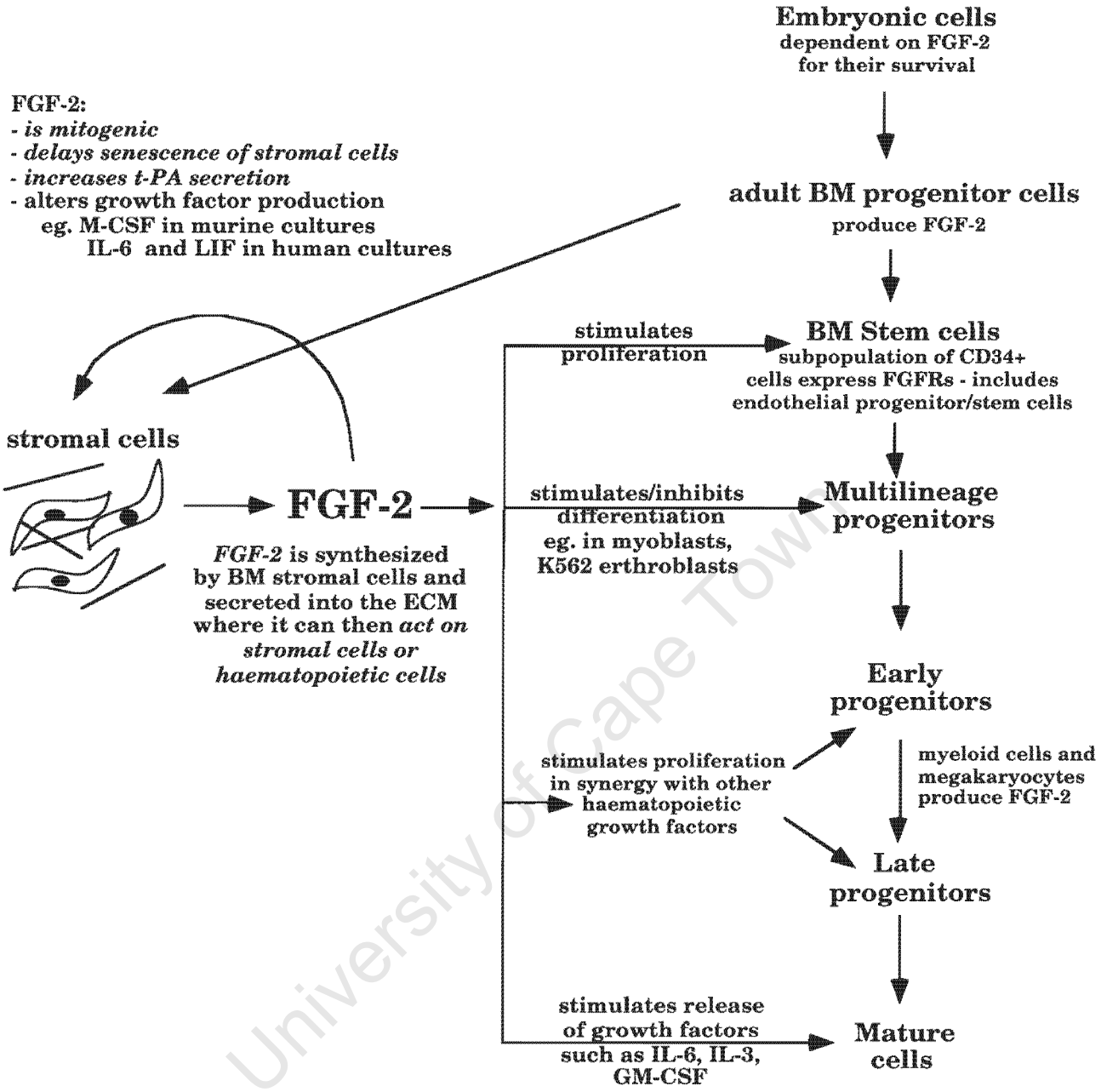


Fig. 1. Schematic representation illustrating the role of FGF-2 in haematopoiesis. My findings are highlighted in italics.

that this growth factor may form part of the complex cytokine network that regulates haematopoiesis.

2. Role of FGF-2 in clinical haematology.

One of the most intriguing challenges in haematology today is the *in vitro* expansion of human haematopoietic stem cells. The generation of large numbers of

haematopoietic stem cells *in vitro* will lead to improved clinical protocols for BM transplantation and gene therapy. This is even more relevant today as it is now apparent that MAPCs and MSCs within the BM possess developmental potency far greater than was original thought (Mertelsmann, 2000; Stocum, 2001; Krause, 2002). These cells have the potential to differentiate into mature cells of the heart, liver, kidney, lungs, skin, bone, muscle, cartilage, fat, endothelium and brain (Herzog et al., 2003; Mertelsmann, 2000; Stocum, 2001; Krause, 2002). Whether this process occurs under normal physiological conditions is debatable (Wagers et al., 2002) but the 'plasticity' of MSCs makes them potentially useful for replacing tissues, via transplantation (Stocum, 2001; Horowitz et al., 1999; Goodell et al., 2001).

Current methods for expanding primitive human progenitor cells utilise LT cultures (Gan et al., 1997; Gupta et al., 2000; Lewis and Verfaillie, 2000) but, defined (serum free, stromal free, cytokine cocktail) liquid culture systems for these cells are also used (Poloni et al., 1997; Rebel et al., 1994; Lansdorp and Dragowska, 1993; Pelosi et al., 1992). The use of FGF-2 or other FGFs (e.g. FGF-4) may improve both methods of stem cell expansion, stromal mediated as well as liquid culture systems since FGF-2 can act directly on haematopoietic cells at early stages of differentiation. My results showed that FGF-2 maintained an increased number of primitive haematopoietic cells in LTBM cultures which may reflect an augmented output of primitive progenitor cells. Similar results have also been noted with FGF-4 (Quito et al., 1996).

2.1. *BM transplantation.*

Allogeneic BM transplantation is a technique currently used for treating patients suffering from a variety of genetic diseases including immunological disorders such as adenosine deaminase deficient disease, chronic granulomatous disease, and SCID, haematological disorders such as sickle cell disease and thalassemia, as well as metabolic disorders such as gaucher's disease and osteopetrosis (Fleischman, 1991; Karlsson, 1991). Furthermore, autologous BM transplants are sometimes given to patients who have undergone chemotherapy for treatment of cancer because the chemical agents also destroy most of the proliferating haematopoietic cells in the BM. There may be an additional use for FGFs in patients undergoing chemotherapy. Low concentrations of FGF-2 inhibit radiation-induced apoptosis, and radioprotect a number of tissues including haematopoietic cells without increasing the rates of tumour growth or metastases or decreasing the radiosensitivity of tumours (Ding et al., 1997 and 1996).

The expansion of stem cells and progenitor cells *in vitro* by FGFs might facilitate the efficiency of BM transplants by yielding enlarged numbers of enriched stem cells and possibly improving their engraftment into the host marrow. Engrafting a larger number of stem cells and progenitor cells into the patient's BM could be of benefit to the patient because conditions for autografting would be improved allowing normal haematopoietic activity to be more rapidly restored which would shorten the patient's recovery period.

2.2. *Gene Transfer.*

The addition of FGF-2 to LTBM cultures may also prove to be a useful technique for improving the conditions for gene transfer. Transfer of cloned human genes into target cells by retroviral vectors is a potential new treatment for a variety of inherited genetic disorders such as ß-thalassemia, adenosine deaminase deficiency, purine nucleoside phosphoylase deficiency, chronic granulomatous disease, gaucher's disease, cystic fibrosis, and hemophilia currently treated by BM transplant or for which their is no treatment available (Fleischman, 1991; Karlsson, 1991). For successful gene therapy the genetic defect should be expressed in target cells that are easily manipulated *in vitro* and that, after transplantation back into the recipient, are capable of self-renewal and differentiation into progeny cells that can generate a large cell pool that contains the transferred gene for the entire life-span of the patient. Thus, MAPCs or MSCs are potentially ideal target cells for gene therapy in genetic or acquired diseases of the haematopoietic system and potentially other disorders or even cancers (van Damme et al., 2002).

Cell division is an absolute requirement for the integration of the retroviral DNA construct into the target cell (Miller et al., 1990), however, haematopoietic stem cells exist primarily in a non-dividing or dormant state (Cashman et al., 1985). Another problem is that human haematopoietic stem cells cultured *in vitro* become committed to differentiation and thus lose their capacity to self-renew and repopulate the BM. A third problem is that stem cells in the BM are rare (estimated to represent less than 0.07 % of nucleated BM cells) (Brandt et al., 1990) resulting in low efficiencies of infection. The observation that the addition of FGF-2 to LTBM cultures increased the number of progenitor cells in the adherent layer and in the supernatant suggests that this culture system may be useful for increasing the success of gene therapy. FGF-2 may induce dormant progenitor cells in LTBM cultures to divide and proliferate, leading to a larger number of progenitor cells incorporating the retroviral vector. Indeed, peripheral blood haematopoietic progenitor cells treated with FGF-2 in combination with

IL-3, IL-6, and SCF showed increased levels of retroviral transduction (Dilber et al., 1994).

3. Conclusions.

Although, FGF-2 may not be an essential growth factor for haematopoiesis there is, nevertheless, a large body of *in vitro* evidence indicating that it plays a relevant role in the complex network of growth factors that exquisitely regulate the development of blood cells. With the discovery in recent years of many new members of the FGF family, the involvement of FGFs and their receptors (of which there are multiple isoforms) in haematopoiesis most likely extends beyond what is now known. There are therefore many possible combinations of FGFs, FGFRs and HSPG cofactors which would allow this family of growth factors to differentially regulate the self-renewal, proliferation, and differentiation of BM cells. Solving the complexity of the FGF/FGFR family in haematopoiesis will provide a greater understanding of BM physiology and ultimately could provide improved techniques for correcting disorders of haematopoietic origin.

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Appendix.

1. Chemicals and Reagents.

1.1. *Protease- and Inhibitor-Free BSA.*

BSA was dissolved in water to give a final concentration of 8 mg/ml. Acid-labile protease inhibitors were removed by adjusting the solution to pH 3.0 with 0.1 M HCl and incubating at room temperature for two hours. The solution was then neutralised with 0.1 M NaOH. Protease activity was removed with diisopropyl fluorophosphate (DFP), as described in the preparation of plasminogen (Appendix 1.3). Three ml aliquots of protease- and inhibitor-free BSA were stored at -20°C.

1.2. *Cytokines.*

Cytokine dilutions (FGF-2, TGF-ß, GM-CSF, G-CSF, IL-1ß, and PDGF) were made immediately prior to use into either serum-free RPMI medium containing 0.4 mg/ml protease- and inhibitor-free BSA or RPMI medium containing 10% FCS.

1.3. *Human plasminogen.*

Plasminogen was purified from human plasma by passing filtered human plasma through a lysine sepharose affinity column at 4°C (Deutsch and Mertz, 1970). The absorbed plasminogen was eluted with 0.2 M 6-aminocaproic acid in 0.1 M potassium phosphate buffer, pH 7.3. The optical density of each fraction was determined at 280 nm. The solution in the tubes that comprised the peak were pooled. The solution was then dialysed against PBS. To inactivate traces of contaminating plasmin, the plasminogen was treated with DFP at a final concentration of 10 mM for one hour at 37°C. This treatment was repeated again before extensive dialysis against PBS to remove unreacted DFP. The plasminogen was aliquoted and stored at -20°C.

1.4. *Urokinase.*

Lyophilised human u-PA was dissolved in 0.1 M Tris-HCl, pH 8.1, containing 0.4 mg/ml BSA to give a final stock solution of 2000 Ploug units/ml. The solution was aliquoted and stored at -80°C. Each aliquot was used only once. Each new preparation of u-PA was standardised against the previous preparation.

1.5. *Fibrinogen.*

To purifiy, lyophilised fibrinogen was dissolved with gentle stirring in 200 ml of sterile H_2O and then precipitated with 70 ml saturated ammonium sulphate (Laki, 1951). The "gummy" pellet was dissolved in 50 ml of 0.6 M NaCl and the pH adjusted to 7.4 with dilute NH_4OH. The solution was dialysed at room temperature against 3 changes of 0.6 M NaCl containing antibiotics (77 U/ml penicillin and 31 µg/ml streptomycin). The optical density of the fibrinogen solution was determined at 280 nm and the concentration adjusted to 10 mg/ml (a 1 mg/ml solution has an optical density of 1.3 at OD_{280}).

Further purification of the fibrinogen was required before it could be labelled with ^{125}I. To do this, one part of fibrinogen was mixed with 5 parts of 0.12 M lysine in 0.005 M NaH_2PO_4, pH 7.0. The solution was cooled to 0°C on an ice/salt bath and the fibrinogen precipitated with 8ml of 0°C ethanol (95%) (Mosesson, 1962). The precipitate was collected by centrifugation and dissolved in 30 ml of 0.6 M NaCl. The fibrinogen was precipitated a second time. After centrifugation, the precipitate was dissolved in 20 ml of 0.6 M NaCl and dialysed at room temperature against PBS containing antibiotics (77 U/ml penicillin and 31 µg/ml streptomycin). The concentration of the fibrinogen was adjusted to 10 mg/ml. The solution was then aliquoted and stored at -20°C.

Purified fibrinogen was then iodinated using a 3 M excess of iodine monochloride (Helmkamp et al., 1960). Briefly, 2 ml of a 10 mg/ml fibrinogen solution was mixed with an equal volume of 2x borate buffer (0.32 M NaCl; 0.4 M H_3BO_3, pH 7.65). One ml of 0.0003 M ICl was then added to 1 ml of 10 mCi I^{125}, mixed well and rapidly added to the fibrinogen solution. The solution was then passed through a Dowex column (AG1-XB 200-400 mesh) and the eluate dialysed at room temperature against 3 changes of PBS containing antibiotics (77 U/ml penicillin and 31 µg/ml streptomycin). The concentration of the fibrinogen was determined (OD_{280}: 1 mg/ml = 1.3) and the number of cpm in an aliquot was counted using a gamma counter. The labelled fibrinogen was aliquoted and stored at 4°C.

1.6. *Antibodies.*

Sheep anti-human u-PA and anti-human t-PA antibodies (Dept. of Immunology, UCT), irrelevant sheep IgG from non-immune sheep serum (Sigma), rabbit anti PAI-1 antibodies (Dept. of Cell Biology, NYU Medical Center), and irrelevant rabbit IgG from non-immune serum (Dept. of Cell Biology, NYU Medical Center) were purified using a protein-G sepharose column. The antibodies were eluted from the column with 0.2 M

glycine, pH 3.0. The solution in each fraction was neutralised with 2 M Tris-HCl, pH 9.0. The optical density at 280 nm of each fraction was determined and the solution in the tubes that comprised the peak were pooled. The sample was then dialysed against 3 changes of PBS. The samples were sterilised by filtration. The concentration of each antibody solution was determined (a 1 mg/ml solution has an optical density of 1.3 at OD_{280}). The samples were then aliquoted and stored at -20°C.

1.7. Buffers for *SDS-Polyacrylamide Gel Electrophoresis.*

1.7.1. *Separating gel buffer.*

The separating gel buffer, 4x, consisted of 0.4% SDS in 1.5 M Tris-HCl, pH 8.8.

1.7.2. *Stacking gel buffer.*

The stacking gel buffer, 4x, consisted of 0.4% SDS in 0.5 M Tris-HCl, pH 6.8.

1.7.3. *Reservoir buffer.*

The reservoir buffer consisted of 0.1% SDS and 0.192 M glycine in 0.025 M Tris-HCl, pH 8.5.

1.7.4. *Sample buffer.*

The sample buffer, 4x, consisted of 12% SDS and 40% glycerol in 0.25 M Tris-HCl, pH 6.8.

1.7.5. *Reducing sample buffer.*

The reducing sample buffer, 4x, consisted of 12% SDS, 30% glycerol, and 20% ß-mercaptoethanol in 0.25 M Tris-HCl, pH 6.8.

1.8. *Versene buffer, 5x.*

Versene buffer, 5x was prepared by dissolving 650 mg of EDTA, 500 mg of KCl, 20 g of NaCl, 4.23 g of $Na_2HPO_4 \cdot 7H_2O$, and 500 mg of KH_2PO_4 in 900 ml of H_2O. The pH of the solution was adjusted to 7.3. The volume of the solution was adjusted to 1 litre and sterilised by autoclaving.

2. Methods.

2.1. *Preparation of ^{125}I-Fibrin coated Linbro plates.*

^{125}I-fibrin coated Linbro plates were prepared with purified human fibrinogen (Appendix 1.5). Sufficient iodinated fibrinogen was added such that each well contained 240 000 cpm. Thirty µg fibrinogen total was added per well. The plates were dried at 37°C for at least three days before use and stored at 37°C. ^{125}I-fibrinogen was converted to ^{125}I-fibrin by the addition of 1 ml alpha minimal essential medium (αMEM) to each well and incubating at 37°C. After two hours the wells were washed twice with PBS, pH 7.0 and once with 0.1 M Tris-HCl, pH 8.1.

2.2. *SDS-Polyacrylamide Gel Electrophoresis.*

The procedures followed were as described by Granelli-Piperno and Reich, 1978. In general an 11% polyacrylamide separating gel with a 4% polyacrylamide stacking gel was prepared and used to separate proteins from BM stromal cell cultures. Both the separating gel (Appendix 1.7.1) and the stacking gel (Appendix 1.7.2) contained a final SDS concentration of 0.1%. To initiate polymerisation, catalysts were added: Temed (to give a final concentration of 0.055% in the separating gel and 0.2% in the stacking gel) and ammonium persulphate (to give a final concentration of 0.5% in the separating gel and 0.4% in the stacking gel). The samples to be loaded onto the gel were diluted with sample buffer (Appendix 1.7.4 and 1.7.5) at a ratio of 4:1 respectively. Electrophoresis was performed at a constant current of 25 mA.

2.3. *Western Blotting.*

Following electrophoresis the proteins in the separating gel were transferred onto a nitrocellulose membrane using semi-wet blotting (LKB apparatus) in blotting buffer (39 mM glycine, 48 mM Tris, pH 8.3, 0.0375% (w/v) SDS, 4% methanol) at 0.8mA/cm^2 for 2.5 hours. After transfer, the nitrocellulose membrane was washed with PBS to remove all traces of the gel. Non specific binding sites were then blocked by immersing the membrane for 30 minutes in a 5% (w/v) solution of dry milk (Carnation) in PBS.

Anti-PAI-1 antibodies, at a concentration of 5.6 mg/ml, were added to the membrane in a fresh aliquot of a 5% solution of dry milk in PBS (the smallest volume needed to cover the membrane was used). The membrane was incubated at room temperature for 30 minutes with gentle rocking. The membrane was then washed sequentially in the following buffers for 10 minutes each time: PBS containing 0.1% dry milk and 0.1% Tween-20; PBS containing 0.1% dry milk and 0.5% Tween-20; PBS

containing 0.1% dry milk and 0.1% Tween-20; PBS containing 0.1% dry milk, 0.1% Tween-20 and 0.35 M NaCl; and PBS containing 0.1% dry milk and 0.1% Tween-20.

^{125}I-protein-A (10^5 - 10^6 cpm/ml) in a 5% solution of dry milk in PBS was added to membranes for 30 minutes at room temperature. The washing procedure was then repeated after which then membrane was exposed to X-ray film at -70°C.

2.4. *Preparation of the Fibrin-Plasminogen Agar Gel Slabs.*

The fibrin-plasminogen agar gels were cast between two clean glass plates, held 0.8 mm apart by thin wires (Fig 1). The entire assembly was clamped together and warmed in a 45°C incubator.

A stock solution of 2.5% (w/v) of agar in H_2O was prepared by dissolving the agar using a microwave. The solution was then transferred to a 45°C waterbath. A fibrinogen/plasminogen/thrombin/Tris-HCl solution (2 mg/ml of purified bovine fibrinogen, 50 µg/ml of purified human plasminogen and 0.03 units/ml of thrombin in 0.1 M Tris-HCl, pH 8.1) was prepared and incubated at 37°C. Sufficient agar was then rapidly added to give a final concentration of 1.25%. The mixture was mixed well and pippetted between the two pre-warmed glass plates with care being taken to avoid air bubbles. The gel was allowed to solidify at room temperature, then kept in a humid chamber at 4°C until needed. For reverse zymography, 3 mU u-PA was included in the fibrinogen/plasminogen/thrombin solution.

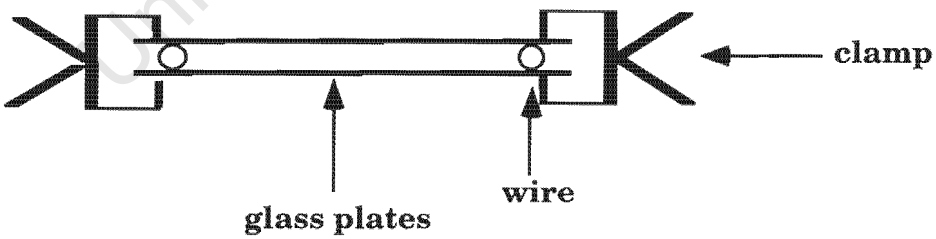

Fig 1. Apparatus required for pouring fibrin-plasminogen agar gels.

3. Suppliers.

All chemical reagents of the highest possible grade were purchased from Sigma, St Louis, MO, Aldrich Chemical Company, Milwaukee, USA, Baker, NJ, USA, or Fisher Scientific, PA, USA.

Tissue culture plastic ware was purchased from Falcon, Becton Dickinson, NJ, USA or Fisher Scientific, PA, USA.

Other reagents were purchased as follows:

American Diagnostica, Greenwich, CT, USA:
PAI-1 ELISA kits; PAI-2 ELISA kits.

Amgen, Thousand Oaks, CA, USA:
Human recombinant GM-CSF; human recombinant G-CSF (specific activity 1 x 10^8 U/mg in a BM colony assay); G-CSF ELISA kits.

Baxter, Miami, FL, USA:
Diff Quik.

Bethesda Res Labs, MD:
Protein molecular weight markers.

Bio-Rad USA:
Protein assay kit.

Calbiochem, La Jolla, CA, USA:
Hydrocortisone.

Leo Pharmaceutical Products, Denmark:
Lyophilised, purified human u-PA.

Flow Labs, McLean, VA, USA:
Linbro 24 well plates.

Genentech, San Francisco, CA, USA:
Human recombinant TGF-ß1.

Gibco BRL, Life Technologies, MD, USA:
McCoy's 5A medium; RPMI medium; nonessential amino acids; L-glutamine; vitamins; sodium pyruvate; essential amino acids.

Hyclone, Logan, UT:
FCS (pretested batch).

International Laboratory for Biological Standards, London, UK:
International u-PA standards.

J.T. Baker, Philipsburg, NJ, USA:
TritonX-100.

Kabi, Stockholm, Sweden:
Lyophilised human fibrinogen.

Kodak, Rochester, NY, USA:
ß-mercaptoethanol; xylene cyanol FF; XAR film.

Medical Resources, Surry Hill, Australia:
GM-CSF ELISA kits.

Pharmacia, Piscataway, NJ, USA:
Ficoll (type 400); Ficoll-Hypaque (1.077 g/cm^3); Protein A and G.

Pierce, USA:
BCA protein assay.

Polaroid, MA, USA:
Polaroid no. 667 film.

Schleicher and Schuell, USA:
Nylon membrane, 0.45µm.

Sigma, St Louis, MO:
Ampicillin; human recombinant PDGF; Bovine serum albumin (fraction V); Sarcosyl.

Synergen, Boulder, CO, USA:
Human recombinant FGF-2.

Syntex, Palo Alto, CA, USA:
Human recombinant IL-1ß.