THE ROLE OF THE HAEMATOPOIETIC MICROENVIRONMENT IN MEGAKARYOCYTOPOIESIS

By

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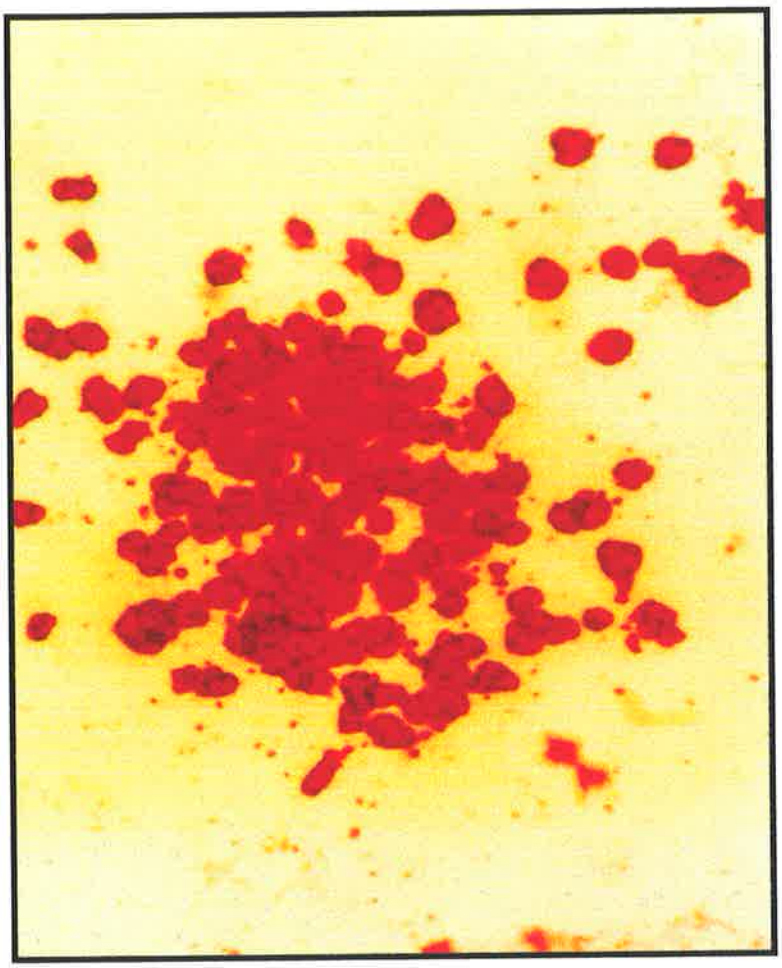

MEGAKARYOCYTE COLONY

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DECLARATION :

The work presented in this does not include any material previously submitted for a degree or diploma in any University. Further, to the best of my knowledge, in does not contain any material previously published by another person unless due reference is given.

I give consent to this copy of my thesis, when deposited in the University libraries, being available for photocopying and loan.

XIAO HUI GUO

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LIST OF ABBREVIATIONS

BFU-E	burst forming unit-erothoid
Bl	blast
BM	bone marrow
CAM	cell adhesion molecule
CB	cord blood
CD	cluster of differentiation
CFC	colony forming cell
CFU	colony forming unit
CSA	cytokine stimulate activity
CS-1	cytokine stimulate-1
CSF	colony stimulate factor
ECM	extracellular matrix molecule
ELAM	endothelial leukocyte adhesion molecule
ME-ETOH	Methanol
Eos.	Eosinophils
FACS	fluorescence activated cell sorting
FC	fluorescein conjugated
FITC	fluorescein isothiocyanate (for a dye of flow cytometry)
FN	fibronectin
FSC	forward scatter
G	granulocyte
GEMM	granulocyte/erythocyte/megakaryocyte/ mactophage
GM	granulocyte/macrophage
GMP	grannule-membrane protein
G0	not cycling
GP	glycoprotein
HSC	hematopoirtic stem cells
ICAM	intercellular cytoadhesion molecule

IMDM	Iscove's modified Dulbecco's medium
LEC	lectin endothrlialcomplement domains
LEU	leucocyte
LFA	leukocyte function
IgG	immunoglobulin
IL	interleukin
LTBMC	long term bone marrow culture
M	macrophage
MAC	macrophage
Meg	megakaryocyte
MEL	leukocyte adhesion molecule
MFI	median fluorescence intensity
MGDF	megakaryocyte growth and development factor
MHC	major histocompatibility class
Min	minutes
MK	megakaryocyte
MoAb	monoclonal antibody
MS-5	Mouse stromal 5 cells
NA	not applicable
NSA	normal serum albumin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PE	Phycoerythrin (use for flow cytometry)
Plt	platelet
RGD	arg-gly-asp
RGDS	Arg-Gly-Asp-Ser
rh	recombinant human
S	spleen
SF	superfamily
SCF	stem cell factor
SLF	steel factor
SSC	side scatter
TEMED	tetramethylethylene diamine

Tetraspan-Plt	tetraspan-platelet
TGF	Thrombopoietin growth factor
TM4	transmembrane 4 superfamily
TPO	thrombopoietin
TSP	throbospondin
VCAM	vascular cytoadhesion molecule
VLA	very late antigen
VNR	vitronectin
VWF	von willebrnad factor

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ABSTRACT

Megakaryopoiesis occurs within the bone marrow microenviroement under the interactive influence of stromal cells, growth factors and extracellular matrix (ECM) on hematopoietic stem cells. The mechanisms regulating megakaryopoiesis are currently not well studied. The extracellular matrix components stimulate stromal and hematopoietic cell proliferation, localise progenitor cells regionally and effect hematopoietic differentiation. An important component of ECM is fibronectin whose ligands are the β1 integrins. The integrins are not signal transducers but likely transduce signals internally via the associated tetraspan molecules.

This study demonstrated that both fibronectin and stromal cells increased and maintained $CD34^+$ cells in culture. Both increased the differentiation of megakaryocyte precursors as indicated by the increase in expression of CD61 and CD41a, and CFU-MKs. The role of integrins CD49d and CD49e, with their associated tetraspan molecules CD63 and the recently described CD151, in megakaryopoiesis activated by fibronectin and stromal cells was then studied. Using immunofluorescence flow cytometry, the majority (94.5%) of $CD34^+$ cells expressed CD49d, whereas a minority (5.4%) expressed CD49e. With the exception of tetraspan CD63, which was positive 21% on CD34+ cells, the other tetraspan molecules (CD9, CD53 and CD15) examined were positive in less than 6% of $CD34^+$ cells. Fibronectin produced an initial fall in CD49d expression which subsequently increased with continued culture, whereas fibronectin increased CD49e expression from the initiation of culture. Preliminary experiments showed that fibronectin led to an increase in tetraspans CD63 and CD151. These were found to be co-expressed with CD49d and CD49e on $CD34^+$ and $CD34^-$ cells. The physical association of CD63 and CD151 was confirmed by immunoprecipitation. Together, these data suggest that interaction of fibronectin with the integrins upregulated their signal transduction molecules. Correlating these changes with the increase in megakaryocyte progenitor differentiation indicates a role for CD49d and CD49e and associated tetraspans in megakaryopoiesis which was induced by fibronectin.

The role of these molecules in megakaryopoiesis was further confirmed in a mouse stromal cell culture model. Antibodies to CD49d and CD49e as well as to CD151

inhibited stromal cell induced megakaryopoiesis as monitored by CD61 and CD41a expression and CFU-MK assay.

CD151 was shown to be physically associated with CD61 by immunoprecipitation. It is important in platelet-platelet interaction as an agonist antibody to CD151-induced platelet aggregation. The data indicate that CD151 associated with CD61 early in megakaryocyte development. Inhibition of the function of CD151 led to decrease in megakaryocytic differentiation highlighting its role in megakaryopoiesis as a signal transducer for the integrins CD49d and CD49e.

This study had shed new light on the factor involved in the complex process of megakaryopoiesis.

Understanding this complex may ultimately led to introduce of strategies to minimise clinical thrombocytopoiesis.

CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION

A major focus in the field of haematology continues to be the identification of the early haematopoietic stem cell (HSC). These may be considered as a set of immature cells located after birth in bone marrow (BM). In the BM, HSC proliferate and differentiate within a microenvironment consisting of stromal cells, extracellular matrix (ECM) and different secreted cytokines, each interacts to direct and coordinate regulation of blood cell development. Adhesion molecules involved in the interaction of HSC with sinusoidal endothelium and stromal cells in BM, may play a cental role in many biological processes. Cell-cell and cell-extracellular matrix interactions are fundamental processes which regulate the proliferation and differentiation of HSC. These adhesive interactions are mediated by specific pairs of cell surface receptors and counter receptors (ligands) including integrin superfamily, immunoglobulin superfamily, p-selectin and cadhesins.

Another important group of molecules is the tetraspans or transmembrane 4 superfamily (TM4) which have two unequal extracellular domains separated by four transmembrane domains. These molecules are associated on the cell surface with other partner molecules, in particular members of the β integrin superfamily. Numerous data suggest a possible functional connection between integrins and tetraspan proteins to form an active complex following integrin-ligand interactions (Rubinstein et al., 1994; Wright et al., 1994; Shaw et al., 1995). Although a function in the regulation of cell proliferation and activation seem likely from some data, unlike the other protein superfamilies, the tetraspan superfamily function is not yet clear (Wright et al., 1994).

The wide clinical and research use of the CD34 antigen as a marker of stem and progenitor cells has contributed to the blanket application of the term "stem cell" to refer to all $CD34^+$ cells in the BM. In fact less then one percent of BM mononuclear cells is $CD34^+$.

Megakaryocytes (MK) are derived from the haematopoietic stem cells. These cells give rise to platelets which play an important role in haemostasis. Most MK progenitors are thought to originate in the BM, where they normally comprise between 0.037 and 0.37 of all marrow cells (Golde et al., 1985). Little is known about the mechanisms which promote proliferation and differentiation of HSC to MK.

Thisstudy was presented the initial aim of isolating of $CD34^+$ cells from cord blood, which were further cultured in different culture systems *in vitro*, such as serum free medium alone, in the presence or absence of fibronectin, stromal cells and specific cytokines to assess the role of β integrins and tetraspan molecules in megakaryopoiesis. This introduction comprehensively reviews the process of haematopoiesis, the BM microenvironment, $CD34^+$ cells and adhesion molecules, with tetraspan superfamily relevant to MK proliferation and differentiation.

1.2.THE PROCESS OF HAEMATOPOIESIS: AN OVERVIEW

1.2.1 BONE MARROW ANATOMY

The bone marrow, one of the largest organs in the human body, is the principal site for blood cell formation. In the normal adult its daily production amounts to about 2.5 billion red cells, 2.5 billion platelets and 2.0 billion granulocytes per kilogram of body weight (Nathan et al.,1987).

Bone marrow is an extremely complex tissue and sustained cellular production depends on the presence of pools of primordial cells and stem cells, capable of both self-replication and differentiation. The most primitive pool consists of pluripotential stem cells with the capacity for continuous self-renewal. The more mature pools consist of differentiated progenitor cells with maturation restricted to single cell type and with no capacity for self-renewal. The proliferative activity of these pools involves humoral feedback from peripheral target tissues as well as cell-cell interactions within the microenvironment of the marrow. The microenvironment of the marrow cavity (Weiss, 1976; Wolf, 1979) is a vast network of vascular channels or sinusoids that separate clumps of haematopoietic cells, including fat cells which are found in the intrasinusoidal spaces. The vascular and haematopoietic compartments are joined by reticular fibroblastoid cells that form the adventitial surfaces of the vascular sinuses and extend cytoplasmic processes to create a lattice on which blood cells are found. The location of the different haematopoietic cells is not random. For example, clumps of megakaryocytes (MK) are found adjacent to marrow sinuses. They shed platelets, the fragments of their cytoplasm, directly into the lumen.

1.2.2 HAEMATOPOIESIS: ANATOMY AND PHYSIOLOGY

Haematopoiesis is the complex process that results in the continuous production of the full spectrum of erythroid, myeloid and lymphoid cells throughout the lifespan of an organism. During human development, haematopoiesis originates in the embryonic yolk sac but shifts to the fetal liver and spleen, and later to the fetal bone marrow (Nathan et al., 1987).

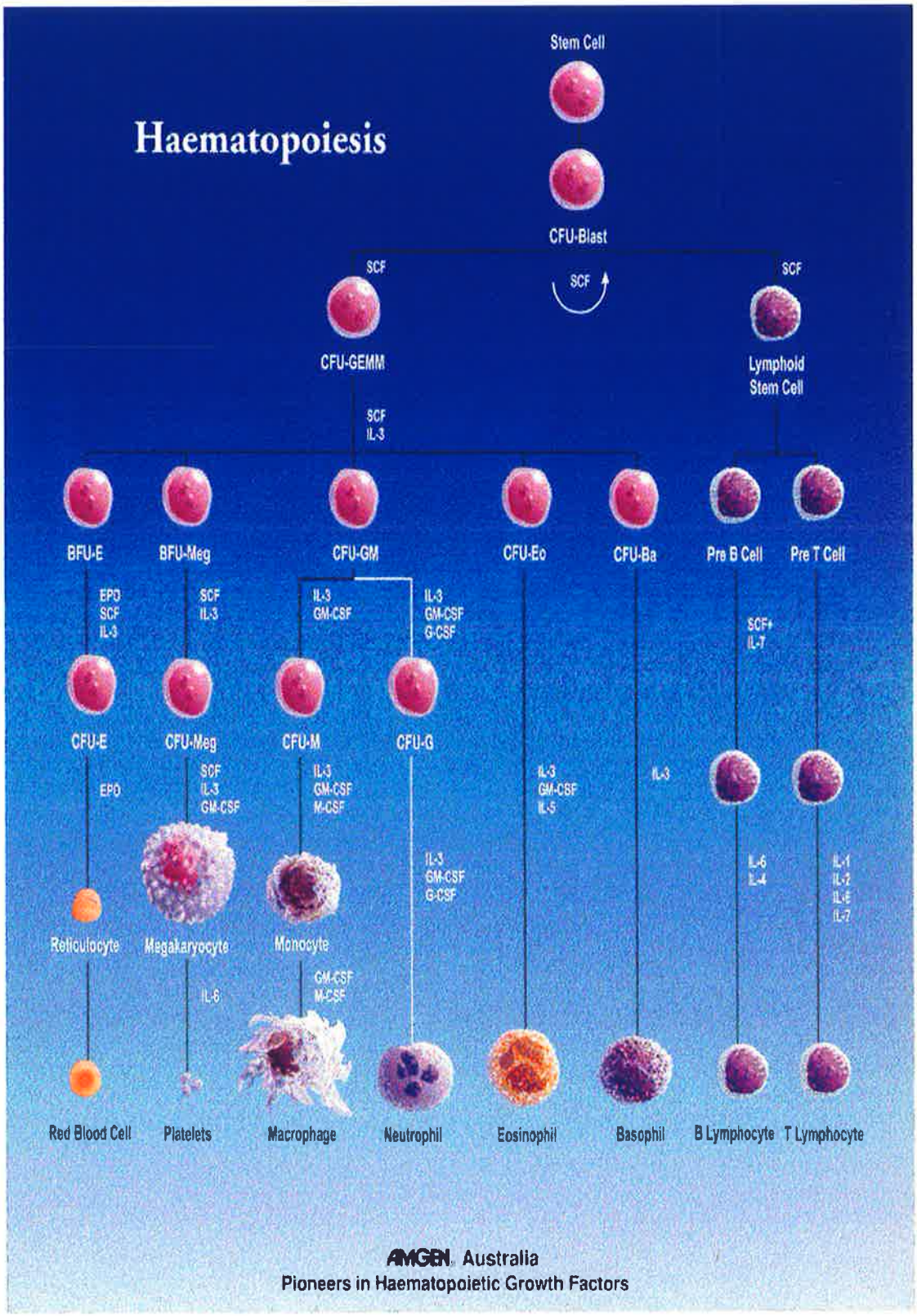
The haematopoietic system is remarkable not only for its huge quantitative output of cells, but also for its ability to respond efficiently to a variety of physiological stimuli, including blood loss, tissue injury and immunologic challenge. This system is supported by a relatively small pool of primitive cells, the pluripotent haematopoietic stem cells, which have estimated frequency of approximately 2 in 100,000 bone marrow mononuclear cells (Nathan et al., 1987). This pluripotent stem cell, capable of both self-renewal and differentiation into all haematopoietic lineages, still forms the basis of current models of haematopoiesis (Lichtman et al., 1981). Differentiation of the pluripotent stem cell results in the production of increasingly mature pools of progeny, called haematopoietic progenitor cells. In the adult the primary site of haematopoiesis is the bone marrow, where haematopoietic stem and progenitor cells are closely associated with stroma, a heterogeneous population of connective and vascular tissue cells, including smooth muscle, reticular cells, adipocytes, osteoblasts, and vascular endothelial cells (Weiss, 1976).

1.2.3 PROLIFERATION AND DIFFERENTIATION OF HAEMATOPOIETIC STEM CELLS

Most haematopoietic stem cells are quiescent in the G_0 stage of the cell cycle. A complex system of events is required by which haematopoietic stem cells begin to proliferate and either self-renew or commit to generate the large population of mature blood cells continuously required during life Fig.1.1. However, the most mature progenitor cell populations cycle more actively and are capable of extensive proliferation and expansion in response to a number of interacting cytokines, and in bone marrow, where stem cells and progenitor cells are closely associated with the bone marrow microenvironment.

1.2.3.1 THE CD34 PHENOTYPE OF STEM CELLS

The discovery and characterisation of the glycophosphoprotein cell surface antigen CD34 opened the door to numerous laboratory and clinical therapeutic studies by providing a basis for phenotypic characterisation, quantitation, and isolation of haematopoietic cells (Krause et al.,1996). [CD stands for 'cluster of differentiation' and denoted originally a


Haematopoiesis
Stem Cell
CFU-Blast
SCF
SCF
SCF
CFU-GEMM
Lymphoid Stem Cell
SCF
IL-3
BFU-E
BFU-Meg
CFU-GM
CFU-Eo
CFU-Ba
Pre B Cell
Pre T Cell
EPO
SCF
IL-3
SCF
IL-3
IL-3
GM-CSF
IL-3
GM-CSF
G-CSF
SCF+
IL-7
CFU-E
CFU-Meg
CFU-M
CFU-G
EPO
SCF
IL-3
GM-CSF
IL-3
GM-CSF
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GM-CSF
G-CSF
IL-6
IL-4
IL-1
IL-2
IL-6
IL-7
Reticulocyte
Megakaryocyte
Monocyte
GM-CSF
M-CSF
IL-6
Red Blood Cell
Platelets
Macrophage
Neutrophil
Eosinophil
Basophil
B Lymphocyte
T Lymphocyte
AMGEN Australia
Pioneers in Haematopoietic Growth Factors

FIG. 1.1 HAEMATOPOIETIC STEM CELLS PROLIFERATION AND DIFFERENTIATION. (Copy from AMGEN).

cluster of monoclonal antibodies that was found during workshop studies to react similarly in various tests, ultimately therefore recognising a common cell-surface antigen.]

CD34 is expressed on developmentally early lymphohaematopoietic stem and progenitor cells (Civin et al., 1984), small-vessel endothelial cells, and embryonic fibroblasts. $CD34^+$ bone marrow cells comprise only 1.5% of marrow mononuclear cells, but contain progenitor cells for all lymphohaematopoietic lineages, as evidenced by the finding that $CD34^+$ cells purified from marrow can reconstitute haematopoiesis of primates, humans, or mice undergoing autologous marrow reinfusion after BM transplantation (Schmitz et al., 1995). CD34 haematopoietic cells obtained from marrow or blood are in clinical use in transplantation and gene therapy studies, including ongoing attempts to expand haematopoietic stem/progenitor cells ex vivo. Although CD34 is an important marker of early haematopoietic stem/progenitor cells in experimental and clinical haematopoiesis, the function of CD34 is not yet clear. CD34 may have a role in such fundamental processes as haematopoietic progenitor cell development and inflammation. Recent experiments on the function of CD34 indicate that CD34 expressed on endothelial cells may play a role in leukocyte adhesion and "homing" during the inflammatory process, and it has been hypothesised that CD34 plays a role in stem/progenitor cell localisation/adhesion in the BM. (Civin et al.,1987).

1.2.3.2 FREQUENCY AND PHENOTYPE OF $CD34^+$ CELLS

CD34 was discovered as the result of a strategy to develop antibodies that specifically recognise small subsets of human marrow cells but not mature blood or lymphoid cells (Civin et al.,1984). In recent years the determination of the number of $CD34^+$ cells has become the standard for the estimation of the number of haematopoietic stem cells.

CD34 antibodies specifically detect an average of only 1.5% of low-density mononuclear cells from BM aspirates of normal donors (Civin et al., 1984). In contrast, there is less than 0.5% CD34 labelling of peripheral blood (PB) cells and approximately 1% of the mononuclear cells in cord blood (CB) (Civin et al., 1987). The $CD34^+$ cell population from normal human BM is enriched in morphologic blast cells, in contrast to the $CD34^-$ cell fraction, which contains predominantly recognisable precursor cells at diverse maturational stages of haematopoiesis.

The discovery of CD34 as a haematopoietic cells-surface antigen has transformed and accelerated studies into developmental haematopoiesis. Immunoaffinity-purified $CD34^+$ marrow and cord blood cells are 10- to 100-fold enriched in colony-forming units (CFUs) whereas $CD34^-$ cells population are depleted in CFUs. The $CD34^-$ cells include CFU-macrophage (CFU-M), CFU-granulocyte (CFU-G), CFU-GM, burst-forming units-erythoid (BFU-E), and are particularly enriched in the earliest types of colony-forming cells, such as CFU-mix and CFU-blast (Straauss et al., 1986). A fraction of most mature unipotent CFU, such as CFU-E and CFU-G, can be found in the $CD34^-$cell fraction. There are two distinct populations of $CD34^+$ BM cells that differ in their relative levels of surface CD34 expression by flow cytometric analysis:

1. The CD34 ^{bright} population contains the majority of the immature haematopoietic progenitor cells,

2. The CD34 ^{dim} population contains more lineage-committed progenitors as assayed *in vitro*. The CD34 ^{bright} populations enriched for unipotent progenitors, including CFU-GM, BFU-E, CFU-E, CFU-MK and even more highly enriched for the developmentally early multipotential CFU-GEMM (CFU-mix), CFU-blast, and long-term culture initiating cells. The very immature lymphoid cells are also $CD34^+$. The stage-specific expression of CD34 in both human and murine haematopoietic stem/progenitor cells suggests a potential regulatory role for CD34 in the early phase of developmental haematopoiesis.

1.2.3.3 *IN VITRO* QUANTITATION OF HAEMOPOIETIC STEM CELLS

Siena and coworker were the first group to describe CD34 measurement by flow cytometry as a measurement of HSC in mobilised peripheral blood (Siena et al., 1989). CD34 measurement was found to have obvious advantages over traditional CFU-GM assay, in particular the result is available the same day instead of 14 days later. Now, flow cytometric analysis of CD34 immunostained blood or marrow preparations is the standard quality control method for harvested BM.

The first *in vitro* functional assays were the haematopoietic colony assays that evaluate ability of committed progenitor cells to produce one or more haematopoietic lineages after culture in permissive conditions (Dexter et al., 1974). The assays for erythriod and myeloid

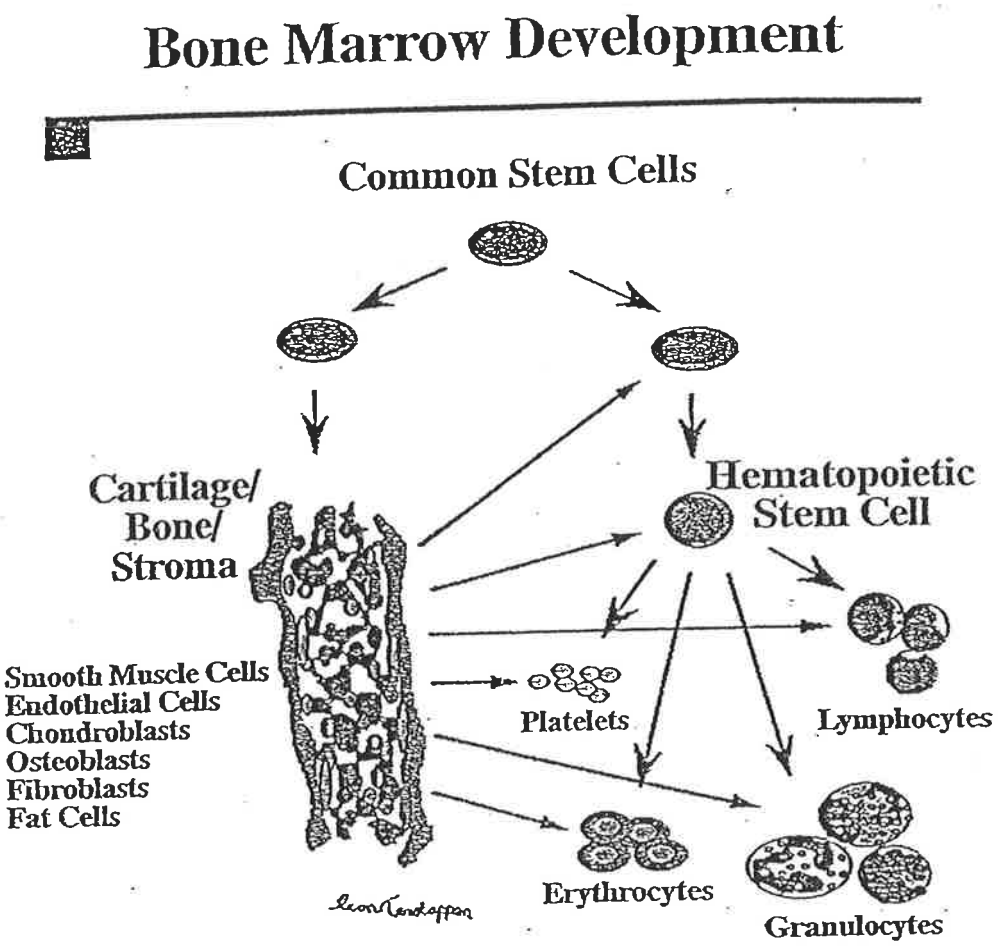
progenitors require quantification of morphologically distinct colonies 7 to 20 days after plating in semi-solid media supplemented with cytokines. These include assays for colony forming unit (CFU)-granulocyte macrophage (CFU-GM), burst-forming units (BFU)- and CFU-erythiod, CFU-megakaryocytes (MK) and CFU -granulocyte/erythrocyte/ monocyte/megakaryocyte (GEMM). The long-term culture system assay can detect an even more primitive progenitor associated with long-term repopulating ability. This cell is defined by its ability to produce CFU-GM, BFU-E, and CFU-GEMM at 5 week or more than ten weeks when co-cultured with marrow stromal cells in appropriate liquid culture system (Sutherland et al., 1989).

1.2.4 HAEMATOPOIETIC MICROENVIRONMENT

The haematopoietic microenvironment can be categorised into three main areas, the cellular elements, the haematopoietic cytokines, and the extracellular matrix molecules. The cellular elements consist of haematopoietic stem cells and progenitor cells that gave rise to mature blood cells and various types of connective tissue cells that comprise the bone marrow stroma (fibroblasts, macrophages, endothelial cells and fat cells). The haematopoietic cytokines consist of a variety of growth factors: stem cell growth factor, macrophage colony-stimulating factor, various interleukin growth factors and especially megakaryocyte growth development factor (MGDF). All of these cytokines have been cloned and (for many) their receptors identified (Migliaccio 1996). The extracellular matrix was long considered to be a structural scaffolding for many tissues. It is appreciated as a dynamic, complex structure that plays an important role in the development, adhesion, and migration of cells. Matrix components stimulate stromal cell proliferation, augment the regional localisation of progenitor cells, and effect haematopoietic cells proliferation and differentiation. Likewise, complex interactions between human cytokines and extracellular matrix seemingly play a role both in compartmentalising haematopoietic cytokines and in presenting complex developmental signals to developing blood cells (Wunder et al., 1994) Fig1.2.

FIG. 1.2 HAEMATOPOIETIC MICROENVIRONMENT

The haematopoietic microenvironment has functional significance for mature and developing blood cells. In this process involves cell: cell, cell:ECM, and cell growth factors interactions.



1.2.4.1 STROMAL CELLS

Proliferation and differentiation of normal haematopoietic stem cells (HSC) occurs within the human bone marrow microenvironment, which comprises stromal cells including fibroblasts, adipocytes, macrophages, smooth muscle and endothelial cells. The importance of stromal cells for the proliferation of early haematopoietic cells has been demonstrated using precursors of colony forming unit (CFU) which only proliferate and produce CFC in the presence of stromal cells (Andrews et al., 1989). These stromal cells also influence haematopoietic cells differentiation. Alterations of culture conditions in murine LTBMCs can determine whether myloid or pre-B cells are produced.(Whitlock et al., 1982).

The development of haematopoietic stem cells *in vivo* and *in vitro*, demonstrates that the haematopoietic microenvironment of the haematopoietic organs is mediated largely by this phenotypically and perhaps functionally diverse population of stromal cells that endow these organs with the unique capacity to support haematopoiesis (Dexter et al., 1977; Whitlock et al., 1982). Much effort has been devoted to understanding the mechanisms responsible for the ability of stromal cells to promote the survival and differentiation of primitive HSC. From most studies it appears that stromal cells from bone marrow not only release a wide variety of cytokines with a range of biological activities on HSC (Alberico et al., 1987; Donshkind et al., 1990) but also express a broad repertoire of adhesion molecules that serve to mediate specific interactions with HSC (Tavassoli et al., 1968).

It has been known for many years that the stromal tissues of the haematopoietic organs are necessary for haematopoietic support and that they contribute to the direction of the type of haematopoiesis which occurs *in situ* (Chabannon et al., 1992). Beginning with the early studies of Dexter and coworkers it has been shown that a murine liquid marrow culture system that allowed both colony-forming and reconstituting haematopoietic progenitors to be propagated for many months. It was stromal dependent and called the long-term bone marrow culture (LTBMC) system that support either myelopoiesis or lymphopoiesis (Whitlook et al.,1982; Tavassoli et al., 1968). Long-term cell production in each case depends on formation of an adhesion layer of cells which may be an *in vitro* counterpart to the *in vivo* haematopoietic microenvironment that serves as a nurturing base for proliferation and differentiation. In these culture, the HSC with the highest renewal

potential either rest or and adhere to the stromal cells that are attached to the plastic substrate (Andrews et al., 1989).

1.2.4.2 STROMAL CELL TYPES

The cell population comprising the stroma in murine Dexter cultures consists of several defined cell types as well as an uncharacterised fraction. The predominant plastic-adherent cells in murine Dexter stroma appears to be the macrophage, comprising approximately 70-80% of adhesion cells (Gualtieri et al., 1984). The next most frequent is an alkaline phosphatase-positive, pradipocytic cell that is probably a fibroblast and may be the *in vitro* analogue of the adventitial reticular cells described *in vivo* (Moore, 1975). In addition Wolf and colleagues (1968) investigated that endothelial cells, another component of stroma. However, there is a small number of plastic-adhesion cells (<5%) that cannot be classified specifically; the role of these cells in haematopoiesis is still undetermined.

1.2.4.3 PRODUCTION OF CYTOKINES BY STROMAL CELLS

In vitro the role of stromal growth factor in regulating haematopoiesis in Dexter cultures was controversial. Stromal cells produce a wide variety of defined cytokines. Initially, investigators had difficulty ascertaining colony-stimulating activities in the Dexter culture but finally, CSF-1 and undefined colony-stimulating activities were detected in conditioned culture. A number of studies followed and showed that stromal cells were capable of producing a wide variety of cytokines (Long et al., 1992). These are presented in Table 1.1.

1.2.5 EXTRACELLULAR MATRIX MOLECULES

There is a fundamental postulate in cell biology that cells normally adherent *in vivo* require attachment and spreading *in vitro* to proliferate and to differentiate. Much research has focused attention on the nature of adhesive macromolecules in the extracellular matrix (ECM) and the characteristics and consequences of the interaction with cell-surface receptors (Burridge et al., 1988). Numerous bone marrow ECM molecules are produced and secreted by epithelial cells, macrophages, fibroblasts (reticular, adventitial and barrier cells), bone-lining mesenchymal cells, and hematopoietic cells present in the extravascular haematopoietic cords (Burridge et al.,1988). The primary constituents of bone marrow

TABLE 1.1 PRODUCTION OF CYTOKINES BY BM STROMAL CELLS

BM stromal cells production of cytokines. Table 1.1 provides all definition for abbreviations. Copy from long (eds), 1992 table 3.8, page of 60.

Cytokine	Cell Source	Induction	Study
GM-CSF	Murine stroma	Detectable in normal and irradiated stroma and further induced by lectin, IL-1, and lithium	Kittler et al.[154] Alberico et al. 1989[163] McGrath et al. 1987[164]
	Clone murine stromal line	IL-1 and LPS induce, not constitutive	Rennick et al. 1987[54]
	Human stroma	IL-1 increased by inducing ribonuclease activity	Bagby et al. 1989[165]
G-CSF	Murine stroma	In irradiated and normal stroma and induced by IL-1, pokeweed mitogen and lithium	Kitler et al.[154]
	Human marrow	Baseline and further induced with IL-1	Fibbe et al. 1988[47]
	Clone murine stromal line	IL-1 and LPS induce, not constitutive	Rennick et al. 1987[54]
CSF-1	Murine	RIA detectable, irradiated and nonirradiated	
	Human marrow	RIA detectable	Fibbe et al. 1988[47]
IL-1β	Long-term human culture	ELISA, induced by IL-6	Kurihara et al. 1990[39]
IL-3	Normal human stroma	Polymerase chain reaction	Barge et al. 1989[166]
	Normal or irradiated murine stroma	Polymerase chain reaction	Kittler et al.[154]
IL-11	Primate stromal line and PV-34		Paul et al. 1990[57]
c-*kit* ligand	Murine		Kittler et al.[154]
TNF	Bone marrow stroma	IL-4 (did not upregulate expression of CSF-1 or TGF-β).	
		Il-4 pretreatment for 5 to 7 days induced inhibitory activity across an agar layer but not in supernatants (reversible with IL-3, also hydrocortisone) (139)	Peschel et al. 1989[167]
IL-6	Human	No constitutive, increase by IL-1α or TNF-α	Nemunaitis et al. 1989[4]

ECM include collagens (type I, II, III, V, and XI), glycoproteins (fibronectin, laminin, thrombospondin, haemonectin, and tenascin), and glycosaminoglycans (hyaluronic acid and chondroitin dermatan, and heparan sulfates) (Williams et al., 1991). It is thought that the process of cell differentiation requires a number of cell divisions, and cell movement involving the making and breaking of interactions with ECM. By serving as dynamic physical intercellular "bridges," ECM molecules assist in facilitating cell-cell communication by directly conveying information to haematopoietic cells though specific cell-surface receptors and by co-localising and stabilising growth factor interactions between stromal and haematopoietic cells (Yoder et al., 1995; Verfaillier et al., 1994;Williams 1993).

1.2.5.1 FIBRONECTIN

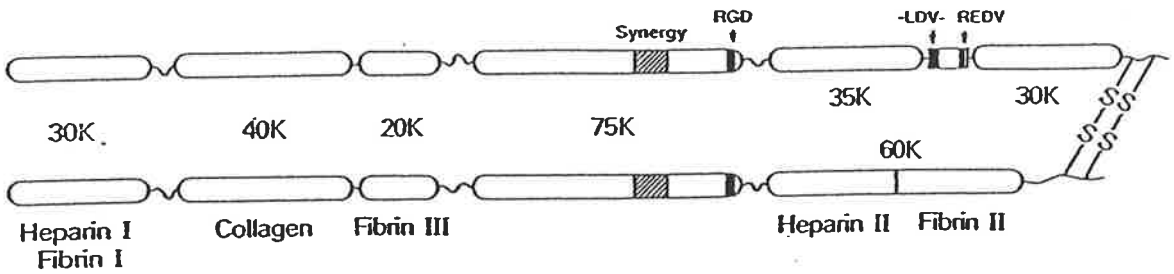
Fibronectin, a major component of bone marrow ECM, is considered to have an important role in haematopoiesis (Dexter,1982). It is a multifunctional glycoprotein that is essential for embryogenesis (Potts et al., 1994) and is involved in cell migration, adhesion, proliferation, differentiation and interaction. Fibronectin is present in both soluble and insoluble forms in body fluids and in tissue ECM. Fibronectin exists in a number of variant forms that differ in sequence at three general regions of alternative splicing of its precursor mRNA; for example, there can be 20 different forms of human fibronectin subunits. All fibronectin molecules appear to contain the same basic functional domains shown in Fig 1.3.

Fibronectin is present in the bone marrow microenvironment, although significant differences across species may exist in its degree of expression (Potts et al., 1994). In contrast, fibronectin expression appears limited to platelets, megakaryocytes, and walls of blood vessels in normal human bone marrow biopsy specimens (Lerat et al., 1993).

HSC and committed progenitor cells predominantly interact with fibronectin through expression of cell-surface receptors belonging to the integrin superfamily. Schick et al, 1998, demonstrated a role for fibronectin in MKs maturation and platelet production. Fibronectin was secreted by MKs and adhered to their surface. They also identified the β integrins VLA-5 on the MKs surface as the major integrin mediating the binding of MKs to fibronectin. In addition, Sugahara and co-workers (1994) investigated the effects of

FIG. 1.3 FIBRONECTIN AND OTHER CELL INTERACTIVE GLYCOPROTEINS

Function domains of fibronectin. This large glycoprotein is depicted as a dimer : of similar but nonidentical subunits linked by a pair of carboxyl-terminal disulfide bonds. Cell-binding regions of sequence are indicated along the top; the 75KD central cell-binding domain contains the Arg-Gly-Sap (RGD) sequence and a synergy region that interacts synergistically with the GGD sequence to mediate adhesion; the IIICS region contains the minimal active sequence Leu-Asp-Val (LDV) as well as Arg-Glu-Val (REDV). Copy from Hay, 1991. Fig. 4.2, page, 111.



fibronectin on the proliferation of a myloid leukemia cell line, M07E, and showed that fibronectin is capable of inducing apoptosis via its interaction with VLA-5, thus raising the possibility that the fibronectin-VLA interaction may contribute, at least in part, to a negative regulation of haematopoiesis.

1.2.6 HAEMATOPOIETIC CYTOKINES

It is well understood that haematopoietic cells proliferation and differentiation is controlled by polypeptide growth factors (cytokines). Within the past decade, approximately 16 cytokines (11 interleukins, macrophage colony stimulating factor, erythropoietin, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, c-kit ligand and γ-interferon) have been identified, cloned and examined for their function in haematopoietic cell development (Testa et al., 1993). The haematopoietic cytokines consist of a large and growing number of molecules, predominantly identified to have stimulatory or inhibitory effects on haematopoiesis. These cytokines include the colony forming-stimulating factors (CSFs), which were first defined in clonal culture systems, the interleukins (IL), which were first described in immunologic studies, and other protein growth factors (Ogawa, 1993). A large body of literature has shown that these cytokines are produced by a variety of haematopoietic and stromal cells and have overlapping or redundant effects on a variety of haematopoietic cells (Metcalf, 1993). Ogawa has categorised the CSFs and ILs in terms of the relative maturity and lineage specificity of the haematopoietic cells on which they have an effect (Ogawa, 1993). The early acting factors include stem cell factor SCF, c-kit ligand, steel factor, IL-6, granulocyte CSF (G-CSF), IL-11, Il-12 and leukaemia inhibitory factors. The intermediate-acting, lineage-nonspecific factors include SCF, IL-3, granulocyte-macrophage-CSF (GM-CSF), and IL-4. The late-acting, lineage-specific factors include G-CSF, IL-5, and erythropoietin.

SCF. During the last few years, several groups have identified a novel cytokine termed c-kit ligand, stem cell factor, mast cell growth factor or steel factor which is known to bind to immature haematopoietic cells and to act synergistically with other haematopoietic growth factors.

IL-3. Interleukin-3 (IL-3) was originally defined in mice as a factor that was released by a certain monocytic leukaemia cell line (WEHI-3B) or by activated T cell, that stimulated growth and differentiation of mast cells and precursors of all haematopoietic lineages. *In vitro*, effects of IL-3 are probably restricted to the pluripotential haematopoietic stem cell and its derivatives, including the mature effector cells of certain lineages. *In vivo*, IL-3 functions as a link between the immune and haematopoietic systems, stimulating the generation and the function of blood cells.

IL-6. Testa and co-workers (1993) has described IL-6 as a multifunctional cytokine acting on a wide variety of cells. It has been called by various names related to the biological function. *In vitro*, IL-6 induces megakaryocyte maturation and T cell proliferation (Arai et al.,1990). *In vivo*, it induces megakaryocyte maturation in mice and monkeys.

Thrombopoietin. Thrombopoietin or Megakaryocyte Growth and Development Factor (MGDF) has a key role in the development of megakaryocytes. Its role will be discussed in detail in the section on Megakaryopoiesis Fig.1.4.

TABLE 1.2 THE MAJOR HAEMATOPOIETIC GROWTH FACTORS

Growth factor	Target cell(s)
SCF	Early stem/progenitor cells
IL-3	CFU-GEMM, BFU-E, CFU-GM, CFU-Meg, CFU-Eos, CFU-M,GM-CSF,CFU-GM, BFU-E, CFU-GEMM, CFU-Eos, CFU- Meg, CFU-G, CFU-M, maturing neutrophils, monocytes, eosinophils
IL-6	B cells, plasma cells
MGDF	Early stem/progenitor cells, megakaryocytes

CSF, colony stimulation factor; CFU, colony forming unit; GEMM, granulocyte/erythocyte/megakarycyte/macrophage; Eos, eosinphils; GM, granulocyte/macrophage; BFU, burst forming unit. (Copy from table 1-1 of .Robinson, 1990).

1.3.IMPORTANCE OF INTERACTION WITHIN THE HAEMATOPOIETIC MICROENVIRONMENT

In the bone marrow, haematopoietic progenitor cells proliferate and differentiate within a specific microenvironment consisting of stromal cells and their associated extracellular matrix and various secreted cytokines. Each has functional significance for both mature and developing blood cells. In essence, the interactions between the HSCs and their microenvironment can be simplified into three categories: cell-cell, cell-extracellular matrix, and cell-cytokine.

1.3.1 HAEMATOPOIETIC STEM CELL AND STROMAL CELL INTERACTIONS

The method by which stromal cells regulate haematopoiesis is believed to occur through cell-cell contact interactions and release of growth factors. Cell-cell interactions are concern with the cytoadhesive capacities of haematopoietic progenitor cells for stromal cells layers and vice versa (Dexter, 1982). The proliferation and in vitro expansion of HSC has been the focus of many investigations and cell culture systems. The Dexter culture system (Dexter et al., 1977) provides an important tool to study the mechanisms of haematopoietic-stromal cells interactions. Heterogenous adherent cell layers derived from BM stroma (endothelial cells, macrophages/monocytes, fibroblasts, adipocytes etc) have been shown to support long-term bone marrow culture. This culture system showed that the proliferation and differentiation of haematopoietic progenitor cells involves cell-cell interaction (Verfaillie et al.,1990).

Lichtman was one of the first to observe stromal cell-haematopoietic cell interactions as seen in the developing erythroblastic islands, which consist of a central macrophage surrounded by maturing erythroblasts (Lichtman 1984). This tight adhesion results from fibronectin-mediated attachment (Tsai et al.,1987). Simmons and co-workers described vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the

binding of haematopoietic progenitor cells (Simmons et al., 1992). At the primitive haematopoietic cell level, studies by Gordon and colleagues documented that human blast-colony forming cells adhere to preformed stromal cell layers (Gordon et al., 1990). Other studies have shown that CD34 selected marrow cell populations attached to stromal cell layers, and that the attached cells are enriched for granulocyte/macrophage progenitor cells (Liesveld et al., 1989). In the murine system, highly enriched spleen colony-forming cells (CFU-S), cultured on stromal cell layers, proliferate and differentiate into haematopoietic cells (Lichtman, 1981). Such *in vitro* studies highlight the ability of the stromal microenvironment to regulate haematopoiesis through a complex interplay of haematopoietic cells with soluble cytokines, ECM, and stromal cells. Stromal cells play a important role in the survival, proliferation and differentiation of $CD34^+$ progenitor cells and potentially influence the self-renewal of pluripotent progenitors cells.

MKs progenitors grow and differentiate in bone marrow, where the microenvironment play a crucial role in megakaryopoiesis. A specific association noted in marrow is the connection of megakaryocytes with the venous sinus endothelial cells (Liesveld et al., 1989). This relationship may be mediated by thrombospondin (a multifunctional adhesion protein) in the extracellular matrix. Thrombospondin can bind fibrinogen and together thereby bridge the platelet glycoprotein IIb/IIIa integrin receptors on megakaryocyte and their progenitors with stromal cells Table 1.3. Although the importance of adhesive interactions in haematopoiesis has been well studied, little is known about the role of adhesion molecules in MK differentiation. The integrins and associated tetraspan super family molecules may be critical for the interactions between MKs progenitors and stromal cells during megakaryopoiesis.

1.3.2 HAEMATOPOIETIC STEM CELL AND EXTRACELLULAR MATRIX (ECM) INTERACTIONS

It is widely thought that the interaction of HSCs and bone marrow extracellular matrix is crucial for the development of mature blood cells. Experimental proof of this hypothesis focused attention on the nature of adhesive macromolecules in the ECM and the characteristics and consequences of their interaction with cell-surface receptors. The major

TABLE 1.3 HAEMATOPOIETIC CELL: CELL INTERACTIONS

Table 1.3 summarises haematopoietic cell: cell interactions. These cytoadhesion molecules mediate both antigen recognition and cell-cell communication. Both the immunoglobulin and integrins superfamilies are involved. Copy from Long (eds), 1992. Table 2.2, page 26.

Molecules mediating specific interactions			
Cell receptor	Receptor cell expression	Ligand, co-, or counter-receptor	Ligand,co- orcounter receptor,expression
Immunoglobulin superfamily			
MHC I	macrophage, T cell	CD8, TCR	T cells
MHC II	macrophage, T cell	CD4, TCR	T cells
	endothelial cells, neutrophils HPC, Bcells,T cells, macrophages	LFA1	monocytes,T &Bcells
ICAM-2	endothelial cells,	LFA1	monocytes,T&B cells
LFA2	Tcells	LFA3	T cells, erythrocytes
Integrins			
MAC1	macrophages, neutrophils	fibrinogen, C3bi	endotheilal cells, platelets
LFA1	macrophages, neutrophils	fibrinogen, C3bi	endotheilal cells, platelets
VCAM	endothelial cells	VLA-4	lymphocytes, monocytes,B prrogenitors
gp150,95	macrophages, neutrophils	VLA-4	
Fibronectin receptor	BFC-E though reticulocytes	fibronectin	NA
IIb/IIIa	platlets, megakaryocytes	fibronectin, TSP, vitronectin,vWF	endthelial cells
Selectin/LEC-CAM			
LEC-CAM-1(MEL14)	endothelial cells	addressins, negativ charged oligosaccharides	lymphocytes
ELAM-1 (LEC-CAM-2)	endothelial cells	sialyl-Lewis x2	endothelial cells neutrophils, tumor
LEC-CAM-3 (GMP140)	Platelet granules, Weible-Palade bodies, endothelial cells	Lewis x (CD15)	cells,endothelial cells neutrophils

adhesive macromolecules for epithelial and mesenchymal cells include fibronectin (FN), vitronectin, laminin, entactin, the fibrillar collagens (types I, II, III, V, and XI) and the collagen in basement membranes (type IV); these component interactions depend on various cell-surface receptors.

Early *in vivo* work by Wolf and Trenton (Zuckerman et al., 1983) provided evidence that components of the microenvironment are responsible for the granulocytic predominance of bone marrow haematopoiesis and the erythrocytic predominance of the spleen. Dexter and co-workers showed that *in vitro* development of a suitable adherent population of bone marrow-derived cells is essential for the maintenance of cell proliferation and differentiation in long-term bone marrow culture system. Zuckerman and colleagues (Zuckerman et al., 1983) demonstrated that specific ECM components such as fibronectin, laminin and various collagens and proteoglycans were present in long-term marrow cultures and the deposition of these proteins coincided with the onset of hematopoietic cell production. ECM components such as fibronectin have been detected by immunohistochemical techniques on substrate attachment surfaces of mouse marrow stromal cells and at the sites of interaction between these cells and developing granulocytes/monocytes (Tsai et al., 1987). Tsai also reports that primitive erythroid progenitors have been found to bind to the cell-binding domain. A number of investigations point to the influence of ECM in promoting cellular development. Gospodarowicz (1980) demonstrated that ECM greatly affects corneal endothelial cell proliferation *in vitro*. Table 1.4 shows the function of both previously known and newly identified ECM components in haematopoietic cell production. Of major importance to the present study is the interaction of fibronectin with megakaryocytes via the β integrins.

1.3.3 HAEMATOPOIETIC STEM CELL AND GROWTH FACTOR INTERACTIONS

Most haematopoietic stem cells are quiescent in the G_0 stage of the cell cycle. Certain haematopoietic cytokines can bind to their corresponding receptors on the stem cell surface, shortening the G_0 stage of the cell cycle, and stimulating cell division. (Arai et al., 1990). More recently, two early acting synergistic cytokines, derived from stroma (IL-11 and

TABLE 1.4 HAEMATOPOIETIC CELL: EXTRACELLULAR MATRIX INTERACTIONS

Matrix component	cell surface receptor	Cellular expression
Fibronectin	FNR	Erythroid
	BFC-E	
	B cells	
	Lymphoid cell lines	
	HL60	
	IIb/IIIa	platelets and MKs
Thrombospondin	TSP-R	monocytes and platelets
		Human CFC,CFC-GEMM
Hyaluronic acid	CD44	T and B cells
		Neutrophils
		Tum or cells
Hemonectin	Unknown	CFC-GM, BFU-E
		immature neutrophils
		BFU-E
Proteoglycans heparan sulfate	Unknown	Bl-CFC
Unfractured ECM	Unknown	Bl-CFC, bone marrow stroma

Table 1.4 shows haematopoietic cell and extracellular matrix interactions. This study identified the function of matrix components in haematopoietic cell cytoadhesion. Copy from Long (eds), 1992.

SCF), have been shown to shorten the G_0 period of early progenitors (Tsuji et al., 1992). Within the past decade, 16 haematopoietic cytokines have been identified. These include 11 interleukins, macrophage-CSF, erythropoietin, granulocyte (G)-CSF, GM-CSF, and gamma-interferon. The recent cloning of the receptors for these cytokines has indicated that a number of these receptors have amino acid homologies, suggesting that they are members of one or more gene families (Arai et al., 1990). Like other cell surface receptors, haematopoietic cytokine receptors structurally contain a large extracellular domain, a transmembrane region, and a cytoplasmic domain, the sequence of which seems to be unique (Arai et al., 1990). The extracellular domains of IL-1, IL-6, and gamma-interferon receptors contain sequence homologies with the immunoglobulin gene superfamily. Weak but significant amino acid sequence similarities exist among the IL-2 (β chain), IL-6, IL-3, IL-4, erythropoietin, and GM-CSF receptors (Arai et al., 1990).

1.4 MEGAKARYOCYTE DIFFERENTIATION AND PROLIFERATION

1.4.1 INTRODUCTION

Megakaryocytes (MKs) are derived from an undifferentiated HSC, a single MK giving rise to approximately 2000 platelets which play an important role in blood coagulation (Alan 1995). MKs represent only 0.05% of the cells in bone marrow (Harker, 1968). In megakaryocytopoiesis, committed MK progenitor cells (CFU-MK) proliferate and differentiate into MKs. MK development in turn is characterised by nuclear polyploidisation and cytoplasmic maturation which eventually leads to platelet release (Corash et al., 1989). This process has long been thought to be regulated by a lineage-specific humoral factor called thrombopoietin (Tavassoli et al., 1968). Even though the role of interactions of HSC with bone marrow stroma has been widely studied in the differentiation of erythroid and myelomonocytic cells, there have been no studies of the effects of stromal cells or ECM on the differentiation of MKs. It is clear that a knowledge of megakaryopoiesis is important for understanding the pathophysiology of acquired and congenital qualitative and quantitative platelet disorders. During the last 15 years the study of megakaryopoiesis has increasingly shifted away from morphologically based studies and become more sophisticated. The understanding of the development of MKs has been extended by culture of MK progenitors *in vitro* (Vainchenker et al., 1979), the isolation and enrichment of relatively pure, viable populations of MKs and the use of molecular methods such as the polymerase chain reaction and *in situ* hybridisation (Gewirtz, 1990). A major advance has been the use of recombinant human haematopoietic growth factors *in vitro* to generate MK progenitor cells from stem cell population. However, little is known about the mechanisms which regulate platelet production from MKs.

The c-Mpl receptor ligand, thrombopoietin (TPO), has a significant role in megakaryocytopoiesis and platelet production as shown by several *in vitro* and *in vivo* studies (Kaushansky, 1995; Wendling et al., 1994; De sauvage et al., 1994). Its recent cloning has greatly enhanced progress in the understanding of MK development. Isolation and culture of $CD34^+$ cells with TPO has been used successfully to expand the MKs

population by culturing $CD34^+$ cells derived from bone marrow, cord blood and peripheral blood.

The role of adhesive interactions with the extracellular matrix of the bone marrow stroma has not been widely studied in the MK lineage. Recently, Pascal and co-workers (1997) reported on the expression and function of receptors for extracellular matrix molecules in the differentiation of human MKs *in vitro.* The results of this study suggested that a proportion of CFU-MK are adherent to fibronectin, indicating that receptors (most probably VLA-4) for this molecule are already present. VLA-4 may be involved in the adhesion of immature MKs on fibronectin and may then be replaced by VLA-5 in the final stages of maturation. Although the expression and function of adhesion molecule receptors (integrins) of the β1 family have been studied in the differentiation of erythroid cells, very little is known concerning their role in the differentiation of megakaryocytic lineage cells. The development of an efficient *in vitro* serum-free liquid culture technique for the production of MKs enables the study of the expression and function of adhesion molecule for extracellular matrix and stromal cells in the differentiation of MKs.

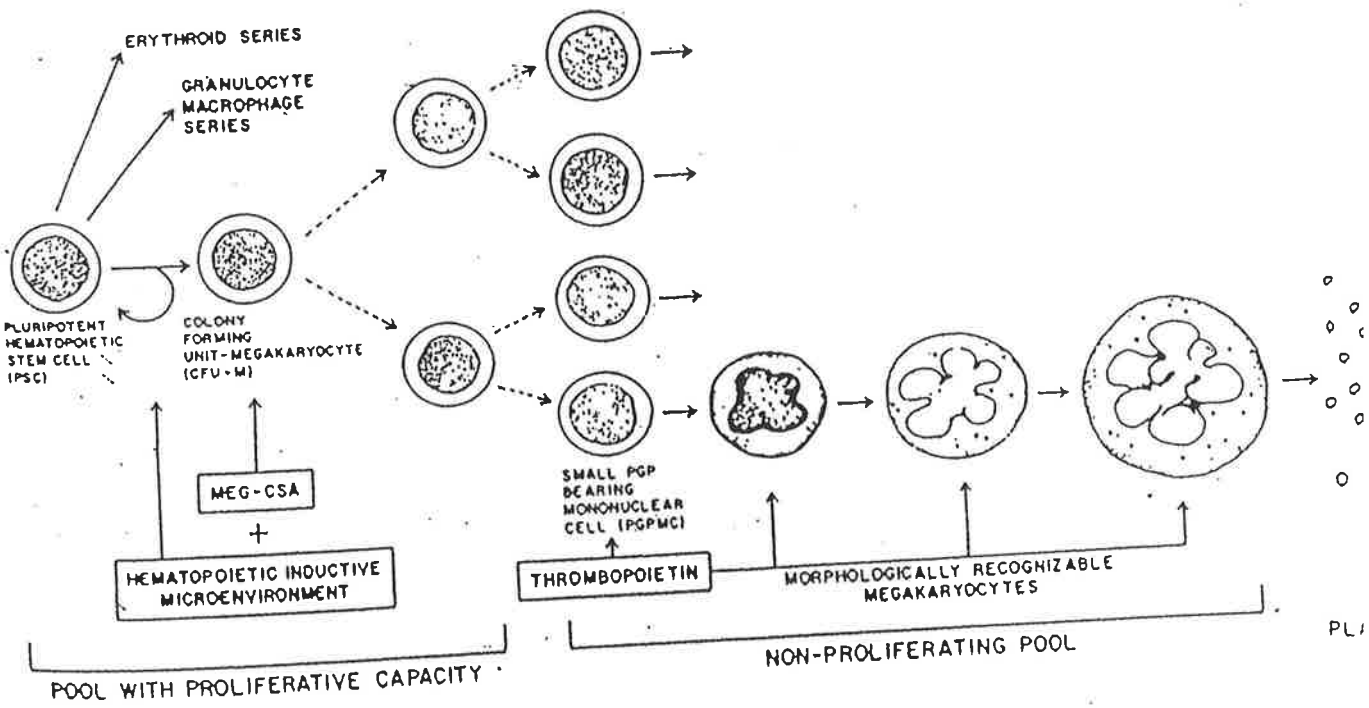
1.4.2 MEGAKARYOCYTE MATURATION *IN VITRO*

MKs are derived from an undifferentiated pluripotent haematopoietic stem cell (Visser et al., 1984). Once lineage commitment occurs, MK progenitor cells occupy the first "compartment." Further proliferation and differentiation amplify the numbers of MKs in the marrow (Goodman, 1960). Only a very small numbers of lineage committed progenitor cells are present in the bone marrow Fig1.4.

Progenitors of MKs can not be identified directly on the basis of their morphological features, but can be detected as colonies of differentiated progeny in various culture systems. The most immature progenitor cell is the megakaryocyte Burst Forming Unit (BFU-MK). This colony requires about 21 days in culture to develop and is composed of multiple clusters of megakaryocytic cells which often number in the hundreds (Long, et al., 1985). The next most immature progenitor cell is the Megakaryocyte Colony Forming Unit (CFU-MK). The CFU-MK is a more mature cell than the BFU-MK and was the first MK progenitor cell to be identified. (Mazur et al., 1981). CFU-MK gives rise to single cluster

FIG. 1.4 PROPOSED SCHEMA OF HUMAN MEGAKARYOCYTE MATURATION

Fig. 1.4 presents a schematic diagram of the events related to the development of MK in human. Copy from Golde (eds),1985.1st).



colonies in 10-12 days. CFU-MK cloning techniques were developed by Mazur and his coworkers with identification of colonies using an immunochemical identification. The MKs identified included those not yet morphologically identifiable (Mazur et al., 1981). This method utilised a highly specific rabbit anti-platelet glycoprotein antiserum in an indirect immuno-fluorescence assay.

1.4.3 REGULATION OF MEGAKARYOCYTE DEVELOPMENT

The regulation of MK development has been discussed in terms of regulators that act early in proliferation at the level of the progenitor cells, and those that act late upon terminally differentiating cells (Williams et al., 1982). The activity responsible for this stimulation was termed "megakaryocyte colony stimulating factor or activity". Now known as thrombopoietin (TPO) or megakaryocyte growth and development factor (MGDF), this activity plays a major role in regulating megakaryocyte production. Other cytokines known to impact on cells of this lineage include SCF, IL-3, IL-6 and IL-11.

For over thirty years, many investigators had been searching for a lineage-specific megakaryocyte proliferation and differentiation growth factor. Although a variety of cytokines do have megakaryocyte stimulating activity (MK-CSA), none was found to be a specific MK stimulant and none had the potency of thrombocytopenic plasma. In the past, many investigators believed that they had discovered the identity of this growth factor, termed thrombopoietin, but efforts to purity this substance to homogeneity from plasma proved unsuccessful (Hoffman et al., 1985). However, in 1994 three groups successfully isolated, cloned and characterised a specific MK growth and development factor which was called c-Mpl ligand or thrombopoietin (TPO). The commercial production of a recombinant form, such as Amgen's megakaryocyte growth and development factor (MGDF) has been a natural outcome of these discoveries.

The use of recombinant c-Mpl ligand in experiments with factor dependent MK cell lines in culture has provided *in vitro* proof of its thrombopoietic activity. *In vitro*, it has been shown that c-Mpl ligand is capable of inducing MK colony formation and enhancing MK maturation. c-Mpl ligand has been shown to specifically induce (CFU-MK) formation from

unfractionated murine bone marrow cells, murine fetal liver cells, and human bone marrow and peripheral blood CD34+ cells. Other studies (Sauvage et al., 1994) indicated that the serum of irradiated mice was able to induce proliferative activity in a MK cell line transduced with the c-Mpl gene. However, when a soluble c-Mpl receptor molecule was added to the aplastic murine serum, its MK-CSA was eliminated. This implied that the specific MK-CSA of the aplastic serum was mediated at least in part by the c-Mpl ligand (Wending et al., 1994). In 1994, Gurney demonstrated that the specific role of the c-Mpl ligand in thrombopoiesis has come from a mouse model *in vivo* (Gurney et al., 1994). This group generated c-Mpl deficient mice by gene targeting and indicated that these mice had an 85% reduction in their platelet counts compared to normal controls, while the other cells such as red cells, and total and differential white cell counts were unaffected. Furthermore, these mice had elevated levels of thrombopoietin (c-Mpl ligand), but Il-3, IL-6 and GM-CSF were not increased. These results strongly indicated that megakaryocyte growth and development factor is a lineage-specific regulatory factor for megakaryopoiesis and platelet production.

In vitro studies suggest that c-Mpl ligand has the properties of MK colony stimulating factor and TPO for the following reasons:

(a) The c-Mpl ligand is capable of inducing MK colony formation and MK proliferation of $CD34^+$ of human and murine haematopoietic stem cells (Kaushansky, 1995).

(b) The biologic level of the molecule is increased in plasma from animals made thrombocytopenic either by irradiation or by immune platelet destruction (Wending et al., 1994).

Evidence for the humoral regulation of megakaryopoiesis was first obtained from observation that serum or plasma, derived from animals rendered thrombocytopenic by irradiation, was capable of stimulating MK progenitor cell proliferation and maturation. Although MGDF appears to play a major role in regulating MK and platelet production, it has been known that other cytokines such as IL-3 (Bruno et al., 1989), IL-6 (Briddell et al., 1992), SCF (Ishbashi et al., 1990), GM-CSF (Xu et al., 1994), IL-11 (Waldburger et al., 1994), and IL-12 (Lotem et al., 1989) can regulate MK development. However, it was not believed that any of these cytokines acted specifically on the MK cells. IL-3 seems to be the most potent of these cytokines, at least in murine systems where the MK-CSA of

mitogen stimulated spleen cell conditioned media is neutralised if anti-IL-3 antibodies are added.

IL-6 has been shown to induce MK progenitor cell proliferation as well as maturation. Some reports showed that MK-CSA of IL-3 mediated by IL-6 as antibody against murine IL-6 neutralised the MK-CSA of murine IL-3 (Demetri et al., 1993). In patients receiving high dose chemotherapy for metastatic sarcoma and lung cancer, IL-6 has reduced the decline in platelet count and hastened the recovery of platelets to baseline level (Tanaka et al., 1992).

Known as steel factor (SLF) or c-kit ligand, SCF is expressed by bone marrow stromal cells, fibroblasts and fetal liver cells. SCF shows modest effects on early myeloid and lymphoid cells, but it is a multi-lineage factor which synergises dramatically with other cytokines. Rasko and colleges indicated that SCF significantly stimulated the proliferative process in both murine and human CFU-MK in serum-free culture with IL-3, and that the main activity of IL-6 is the promotion of MK maturation (Rasko et al., 1997).

1.4.4 MEGAKARYOCYTE PHENOTYPE

The central and most powerful advancement in the analysis of cell surface marker expression was the development of highly specific monoclonal antibodies (MoAbs) from 1975. MoAbs suitable for the identification of MKs are now available.

1. CD61. The CD 61 antigen (platelet glycoprotein GpIIIa) is the 110 kDa integrin β_3 subunit which is involved in platelet aggregation and adhesion to extracellular matrix. CD 61 is expressed on megakaryocytes and platelets and also on monocytes, macrophages and endothelial cells (Vinci et al., 1984). Evaluation of MKs lineage markers showed that the antigenic expression of lineage markers are distinctive in the developmental seqence of MKs cells. CD61 is an early lineage marker and its expression preceded both GpIIb/IIIa (CD41) and GpIb (CD42b). CD61 is considered the specific marker for the MKs lineage (Tao et al., 1999).

2. CD41. The CD41 antigen is the integrin αIIb chain of platelet glycoprotein GPIIb/IIIa. It is the major integrin on platelets and is important for MK and platelet adhesion and

aggregation. CD41 binds to multiple sites on fibronectin and is important for platelet adhesion, it is believed to be a specific antigen for MKs (Tomer et al., 1988).

3.CD42b. CD42 (GpIb) is a 160 kDa protein, mediating platelet adherence and aggregation at sites of vascular damage. CD42 binds to von Willebrand factor, and is also a receptor for thrombin (Berridge et al., 1988). Interaction of subendothelial vWF and Gp Ib results in both the adhesion and subsequent activation of platelets and MKs (Bartha et al., 1993)

1.5 ADHESION MOLECULES AND HAEMATOPOIESIS

1.5.1 INTRODUCTION : THE FUNCTION OF ADHESION MOLECULES

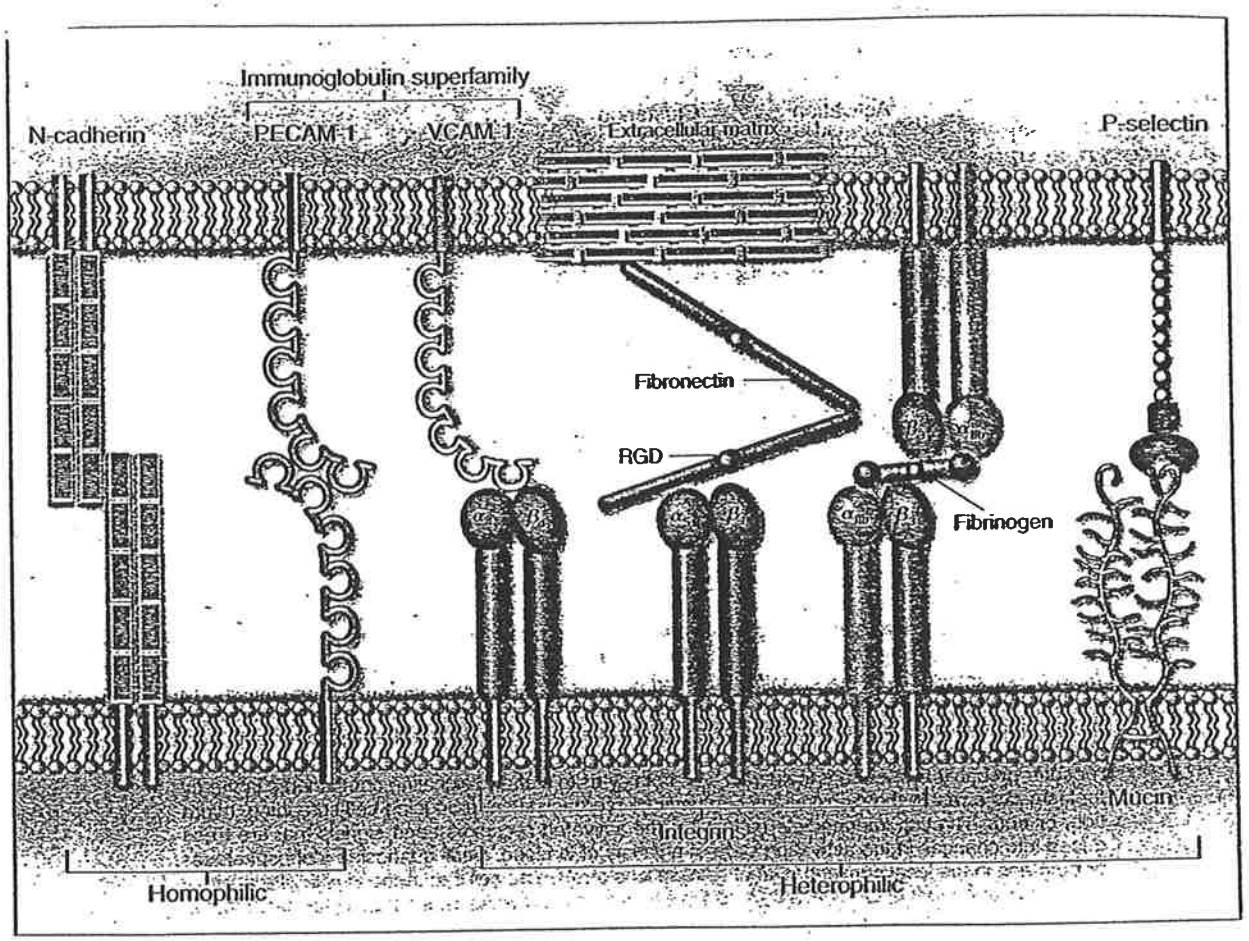
Cellular adhesion plays a central role in many biological processes such as cell migration, cell-cell interaction, and morphogenesis (Edelman, 1985). Adhesion is of fundamental importance to a cell. It provides anchorage, cues for migration, and signals for growth and differentiation. There are two basic types of cell adhesion: ECM adhesion and cell-cell adhesion. Adhesion is involved in many clinical diseases, for instance, vascular thrombosis, inflammatory process, tumour metastasis, bacterial and parasitic infections.

Adhesion is mediated by adhesion molecules which are essential for the above functions. They are also believed to be important in the regulation of haematopoiesis (Hanspal, 1997). Adhesion molecules are the cell surface structures that play a direct mechanical role in cell binding. Cell adhesion occurs when a plasma membrane adhesion receptor interacts with a molecule in the extracellular matrix or on the neighbouring cell and when the liganded receptor forms a connection with the cell's own cytoskeleton.(Edelmen et al., 1991).

Over the last decade a major effort has been made to identify many transmembrane glycoprotein receptors or cell adhesion molecules (CAMs). These mediate the adhesion between cell and cell, cell and ECM proteins (Edelman et al., 1991). Various adhesion molecules of different families have been implicated in the interactions of developing haematopoietic progenitors and their microenvironment. Four main families of adhesion molecules, the integrin superfamily, cadherin, immunoglobulin superfamily and the selectins, have been reported (Hynes, 1992) Fig 1.5. Only the integrin superfamily was studied in the present work. Numerous groups have studied integrin and Ig superfamies of adhesion molecules, particularly, and some studies have indicated that the β_1 (VLA) family of adhesion molecules might be important in the regulation of haematopoiesis (Miyaka et al., 1991; Yanai et al., 1996).

FIG. 1.5 THE FOUR MAJOR CLASSES OF ADHESION RECEPTORS

N-cadherin is one of the adhesion molecules mediating the outgrowth of neurites. The second major class, the immunoblobulin superfamily, may also mediate homophilic interactions. The third major class, integrins are heterodimers whose two chains contribute to ligand binding, finally, the selectins, which have a distal calcium dependent lectin interact with carbohydrate group. Copy from Frenette, 1996.



Because adhesion molecules play a major part in cell-cell and cell-matrix interaction, these adhesive interactions are important in the regulation of HSC maturation, and the direction of HSC migration through tissues. Other studies have shown that CAMs participate in the regulation of proliferation and differentiation (Rosales et al., 1995).

In the bone marrow, haematopoietic progenitor cells proliferate and differentiate within a specific microenvironment consisting of stromal cells, extracellular matrix (ECM) and various secreted cytokines (Donshkind, 1990). Close interactions of the developing progenitor cells with this microenvironment is needed to control the haematopoietic cells proliferation and differentiation (Klein, 1995).

1.5.2 INTEGRIN SUPERFAMILY

Integrins are a family of cell surface proteins that mediate cell adhesion. The integrin family appears to be the primary mediators of cellular-extracellular matrix adhesion, and also serves as one of the many families of molecules active in cell-cell adhesion.

Integrins are αβ heterodimeric transmembrane proteins comprising of at least 14 α chains and 8 β chains which are expressed by epithelial cells as well as other cell types. Table1.5. Integrins are classified according to their type of beta subunits (β_1, β_2, β_3), based on the sharing of a common β chain. It is clear that some α chains can associate with more than one β chain.

The β_1 or very late activation antigen (VLA) family consists of at least six different heterodimers with a common 130 kd β1 chain and six distinct α subunits. They are generally involved in adhesion with ECM molecules and may function as receptors for collagen (VLA-2, VLA-3), fibronectin (VLA-3, VLA-4, VLA-5) and laminin (VLA-6) (Hynes, 1992).

In the β_2 or leucocyte cell adhesion molecule (Leu-CAM) family there are three members: LFA-1 (CD11a), MAC-1 (CD11b) and p150·95 (CD11c). These have a common β2 chain and α chains of 175, 165 and 150 kd respectively). They mediate leucocyte homotypic adhesion through a specific interaction with ICAM-1 (LFA-1) (Hogg et al., 1989). The effective activation stimuli for β2integrins vary depending on cell type. The earliest

TABLE 1.5 THE INTEGRIN RECEPTORS FAMILY

The Integrin Receptor Family			
Subunits		Ligands and Counterreceptors	Binding Site
β_1[a]	α_1	Collagens, laminin	DGEA[h]
	α_2	Collagens, laminin	RGD ± ?[i]
	α_3[a]	Fibronectin, laminin, collagens[c]	EILDV[k]
	α_4	Fibronectin (V25), VCAM-1	RGD
	α_5	Fibronectin (RGD)	
	α_6[a]	Laminin	
	α_7	Laminin	
	α_8	?	RGD
	α_V	Vitronectin, fibronectin (?)[d]	
β_2	α_L	ICAM-1, ICAM-2	
	α_M	C3b component of complement (inactivated), fibrinogen, factor X, ICAM-1	GPRP
	α_X	Fibrinogen, C3b component of complement (inactivated)?	
β_3[a]	α_{IIb}	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin	RGD, KQA(
	α_V	Vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, osteopontin, collagen	RGD
β_4[a]	α_6[a]	Laminin ??[e]	RGD
β_5	α_V	Vitronectin	RGD
β_6	α_V	Fibronectin[f]	EILDV[k]
β_7(=β_P?)	α_4	Fibronectin (V25), VCAM-1[g]	
	$\alpha_{E(L)}$[b]	?	
β_8	α_V	?	

The current spectrum and interactions of vertebrate integrins are list.

Copy from Hynes, 1992.

observations were on monocytes, in which $\alpha_M\beta_2$ can be activated by adherence to fibronectin.

The β_3, or cytoadhesion family, includes at least gpIIb/IIIa, the platelet receptor for fibronectin, fibrinogen, and von Willebrand factor, and the receptor for vitronectin (VNR); these molecules also consist of a common β3 subunit (VNRβ/gpIIIa) and of distinct α subunits (Ginsberg et al., 1988)

Another important function of the integrin heterodimers is that they are likely to participate in the signal transduction pathways mediating information about the structure of the cells (Schwartz, 1992). The exact molecular mechanism of signal transduction via integrins is still not clear. However, the fact that the cell surface integrin pattern does not only regulate the adhesion properties of a cell but can also significantly influence cell behaviour increases the interest in studying the factors regulating integrin expression.

The integrin molecules mediate adhesion by three mechanisms of interaction: cell-cell, cell-matrix and cell-soluble cytokines. Integrins function as receptors for extracellular matrix proteins including collagens, fibronectin and laminin (Hynes, 1992). Evidence is emerging that individual integrin receptors mediate distinct functions and trigger distinct signalling pathways (Hynes, 1992).

Most integrin receptors recognise a tripeptide sequence RGD on their respective ligands with the exception of CD18/CD11a (LFA-1) (Ruoslahti et al., 1987). The precise mechanism of how this simple tripeptide is utilised by so many receptors for different adhesion and signalling processes is not known. This thesis only describes the β_1 or very later antigen (VLA) and mainly focuses on VLA-4 and VLA-5, and CD 61 (integrin β_3 subunit) as referred to previously.

1.5.2.1 VLA-4 (CD49d, $\alpha_4\beta_1$)

The VLA-4 (CD49d) is the major mediator of cell-extracellular matrix interactions and homing. CD49d is now known to be highly expressed on T cells, B cells, and monocytes. The VLA-4 is different from other VLA proteins and has been implicated as a cell-cell adhesion receptor (Hemler, 1990). The VLA-4 integrin shows an interesting dual specificity in that it can also bind to fibronectin (Mould et al., 1990). VLA-4 ($\alpha_4\beta_1$) which

binds to the GGD segment of fibronectin, are believed to be important mediators of direct interactions between haematopoietic progenitor cells and BM microenvironment (Teixido et al., 1992; Levesque et al., 1995). Recent work by Schick and coworkers (1998) has shown that the integrin involved in the adhesion of MKs to fibronectin and fibrinogen, VLA-4 and its co- or counterreceptor, vascular cytoadhesion molecule (VCAM)-1, play an important role in a wide variety of interactions. Adhesion molecules in addition to VLA-4 ($\alpha_4\beta_1$) have been described on $CD34^+$ cells, stromal cell-derived proteins such as haemonectin and proteoglycans or cell-cell interaction mediated by endogenous lectin (Simmons et al., 1992). Pascal and his colleagues have reported that expression and function of receptors for extracellular matrix molecules are involved in the differentiation of human megakaryocytes *in vitro*. In particular VLA-4 seems to be involved in the adhesion of immature MKs to fibronectin and is then replaced by VLA-5 in the final stages of maturation (Pascal et al., 1997).

1.5.2.2 VLA-5 (CD49e, $\alpha_5\beta_1$)

The role of VLA-5 in attachment of monocytes to fibronectin is well established (Wayner et al., 1989). VLA-5-like structures of human monocytes have been eluted with RGD (Arg-Gly-Asp) peptides from fibronectin fragment affinity columns. VLA-5 property is the prototype fibronectin receptor initially isolated from an osteosarcoma cell line by absorption on fibronectin Sepharose and elution with RGD peptide. VLA-5 was independently characterised on the leukaemic cell lines K-562 and U-937, where it was shown to be the fifth distinct member of the VLA protein family (Wayner et al., 1988). Rosemblatt and coworkers (1991) have demonstrated that co-expression of two fibronectin receptors, VLA-4 and VLA-5, by immature human erythroblastic precursor cells, suggested that the adhesion to fibronectin first increases during early stages of erythroid differentiation, and is subsequently lost with terminal maturation.

Rosemblatt and coworkers, (1991), elucidated the role of the two fibronectin receptors, VLA-4 and VLA-5. Both VLA-4 and VLA-5 bind fibronectin. VLA-4 acts as a receptor for the CS-1 region of this molecule and recognises VCAM-1 on activated endothelial cells. VLA-5 binds specifically to the Arg-Gly-Asp-ser (RGDS) sequence of fibronectin.

1.5 2.3 CD61 (β^3)

See section 1.4.4.

1.6 TRANSMEMBRANE 4 OR TETRASPAN SUPERFAMILY (TM4SF)

1.6.1 INTRODUCTION

It has recently been shown that the transmembrane 4 superfamily (TM4SF) of membrane proteins (also called tetraspan superfamily) comprises a group of cell-surface proteins that are characterised by the presence of four hydrophobic domains, which are presumed to be membrane spanning (Wright et al., 1994). It seems likely that they mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility (Berditchevski et al., 1995). Unlike the other protein superfamilies, such as immunoglobulin and integrin superfamilies involved in cell-surface recognition and adhesion events, the TM4 SF role is not yet clear. This current work will examine any significant interactions between TM4SF and the integrin superfamily and whether or not the TM4SF associates with β1 integrin. (Wright et al., 1994).

1.6.1.1 THE TETRASPAN SUPER FAMILY FUNCTION

The precise biochemical function of the TM4SF is not known, although the data currently available summarised in Table 1.6 suggest a role for this superfamily in the regulation of cell development, proliferation, activation and motility. Roberts and his co-workers, (1995), have shown that Anti-CD9 MAb are able to induce platelet activation and mediator release by crosslinking CD9. Olweus and his colleagues also demonstrated that expression of signal transduction events has been modified in B-cells, granulocytes, and monocytes by anti-CD53 monoclonal antibody (Olweus et al., 1993).

A potential role for tetraspans in modulating adhesion events has been further supported by subsequent studies highlighting their associations with integrins of the β_1 family. CD9 is a 24 kDa membrane glycoprotein expressed on the surface of human platelets and potentially involved in cellular activation and adhesion functions. Slupsky and his co-workers have shown that CD9 is associated with the $\alpha IIb\beta_3$ complex in platelets (Slupsky et al., 1989). Thus CD9 is reported to associate with small GTP binding proteins in platelets and

TABLE 1.6 THE TANSMEMBERANE 4 SUPERFAMILY

The TM4SF molecules remain a superfamily in search of a function: possible roles include coupling to signal trasduction pathways or involvement in cell proliferation and activation. Copy from Wright et al., 1994.

Antigen	Tissue distribution	Functional data	Associated molecules
CD9 (MRP-1, DRAP27)[a]	Platelets, early B cells, activated T cells, eosinophils and basophils; not on resting B and T cells; marker for 90% non-T acute lymphoblastic leukaemic cells and 50% acute myeloid and chronic lymphoid leukaemias; also found on a variety of non-lymphoid cells[27]	Signal transduction in platelets[39]; cell adhesion in platelets and pre-B-cell lines[39]; cell motility and metastasis in a lung adenocarcinoma line[34,35]	mAbs induce association wit integrin CD41/CD61 (Ref. 5 monkey CD9 associates with the diphtheria toxin receptor
D37	Mature B cells; not on pre-B or plasma cells; low expression on T cells, monocytes and macrophages; large proportion is expressed intracellularly[28]	mAb modulates B-cell proliferation[32]; downregulated on B-cell activation[32]	CD53, CD81, CD82, MHC class II, CD19 and CD21 in B cells[24]
CD53 (OX-44)[b]	Thymocytes (90% in human), B and T cells, monocytes, macrophages and granulocytes[1]; 12% of thymocytes in the rat; including the $CD4^-CD8^-$ subset and mature phenotype cells[26]	Signal transduction in B cells, monocytes and granulocytes[42], rat macrophages[41], NK and T cells[40]	CD2 in a rat NK-cell line and cells[40]; CD37, CD81, CD82, MHC class II, CD19 and and CD21 in B cells[24]
CD63 (ME491, MLA1, PTLGP40)	Activated platelets; expressed on lysosomal and dense granule membranes in resting platelets; monocytes and macrophages; weakly on B and T cells and granulocytes; widespread on non-lymphoid cells[1]	Role in cell adhesion of platelets and endothelial cells[54]	Unknown
CD81 (M38, TAPA-1)	B and T cells; low on monocytes and granulocytes; marker on leukaemia and lymphoma lines, also neuroblastoma and melanoma lines[4]	Signal transduction in B cells[45]; cell adhesion in B-, T- and non-lymphoid cell lines[55]; syncytium formation in HTLV-1-infected T cells[25]	Component of the B-cell CD19/CD21/CD81/Leu-13 signalling complex[45]; CD37, CD53, CD82, MHC class II, CD19 and CD21 in B CD4, CD8 and CD82 in T cel
CD82 (C33, IA4, R2, 4F9)	B and T cells, monocytes and granulocytes; expression upregulated on activation[31]	Signal transduction in B cells[44]; syncytium formation in HTLV-1-infected T cells[25]; activation marker[31]	CD37, CD53, CD81, MHC class II, CD19 and CD21 in B cells[25]; CD4, CD8 and CD8 in T cells[25]
A15	Isolated as an immature T-cell antigen; wider distribution not known[29]	None	Unknown

Abbreviations: mAb, monoclonal antibody; NK, natural killer; HTLV-1, human T-cell leukaemia virus 1; MHC, major histocom complex.

[a]DRAP27 refers to the monkey antigen.

[b]OX-44 refers to the rat antigen.

transfection of CD9 into Raji cells induces phosphorylation of a 110 kD protein when cells are plated on integrin ligands (Shaw et al., 1995).

CD53 is a pan-leukocyte glycoprotein which is another member of the recently described TM4SF of membrane. In the rat, CD53 is expressed on all peripheral leukocytes but on only 12% of thymocytes. Remarkably, expression is absent on the vast majority of $CD4^+CD8^+$ thymocytes, which are cells that are destined to die in the thymus. The immature CD4-CD8- cells, and the mature single positive cells, do express CD53. For example, signal transduction through CD53 has been demonstrated by the use of human anti-CD53 monoclonal antibodies which cause calcium fluxes in B cells, monocytes and granulocytes (Olweus et al., 1993). It is not known from these studies whether signalling through CD53 requires co-ligation. Since $CD53^+$ cells account for all thymocytes that proliferate in response to alloantigens and lectins, CD53 may mark those thymocytes that are on the differentiation pathway to forming immunocompetent T cells (Wright et al., 1994).

CD63 belongs to the TM4 superfamily of membrane proteins. Previous results have suggested that CD63 may play an important role in the regulation of melanoma (Radford et al., 1996). CD63 is one of the major components of lysosome membranes. It has a 53 kDa portion that is translocated to the plasma membrane after platelet activation. CD63 is a marker for early stages of tumour progression of melanoma, but is not detected on normal tissue melanocytes and is weak or absent in advanced stages of melanoma (Radford et al., 1996).

CD151 (tetraspan-platelet) also is a member of the tetraspan superfamily, and is expressed on MK and platelets. Recently a cDNA clone encoding a 27-kD protein PETA-3 (platelet endothelial tetraspan antigen-3) was isolated in Ashman's laboratory (Fitter et al., 1995). The function is similar to other tetraspan superfamily and it induces Fc dependent platelet aggregation and mediator release (Roberts et al., 1995). Ashman's group has shown that the association of CD151 with CD49c on endothelia cells and capillary formation *in vitro,* indicated that CD151 might induce cell-cell adhesion by activating "outside-in" signalling pathways that indirectly up-regulated other adhesive mechanisms. CD151 may play a role in modulating endothelial cell function.

Expression of certain TM4SFs is altered when cells are growing or activated, suggesting a function in regulation of cell growth. CD9, CD53, and CD63 are all up regulated on activation of lymphocytes. Four of the TM4SF members have been shown to couple to signal transduction pathways (Wright et al., 1994)

1.6.1.2 CHARACTERISATION OF THE CELL SURFACE RELATIONSHIP BETWEEN INTEGRINS AND TETRASPAN SUPERFAMILY

The relationship of integrin signalling to tetraspan superfamily is not fully understood. It has recently been shown that several members of the tetraspan superfamily, including CD9, are associated withVLA-4 (Rubinstein et al., 1994) and for CD63, associated with each other and with β_1 integrins (Berditchevski et al., 1995). Furthermore, CD9, CD63 and CD81 were demonstrated to associate with each other and $\alpha_3\beta_1$ to form tetraspan/ integrin and tetraspan/tetraspan/integrin complexes by immunoprecipitation. (Berditchevski et al., 1996).

More recently, Schick and colleges reported that integrins are involved in the adhesion of megakaryocytes to fibronectin and fibrinogen, and showed that the adhesion of MK to fibronectin was primarily inhibited by an anti-VLA-5 antibody. Thus, VLA-5 is the major integrin involved in the binding of MK to fibronectin (Schick et al., 1998).

Yanai and co-works (1994), demonstrated a role for integrin VLA-4 in stroma-dependent erythropoiesis. The development of the erythroid cells on stroma cells was inhibited by anti-VLA-4 integrin antibody, but not by VLA-5 antibody although the erythroid cells express both VLA-4 and VLA-5.

However, as far as can be ascertained the relationship between integrins and tetraspan superfamily on MKs development has not been reported. The aim of this thesis is to provide more information on this topic.

1.7 RATIONALE AND AIM OF PRESENT STUDY

It is evident from the literature review that the role of the haematopoietic microenvironment in megakaryopoiesis is not well studied. The objectives of the present study were:

1. to establish a serum free, cytokine supplemented $CD34^+$ cell culture system to investigate the role of various components of the haematopoietic microenvironment in supporting megakaryocytic progenitor cells derived from cord blood stem cells;
2. to characterise *in vitro* the differentiation of MK progenitor cells in liquid culture with or without components of the extracellular matrix;
3. to establish the co-culture system with mouse bone marrow stromal cells (M-5) to study the effects of cellular interactions in the differentiation of MKs; and
4. to assess the roles of adhesion molecules, such as integrins, and signalling molecules, such as tetraspans, in the process of megakaryopoiesis.

CHAPTER 2
MATERIALS & METHODS

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 ISOLATION OF MONONUCLEAR CELLS

(5%) Human albumin (NSA)	CSL Parkville, Australia
Lypho prep	Nycomed Pharma AS, Oslo, Norway
Red out	Robbins Scientific, Sunnyvale, CA USA

2.1.2 ISOLATION OF CD34$^+$ PROGENITOR CELLS

CD34 progenitor cell isolation kit	MACS, Miltenyi Biotec, Gemany
Reagent A1:	human Ig FcR block reagent
Reagent A2:	monoclonal hapten-conjugated
CD34 antibody. Clone:	QBEND/10.Isotype: mouse IgG1.
Reagent B:	Microbeads conjugated to an anti-hapten antibody.
MACS positive selection columns	Type: MS$^+$/RS$^+$ VS$^+$ Miltenyi Biotec, Gemany
Magnetic cell separator:	MiniMACS, and MACS/virioMACS.
Precolumn separations 30 m filter	MACS Miltenyi Biotec, Gemany

2.1.3 SEMI-SOLID AGAR CULTURE MEDIUM

(0.66%) Bacto-agar	Difco, Michigan, USA
Fetal bovine serum (FBS)	Trace Biosciences Pty Ltd, Castle Hill,NSW, Australia
Mercaptoethanol	AJAX Chemicals NSW, Australia
NaHCO3	AJAX Chemicals NSW, Australia

2.1.4 LIQUID CULTURE MEDIUM

Stem Pro^{TM} 34 serum free medium	GIBCO BRL, Life Technologies Inc.
Horse serum	GIBCO BRL, Life Technologies Inc.
Single strength Iscove's modified	GIBCO BRL, Life Technologies Inc.
Dulbecco's medium (IMDM)	GIBCO BRL, Life Technologies Inc

2.1.5 CYTOKINES FOR CULTURE

Recombinant Human Interleukin-3 (rhIL-3)	R&D SYSTEMS Minneapolis, MN USA
Recombinant Human Interleukin-6 (rhIL-6)	R&D SYSTEMS Minneapolis, MN USA
Recombinant human Megakaryocyte Growth Development Factor (rhMGDF)	A gift from Amgen (Kew,Victoria), Australia
Recombinant Human Stem Cell Factor (SCF)	Novartis Pharmaceuticals . North Ryde, NSW, Australia

2.1.6 ALKALINE PHOSPHATASE ANTI-ALKALINE PHOSPHATASE (APAAP) STAINING

Rabbit serum	DAKO A/S, Denmark
Mouse serum	DAKO A/S, Denmark

Methanol-Acetone	1:3 work solution
Mouse anti-human GpIIIa (CD61)	DAKO A/S Denmark
Mouse anti-human IIB (CD41)	DAKO A/S Denmark
Rabbit anti-mouse IgG antibody	DAKO A/S Denmark
APAAP complex	DAKO A/S Denmark
Fast red TR salt	Sigma Co. USA

2.1.7 FLOW CYTOMETRY REAGENT

Monoclonal antibodies (MoAbs)

Direct antibodies

CD34-PEcy5, CD49d-FITC, 49e-FITC	Immunotech, USA
CD63-PE, Mouse IgG1-FITC/IgG1-PE	Becton Dickinson, USA
CD34-PE, CD61-FITC	Pharmingen Inc., USA
CD53-PE	
CD41-FITC,CD45-FITC	Coulter Immunology, USA

Indirect antibodies:

Streptavidin-R-PE conjugate	Sigma Co, USA
Biotin anti-mouse (IgG2a)	Pharmingen, Inc, USA
Mouse IgG2a (negative control)	DAKO A/S, Denmark
Tetraspan Plt-(CD151)	Gift of Dr. L. Ashman, Adelaide University, Australia

Flow cytometry wash and fix reagents

Intragam normal human immunoglobulin	(working solution 5% to block non-specific binding
	CSL, Parkville, Australia
Isoton II	Coulter Electronics, Brookvale, NSW, Australia
Paraformaldehyde	(1%) in Isoton II, AJAX Chemicals, NSW Australia

2.1.8 ELECTROPHORESIS

Acrylamide	Bio-Rad, USA
Ammonium persulfate	Bio-Rad, USA
N,N,N1,N1-tetramethyl ethylenediamine (TEMED)	Bio-Rad, USA
2-mercaptoethanol	5% in 0.02 M Tris-HCl pH 7, Bio-Rad, USA
Low molecule weight marker	Bio-Rad, USA
Bromophenol Blue	BDH Chemicals Ltd. England
X-ray film	Kodak film

2.1.9 IMMUNOPRECIPITATION

Immunoprecipitation kit (protein A)	Boehringer Mannheim, Germany
ECL western blotting kit	Amersham Life Science, UK
Peroxidase-conjugated rabbit	DAKO Denmark
Unconjugated monoclonal antibodies:	
CD49d, CD49e, CD61, CD53, CD63	Immunotech, USA

2.1.10 BUFFERS AND SOLUTIONS

Phosphate buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 4.3mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH7.4
PBS^+EDTA	PBS PLUS 0.02% EDTA
2.5x IMDM	One packet IMDM (Gibco), 390 ml water injection, 1.5 ml DEAE-(50mg/ml) 0.2 g L-asparagine 5,000u penicillin,5,000u streptomycin (Gibco).
Tris-HCl buffer	0.1M, pH 7.6

Alkaline phosphatase substrate	0.1M Tris buffer pH 8.2, 0.02 g, Naphthol 2ml dimethyl formamide, 100µl (1M,0.24g) Levamisole.
Evan Blue	work solution 1:25 in 0.1 Tris-HCL pH 7.6.
SDS-PAGE electrophoresis buffer	25mM Tris-HCl, 0.192M glycine, PH8.3
Western blotting transfer buffer I	0.3M Tris-HCL, 20% Methanol, pH 10.4
Western blotting transfer bufer II	0.025M Tris-HCl, 20% Methanol, pH 10.4
Western blotting transfer buffer III	0.025M Tris-HCl, 0.004 M, aminohexanoic acid, 20%Methanol, pH 9.4.
Immunoprecipitation kit (Protein A) buffer 1	50 mM Tris-HCl, pH7.5, 150 mM NaCL 1% NP40, 0.5% sodium deoxycholate. one complete™ tablet/25ml
Immunoprecipitation kit (Protein A) buffer 2	50mM Tris-HCL, pH7,5, 500 mM NACL, 0.1% NP40, 0.05% sosium deoxycholate.
Immunoprecipitation kit (Protein A) buffer 3	50mM Tris-HCL, pH7.5, 0.1% NP40, 0.05% sodium deoxycholate.

2.1.11 MISCELLANEOUS MATERIALS, CHEMICALS

Cell count machine	Performed on a Cell-Dyn 3500
10ml Falcon tubes	Becton Dickinson, CA
24 wells Multidish	Nunclon A/S Denmark
Tissue culture dish	(35x10 mm) Corning Lab. Sci. Co.

2.2 GENERAL METHODS

2.2.1 CORD BLOOD COLLECTION

30 to 60ml placental cord blood of full-term deliveries was collected into sterile tubes containing 20-25u/ml of heparin. The blood sample was processed within 5 hours.

2.2.2 ISOLATION OF MONONUCLEAR CELLS (MNC)

Anticoagulated cord blood was diluted 1:3 with PBS (Ca^{++} and Mg^{++} free) containing 2 mM EDTA and 0.5 % NSA. 35ml of the diluted cell suspension was then carefully layed over 15ml of Lymphoprep^{TM} (density 1.077g/ml) and centrifuged at 20^0C at 400g for 35min in a swinging-bucket rotor (without brake). After centrifugation, the interface fraction was aspirated, and the isolated cells washed once in PBS/EDTA and re-centrifuged at 200g, 10min, 20^0C. After two washing, the cell pellet was resuspended in 10ml PBS/EDTA buffer and a cell count performed on a Cell-Dyn 3500. The cells were resuspended to a maximum final concentration of $1x10^8$ /ml.

2.2.3 POSITIVE SELECTION OF $CD34^+$ CELLS

The $CD34^+$ cells were present at a frequency of 0.1-0.5% in cord blood. Isolation of hematopoietic progenitor cells was performed by positive selection of $CD34^+$ cells.

CD 34^+ hematopoietic progenitor cells were indirectly magnetically labelled using a hapten-conjugated primary monoclonal antibody and anti-hapten antibody coupled to MACS microbeads, a maximum of $1x10^8$ MNC was resuspended in 300µl isolation buffer, incubated with 100µl Reagent A1 (anti-CD34 antibody (QBEND 10) and 100µl Reagent A2 (human IgG to prevent nonspecific binding) mixed well and incubated for 15min at 12^0C. Cells were washed carefully in 10ml buffer, centrifuged and the supernatant was completely removed. The cell pellet was resuspended carefully and diluted with buffer to a final volume of 400µl per $1x10^8$ total cells. 100 µl Reagent B per $1x10^8$ total cells was added, mixed well and incubated for 15 min at 12^0C. The cells were washed once by adding 10ml isolation buffer and the cell pellet was resuspended in 0.5ml buffer per 10^8 total cells.

A positive selection column was selected (size according to the number of total unseparated cells) and placed in the magnetic field of the separator. The column was rinsed with buffer (MS^+/RS^+, 0.5ml; VS^+, 3ml). The cells were carefully passed through a 30µl filter to remove clumps and applied to the column, and held in the column which rinsed with buffer (MS^+/RS^+ 4x0.5ml, VS^+ 4x3ml) to remove unbound cells.

$CD34^+$ cells were collected by placement in a new tube. 1ml isolation buffer was loaded onto the top of the column and the retained cells were eluted using the plunger supplied with the column. The magnetic separation step was then repeated in order to achieve 60-90% of purity of $CD34^+$ cells. Cells were gently washed 2x in StemPro™ 34 serum free medium at room temperature.

2.2.4 MEASUREMENT OF $CD34^+$ CELLS PURITY AND RECOVERY

Flow cytometry was used to measure CD34 cell purity. $1x10^4$ isolated cells were directly stained by 2.5µl CD34-PE and CD45-FITC monoclonal antibodies, incubated for 15min on ice and then washed once with Isoton II. At least 5000 events were acquired by flow cytometry and analysed using the Lysis II software. Mouse IgG1-FITC and IgG1-PE were used as isotype controls.

2.2.5 CELL COUNT AND VIABILITY

Manual count

For manual cell counts, blood samples or cells were mixed gently and 0.05ml were pipetted into a plain tube, and 0.01ml of toluidine blue (0.25% in PBS) was added and then mixed. Cells were counted in $5mm^2$ of a hemocytometer. The number of cells (N)/mm3 = No. of cell counted x 0.2 x 10 x1.2. (N was to be reported as $x10^6$/L).

Automated white cell counts were performed using a cell viability count. Trypan blue dye exclusion was used to determine cell viability. 0.2ml cell suspension in medium was added to 0.3ml (0.4% w/v) trypan blue and 0.3ml medium, mixed and allowed to stand 5-15min. Cells were counted in 5mm2 of a Hemocytometer.

Cell viability was calculated by the formula:

% viability = number of viable cells (total viable cells (unstained) x100%

total cells (stained and unstained)

2.2.6 CELLS CYTOSPIN

To 250µl of the sample, 50µl 5% normal serum albumin was added and centrifuged at 600 rpm, for 5 min in a Shandon Cytospin 3 cytofuge to prepare a cytospin sample. After air drying, slides were stained in May Grunwald Geimsa.

2.2.7 MEGAKARYOCYTE COLONY-FORMING UNIT (CFU-MK)

Clonogenic progenitors, committed and relatively late progenitors with little self-renewal potential, can be identified by the colony assay Semi-solid agar culture. 1-3x10^3 CD 34^+ cells in 1-10µl StemPro^{TM}34 serum free medium were added to each of 35mm petri dish. The cells were added together with 0.5ml medium (2.5x IMDM + 10% FCS) with the appropriate cytokines. 0.5ml agar (0.66%), liquefied in a microwave oven and cooled to 45^0C, and quickly but gently poured into each petri dish, mixed by swirling and spread evenly over the surface of the petri dish to avoid any bubbles. Cultures were incubated at 37^0C in a fully humidified atmosphere of 5% CO_2 for 12-14 days.

After 12-14 days, scoring of all colony types was performed with the 4x and 10x objective lens to examine colonies in the petri dishes. The dishes were allowed to air drying at room temperature, then stored at -20^0C prior to APAAP staining.

2.2.8 ALKALINE PHOSPHATASE ANTI-ALKALINE PHOSPHATASE (APAAP)

MK lineage colonies were further identified by the APAAP immunostaining procedure. This *in situ* technique utilises primary antibodies which directly bind to platelet glycoprotein IIb/IIIa (CD41) and IIIa (CD61) surface antigens which are commonly expressed on platelets and their precursors. CFU-MK lineage colonies appear red and other colonies appear blue.

The colonies were fixed using 2ml of methanol: acetone (1:3) mixture for 20 min. Excess fixative was aspirated and the dish washed twice with Tris-HCl buffer pH 7.6.

Non-specific binding was blocked with rabbit serum (1:10 in dilution in Tris-HCl buffer pH 7.6) for 20 min. The dishes were then washed 4x with Tris-HCl buffer, pH7.6. The primary antibodies CD41 (1:50 dilution with Tris-HCl buffer) and CD61 (1:100 dilution in Tris HCl buffer) were added to the dishes, except to the negative control dish to which was added mouse serum (1:5 dilution). The dishes were incubated for 30min, and washed 4x with Tris-HCl buffer.

The secondary antibody, rabbit anti-mouse at 1:25 dilution, was added to all the dishes which were then incubated for 30 min and washed 4x with Tris-HCl buffer. 0.5 ml of mouse APAAP reagent (1:25 dilution) was added to the dishes and incubated for another 30 min, and washed 4x with buffer.

The filtered substrate solution 0.5ml was then added and incubated for 20 min and then washed 2x with buffer. Following counterstaining for 5 min in Evans Blue, excess was gently rinsed off with distilled water and the plates were allowed to air dry. Plates were mounted with aqueous warmed glycergel medium and examined for colony formation.

Three categories of MK colonies were identified in the agar culture:

CFU-MK - pure megakaryocyte colonies. CFU-MK ranging in size from 3 to several hundred MKs per colony.

CFU-Mkmix - mixed MK colonies (containing other lineages in addition to MK).

NON MK - non megakaryocyte colonies. They were usually granulocyte and monocyte lineages.

MK and platelets which express glycoprotein IIb/IIIa (CD41) and IIIa (CD61) appeared pink following fixation and staining. Counterstaining with Evans blue caused the nuclei of all cells to appear pale blue regardless of lineage.

Because CFU-MK ranged in size from 3 to 100s of MKs per colony, it was convenient to subdivide them by colony size:

- **Small colonies** 3-20 cells/colony.
- **Medium colonies** 21-49 cells/colony.
- **Large colonies** 50-over 100cells/colony.

2.2.9 CELL CULTURE

The growth of megakaryocytic progenitor cells derived from CB $CD34^+$ cells were cultured using three different culture conditions.

1. Liquid culture. A Stem Pro™ 34 serum free medium was used as a basic culture medium with IL-3, IL-6 (20ng/ml), SCF (50ng/ml) $^+$/- MGDF (50ng/ml) cytokines.

2. Fibronectin culture. Lyophilised human fibronectin was dissolved in sterile distilled water to a final concentration of 1mg/ml. 0.25 ml of this solution was used to coat each well (24 wells/plate). The wells were refilled with StemPro™34 free medium with cytokines.

3. Stromal culture. Mouse stromal cells (MS-5) were a gift from Dr. Inoue Hideo. The cells were cultured in IMDM, supplemented with 10% horse serum, and penicillin-streptomycin (Coulombel et al., 1983). 1ml aliquot of the suspension containing $1x10^6$ stromal cells were plated in a 25cm flask, and placed in an incubator and cultured at 37^0C, 5% CO_2 and 90% humidity. Cells were fed every 7 days by replacing 50% of the supernatant with fresh medium. A confluent layer of stromal cells formed in 2-4 weeks.

Stromal cells were washed twicewith PBS detached by treatment with 0.05% trypsin solution for 5min and resuspended in Stem Pro™ 34 serum free medium with cytokines, 1 $x10^4$ cells were distributed into 24 wells plates, until the stromal cell confluence was reached in 2 days.

Purified $CD34^+$ cells were seeded at a concentration of $2x10^4$/ml in 24-well tissue culture plates at 1 ml Stem-Pro™ 34 serum free medium with cytokine per well. The cultures were incubated at 37^0C in a fully humidified atmosphere containing 5% CO_2 up to 4 and 8 days. After 4 days and 8 days cells were gently mixed, collected and processed as follows:

- an aliquot cell count performed on the automatic Cell-Dyn 3500 machine or manually, depending on the cell numbers.

- a 100 -200 µl aliquot for cytospin to assay cells differentiation and morphology.

- an aliquot to monitor the megakaryocytic progenitor cells:

1.CFU-MK assay

2. Immunophenoteping for MK differentiation using immunoflourescence flow cytometry

2.2.10 FOR IMMUNOPHENOTYPING USING FLOW CYTOMETRY

SET CONTROL

The control was always a monoclonal antibody using in the experiment and same subclass. 2.5 µl isotype matched IgG1/IgG2 monoclonal antibodies were used. This control help recognitive the stained cells considered to be positive.

DIRECT STAINIG

$1x10^6$/ml cells were blocked by Intragam and incubated at 22^0C, 30min 2.5 µl of the monoclonal antibodies were added to each tube and incubated 15min, 4^0C in the dark. Cells were washed once with 2ml of Isoton II and 1% NSA at 300g centrifuge for 1min. After vortex, the cells were fixed in 0.3 ml of 1% paraformaldehyde in Isoton II. The cells were kept at 4^0C in the dark and analysed within 24hr on the FACScan using Lysis II software (Becton Dickinson).

SUMMARY OF CELLS SURFACE MARKERS

Surface markers used are listed in Table 2.1.

Table 2.1. Monoclonal Antibodies Used in Surface Marker Analysis

Antibody	Expression	clone details
CD34	very early antigen, for stem cells	581-PE-CY5
CD49d (41integrin)	very late antigen (VLA-4)	(Hp2)- FITC
CD49e(51integrin)	very late antigen (VLA-5)	SAM1-FITC
CD61(3integrin)	MK antigen	Plt glycoprotein IIIa, (Y2/51-FITC)
CD42b	MK antigen	Plt.glycoprotein-1b-FITC
CD53	tetraspan protein for membrane	(H129)-PE
CD63	tetraspan platelet protein	(CantibGran/12)-PE
CD9	tetraspan platelet protein	(M-L13)-PE
CD151	tetraspan Plt	G4-PE
IgG/IgG	control	PE/FITC

2.2.11 FLOW CYTOMETRY ANALYSIS

Flow cytometric analysis was performed on a FACScan (Becton-Dickinsin). A range of 5000-10,000 cells was collected in list mode format using B D LYSIS II software.

2.2.12 GATING STRATEGY

Dead cells were gated out by 7AAD staining and the percentage of cells positive for various markers was determined from the total live population. Then a gating from 7AAD/FSC was used to determine the cells within a FSC/SSC (forward scatter/side scatter light) lymphocytes gate R1, Fig.2.1.A. The combination cells from gate R1 were further analysed for R2 CD34 positive ($CD34^+$) and cells in region R3 was CD34 negative (34^-, Fig.2.1.B. The bright $CD34^+$ cells and negative $CD34^-$ cells were analysed for positive staining other markers. Pairwise combinations of fluorescently labelled monoclonal antibodies against the integrins (CD49d, CD49e, CD61) and the three tetraspans (CD53, CD63, CD151) were used to determine the co-expression of the respective integrin/tetraspan pairs.

2.2.13 IMMUNOPRECIPITATION

In this study, we further determined association of the respective integrins and tetraspans complexes on the cell surface by immunoprecipitation. Following liquid culture, the $CD34^+$ cells ($2x10^4$/per well) were grown with or without fibronectin for 4 days. $1x10^6$-$10x10^6$/ml cells were collected and washed once with PBS buffer and centrifuged 1876g, 10 min. (Samples were generally prepared according to the Protein A Immunoprecipitation Kit Boehringer-Mannheim package insert). Briefly, the cell pellet was suspended in lysis buffer, cells were frozen on dry ice for 10min then thawed at 37°C for 10min. This process was repeated 3 times. The suspension was centrifuged at 12000g, 10 min, 4^0C to remove debris. After transfer of the supernatant to a Eppendorf 1.5ml microfuge tube, each 1ml of solution was pre-cleared by the addition of 50 µl of homogeneous protein A-agarose, and incubated overnight at 4^0C with rocking. The agarose was removed by centrifugation at 12,000g for 20 seconds in an Eppendorf microfuge. 0.2µg of the appropriate purified monoclonal antibody against CD49d, CD49e and CD61 was added to each 1ml supernatant

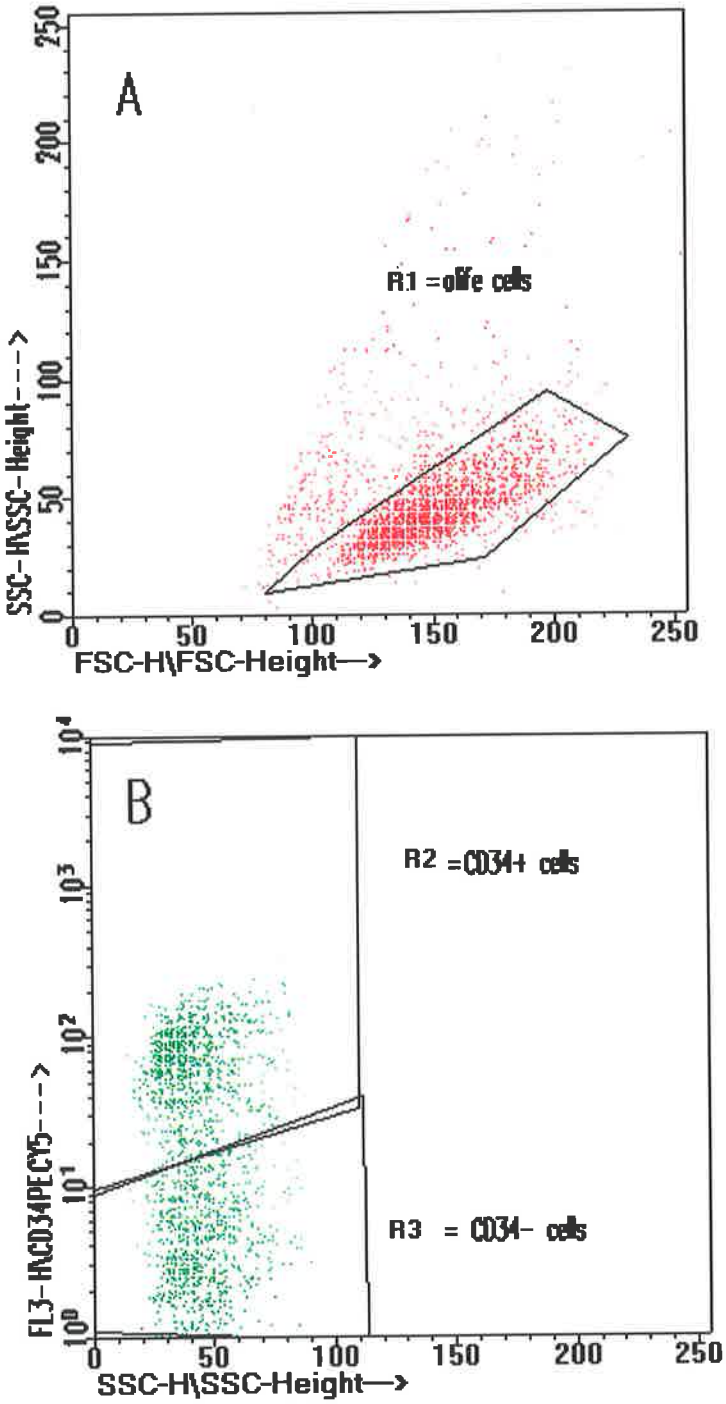


FIG. 2.1 Gating strategy. A=(R1) backing from 7AAD/FSC to a FSC/SSC lymphocytes gate. B= a backing gate from R1 of FL3/SSC to a $CD34^{+}$ cells gate (R2) and a $CD34^{-}$ cells gate (R3).

and incubated at 4^0C for 1hr with rocking. Homogeneous protein agarose (50µl) was added to each sample, incubated at 4^0C for a further 24hr with rocking. The pellet, which contained the integrins -protein A complexes, was washed once in 1 ml buffer 1 (Protein A kit) and incubated at 4^0C for 20 min on a rocking platform. The process was repeated once with the same wash buffer 1. To the collected complexes was added 1 ml of wash buffer 2 (protein A kit). Complexes were resuspended, and incubated at 4^0C for 20min on a rocking platform. Beads were again pelleted and the supernatant removed. The pellet was resuspended in 1ml of wash buffer 3 (protein A kit) and incubated for 20 min at 4^0C on a rocking plaform. The respective pellet, which contained the CD49d, CD49e, and CD61-antibody-protein-A complexes, was then resuspended in 40µl gel-loading buffer (1% w/v) SDS, (10% w/v), 0.02 M Tris-HCl pH 7, 2-mercaptoethanol (5%), 0.1% (w/v) Bromophenol Blue. Samples were boiled for 5 min, centrifuged at 12,000g for 20 sec at room temperature and supernatant was transfer to a fresh tube. Samples were analysed by SDS-PAGE and Western Blotting.

Protein samples were separated on a mini SDS-PAGE gel containing 10% polyacrylamide and transferred onto Hybond-C nitrocellulose using the semi-dry electroblotter at 64 mA for 2 h. The membrane was blocked with 5% skim milk at room temperature for 2 h, the membrane was washed 3 times in PBS containing 1% (w/v) BSA and 0.1% Tween 20 and then incubated overnight with 10ml of a 1:500 dilution in PBS of tetraspan monoclonal antibodies (CD63 or CD151) at 4^0C with rocking. After washing with PBS buffer 3 times, the membrane was incubated with HRP-conjugated mouse anti-IgG (1:10000) in PBS for 1hr at room temperature with rocking. The blot was washed 1x15 min and 4x5 min in fresh changes of buffer. The membrane was exposed in equal volume of 10 ml of detection solutions 1 and 2 at 1 min, the excess solution was poured off and the membrane which was then wrapped in *Cling-Wrap* and exposed to a X-ray film in a light-tight cassette. The exposure time varied from 1 min to 30 min, depending on the amount of target protein applied in the membrane.

2.2.14 STATISTICAL ANALYSIS

The data were expressed as mean±standard error of the mean (SD). Statistical analysis was performed using a non-parametric Mann-Whitney U Test. The values of $P<0.05$ were considered to be statistically significant.

CHAPTER 3
RESULTS

RESULTS

3.1 THE ROLE OF MGDF IN MEGAKARYOCYTE PROGENITOR CELL DEVELOPMENT IN *IN VITRO* CULTURE

INTRODUCTION

Regulation of megakaryopoiesis is controlled by a complex series of interacting cytokines *in vitro.* MGDF was shown to induce thrombocytosis *in vivo* and stimulate MK colony formation as well as MK maturation *in vitro* (de Sauvage et al, 1994). MGDF is thought to be the major regulator of megakaryopoiesis.

Megakaryocyte development is possibly the net effect of interactions of $CD34^+$ precursors with extracellular matrix and secreted cytokines in particularly MGDF. The mechanism by which MGDF regulates megakaryocyte development is not well defined. This study investigates the possible interactive mechanisms of MGDF and extracellular matrix in megakaryopoiesis by:

(1) examining the effects of MGDF on $CD34^+$ cell proliferation;

(2) analysing changes in the expression of adhesion molecule receptors CD49d and CD49e on MK lineage in the presence of MGDF;

(3) determining the functional role of integrins CD49d and CD49e in megakaryopoiesis in the presence of MGDF and/or fibronectin and/or stromal cells.

Isolated $CD34^+$ cells from CB were used as the starting cells.

3.1.1 ISOLATION OF UMBILICAL CORD BLOOD CD34 POSITIVE CELLS ($CD34^+$)

0.1%-0.5% of CB cells are $CD34^+$. Immunomagnetic separation with the MACS system increased the proportion of $CD34^+$ progenitor cells in the CB to over 90%. Non-separated and isolated $CD34^+$ cells were analysed by flow cytometry. $CD34^+$ cells were gated. A typical dot plot and histogram of pre and post isolation $CD34^+$ cells directly labelled with

CD34-PE and CD45-FITC is shown in Fig 3.1.1. In the example 0.17% of unseparated $CD45CD34^+$ cells and 94% of MACS system isolated cells stained with CD34-PE. The enrichment of CD34 positive cells was 55-fold from mononuclear cells, and 188-fold from whole CB.

The degree of enrichment of $CD34^+$ cells in the 77 individual cord blood samples used in this study ranged from 60% to 98% but was generally in the range 85%-98% (Table 3.1.1).

As expected the isolated $CD34^+$ progenitor cells were morphologically a relatively homogenous population of large cells with little evidence of cytoplasmic maturation Fig. 3.1.2.

3.1.2 THE EFFECT OF MGDF ON TOTAL CELL COUNT IN LIQUID CULTURE

The effect of MGDF on $CD34^+$ proliferation in liquid culture with standard cytokines (SCF, IL-3 and IL-6) was assessed at day 4 and 8 of culture. The total white cell count was performed. As shown in table 3.1.2. MGDF with standard cytokines has minimal effective support for $CD34^+$ cell proliferation at day 4 and 8. With or without MGDF there was a 9.5-10 fold increase by day 4, and an increase of 38-49 fold cells over starting cell number ($2x10^4$/ml) by day 8.

3.1.3 THE EFFECT OF CYTOKINES ON CFU-MK IN AGAR CULTURE

$1x10^3$ $CD34^+$ cells were incubated with MGDF alone or with individual cytokines SCF, IL-3, IL-6, and with combinations of cytokines to determine the effects on MK colony formation. The effect of cytokines on the number of CFU-MKs is shown in Fig. 3.1.3. MGDF alone produced CFU-MK colonies. However, MGDF and IL-3 or IL-6 produced less colonies than MGDF alone. The three cytokines, SCF, IL-3, and IL-6 in combination, supported CFU-MK growth better than the two cytokine combination including MGDF alone. The combination of all cytokines stimulated the greatest increase in the CFU-MK from $CD34^+$ progenitor cells

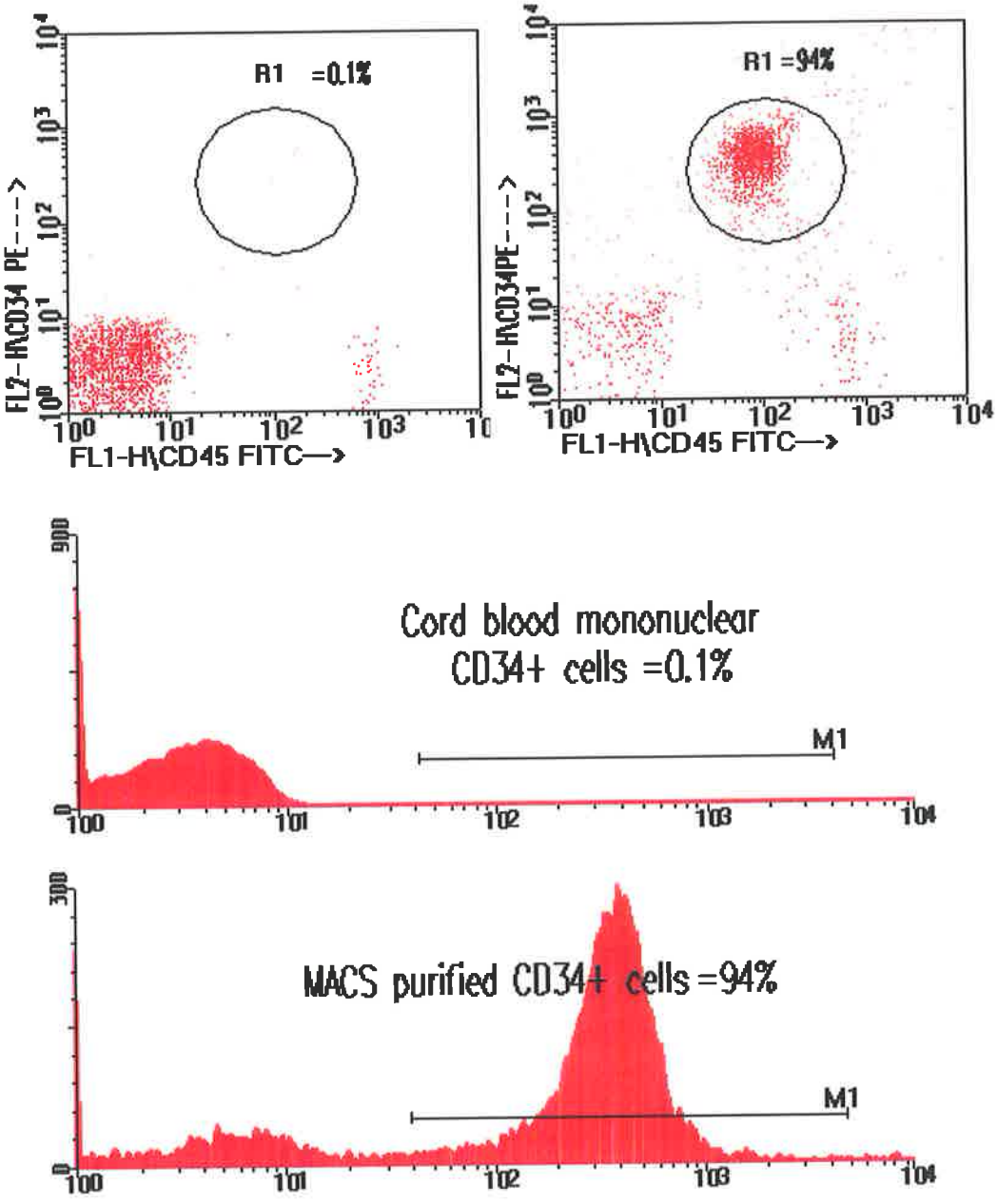


FIG.3.1.1. FACS analysis of mononuclear cells from cord blood using MACS CD34 isolation kit. Using direct staining with CD34-PE (vertical) and FITC-CD45 (horizontal axis). R1 (region 1) and marker M1 define $CD34^+$ cells from mononuclear cells of the cord blood (A), MACS purified and isolated $CD34^+$ progenitor cells (B). A histogram of fluorescence shows the same data (C and D).

Table 3.1.1. Percentage of CD34 positive cells. Table shows the percentage of MACS purified $CD34^+$ progenitor cells by histogram analysis.

Experiment No.	(%) $CD34^+$ cells
1	90
2	98
3	92
4	93
5	90
6	92
7	95
8	87
9	94
10	98

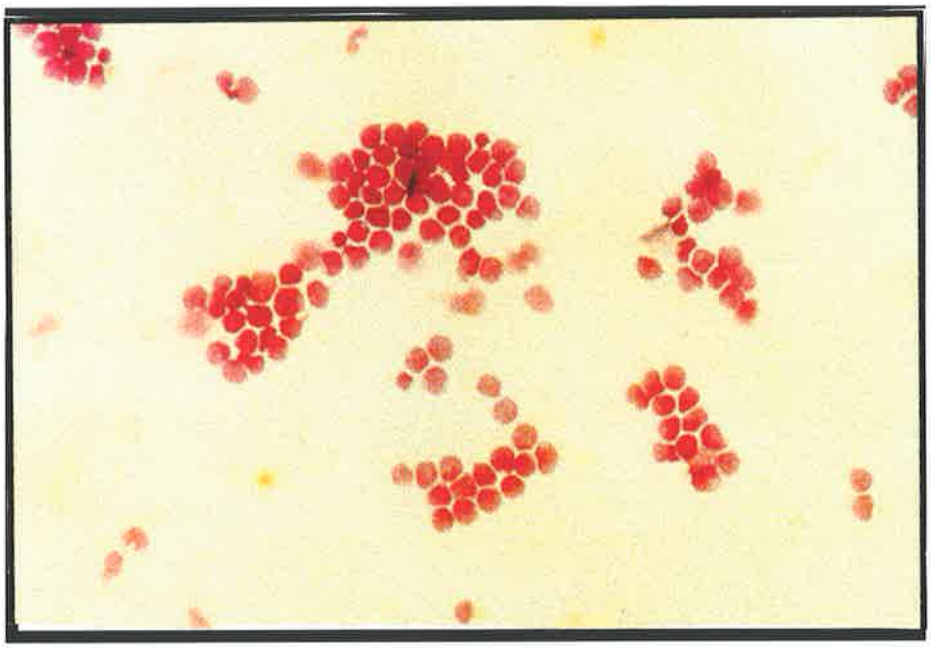


FIG. 3.1.2 Stained $CD34^+$ cell progenitor cells. The $CD34^+$ progenitor cells were cytofuged and further assessed by Wright-Giemsa staining. Original magnification x120.

Table. 3.1.2 Effect of MGDF on cell count in liquid culture.

	Viable cell count ($1x10^6$/ml)	
Days	MGDF	No MGDF
Day 0	0.02	0.02
Day 4	10±0.03	10±0.08
Day 8	37±0.17	34±0.14

Data are the mean ±SD (n=5).

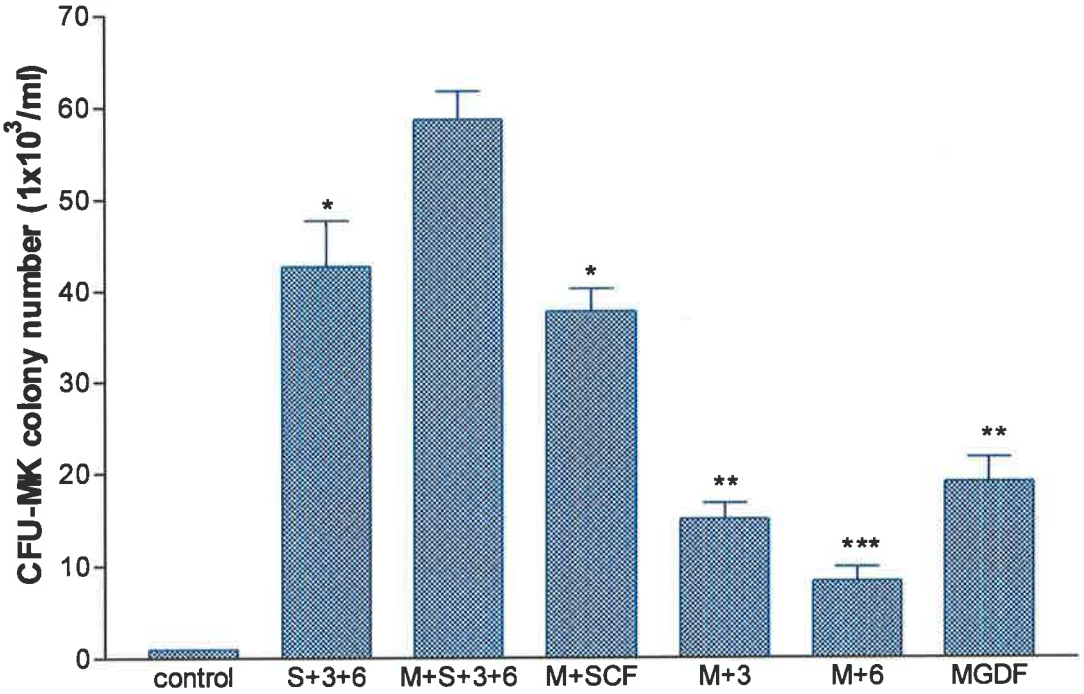


FIG. 3.1.3. Effects of cytokines on CFU-MK colony number. $CD34^+$ cells were cultured in semi-solid agar within different cytokines for 21 days.

The results shown as the mean ±SD (n=3). M=MGDF, S=SCF, 3=IL3, 6=IL6. Control was medium alone.

*p=0.043 significantly different compared with (M+S+3+6).

**p<0.014 significantly different compared with (M+S+3+6).

***p<0.011 significantly different compared with (M+S+3+6).

3.2 DETECTION OF ADHESION AND TETRASPAN MOLECULES ON $CD34^+$ CELLS

Little is known about the expression and functional role of adhesion molecules of the integrin β_1 family and the tetraspan superfamily on megakaryopoiesis. The percentage of $CD34^+$ cells expressing several of the integrins (CD49b, CD49d, CD49e, CD18, CD11a, CD41a, CD51, and CD61), and tetraspans (CD53, CD63, CD9) was determined. Results of their expression are shown in Table 3.2.1. In the integrin family, expression of CD49d positive CD34 cells was the highest (94.0%±1.8) and CD49e positive CD34 cells the lowest (5.4%±3.8). The percentage of expression of the other integrins was: CD61 (39.0%±3.1), CD41a (32.3%±5.1), CD11a (93.8%±3.9), CD18 (73.3%±5.1), CD51 (27.3±2.1) and CD49b (39.3±3.3). In the tetraspan family, 21.1%±1.2 of $CD34^+$ cells expressed CD63 whereas $CD34^+$ cells expressed low levels of CD9, CD53 and CD151 (3.5-6.5%).

3.2.1 EXPRESSION OF CD49D AND CD49E ON $CD34^+$ CELLS CULTURED WITH OR WITHOUT MGDF

$CD34^+$ cells were cultured in liquid culture with standard cytokines with or without MGDF over 8 days. The expression of CD49d and CD49e was evaluated Fig 3.2.1. Majority (94.5%) of $CD34^+$ cells expressed CD49d at day 0 in both culture conditions, the percentage of expression of CD49d was significantly reduced at day 4 ($p=0.021$) to the same extent but was increased at day 8 ($p=0.021$) irrespective of the addition of MGDF. However, at day 8 there was a slightly higher percentage of $CD34^+$ cells expressing CD49d with MGDF than without MGDF (73.5% vs 66.9%, $p>0.05$).

By contrast to CD49d, only a small percentage (5.4%) of cells expressed CD49e at day 0 and the expression of CD49e decreased further irrespective of the addition of MGDF at day 4 Fig. 3.2.2. However, the percentage of CD49e positive cells was significantly increased by day 8. Also at day 8, the addition of MGDF increased the percentage of CD49e on $CD34^+$ cells significantly compared to those cultured in the absence of MGDF (22% vs 12%, $p=0.02$).

Table 3.2 1 Expression of integrin and trtraspan molecules on $CD34^+$ cells at day 0

Integrin family	expression (%)
CD49d	94.0% ± 1.8
CD49e	5.4% ± 3.8
CD49b	39.3% ±3.3
CD61	39.0% ± 3.1
CD41a	32.2% ± 5.1
CD11a	93.8% ± 3.9
CD18	73.3% ± 5.1
CD51	27.3% ± 2.1
Tetraspan family	
CD9	5.7% ± 0.8
CD53	3.5% ± 0.5
CD63	21.1% ± 1.2
CD151	6.5% ± 1.4

Values of percentage expression on $CD34^+$ cells represent mean ± SD (n=4).

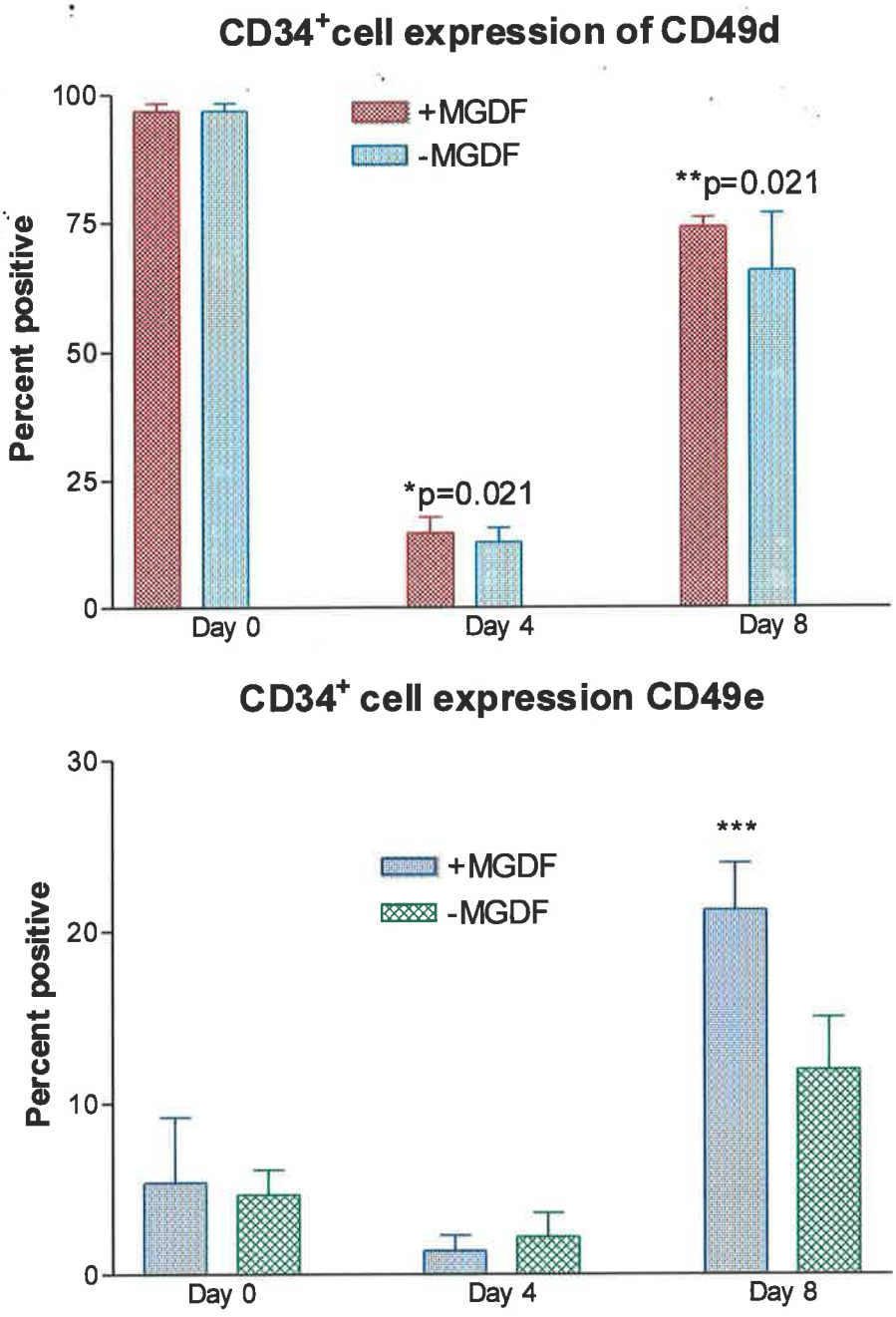


FIG. 3.2.1. Expression of CD49d and CD49e on $CD34^+$ cells following culture with or without MGDF during day 0 to day 8. Each value represents the mean ±SD (n=4).

*p=0.021 significantly reduce compared with day 0.

**p=0.021 significantly increase compared with day 4.

***p=0.043 significantly increase compared without MGDF at day 8.

3.2.2 THE EFFECTS OF MGDF ON EXPRESSION OF MEGAKARYOCYTIC LINEAGE MARKER CD61

CD61 belongs to the β_3 integrin superfamily and is a specific lineage marker of MKs and platelets and also on macrophage and endothelia cells. Lineage can be assessed by flow cytometry using expression of CD61. $CD34^+$ cells were cultured with standard cytokines in the presence or absence of MGDF in liquid culture for up to 8 days. The results are summarised in Fig 3.2.2 (I). Expression of CD61 on $CD34^+$ cells was 39%±3.3 at day 0. With culture there was a significant reduction of expression of CD61 by day 4 ($p=0.02$). However, MGDF maintained a slightly higher percentage of expression CD61 on $CD34^+$ cells, (9.0% ±0.5 vs 7.8% ±1.2). At day 8, CD61 expression increased further with MGDF maintaining a significantly higher percentage of expression ($p=0.043$). CD61 expression on day 4 and day 8, however, remained lower than that of day 0 ($p=0.021$).

The expression of CD61 on $CD34^-$ cells was investigated in culture presence and absence of MGDF over 8 days incubation, Fig. 3.2.2 (ii). The initial expression of CD61 on $CD34^-$ was low (12%±0.2) at day 0, with over 90% of cells being $CD34^+$ at day 0. Similarly to $CD34^+$ cells at day 4, there was significant reduction of expression of CD61 ($p=0.02$) compared with day 0 levels, in both presence and absence of MGDF. However by day 8, CD61 levels had risen to 24%±3.1 at day 8 and to 19%±2.3 in the presence and absence of MGDF respectively. These levels were significantly higher ($p=0.043$) than those measured at day 0.

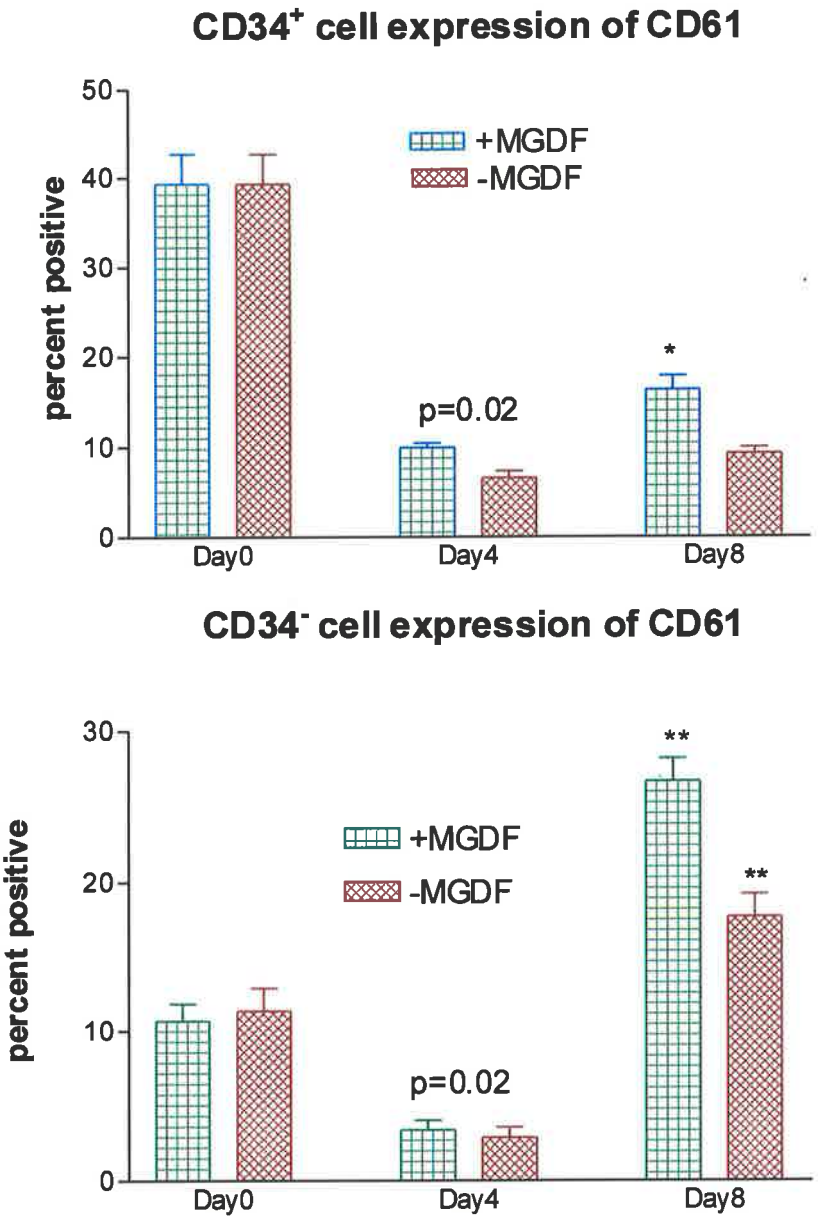


FIG. 3.2.2. The effects of MGDF on expression of CD61 lineage marker on $CD34^+$ cells and $CD34^-$ cells in liquid culture at day 0 to day 8. The data are expressed as the mean ±SD (n=3).

P=0.02 significantly decrease compared with day 0.

*p=0.043 significantly increased compared to culture without MGDF at day 8.

**p=0.043 significantly increased compared to day 0 on $CD34^-$ cells.

3.3 EFFECT OF FIBRONECTIN ON MEGAKARYOCYTE DIFFERENTIATION

INTRODUCTION

The effect of fibronectin on the development of MK progenitor cells was investigated by i) the expression of MK lineage markers CD61 and CD41a, ii) MK progenitor colony assay following subculture and iii) co-expression of β_1 integrins and tetraspans in the presence of fibronectin.

3.3.1 EFFECT OF FIBRONECTIN ON CD34$^+$ CELL NUMBER AND PERCENTAGE OF CD34$^+$ CELLS IN CULTURE

The effect of addition of fibronectin to cell culture on total and CD34$^+$ cell number is shown in Table 3.3.1. At day 4 in liquid culture, the total cell number was increased 4.4 fold over day 0 and in the presence of fibronectin 6.8 fold. Assuming 90% of cell on day 0 were CD34$^+$ cells, the number of CD34$^+$ cells was increased 1.5 fold in liquid culture and 2.0 fold in fibronectin over day 0 levels. By day 8, there were further significant increases in both total cell and CD34$^+$ cell counts in both liquid and fibronectin cultures. However, the rise in both these counts at both days 4 and day 8, was significantly greater in the presence of fibronectin than in liquid culture alone ($p=0.021$). At day 8, fibronectin culture showed increase in total cell number of 15 fold and in CD34$^+$ cell number of 2.9 fold.

The percentage of CD34$^+$ cells was investigated in both cultures at day 0, 4, 8. In the example given in Fig. 3.3.1 76% of cells at day 0 were CD34$^+$. By day 4, the percentage of CD34$^+$ cell had decreased to 65% in fibronectin culture and even further (50%) in liquid culture. By day 8, the percentage of CD34$^+$ cells was reduced to 14.5% in fibronectin culture and 5.4% in liquid culture. CD34$^+$ cell numbers in liquid versus fibronectin cultures were significant different ($p=0.02$). Fibronectin may have the potential to increase CD34$^+$ cell number. ECM may have a "secret preserver" of CD34$^+$ cells during cell culture.

Table 3.3.1 Effect of fibronectin on $CD34^+$ cell number during cell culture

	Total cell number ($x10^4$)			CD34+ cell number ($x10^4$)		
	Day 0	**Day 4**	**Day 8**	**Day 0**	**Day 4**	**Day 8**
LI	2.0	8.8±6.7	12.2.±8.9	1.8	2.8±1.8	3.2±2.6
FIN	2.0	13.8±7.9	*30.0±10.1	1.8	3.6±1.7	5.3±3.6

Data represent mean±SD (n=4). LI= liquid culture, FIN= fibronectin culture.

* p=0.021 significantly different from liquid culture at day 8.

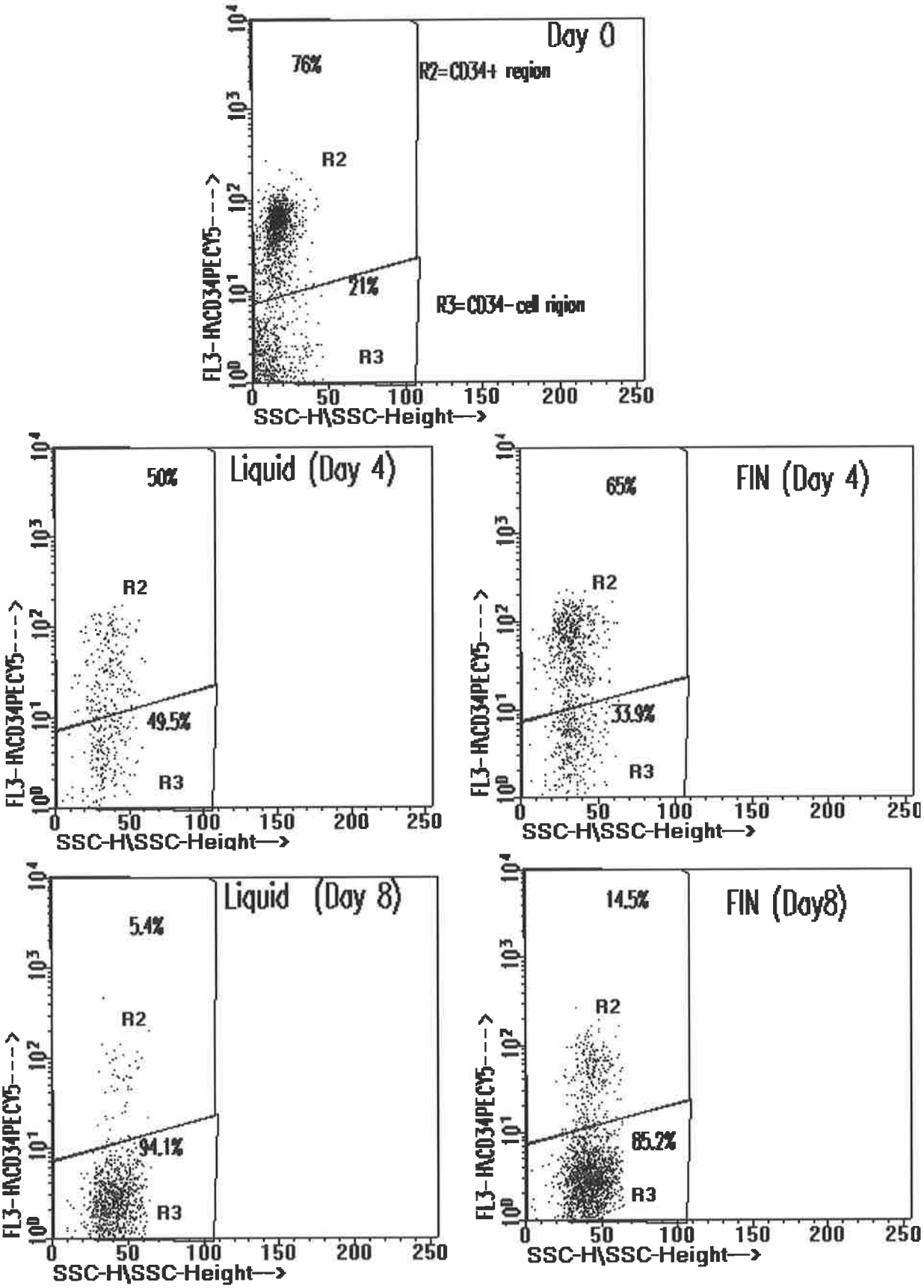


FIG. 3.3.1. The effect of fibronectin on proportion of $CD34^+$ and $CD34^-$ cells from day 0 to day 8. FIN= fibronectin.

3.3.2 MICROSCOPIC STUDY OF CD34$^+$ CELLS CULTURED ON FIBRONECTIN

A typical photomicrograph of cell growth with or without fibronectin is shown in Fig. 3.3.2. In the presence of fibronectin, cells were frequently seen clustered around fibronectin fibres, whereas in liquid culture, colony-like clusters of cells were seen.

3.3.3 THE EFFECT OF FIBRONECTIN ON MK PROGENITOR CELL GENERATION USING IMMUNOPHENOTYPING

At day 0, CD34$^+$ cell co-expression of CD61 (GpIIIa–early marker) and CD41a (GpIIb-intermediate marker) was 39% and 32% respectively Table 3.2.1. Both lineage markers altered expression on CD34$^+$ and CD34$^-$ cells during culture. Furthermore, CD61 has been detected on endothlial cells, monocytes, and fibroblasts, as well as megakaryocytes (Bai et al., 1984), CD61 is a sensitive, but not specific marker for the megakaryocytic lineage

Fig. 3.3.3 shows the effect of fibronectin on co-expression of MK lineage markers CD61 and CD41a on CD34$^+$ cells at days 4 and 8. In the presence of fibronectin, CD34$^+$ cells significantly increased expression of CD61 ($p=0.043$) and CD41a ($p=0.021$) compared with liquid culture at day 4. At day 8, the percentage of CD34$^+$ cells with co-expression of CD61 was significantly increased in the presence of fibronectin ($p=0.021$) but there was no significant difference in expression of CD41a between the two culture systems. In fact there was a decrease in expression of CD41a on CD34$^+$ cells in the presence of fibronectin at day 8.

Figure 3.3.4 shows the expression of CD61 and CD41a on CD34$^-$ cells in both culture systems at day 4 and day 8. Both CD61 and CD41a expression were significantly higher on CD34$^-$ cells, a 5.7 fold increase in percentage expression of CD61 and a 3.5 fold higher expression of CD41 were noted in the presence of fibronectin at day 4 ($p=0.021$). At day 8, in the presence of fibronectin, the percentage of expression of both CD61 and CD41a on CD34$^+$ cells was significantly higher ($p=0.043$) than CD34$^-$ cells in liquid culture. Taken together the results indicate that fibronectin had a significant early differentiating effect on megakaryocyte development.

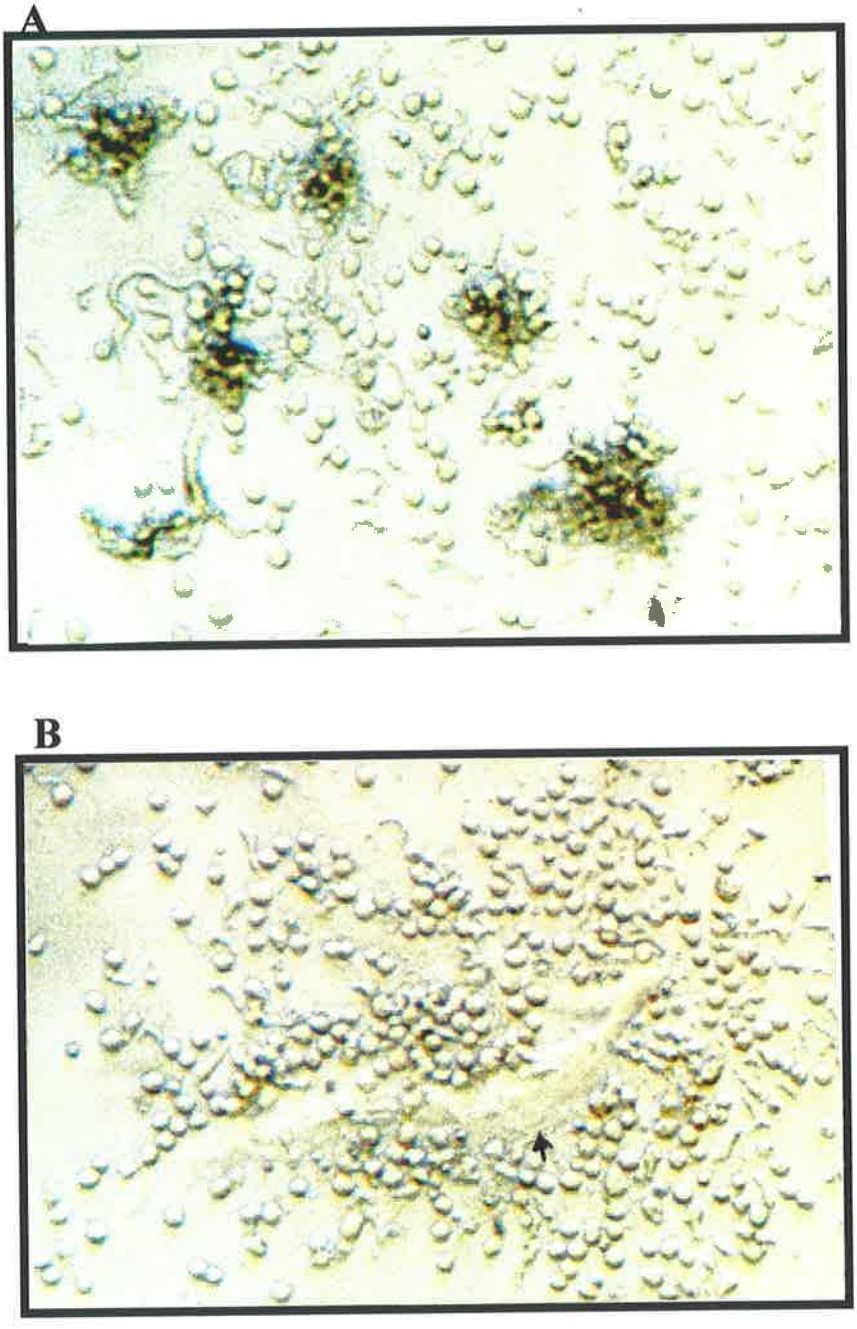


FIG. 3.3.2 A photomicrograph of the $CD34^+$ cells cultured without fibronectin (A), or with fibronectin (B). In the presence of fibronectin, $CD34^+$ cells were seen clustered around fibres (indicated by arrow). Original magnification x250.

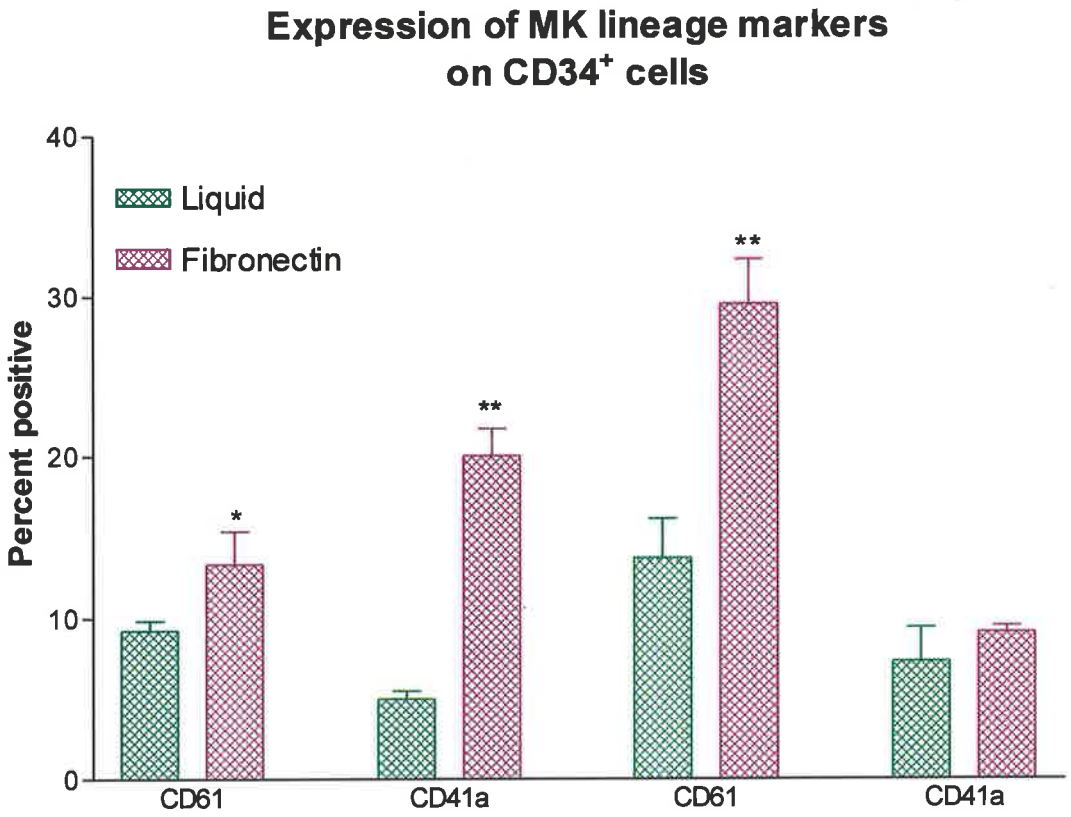


FIG. 3.3.3. The effects of fibronectin on expression of MK lineage markers on $CD34^+$ cells. Expression of CD61 results are as the mean±SD (n=4).

*p=0.043 significantly increased compared with liquid culture.

**p= 0.020 significantly increased compared with liquid culture.

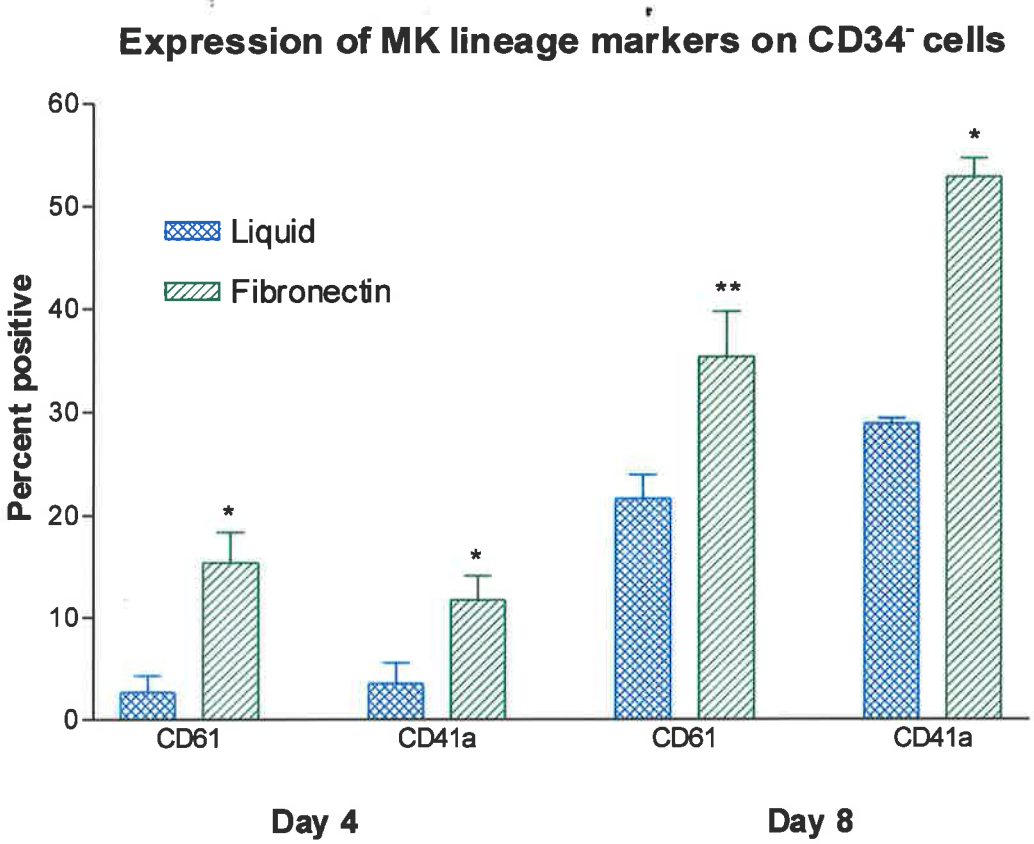


FIG. 3.3.4. The effects of fibronectin on $CD34^-$ cell expression of MK markers during culture period.

The results are shown as the mean±SD (n=4).

* p= 0.021 significantly increased compared with liquid culture at day 4.

**p=0.043 significantly increased compared with liquid culture at day 8.

3.3.4 EFFECT OF FIBRONECTIN ON MK PROGENITOR CELL GENERATION

At day 4 and 8 of culture, cells were subcultured into semi-solid agar for CFU-MK colony assay. CFU-MK colony numbers were increased in the presence of fibronectin but, the increase was not statistically significant ($p=0.08$) at day 4 Fig.3.3.5. However, at day 8 the number of CFU-MK colonies was significantly increased in the presence of fibronectin ($p=0.029$). The CFU-MK results indicated that fibronectin increases commitment of precursor cells to the megakaryocytic lineage.

3.3.5 EFFECT OF FIBRONECTIN ON MK PROLIFERATION

The effect of fibronectin on proliferation of MK progenitors was assessed by examination of three individual experiments on MK colony size (number of cells per colony) in semi-solid agar culture. The size of CFU-MKs was slightly increased in the presence of fibronectin, the liquid culture showed a slight bias to the smaller (1-5 cell) colonies (21% of total colony CFU-MKs vs 10% for fibronectin) whereas fibronectin showed a slight bias to larger (>100 cell) colonies (29% of total colony CFU-MKs vs 21% for liquid) Table 3.3.2. These data suggest that fibronectin has some effect on proliferation of differentiating MK progenitor cells.

3.3.6 THE EFFECT OF FIBRONECTIN ON EXPRESSION OF INTEGRINS AND TETRASPANS ON $CD34^+$ CELLS

The mechanisms by which fibronectin increases megakaryocyte commitment were studied by assessing its effect on integrin and tetraspan expression and their molecular interaction. $CD34^+$ cells do not constitutively express CD49d and CD49e integrins. With culture $CD34^+$ cells increased their expression of these two integrins. The effect of fibronectin on the expression of the integrins CD49d and CD49e on $CD34^+$ and $CD34^-$ cells is

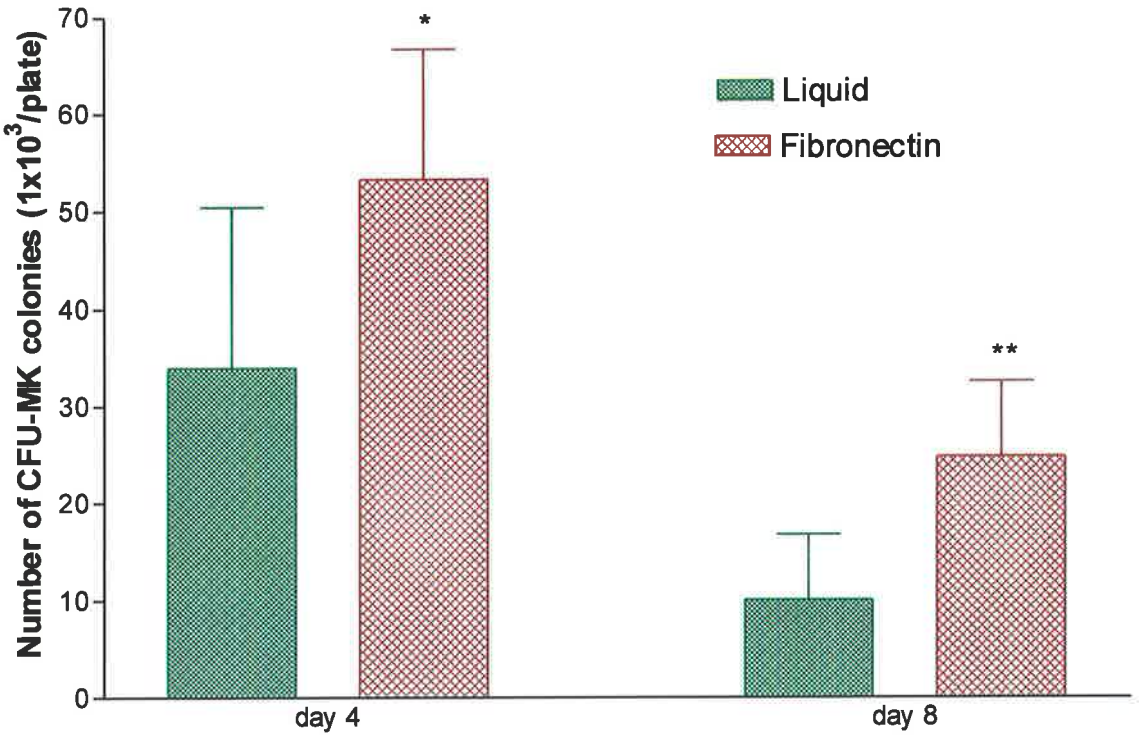


FIG. 3.3.5. The effect of fibronectin on MK lineage commitment. $CD34^+$ cells were co-cultured with or without fibronectin for 8 days. On day 4 and day 8, cells were subcultured in semi-solid agar for a further 14 days to identify CFU-MK. The results are shown as the mean±SD (n=3).

*P=0.043 significantly increased compared with liquid culture.

** p= 0.029 significantly increased compared with liquid culture.

Table. 3.3.2. Effect of fibronectin on MK colony size at day 4.

	Total colony (No.)	Range of colony size on day 4 (cell no.)		
		1-5	5-50	0ver 100
Liquid Culture	61	13±0.5	35±5.0	13±2.0
Percent colony		(21%)	(57%)	(21%)
Fibronectin culture	98	10±1.4	60±6.0	28±4.0
Percent colony		(10%)	(61%)	(29%)

$CD34^+$ cells ($1x10^4$/ml) were cultured in the presence of fibronectin or liquid culture. At day 4, $1x10^3$/ml cells were further subcultured in semi-solid agar for 14 days. Data represent values from three independent experiments.

There was no significant difference on MK colony size in either cell culture system.

summarised in Table 3.3.3, and representative dot plot of $CD34^+$ cell expression of CD49d and CD49e at days 4 and 8 of cell culture Fig.3.3.6 and Fig. 3.3.7.

A significantly higher expression of CD49d and CD49e ($p=0.043$) was noted in the presence of fibronectin compared to liquid culture by day 4. At day 8, $CD34^+$ cells increased their expression of CD49d with a higher percentage of cells expressing it in the presence of fibronectin ($p=0.04$). A significant increase in expression of CD49e ($p=0.021$) was also noted in the presence of fibronectin compared to liquid culture.

When the more differentiated $CD34^-$ cells were studied, it was similarly found that by day 4 fibronectin resulted in an increase in expression of CD49d and CD49e ($p=0.02$). However, the expression was less than that observed with $CD34^+$ cells Table 3.3 3.

By day 8, $CD34^-$ cells increased further their expression of CD49d with a higher percentage of cells expressing it in the presence of fibronectin ($p=0.043$). The expression of CD49e was similarly increased on $CD34^-$ cells by fibronectin. However, the CD49d expression on $CD34^-$ cells was as high as that of $CD34^+$ cells at this time point (97.9% vs 96.8% respectively). These results suggest that fibronectin maintained and enhanced expression of CD49d and CD49e on $CD34^+$ cells.

Tetraspan molecules are associated with integrins and are considered to be important in signal transduction for the integrins (Schwartz et al, 1995). There are several candidate tetraspan molecules and preliminary experiments showed a co-expression of CD63 and CD151 with CD49d and CD49e on $CD34^+$ cells. Furthermore, CD151 was found on megakaryocytes and platelets but its role in megakaryopoiesis is unknown. Therefore the effect of fibronectin on expression of the tetraspans CD63 and CD151 was investigated.

The results are shown in Fig. 3.3.8. At day 0, $CD34^+$ cells co-expression of CD63 was 21.1%± 1.2. There was a 75%-85% of $CD34^+$ and $CD34^-$ cells co-expressed CD63 in both cultures at day 4. Expression of CD63 was Similarly obtained in both cell cultures at day 8. However, as with day 4, the percentage expression of CD63 was not altered by fibronectin at day 8.

The effect of fibronectin on expression of CD151 can be seen in Fig.3.3.8. $CD34^+$ cells have a low expression of CD151 at day 0, but by day 4 fibronectin increased slightly the expression of CD151 on $CD34^+$ and $CD34^-$ cells. The percentage expression of CD151 was higher on $CD34^-$ cells in both cultures. However, fibronectin significantly increased

Table 3.3.3 The effect of fibronectin on expression of integrins on $CD34^+$ and $CD34^-$ cells.

Day 4 Integrin	$CD34^+$ cell (%)		$CD34^-$ cell (%)	
	LI	FIN	LI	FIN
CD49d	13.2±2.6	*48.3±5.4	2.3±1.7	***8.6±1.0
CD49e	1.4±0.9	*10.7±3.2	0.6±0.4	***3.2±1.1
Day 8 Integrin	**$CD34^+$ cell (%)**		**$CD34^-$ cell (%)**	
	LI	FIN	LI	FIN
CD49d	75.2±5.0	**96.8±2.1	78.0±2.0	**97.9±1.3
CD49e	21.6±2.1	***51.4±4.0	14.3±2.4	**27.3±0.7

The results show expression level as the mean ± SD (n=3). FIN=fibronectin, LI=liquid.

* p=0.043 significantly different from liquid culture.

**p=0.043 significantly different from liquid culture.

***p=0.021 significantly different from liquid culture.

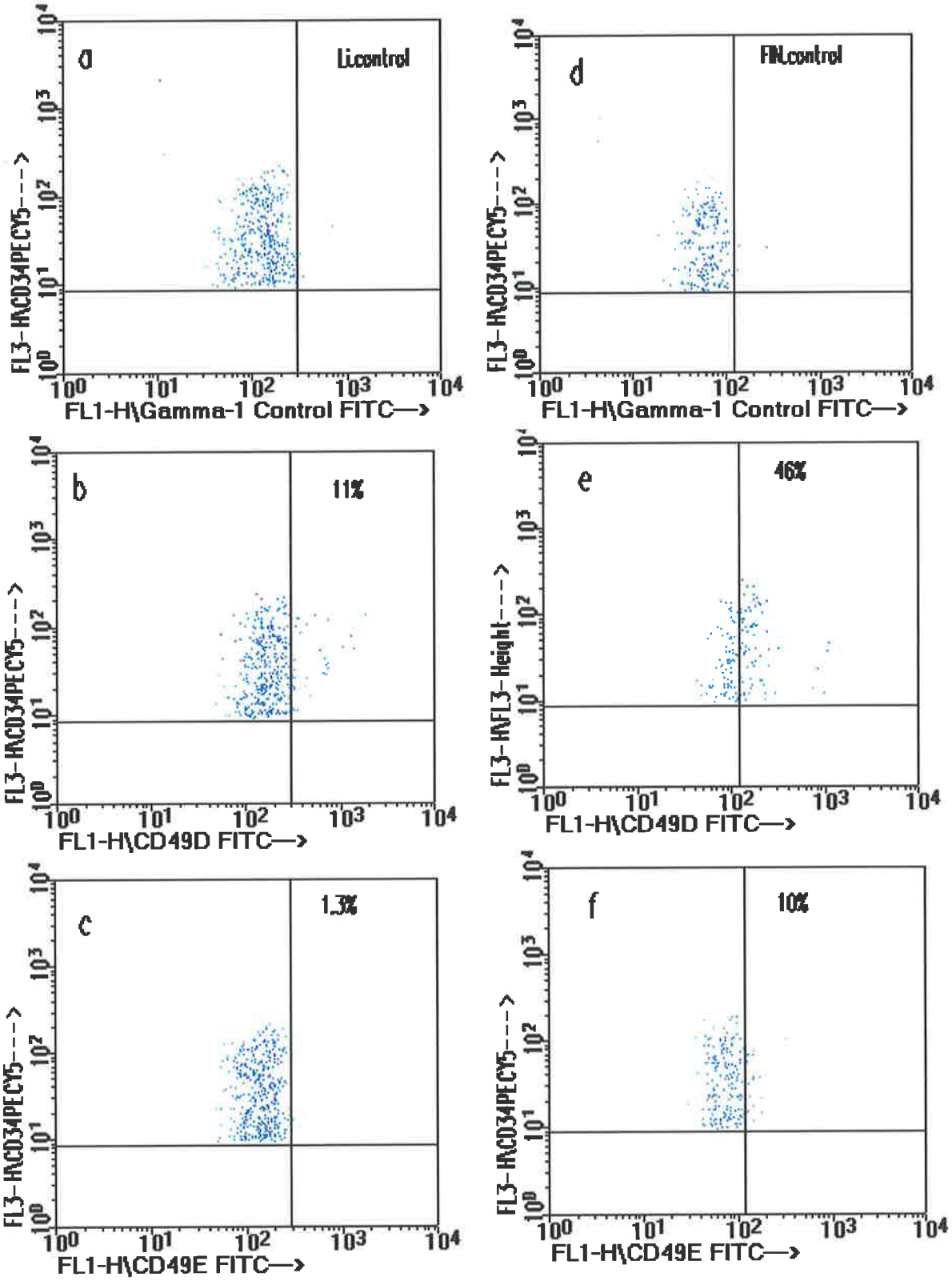


FIG.3.3 6. Representative dot plots showing the effect of fibronectin on $CD34^{+}$ cell co-expression of CD49d and CD49e at day 4. LI. control = <1% positive events of liquid on control plot (a). FIN control = <1% positive events of fibronectin on control plot (d).

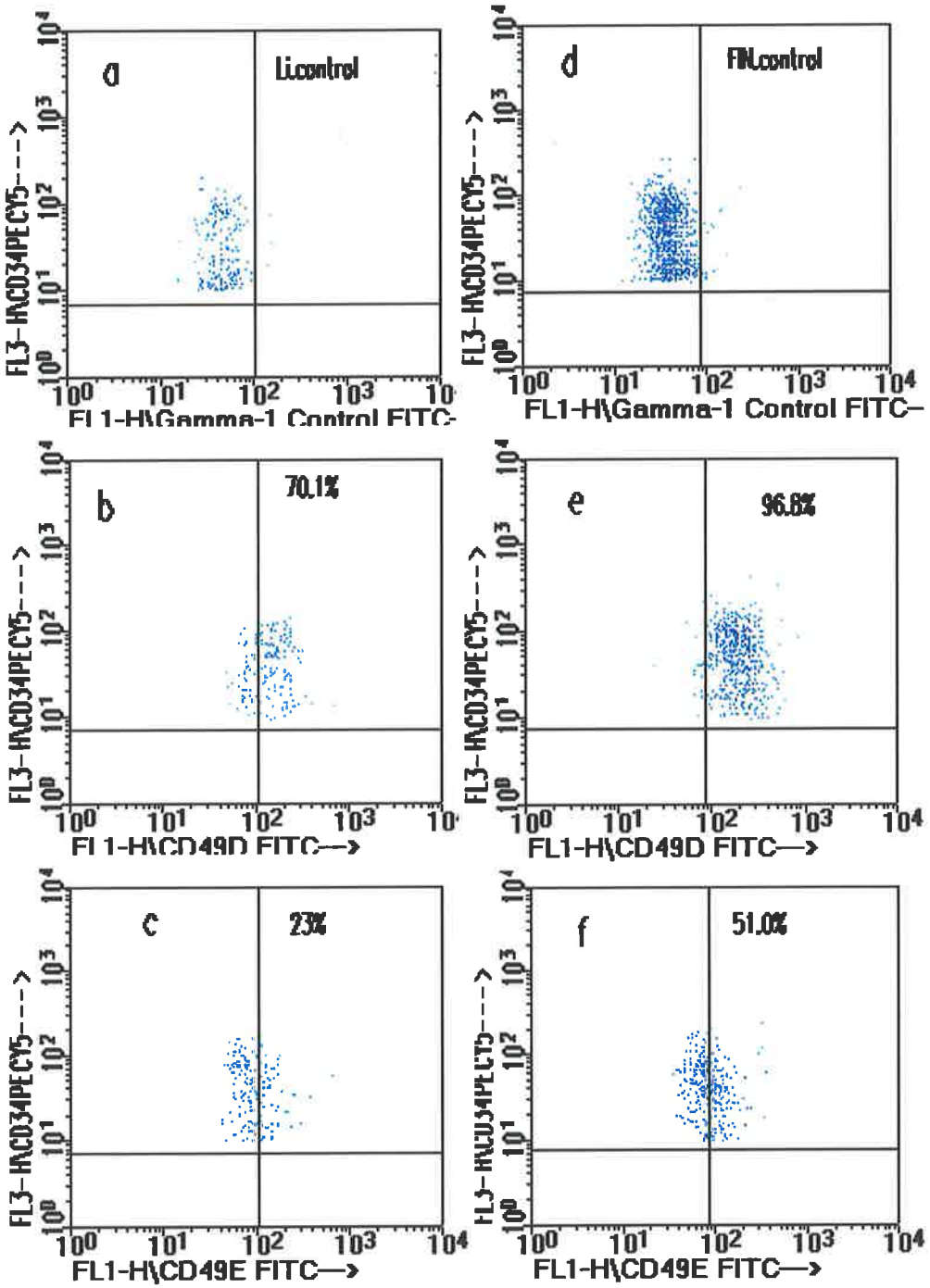


FIG. 3.3.7. Representative dot plots showing the effect of fibronectin on expression of CD49d and CD49e on $CD34^{+}$ cell at day 8.

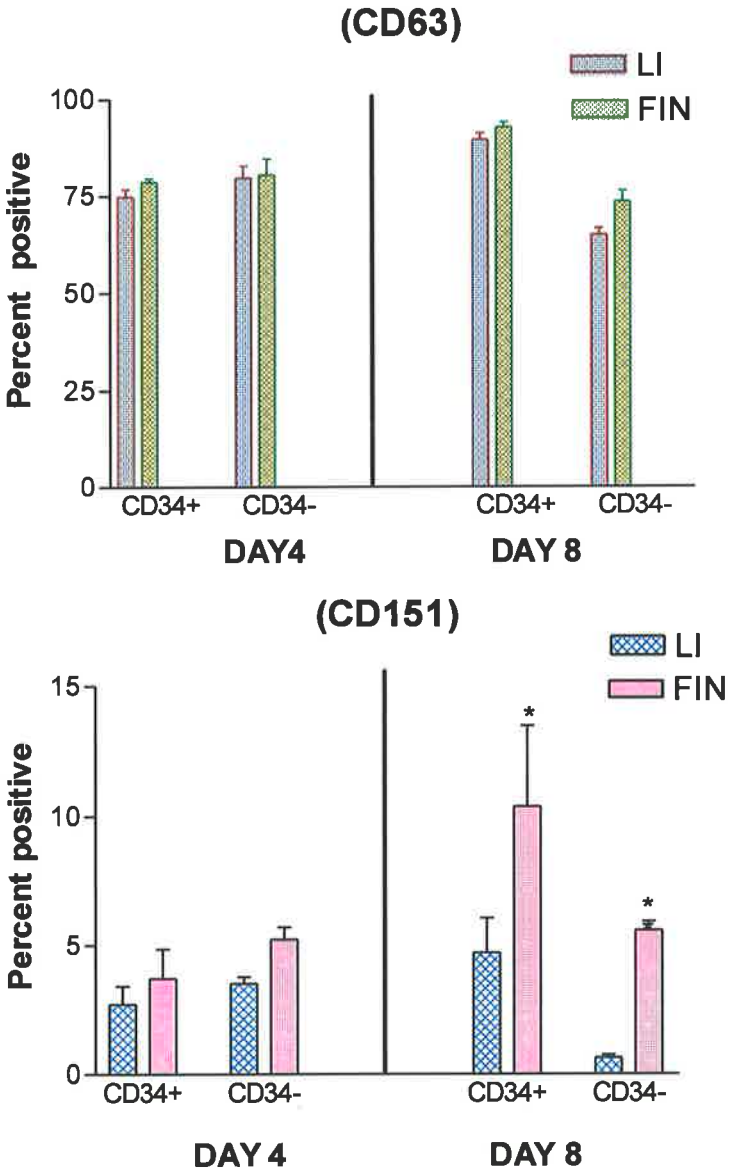


FIG. 3.3.8. Effect of fibronectin on expression of tetraspans on $CD34^+$ and $CD34^-$ cells during cell culture over 8 days. The results were shown as the mean±SD (n=3). There was no significantly different in CD63 expression on $CD34^+$ or $CD34^-$ cells using either culture system. The percentage expression of CD151 was significantly increased on $CD34^+$ and $CD34^-$ cells in the presence of fibronectin at day 8.

LI=liquid, FIN=fibronectin.

*p=0.021 significantly different from liquid culture.

expression of CD151 at day 8 on both $CD34^+$ and $CD34^-$ cells (p=0.02). These results indicate that fibronectin has little influence on the expression of CD63 on $CD34^+$ and $CD34^-$ cells in contrast to the expression of CD151.

3.3.7 THE EFFECT OF FIBRONECTIN ON INTEGRIN AND TETRASPAN CO-EXPRESSION

To define the relationship of the tetraspans with the integrins, $CD34^+$ cell co-expression of integrins CD49d, CD49e and CD 61 (MK marker) with tetraspans CD63 and CD 151 was determined.

At day 0, there was no co-expression of CD49d, CD49e and CD61 with CD63 on $CD34^+$ cells. However, culture of the cells led to an increase in their co-expression on both $CD34^+$ and $CD34^-$ cells.

$CD34^+$ cell co-expression of integrins CD49d, CD49e and CD61 (MK marker) with tetraspan CD63 was analysed by contour plots Table 3.3.4 and representative contour plot in Fig.3.3.9.

At day 4, co-expression of CD49d/CD63 on $CD34^+$ cells in fibronectin was significantly higher than on these cells in liquid (control) culture (p=0.043). However, the presence of fibronectin resulted in reduced co-expression of CD49e/CD63 and significantly reduced co-expression of CD61/CD63 (p=0.043) on these cells at day 4.

At day 8 on $CD34^+$cells the presence of fibronectin resulted in a slightly greater co-expression of CD49d/CD63, and a significantly (p=0.021) higher co-expression of CD49e/CD63 and CD61/CD63 compared with liquid culture control representative contour plot in Fig.3.3.10.

When day 4 and 8 fibronectin cultures were compared, it was found that there was increased co-expression of CD49d/CD63 (91.8% vs 60.9%), CD49e/CD63 (61.8% vs12.7%) and CD61/CD63 (37.4%vs 7.8%) on $CD34^+$ cells at day 8. When day 4 and day 8 liquid culture were compared, there was an increase co-expression of CD49d/CD63 (71.9% vs 36.9%) on the $CD34^+$ cells on day 8 as compared to day 4. However, the percentage co-

Table 3.3.4. Co-expression of integrins on $CD34^+$ cell CD49d, CD49e and CD61 (MK marker) with tetraspan (CD63) at day 4 and day 8 of cell culture.

DAY 4

Activity marker	LI (%)	FIN(%)
CD49d/CD63	36.9±7.0	*60.9±6.1
CD49e/CD63	20.9±1.8	12.7±6.2
CD61/CD63	***25.8±7.9	7.8±1.4

DAY 8

Activity marker	LI (%)	FIN (%)
CD49d/CD63	71.9±7.7	91.8±0.7
CD49e/CD63	18.3±12.2	**61.0±2.4
CD61/ CD63	7.8±2.5	**37.4±7.6

The data was obtained from analysis of contour plots. Results expressed as the mean±SD (n=3). LI= liquid culture, FIN= fibronectin culture.

* p=0.043 significantly different compared with liquid culture.

**p= 0.021 significantly different compared with liquid culture.

*** p=0.02 significantly different compared with fibronectin.

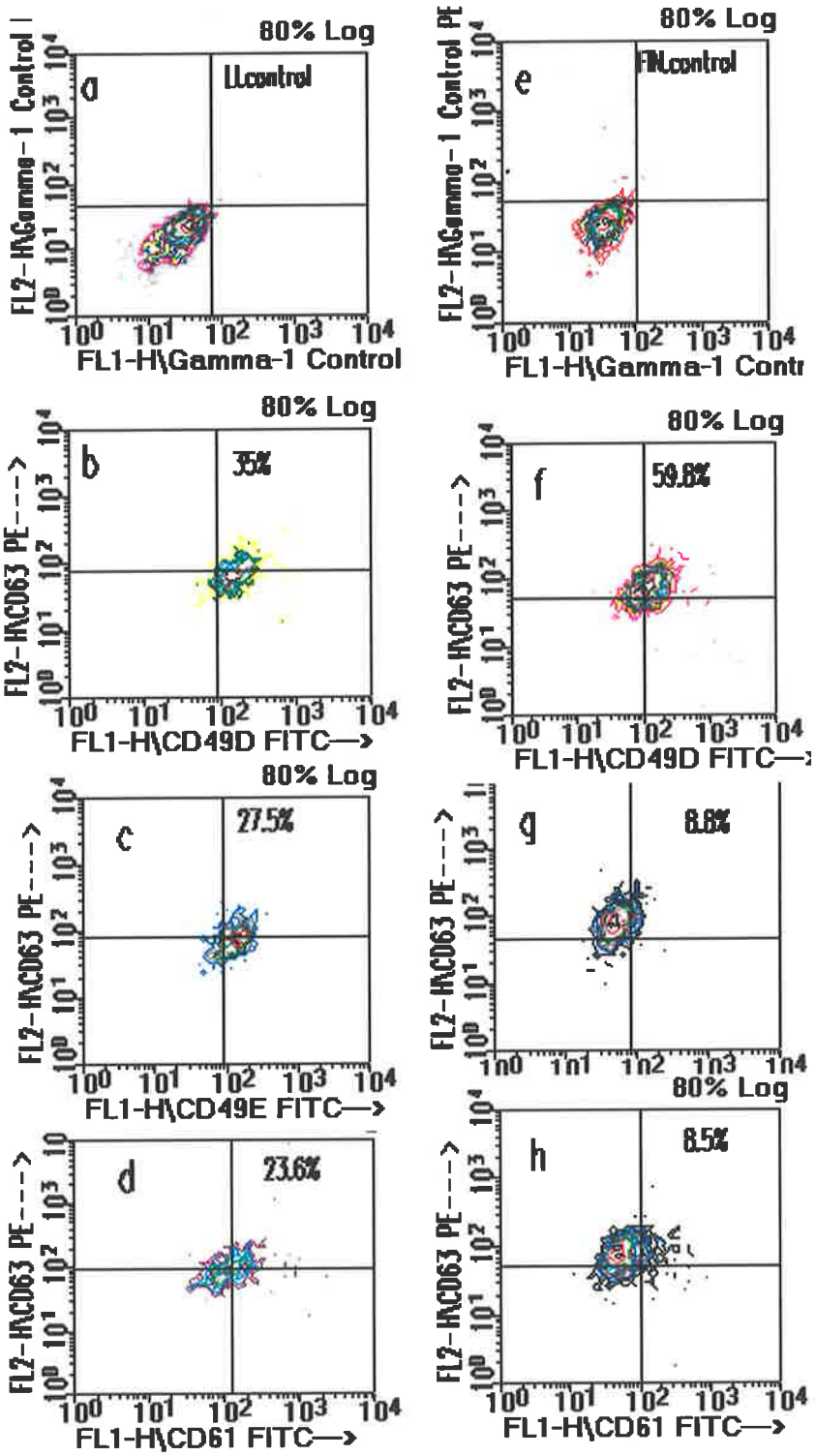


FIG. 3.3.9. Contour plots showing the effects of fibronectin on co-expression of CD49d/CD63, CD49e/CD63 and CD61/CD63 on $CD34^{+}$ cells at day 4. (a) and (e) were isotype control.

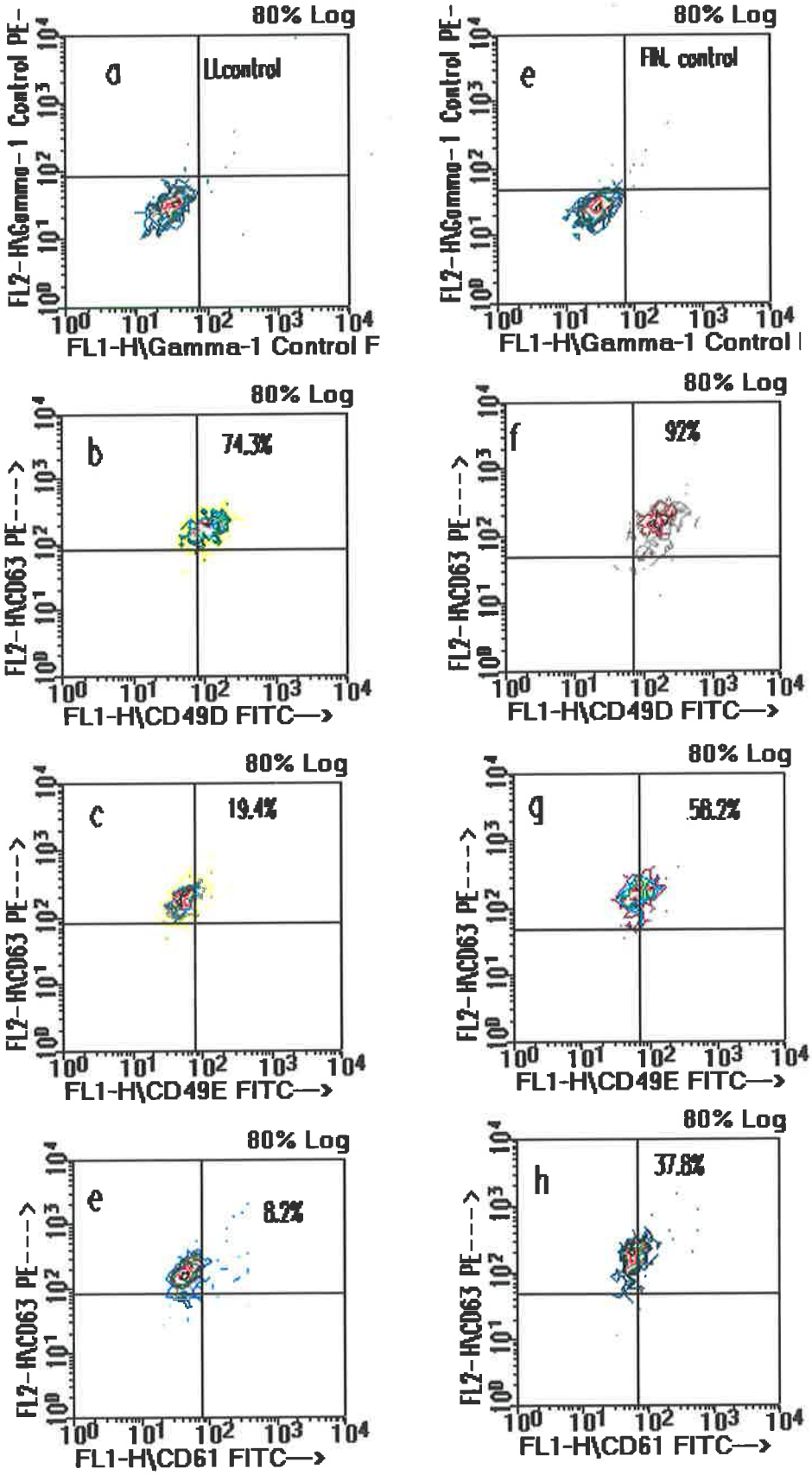


FIG. 3.3.10. Contour plots showing the effects of fibronectin on co-expression of CD49d/CD63, CD49e/CD63 and CD61/CD63 on $CD34^{+}$ cells at day 8. (a) and (e) were isotype control.

expression of CD49e/CD63 and CD61/CD63 was significantly decreased in the liquid culture at day 8.

$CD34^-$ cells also showed co-expression of CD49d, CD49e and CD61 with CD63. The result was shown in Table 3.3.5 and representative contour plot Fig.3.3.11 and 3.3.12.

At day 4, the data showed that co-expression of CD49d/CD63 on $CD34^-$ cells in the presence of fibronectin was significantly increased ($p=0.043$) but the presence of fibronectin resulted in a decreased co-expression of CD49e/CD63 and no change of CD61/CD63 at day 4.

However, at day 8, the presence of fibronectin resulted in a slight increase in co-expression of CD49d/CD63 on $CD34^-$ cells and a significant ($p=0.043$) increase in co-expression of CD49e/CD63 (39.8% vs 15.1%) and CD61/CD63 (41.4% vs 16.5%) as compared to liquid culture at day 8.

Furthermore, on $CD34^-$ cell, when day 4 and day 8 fibronectin cultures were compared, there were significantly ($p=0.043$) increase co-expressions of CD49d/CD63 (80.5% ±6.9 vs 44.1%±8.9),) CD49e/CD63 (39.8%±6.8 vs 13.1%±2.7) and CD61/CD63 (41.4%±5.5 vs 23.4%±7.1) on $CD34^-$ cells at day 8 as compared with day 4.

When day 4 and 8 liquid cell culture were compared, co-expression of CD49d/CD63 was increased (65.7%±4.6 vs 27.0%±6.0). However, CD49e/CD63 (15.1%±7.5 vs 19.4%±5.1) and CD61/CD63 (16.5%±5.2 vs 23.4±8.4%) were decreased at day 8 on $CD34^-$ cells as compared with day 4.

Taken together, these results suggest that fibronectin may play an important upregulatory role for integrin and tetraspan co-expression on both $CD34^+$ and $CD34^-$ cells. Furthermore, fibronectin may be responsible for co-expression of CD49d/CD63 at an early rather than late stage. However, the effect of fibronectin on co-expression of CD49e/CD63 and CD61/CD63 were mostly at a late stage of cell growth.

As shown in Fig. 3.3.13, the effects of fibronectin on co-expression of CD49d, CD49e and CD61 respectively with tetraspan CD151 were investigated at days 4 and 8. The percentage co-expression of CD49d, CD49e and CD61 with CD151 was not significantly increased at day 0 (data not shown).

At day 4 on $CD34^+$ cells, co-expression of CD61/CD151 was significantly increased ($p=0.043$) in the presence of fibronectin as compared with liquid culture. However, in both

Table 3.3.5. Co-expression of integrins on $CD34^-$ cells CD49d, CD49e and CD61 (MK marker) with tetraspan (CD63) at day 4 and day 8 of cell culture.

DAY 4

Activity marker	LI (%)	FIN (%)
CD49d/CD63	27.0±6.0	*44.1±8.9
CD49e/CD63	19.4±5.1	13.1±2.7
CD61/CD63	23.4±8.4	23.4 ±7.1

DAY 8

Activity marker	LI (%)	FIN (%)
CD49d/CD63	65.7±4.6	80.5±6.9
CD49e/CD63	15.1±7.5	*39.8±6.8
CD61/CD63	16.5±5.2	*41.4±5.5

The data were obtained from analysis contour plots. Results expressed as the mean ±SD (n=3). LI= liquid culture, FIN= fibronectin culture.

*p=0.043 significantly different from liquid culture.

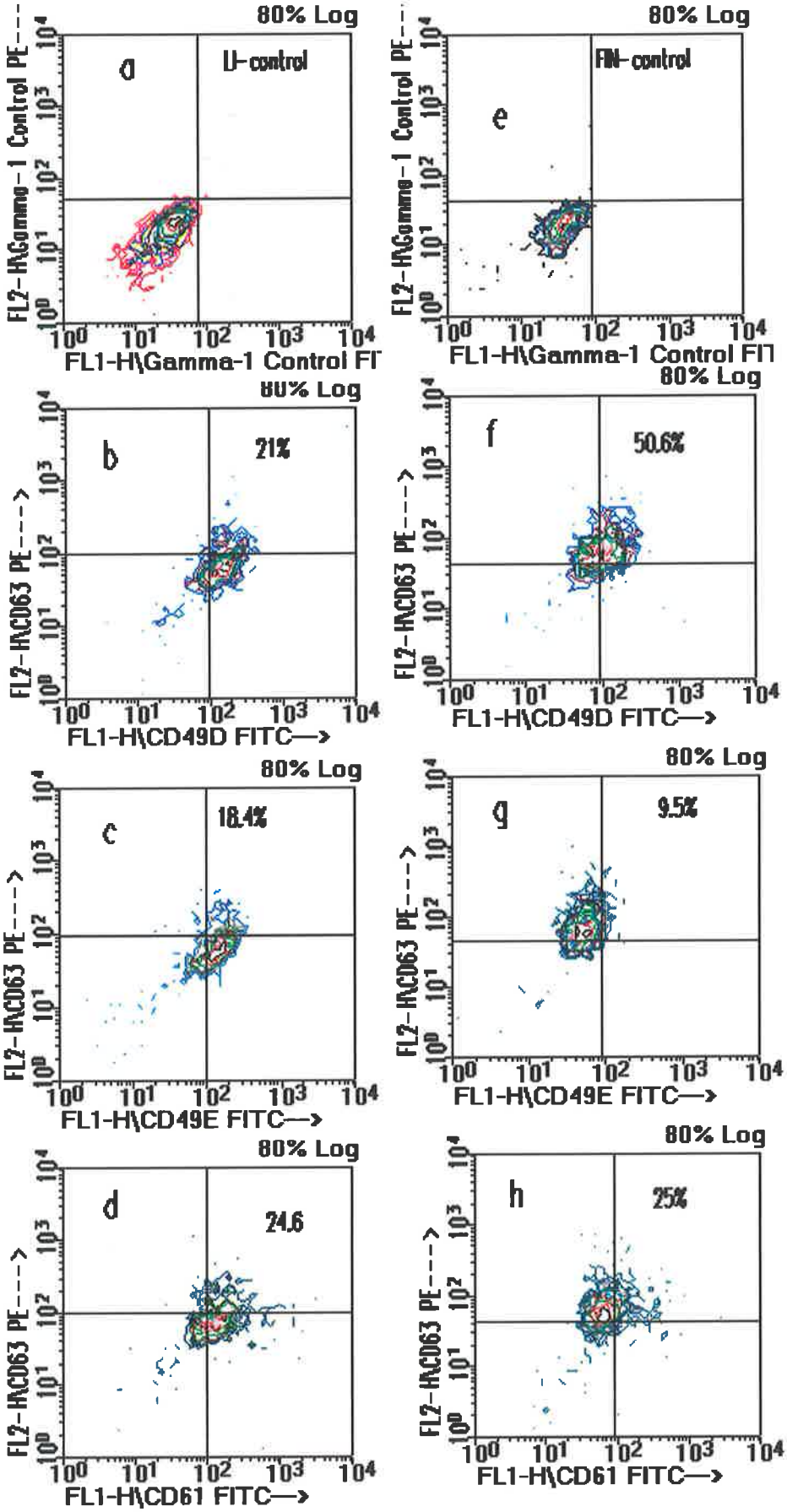


FIG. 3.3.11. Contour plots showing the effect of fibronectin on co-expression of CD49d, CD49e, CD61/CD63 on $CD34^-$ cells at day 4 of cell culture. a and e = isotype control, b and f =CD49d/CD63, c and g =CD49e/CD63 d & h =CD61/CD63.

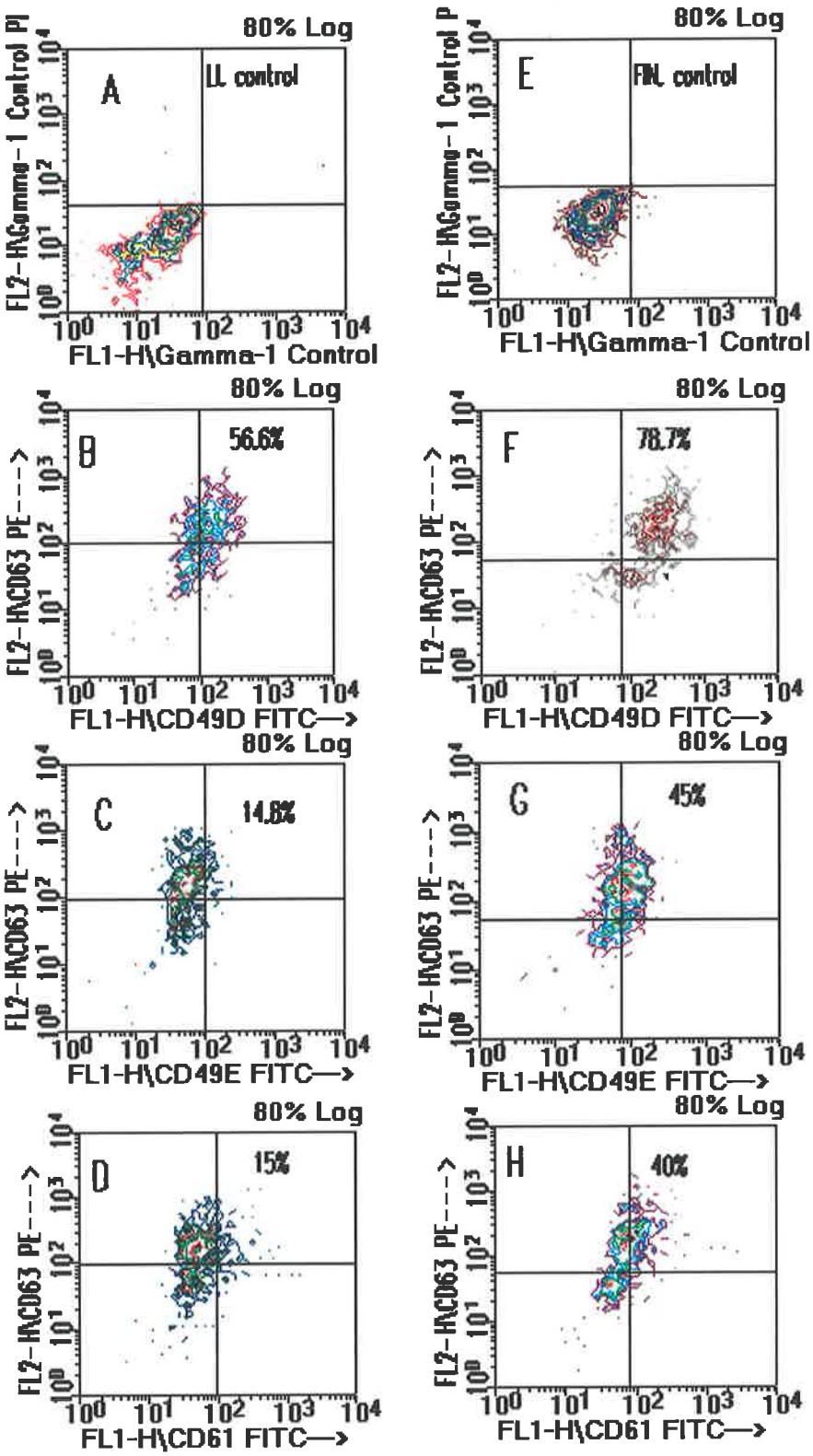


FIG. 3.3.12. Contour plots showing the effects of fibronectin on co-expression of CD49d/CD63, CD49e/CD63 and CD61/CD63 on $CD34^-$ cells at day 8 of cell culture. A and E were isotype control.

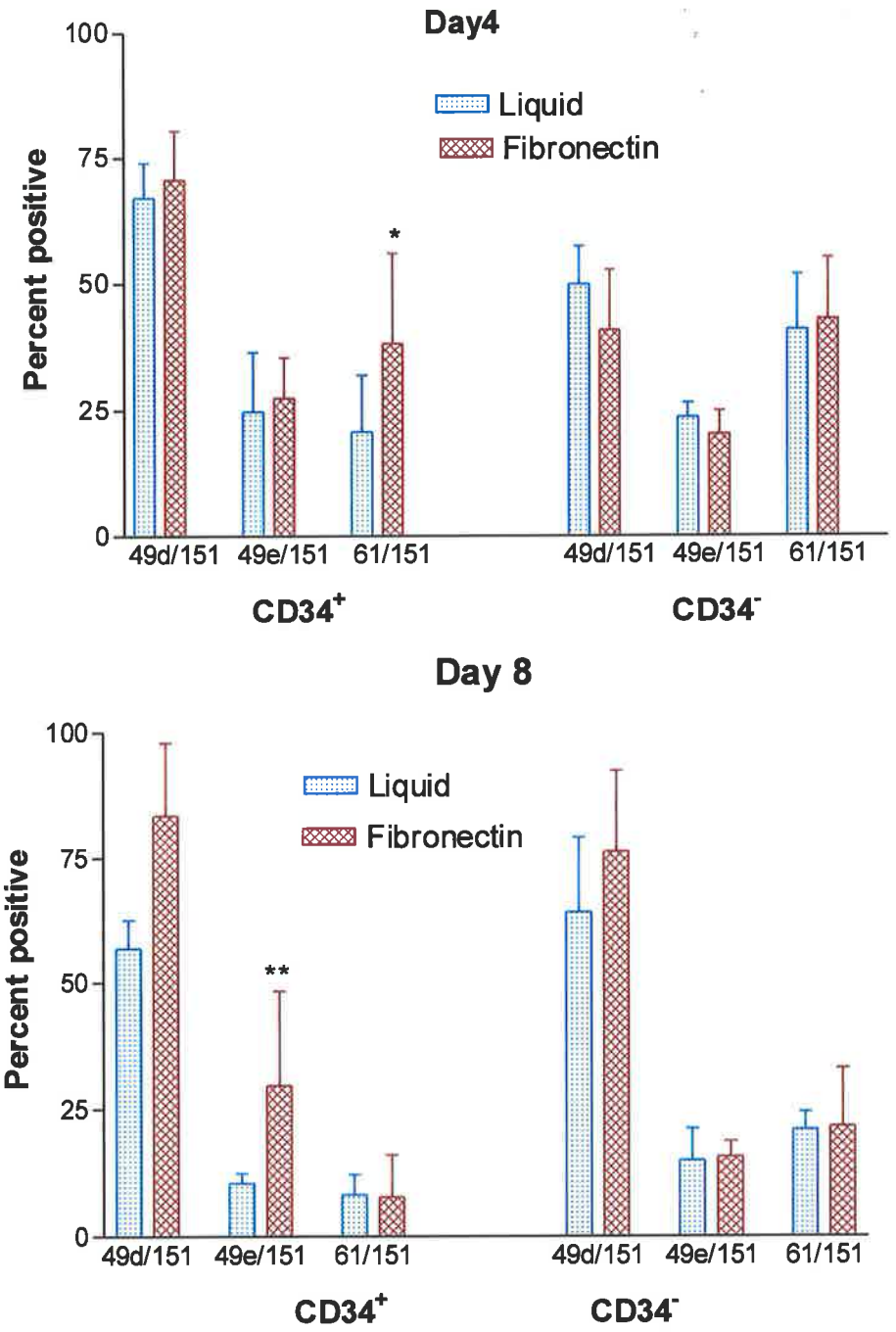


FIG. 3.3.13. Effects of fibronectin on co-expression of CD49d/CD151, CD49e/CD151 and CD61/CD151 on $CD34^+$ and $CD34^+$ cells at day 4 and day 8 of culture. Results are shown as the mean±SD (n=4).

*p=0.043 significantly higher than the liquid culture.

**p=0.039 significantly higher than liquid culture.

of the cultures, the percentage of CD49d/CD151 and CD49e/CD151 was not different at day 4.

At day 8 on $CD34^+$ cells, the presence of fibronectin resulted in a slightly increased co-expression of CD49d/CD151 (79%±15 vs 55%±4.3) as compared with liquid culture. The co-expression of CD49e/CD151 (27%±12 vs 10%±1.6) on CD34+ cells was increased significantly in the presence of fibronectin ((p=0.02) as shown in Fig. 3.3.13. The co-expression of CD61/CD151 was no different in both cultures at this time point.

When compared day 4 and day 8, $CD34^+$ cells were cultured with fibronectin, it was noted that co-expression of CD49d/CD151, CD49e/CD151 was not significantly different between the two time points. However, co-expression of CD61/CD151 was significantly decreased (38% ±10 vs 9.0%±2.1) as compared with day 4 of culture.

For further compare of day 4 and day 8 liquid cultures on $CD34^+$ cells, it was found that co-expression of CD49d/CD151 on $CD34^+$ not significantly changed. However, CD49e/CD151 (9%±2.1 vs 25%±7.8) and CD61/CD151 (9%±2.1 vs 23%±5.3) co-expression were significantly decreased at day 8 compared to day 4.

$CD34^-$ cell co-expression of CD49d/CD151, CD49e/CD151 and CD61/CD151 also were investigated at 4 and 8 days of culture. At day 4, in both cultures, the percentage of co-expression was not different in CD49d/CD151, CD49e/CD151 and CD61/CD151.

At day 8, There was a similar of co-expression in both cultures. When compared day 4 and day 8 culture in presence of fibronectin on $CD34^-$ cells, the results shown that co-expression of CD49d/CD151 was increased at day 8 as compared with day 4. However, co-expression of CD49e/CD151 and CD61/CD151 were decreased at day 8 as compared with day 4.

By contrast, when day 4 and 8 liquid cultures of $CD34^-$ cells were compared, no difference was obtained between co-expression of CD49d/CD151 at these two times. However, in comparing to day 4, co-expression of CD49e/CD151 and CD61/CD151 were reduced by day 8.

These results indicate that the effect of fibronectin on co-expression of CD61/CD151 on $CD34^+$ cells was mainly evident at day 4. However, the effect of co-expression on CD49d/CD151 and CD49e/CD151 was at a later stage.

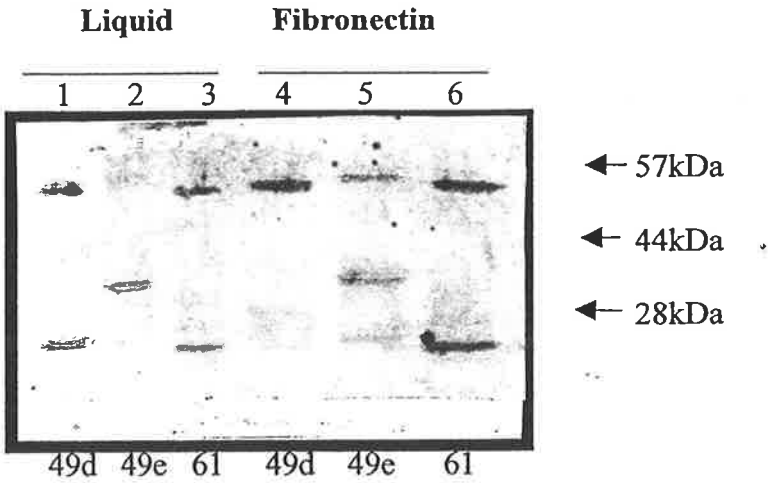
3.3.8 DETECTION OF INTEGRIN TETRASPAN CO-EXPRESSION ON CD34$^+$ CELLS BY IMMUNOPRECIPITATION

Immunoprecipitation was used to assess physical co-location of integrins and tetraspans in cultured CD34$^+$ cells. CD34$^+$ cells were cultured in liquid or with fibronectin in medium containing the standard cytokines for 4 days. The cells were then collected and immunoprecipitation procedures performed. The tetraspan antibodies were used as the primary immunoprecipitating antibody. The integrin antibodies were used to identify the whether the respective integrin co-precipitated with the tetraspan. The results of immunoprecipitation of integrin CD49d and CD49e proteins with CD63 and CD151 proteins are shown in Fig.3.3.14. Two bands were evident from the CD49d/CD63 immunoprecipitate derived from cells of both liquid and fibronectin culture Fig 3.3.14**A**, lane 1 and 4: MW bands of 57kDa and 28kDa. The second band of 28kDa, however, was more easily identified from cells grown in liquid culture.

CD49e/CD63 immunoprecipitate Fig.3.3.14 **a**, lane 2 and lane 5 was identified as a MW band of 44kDa. CD61 (β_3) also immonoprecipitated with CD63 Fig 3.3.14 **a**, lane 3 and lane 6 with two MW bands, 57kDa and 28kDa.

CD49d/CD151 immunoprecipitate had a similar MW band of 57kDa to CD49d/CD63 (Fig.3.3.14 **b** lane 1 and lane 4. Similarly, the CD49e/CD151 immunoprecipitate has a MW band of 44kDa similar to that of CD49e/CD63 immunoprecipitate (Fig3.3.14 **b**, lane 2 and lane 5. The CD61/CD151 immunoprecipitate had two MW bands of 57kDa and 28kDa similar to that of CD61/CD63 immunoprecipitate Fig.3.3.14 **b**, lane 3 and lane 6. However, in this study, precipitation of integrins/CD151 molecules in the rage 110-150kDa (Fitter et al., 1999) was not detected by immunoprecipitation from anti-CD151. Furthermore, the large of MW band (110-150kDa) precipitation of integrins/CD63 from anti-CD63 immunoprecipitation was not detected either. The failure to detect the large molecule could be the experiment containing 5% of 2-mercaptoethenal in the loading buffer to run SDS/PAGE. The data suggest that tetraspans CD63 and CD151 physically co-locate with the two integrin families represented by CD49d, CD49e and CD61 respectively.

CD63 immunoprecipitation



CD151 immunoprecipitation

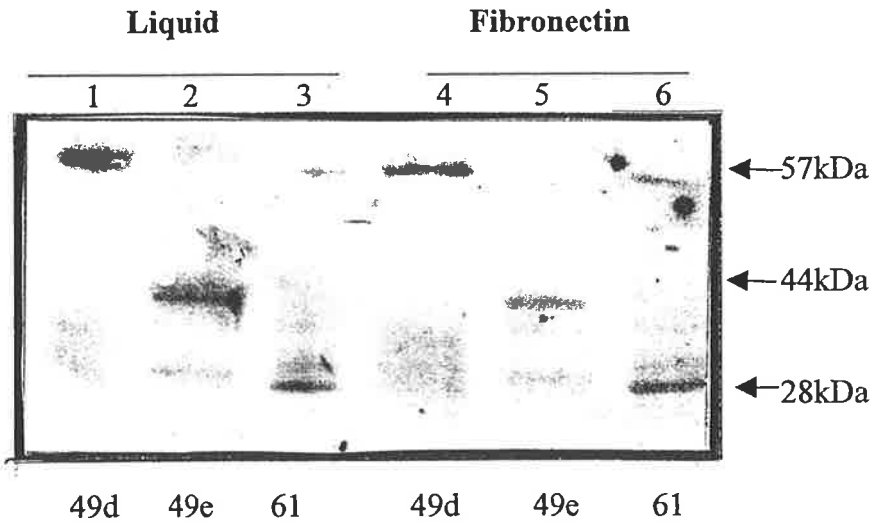


FIG. 3.3.14 Immunoprecipitation of integrins associated with tetraspans. Western blots of SDS-PAGE are showing immuno-precipitation of integrins CD49d and CD49d and CD61 with tetraspans CD63 and CD151 from liquid and fibronectin cell cultures. The method of western blots and immunopre-cipitation performed as described in chapter 2.

3.4 THE EFFECT OF INTEGRIN AND TETRASPAN ANTIBODIES ON MK PROGENITOR CELLS IN STROMAL CELL CUTURE

INTRODUCTION

Bone marrow stromal cells are known to provide essential environmental factors *in vivo* for the growth of haemopoietic cells. These factors include extracellular matrix (ECM) like fibronectin, and haemopoietic growth factors. An *in vitro* $CD34^+$ cell microenvironment was developed using a mouse stromal cell line which provided direct cell-to-cell and/or cell-ECM contact in the presence of standard cytokines and MGDF. $CD34^+$ cells were co-cultured on confluent stromal cells to evaluate the ability of stromal cell to support CD34+ cells growth *in vitro*.

In order to examine the role of integrins and tetraspan in the growth of MK progenitor cells, $CD34^+$ cells were cultured in the presence and absence of the blocking antibodies anti-CD49d, anti-CD49e and anti-CD151. Non-blocked and blocked cells were further co-cultured with stromal cells to examine the effect of blocking and stromal cells on cell proliferation and differentiation. CFU-MK assay and expression of adhesion and tetraspan molecules were used to assess the role of integrins and tetraspan in stromal dependent megakaryocytopoiesis.

3.4.1 EFFECT OF STROMAL CELL CULTURE ON THE PROPORTION OF $CD34^+$ CELLS

$CD34^+$ cells were co-cultured with stromal cells or in stromal free culture over 8 days. There were a significant increase in percentage of $CD34^+$ cells in the presence of stromal cells ($p=0.009$) compared to stromal free culture, 24.5% ±3.3 vs 5.7% ±2.8 (mean ±SD, n=3) Fig. 3.4.1. These data confirmed that stromal cells have an interactive role in and/or provide a microenvironment for $CD34^+$ cell proliferation.

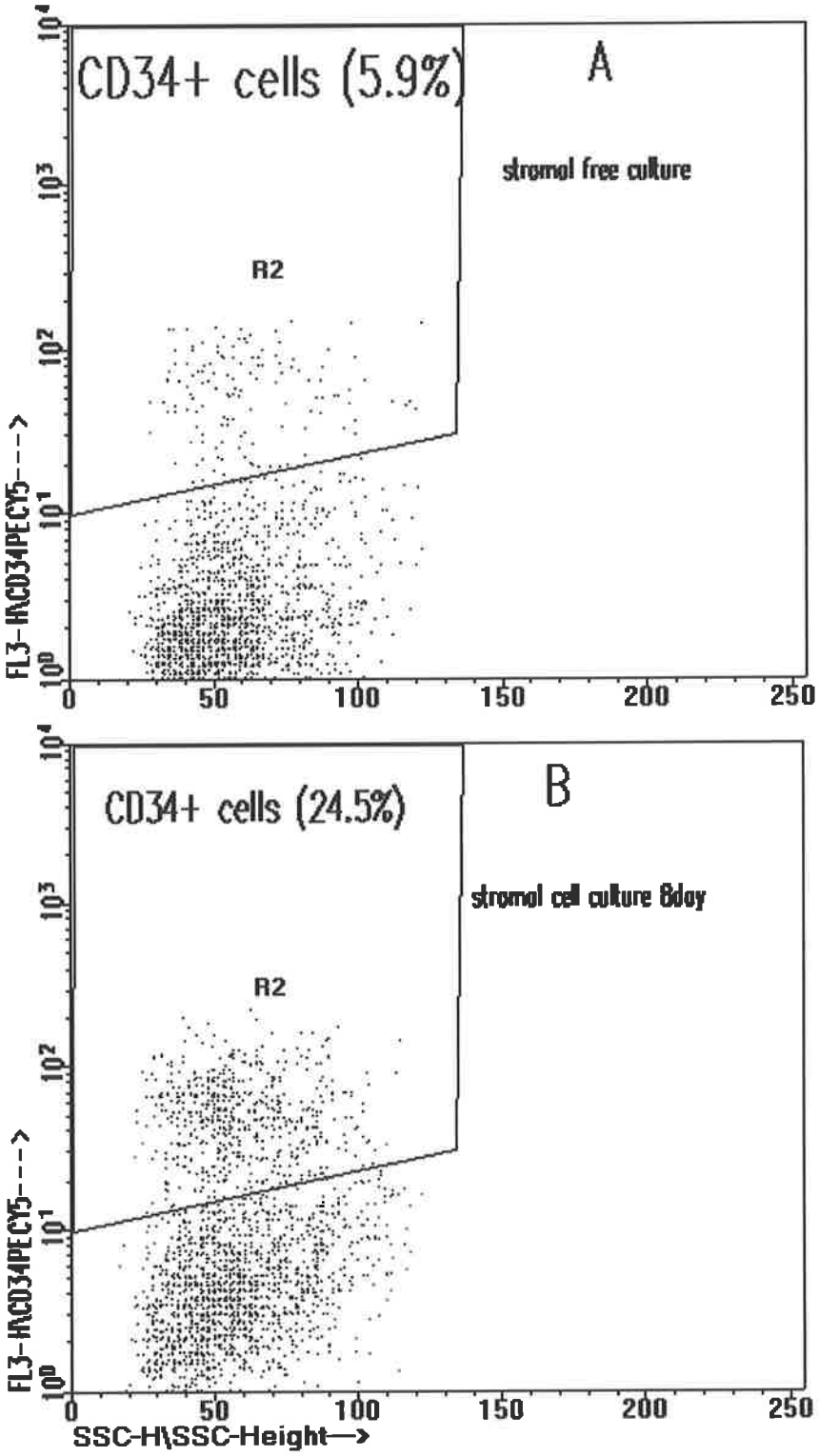


FIG. 3.4.1. The effect of stromal cells on CD34 cell proliferation. The $CD34^+$ cells were cultured in stromal free or stromal cell culture for 8 days. $CD34^+$cells were analysed by flow cytometry. $CD34^+$ cell numbers were significantly higher in stromal cell culture ($p=0.009$). A= stromal free culture. B=stromal cell culture.

3.4.2 EFFECTS OF INTEGRIN AND TETRASPAN BLOCKING ON TOTAL CELL COUNT

In order to examine the role of integrin and tetraspan on CD34+ cell proliferation and differentiation to MK progenitors, the effect of blocking antibodies to these molecules was investigated. CD34+ cells were incubated with monoclonal antibodies against CD49d, CD49e and CD151 for 30min at 4^0C. These cells were further cultured with confluent stromal cells for 4 and 8 days.

The results are shown in Fig 3.4.2. A total of $2x10^4$ non-blocked cell increased 26.5 ±4.3-fold (mean ±SD, n=3) by day 4, and 58±7.2-fold (mean ±SD, n=3) by day 8.

Effect of anti-CD49d on $CD34^+$ cell count. With blocking of CD49d cell count increased 10.5±3.6-fold (mean ±SD, n=3) by day 4, with declining to 8.5 ±4.4-fold increase (mean ±SD, n=3) by day 8. In the presence of CD49d blocked with antibody, cell proliferation was significantly inhibited at both day 4 (p=0.04) and day 8 (p=0.0021).

Effect of anti-CD49e on $CD34^+$ cell count. Similar results were seen with blocking CD49e function. On day 4, there was an 8.5±1.2-fold (mean ±SD, n=3) increase and by day 8 a 9.5±2.2-fold (mean ±SD, n=3) increase. The inhibition of cell proliferation was significant at day 4 (p=0.04) and also at day 8 (p=0.021).

Effect of anti-CD151 on $CD34^+$ cell count. On day 4, there was a 9.2± 0.9-fold (mean ±SD, n=3) increase in $CD34^+$ cell count and by day 8, a 10.3±3.7 fold (mean ±SD, n=3) increase compared with day 0. The inhibition of cell proliferation was significant (p=0.04) at day 4 and (p=0.021) at day 8. Thus, these results suggest that integrins, CD49d, CD49e and tetraspan CD151 are required by $CD34^+$ cells for proliferation.

3.4.3 THE EFFECT OF INTERGRIN AND TETRASPAN BLOCKING ON PROPORTION OF $CD34^+$ CELLS

Fig. 3.4.3 is a representative contour plot showing the percentage of $CD34^+$ at day 8 in stromal cell cultures containing $CD34^+$ cells inhibited by the respective antibody to CD49d, CD49e and CD151. The percentage of CD 34^+ cells was 24.5% ±7.2 in the control (non-blocking) Fig. 3.5.3, and the percentage of $CD34^+$ cells was higher in the cultures

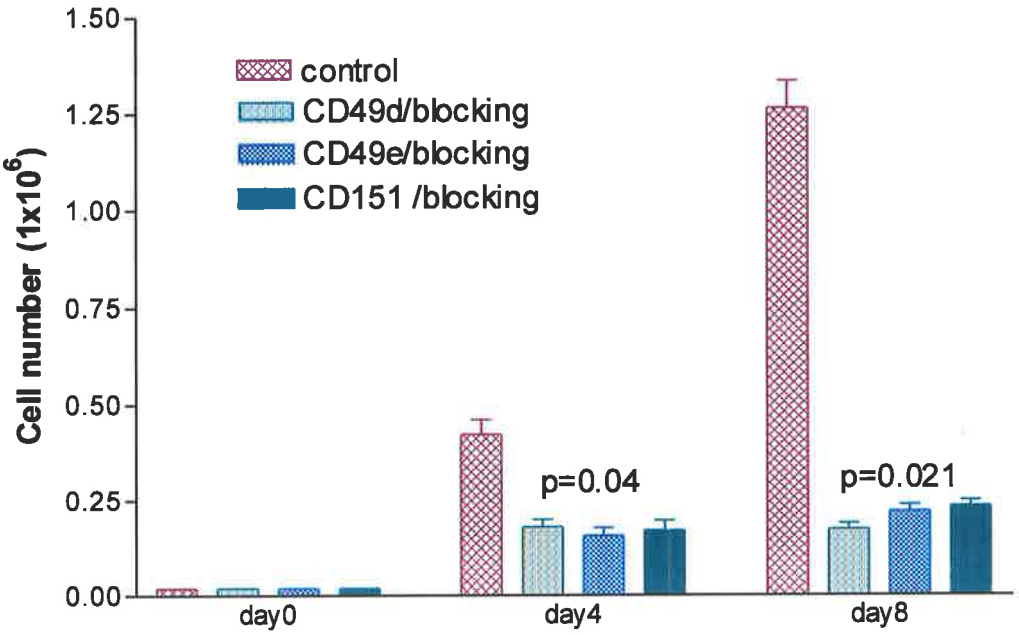


FIG. 3.4.2. Effect of anti-CD49d, CD49e and anti-CD151 on total cell count. $CD34^+$ cells were blocked by anti-CD49d, CD49e and CD151 for 30 min at 4^0C, then co-cultured with stromal cells for 4 and 8 days. In this experiment non-blocked $CD34^+$ cells were used as control. The results are presented as mean±SD (n=4).

p=0.04 three of different anti-blocking cell count were significantly low than non-blocked cells at day 4.

p=0.021 three of different anti-blocking cell count were significantly low than non-blocked cells at day 8.

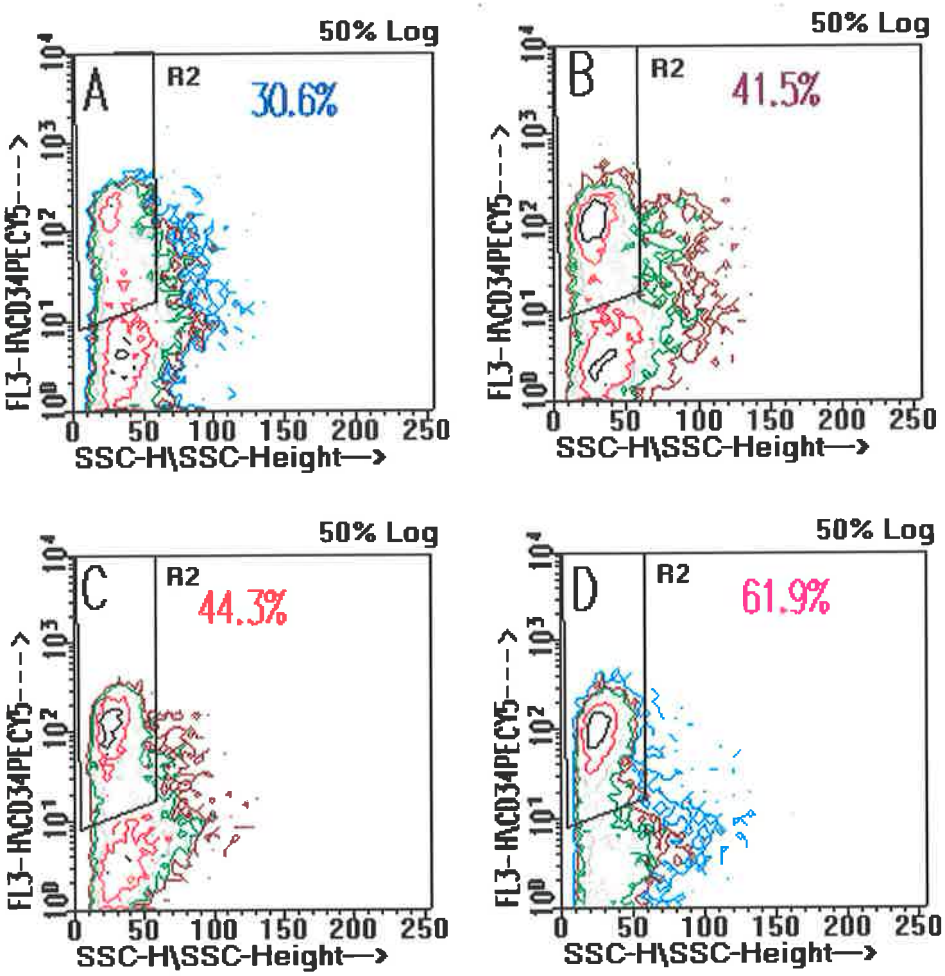


FIG.3.4.3. Representative effects of blocking antibody on the percentage of $CD34^+$ cells at day 8. Results are shown in the contour plots. A=non-blocked cells (30.6%), B=anti-CD49d blocking (41.5%), C=anti-CD49e blocking (44.3%), D=anti-CD151 blocking (61.9%). $CD34^+$ cell numbers were significantly higher in blocking than in non-blocked control ($p=0.043$).

containing the inhibiting antibodies. In the previous section, the data showed an inhibition of proliferation of $CD34^+$ cells by the inhibiting antibodies. The data from both sections taken together suggest that blocking of integrin/tetraspan function results in inhibition of cell proliferation and maturation and that inhibition of tetraspan has a greater effect on maturation.

3.4.4 EFFECT OF INTEGRIN AND TETRASPAN BLOCKING ON MK PROGENITOR DEVELOPMENT AS ASSESSED BY EXPRESSION OF MK MARKERS (CD61, CD42b) AND ON EXPRESSION OF TETRASPAN CD151

To clarify the important roles of CD49d, CD49e and CD151 in mediating MK progenitor cell development, the expression of MK markers CD61 and CD42b were studied following blocking by anti-49d and anti-49e antibodies. Expression of CD151 was also performed at day 4 and 8.

Anti-CD49d blocking resulted in a significantly reduced CD61 MFI at day 4 (16.5±4.2 vs 30±2.0 mean ±SD n=4) (p=0.043), and CD61 remained to reduce MFI (22.6±0.8 vs 29.6±0.7) at day 8. However, at day 8 the difference was not significant compared to non-blocking cells respectively Fig 3.4.4 (a). No significant difference for CD42b MFI was noted at day 4 and 8.Fig 3.4.4(b). Anti-CD49d blocking significantly reduced CD151 MFI (27±0.6 vs 48±2.7, mean±SD n=4) (p=0.043) at day 4. However, by day 8 CD151 MFI has recovered Fig 3.4.4(c).

Similarly, CD61 MFI was reduced at day 4 by blocking CD49e (21±7.2 vs 30±2.2 mean±SD n=4), CD61 MFI remained reduced at day 8. The MFI of CD42b and CD151 was not altered by blocking CD49e over 8 days of culture Fig.3.4.5. The reduction of CD61 MFI by blocking CD49d and CD49e was greater than CD42b. However, the effect of blocking CD49d on CD151 was seen early at day 4. Integrin CD49d but not CD49e plays an important role for MK differentiation. CD151 functions more closely with CD49d than CD49e.

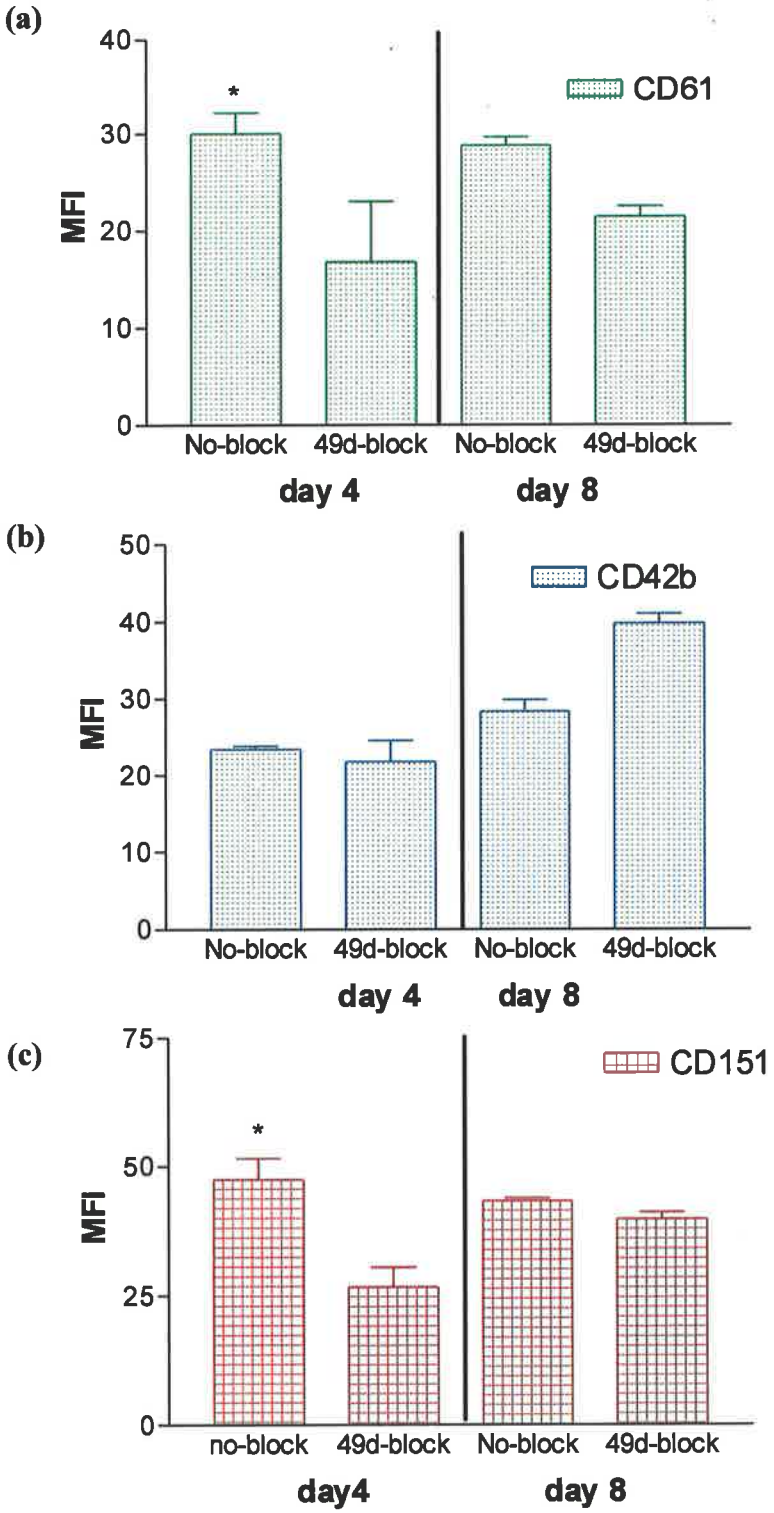


FIG. 3.4.4 Effect of CD49d blocking on expression of MK markers. Histograms MFI of CD61, CD42b and CD151 expression with or without blocking.

*p=0.043 no-blocked significantly increased expression compared with blocking.

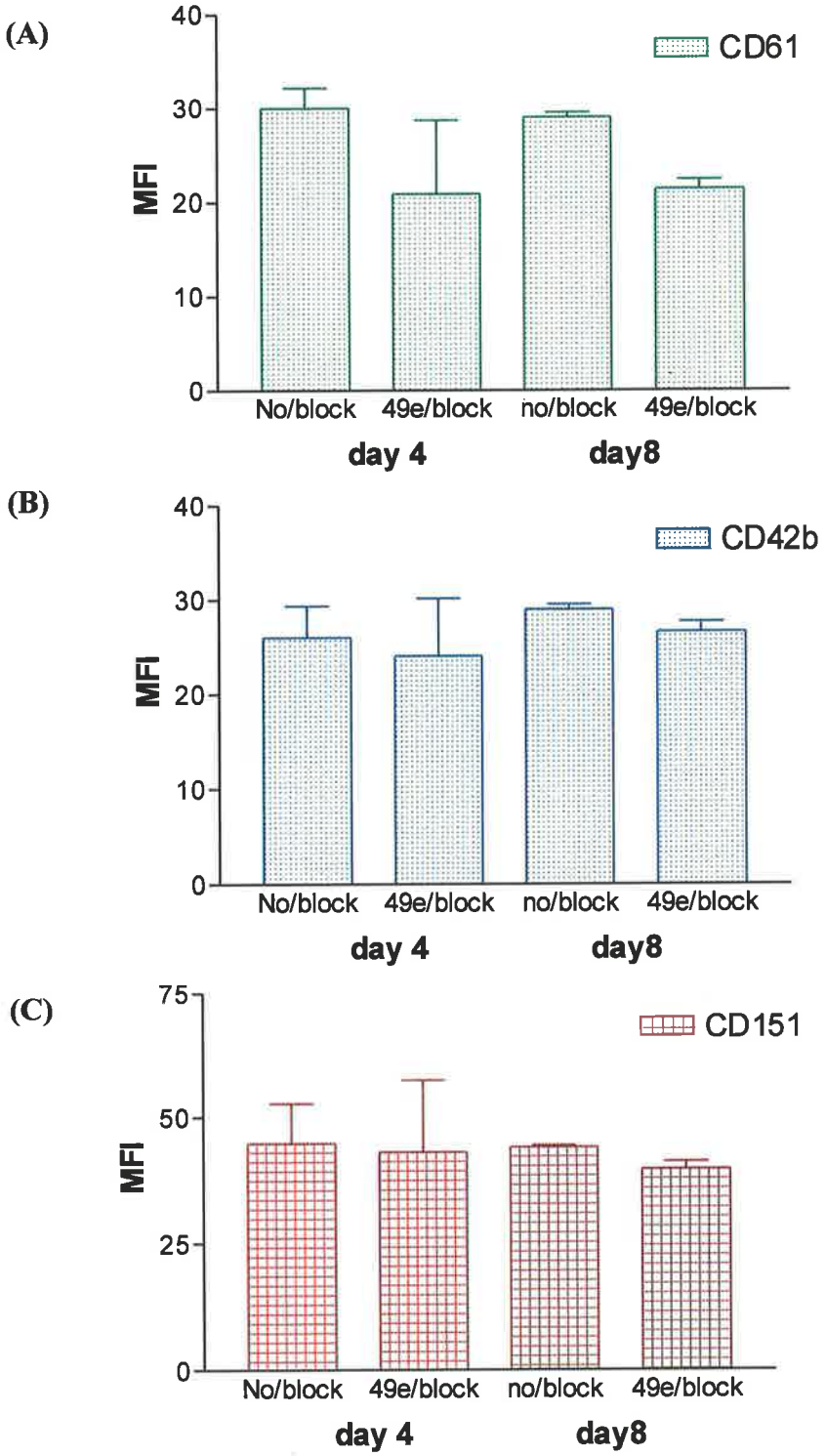


FIG. 3.4.5. Effect of CD49e blocking on expression of MK markers. Histograms showed the MFI of CD61, CD42b and CD151 expression with or without blocking. There was no significant difference in MK marker expression following blocking.

3.4 5 EFFECTS OF INTEGRIN AND TETRASPAN BLOCKING ON CFU-MK FORMATION

The influences of anti-CD49d, CD49e, and CD151 blocking on MK progenitor cell proliferation and differentiation were evaluated by using the CFU-MK assay. The $CD34^+$ cells were incubated for 30 min with or without anti-CD49d, anti-CD49e and anti-CD151 and were then co-cultured with stromal cells. After 4 days, cells were collected and subcultured in semi-solid agar for a further 14 days, when the CFU-MK colonies were identified and counted.

The control cultures (no blocking of $CD34^+$ cells) showed significantly higher CFU-MK colonies than any of the blocked cultures Fig.3.4.6 ($p=0.029$). Based on CFU-MK assay, integrins CD49d, CD49e and the associated tetraspan CD151 play an important role in MK progenitor cell proliferation and differentiation.

3.4.6 MORPHOLOGY AND DEVELOPMENT OF MK COLONIES WITH INTEGRIN AND TETRASPAN BLOCKING

CFU-MK progenitor cells were detected by their capability to form MK colonies in semi-solid culture. $CD34^+$ cells, unblocked or blocked by anti-CD49d, anti-CD49e and anti-CD151 were co-cultured on stromal cells and at days 4 and day 8 subcultured to semi-solid agar culture for a further 12 days. The frequency of MK was identified by APAAP staining, the colonies labelled with the anti-GPIIbIIIa and anti-GPIIIa monoclonal antibodies. The distribution of MK colonies is shown in Fig 3.4.7. In cells derived from 4 days of culture, the anti-CD49d, anti-CD49e and anti-CD151 blocked $CD34^+$ cells showed significantly reduced numbers of MK colonies Fig 3.4.7 A, B, C, compared with the $CD34^+$ control: from 20-50 vs 50-100 at day 12 of colony growth Fig 3.4.7 D. Subculturing from day 8 cultures, the MK colony generation was similar to that from day 4 cultures, with the control cells showing significantly higher colony number (Fig 3.4.7 H), than the anti-CD49d, CD49e and CD151 blocked cells Fig. 3.4.7 E, F, G. MK-colony formation from $CD34^+$ cells was clearly affected by blocking integrins and the associated tetraspan.

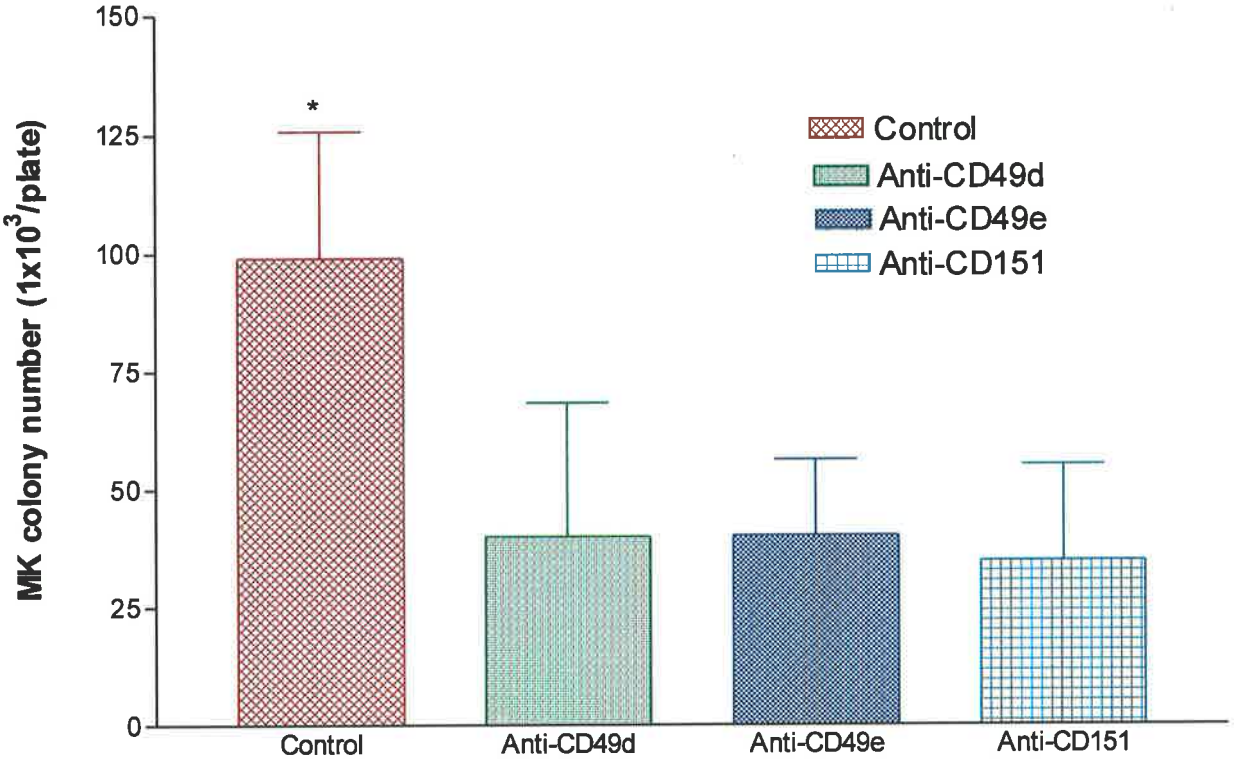


FIG.3.4.6. Effect of anti-CD49d, anti-CD49e and anti-CD151 blocking on MK commitment lineage. $CD34^+$ cells were blocked by anti-CD49d, anti-CD49e and anti-CD151 for 30 min at 4^0C, then co-cultured on stromal cells for 4 days. $1x10^3$ cells were subcultured in semi-solid agar for further 12 days to identify CFU-MK. The results shown as the mean±SD (n=4).

*p=0.029 Significantly higher CFU-MK than three of anti-blocking of CFU-MK.

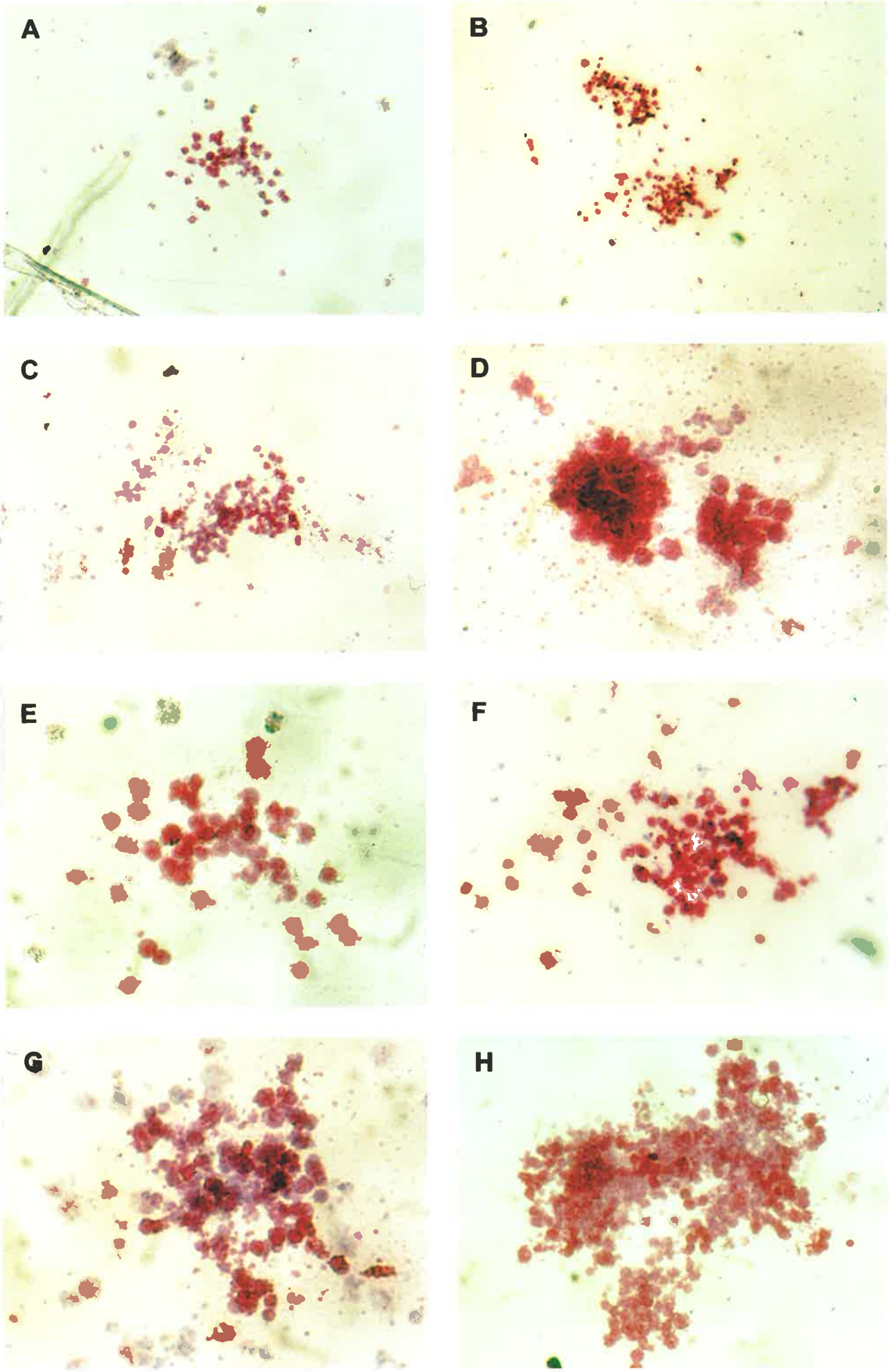

A
B
C
D
E
F
G
H

FIG. 3.4.7 Microphotographs of CFU-MK. $CD34^+$ cells were blocked by anti-CD49d, anti-CD49e and anti-CD151 for 30 min at 4^0C, and co-cultured in stromal cells in 4, 8 days. The cells were collected and cultured for further 14 days in semi-solid agar culture with standard cytokines and MGDF. The experiment was added control as well. The CFU-MK colonies were photographed after APAAP staining. Anti-CD49d blocking (A), anti-CD49e blocking (B), anti-CD151 blocking (C), and Control (D), at day 4 in the stroma cells. Original magnification x 250.

Anti-CD49d (E), anti-CD49e (F), anti-CD151 (G), and control (H), at day 8 in the stromal cell culture. Original magnification x 500.

CHAPTER 4
DISCUSSION

DISCUSSION

Normal haemopoiesis is regulated by a complex set of factors of the haemopoietic microenvironment. These factors consist of haemopoietic growth factors, ECM molecules and stromal cells each interacting with haemopoietic stem cells to direct the coordinate regulation of blood cell development. The role of interaction of ECM and BM stromal cells in the differentiation of erythroid and myelomonocytic cells has been reported (Williams et al, 1991; Yanai et al, 1994), but there is a dearth of study in regard to megakaryopoiesis. In this study, using CB blood as a source of $CD34^+$ stem cells the role of BM microenvironment on MK differentiation and proliferation was studied *in vitro*. Understanding the mechanisms of regulation of MK progenitor differentiation may help in the development of optimal *in vitro* culture conditions for the expansion of MK progenitors for transplantation therapy.

Several studies have shown that ECM proteins are important in regulating haempoiesis (Simmons et al, 1994; Dexter et al, 1997; Yoder and Williams, 1995; Verfaille et al, 1994). The integrin superfamily molecules which are receptors for ECM are expressed on HSC and on stromal cells (Simmons et al, 1994;. Verfaille et al, 1994). The β_1 integrins may play a role in regulating haemopoiesis (Miyake et,1991; Williams et al, 1991; Yanai et al, 1996; Rosemblatt et al, 1991). The integrins CD49d and CD49e, both of which are fibronectin receptors, are believed to be important mediators of direct interaction of haemopoietic progenitors and ECM (Miyake et,1991; Williams et al, 1991; Yanai et al, 1996; Kerst et al, 1993; Teixido et al, 1992). The study has confirmed that CD49d was highly expressed on fresh $CD34^+$ cells but CD49e was weakly expressed. There are literature evidence that CD49d and CD49e associate with the tetraspan family which may have a role in the modulation of integrin function possibly involved in transmembrane signalling (Paul, M. et al 1997). Expression of a panel of tetraspan molecules on $CD34^+$ cells were examined: CD9, CD53, CD63 and CD151. In this study, CD63 and CD151 were chosen for further study because the expression of CD63 was higher (21.1% ±1.2) than the other tetraspans on $CD34^+$ cells and in culture with cytokines its expression increased further. Although fresh $CD34^+$ cells expressed low level of CD151 (6.5% ±1.4), however, CD151 is expressed on megakaryocytes and platelet (Fitter et al., 1999). Also CD151 was

found on haematopoietic cell lines, M07e, HEL and K562 (Fitter et al., 1999). CD9 and CD53 were expressed at level lower than CD63 and CD151 on $CD34^+$ cells at day 0 and their expression did not increase with culture and were, therefore, excluded from further study.

A serum-free culture medium was used to study the role of ECM in regulating MK progenitor cells development. The base medium contained IL-3, IL-6 and SCF which were found to maintain well $CD34^+$ in culture. The addition of MGDF in combination with IL-3, IL-6 and SCF was a potent stimulator of MK progenitor cells differentiation. MK progenitors differentiation was monitored using the CFU-MK colony assay and by flow cytometry for expression of CD61 and CD41a, the MK lineage markers. 39% of $CD34^+$ cells expressed CD61 at day 0. However, with in vitro culture, both in the presence and absence of MGDF, expression of CD61 on $CD34^+$ cells had fallen to <10%, by day 4. However, expression of CD61 was slightly higher in the presence of MGDF. $CD34^+$ cells expression of CD61 recovered significantly in the presence of MGDF by day 8. On the other hand, the expression of CD61, which was just above 10% on $CD34^-$ cells at initiation of culture, increased to above 20% by day 8. Thiagarajan and co-workers (1985) reported that CD61 was detected on endothelial cells, monocytes and fibroblasts. It appears that CD61 though an early MK lineage marker is not a specific marker for the megakaryocytic lineage. Although cord blood contains MK progenitors, the initial expression of CD61 on $CD34^+$ cells probably does not reflect MK differentiation entirely. In this study, CD41 expression was not examined. In later studies involving fibronectin or stromal cells, the late increased in CD61 expression was associated with increased CD41 expression. Hence, the subsequent rise in CD61 in both $CD34^+$ and $CD34^-$ cells most likely reflected maturation of MK progenitors.

This study confirmed that MGDF is an important physiological regulator of megakaryopoiesis. MGDF, as a single growth factor, can stimulate growth of CFU-MK colonies from CB. However, MGDF with either IL3 or IL6 alone respectively in culture was found to produce less CFU-MK colonies than MGDF alone. This agrees with the finding of Nichol et al (1995) that IL-3 and IL-6 may be inhibitory of MGDF-induced MK colony formation *in vitro*. The cytokine combination of SCF, IL3 and IL6 supported MK progenitors development. Addition of MGDF with these cytokines increased MK

progenitor differentiation but did not alter $CD34^+$ cell proliferation. This increase of MK progenitors was confirmed by the CFU-MK assay (Fig3.1.3).

Several studies have indicated that β_1-integrins play a role in erythropoiesis and myelomonocytic differentiation. This study provides information regarding the role of these integrins and ECM in megakaryopoiesis. MGDF altered the expression of CD49d and CD49e suggesting that these β_1-integrins may play a role in megakaryopoiesis. MGDF initially decreased the high expression of CD49d on $CD34^+$ cells but its expression increased to moderate level by day 8. In contrast, MGDF increased the expression of CD49e on $CD34^+$ cells over the 8 day culture. Cui et al (1997) demonstrated that MGDF increased the adhesion of the Mk leukaemic cell line Mo7e to fibronectin as well as vascular cell adhesion molecule-1 through activation of CD49d and CD49e. The observation in this study that both CD63 and CD151 coexpression with CD49d was increased by MGDF is consistent with the activation of CD49d. MGDF has little effect on the co-expression of CD63 and CD151 with CD49e indicating that it did not activate CD49e.

The effect of fibronectin was used a model for the effect of ECM on MK differentiation. Fibronectin had no effect on total cell number but did increased the number of $CD34^+$ cells over culture indicating that fibronectin preserved $CD34^+$ cells during culture. Fibronectin increased the differentiation of MK progenitors as evident by the increased expression of CD61 and CD41a on $CD34^+$ cells and $CD34^-$ cells. This was confirmed by the CFU-MK assay. Fibronectin upregulated the expression of CD49d and CD49e on both $CD34^+$ and $CD34^-$ cells. Thus, these data suggest that ECM eg fibronectin augmented the differentiation to MK precursors of $CD34^+$ cells through its adhesive interaction with $CD34^+$ cells. Finally, that fibronectin regulates MK differentiation through interaction with β_1-integrins is strengthened by its upregulation of the expression of both CD49d and CD49e with the associated tetraspans CD63 and CD151, on $CD34^+$ and $CD34^-$ cells. That tetraspans CD63 and CD151 are most likely the signal transducers of CD49d and CD49e is confirmed by the following observations : i) their co-expression with CD49d and CD49e, ii) the upregulation of the coexpressions by fibronectin and iii) their physical association with CD49d and CD49e as indicated by their co-precipitation. This study is the first report describing the presence of integrin/tetraspan complexes on CD34 cells and their

upregulation by fibronectin. Fibronectin increased MK differentiation is most likely through a β_1-integrin-ligand interaction.

A mouse bone marrow stromal cell line, MS-5, was used to mimic a HSC microenvironment to study MK-progenitor differentiation *in vitro* and the role of β_1-integrins in this differentiation. MS-5 stromal cells increased total and $CD34^+$ cell counts confirming their supportive role for haemopoiesis. The role of integrins CD49d, CD49e, and the associated tetraspan CD151 in MK differentiation was studied in this stromal cell microenvironment. Anti-CD49d, anti-CD49e and anti-CD151 significantly inhibited $CD34^+$ cells proliferation. Thus, interaction with CD49d, CD49e and CD151 potentially regulates $CD34^+$ cells proliferation. Blocking of the β_1-integrin CD49d affected expression of MK lineage markers CD61 and tetraspan CD151 but not CD49e. However, anti-CD49d, anti-CD49e and anti-CD151 inhibited stromal cells induced MK-progenitor differentiation as monitored by CFU-MK assay. The results indicate that adhesive interaction of $CD34^+$ cells with stromal microenvironment most likely fibronectin is an important mechanism for megakaryopoiesis. CD151 was first identified as a novel human platelet surface glycoprotein, and has co-localisation with β_1-integrins (Sincock et al, 1997; Fitter et al, 1999). It has a role in platelet aggregation (Roberts et al, 1995). This study provides the first evidence that the tetraspan CD151 is a signal transducer for the β_1-integrins in the induction of megakaryopoiesis. CD61 is also important in platelet aggregation through binding of fibrinogen. An agonist antibody to CD151 induced platelet aggregation indicating that it is the likely signal transducer for CD61 following its interaction with fibrinogen. This study showed that CD151 is already associated with CD61 (GpIIIa) at the progenitor level. However, the role of CD61 in megakaryopoiesis was not studied, but as MGDF did upregulate the co-expression of CD151 and CD61 early, future study should examine the role of CD61 in megakaryopoiesis.

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