ROLE OF CD44-DEPENDENT PATHWAY IN THE ADHESION AND HOMING OF HUMAN HAEMOPOIETIC STEM CELLS AND PROGENITORS.

BY

MATHEW MWATA MUSAMI

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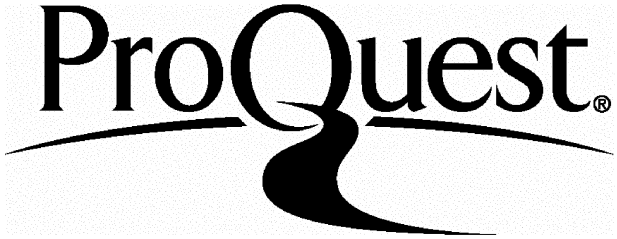

ABSTRACT

Although the presence of cell adhesion molecules on primitive haemopoietic cells are known and well characterised, adhesion pathways mediated by many of these CAMs remain a matter of intense investigation. This thesis explores the adhesive interactions of haemopoietic stem cells (HSC) and progenitors with ECM ligands and stroma with particular emphasis on characterising the CD44:HA adhesion pathways.

The myeloid cell line, KG1a, expressing the CD34 molecule demonstrated significant binding to stroma and less so to HA, this was mediated by CD44.

Long-term culture-initiating cell (LTC-IC) and plastic adherent (PΔ) cells (defined as stem cells) were quantified, in adult BM and peripheral blood (PB), using either limiting dilution assay (LDA) or single dilutions and both showed frequencies similar to previous studies.

Adhesion assays using BM MNC and PB $CD34^+$ cells on ligand-coated surfaces, showed that the frequencies of stem cells (either LTC-IC or PΔ cells), in the nonadherent fraction were less compared with those in the original cell suspension.

These data suggest that there was 2-3 fold enrichment of of the two stem cell population by HA.

The role of CD44 in the adherence of the stem cells to the ligands was investigated by including anti-CD44 moAb in adhesion assays and in two experiments both stem cells populations showed partial inhibition of adhesion to HA, compared with control antibody.

Adhesion of these stem cell populations to fibronectin were also studied. Similar to HA, the frequencies in the two stem cells were higher in the original than in the nonadherent cell suspension suggesting enrichement by FN. The adhesion was blocked by anti-integrin β_1 antibody but not by anti-CD44 antibody.

CFU-GM progeny assayed from PB $CD34^+$ cells were shown to adhere to HA, furthermore this adhesion was partially inhibited by anti-CD44 antibody. Similarly the

adhesion of CFU-C progeny assayed from UCB cell fractions was inhibited by the antibody.

These findings suggest that the majority of stem cells in BM, PB and progeny in UCB cell populations are adherent to HA and that this is dependent, at least in part, on CD44.

Declaration

The work contained in this thesis is the result of original research carried out by myself under the supervision of Dr. K.L.Yong, unless otherwise stated. All sources of information have been specifically acknowledged by means of reference. None of the work contained in this thesis has been used in any previous application for a degree.

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To the memory of my father and mother

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ABBREVIATIONS

ACD	Acid Citrate Dextrose
BFU-E	Erythroid burst forming units
BM	Bone marrow
BMEC	Bone marrow endothelial cells
BMT	bone marrow transplant
BMMNC	Bone marrow mononuclear cells
BSA	bovine serum albumin
CAFC	Cobblestone area forming cells
CAMs	Cell adhesion molecules
CD	cluster of differentiation
cDNA	complementary DNA
CFC	Colony forming cells
CFU-C	Colony forming unit cells
CFU-GM	Granulocyte-macrophages colony-forming units-
CFU-MK	Megakaryocyte colony-forming units
cGy	Centigrays
CPM	Counts per minute
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EPO	Erythropoietin
FACS	fluorescence-activated cell scanner
FCS	foetal calf serum

FN	Fibronectin
GAG	Glycosaminoglycans
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
HA	Hyaluronic acid
HBFP	Heparin-binding fragment peptide
HBSS	Hank's balanced salts solution
HPC	Haemopoietic progenitor cells
HSC	Haemopoietic stem cells
ICAM	Intracellular cell adhesion molecule
IFN	interferon
Ig	Immunoglobulin
IL	interleukin
IMDM	Iscove's modified dulbecco's medium
kDa	kilodaltons
LDA	Limiting dilution analysis
LFA	Lymphocyte function-associated antigen
LTBMC	Long-term bone marrow culture
LTC	Long-term bone marrow culture
LTC-IC	Long-term culture-initiating cells
M-CSF	Macrophage colony stimulating factor
MNC	Mononuclear cells
mRNA	messenger RNA
PBMNC	Peripheral blood mononuclear cells
PBPC	Peripheral blood derived progenitor cells
PBS	phosphate-buffered saline
PECAM	Platelet-Endothelial cell adhesion molecule
PHSC	Pluripotent haemopoietic stem cell
PMA	phorbol myristate acetate
RGD	Arginine-Glycine-Aspartic acid sequence of fibronectin

RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SCF	Stem cell factor
SCID	Severe combined immunodeficiency disease
SDA	Single dilution analysis
SRC	SCID repopulating cells
TdT	terminal deoxynucleotide transferase
TNF	Tumour necrosis factor
tRNA	transfer RNA
UCB	Umbilical cord blood
UCBMNC	Umbilical cord blood mononuclear cell
uv	ultraviolet
VLA	Very late antigen
VCAM	Vascular cell adhesion molecule

CHAPTER 1 GENERAL INTRODUCTION

1.1 Haemopoietic stem cells and progenitors

Haemopoiesis takes place in the bone marrow via a self-renewing pluripotent stem cell which in turn gives rise to lineage-committed progenitor cells and ultimately to the mature functional cells that circulate in peripheral blood. Defining a pluripotent haemopoietic stem cell (PHSC) is fraught with difficulties. Early definitions of the properties of PHSC were based largely on observation that these cells had extensive proliferative potential, and a capacity to differentiate into all haemopoietic lineages (Lajtha, McColluch).

More information has now accumulated regarding the properties of these cells. This has been facilitated by the development of numerous in vitro and in vivo assays for haemopoiesis, and the development of strategies for enriching subpopulations of haemopoietic cells.

1.2 Characteristics and properties of stem cells and progenitors

The primitive pluripotent stem cells are defined by their capacity for self renewal as well as their ability to differentiate into cells of the different lineages. These properties endow these cells with their remarkable ability to repopulate the haemopoietic tissue of, for example, lethally irradiated or genetically defective recipients. A number of purification strategies have aided in defining the properties of stem cells. Normally the majority of primitive stem cells in vivo are in the G_0 phase of the cell cycle (Lajtha, 1979), as demonstrated by treating cells with S-phase-specific or cycle dependent agents (^{3}HTdR, 5-fluorouracil, 4-Hydroperoxycyclosphamide, and cytosine arabinoside) prior to an assay. HSC have a capacity to self renew in vivo as can be demonstrated by collecting the progeny of candidate stem cells and replating them in conditions used for primary culture.

In addition HSC can generate cells belonging to all lineages of haemopoietic cell differentiation which is demonstrated by plating stem cell progeny in clonogenic assays for granulocyte-macrophage colony forming cells (CFU-GM), erythroid burst forming units (BFU-E) and megakaryocyte colony-forming cells (CFU-MK).

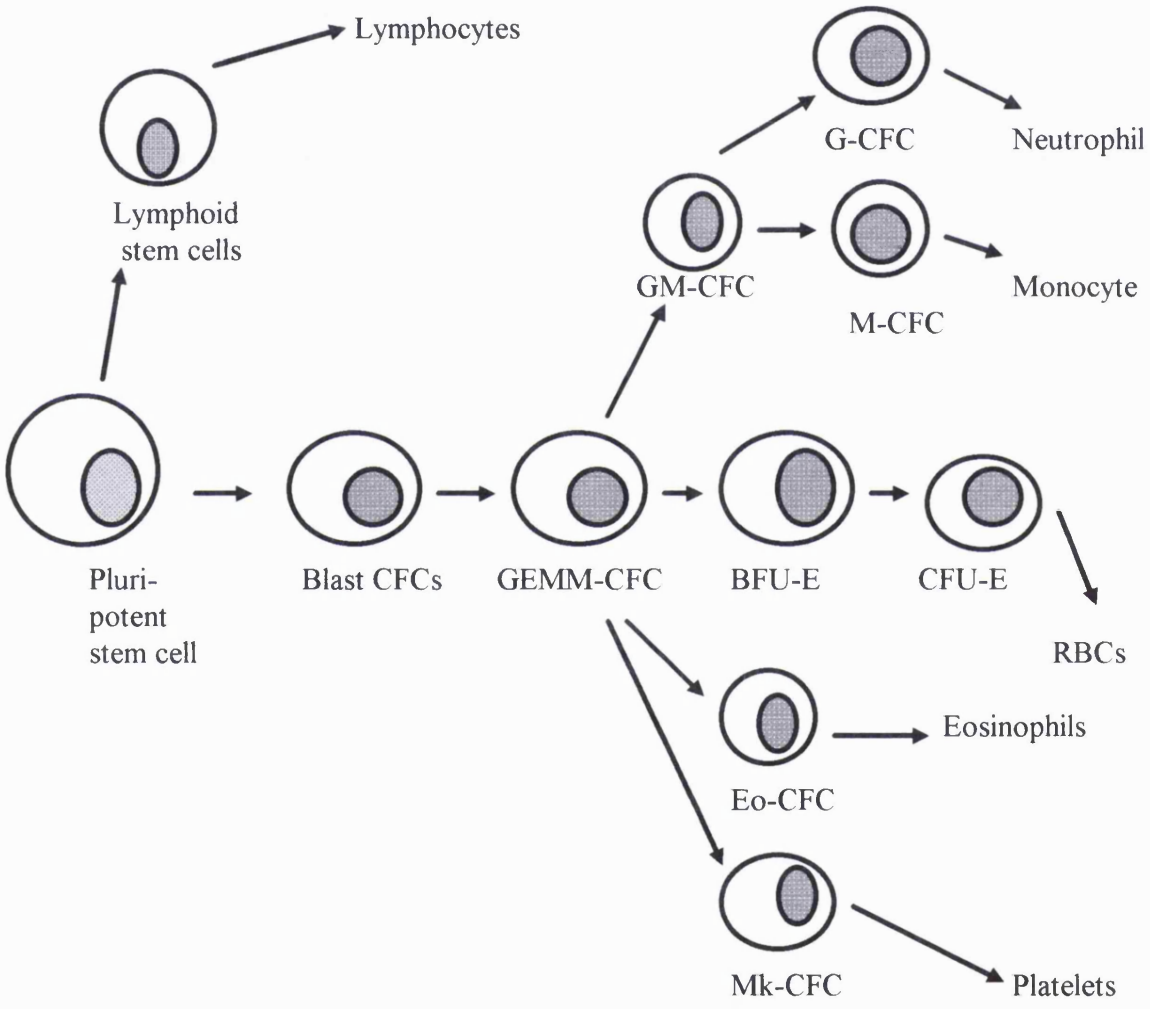


Figure1.1 Proposed hierarchy of haemopoietic stem and progenitor cells

Immunophenotypic characterisation of these cells shows expression of the stem cell antigen CD34 as a defining hallmark of haemopoietic stem cells and progenitors (Civin et al, 1987). Other phenotypic properties which could be exploited in stem cell purification and enrichment procedures include expression of Thy-1, $CD34^+$, $CD33^-$, $CD38^-$, human lymphocyte antigen (HLA) DR^- negative lineage-specific antigen (Craig et al, 1993), and resistance to the antimetabolite 5-fluorouracil (5-FU) (Lerner et al, 1990). Primitive haemopoietic progenitors are also distinguishable from their progeny by their abilities to adhere to cultured stromal monolayers (Mauch, 1980) and to tissue culture plastic surfaces (Gordon et al, 1988).

1.2.1 Human CD34-negative adult marrow cells and long-term engraftment

Many studies support expression of CD34 antigen as a requisite marker of long-term repopulating cells (Kawasaki et al, 1996). The majority of data supporting $CD34^+$ expression on human HSC is derived from in vitro assays that phenotypically characterise cells capable of haemopoietic colony formation after long-term culture i.e. LTC-IC. These studies have supported CD34 expression, lack of CD38 expression, and absence of lineage differentiation markers as a surface phenotype of human HSC (Haung et al, 1994).

The demonstration that CD34 enriched cells could provide rescue and long-term reconstitution in lethally irradiated baboons (Berenson et al, 1988) and that $CD34^+$ cells could engraft in a variety of immunodeficiency mouse and pre immune fetal sheep models (Cashman et al, 1997) has provided further in vivo support for CD34 as a human stem cell marker.

However, the relationship between LTC-ICs and cells with in vivo long-term reconstituting ability have recently been questioned. In gene marking studies, (Larochelle et al, 1996) it has been shown that human cells capable of long-term engraftment in the NOD/SCID model are at least partially distinct from LTC-IC. In addition, recent studies in the mouse have supported the presence of a $CD34^-$ population that contains progenitors capable of differentiation into $CD34^+$ cells, are

highly enriched in HSC and have competitive long-term repopulating capacity in vivo (Osawa et al, 1996).

The studies demonstrated that transplantation of human $CD34^-$, Lin^- populations resulted in long-term, multilineage human engraftment in human/sheep model.

This study therefore provide further support for the existence of a primitive $CD34^-$ human cell population that contains in vivo long-term repopulating potential and is a precursor to $CD34^+$ hematopoietic progenitors

Table 1.1 Phenotype of adult BM stem cells and progenitors

Stem cells	**Progenitor cells**
$CD34^+$(Peault et al, 1993)	$CD34^+$ $CD38^+$ (100%)
Lin^- (Terstappen et al, 1991)	$CD33^+$ $CD54^+$, $CD7^+$, $CD19^+$, $CD24^+$
Thy-1^+ (Peault et al, 1993)	Thy-1^+ (5% to 25%)
C-Kit^+	C-Kit^+ (70% to 80%)
Flk-2^+	Flk-2^+ (20% to 50%)

1.3 Assay methods for candidate stem cell

The identification of very primitive human haemopoietic stem cells relies on both in vitro and in vivo assay systems. A number of these systems have been devised for these early primitive haemopoietic cells .

They can be broadly divided into three classes; 1. Direct clonogenic assay; 2. Assays where non-clonogenic cells are detected by their ability to produce more mature clonogenic progeny, and Transplantation of human haemopoietic cells into immunocompromised (SCID) mice. These assays are important in that they can be designed to provide information regarding various regulatory mechanisms of haemopoietic stem cell function in addition to providing qualitative and quantitative

information about stem cell numbers and identity. The long-term bone marrow culture system which falls in the second category was originally devised from studies of murine haemopoiesis in vitro (Dexter , 1979) and later adapted for studies of human haemopoiesis (Gartner et al, 1980).

These cultures have shown the presence of cells capable of generating sustained haemopoiesis in vitro. Long-term culture-initiating cells have been identified as the best available approximation of stem cells in humans in this assay (Sutherland et al, 1990). Primitive haemopoietic progenitors can also be distinguished from their progenies by their different adhesive abilities to bind to cultured stromal layers and to tissue culture plastics (Kerk et al, 1985; Gordon et al, 1987).

1.3.1 Long-term bone marrow cultures

One approach to the quantitative measure of the number of primitive cells [long-term culture-initiating cells (LTC-IC)] may be obtained by culturing the LTC-IC at limiting dilution in the wells of microtitre plates so that the frequency of LTC-IC in a sample can be determined using Poisson statistics (Taswell, 1984).

These assays are set up in two stages. The first involves the culture of a confluent layer of stromal cells derived from precursors in BM. For the second stage, the confluent stromal culture is used as a feeder layer for the sample to be tested.

Primitive cells in the test sample migrate into and adhere to the stromal layer wherein they can remain quiescent in culture for up to 8 weeks during which time the pre-existing progeny will have disappeared and all the resulting progeny after the period of incubation will have been produced by the primitive cells adherent to the stromal layer. For this reason, primitive cell activity is usually evaluated 5-8 weeks after initiating of the culture (Eaves et al, 1991).

Estimating of the number of haemopoietic cells is done by limiting dilution analysis. A number of replicate wells for each of a range of cell dilution in microtitre plates is

required, with the aim of covering the range between an average of more that one progenitor cell per well to an average of less than one progenitor cell per well.

Each well is then assessed for progenitor cell activity and, by interpolating, the dilution that would result in 37% of the wells containing no activity can be obtained.

This is the proportion of inactive (empty) wells to be expected from the Poisson distribution when there is on average one progenitor cell per well. In practice, a plot of the logarithm of the proportion of empty wells against cells plated should give a straight line and the concentration of progenitor cells in the original sample can then be calculated (Sutherland et al, 1990).

As well as providing a measurement of progenitor frequency, limiting dilution analysis can provide a measurement of the ability of individual progenitor cells to form progeny such as CFU-GM (Gordon et al, 1995). Determination of the average number of progeny (CFU-C or CFU-GM) produced by individual primitive progenitor cells is obtained from the estimates of the average number of progenitors per well and the average number of progenies per well.

1.3.2 Delta assay of plastic-adherent cell cultures

Another method of stem cell quantitation is based on the observation by Gordon and co-workers of the ability by primitive haemopoietic progenitors to adhere to tissue culture plastic.

The existence of these primitive plastic-adherent haemopoietic progenitor cells have been evaluated in studies by Gordon and co-workers. These cells are detected in a delta type assay system by quantifying the progeny (CFU-GM) released into the supernatant and have been referred to as PΔ progenitor cells (Gordon, 1993). The PΔ progenitor cells are capable of sustaining long-term haemopoiesis in vitro and produce non-adherent progeny (Gordon, 1994).

Phenotypically, these PΔ progenitors express features characteristic of haemopoietic stem cells such as the CD34 antigen and Thy-1 and a relative resistance to treatment

with 5-Fluoruracil (Gordon et al, 1996). The presence of these features does therefore suggests that the plastic-adherent fraction of bone marrow contain very primitive haemopoietic progenitor cells. The frequency of these PΔ progenitor cell population can be estimated, as for LTC-IC, by limiting dilution assays.

The assay involves incubating of isolated tissue culture plastic-adherent marrow cells in liquid culture for one week in the presence of growth factors, harvesting the nonadherent cells that are produced and plating them in a semi-solid CFU-GM assay.

The adherent progenitors present in each well can then be quantified by limiting dilution analysis and use of Poisson statistics and the ability of individual progenitors to produce CFU-GM then measured (Blackett et al, 1996).

By plating the adherent cells under limiting dilution conditions with a high probability that the progeny in each well will be produced by a single adherent progenitor, it is possible to analyse the CFU-GM content of individual clones.

Due to variability in the lengths of maturation and that CFU-GM-derived colonies form at different times in culture and achieve different maximum sizes, the plastic-adherent cell-derived clones are scored at three different time intervals (Gordon et al, 1994). The assay can also be set up using single dilutions and in this case, the average number of progenitors per well, and hence their frequency is obtained by simple calculations involving taking the natural logarithm of the percentage of empty wells and ignoring the negative sign. It has been demonstrated that using a single dilution protocol consisting of a large enough number of replicates of the same number of cells is able to provide an estimate of progenitor cell frequency (Blackett et al, 1996)

1.3.3 Assay for primitive human SCID repopulating cells (SRC's) in vivo.

The engraftment of normal human haemopoietic cells in immune-deficient mice provides a system for developing an assay that measures the repopulating capacity of human stem cells. It has been previously reported that intravenous injection of human BM or cord blood into mice with severe combined immunodeficiency disease (SCID

mice) or non-obese diabetic (NOD) SCID results in the engraftment of a small number of primitive cells that proliferate and differentiate in the murine BM producing large numbers of LTC-IC, CFC, immature $CD34^+$ Thy^+ and $CD34^+$ $CD38^-$ cells (Cashman J et al, 1994). The primitive cells that initiate the graft were operationally defined as SCID-repopulating cells (SRC's).

The assay is based on the ability of the SRC's to repopulate the BM of irradiated SCID or NOD/SCID mice to yield high levels of both myeloid and lymphoid cells. The SRC assay involved intravenous injection of cells into sublethally irradiated NOD/SCID mice using standard conditions. Four to 5 weeks after transplantation, the murine BM suspension was processed for DNA analysis using southern blot analysis to determine the level of human cell engraftment and by methylcellulose plating to assess the presence of multilineage human CFC (estimated by the presence of CFU-GM, BFU-E and CFU-mix colonies).

The cells have however not been characterised and the relationship between SRC and in vitro progenitors such as LTC-IC and CFC is not known; nor is it known whether LTC-IC and CFC them selves have SRC activity. Using retroviral gene marking of human haemopoietic cells and cell purification based on surface marker expression to characterise the SRC and to determine it's relation to LTC-IC and CFC, Both methods demonstrated that most LTC-IC and CFC were incapable of engrafting NOD/SCID CFC, providing strong evidence that the SRC is a more primitive and distinct cell population.

1.4 Adhesion interactions in haemopoiesis

In the adult population, the bone marrow (BM) is the main site of the continuos regeneration of blood cells. The earliest step in the haemopoietic process is the binding of haemopoietic progenitor cells (HPC) to stroma. The precise definition of the nature of these interactions at a molecular level remains a subject of intense study by many investigators. Under steady state conditions the majority of primitive haemopoietic cells appear to reside in the bone marrow microenvironment where they and their

progeny develop in association with a phenotypically and probably functionally heterogeneous population of stromal cells (Tavassoli et al, 1983). The various cellular elements of the stroma, which include endothelial cells, macrophages, adipocytes and fibroblasts, together with their associated biosynthetic products including extracellular matrix (ECM) components and haemopoietic growth factor constitute this haemopoietic microenvironment of the bone marrow (Wolf et al, 1979).

There is accumulating evidence that the localisation of haemopoiesis to the bone marrow involves developmentally regulated adhesive interactions between primitive haemopoietic cells and their complex stromal cells-mediated haemopoietic microenvironment (Clark et al, 1992). This evidence is based on the findings that primitive progenitor cells in human marrow bind to pre-formed, marrow-derived stromal cells in vitro and subsequently proliferate to form colonies (Gordon et al, 1987a; and that the binding of lymphoid precursor cells to stromal element and their subsequent proliferation involves well-defined adhesion molecules and growth factors (Kincade et al, 1989). The net result of the adhesive interactions is the firm attachment of the progenitor cells to the stromal cells.

1.5 Cell adhesion molecules on haemopoietic stem and progenitor cells

Cell adhesion molecules (CAMs) are cell surface proteins thought to be responsible for the localisation of the haemopoietic progenitors in the bone marrow microenvironment through adhesion interactions.

Several distinct superfamilies of CAMs have been identified, however investigating the functions and contribution of particular CAMs to the interaction of HPC is complicated by the diversity of potential ligands for many of these CAMs.

Adhesive mechanisms identifying CAMs-ligand pairing that is essential for localisation of HPC to the bone marrow have been discussed in numerous reviews. According to structural and functional similarities these adhesion molecules can be grouped into various families, including the integrins, selectins, immunoglobulin gene superfamily,

cadherins, syndecans, and the CD44 family (Table 1.2). These cellular adhesion molecules are involved in cell-cell and cell-matrix interaction. Many of the CAMs are constitutively expressed on haemopoietic stem cells and progenitors (Simmons et al, 1994)

1.5.1 The Integrins

Integrins are a large family of CAMs with well documented roles in a variety of cellular functions. Integrins primarily are mediators of cell-ECM interactions and of some cell-cell adhesive events (Albelda et al, 1990). They are so named because of their ability to integrate the intracellular cytoskeleton with ECM.

Structurally, they represent a conserved family of integral membrane glycoproteins consisting of at least 20 distinct heterodimers formed by covalent association between 14 α subunits and 8 different β subunits, each with distinct ligand-binding properties (Hynes et al, 1992). The integrins are subdivided on the basis of β-chain composition although it is important to note that some α-chains (particularly α_v) can associate with more than one β subunit (Clark et al, 1992). The β_1 (CD29) or very late antigen (VLA) integrins comprise the largest subfamily and mediate cell-ECM and cell-cell adhesion. The β_2 (CD18) or leukocyte integrins bind to cell surface counter-receptor of the immunoglobulin gene superfamily and therefore mediate cell-cell adhesion (Arnoaout, 1990).

The β_3 (CD61) or cytoadhesion integrins predominantly bind to ECM proteins.

Currently the majority of published studies have examined the expression of only the β_1, β_2, and β_3 integrins in human bone marrow (Simmons et al, 1992,). Of these, β_3 integrin is present on a minor proportion of $CD34^+$ cells (approximately 10%) which are thought to include megakaryocytic precursors (Liesveld et al, 1993).

The expression of β_3 by the more primitive HPC population with the capacity to initiate and sustain haemopoiesis in long-term marrow culture has not been determined. Of the β_2 (CD18) integrin family, CD11a has been detected on $CD34^+$

cells (approximately 80%) (Saeland et al, 1992). LTC-IC however are restricted to the Cd11a/CD18$^-$ subpopulation (Gunji et al, 1992).

There is good evidence to suggest the expression of the β_1 (CD29) integrins, $\alpha_4\beta_1$ (CD49d) and $\alpha_5\beta_1$ (CD49e) on human progenitor cells that are characterised by the CD34 antigen (Teixido et al, 1992). The pioneering work of Kinkade and colleagues first demonstrated the importance of $\alpha_4\beta_1$ in regulating haemopoiesis in vitro where addition of an anti-α_4 subunit monoclonal antibody to murine long-term bone marrow culture (LTBMC) retarded myelopoiesis. Moreover, medullary haemopoiesis was inhibited after intravenous infusion of β_1 polyclonal antibodies treated bone marrow cells into lethally irradiated mice (William et al, 1991).

Recent studies in humans have suggested that certain β_1 integrins may be involved in mediating the adhesion interaction of haemopoietic cells with the ECM matrix. It is now generally accepted that the $\alpha_4\beta_1$ integrin plays a role in the adhesive interaction between human primitive and committed haemopoietic progenitors and bone marrow stromal microenvironment.

Very late activation-5 (VLA-5) $\alpha5\beta1$ like VLA-4, does participate in this adhesion interaction, however whereas VLA-4 ($\alpha4\beta1$) may bind either to vascular adhesion molecule VCAM-1 on the surface of activated endothelial cells or to the COOH-terminal heparin-binding domain of fibronectin, VLA-5 has as its ligand the sequence arginine-glycine-aspartic acid (RGD) cell binding domain of fibronectin (Minguell et al, 1993). This therefore suggests, that human CD34 cells use multiple integrin-mediated pathways to different extents, including VLA-4 and VLA-5 and $\beta2$ integrins in adherence to marrow stroma (Teixido et al, 1992).

Differential expression of VLA-4 and VLA-5 during myelopoiesis also was found , underscoring the importance of these integrins as mediators of critical stem cell-Stromal interactions during differentiation (Kerst et al, 1993).

Two more recent studies of great interest in integrin biology involve VLA integrins. In one study, Bhatia et al restored adherence of chronic myelogenous leukaemia progenitors to marrow stroma with interferon-α-induced stimulation of β1 integrins.

Normal progenitors adhere to stroma through a variety of cell surface adhesion receptors including $\alpha_4\beta1$ and $\alpha_5\beta1$. However unlike normal progenitors, CML progenitors fail to adhere to normal stromal layers (Gordon et al, 1987) and to fibronectin or its fragments (Verfaillie et al, 1992) despite the normal levels of these receptors on CML progenitors suggesting that the function of these receptors may be impaired in CML. Treatment with interferon-α, used successfully in the treatment of CML, induced a significant, dose-dependent increase in the adhesion of primitive long-term culture initiating cells and committed colony-forming cells (CFC) from CML bone marrow to normal stroma. The effect of interferon-α did not appear to proceed through the up-regulation of integrin expression but rather through restoration of integrin function.

In a second study, researchers examined the role of VLA-4 integrin and its ligand VCAM-1 in the immediate stages of homing and whether treatment of transplanted haemopoietic cells with adhesion-blocking antibodies or treatment of recipients with anti-VCAM-1 antibody could influence haemopoietic cell lodgement after transplantation in mice (Papayannopoulou et al, 1997).

Donor cells were incubated with anti-murine VLA-4, injected intravenously into primary recipient mice which were then sacrificed and mononuclear cells then obtained from peripheral blood, bone marrow, and spleen assayed for CFU-C and CFU-S.

There was a reduction in the homing of progenitor cells to the bone marrow with an accompanying increase in the numbers of progenitors in the spleen and peripheral blood. They conversely studied HPC homing in the presence of a rat anti-murine VCAM-1 antibody injected into the mice prior to transplantation. The results in both cases were similar. The two experiments suggested a role for the VLA-4- VCAM-1 in mediating homing of HPC.

1.5.2 The Selectins

Selectins are integral membrane glycoproteins characterised by an N-terminal lectin domain and a variable number of repetitive units (two in L-selectins). The lectin domains of these proteins directs their adhesion to carbohydrate molecule present on the cell surface (Bevilacqua et al,1991). The selectin (CD62 family) of adhesion receptors consists of three membrane glycoproteins that mediate Leukocyte - endothelial cell interactions by binding to carbohydrate ligand on the opposing cell surface.

The homing receptor L-selectin is expressed on mature myeloid cells (neutrophils, eosinophils and monocytes) and most lymphocytes and mediates the adhesion of leukocytes to the endothelium during Neutrophil emigration at inflammatory sites and homing of lymphocytes to peripheral lymph nodes (Tedder et al, 1990). A second selectin P-selectin can be found in α granules of platelets and Weibel-Palade bodies of endothelial cells. P-selectin is brought to the cell surface after thrombin or histamine stimulation allowing myeloid and lymphoid cells to bind (Bevilacqua et al, 1993).

A third member, E-selectin is a glycoprotein synthesised by endothelial cells in response to inflammatory agents such as endotoxins, TNF-α and IL-1 and promotes

adhesion of myeloid cells and memory T-lymphocytes. All three selectins can bind to sialylated, fucosylated glycoconjugates (Springer et al 1991).

The role of these adhesion receptors in haemopoiesis is less well understood. However their interest in relation to haemopoietic function is increasing. Studies by Simmons and co-workers demonstrated expression of L-selectin by approximately 75% of $CD34^+$, which included >90% of CFU-GM and 60% of BFU-E, confirming earlier studies undertaken by Griffin and co.

The group in addition, found low expression of L-selectin by LTCIC which were adherent to an TNFα-inducible ligand on marrow stromal cells (Simmons et al, 1992).

As a result of the interest in the role of L-selectin in haemopoiesis preliminary data has clearly been able to demonstrate an association between L-selectin expression and

increased clonogenic capacity as evidenced in studies where expression of L-selectin correlated with higher clonogenic potential of progenitors in vitro clonogenic assays (Koenig et al 1994) and that cross-linking of L-selectin on stem cell membranes prior to cultures increased the clonogenic capacity of the cell (Terstappen et al 1993).

However chronic addition of an Anti- L-selectin antibody to colony assay systems of both methylcellulose and long-term bone marrow culture suppresses colony formation (Gunji et al 1992), suggesting that L-selectin-mediated adhesive interactions between cells and /or cells and matrix are critical for optimum clonogenic growth. It is possible that the endothelial selectins participate directly in homing, proliferation or differentiation of haemopoietic cells as have been demonstrated in experiments using mice deficient in both P- and E-selectin (Frenette et al, 1996)

Primitive haemopoietic progenitors have been reported to bind P-selectin glycoprotein ligand-1 (PSGL-1) (Zannetino et al, 1995). Since PSGL-1 is a signalling molecule regulating cytokine production in monocytes (Weyrich et al, 1995) and is a common ligand for both P- and E- selectins (Asa et al, 1995), it might have a role in regulation of haemopoietic function

Finally, CD15 contains the SLE^x ligand recognised by either P-selectin or L-selectin (Larsen et al, 1990). This ligand is present on maturing myeloid progenitors and precursors, a small fraction of the more primitive $CD34^+$ population (Brandt et al, 1990), but is absent from erythroid progenitors. The role of CD15 present on immature progenitors and maturing myeloid precursors is unknown.

1.5.3 Immunoglobulin superfamily

The Ig superfamily has over 70 members with varied functions and primary structures, they, however, share Ig-domains, and a common tertiary structure. These molecules are involved in cell-cell, rather than cell-matrix, interactions and mediate homophylic (like-with-like) as well as heterophylic adhesion.

The group includes intracellular adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAM-1), platelet-endothelial cell adhesion molecules (PECAM-1) and neural cell adhesion molecule. Several of these immunoglobulin superfamily CAMs are expressed by HPC's including ICAM-1 (CD54), PECAM-1 (CD31), and LFA-3 (CD58) (Turner et al, 1995). ICAM-1 has recently been shown to be present on a subset of HPC'S, however these studies have demonstrated that ICAM-1, is not readily detectable on HPC'S under basal conditions.

Table 1.2 Reported adhesion receptor expression on haemopoietic progenitor cells.

RECEPTORS	LIGANDS
Integrins	
CD49d (VLA-4)	Fibronectin (CS-1 region), VCAM-1
CD49e (VLA-5)	Fibronectin (RGD site)
CD29 (β_1 integrin chain)	Collagen, Laminin, Fibronectin
CD18 (β_2 integrin chain)	ICAM-1
CD11a (LFA-1)	ICAM-1, ICAM-2, ICAM-3
Immunoglobulins	
CD54 (ICAM-1)	β_2 integrin (LFA-1, Mac-1)
ICAM-3	? β_2 integrin
PECAM-1 (CD31)	PECAM-1, $\alpha_v\beta_3$
Mucins	
LFA-3 (CD58)	CD2
Selectins	
L-Selectins	CD34, Glycam1, Sialyl LeX
Proteoglycans	
CD44	Hyaluronate, serglycin, Fn, Collagen Fibrillar collagen, Fn, Thrombospondin, Tenascin.
Syndecan-1	

However following induction with appropriate stimuli the receptor is detectable on haemopoietic progenitor cells (Arkin et al, 1995). ICAM-1 and VCAM-1 are also present on marrow stromal cells, as is N-CAM and participate in adhesive interactions with HPC.

Platelet-endothelial cell adhesion molecule-1 (PECAM-1) or CD31 is an immunoglobulin gene superfamily member of 130-kD that is expressed on endothelium, platelets, granulocytes, monocytes and a subset of lymphocytes (Albelda et al, 1991). Although the precise function of CD31 is not known in all cell types that express it, a number of studies clearly document its ability to function as a CAM (Muller et al, 1992). CD34 cells express CD31; BFU-E at low level, CFU-GM at high level and LTCIC at an intermediate level. Anti-CD31 Fab fragment partially inhibit adhesion of CD34 cells to marrow stroma (Watt et al, 1993).

PECAM-1 is considered to engage in homotypic interaction involving haemopoietic progenitor cells and stromal macrophages or T-cells or with heparin sulfate proteoglycans on stromal reticular cells or in the extracellular matrix in the bone marrow environment in a heterotypic manner (Watt et al, 1995).

PECAM-1 may also regulate progenitor cell adhesion in a subtle way through its ability to trigger adhesion mediated by β1 and β2 integrins (Tanaka et al, 1992).

It is also possible that PECAM-1 may be involved in homotypic or heterotypic interactions of megakarycytes, peri-sinal macrophages, adventitial reticular cells and adipocytic cells with the luminal surface of the vascular sinus endothelia, thereby contributing to the formation of a blood-marrow barrier and to regulate the release of haemopoietic cells from the marrow space (Watt et al, 1995).

1.5.4 Syndecans

The syndecan comprise a family of integral membrane heparin sulfate proteoglycans, characterised by a well conserved cytoplasmic and transmembrane protein domain and dissimilar glycosaminoglycan extracellular domains (Elenius et al, 1994).

So far, four members of this family have been fully identified, with syndecan-1 best characterised of all.

Recent studies of myeloma cells have suggested that syndecans may participate in the homing of tumour cells to the marrow, or following initial cell attachment, syndecans may aid in retaining myeloma cells within the marrow (Ridley et al, 1993).

Syndecan-1 is almost exclusively expressed by epithelial cells in mature tissues, but, as an exception to this rule it is expressed by B lymphocytes during maturation in bone marrow. Syndecan-1 can function as a matrix receptor for Fibrillar collagens, thrombospondin and tenascin and may also, through yet unresolved mechanisms, be involved in cell-cell interactions (Salmivirta et al, 1995).

1.5.5 Mucins

The sialomucin adhesion family are a recent addition of the adhesion molecules that have mucin-like structure. Their role has so far been attributed mainly to act as scaffold that presents selectin carbohydrate ligands in a clustered tissue specific manner thus allowing for higher avidity interactions between leukocytes and endothelial cells during the inflammatory process (Lasky et al, 1994). The role of these family of adhesion molecules in haemopoiesis are yet to be evaluated.

1.5.6 CD44 Receptor

CD44 is a transmembrane cell surface glycoproteins which possesses multiple isoforms and is present on a range of different cell types including haemopoietic cells. It is encoded by a single gene of approximately 60kb located on chromosome 11p13 and comprising at least 20 exons (Heider et al, 1993). Genome and cDNA cloning data have demonstrated that CD44 isoform diversity is generated by alternate splicing (Mackay et al, 1994).

This process involves the exclusion or inclusion of up to 10 exons from the genom in the final mRNA transcript to generate several related proteins from a single gene.

Splicing of this CD44 gene occurs at two different regions, the membrane proximal extracellular domain and the cytoplasmic carboxyl tail region, suggesting that the inclusion of one or more of these exons may confer changes in ligand affinity or changes in signal transduction of the final of CD44 molecule (Hofmann et al, 1991).

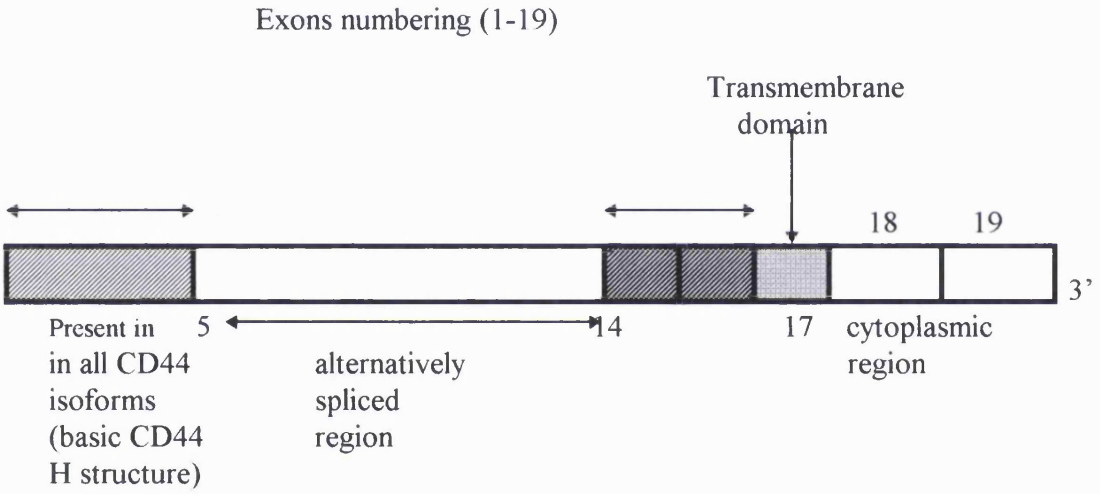


Figure 1.2 CD44 gene structure.

The haemopoietic CD44 isoforms, also known as the standard form of the molecule (CD44s) is encoded by ten exons (designed exon 1s to 10s). The molecule is synthesised as a 37kD molecule which is processed to a 80-90kD molecule by glycosylation on its extracellular domain with N- and O-linked oligosaccharides as well as chondroitin sulfate chains (Goldstein et al, 1989). Variation in the degree of glycosylation give rise to multiple molecular mass forms of CD44 (Goldstein et al, 1989).

CD44 has been implicated in a number of functions including regulation of normal haemopoiesis. It was originally described as a homing receptor. The molecule seems to function by mediating cell-cell or cell-substrate interactions through recognition of elements of the extracellular matrix. It is an integral membrane protein with an NH2 extracellular domain homologous to the cartilage link protein and proteoglycan core proteins found in connective tissue (Goldstein et al, 1989). The two isoforms of CD44 differ in that CD44H can bind to hyaluronic acid (HA) and mediate various cellular interactions, while CD44E does not (Brown et al, 1991).

In the case of the CD44H isoform, the molecular mass range from 80-100 kDa, while for CD44E isoform this value is from 100 to 160 kDa in addition chondroitin and heparin sulphate can be linked to CD44, increasing its apparent molecular mass to the range of 180-200 kDa (Brown et al, 1991).

Evidence has provided that the addition of glycosaminoglycan (GAG) side chains is required for binding to non-HA extracellular matrix ligands such as fibronectin, and collagen, this interaction appears to be spatially distinct from the interaction of CD44 with hyaluronan (Gallagher et al, 1987).

Both functionally and physically, the CD44 molecule can be divided into three major domains; the cytoplasmic domain, which is highly conserved and speculated to be involved in signal transduction, the transmembrane domain and the variable extracellular portion containing the amino-terminal domain which binds to HA (Zhou et al, 1989). Both the expression and binding properties of CD44 on normal cells appear to be subject to regulation .

This regulation may provide specificity for what would otherwise be an uncontrolled interaction between a broadly distributed cell surface molecule and common components of the extracellular matrix environment.

Hyaluronic acid is the most widely recognised ligand of CD44, but evidence of binding to fibronectin, collagen and serglycin has also been reported (Underhill et al, 1992).

Table 1.3 Characteristics of the different isoforms of CD44

Isoform	Structural Characteristic	Location	Relative amount
CD44H	Most common form, core protein is 37kDa	Haemopoietic cells, leukocytes and other mesodermal cells	Major
CD44TR	Lacks cytoplasmic domain	Haemopoietic and other cell lines	Minor
CD44E (R1)	132 amino acid insert in middle domain	Epithelia and other cells	Major
CD44E (R2)	69 amino acid insert in middle domain	Epithelia, granulocyte and mononuclear cells	Minor
CD44M	162 amino acid insert in middle domain	Malignant rat tumour cell lines	Unknown

It is a polycarboxylic GAG with a high molecular mass (10^6) and is synthesised and secreted in large quantities by stromal cells (Gallagher et al, 1983; Gordon, 1988) The binding between HA and CD44 depends to greater extend on an ionic interaction between negatively charged carboxyl groups of the GAG and two regions from CD44 extracellular domain, containing clusters of positively charged basic amino acid (Underhill, 1992; Peach et al, 1993).

A large number of higher molecular weight isoforms may also be produced in specific cell types or under specific conditions as a result of the alternative splicing of at least 10 contiguous exons within the CD44 gene (Mackay CR et al, 1994).

CD44R1 is one of several v10-containing CD44 cDNAs. It contains in the extracellular region of the molecule an insertion of 132 amino acids. CD44R2 is a v10-

containing isoform of CD44 that shares only the last 69 amino acids present in the unique region of CD44R1 (Dougherty et al, 1994).

The presence of CD44 receptor does not necessarily indicate that the receptor has functional significance, thus although almost all haemopoietic cells express the CD44 receptor only a fraction adheres to HA coated on wells (Morimoto K et al, 1994).

Accumulating evidence has given credence to the effect of an anti-CD44 monoclonal antibody, H90 which is thought to increase the adhesion of $CD34^+$ progenitors to HA, through increasing its expression which may indicate that CD44 is in a low affinity state (Smadja-Joffe et al, 1996). Most of the work in this area is still preliminary. Other similar findings include activation of CD44 on KG1a cells by phorbol esters (Morimoto et al, 1994)

CD44 role in haemopoiesis

The CD44 receptor is present on almost all cells of haemopoietic origin. Expression of CD44 on haemopoietic cells has been mostly studied using monoclonal antibodies that recognise indistinctly standard and variant CD44 molecules (Miyake et al, 1990).

Recently it has been shown that $CD34^+$ myeloid precursor cells spontaneously bind to HA via CD44, suggesting CD44 is involved in the interaction of these precursor cells with the BM stroma. (Morimoto et al , 1994). Other studies implicating CD44 in the interaction of haemopoietic progenitors with the BM microenvironment were done by Miyake et al, that the addition of anti-CD44 moAb to LTC can cause a marked and sustained decrease in the number of mature cells subsequently found in the nonadherent fraction of these cultures.

In humans, treatment of stromal layers with hyaluronidase decreased the adhesion of immature blast colony forming cells during a 2 hour adhesion assay (Gordon et al, 1988). Additional preliminary studies suggest that antibodies against the CD44 receptor (Hemes III and 50B4) can inhibit adhesion of BFU-E and CFU-GM to

substrata coated with the FN-C/H-II peptide, a cell adhesion site in the C-terminal heparin binding domain of fibronectin (Verfaillie et al, 1994).

1.5.7 Other CAMs involved in HPC interactions

There are many more CAMs involved in the adhesion interactions between components of the stromal monolayer and HPCs, many of these CAMs do not appear to play a major role in adhesion interactions and subsequent retention of these cells.

1.6 The Extracellular Matrix in stem and progenitor cell adhesion

The haemopoietic microenvironment consists of various stromal cell elements, secreted and membrane-bound cytokines and a complex extracellular matrix (ECM).

The heterogeneous population of stromal cells includes fibroblasts-like cells, adipocytes, and macrophages and endothelial cells (Clark et al, 1991).

The stromal cells synthesise and secrete various cytokines including colony stimulating factors, interferons, and interleukins. The growth factors may either directly interact with the developing haemopoietic cells or they may be secreted and stored in the ECM which then presents the factors to the developing haemopoietic cells in a biologically active form (Campell et al, 1988).

The ECM of the different species has been characterised and has been shown to include members of all three categories of matrix molecules: collagen, glycoproteins, and proteoglycans.

These matrix components do not form an inert scaffolding, but play an active role in control of cell adhesion and migration within the bone marrow, presentation of cytokines and directly or indirectly, in proliferation processes (Gordon et al, 1988).

Various ECM components of the haemopoietic microenvironment were first identified in long-term cultures by biochemical, immunohistological or molecular biology methods.

1.6.1 Fibronectin

Fibronectin (FN), a ubiquitous ECM molecule, is a multifunctional glycoprotein involved in cell adhesion, migration, proliferation, and differentiation. It is composed of two similar subunits A and B joined by a pair of disulphide bonds. Due to alternative splicing of the mRNA this matrix component exist in a variety of isoforms (Hynes, 1990).

FN is found in the native bone marrow and is also synthesised and deposited in an extracellular network by bone marrow stromal cells (Bentley et al, 1983). Early work of patel, lodish and co-workers showed that fibronectin is involved in adhesion and maturation of the erythroid lineage (Patel et al, 1986).

More recent work has demonstrated that multipotent HPC's adhere to fibronectin, and the regions within the fibronectin subunits responsible for these interactions were characterised (Verfaillie et al, 1991).

The modular FN molecule contains several attachment sites. Several of these domains have been identified by cleaving the molecule using proteolytic enzymes. The central, 75 kDa RGD-containing domain can interact with cells via the $\alpha 5\beta 1$ integrin receptor.

An RGD-independent cell adhesion site is located in the COOH-terminal 33/66 kDa fragment which is also known as the 'heparin-binding domain'. This fragment harbours a sequence known as III-CS. A peptide derived from the III-CS sequence and designated CS-1 contains the LDV sequence which is known to interact with the $\alpha 4\beta 1$ integrin (Guan at al, 1990). Two flanking regions of the CS-1 peptide termed FN-C/H-1 and FN-C/H-II can interact with either the $\alpha 4\beta 1$ integrin or with membrane-bound proteoglycans (Haugen et al, 1990).

Bone marrow endothelial cells and fibroblasts can generate fibronectin which contains all three cell adhesion promoting sites in the alternative spliced heparin-binding domain of fibronectin. Primitive murine haemopoietic cells (CFU-S12) can adhere to the CS-1 sequence in an $\alpha 4\beta 1$ dependent fashion (Williams et al, 1991). Human multipotent progenitors also adhere to the 33/66 kDa fragment and, to a lesser extend, to the 75

kDa RGD-containing domain. In contrast, more differentiated clonogenic progenitors adhere equally well to both fragments (Verfaillie et al, 1991).

1.6.2 Collagens

The collagen family which shares triple helical domains as a common structural motif contains nineteen different characterised collagen types (Mayne et al, 1993). These collagens can be subgrouped according to their supramolecular structures: collagen types I-III and V form large fibrils, types VI and VII form microfibrils and anchoring fibrils, respectively. Various studies have demonstrated that collagen types I-V are abundantly synthesised and deposited in the BM stroma (Bentley et al, 1981). In addition, it has recently been shown that the microfibrillar collagen type VI is also a major constituent of haemopoietic stroma (Klein et al, 1995).

Inhibition of collagen synthesis by the proline analogue *cis*-hydroxyproline in murine LTBMC leads to a reduction of stem and committed progenitor cell proliferation, suggesting a role of the collagens in haemopoiesis (Zuckerman et al, 1985). Koenigsmann et al, 1992, identified collagen type I as an adhesive substrate for erythroid (BFU-E) and myeloid (CFU-GM) progenitor cells, and the specificity of these interactions was demonstrated by digestion with collagenase.

Functional analyses with the microfibrillar collagen type VI have shown that this collagen type is an even stronger adhesive substrate than collagen type I for various haemopoietic cells, and that these interactions are not integrin-mediated. Again, inhibition of haemopoietic cell attachment to collagen VI by heparin suggested a role for transmembrane glycoproteins (Klein et al, 1995).

1.6.3 Tenascin and Thrombospondin

Tenascin and Thrombospondin (TSP) share a similar restricted pattern of expression outside the bone marrow, typically appearing transiently at sites of tissue morphogenesis or remodelling (Sage et al, 1991).

Furthermore both proteins may exert 'anti-adhesive' functions in some contexts, such as the ability to promote cell rounding and detachment. Tenascin has been detected by immunofluorescence in human LTBMC and BM cryosections (Klein et al, 1993).

Erythroid and myeloid cell lines and bone marrow mononuclear cells bind to purified tenascin, and treatment of stromal monolayer with antitenascin antiserum appeared to reduce their ability to adsorb CFU-GM (Klein et al, 1993).

Thrombospondin, a multifunctional homotrimeric glycoprotein of 450 kDa, consists of identical subunits which are covalently cross-linked at their NH_2-terminal ends by disulphide bonds. with several domains. It is present in the BME localised in megakaryocytes, fibroblasts and the extracellular matrix associated with active haemopoiesis and serves as an adhesive ligand for committed progenitor cells.

1.6.4 Hemonectins

Hemonectin a 60 kDa extracellular matrix protein recognised in the BME is present among developing haemopoietic cells in association with the processes of adventitial reticular cells.

Approximately 90% of the cells that bind to purified hemonectin are granulocytic, with a significant enrichment of immature forms (Campbell et al, 1987). Binding to hemonectin by CFU-GM, BFU-E and day 14 spleen colony-forming units (CFU-S) has also been observed (Anklesaria et al, 1991). The adhesion of BMMNC's to hemonectin has recently been shown to involve interaction of the cells with mannose and galactose in hemonectin-associated carbohydrates (Sullenbarger et al, 1995)

1.6.5 Proteoglycans

The proteoglycans group of adhesion molecules is made up of core proteins with covalently linked GAG side chains., negatively charged, unbranched polysaccharide chains composed of repeating sulphated disaccharide units

Most of these GAG chains are usually very long except HA which in addition contains no core protein. Four major proteoglycan classes are distinguished, based on their differing disaccharide units. They include Chondroitin and Dermatan sulphate, heparan sulphate and keratan sulphate.

These PG and the GAG hyaluronic acid are all found in the BME, either in a membrane-bound form or extracellularly with the exception of keratan sulphate PG (Gallagher et al, 1983). They are also abundantly present in LTBMC (Wright et al, 1986). Different heparan sulphate, dermatan and chondroitin sulphate PGs are produced by both haemopoietic cells and BM stromal cells (Minguell et al, 1989).

Gordon and colleagues (Siczkowski et al, 1992) demonstrated that heparan sulphates, which are shed into the long-term culture medium, are involved in the adhesive interactions with the haemopoietic progenitor cells.

HA and chondroitin sulphate are present both attached to the cells composing the adherent layers of LTBMC's and in the culture supernatant (Gordon et al, 1987), they both play an important role in haemopoietic cell adhesion , for HA through the CD44 receptor as outlined in the last section.

β-d-xyloside, an agent that uncouples the chondroitin sulphate GAG synthesis from the proteoglycan synthesis, when added to LTBMC induced a significant increase in chondroitin/dermatan sulphate GAGs in stromal supernatant (Spooncer et al, 1983).

Haemopoiesis in *βd*-xyloside treated LTBMC's is significantly increased, suggesting a role for chondroitin sulphate in haemopoiesis. Proteoglycans in the marrow also play a role in the binding and subsequent presentation of cytokines to haemopoietic cells.

Studies have shown that BM isolated GAGs were capable of binding exogenous granulocyte-macrophage colony stimulating factor (GM-CSF) (Gordon et al, 1987).

Heparan sulphates was identified as the components responsible for the binding of GM-CSF and interleukin-3 and their biologically active presentation of haemopoietic progenitors (Roberts et al, 1988).

However specific receptors for the interaction of these cytokines with heparan and chondroitin sulphate proteoglycans on haemopoietic cells are as yet unidentified.

Although the characterisation of such receptors is to be expected, one has to keep in mind that many other extracellular matrix molecules (e.g. fibronectin, collagen, laminin) have GAG binding sites.

Some of the interactions observed for proteoglycans may, therefore, involve indirect binding of haemopoietic cells to macromolecular structures of the bone marrow stroma which are in intimate contact with the proteoglycans.

1.7 Role of Chemokines in adhesion and homing of haemopoietic stem cells

Chemokines are cytokines that are best known for their ability to selectively attract subsets of leukocytes to sites of inflammation. The role that chemokines and their receptors play in homing and repopulating of human stem cells is not fully understood. There may be as many as 40 to 50 human cytokines. Chemokines are small with molecular weights in the range of 8 to 12 KD, but there are exceptions which involve proteins composed of multiple domains, one of which looks like a chemokine. They are divided into families based on structural and genetic consideration. All chemokines are structurally similar, having at least three β-pleated sheets (indicated as β1-3) and a C-terminal α-helix. Most chemokines also have at least four cystein residues in conserved positions. The main chemokine families include: CXC chemokines family, CC chemokines family, C- chemokines family and CX_3C chemokine (neurostatin). SDF-1 is a member of the CXC subfamily of chemokines, produced by stromal cells. The chemotactic effect of SDF-1 is mediated by binding to the chemokine receptor CXCR-4. The signalling through CXCR4 provides a potent chemotactic stimulus for $CD34^+$ haemopoietic cells. SDF-1 mediates transmembrane and transendothelial chemotaxis of both 2-week and 5-week cobblestone area colony forming cells (CAFC) and LTC-IC (Möhle et al, 1998). It's also reported that the chemokine SDF-1 and it's receptor CXCR4 are essential for murine BM engraftment by immature human $CD34^+$ cells (Lapidot et al, 1997). Recent data has also emerged to show that CXCR4-

dependent migration to SDF-1 is essential for human stem cell function in NOD/SCID mice. SRC were further characterised as $CD34^+CD38^-CXCR4^+$ stem cells and $CXCR4^-$ cells could be converted into functional $CXCR4^+$ stem cells by cytokine treatment (Peled et al, 1999)

The CC chemokine MIP-3β also demonstrates chemotactic activity for $CD34^+$ cells. Chemoattraction by MIP-3β is highly specific to subtypes of myeloid progenitors in BM and CB $CD34^+$ cells. MIP-3β has recently been described as a ligand for the CCR7 receptor, and shows specific chemotactic activity for HPC restricted to macrophage differentiation. The mechanisms underlying differences in chemotactic specificity between SDF-1 and MIP-3β are not known. However it is speculated that MIP-3β may influence trafficking of CFU-GM to/from BM under inflammatory conditions (Kim et al, 1998)

1.8 AIM OF THE STUDY

The aim of this project is to investigate the adhesive interaction of haemopoietic stem cells (HSC) with ECM components and stroma with particular emphasis on the CD44 dependent pathways.

Such adhesive interactions are postulated to be important in homing and anchorage, as well as in the regulation of HSC survival and proliferation.

The plan of investigation can be divided into three:

1. Studies on adhesion of the KG1a cell line which express CD44, in order to test blocking strategies by employing monoclonal antibodies and soluble peptides. Preliminary studies could then be used to investigate the CD44-HA adhesion pathway.

2. Setting up of a reproducible stem cell assay which can be used to quantify the number of stem cell in an aliquot of peripheral blood/bone marrow stem cells and thus to investigate the specific adhesion of such stem cells to various ligands for CD44.

Preliminary studies were carried out to evaluate the above in vitro assays for the quantification of stem cells in bone marrow and peripheral blood derived progenitor cell suspensions and to optimise the use of such assays in studying the adhesive interactions of haemopoietic stem cells.

3. Application of these blocking strategies in order to evaluate the adhesion pathways with respect to the candidate stem cells; LTC-IC and adherent PΔ progenitor cells, as well as adhesive interactions of UCB progenitor cells and peripheral blood derived progenitor cells.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 GENERAL MATERIALS

MATERIALS AND SUPPLIERS

Equipment	suppliers
37^0C/5% CO_2 Incubator	
Vertical laminar flow hood	
Neibauer haemocytometer	
Centrifuge	
Pipetteman	
Inverted microscope	
Water bath	

STERILE DISPOSABLE PLASTICS

30ml universal containers	Sterilin Ltd. Feltham,
7ml bijou bottles	
50ml conical-bottom test tubes	Greineir.
5ml, 10ml & 25ml graduated pipettes	Sterilin Ltd
Pasteur pipette, 2ml bulb	
1.8ml cryovials	Corning Paisley, Scotland
Tissue culture flasks, $25cm^2$	Nunc.
Tissue culture flasks, $75cm^2$	Falcon Ware, U.K Ltd
	Cowley, Oxford
15ml graduated V-bottomed tubes	Flow Laboratories Ltd.
Tissue culture plates, 96 well flat bottomed	Becton Dickinson

Falcon 6, 12, 24, 48 well plates	
0.22μm and 0.45μm cellulose acetate filters	Sartorius Ltd. Belmont, Surrey
Syringes (1 - 50 ml capacity)	Sabre International Products Ltd. Middlesex.
Quill filling tubes	Avon medical, Redditch Worcestershire.

Disposable Glassware

Sterile 1ml pipettes	BDH, Poole U.K
Cover slips	Fisher

REAGENTS/CHEMICALS

Lympho prep (1.077)	Nycomed Birmingham
Heparin, preservative free	Amersham
Hydrocortisone hemisuccinate	sigma
^{51}Chromium	Amersham
Trypsin/ethylenediaminetetraacetic acid (EDTA)	sigma
Penicillin and streptomycin	Sigma
Hyaluronic acid	Sigma
Fibronectin (FN)	Sigma
Hyaluronidase	Sigma
Iscove's Modified Dulbecco's Medium (IMDM)	Gibco
Roswell Park Memorial Institute (RPMI)	Gibco
Heparin-binding fragment peptide of fibronectin	Sigma
Alpha medium	Gibco
Bovine serum albumin (BSA)	Gibco

Hank's balanced salt solution (HBSS)	Gibco
Phosphate buffered saline (PBS)	Gibco
Tryphan blue	Sigma
Methylcellulose(Methocult H4230)	Stem cell technologies
Myeloid-LTC-media (Myelocult H5100)	Stem cell technologies
Fetal Calf Serum (FCS)	Gibco
human erythropoietin (EPO)	Chugai pharmaceuticals
Stem cell factor	First link UK Ltd
Dimethylsulfoxide (DMSO)	Sigma
NP40, detergent	

ANTIBODIES

HCAM, PAN/CD44 Ab-2	Neomarkers
BU52 Anti-CD44 Ab	Binding site UK Ltd Birmingham
Anti-CD29 Ab	Becton Dickinson

STOCK SOLUTIONS:

Extracellular matrix proteins:

Hyaluronic acid from umbilical cord, was dissolved in PBS and sterilised using a 0.22μm filter it was then aliquoted and stored at -20^{0}C and diluted with PBS to the required concentration before use in cytoadhesion assays.

Fibronectin was made up in sterile water and stored in aliquots at -20^{0}C it was diluted with HBSS to the required concentration before use in cytoadhesion assays.

Testicular Hyaluronidase was up in PBS (pH 7.2) plus 0.1% BSA to stabilise the protein, stock solution was made up at 4000units/ml

Heparin-binding fragment peptide of fibronectin was made up in sterile water at 1 mg/ml before dilution to the appropriate concentrations. Aliquots were stored at -20^{0}C.

Tissue culture media and Serum

RPMI with L-glutamine was used for culturing and freezing cell lines.

Iscove's Modified Dulbecco's Medium (IMDM) was used for progenitor cell handling and liquid cultures

Alpha medium was used for liquid cultures

Vancouver medium: comprising an enriched version of alpha medium (MEM) supplemented with 400 mg/ml Glutamine, 40 mg/ml inositol, 10 mg/ml Folic acid, 10^{-4} M 2-mercaptoethanol, 12.5% horse serum, 12.5% fetal calf serum and 10^{-6} M/l Hydrocortisone sodium succinate. Medium were made up in large amounts aliquoted in convenient volumes and kept frozen at -20^{0}C for months.

Myeloid-LTC-media (Myelocult H5100): containing horse serum (12.5%), fetal bovine serum (12.5%), and 2-mercaptoethanol (10^{-4} M) in alpha medium and supplemented with L-Glutamine (2mM), Inositol (0.2mM) and folic acid (20μM) to which fleshly dissolved hydrocortisone sodium hemisuccinate was added just before use to give a final concentration of 10^{-6} mol/l) at 10^6 cells/ml

The components were all pre-tested and selected for their ability to optimally initiate and maintain myelopoiesis in long-term cultures of human bone marrow. The media was used following addition of appropriate concentration of growth factors.

Fetal Calf Serum (FCS): Batches of FCS were tested for the ability to support optimal cell growth in both proliferation and clonogenic assay systems. It was heat inactivated at 56°C for 30 minutes, aliquoted into polypropylene tubes and stored at -20°C.

Hank's balanced salt solution was used for cell washing and various dilutions

Methycellulose stock: was purchased as pre-tested mixture of methylcellulose, fetal bovine serum and bovine serum albumin in iscoves medium. Growth factors (mixture dependent on the type of assay) and Iscoves were added to achieve final concentration of 0.9% methylcellulose, 30% FBS and 1% BSA, 2mM glutamine and 10^{-4} M mercaptoethanol. The 'ready to use' mixture was aliquoted into 7ml bijou's and stored at -20°C.

Growth factors: The following recombinant growth factors were used in the various assays used; human erythropoietin (EPO), IL-3, and, GM-CSF were all obtained, as donations, from advanced protein products and were reconstituted in 2% HBSS to stock concentrations of 30μg/ml, 30μg/ml and 25μg/ml respectively, aliquoted and stored at -20°C. Human recombinant stem cell factor was reconstituted in sterile water to a final concentration of 10μg/ml aliquoted and stored at -20°C.

GENERAL METHODS

2.2 CELL PREPARATION AND SEPARATION

2.2.1 Preparation of mononuclear cells fractions from BM and UCB .

Normal human bone marrow samples were obtained from donors from the Royal Free Hospital, Anthony Nolan bone marrow harvests and all UCB specimens were obtained from the London cord blood bank. Specimens were collected in blood bags containing ACD and cell separation undertaken immediately on receipt of the blood samples. The cord blood was aspirated from the bag with a 50-ml syringe under sterile conditions.

The human bone marrow and umbilical cord blood sample were diluted with an equal volume of serum-free HBSS (to reduce viscosity) , and carefully layered onto an equal volume of Lymphoprep in a round bottomed plastic centrifuge tube. The tubes were centrifuged at 2000rpm for 30 minutes at room temperature (25^0C) with slow acceleration and deceleration, and no vibration. Following centrifugation the mononuclear cells became layered between HBSS at the top and ficoll cushion below.

These cells were carefully pipetted off using a pasteur pipette, washed twice in HBSS containing 2% FCS at 1500 rpm for 10 minutes at room temperature. The pellet was then resuspended in IMDM containing 20% serum and the cells (diluted 1:10 with acetic acid) were counted using a haemocytometer.

2.2.2 Isolation and purification of $CD34^+$ haemopoietic progenitors from peripheral blood

$CD34^+$ selected cells were obtained from patients with relapsed or resistant lymphoma undergoing peripheral blood stem cell transplantation at UCH. Informed consent was obtained from all patients prior to selection. All subjects were mobilised with a protocol involving low dose cyclophosphamde (1.5 g/m^2) given on day 1 followed by

G-CSF given subcutaneously at 10 μg/kg (filgrastim) or a single vial of lenograstin (263 μg/vial) 24 hours afterwards and daily thereafter until harvesting was completed (Socinski et al, 1988).

One to three apheresis collections were performed on days 8 to 12, on a Cobe Spectra commencing when the recovery white blood cell count was around 5.0 X 10^9/L. Single apheresis collections were processed for clinical scale $CD34^+$ cell purification using a Ceprate SC immunoaffinity column and associated microprocessor as described by Watts, 1995.

Cytocentrifuge preparations of the final products were made for blast cell morphology (fig 2.1) and the percentage of $CD34^+$ cell were evaluated by alkaline phosphatase - antialkaline phosphatase immunoenzymatic staining (fig 2.2) as well as by dual immunofluorescence staining with anti-CD45-FITC and anti-CD34-PE and flow cytometry. The cell preparation had a median purity of 80% (range 52% to 95%).

2.2.3 Assessment of cell viability

Prior to their use in an experiment or following treatment during an experiment, cells were assessed for the number of viable cells per millilitre of medium. This was done by the dye exclusion test which relies on the ability of living cells to exclude certain stains from crossing the membrane. Trypan blue was added to a small aliquot of cells before counting on a haemocytometer. The percentage viability was calculated as

$$\% \text{ viability} = \frac{\text{Number of cells unstained}}{\text{Total number of cells}} \times 100$$

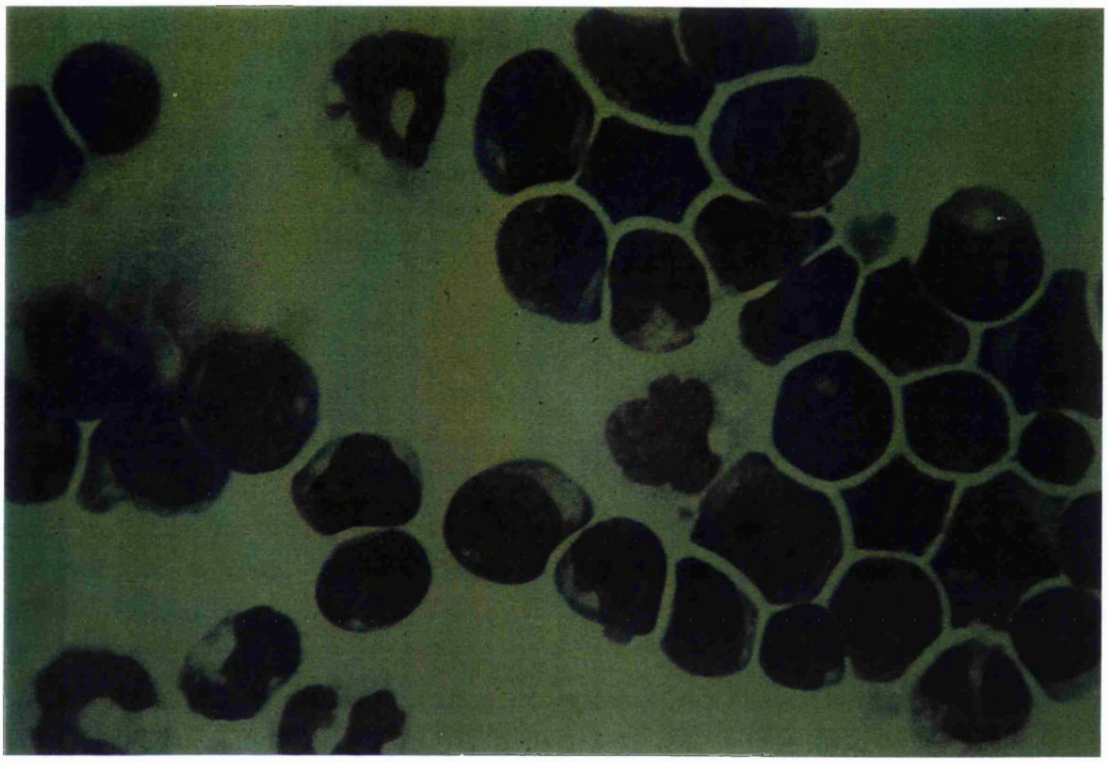


Figure 2.1 Morphology of peripheral blood $CD34^+$ selected cells, prior to use in various assays. Photomicrograph of May-Grunwald-Giemsa stained cytospin showing the morphology of peripheral blood $CD34^+$ selected cells prior to use in various assays. Blast-like myeloid cells were observed. Magnification x400.

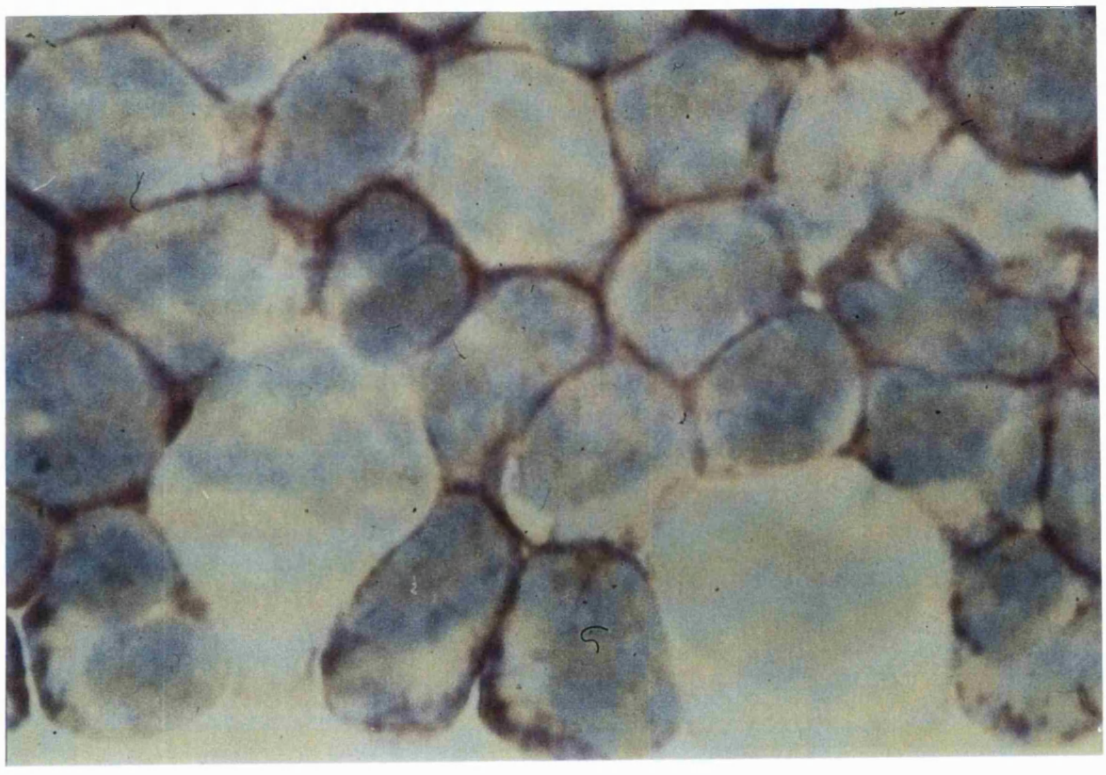


Figure 2.2 Blast-cell morphology of PB $CD34^+$ selected cells. $CD34^+$ cells were centrifuged and stained with alkaline phosphatase-antialkaline phosphatase immuno enzyme (APAAP).

2.3 PREPARATION OF ADHESIVE SURFACES.

Preparation of Hyaluronic acid coated culture surface: Tissue culture wells were pre-coated with 2 mg/ml HA in PBS pH 5.6 and left overnight at room temperature. The solution was removed the following morning and the wells washed twice with PBS and used immediately.

Preparation of Fibronectin (FN) coated culture surfaces: Tissue culture wells used in different experiments were coated with a solution of 0.1 mg/ml fibronectin (FN), and left for 1-2 hours, or longer depending on the number of times the Fn batch had been used. Fibronectin was aspirated off and the well surface left to dry.

Preparation of Heparin binding fragment peptide coated culture surfaces:
The synthetic peptide was made up in sterile water to 1 mg/ml and then diluted to the appropriate concentrations in PBS. Ovalbumin-coupled synthetic peptide was then adsorbed to culture vessel surface, by incubating overnight in incubator (37^0C/5% CO_2). Washing with PBS and 2% FCS was then done to remove non-adsorbed proteins whilst blocking non-specific sites.

2.4 CELL CULTURES

2.4.1 Preparation of stromal cells

Human bone marrow stromal cells were established from normal donors as follows. Mononuclear cells were separated from samples of BM (as outlined in section 2.2.1) and re-suspended at 10^6 cells/ml of Myeloid LTC medium in 25 cm^3 tissue culture flasks. The adherent stromal cells were Fed weekly till confluent, usually within 7 to 14 days.

Trypsinisation of the confluent stromal cells

Confluent stromal cells were passaged as follows. All non-adherent cells and growth media were removed from the flask and the adherent layer rinsed carefully twice with warm PBS. 2 ml of warm 0.25% trypsin /EDTA were added to the flask and incubated for 5 minutes at 37^0C, 500 µl of FCS were added to inactivate the trypsin (1/4 of the volume of trypsin). Gentle pipetting up and down across the bottom of the flask to recover any remaining adherent cells and to suspend and break up clumps of cells was done with a Pasteur pipette,.

The cells were then transferred to tubes and washed twice with IMDM containing 2% fcs at 1000rpm for 5-10 minutes and resuspended in LTC media and counted using tryphan blue (as diluent) to assess viability. The stromal cells were passaged up to four times before either being frozen in liquid nitrogen or used immediately in the appropriate assays.

Preparation of stromal feeders for use in LTC-IC assays

Stromal cells were used for LTC-IC assay at second passage or more. 96-well microtitre plates were seeded with 1.5 x 10^4 cells in 0.1 ml of LTC media per well. The seeded stromal cells grew to confluence in 2 to 3 days, and morphologically comprised mainly of fibroblast-like cells and adipocytes. Confluent stromal cell layers were then irradiated with a ^{137}Cs-gamma source with 1500 cGy at a dose of 3 to 4 Gy/min after which fresh media was added onto the cells. The stromal plates were then used within two to five days following irradiation.

Preparation of stromal monolayers for *in vitro* adhesion: Stromal cells for *in vitro* adhesion assay were used at third passage. 48-well plates were seeded with 2.5 x 10^4 cells in 0.2 ml LTC medium per well, and used when confluent (Stromal layers were not irradiated prior to being used in adhesion assays).

2.4.2 Culture of KG1a cell lines

Human haemopoietic cell line KG1a (undifferentiated myeloblasts), obtained from the ATCC (American type culture collection, Rockville, MD, USA) and cultured in RPMI medium supplemented with 10% fetal calf serum and antibiotics, at a concentration of 10^5/ml in 80 cm^2 flasks.

The growth characteristic was assessed using a growth curve (appendix one), cells were only used for studies during the exponential (log) growth phase as determined by cell counts.

2.4.3 Freezing and thawing of cells.

Cells were frozen using the cryopresevative agent, DMSO. Cells were pelleted by centrifugation (1600 RPM for 10 minutes for KG1a), and resuspended between 5 x 10^6 cells/ml and 2 x 10^7 cells/ml (depending on cell size), in appropriate media containing 20% FCS.

The cell suspension and the freezing mixture were placed in ice for 5 minutes (Typical freezing mixture consisted of culture medium containing; I). 10-20% serum; ii). 5-10% DMSO. The cell suspension was then slowly dripped onto the freezing medium mixing regularly. Approximately 1.5 ml aliquots were then dispensed into sterile vials and stored at -70^{0}C for up to 24 hours before transferring to liquid nitrogen (-196^{0}C). The efficacy was checked by doing viability test before freezing and after thawing

Vials containing cells were removed from the liquid nitrogen and thawed in a 37^{0}C water bath as rapidly as possible using gentle hand agitation. The vial was then sprayed with 70% alcohol to sterilise the outside and the contents of the vial transferred into a universal. A few drops of preheated HBSS (37^{0}C) with 10% FCS were dripped onto the cells before dispensing the rest of the media into the tube. Cells were then washed, resuspended, counted and viability checked before being seeded into culture.

2.4.4 HAEMOPOIETIC PROGENITOR CELL ASSAYS

Methylcellulose cultures.

Two assay methods for progenitor cell detection were employed in the studies

Assay for colony-forming unit cells

In this assay, cells were suspended in 10% IMDM at 10^6 cells/ml. Methylcellulose mixture containing growth factors IL-3, SCF, EPO, and GM-CSF all at 100 ng/ml was plated at a volume of 0.5 ml per well of 24 well plates followed by 0.05 ml of MNC suspension added to the wells in triplicates. The cell suspension was mixed with the MC by side ways tilting of the plates and then incubated for two weeks at 37^0C/ 5% CO_2. Colonies were then counted on day 14 and expressed as colony-forming unit cells.

Assay for colony-forming units-granulocytic and monocytic progeny

In this assay test cells were suspended in IMDM/10% FCS at 10^6 cells/ml.

A methylcellulose mixture containing the following growth factors, IL-3 (10 ng/ml); SCF (20 ng/ml); G-CSF (100 ng/ml); GM-CSF (1 ng/ml) was plated at volume 0.5 ml in wells of a 24 well plate followed by 0.05 ml of cell suspension added to each well in triplicates. The cells were dispensed into the MC mixed by side ways tilting of the plates, and each incubated for three weeks. Colonies formed by CFU-GM were scored after 7 (> 50 cells), 14 (> 200 cells) or 21 (> 500 cells) days of incubation with the progenitor output being the cumulative total of the three readings. Table 2.1 shows the optimum cell concentration, of various cell types, seeded into the methylcellulose mixture in order to set-up clonogenic progenitor assays.

Table 2.1 Optimal cell concentration of input cells in methylcellulose culture of different cell sources

Culture conditions	Cell concentration per well in culture			
		Umbilical cord blood		
	BMMNC	UCBMNC	UCBPC	PBPC
EPO/IL-3/SCF/GM-CSF	5×10^4	2×10^4	200	400
G-CSF/IL-3/SCF/GM-CSF	5×10^4	2×10^4	200	400

2.4.5 HAEMOPOIETIC STEM CELL ASSAYS

LTC-IC assay of nonadherent BMMN and CD34 cells by limiting dilution cultures

The LTC-IC assay was performed according to a modification of the technique developed by Sutherland et al, 1991.

Isolated bone marrow mononuclear cells (BMMNC) and peripheral blood derived progenitor cell (PBPC), prepared as outlined in section 2.2.2, were resuspended in Myeloid long-term culture medium (an enriched alpha medium) and then seeded on irradiated confluent stromal adherent monolayers. The cells were seeded at decreasing dilutions, between 5×10^3 and 10^5 cells per 0.1ml using 15-30 replicate wells per dilution step.

The plates were then cultured for 5 weeks at 37^0 C and fed thrice weekly by replacement of half of the medium with the same volume of fresh long-term media.

After 5 weeks the plates were centrifuged and the LTC-M media aspirated gently with a pipette. The wells were subsequently covered with 0.1ml Methylcellulose mixture with added growth factor combinations of erythropoietin (2u/ml), stem cell factor (10ng/ml), IL-3 (10ng/ml) and GM-CSF (10ng/ml).

Plates were then incubated for up to 3 weeks and scoring was performed by scanning the surface of each well at 100x magnification, using an inverted microscope.

A well was scored as positive if it contained at least one CFU-C identified as clusters adhering to and in the same plane as the stromal layer (Greenberger, 1979) the scoring was done between 14 - 21 days.

Delta assay of plastic-adherent PΔ progenitor cells in BMMN and CD34 cells using limiting dilution and/or single dilution cultures

Bone marrow mononuclear cells, and/or peripheral blood derived progenitor cells suspended in 15% alpha media, were seeded in 20 - 40 replicate wells of 96-well microtitre plates (100μl volume) at decreasing dilutions between 5×10^3 and 10^5 /well.

The plates were then incubated for 2 hours at 37^0C, after which nonadherent cells were completely removed by washing at least three times with serum free balanced salt solution. The medium was then replaced with Myeloid-LTC-media containing the growth factors; IL-3, 10 ng/ml; GM-CSF, 1 ng/ml; G-CSF, 100 ng/ml and stem cell factor, 20 ng/ml to which freshly made hydrocortisone, 10^{-6} M was added.

The cultures were incubated for 1 week and the nonadherent cells within the supernatant were harvested and plated in semi-solid assays. The contents of each individual well were plated into two 0.5 ml semi-solid MC cultures in 24-well plates for a total of 3 weeks. Colonies formed by CFU-GM were scored after 7 (> 50 cells), 14 (> 200 cells) or 21 (> 500 cells) days of incubation (Gordon et al, 1994).

Scoring/Frequency estimation

Poisson statistics indicate that the mean numbers of LTC-IC and/or PΔ cells per well is 1 at a dilution of 37% empty wells i.e. wells with no secondary production of CFU-C and/or CFU-GM. Therefore by determining the proportion of empty wells at each dilution, the dilution that would theoretically correspond to one LTC-IC/PΔ cell/well could be determined by interpolation and hence the LTC-IC /PΔ frequency in the initial cell population.

2.5 ADHESION ASSAYS

2.5.1 KG1a Cell adhesion.

^{51}Chromium Radiolabeled assay for KG1a cell adhesion to stromal monolayers and ECM substratum.

KG1a cell adhesion assays were set up as follows; KG1a cells suspended in RPMI supplemented with 10% FCS, were radiolabeled with ^{51}Cr at 1μCi/10^6 by incubating for 1 hour at 37^0 C and then washing three times with 2% HBSS.

The chromium labelled cells were then seeded at 2 x 10^5/200μl per well onto confluent second to fourth passage stromal monolayer and either Hyaluronic acid or fibronectin coated surfaces set-up in 48 well plates, and incubated for 1 hour at 37^0 C.

Nonadherent cells were removed by aspirating with a pasteur pipette after gently shaking plates. The bound cells were then lysed by adding 0.5 ml of the solubilising agent 1% NP-40 and the contents of each well then placed in radioactive tubes (LP4). The radioactivity of each lysate was measured with a gamma counter.

The percentage of bound cells was calculated from the difference between the mean counts per minute (Cpm) in monolayer/ECM-coated surface, and mean background released (blank) Cpm divided by the total Cpm and expressed as a percentage.

Adhesion of KG1a cells to stromal monolayers treated with hyaluronidase

Stromal monolayers were treated with Hyaluronidase by incubating the stroma with 200µl hyaluronidase at 4000 u/ml for 1 hour at 37C. The monolayers were then washed three times with HBSS/1% FCS and reinfused with fresh media before the adhesion assay.

2.5.2 Effect of anti-CD44 on the Adhesion of KG1a cells to stromal monolayers and ECM substratum.

Antibody blocking assays were carried out by pre-incubating KG1a cells with the anti-CD44 monoclonal antibody for 30 minutes at room temperature. Monoclonal antibody was used at a concentration of 20µg/ml, and the control isotype, IgG1, was used at 10 µg/ml. Hyaluronic acid blocking assays were performed by incubating KG1a cells with 0.2 mg/ml HA prior to the assay for 30 minutes at room temperature.

2.5.3 UCB mononuclear and $CD34^+$ cell adhesion to HA/Fn:

Mononuclear and CD34+ cells were washed three times in HBSS supplemented with 5% FCS and resuspended in 1ml IMDM/10% FCS. The cells were then placed in 12 or 24 well plates coated with either HA or Fn and incubated for 1hour at 37^0C to permit adhesion of cells.

The wells were then washed three times using 10% IMDM and the pooled nonadherent cells were then counted using a counting chamber.

Assay of UCB mononuclear and $CD34^+$ HA-nonadherent cell fractions for colony-forming unit cells (CFU-C)

The nonadherent cells collected from the adhesion assay were pelleted and resuspended before being pipetted into 2.5ml bijou's of methylcellulose containing IL-3, GM-CSF and SCF (10ng/ml each) and 40 U/ml EPO. The MC was then

dispensed so that 4 wells of a 24 well plate each contained a volume of 0.5 ml methylcellulose with a roughly equal number of cells.

In parallel, cells from the original suspension were directly placed in another bijou containing 2.5ml methylcellulose before being plated and incubated in order to measure the number of colony-forming unit cells in the original cell population.

Effect of anti-CD44 and anti-CD29 moAb on adhesion of UCB to HA and FN.

Monoclonal antibodies were added to the cell aliquots as follows: 10^5 cells were incubated for 20 minutes at 37^0C in 500µl of adhesion assay medium containing either 10µg/ml of anti-CD44 or anti-CD29 mAbs.

Control cells were incubated with 10µg/ml control IgG1 isotype, with the azide dialysed off using the slide-a-lyser method (see general methods) before the moAbs were used in cell cultures. The various cell aliquots were then added to both HA and FN-coated surfaces and incubated as above. Non-adherent cell populations were harvested by gently pipetting off medium from the wells. Efficacy of the wash was visually verified on an inverted microscope in order to ensure all nonadherent cells were removed. The cells were then counted and assessed for viability and subsequently grown in MC for progenitor assay analysis. After 14 days at 37^0C in humidified 5% CO_2 in air , colonies were scored according to the standard criteria all assays were performed in quadruplicates.

2.5.4 Adhesion of Peripheral blood (PB) $CD34^+$ selected cells to HA, Fn and Heparin binding peptide.

PB $CD34^+$ cells were washed three times in serum free HBSS and resuspended in IMDM/10% FCS. They were then placed in HA and Fn-coated wells and incubated for 1hour at 37^0C to allow for adhesion of cells to the coated wells. Plates were washed 3 times in adhesion assay medium and the pooled nonadherent cells were pelleted and resuspended in 1ml of IMDM/10% FCS before being counted.

Assay for CFU-GM in nonadherent cell fraction of PB $CD34^+$ haemopoietic progenitor cells.

The counted non-adherent cells were then dispensed into 2ml bijou's of methylcellulose (see table 2.1 for seeding concentration).

In parallel, cells from the original suspension were directly placed in another bijou containing methylcellulose and plated to measure the number of CFC and the different committed progenitors in the input cell suspension. All assays were performed in quadruplicates.

After 14 days at 37^0C in humidified 5% CO_2 in air, colonies were scored according to the standard criteria.

Effect of Anti-CD44 and Anti-CD29 mAb on the adhesion of PB $CD34^+$ cell to HA/Fn, and clonogenic features of $CD34^+$ HPC nonadherent to HA/Fn.

To analyse the effect of the above monoclonal antibodies on the adhesion of CD34+ cells to immobilised HA and Fn, 10^5 cells were incubated for 20 minutes at 4^0C in 500µl of cell adhesion medium containing 10µg/ml of anti-CD44 or anti-CD29 mAbs. Control cells were incubated with 5µg/ml IgG1, and cells were then processed for adhesion assay as described above.

To verify whether pre-incubation with the various mAb might alter the proliferation of clonogenic progenitors, an aliquot of antibody treated CD34+ cells was immediately seeded in the short-term methyl cellulose assay, without prior adhesion to the ligand surfaces.

2.6 STEM CELL ADHESION

2.6.1 Assay of Nonadherent BMMNC and CD34 cells for LTC-IC

Aliquots of input cells suspended at 10^6 cells/ml in alpha medium with 15% FCS, were incubated, on HA and/or Fn coated culture vessel surfaces (as described above), for 1 hour at 37^0C/5% CO_2.

Cell aliquots were pre-incubated either with or without antibodies against β_1, antibodies against the CD44 receptors or control IgG (1-mg/ml) for 30 minutes at 37^0C/5% CO_2. The antibodies were used at a concentration of 10μg/ml.

In addition, aliquots of cells were added to surfaces coated with the fibronectin fragment containing the heparin-binding domain of fibronectin, the peptide fragment used was coupled to ovalbumin.

Nonadherent cells were then removed by gentle washes with warm balanced salt solution, pooled and counted to determined percentage of cells adherent to each substratum.

The mononuclear cells were centrifuged and resuspended in myeloid LTC medium before use as input cells in the LTC system set up in limiting dilution cultures in order to enumerate LTC-IC frequencies of these various cell fractions and in plastic adherent PΔ progenitor cells in delta type cultures

2.6.2 Assay of Nonadherent BMMNC and $CD34^+$ cells for PΔ cells

Isolated bone marrow mononuclear cells suspended in 15% alpha mem at 10^6 cells/ml were added to various tissue culture vessels (25 cm^3 flasks or 6-12 well tissue culture plates) pre-coated with either HA or FN. Control flask containing no ligand coating were also included. The cells were incubated on the ligand coated surfaces for one hour at 37^0C/5% CO_2.

Nonadherent cells were removed by two gentle washes with warm serum free medium which was pooled and the cells counted to determine the percentage of cells adherent to each substratum. Cells were then washed twice with 2% HBSS resuspended, and then assessed for viability. The nonadherent cell fraction was suspended in myeloid LTC medium and then used to set up Delta assays of plastic adherent PΔ cells, either using limiting dilution or single dilution cultures.

Statistics. Results are expressed as Mean ± SEM. LTC-IC and PΔ stem cell frequencies were determined by either limiting dilution analysis (LDA) with Poisson statistics or using single dilution analysis (SDA) by taking the natural logarithm of the proportion of negative cultures and ignore the negative sign.

RESULTS 3 ESTIMATION OF FREQUENCIES OF HAEMOPOIETIC STEM CELLS USING LONG-TERM CULTURE-INITIATING CELLS AND PLASTIC-ADHERENT PΛ CELL ASSAYS

3.1 INTRODUCTION

In order to investigate the adhesive properties of haemopoietic stem cells, it is important to have access to an enriched population of these cells. The identification of human haemopoietic stem cells in different cell suspensions can be undertaken using various methods including in vitro assays.

These in vitro assay systems are based on the ability of these haemopoietic stem cell (HSC) to be maintained in long-term cultures for an extended period and to generate committed progenitor cells, which can be enumerated in semi-solid culture systems.

The long-term bone marrow culture system was originally devised from studies of murine haemopoiesis in vitro (Dexter et al, 1977) and has since been adapted for studies of human haemopoiesis (Gartner & Kaplan, 1980). These cultures have demonstrated the presence of cells capable of generating sustained haemopoiesis in vitro. Because LTBMC is a qualitative method, frequency analysis of stem cell subsets with presumed different proliferative ability is not feasible unless carried out in a limiting dilution set up.

The cells assayed in these limiting dilution cultures, referred to as long-term culture-initiating cells (LTC-IC), have been identified as the best available approximation of stem cells in humans (Sutherland et al, 1990). In addition these cells have also been shown in vitro to have several characteristics of quiescent cells including resistance to 5-fluorouracil.

Limiting dilution assays have thus been widely adopted and been applied in estimating the numbers of primitive haemopoietic cells that cannot be measured directly using clonogenic assays.

In this method quantitative measurement of the numbers of long-term culture-initiating cells is carried out by culturing haemopoietic cell suspensions at limiting dilution in 96-well micro-titre plates, pre-seeded with confluent stromal layers, for 5 weeks, after which the frequency of LTC-IC in a haemopoietic cell suspension is then calculated using Poisson statistics described later in the chapter.

Another method of stem cell quantification is the delta assay of plastic-adherent PΔ progenitor cell culture assay based on the finding that removal of plastic-adherent cells from murine bone marrow compromises its ability to repopulate the haemopoietic system when it is transplanted into irradiated recipients (Kerk et al 1985). Further observations were made which established the ability by primitive haemopoietic progenitors to adhere to tissue culture plastic (Gordon et al, 1988). These cells, the plastic-adherent haemopoietic progenitor cells have been evaluated by Gordon and co-workers.

These cells are detected in a delta type assay system in which haemopoietic cell suspensions are incubated with tissue culture plastic for 2 h and then washed free of non-adherent cells. The adherent cells are incubated with medium, serum and growth factors for 1 week and the numbers of CFU-GM produced are enumerated using a semi-solid clonogenic assay. These cells have been referred to as PΔ progenitor cells. The PΔ progenitor cells are capable of sustaining long-term haemopoiesis in vitro and produce non-adherent progeny (Gordon, 1994). Phenotypically, the PΔ progenitors express CD34 antigen and Thy-1 and are relatively resistant to treatment with 5-Fluoruracil (Gordon, 1987).

Overall this information suggests that the plastic-adherent fraction of bone marrow contain very primitive haemopoietic progenitor cells. The frequency of PΔ progenitor

cell population can be estimated, as in LTC-IC assays, by limiting dilution and the frequency calculated using Poisson statistics.

Preliminary studies were carried out to evaluate the above in vitro assays for the quantification of stem cells in bone marrow and peripheral blood derived progenitor cell suspensions and to optimise the use of such assays in studying the adhesive interactions of haemopoietic stem cells.

3.2 RESULTS:

3.2.1 Optimum conditions for LTC-IC assay in limiting dilution culture.

LTC-IC assays were established by co-culture of either ficoll separated bone marrow mononuclear cell fraction from normal donors of allogeneic bone marrow transplantation or peripheral blood-derived progenitor cells, isolated by apheresis method as described in general methods, onto an irradiated confluent stromal adherent layer which were set-up in 96-well plates.

For each limiting dilution assay at least four input mononuclear cell numbers (see general methods) were used with 25 - 40 replicates per each input cell number. Input cell numbers were scaled down by two logarithm when using $CD34^+$-enriched cells. After 5 weeks the plates were centrifuged, media aspirated and the wells filled with methycellulose. After a further incubation for 3 weeks, wells were scored as positive if they contained at least one CFU-C identified as clusters adhering to and in the same plane as the stromal layer (Greeberger, 1979).

The adherent stromal monolayers were set-up as described in general methods. The duration between initial passage to confluence ranged between 3 and 6 days depending on the seeding concentration of the trypsinised stromal cells.

The stromal layers were assessed morphologically before use in the coculture experiment with an inverted microscope. The stromal cell subtypes consisted mainly of homogenous fibroblast-like cells with isolated patches of adipocytes (Figures 3.1-3.3.) In all a total of ten LTC-IC assay experiments were set-up using ficoll-separated BMMNC and in the course of setting up these experiments several problems were experienced including:-

1. Frequent detachment of the adherent stromal layers in the coculture wells, many of which occurred during the second and third week of coculture incubation. The stromal detachment was more marked with stromal cells set-up in larger tissue culture vessels and,
2. Bacterial infection occurring in individual wells

Special modifications of the original procedure were therefore made, in an attempt to optimise the assay as well as to maintain the quality of the bone marrow stroma.

3.2.2 Stromal detachment and deterioration of quality of adherent layer

Experiments were undertaken to compare the maintenance of LTC-IC and the quality of the stromal adherent layer during the culturing period, by varying the frequency with which media exchange was done on the cultures during demi-depopulation.
Increasing the frequency of demi-depopulation from once weekly to every third day resulted in both improvement in quality of the pre-formed stromal layer as well as the overall assay outcome and yield of LTC-initiating cells with the quality of the pre-formed stromal layer remaining consistently good at more frequent media exchange intervals.

In addition there was less detachment of the stromal layers when the seeding concentration of the freshly trypsinised stromal cells was increased. Based on these

results, a feeding schedule of 50% medium exchange every third day was used in all future experiments

3.2.3 Conditions for maintenance of LTC-IC set-up in long-term cultures

Experiments were also carried out comparing LTC-IC maintenance using ready made Long-term culture media (M-LTC-M) from stem cell technology and media prepared in the laboratory (Vancouver media, see general materials). The LTC-IC assays set-up using ready made media resulted in frequencies well within those in other published works with noticeably better quality stroma on microscopic examination.

Six experiments were successfully cultured and scored after 8 weeks, while four experiments were aborted due to stromal detachment between the second and third week of culture. Of the remaining two experiments however, although successfully cultured for eight weeks, no colonies were detected and thus not scored. The reasons for this are not clear, but may relate again, to poor quality stroma. A clonogenic assay was set-up in parallel with every LTC-IC culture carried out and the clonogenic cells output is shown in Table 3.1.

Therefore special modifications of the original procedure were made to optimise the assay as detailed above.

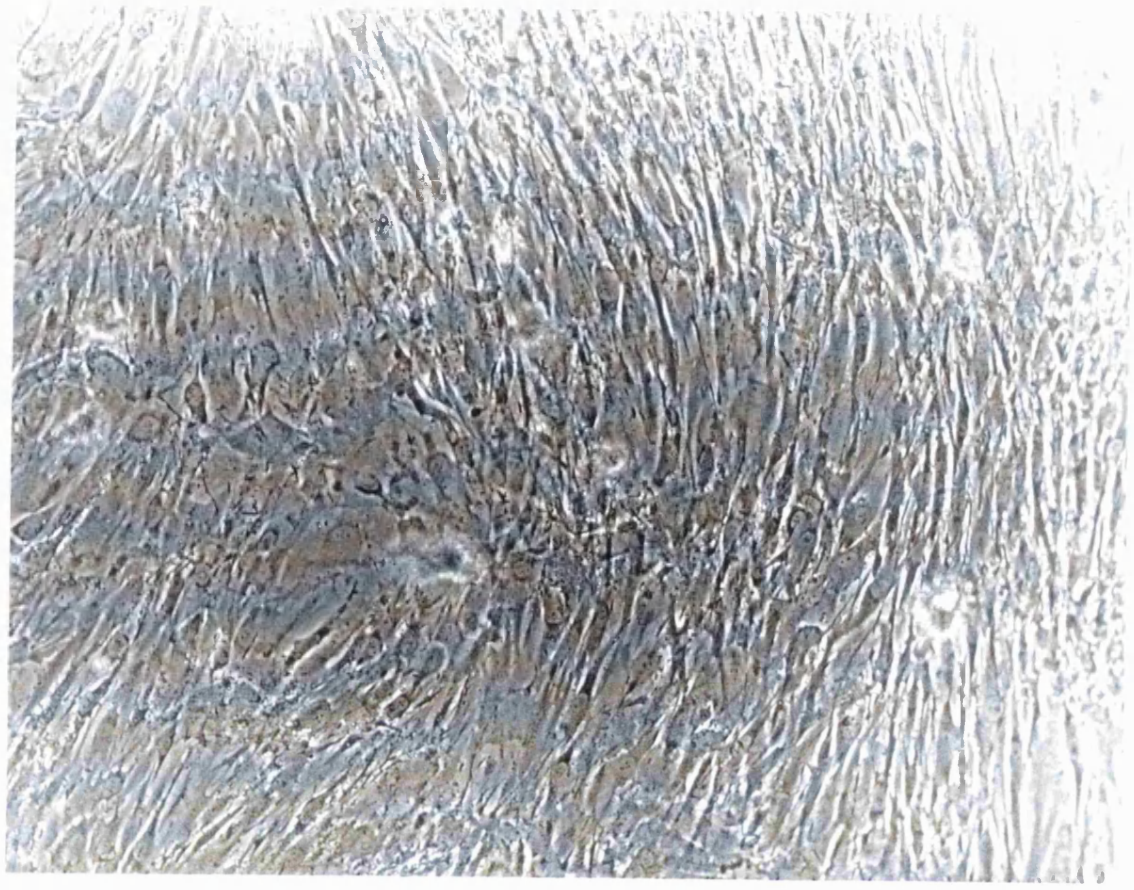


Figure 3.1 Morphology of fresh marrow stromal layer after 1500 cGy irradiation and before co-culture with mononuclear cells and peripheral blood derived progenitor cells. The confluent cultures of stromal monolayers were made from freshly trypsinised bone marrow stroma. Magnification x 400

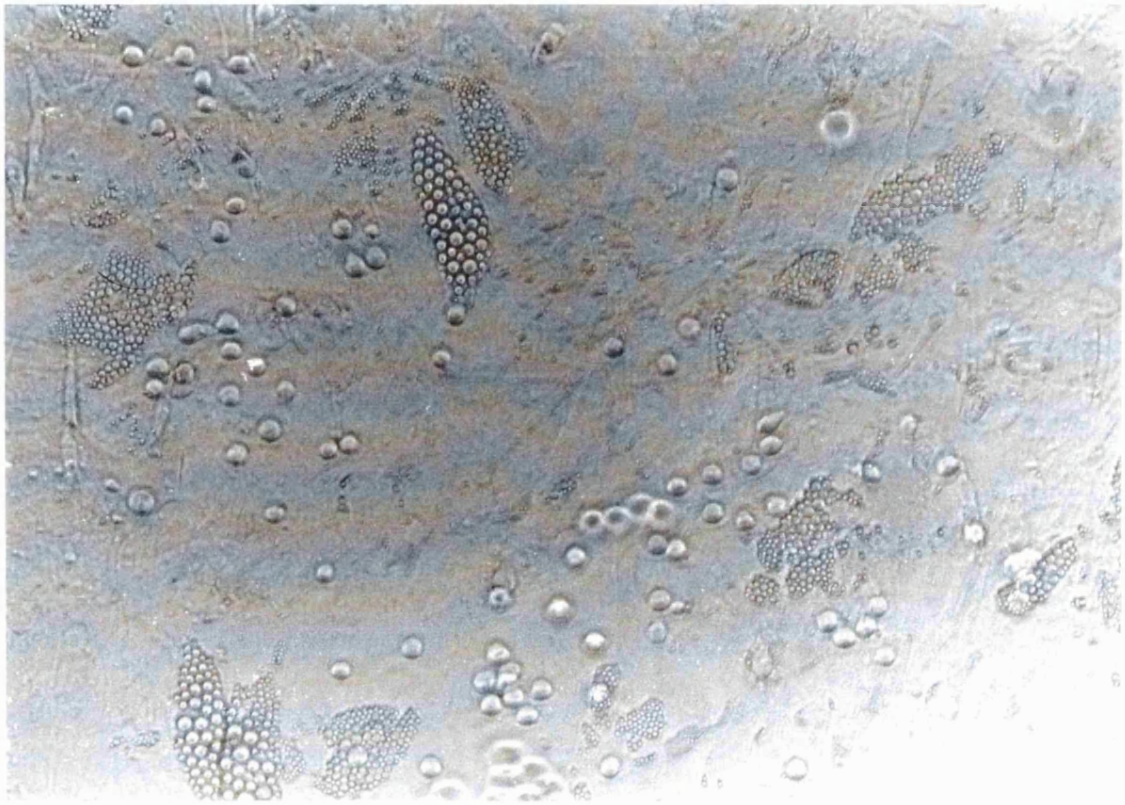


Figure 3.2 Micrograph of stromal cell morphology showing characteristic fibroblast-like cells and small islands of adipocytes. Bone marrow stromal cells were passaged three times before appearance of the adipocytes. Magnification x 400

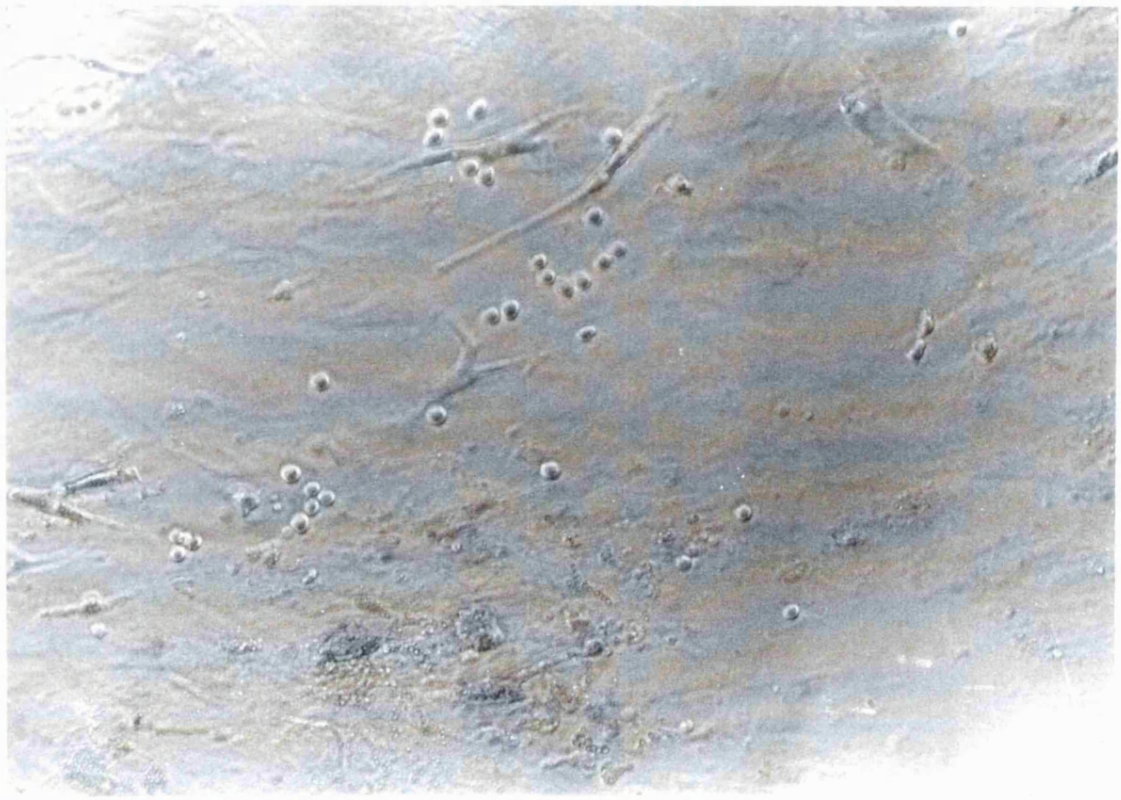


Figure 3.3 Co-culture of BMS with BM.MNC and peripheral blood cell derived progenitor cells. $CD34^+$ /BM.MNC were added to BMS then incubated for 5 weeks with weekly demi-depopulation. After 5 weeks nonadherent cells were replaced with methylcellulose mixture containing GM-CSF, IL-3, SCF and EPO. Magnification x 400

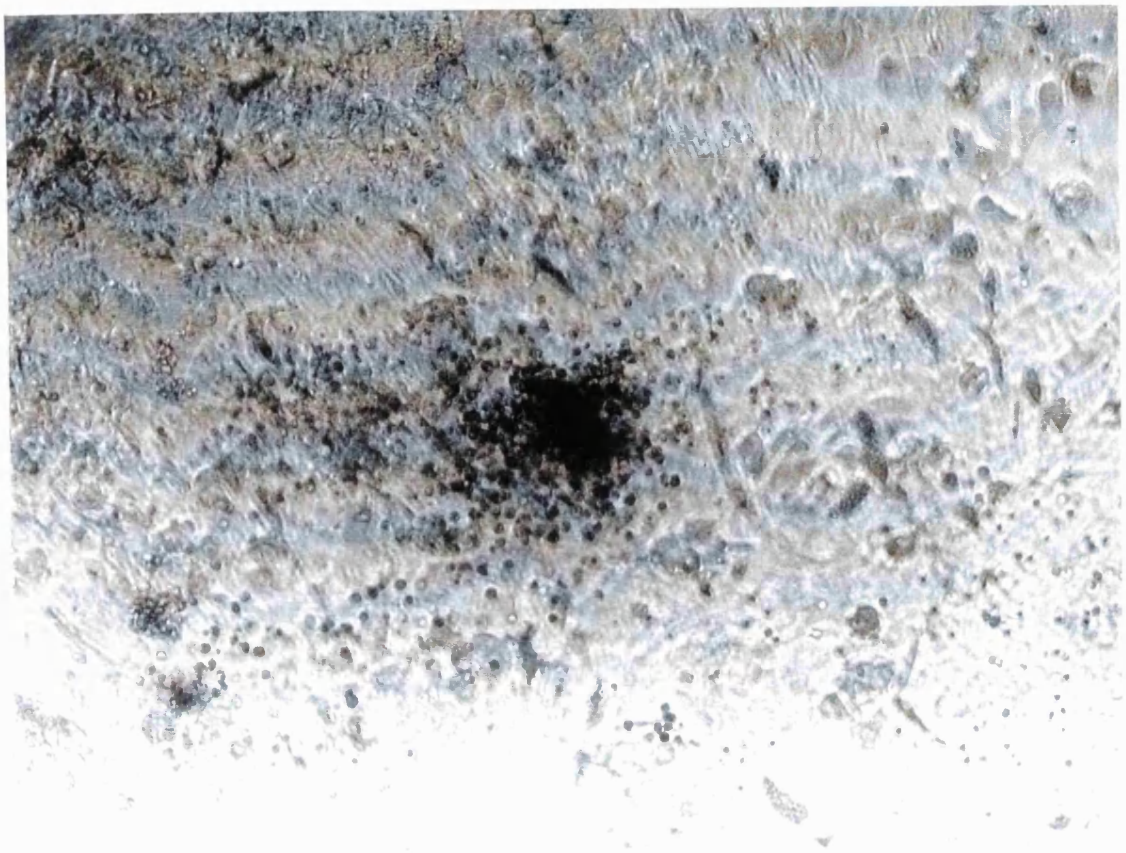


Figure 3.4 Photographs of colony forming unit cells observed after 21 days in culture following coculture of mononuclear and $CD34^+$ cells on confluent irradiated adherent marrow stromal monolayer for 5 weeks followed by addition of growth factor combination. Magnification x 400

3.2.4 Frequencies of LTC-IC in limiting dilution cultures

In the assays successfully cultured after more than 5 weeks in culture, the frequency of LTC-initiating cells in the original test cell suspension was calculated using Poisson statistics (Taswell, 1981). This was done by plotting the proportion of negative wells against the number of input cells on a semi-log graph and by interpolating the cell dilution that would result in 37% of wells containing no activity was then obtained, which is the proportion of empty wells to be expected from Poisson distribution when there is on average one progenitor cell per well (Table 3.2).

An example is given in Table 3.2, where the frequency of LTC-IC in the starting cell population (i.e. the reciprocal of the concentration of test cells that gave 37% negative cultures) was calculated at 1 per 3.7×10^4 cells.

The frequency of LTC-IC in the experiments initiated using ficoll separated bone marrow mononuclear cells was calculated and found to be in the range 1.3 to 2.7 per 10^5 cells (Table 3.3). This agrees with reported frequency of 2.5 - 10 per 10^5 cells (Sutherland , 1990).

Table 3.1. CFU-C Production by BM mononuclear cells. 50,000 BM.MNC from 5 different marrow samples were cultured in 24-well plates of methylcellulose mixture in the presence of growth factors, colonies were then counted after 14 days incubation.

Exp. No	Number of CFU-C colonies counted (mean ± se)
1	122.5 ± 8.6
2	145.5 ± 7.8
3	126.7 ± 14.8
4	84.2 ± 2.8
5	168.3 ± 5.2

Table 3.2. Limiting dilution assay for determination of LTC-IC frequency in unmanipulated Ficoll-separated BM.MNC: Data from a representative experiment in which decreasing number of ficoll separated BM.MNC were seeded onto irradiated marrow feeders on 96-well plates and the number of negative wells scored.

Replicates culture	input cells/well	number of wells	number of negative wells	prop of neg wells
1	4.8×10^4	28	7	.25
2	2.4×10^4	28	16	.57
3	1.2×10^4	28	23	.82
4	6.0×10^3	28	25	.89
5	3.0×10^3	28	28	1

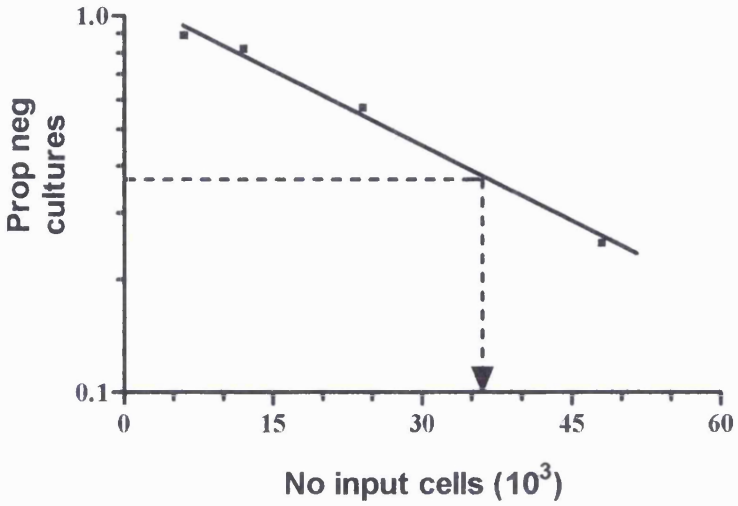


Figure 3.5. Limiting dilution analysis of data from one of the experiment. The percentage of negative wells was calculated after all individual wells were scored for the presence of haemopoietic colonies. The frequency of LTC-IC determined by extrapolation (i.e. the reciprocal of the concentration of test cells that gave 37% negative cultures).

Experiment	No of cells containing one LTC-IC	No of LTC-IC/10^5 cells
1. BM.MNC	7.8×10^4	1.3
2. BM.MNC	3.7×10^4	2.7
3. BM.MNC	3.8×10^4	2.6

Table 3.3 Frequency of Long-term culture-initiating cells in three samples of ficoll-separated bone marrow mononuclear cell populations. Number of LTC-IC per 10^5 were derived from the semi-log graph showing relationship between the proportion of negative wells and input cell concentrations

3.2.5 Frequencies of plastic adherent PΔ stem cells in BM.MNC and peripheral blood $CD34^+$

The LTC-IC assay suffers from several disadvantages in that it requires the use of primary bone marrow stromal cultures making it difficult for the assay to be standardised and also that the lengthy culture protocol takes at least 8 weeks.

The P delta assay was thus adopted for these studies to quantify stem cells in a mononuclear as well as $CD34^+$ cell suspensions as an alternative to the LTC-IC assay.

The advantages of the P delta assay as a method for stem cell quantitation are:

a. The assay only takes a total of 4 weeks (i.e. one week during which the plastic adherent cells in liquid culture are stimulated with the growth factors and three weeks when the harvested nonadherent cells are cultured in methycellulose)

b. Information about the length of maturation pathways within individual clones can be obtained, based on published observation that CFU-GM-derived colonies form at different times in culture (Gordon et al, 1994)

The assay involved incubation of isolated tissue culture plastic-adherent marrow cells in liquid culture for 1 week, and then harvesting of nonadherent cells for CFU-GM assay. The adherent progenitors are then quantitated using limiting dilution analysis and Poisson statistics as outlined in previous section and in single dilution experiments by simple calculation.

Thus as well as providing a measurement of progenitor frequency, limiting dilution analysis does provide a measure of the ability of individual progenitor cells to form CFU-GM progeny.

3.2.6 PΔ stem cells frequencies in BM.MNC

The numbers of CFU-GM produced in each well were counted with an inverted microscope in the limiting dilution experiments, with simultaneous scoring of each

wells for either presence or absence of colonies weekly over a three week duration, to allow for the variability in the time taken for the individual progenitors to produce progeny (Gordon et al, 1994).

The numbers of CFU-GM produced per well on days 7, 14 and 21 in the limiting dilution culture varied in different bone marrow samples with the numbers of CFU-GM counted being highest in the first week and the least numbers of CFU-GM recorded on day 21.

Table 3.4. Shows the distribution of CFU-GM produced in a representative limiting dilution experiment which was set up to determine the frequency of PΔ progenitor cells in a ficoll separated bone marrow mononuclear cell fraction.
There was variability in the numbers of CFU-GM produced per well with the total number of progeny produced being the sum of the three colony counts (figure 3.6).
There was also linearity between the numbers of CFU-GM produced and the numbers of mononuclear cells used to initiate the culture (figure 3.7).

The average number of progenitors per well and hence their frequency was obtained
a). for limiting dilution culture by limiting dilution analysis and Poisson statistics, figure 3.8 and,
b) in a single cell dilution experiments by obtaining the natural logarithm of the percentage of empty wells and ignoring the negative sign.

These elementary calculations have sufficiently demonstrated that a single dilution protocol consisting of a large enough number of replicates of the same number of cells is able to provide an estimate of progenitor cell frequency over as wide a range of progenitor cell concentrations as is likely to be met in practice.

No input cells	CFU-GM produced D7	D21	D14	Total No CFU-GM	Prop Neg wells
4.8×10^4	195	50	45	290	0.15
2.4×10^4	107	72	44	223	0.25
1.2×10^4	96	21	10	127	0.60
0.5×10^4	19	11	5	35	0.85

Table 3.4. Data on one representative delta assay of plastic-adherent PΔ progenitor cell experiment in limiting dilution cultures The frequency of PΔ progenitor cells in the initial MNC suspension calculated using Poisson statistics was 4.5 per 10^5 cells.

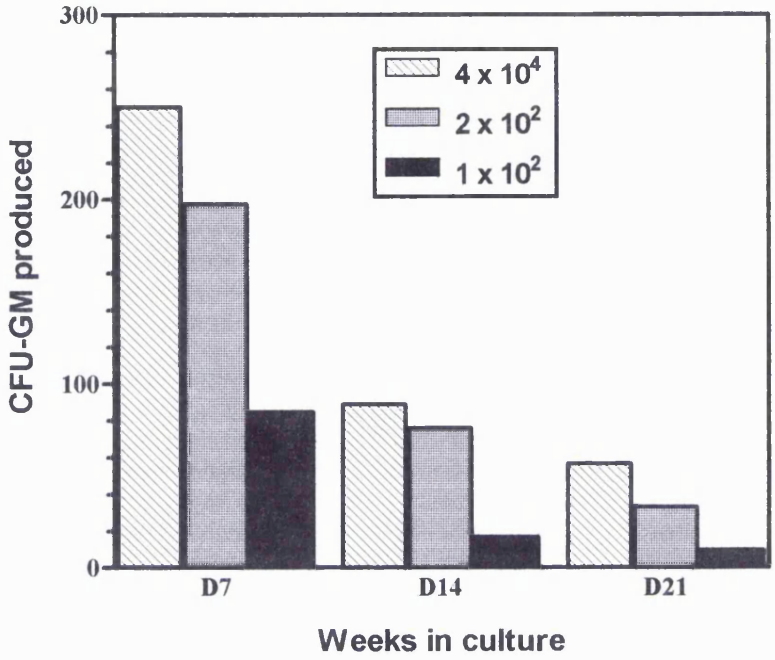


Figure 3.6 Numbers of CFU-GM produced in a limiting dilution experiment set up to determine the frequency of adherent PΔ progenitor cell (representative of three experiments). CFU-GM colonies were scored on days 7, 14 and 21 in an assay set-up using 3 cell concentrations. The number of CFU-GM produced are also linearly related to the input cell concentration.

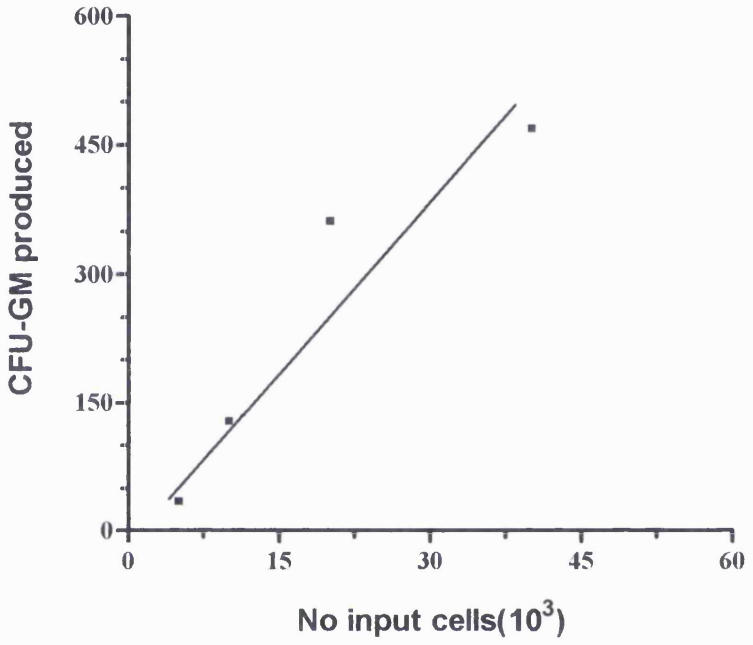


Figure 3.7 CFU-GM produced in adherent PΔ assay. showing linearity between the number of CFU-GM produced by plastic-adherent MNC and the number of input cells, after increasing numbers of MNC were plated in methylcellulose mixture containing growth factors SCF, IL-3, GM-CSF, G-CSF

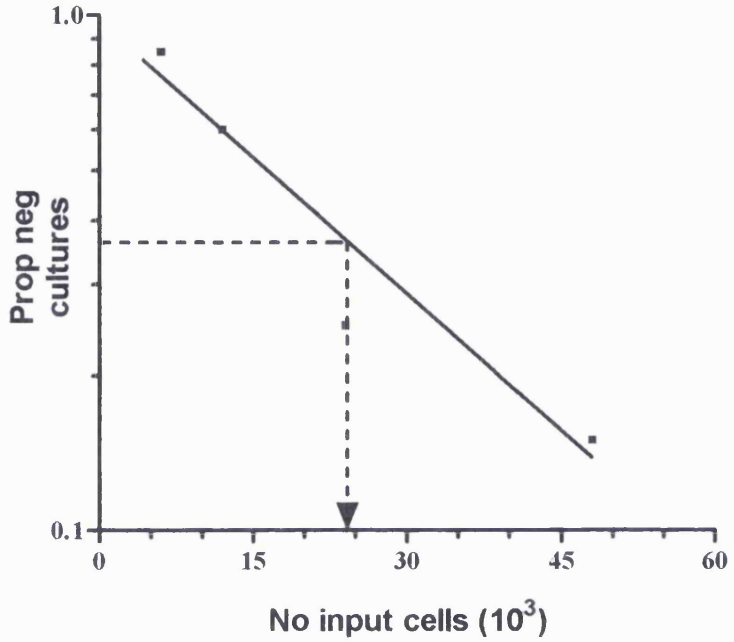


Figure 3.8 Limiting dilution analysis data from a delta assay experiment in which decreasing numbers of plastic adherent mononuclear cells were incubated in liquid cultures for 1 week, nonadherent cells then harvested and set-up in CFU-GM assay. The frequency of the PΔ cell in this experiment is the reciprocal of the concentration of test cells that gave 37% negative cultures.

Table 3.5 and 3.6 show the results for a protocol of three to four dilutions with between 24 - 40 replicates per dilution and a single dilution with 48 - 50 replicates respectively. The frequencies of adherent PΔ progenitors in the two respective protocols as determined by Poisson statistics and calculations outlined above ranged between 2.6 and 7.7 and 2.0 - 3.5 per 10^5 cells (mean for LDA of 5.3 ± 2.2 per 10^5).

There was no significant difference between the means of PΔ progenitor frequency obtained in multiple limiting dilution protocols and that obtained in single dilution protocols ($p=0.1766$).

These experiments show, as has been shown before that the use of single dilution instead of multiple dilution cultures does produce accurate estimates of the frequency of adherent PΔ progenitor population in a given cell fraction (Blackett et al, 1996).
The single dilution protocols are advantageous as they avoid the problems concerned with statistical fitting of the results of multiple dilution protocols when some of the dilutions have all positive or all negative wells.

However it is still necessary to ascertain that the proportion of empty wells is linearly related to the number of cells per well.
In the delta assays experiments carried out the average number of CFU-GM progenies produced by individual primitive progenitor cells was obtained from the estimates of the average number of progeny (CFU-GM) per well and the average number of progeny per well.

Table 3.5 Frequencies of PΔ progenitor cells in limiting dilution cultures

Frequencies of adherent PΔ progenitor cells four different samples of normal human bone marrow mononuclear cells

Exp No	Cell no/ PΔ cells	PΔ cell/10^5
1	1.3×10^4	7.7
2	2.2×10^4	4.5
3	3.6×10^4	2.8
4	1.6×10^4	6.2
5	2.6×10^4	3.8

Table 3.6 Frequency of PΔ progenitor cells in single dilution cultures, showing data from three different adult normal bone marrow samples. The frequency of PΔ progenitor cells was calculated from the natural log of the proportion of negative wells and ignoring the negative sign

Exp. No	input cell	prop neg wells	log neg wells	PΔ/10^5
1	2×10^4	0.67	0.4	2.03
2	2×10^4	0.5	0.693	3.5
3	2×10^4	0.68	0.39	2.0

3.2.7 PΔ stem cells frequencies in PB $CD34^+$ Selected cells

The assays for adherent PΔ progenitor cells in $CD34^+$ cells fraction was set up as for BMMNC in both limiting and single dilution cultures.

Input cells used to initiate the delta assay was scaled down by two logarithms. In the assay of $CD34^+$ to estimate the frequency of plastic-adherent PΔ cells, single dilution was used in all experiments, having ascertained that the proportion of negative wells is linearly related to the numbers of cells per well.

The data generated from the single dilution protocol experiment are shown in table 3.7 and 3.8. The average numbers of CFU-GM progeny produced in 5 experiments was 62 ± 7.7. Analysis of five experiment yielded an average adherent PΔ progenitor frequency of 360 ± 150 per 10^5 cells. The frequency of adherent PΔ progenitor calculated in this study for $CD34^+$ is in agreement with what has been previously reported in studies of Gordon et al using similar fraction of $CD34^+$ cells.

Table 3.7 Experimental data on assay of adherent PΔ progenitor in different $CD34^+$ cell fractions. CFU-GM production were generated following assay of 500 $CD34^+$ cells in methylcellulose culture using single dilution protocol with varying numbers of replicate wells in the different experiments.

Data analysis of single dilution cultures

Exp NO	1	2	3	4	5
Number of culture wells	48	48	48	48	48
Cumulative number of CFU-GM produced/10^5 cells	8400	4800	4500	7100	4600
Cumulative number of negative cultures	39	32	32	16	30
Proportion of negative cultures	0.81	0.67	0.80	0.67	0.75
PΔ frequency/10^5 cells	200	400	220	400	300
Number of CFU-GM produced / PΔ cell	42	12	20.5	17.8	15.3

3.3 DISCUSSION

In this study two quantitative assays for primitive haemopoietic cells have been evaluated in inoculums of human bone marrow as well as peripheral blood cell suspension. The first of these assays involved assaying of cells for clonogenic progenitors after a 5 weeks incubation in an LTC-culture. These clonogenic progenitors present in 5 week old LTC-culture assays and with the capacity to repopulate are referred to as LTC- initiating cells.

As has been stated before, the LTC-IC assay allows the quantitation of very primitive haemopoietic cells by virtue of their ability to generate myeloid colony-forming cells (CFC) for at least 5 weeks when cultured with certain fibroblast-containing stromal feeder layers.

The quantitation of LTC-IC was thus undertaken by measurement of the total number (both adherent and nonadherent) of the clonogenic progenitors present after a culture period of 5 weeks. This was approached by using limiting dilution analysis techniques. Weekly assay of cell suspension in the demi-depopulated cell fraction showed that clonogenic cell numbers were undetectable after 4 weeks of incubation and thus cells assayed after 5 weeks duration of the LTC-culture are cells which in addition to being primitive in nature, also have the capacity to repopulate following stimulation with a combination of growth factors.

This approach was validated by the demonstration of a linear relationship between the end point measured (clonogenic progenitor cell output as assessed after 5 weeks of culture) and the number of LTC-IC in the original cell suspension down to limiting numbers of LTC-IC. Linearity of the LTC-IC assay, even at low input cell numbers has made it possible to use limiting dilution analysis techniques to quantify absolute LTC-IC frequency, in cell suspension from various sources, before and after purification, and before and after maintenance in culture under various conditions.

In the assay, after 5 weeks in culture, the proportion of LTC-IC in three Ficoll-separated marrow mononuclear cell samples was approximately $1:5.1 \times 10^4$ cells.

Taking into account sample variability and the modification of the experimental design, the results are comparable to those obtained in similar studies (sutherland 1990).

Culture performance of LTC-IC assay were found to be dependent on a number of factors, involving modifications to the original assay.
Some of these modifications included increasing the rate of medium exchange.
The frequency of medium exchange was increased to every third day in all the assays which resulted in positive LTC-IC cultures in addition to improving the quality of adherent stromal monolayer.

The second stem cell assay which involved quantitation of adherent PΔ progenitor cells does offer a useful alternative method. The plastic adherent PΔ progenitor cells are primitive, multipotent cells which are easily isolated and detected through their production of clonogenic progeny (CFU-GM) in vitro. The isolation method employed, plastic adherence, does have advantages over the long-term culture method in that the other progenitor cell compartment i.e. CFU-GM, BFU-E etc. pre-existing are removed (Gordon, 1994) at the beginning of the culture period and thus removing the need for prolonged incubation which is aimed at allowing all progenitors present in the assay system mature into non-colony-forming cells.

Estimated frequencies of adherent PΔ progenitor cells in both limiting dilution analysis (LDA) and single dilution analysis (SDA) protocols yielded similar results and thus either protocol will be used in experiments investigating the adhesive interactions of the cells with specific ligands (chapter 5).

4. RESULTS: ADHESION OF KG1A CELLS TO FIBRONECTIN AND HYALURONIC ACID AND ROLE OF CD44

4.1 INTRODUCTION

The difficulties associated with isolation, identification and assay of haemopoietic stem cells (HSC) makes functional studies of these cells even harder. Moreover the numbers of HSC isolated in cell suspensions of bone marrow, peripheral blood and umbilical cord blood are very low, further compounding the difficulties associated with studies of these cells. Numerous studies have been undertaken to outline the adhesion pathway mediated by the interactions of HSC with stromal elements.

Since development of myeloid cells occurs in close proximity with various extracellular matrix molecules produced by stromal cells, studies of the interaction between various cell adhesion molecules present on HSC with extracellular matrix (ECM) molecules helps in elucidating the different adhesion pathways and their roles in haemopoietic processes.

CD44, one of these adhesion molecules, plays a role in adhesion of lymphoid cells to the bone marrow stroma through its binding to the ECM protein, hyaluronic acid (HA), which is abundantly produced by the stromal cells is one of these ECM proteins (Morimoto et al, 1994).

The KG1a cell line, derived from the parent human AML cell line known as KG-1 presents an interesting model of myeloid primitive cells, as their differentiation is arrested at the myeloblast stage of myelopoiesis. The mechanisms by which KG-1a emerged from the parent line is not clear. The cells are growth factor independent (Koeffler et al, 1980).

They are a homogenous population of primitive myeloid cells with characteristics that are found in haemopoietic stem cells such as expression of the CD34 antigen as well as other antigens such as HLA-DR and low CD38 antigen (Koeffler, 1980). They have

also been found to highly express the CD44 receptor (Morimoto et al, 1994). Other adhesion molecules expressed on KG1a cells include CD54, CD49d, CD49e, CD11a and CD31 (Turner et al, 1998). They thus form an interesting model for studying the role of CD44 in mediating HPC binding.

Preliminary studies were carried out to optimise the use of KG1a cells in an adhesion assay which could then be used to investigate the CD44-HA adhesion pathway.

4.2 RESULTS

4.2.1 Optimum conditions for assay of KG1a adhesion

In these assays ^{51}chromium-labelled KG1a myeloid cells were allowed to bind to wells previously coated with fibronectin, hyaluronic acid or to a confluent stromal monolayer for 1 hour at 37^0C. Nonadherent cells were then aspirated, and after three rinses adherent cells and adhesive substrates were lysed and the specific binding was then measured by counting the radioactivity of each lysate in the gamma counter.

To determine the optimum seeding concentration, aliquots of KG1a cells ranging from 5×10^4 and 8×10^5 were seeded onto confluent stroma in a 48 well plate. The percentage adherence peaked at 2×10^5 cells (Fig 4.1). The number 2×10^5 cells/well was thus adopted for standard use because this number of KG1a cells bound to either stroma monolayers or HA and Fibronectin coated surface was maximal at this cell concentration and could easily be visualised by light microscopy when the plates were examined.

A time course study of adherence of KG1a cells to surface monolayer was set-up by incubating the cells for different times ranging from 30 minutes to 2 hours. Adhesion of KG1a cells reached a plateau after an hour (table 4.1).

Preliminary experiments were also done to study the effect of different serum concentrations on the adhesion of KG1a cells. In three experiments carried out there

was no demonstrable difference in the adhesion of KG1a to the stromal monolayer with varying serum concentration used in the assay (figure 4.2).

Finally the concentration of HA used to coat plastic surfaces was optimised. Adhesion of KG1a cells was found to be maximal at 2 mg/ml of HA or greater. This concentration was thus used in all subsequent assays experiments.

Table 4.1 Data from an experiment of the time course for adhesion of KG1a cells to stromal monolayer following addition of 2 x 10^5 cells per well and incubating for an hour at 37^0C

Length of co-culture	% adherence
30	38.2
60	63.8
90	66.7
120	64.7

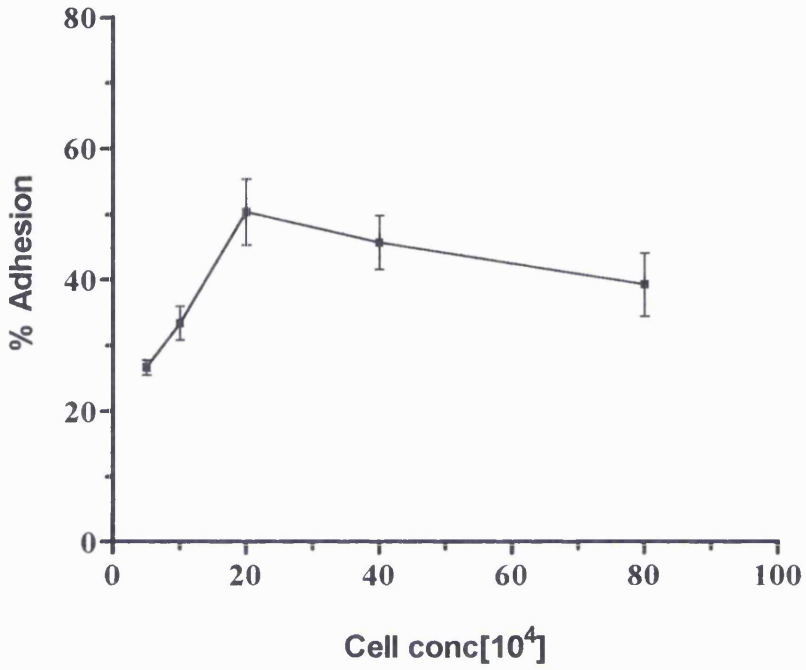


Figure 4.1 Graph showing adhesion of KG1a cells to stromal monolayer. The cells were seeded at increasing cell concentration ranging from 5 x 10^4 and 8 x 10^5 in 48-well plates containing confluent stromal monolayer, and adherent cells were then lysed and counted in a gamma counter, results are mean ± SEM of three experiments.

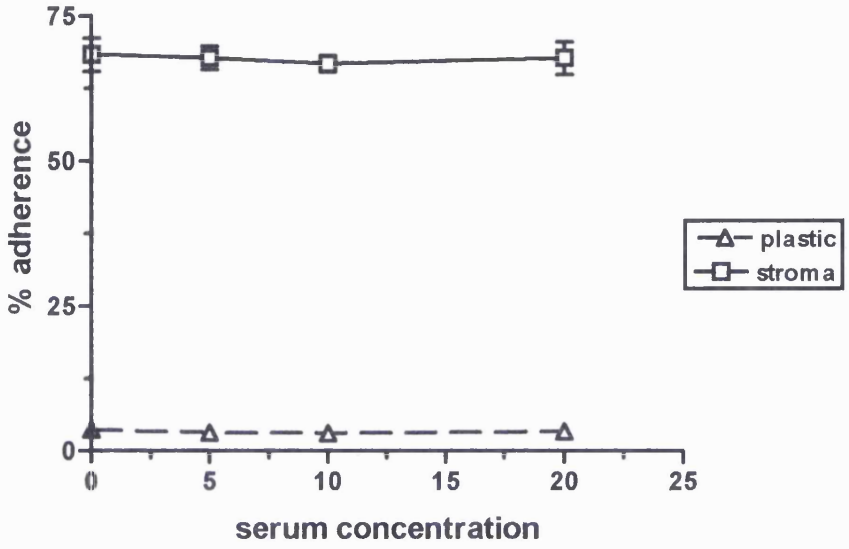


Figure 4.2 Graph showing the percentage adherence of KG1a cells to stroma and plastic using cell aliquots containing varying serum concentrations in the assay medium. Results are of adherence of KG1a cells adherence to stromal monolayer and plastic in 3 experiments (mean ± SEM)

4.2.2 KG1a cell adhesion to stromal and endothelial monolayers

To assess the adhesion of these cells to stroma, KG1a cells were incubated on bone marrow stromal and endothelial monolayers (BMEC), the adherent stromal monolayer was prepared as described in general methods. The adherent layers were used in adhesion assays only after more than three passages and after incubation for 2-5 days to allow formation of confluent monolayers. BMEC in cultures of tissue culture flasks were obtained from Dr K Yong and re-passaged into 48-well plates before being used for adhesion assays.

The percentage adherence to each monolayer was determined as described above. There was marked adherence to bone marrow stroma in three experiments (60.5 ±

6.2%), Figure 4.2. A higher percentage adherence was also noted in two experiment where BMEC was used as the monolayer (66% and 69%), compared with plastic surfaces in which the binding of these cells was significantly low.

4.2.3 KG1a cell adhesion to HA and Fn

The adhesion of KG1a cell to HA and /or Fn coated surfaces was determined as described above. Surface coating of tissue culture plastic surfaces was done as described in general methods.

There was significant binding of KG1a cell to hyaluronic acid coated surfaces(20.6 ± 3.9 %, n = 7) as well as fibronectin coated surfaces (44.4 ± 11.8%, n = 4) compared to plastic surface (4.7 ± 1.1%), with $P<0.05$ in both cases. The adherence to hyaluronic acid occurred in a dose dependent manner (fig 4.4) as determined in preceding dose response experiments. The percentage adhesion to both HA and Fn was comparable to previously reported studies (Morimoto, 1994).

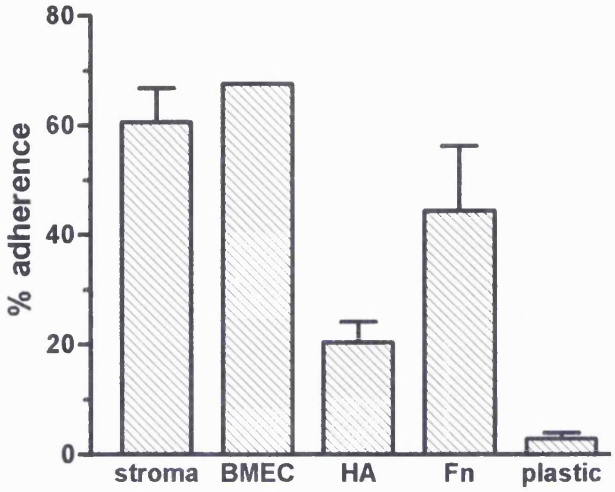


Figure 4.3 Adhesion of KG1a cells to various surfaces and monolayers. KG1a cells were incubated on stromal, BMEC, ECM substratum and plastic for one hour at 37^0C and the percentage bound cells then determined as outlined in general methods. stromal adhesion, n = 5; BMEC, n = 2; HA, n = 7; Fn, n = 4; Plastic, n = 4.

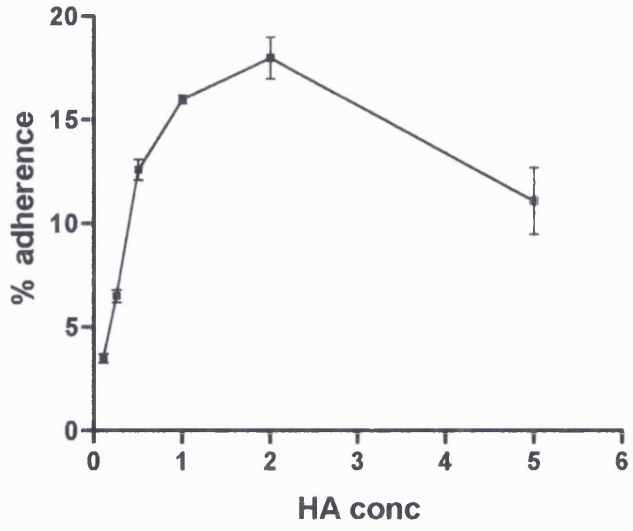


Figure 4.4 Dose response effect of KG1a cell binding to HA. Increasing concentration of HA were coated onto wells of 48-well plates. Adhesion assays of the cells were then carried out. Results are mean ± SEM of three experiments

4.2.4 Enzymatic treatment of stromal monolayer does not reduce the adhesion of KG1a cells

To investigate the effect of enzymatic treatment of stroma on KG1a, stromal monolayers were treated with the enzyme hyaluronidase prior to adhesion assay with KG1a cells. The hyaluronidase treatment was performed by incubating the stromal monolayers with 4000u/ml hyaluronidase for an hour at 37^0 C. The monolayer was then washed before use in the adhesion assay.

Parallel experiments were done by treatment of HA-pre-coated wells with the same concentration of hyaluronidase in order to assess the effectiveness of enzymatic digestion before measuring the binding of KG1a.

There was no effect on the adhesion of KG1a cells to stromal monolayers following treatment with hyaluronidase treatment (figure 4.5). In all experiments parallel assays in which HA-coated surfaces were similarly treated with the enzyme prior to adhesion resulted in reduced adhesion (figure 4.6).

4.2.5 Excess soluble HA partially blocked KG1a cell adhesion

The role of the hyaluronic acid binding site in KG1a cell adhesion was investigated by pre-incubation of the cells with excess solubilised HA before the adhesion assay. As shown in figure 4.7 addition of excess soluble HA inhibited the adhesion of KG1a cells to HA coated surface but not to confluent stromal monolayer. This effect was achieved both when the hyaluronic acid was dissolved in either a condensing agent 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimine or in phosphate buffered saline dissolved at very low concentration of 0.2 mg/ml.

There was however no effect of soluble HA on adhesion to stromal monolayers.

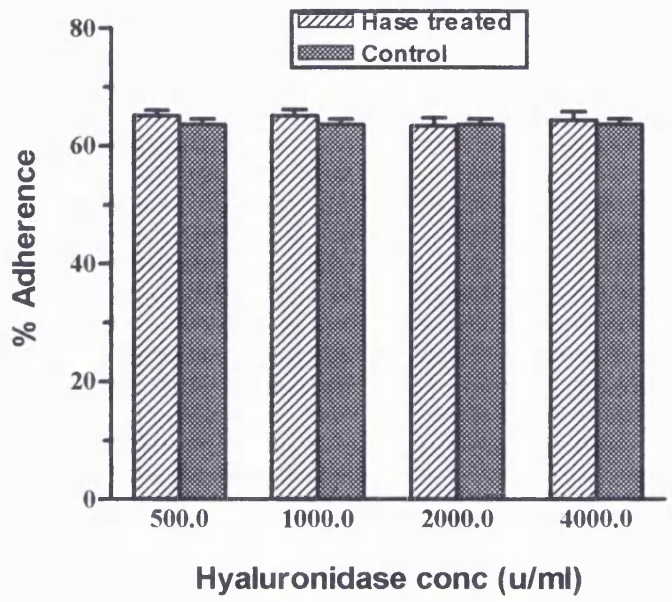


Figure 4.5 Effect of enzymatic treatment on adhesion of KG1a cells to stromal monolayers. Confluent stromal monolayers were treated with varying concentrations of Hyaluronidase prior to adhesion assay, KG1a cells were then added to treated wells and incubated for one hour. Results are mean ± SEM of three experiments.

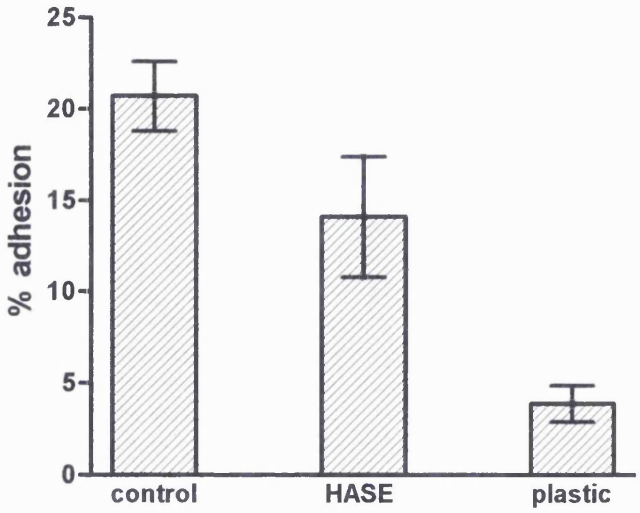


Figure 4.6 Effect of hyaluronidase on the adhesion of KG1a cells to HA. HA-coated wells were incubated for 1 hour at 37^0C with 2000u/ml hyaluronidase prior to the addition ^{51}Cr-labelled KG1a cells. The adherent cells were then lysed and counted. Data are expressed as mean ± SD of percent adhesion from three separate experiments, P>0.05.

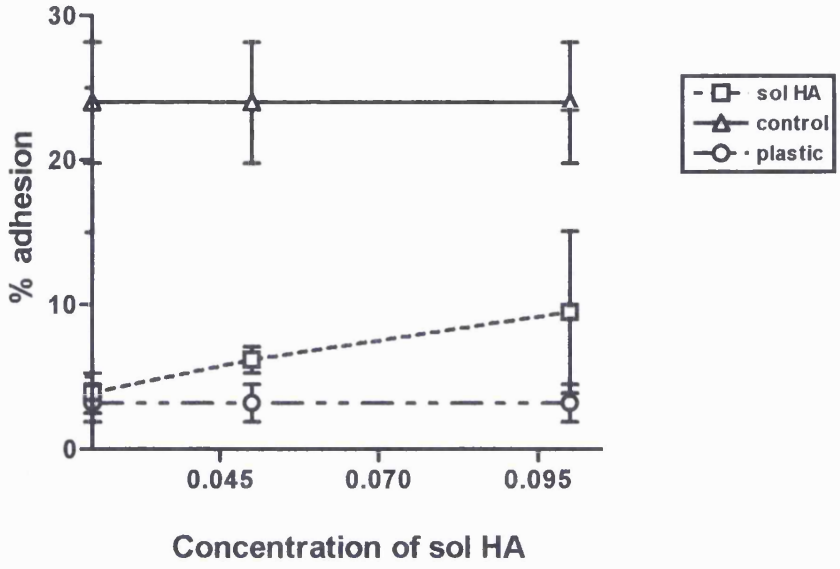


Figure 4.7 : Effect of excess soluble HA on adhesion of KG1a to HA. KG1a cell were treated with soluble HA at 37^0C for 1 hour. Cells were then cultured on HA surface, nonadherent cells were removed and adherent cells lysed and counted using a gamma counter. Results presented as mean ± SEM of three experiments.

4.2.6 Effect of Anti-CD44 monoclonal antibody on the adhesion of KG1a cells to coated surfaces and stromal monolayers.

^{51}Cr labelled KG1a cells were incubated with the Anti-CD44 moAb (BU52) for 30 minutes at room temperature before being added to HA-coated wells.

Dose response experiments demonstrated that the antibody produced maximal inhibition at 20 μg/ml, this dose was thus used in all subsequent experiments (fig 4.8).

In two experiments anti-CD44 (BU52) antibody blocked the adhesion of KG1a cells to HA. The antibody had however no effect on the binding of KG1a cells surfaces coated with fibronectin as well as plastic (figure 4.9).

In contrast, this same moAb has no effect on adhesion of KG1a cells to the stromal monolayer.

4.2.7 Adhesion to Heparin binding fragment peptide of fibronectin

Experiments to evaluate the adhesion of KG1a cells to the Heparin binding fragment peptide of fibronectin (CS1) were also included in this study. Synthetic peptide substrata was prepared by making up the peptides in sterile water to 1 mg/ml and then coupling to the ovalbumin. Having been diluted to the appropriate concentrations in PBS the ovalbumin-coupled synthetic peptides were then adsorbed onto wells of 48-well plate, by incubating wells overnight in a humidified oven at 37^0C. Nonadsorbed proteins were removed and non specific sites blocked with PBS and 2% FCS. KG1a cells were then added to the well and percentage adhesion assessed as before.

As shown in figure 4.10 the background adhesion of the KG1a cells to the peptide was low in two experiments, as a result further experiments to evaluate this adhesion pathway were not pursued any further.

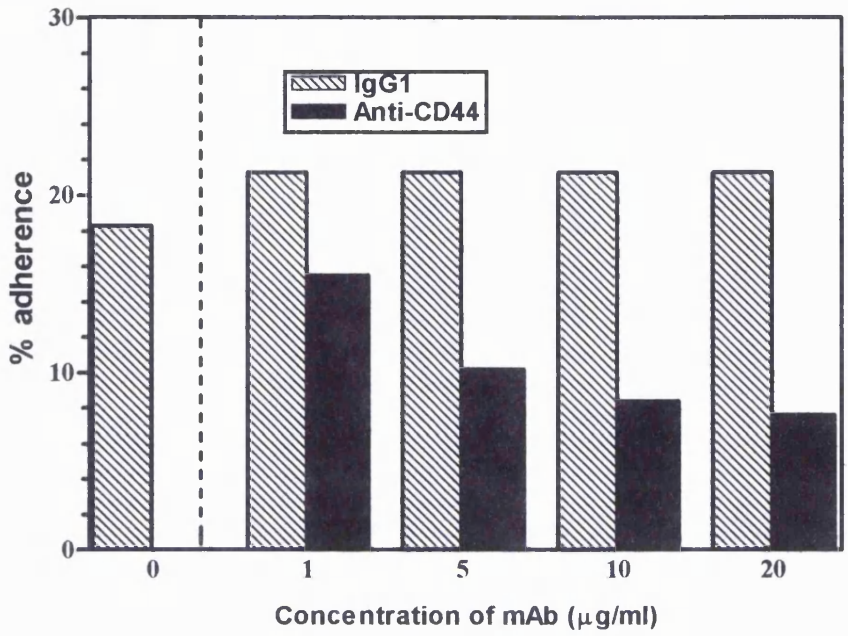
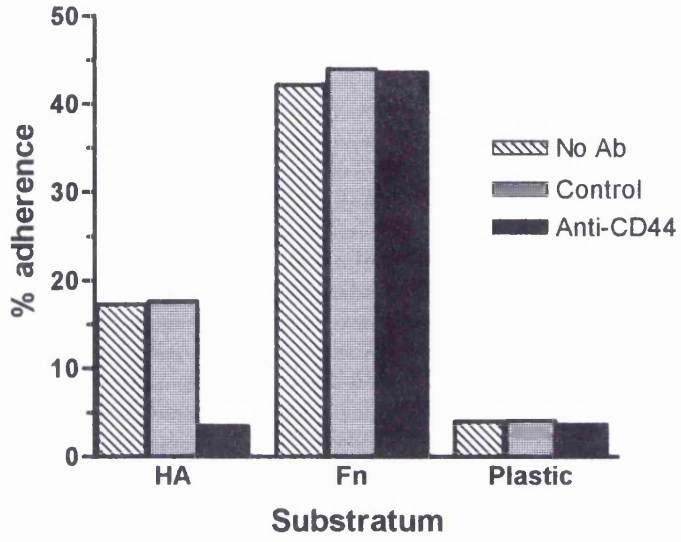


Figure 4.8 Effect of Anti-CD44 antibody treatment on adhesion of KG1a. Data (Mean of duplicates) from one experiment in which KG1a cells were pre-incubated with 1 to 20 μg/ml anti-CD44 or control antibody for 30 minutes at 37^0C prior to the adhesion assay. Nonadherent cells were then removed and adherent cells lysed and measured.

A. Exp. 1



B. Exp. 2

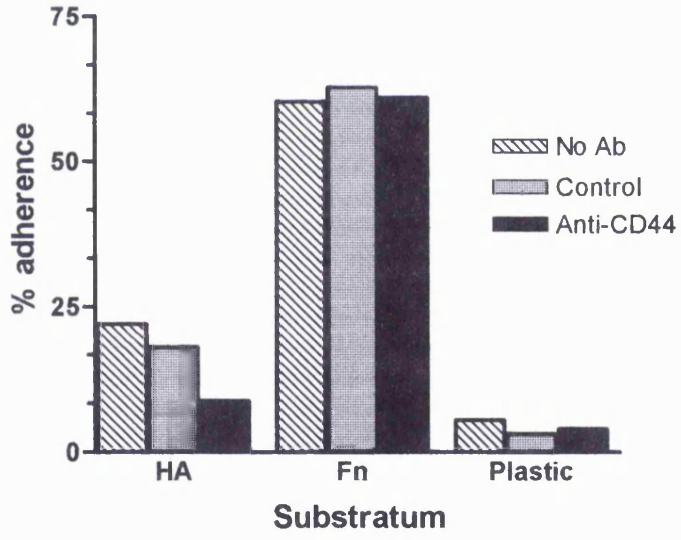


Figure 4.9 Effect of anti-CD44 moAb, BU52 on adhesion of KG1a cells. Graph shows results of two experiments in which ^{51}Cr-labelled cells were pre-incubated with the antibody or its isotype control, and then further incubated on HA, Fn and plastic surfaces for an hour.

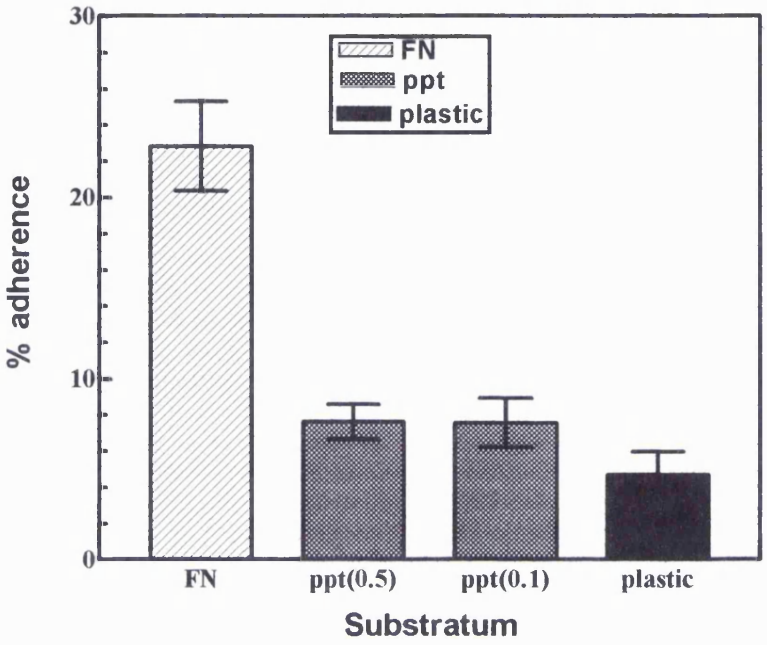


Figure 4.10 Adhesion of KG1a cells to heparin binding peptide fragment. Wells were coated with the synthetic peptide overnight in an oven at 37^0C and then KG1a cells placed on the wells and incubated for 1 hour. Nonadherent cells removed, adherent cells then lysed and counted. Results are of data from three experiments expressed as mean ± SEM.

4.3 DISCUSSION

The human haemopoietic cell line KG1a demonstrates high levels of adhesion to various cellular substrates such as bone marrow stromal and endothelial cells as well as to fibronectin coated surface; and to a lesser extend to HA surfaces under similar culture conditions. The specificity of the binding was assessed by studying the blocking ability of excess soluble hyaluronic acid, hyaluronidase treatment and by anti-CD44 moAb to the adhesion to cell monolayers and to ECM components. Adhesion to HA was inhibited by both excess soluble HA and the antibody.
Treatment of the stromal monolayer with hyaluronidase had no effect on the adhesion. Further more the blocking effect was restricted to surfaces coated with HA and not to Fn surfaces nor plastic. Anti-CD44 antibody was unable to block adhesion of KG1a to stromal monolayers. KG1a cells did demonstrate high levels of adhesion to BMS and BMEC.

Though HA is known to be the ligand of CD44 (Aruffo A, 1990), in my studies treatment of stromal monolayer with hyaluronidase, did not affect the subsequent adhesion of KG1a cells to the treated stromal monolayer. On the other hand soluble HA did partially inhibit the binding of the KG1a cells to HA coated wells.

The ineffectiveness of blockade in adhesion assays carried out using confluent stromal monolayer as the substrate could be due to the participation of other adhesion pathways.

KG1a cells express a wide range array of adhesion receptors including very late antigen-4, LFA-1 and platelet/endothelial cell adhesion molecule-1 (PECAM-1) (Leavesley et al, 1994). BM stromal cells express constitutively both ligands and counter receptors of many of these adhesion molecules including PECAM-1, VCAM-1and ICAM-1(Simmons et al, 1994).

Thus although KG1a cells highly express CD44, the adhesive properties of the CD 34^+ KG1a cells is determined probably by a complex interactions involving any number of the above adhesion molecules.

Experiments carried out using heparin-bind peptide fragment as substrate showed no adhesion of KG1a cells to the Fn-peptide fragment. KG1a cells are known to express both VLA-4 and VLA-5 receptors (Turner et al, 1988). In vitro, VLA-4 interact with two dominant ligands, VCAM-1 and the CS-1 moiety of fibronectin (a carboxy-terminal heparin domain of fibronectin). In addition two flanking regions of the CS-1 peptide, termed FN-C/H-1 and FN-C/H-II can interact with either the VLA-4 integrin or with membrane-bound proteoglycan (Haugen et al, 1990). The inability therefore to demonstrate binding of KG1a cell lines to the heparin-binding fragment peptide (HBFP) could be due to the fact that this domain of fibronectin does not mediate in vitro KG1a cell adhesion.

5. RESULTS: ADHESION OF LTC-IC AND PΔ STEM CELLS TO FIBRONECTIN AND HYALURONIC ACID AND THE ROLE OF CD44

5.1 INTRODUCTION

Adhesive receptors are likely to be important in the retention of haemopoietic stem cells (HSC) within the bone marrow micro-environment (ME) in close proximity with the stromal cells and its associated elements. The cellular interactions between HSC and the ME are not well understood mainly due to the heterogeneity of cells making up the ME. HSC, which loosely refer to any cell that represents an early stage of haemopoietic development, are found at extremely low frequency in normal haemopoietic tissue. Assay of these cells is mainly undertaken in long-term repopulating studies. The long-term culture (LTC) system attempts to recapitulate the long-term maintenance of haemopoiesis, through the establishment of interactions between haemopoietic cells and certain non haemopoietic cells. The cells measured in this assay, the long-term culture initiating cells (LTC-IC), capable of initiating and maintaining haemopoiesis in human LTC, most likely represents the most immature stem cells. HSC, assayed in this system are also associated with a stromal layer of these bone marrow cultures suggesting that certain components of this matrix play a role in haemopoietic maintenance. An alternative stem cell assay method is based on findings that these long-term repopulating stem cells, as opposed to mature cells adhere to plastic tissue culture vessels. The cells assayed in this system are referred to as plastic adherent PΔ progenitor cells.

The mechanism by which HSC interact with bone marrow stroma in vitro is difficult to study. Studies using purified ligands may provide alternative and useful information on adhesive mechanisms, and also helps in defining specific adhesion pathways.

In this study, BMMNC and $CD34^+$ selected PBPC were used to study the adhesive interaction of stem cells to HA and FN. Both LTC-IC and PΔ assays were used to enumerate the number of stem cells in original and nonadherent cell fractions

5.2 RESULTS:

5.2.1 Adhesion of LTC-IC to Fibronectin

The first set of experiments were designed to evaluate the ability of extracellular matrix (ECM) proteins to select an enriched population of LTC-initiating cells from ficoll separated BM.MNC fraction. FN and HA were the ECM proteins used.

To investigate whether LTC-IC selectively adhered to fibronectin and/or hyaluronic acid coated surfaces, of mononuclear cells, an LTC-IC assay in limiting dilution cultures was performed on the nonadherent cells obtained following the incubation of BM.MNC on fibronectin-coated surface.

Briefly, in two experiments, 10^6/ml aliquot of BMMNC suspension were incubated on ligand coated surface, nonadherent cells were then harvested, pooled and counted and then set-up in an LTC-IC assay in parallel with an aliquot of the original BMMNC suspension. The viability of the cells in the nonadherent cell fraction in all the cell fractions was more than 99%, prior to use of these cells in the culture systems.

Adsorption/coating of the ligands to the tissue culture surfaces was verified by adhesion assays carried out using a myeloid cell line KG1a, see chapter 4.

The LTC-IC frequencies obtained from limiting dilution assay of the fibronectin nonadherent BM.MNC fraction and an aliquot of the original BMMNC suspension are shown in table 5.1, with Fig 5.1 showing the graph of the limiting dilution analysis data used to derive the frequencies of the LTC-IC in the cell fractions.

Table 5.1. Data from an LTC-IC assay of the original and fibronectin nonadherent cell fractions of BMMNC population. Cultures were set up in limiting dilution and each well was scored for either presence or absence of colonies. The LTC-IC frequency in this assay were 2.7 per 10^5 cells in the original cell fraction and 1.5 per 10^5 cells in the FN-nonadherent cell fraction.

original cell fraction				
Replicate cultures	No of input cells	No of wells	No negative wells	Prop negative wells
1	4.8×10^4	28	7	0.25
2	2.4×10^4	28	16	0.57
3	1.2×10^4	28	23	0.82
4	0.6×10^4	28	25	0.89
FN-nonadherent cell fraction				
Replicate cultures	Input cells/well	No. of wells	No. of neg wells	Prop neg wells
1	4.8×10^4	28	13	0.46
2	2.4×10^4	28	24	0.86
3	1.2×10^4	28	26	0.93

72% of the Ficoll separated BM.MNC fraction were nonadherent to the fibronectin-coated surface after incubating for the optimum incubating period set in all the experiments. The mean percentage of mononuclear cells nonadherent to fibronectin in coated flasks were consistently reproducible in four experiments set up for this assay.

In one experiment (Experiment 1) the frequencies of LTC-IC in the original (i.e. unmanipulated) and fibronectin-nonadherent fraction, were 2.7 and 1.5 per 10^5 respectively, fig 5.1. Due to the difficulties associated with this type of assay (some of which have been outlined in chapter 3), only two experiments yielded results after 8 weeks of culture. In the second experiment, the frequency of LTC-IC in the original fraction was 2.6 per 10^5 cells while the frequency of LTC-IC in the FN-nonadherent cell fraction in this particular experiment was not obtained due to the problems encountered during the incubation period.

Table 5.2 shows that when the actual numbers of LTC-IC are calculated for each cell fraction in experiment 1 the percentage adhesion of LTC-IC to FN works out as 60%. Actual percentage cell adhesion is 28%. This therefore shows that the adherent cell population is enriched for LTC-IC, as shown in fig 5.2.

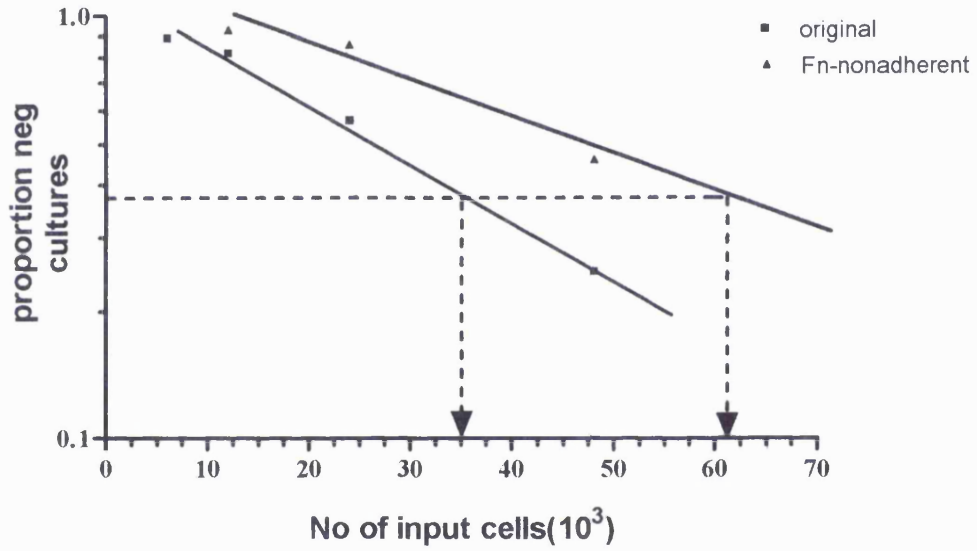


Figure 5.1. Quantification of LTC-IC by limiting dilution analysis (LDA).

Limiting dilution analysis of data from experiment 1 (Table 5.1). BMMNC were inoculated into 96-well flat-bottomed microtiter plates containing irradiated performed stromal layers. The frequency of LTC-IC in the two fractions (original and FN-nonadherent) , being the reciprocal of the concentration of test cells that gave 37% negative cultures, were 2.7 and 1.5 per 10^5 cells respectively.

Table 5.2. Adhesion of LTC-IC to fibronectin. Data is the derived from results in table 5.1

	Actual no of cells	LTC-IC/10^5 cells	Actual no of LTC-IC	% of LTC-IC
Original cell fraction	5 x 10^6	2.7	135	100
Nonadherent cells	3.6 x 10^6	1.5	54	40
Adherent cells			81	**60**

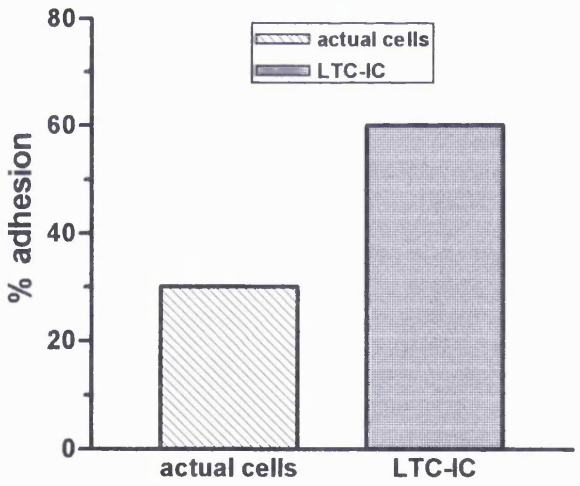


Figure 5.2. Adhesion of BMMNC populations and assayed LTC-IC fractions to fibronectin in a single experiment in which Fn-nonadherent MNC were assayed in limiting dilution cultures for LTC-IC frequency.

5.2.2 Adhesion of LTC-IC to HA

Experiments were also set up to assess the adhesion of LTC-IC to HA. These were carried out in much the same way as for fibronectin adhesion. HA for coating culture vessel surfaces was used at an optimum concentration of 2-mg/ml in all experiments, see chapter 4. Aliquots of BMMNC suspension were incubated on HA surfaces for an hour followed by the harvesting of the nonadherent cells, which were then pooled, counted and assessed for viability after washing. The viability after washing was 95%.

The HA-Nonadherent mononuclear cells were then used to set up LTC-IC assays in limiting dilution cultures and CFU-C assayed as the end point after more than 5 weeks in culture.

To ensure that the HA used in the adhesion assay had no effect on the proliferative potential of the progenitor cells in the mononuclear cell suspension (Hamann et al, 1995), clonogenic assays were set up using mononuclear cells treated with HA for an hour or untreated, prior to the clonogenic assay. There was no difference in the clonogenic cell output between the two cell fractions (Table 5.3).

The frequencies of LTC-IC in the HA-nonadherent fraction was reduced in the two experiments (1.2 and $1.3/10^5$ cells) compared to that obtained in the original cell population (2.7 and $3.1/10^5$ cells respectively, table 5.4.

In two experiments using HA as substrate the percentage of nonadherent mononuclear cells were 82.7% and 85.6% respectively.

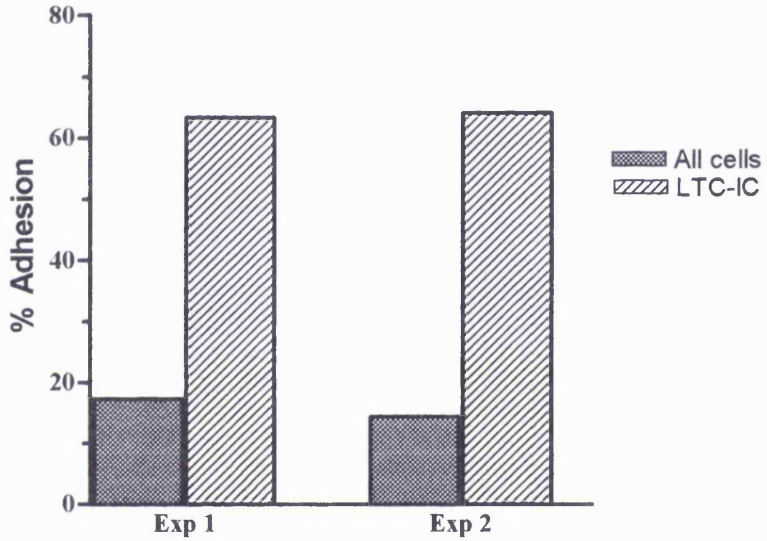
The calculated % adhesion of LTC-IC in these experiments was 64.2% and 63.3% indicating a 2-3 fold enrichment of LTC-IC in the adherent fraction of HA in the two successful experiments (Table 5.4) Figure 5.3.

Table 5.3 Clonogenic output in BMMNC fractions in two experiments. Original cell fractions were assayed for CFU-C with or without HA pre-incubation and colonies scored after 14 days.

	Unmanipulated cell fraction		Non-adherent cell fraction		
EXP	No HA	+ HA	NO Ab	control	Anti-CD44
1	48.7	48	32.5	27.2	51.2
2	29.2	31.2	21.2	20.5	34.2

Table 5.4. Adhesion of LTC-IC to HA. Percentage adhesion to HA of LTC-IC assayed from nonadherent mononuclear cell fraction

Experiment 1				
	Actual no of cells	LTC-IC/10^5 cells	Actual no of LTC-IC	% of LTC-IC
Original cell fraction	6×10^5	2.7	16.2	100
Nonadherent cells	4.96×10^5	1.2	5.95	36.7
Adherent cells			10.25	**63.3**
Experiment 2				
	Actual no of cells	LTC-IC/10^5 cells	Actual no of LTC-IC	% of LTC-IC
Original cell fraction	2×10^6	3.1	62	100
Nonadherent cells	1.71×10^6	1.3	22.2	35.8
Adherent cells			39.8	**64.2**



Figures 5.3. Adhesion to HA by the BMMNC fraction as well as the assayed LTC-IC in the nonadherent cell fraction, set up using limiting dilution cultures.

5.2.3 Effect of anti-CD44 moAb on LTC-IC adhesion to HA.

Two experiments were performed to examine the effect of anti-CD44 mAb on the adhesion of BMMNC and assayed LTC-IC to HA. BMMNC aliquots were pre-treated with the monoclonal antibodies for 30 minutes at 37^0C before being incubated on ligand coated surfaces for an hour in the continued presence of the antibody. The pooled nonadherent cell fractions after counting (to determine the percentage adhesion), were then assayed for LTC-IC frequency.

The cloning efficiency of the assayed CFU-C was not affected by the pre-treatment of the MNC with any of the antibodies used (CFU-C colonies generated in fractions with no Ab, treated with control Ab, or treated with anti-CD44 moAb were 39.2 ± 3.7, 37.7 ± 4.3 and 39.2 ± 2.7 respectively) as shown in table 5.5. In addition there was no obvious effect on the morphological appearance of the stromal cells in the cultures to which the antibody treated cell fraction were added.

Pre-incubation of BMMNC with the anti-CD44 monoclonal antibody, BU52, reduced the adhesion of BMMNC to HA (64.2% and 63.3% adhesion in controls, reducing to 41.6% and 40.3% in anti-CD44 mAb treated LTC-IC in 2 experiments respectively). In contrast the moAb to the β_1 integrin, Anti-CD29 did not reduce the adhesion of the assayed LTC-IC to HA (60.8% and 64.6% respectively), table 5.6.

Table 5.5 Clonogenic output in BMMNC fraction treated either with moAb or without the Ab in two experiments

	CFU-C output in mononuclear cell suspensions				
	NO Ab	IgG1	IgG2a	anti-CD44	ant-CD29
1	31.7	28.5	32.5	29	31.5
2	38.2	49.7	35.5	41.2	41.2

Table 5.6 Effect of anti-CD44 on the adhesion of LTC-IC, assayed from BMMNC, to HA in two experiments. Aliquots of mononuclear cell suspensions were pre-incubated with either anti-CD44 or control isotype, nonadherent cells were then set up in LTC-IC assay

Experiment 1					
		Actual no of cells	LTC-IC/10^5 cells	Actual no of LTC-IC	% of LTC-IC
Original fraction		2×10^6	3.1	62	100
Nonadherent fraction	Cntrl	1.71×10^6	1.3	22.2	35.8
	CD44	1.8×10^6	2.01	36.2	58.4
	CD29	1.61×10^6	1.51	24.3	39.2
Adherent fraction	Cntrl	0.29×10^6	1.8	39.8	**64.2**
	CD44	0.20×10^6	1.09	25.8	**41.6**
	CD29	0.39×10^6	1.59	37.7	**60.8**
Experiment 2					
		Actual no of cells	LTC-IC/10^5 cells	Actual no of LTC-IC	% of LTC-IC
Original fraction		6×10^5	2.7	16.2	100
Nonadherent fraction	Cntrl	4.96×10^5	1.2	5.95	36.7
	CD44	5.32×10^5	1.82	9.68	59.7
	CD29	4.63×10^5	1.24	5.74	35.4
Adherent fraction	Cntrl	1.04×10^5	1.5	10.25	**63.3**
	CD44	0.68×10^5	0.88	6.52	**40.3**
	CD29	1.37×10^5	1.46	10.46	**64.6**

The proteoglycan form of CD44 binds to FN C/H-1 and FN C/H-II, heparin binding peptides present in the carboxy-terminal domain of FN (Verfaillie et al, 1994). Adhesion assays using one of these fibronectin peptides, FN-C/H II (sequence WQPRARI) a 33-kD heparin-binding fragment of fibronectin were also undertaken. However despite coupling of the peptide to ovalbumin (OA), there was very little adhesion by MNC to the OA- coupled peptide coated surface.

No further adhesion studies using the heparin binding fragment as the substrate were pursued due to the low background adhesion by both MNC and $CD34^+$ cells.

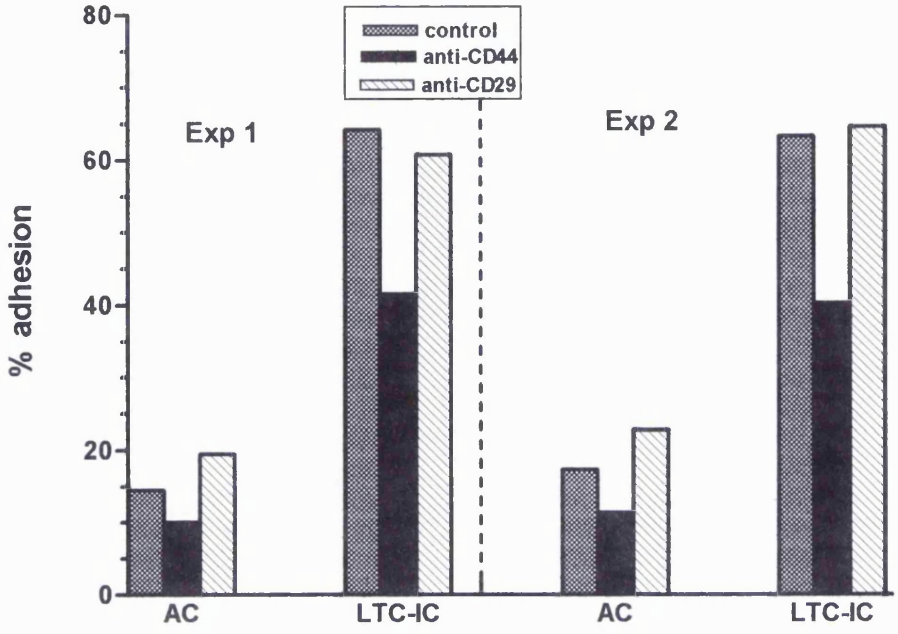


Figure 5.4. Effect of Anti-CD44 and Anti-CD29 moAbs on the adhesion of BMMNC fractions as well as the assayed LTC-IC to HA. The moAbs were pre-incubated with aliquots of BMMNC fractions for 30 minutes at 37^0C prior to addition to the adhesion assay. Nonadherent cells were then assayed for LTC-IC in parallel with original cell fraction (AC - Actual cells).

5.2.4 Use of PΔ stem cell assay to determine adhesion of stem cells to HA.

Original cell fractions and ligand nonadherent cell populations were each used in limiting dilution assays set up to enumerate the frequencies of PΔ stem cells as described in methods.

In two experiments following adhesion to HA, the frequencies of PΔ stem cells assayed in nonadherent fraction (0.7 and $3.12/10^5$ cells) was reduced compared to that assayed in the original population (3.8 and $6.2/10^5$ cells respectively) of BMMNC as shown in table 5.7.

However limiting dilution assays are particularly time consuming and laborious because a second assay, such as a clonogenic CFU-GM assay, on a large number of individual wells is often required to obtain the final result.

Using single dilution assays (SDA) therefore measurements of PΔ stem cell frequencies were carried out in both the original cell fraction and HA-nonadherent fractions of both BMMNC and peripheral blood derived progenitor cells (PBPC).

The frequencies of PΔ stem cells, assayed in single dilution experiments, in BMMNC and PBPC were reduced in the HA-nonadherent cell fraction (0.9 ± 0.5 and 40 ± 10 respectively) compared to the original cell fraction (2.5 ± 0.5 and 360 ± 50, respectively), see table 5.8

Table 5.7 Frequencies of PΔ stem cells in the original and nonadherent fractions of BMMNC in limiting dilution assays

Experiment No	Frequency of P delta cells ($/10^5$) in BM.MNC	
	original fraction	Nonadherent fraction
1	7.7	-
2	4.5	-
3	2.8	-
4	3.8	0.7
5	6.2	3.12
M ± SD	5.3 ± 2.2	

Table 5.8 P delta stem cell frequencies in nonadherent cell populations of single dilution assays

Exp No	Frequency P delta stem cells per 10^5 cells			
	BM.MNC		PB CD34+ cells	
	Original cell	Non-adherent	Original cell	Non-adherent cell
1	2.03	0.2	400	83
2	3.5	0.7	600	58
3	2.0	1.8	100	20
4			400	40
5			300	20
6			300	22
M±SD	2.5 ± 0.5	0.9 ± 0.5	360 ± 50	40 ± 10
Range	2-3.5	.2-1.8	210-600	20-83

The ligand nonadherent cell populations were vigorously washed with serum free balanced salt solution before being used in the delta assay set up either in limiting dilution or single dilution cultures, and in all the assays viability of the cell fraction used was greater than 97% following the washes.

The actual adhesion of BMMNC to HA was 25.9 ± 8. Table 5.9 shows 3 representative experiments in which the actual numbers of PΔ cells are calculated for each cell fraction of BMMNC.

In 2 experiments, PΔ cells demonstrated high levels of adhesion to HA of 93.3% and 86% respectively, while in a third the level of adhesion was lower at 24.2%. The adherent cell fraction was thus enriched for PΔ cells in two of the three experiments, Figure 5.5.

Similarly for PBPC, the percentage adhesion of the actual cells was 33.9 ± 4.7 while the calculated percentage adhesion was 92.05 ± 4.5, table 5.10. The adherent cell population were thus enriched for PΔ cells, figure 5.6

Table 5.11 summarises the data.

Table 5.9 Adhesion of PΔ cells, from bone marrow mononuclear cells, to HA

Exp no		Actual no of cells	PΔ cells/10^5	Actual no PΔ cells	% of PΔ cells
1	Original	3×10^6	2.03	60.9	100
	Nonadherent	2.04×10^6	0.2	4.08	6.7
	Adherent			56	**93.3**
2	Original	8×10^6	3.5	280	100
	Nonadherent	5.6×10^6	0.7	39.2	14
	Adherent			240.8	**86**
3	Original	3×10^6	2.0	60	100
	Nonadherent	2.53×10^6	1.8	45.5	75.8
	Adherent			14.5	**24.2**

Table 5.10. Adhesion of PΔ cells, from PB $CD34^+$ selected cells, to HA

Exp no		Actual no of cells	PΔ cells/10^5	Actual no PΔ cells	% of PΔ cells
1	Original	5×10^5	400	2000	100
	Nonadherent	3.5×10^5	83	290.5	14.5
	Adherent			1709.5	**85.5**
2	Original	4×10^5	600	2400	100
	Nonadherent	2.8×10^5	58	162.4	6.8
	Adherent			2237.6	**93.2**
3	Original	5×10^5	400	2000	100
	Nonadherent	3.2×10^5	40	128	6.4
	Adherent			1872	**93.6**
4	Original	5×10^5	300	1500	100
	Nonadherent	3.02×10^5	20	61	4.1
	Adherent			1439	**95.9**

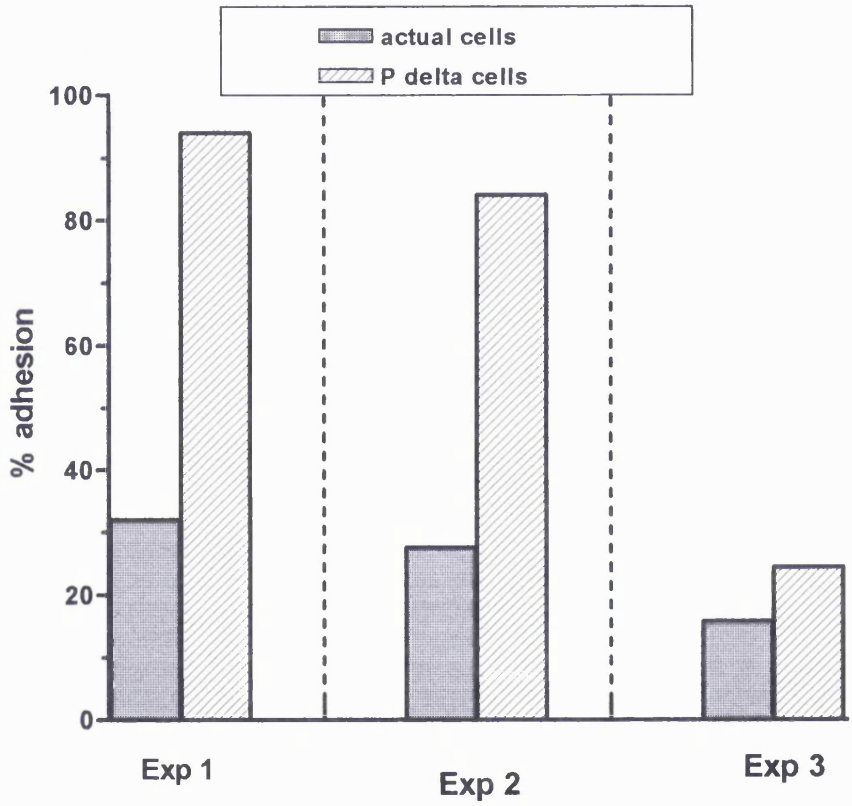


Figure 5.5 Adhesion of BMMNC and assayed PΔ stem cells to HA. Data from three experiments are shown. Percentage adhesion was calculated as shown in table 5.10.

Table 5.11. Percentage adhesion of PΔ cells from BMMNC and PB $CD34^+$ Cells to HA

Exp	Percentage adhesion of PΔ cells	
Number	BMMNC	PB $CD34^+$ Cells
1 2 3 4	93.9 86 24.2	85.5 93.2 93.6 95.9
Mean ± SD	67 ± 38	92 ± 4.5

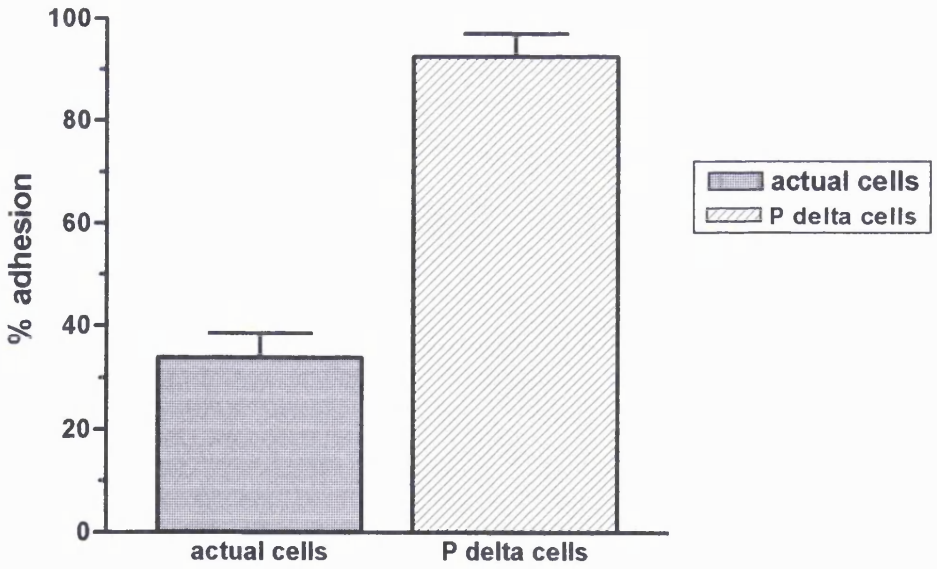


Figure 5.6. Adhesion of PB $CD34^+$ selected cells fractions as well as the assayed PΔ stem cells to HA, the experiments were set up using single dilution cultures.

5.2.5 Inhibition of PΔ stem cell adhesion to HA by blocking antibodies

PB $CD34^+$ cells pre-treated with anti-CD44 monoclonal antibody or control IgG were used to assay for PΔ stem cells following adhesion to HA surface. As shown in figure 5.7, in two experiments pre-incubation of $CD34^+$ cells with the anti-CD44 antibody (BU52) reduced the percentage adhesion of all cells, this was however more marked with the assayed PΔ cells (adhesion was reduced from 76.3% to 25.4%, and 64.6% to 37.4% respectively.

The cloning efficiency of the CFU-GM was not affected by pre-incubation of the $CD34^+$ cells with the antibody.

Adhesion assays using BMMNC and PB $CD34^+$ cell fractions to surfaces coated with the heparin peptide fragment of fibronectin were not carried out due to low background adhesion obtained with both MNC and $CD34^+$

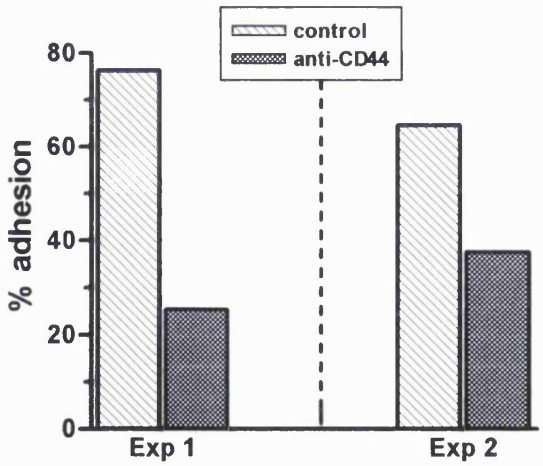


Figure 5.7 Effect of Anti-CD44 moAb on the adhesion of PΔ cells, assayed from PB $CD34^+$ Cells, to HA

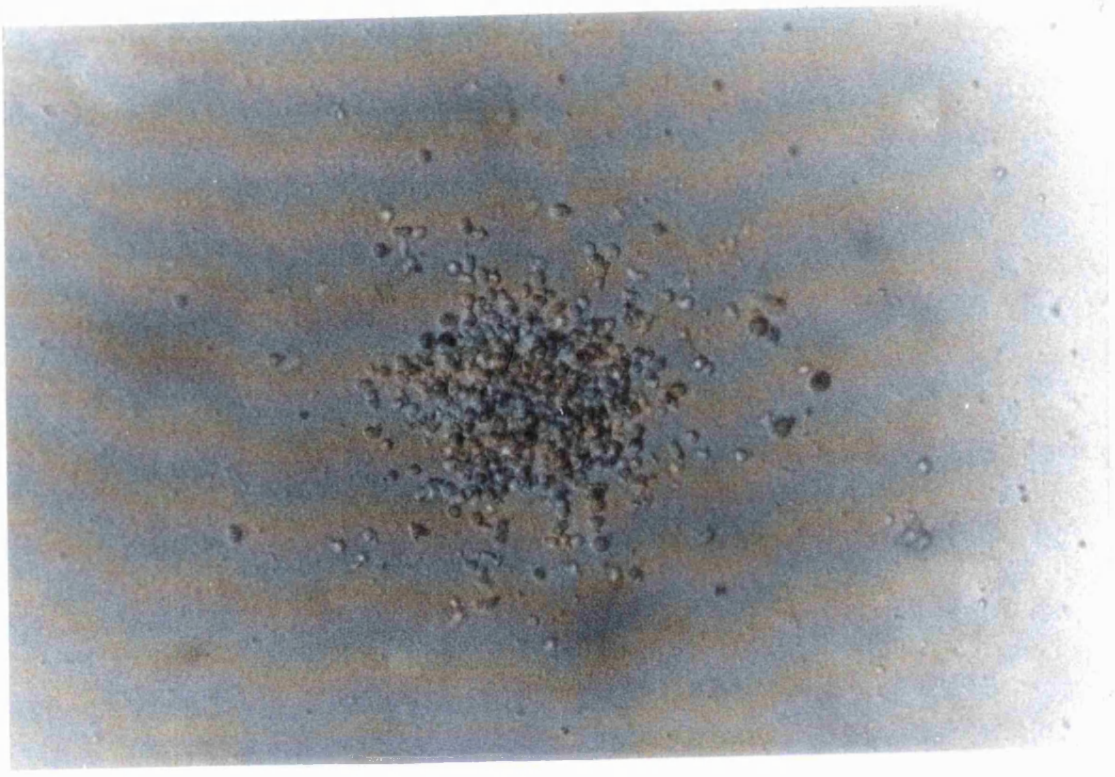


Figure 5.8 Photomicrograph of CFU-GM progenitors observed on days 7, 14, and 21 in methyl cellulose colony assay of MNC in the presence of SCF, IL-3, G-CSF and GM-CSF. The colonies were counted as described in general methods. Magnification x 400

5.4 DISCUSSION

These experiments on stem cell adhesion have used 2 different stem cell assays, the LTC-IC assay and delta assay for plastic adherent PΔ progenitor cells to quantify stem cells. The experiments have also been able to show that the candidate stem cells 'LTC-IC and PΔ stem cells' assayed in the two different protocols could be detected in nonadherent fractions of both BM and PB and that these adhered to HA. The experiments further demonstrated that the adhesion was mediated in part by the adhesion molecule CD44. It was also demonstrated that adhesion to HA enriched for both the LTC-IC and PΔ cells.

The background adhesion levels to both HA and Fn by mononuclear and $CD34^+$ cell population (25.9 ± 8.9%, n=3 and 33.9 ± 4.7%, n=4 respectively) was slightly higher in my experiments compared to previously reported studies in which the background adhesion was 15% (Smadja-joffe et al, 1994). Peripheral blood derived progenitor cell population (PBPC) adhesiveness to the two ligands was similar to that of BMMNC.

The majority of the plastic adherent PΔ progenitor cell population assayed in delta type cultures are adherent to HA, similarly LTC-IC assayed in long-term cultures. However plastic adherent PΔ progenitor cell adhesion to HA in two experiments (93.3 and 86) was greater than that of LTC-IC (63.3 and 64.2), despite the fact that the adhesion of the actual cells (pre-assay cell population) in the two cell populations were similar as shown above. This could be due to a number of reasons, foremost being the fact that the two assays are different in terms of the duration of culture as well as the end point. Subtle difference in intrinsic adhesions of plastic adherent cells compared to LTC-IC (adherent to stroma) could also account for this difference. An effect of bone marrow stroma (BMS) on this assay, resulting in increased frequency of LTC-IC and thus increased nonadherent fraction and conversely reduced adhesion.

The adhesion to HA by all cells as well as assayed candidate stem cells was found to be partly dependent on the CD44 receptor. This was based on the findings in my experiments which showed that addition of the anti-CD44 moAb partially blocked the adhesion to HA by both cells.

The blocking effect could not have been due to the effect on the cloning efficiency of the end point cells, as parallel experiments showed no effect by the blocking antibodies on the cloning potential.

Competitive inhibition using excess soluble ligand as a competitive inhibitor were inconclusive, although this was demonstrated in adhesion assay using the myeloid cell line KG1a cells (see chapter 4).

Further experiments were carried out in an attempt to demonstrate that the CD44 receptor was linked to the adhesion of progenitor cells to the C-terminal heparin-binding domain of fibronectin (Verfaillie, 1994). In my experiments adhesion assay using a synthetic heparin-binding fibronectin peptide fragment showed low levels of adhesion by both MNC and PBPC. Furthermore there was no blocking effect of the peptide fragment on the adhesion of all cells to HA surfaces. Further adhesion assays to evaluate this adhesion pathway were not pursued any further due to the very low background adhesion obtained. The low background adhesion could be attributed to the size of the synthetic peptide used, although there was no effect on the adhesiveness when the peptide fragment was lengthened by coupling to ovallbumin.

In both cell populations assayed therefore the adhesion to HA resulted in demonstration of an enriched population of stem cells in the cell fractions. HA is abundant in human bone marrow, thus CD44 is likely to play a role in mediating stem cell interactions with the BM microenvironment. Interestingly, PB derived stem cells demonstrate similar levels of adhesion to BM counterparts, thus the mobilisation of these 'stem cells' into the peripheral circulation is unlikely to be due to changes in CD44-mediated adhesion.

6. RESULTS: ADHESION OF HUMAN CD34$^+$ HAEMOPOIETIC PROGENITOR CELLS DERIVED FROM PERIPHERAL BLOOD

6.1 INTRODUCTION

Haemopoietic progenitor cells in unstimulated adult peripheral blood exists only in small numbers (McCredie et al, 1971). The existence of these cells has been functionally shown in transplant experiments involving mice, dogs and primates (Korbling et al, 1994). Greater numbers of HPC are contained in human umbilical cord blood and normal bone marrow than in adult peripheral blood (Hows et al, 1992)

These circulating stem and progenitor cells can be increased to significant levels after administration of pharmacological doses of haemopoietic cytokines (Socinski et al, 1988). This provides the basis for peripheral blood stem cell transplantation.
However an unresolved question is whether HPC's obtained in this way are different in terms of their expression and function of adhesion molecules.

CD34+ cells are known to express a number of adhesion molecules, including the CD44 receptor. The CD44 receptor has in turn been noted to play a possible role in the early stages of haemopoiesis, since anti-CD44 moAb abrogates in vitro long-term haemopoiesis on pre-established stroma (Miyake K 1990). The expression and function of the CD44 molecule on PB HPC has as yet not been thoroughly investigated. In this chapter I have investigated the adhesion of peripheral blood derived HPC to HA, and the role of CD44 are investigated

6.2 RESULTS

6.2.1 Adhesion of PB CFU-GM to HA and FN

$CD34^+$ selected peripheral blood derived progenitor cells were incubated on HA and Fn coated surfaces for one hour and the nonadherent cell populations harvested and counted. The efficacy of the wash was verified visually using an inverted microscope. The pooled nonadherent cells were subsequently analysed in semi-solid methylcellulose medium in the presence of IL-3, GM-CSF, G-CSF, and SCF. Progenitor assays on the original cell suspension were set-up in parallel to determine the numbers of CFU-GM originally seeded into the adhesion assays.

After 14 days incubation the numbers of CFU-GM colonies present in the nonadherent fraction (15.7 ± 1.9/400 cells) was significantly lower than that in the original cell fraction (37.8 ± 6.6/400 cells) that was seeded to the adhesion assay
($p<0.05$, n=3 with HA), thus suggesting significant adhesion of clonogenic cells to HA (Table 6.1). Similar findings were obtained with FN-nonadherent fractions (18 ± 1.5/400 cells in the nonadherent fraction compared with 37.8 ± 6.6/400 cells in the original fraction, mean ± SD of three experiments $p<0.05$ table 6.1).

The actual percentage adhesion of CFU-GM was calculated from the CFU- GM generated in the nonadherent fractions as well as the actual number of cells in the adhesion assay (Tables 6.2 and 6.3). PBPC derived CFU-GM demonstrated equivalent adhesion to both substrates, with 68.2 ± 6.6% of adhesion to HA, and 64.6 ± 10% adhesion to FN (Table 6.4). Figure 6.1 shows that the adherent fraction of PB $CD34^+$ cells was enriched for CFU-GM when either HA or FN was used as the adhesive substrate. The data is summarised in table 6.4.

Table 6.1 Numbers of CFU-GM generated by cell fraction nonadherent to either HA or Fn. Cell fractions harvested from adhesion assay were assayed for CFU-GM in methylcellulose cultures

Exp No	Numbers of CFU-GM produced /400 cells		
	Original	HA-nonadherent	FN-nonadherent
1	41.3	14.1	17.2
2	30.2	15.2	20.1
3	42	17.8	16.7
Mean ± SD	37.8 ± 6.6	15.7 ± 1.9	18 ± 1.5

TABLE 6.2 Adhesion of PB $CD34^+$ cells and CFU-GM to HA. Percentage adhesion of CFU-GM to HA was calculated from numbers of cells originally seeded in the adhesion assay, the number of nonadherent cells, and the CFU-GM output of each fraction.

		Actual no of cells	CFU-GM/400 cells	Actual no CFU-GM	% CFU-GM Adherent
1	Original	1.4×10^5	41.3	14,455	100
	nonadherent	1.01×10^5	14.1	3560.2	24.6
	adherent	0.39×10^5		10,894.7	**75.4**
2	Original	6.7×10^4	30.2	5058.5	100
	nonadherent	4.4×10^4	15.2	1672	33.1
	adherent	2.3×10^4		3386.5	**66.9**
3	Original	2×10^5	42	21000	100
	nonadherent	1.78×10^5	17.8	7921	37.7
	adherent	0.22×10^5		13079	**62.3**

Table 6.3 Adhesion of CFU-GM to FN. Percentage adhesion of CFU-GM to FN was calculated as for Table 6.2

		Actual no of cells	CFU-GM/400 cells	Actual no CFU-GM	% CFU-GM Adherent
1	Original	1.4×10^5	41.3	14,455	100
	nonadherent	0.78×10^5	17.7	3451.5	23.9
	adherent	0.62×10^5		11,003.5	**76.1**
2	Original	6.7×10^4	30.2	5058.5	100
	nonadherent	4.0×10^4	21.2	2120	41.9
	adherent	2.7×10^4		2938.5	**58.1**
3	Original	2×10^5	42	21000	100
	nonadherent	1.78×10^5	19	8455	40.3
	adherent	0.22×10^5		12,545	**59.7**

Table 6.4 Adhesion of PB $CD34^+$ cells and assayed CFU-GM to HA and FN. This summarises the data in Tables 6.2, and 6.3, and also gives the % adhesion of all cells (actual cells)

Exp NO	HA		FN	
	Actual cells	CFU-GM	Actual cells	CFU-GM
1	27.8	75.4	44.3	76.1
2	34.3	66.9	40.3	58.1
3	11	62.3	11	59.7
M ± SD	24.4 ± 12	68.2 ± 6.6	31.9 ± 18.2	64.6 ± 10

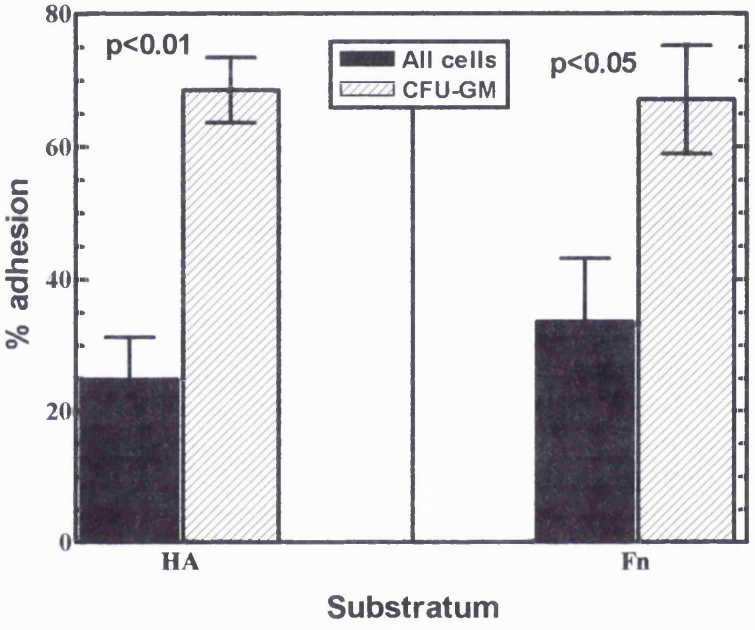


Figure 6.1 Adhesion of peripheral blood derived progenitor cells to HA and Fn. Selected $CD34^+$ PB cells were incubated on HA and Fn surfaces and nonadherent cells then assayed for CFU-GM. The percentage of CFU-GM adherent to the substratum was calculated as described above. Data is mean ± SD of three experiments.

6.2.2 Anti-CD44 moAb addition reduced the adhesion of $CD34^+$ PB cells to HA and not FN

In order to investigate the effect of the anti-CD44 moAb on the adhesion of PB $CD34^+$ cells to HA and subsequent production of CFU-GM in the nonadherent cell fraction, peripheral blood derived progenitor cells were incubated with anti-CD44 moAb, anti-CD29 or IgG control for 30 minutes and then added to ECM ligand- coated plates. All the antibodies were used at 20 μg/ml and were present throughout the adhesion assay.

Fractions of both the nonadherent cell population and original cell fraction were then dispensed into methyl cellulose cultures and evaluated for the numbers of CFU- GM produced. The adhesion of the actual cells to the ligands following the addition of the antibody was determined from the nonadherent cell fractions (Table 6.5).

The moAb used here have been shown in preceding experiments to have a blocking effect on the adhesion of the myeloid cell line KG1a to HA (chapter 4).

The adhesion of the PBCD34^+ cells to HA substrate, determined by counting the nonadherent cell fraction in the adhesion assay, was inhibited by the anti-CD44 moAb (table 6.5 and Figure 6.3). Similarly anti-CD29 moAb reduced the adhesion of actual PBPC to FN (table 6.5). However the anti-CD29 mAb did not inhibit the adhesion of PBPC to HA (Table 6.5). similarly anti-CD44 mAb had no effect on adhesion of PBPC to FN (Table 6.5)

Table 6.5 Adhesion of actual PBPC to HA and FN. Percentage adhesion determined from the counts of the harvested nonadherent cell fraction in the adhesion assay.

Exp no	Percentage adhesion					
	HA			FN		
	CNTRL	CD44	CD29	CNTRL	CD44	CD29
1	19.60	5.70	35.0	40.7	37.2	14.3
2	30.00	17.90	29.9	35.8	34.3	13.4
3	18.50	13.50	22.5	25.7	30.2	13.1
M ± SD	22.7 ± 6.3	12.4 ± 6.2	29.1 ± 6.3	34.1 ± 7.6	33.9 ± 3.5	13.6 ± 0.6

6.2.3 Anti-CD44 moAb addition decreases adhesion of CFU-GM to HA, but not to FN.

The numbers of CFU-GM progeny generated in the PBPC HA nonadherent fraction pre-incubated with anti-CD44 moAb (24.6 ± 3.7/400 cells) were higher compared with that from the nonadherent fraction from wells treated with the control antibody (15.2 ± 3.5/400 cells, n=3, $p<0.05$), Table 6.6.

In preliminary studies, the effect of the anti-CD44 and anti-CD29 mAbs on CFU-GM output in methylcellulose cultures was evaluated. Progenitor recovery values following incubation with either moAb were equivalent to control values, (Fig 6.2). Thus, increased numbers of CFU-GM in the nonadherent fraction treated with mAb was not due to direct effect on CFU-GM output, but to an actual reduction in adherent CFU-GM.

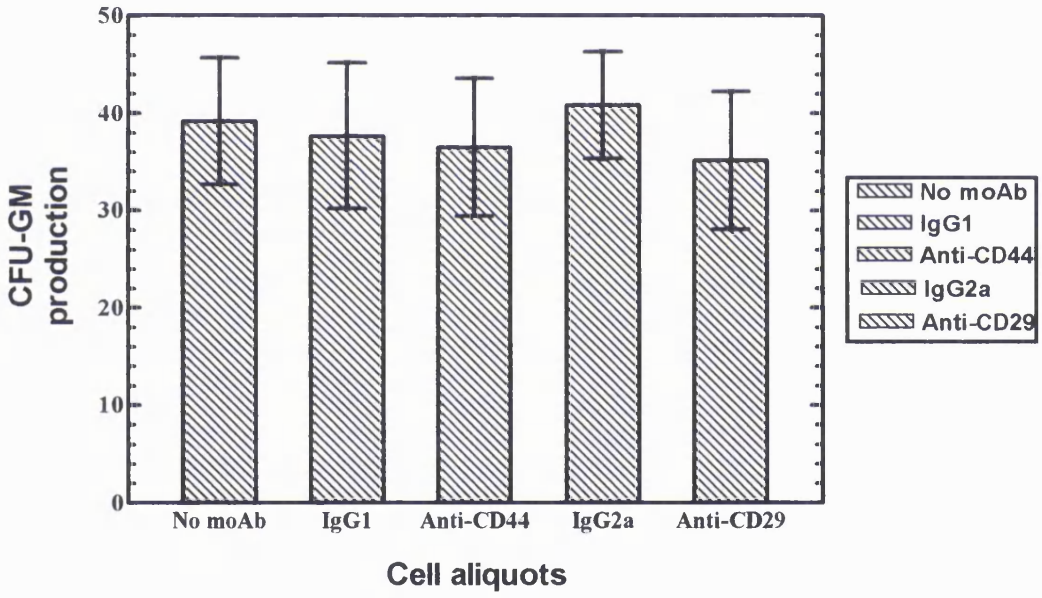


Figure 6.2 CFU-GM production in aliquots of PBPCs. The cell aliquots were incubated with or without antibodies for 30 minutes at 37^0C and each aliquot then assayed for CFU-GM. Results are mean ± SD of three experiments.

Based on the CFU-GM output in nonadherent fractions and original cell population, the calculated % adhesion of PB CFU-GM in three experiments under different conditions is shown in Tables 6.6 and 6.7. The binding of CFU- GM progeny to HA was significantly inhibited by the Anti-CD44 moAb (68.7 ± 7.1 % in control, compared with 42.4 ± 10.4, with anti-CD44, P=0.0223, n=3,), Figure 6.3. The percentage inhibition of CFU- GM progeny adhesion to HA in three experiments was 38.3%.

There was however no significant difference in the percentage adhesion of CFU- GM to HA when the cells were pre-incubated with the anti-CD29 moAb compared with the control (68.6 ± 8.6% and 64.9 ± 14.3% respectively, n=3), Figure 6.4. Adhesion of CFU-GM to FN was inhibited by pre-treatment of the PBPC with the anti-CD29 moAb (68.6 ± 8.6% with control and 38.2 ± 15.5% with the anti-CD29 mAb), Table 6.8, Fig 6.3. Conversely, anti-CD44 had no effect on the adhesion of CFU-GM to FN.

Fig 6.3

Adhesion assays to examine the role of CD44 receptors in the adhesion of CFU- GM to the heparin-binding domain of fibronectin were not carried out due to the difficulties encountered when the synthetic peptide was used as the adhesive substrata.

Table 6.6 Effect of anti-CD44 mAb and anti-CD29 mAb on CFU-GM adhesion to HA.

Exp no			Actual cells	CFU-GM/400 cells	Actual no CFU-GM	% CFU-GM adherent
1	original cell fraction		1.4×10^5	41.3	14,455	100
	Nonadherent fraction	C	1.14×10^5	12	3420	23.6
		CD44	1.32×10^5	20.7	6831	47.2
		CD29	0.91×10^5	13.7	3116.7	21.6
	adherent fraction	C				**76.4**
		CD44				**52.8**
		CD29				**78.4**
2	original cell fraction		6.7×10^4	30.2	5058.5	100
	Nonadherent fraction	C	4.7×10^4	14.5	1703.7	33.7
		CD44	5.5×10^4	25	3437.5	67.9
		CD29	4.7×10^4	21.5	2526.2	50
	adherent fraction	C				**66.3**
		CD44				**32.1**
		CD29				**50**
3	original cell fraction		2.22×10^5	42	23,310	100
	Nonadherent fraction	C	1.81×10^5	19	8597.5	36.9
		CD44	1.92×10^5	28	13440	57.6
		CD29	1.72×10^5	18.2	7826	33.6
	adherent fraction	C				**63.1**
		CD44				**42.4**
		CD29				**66.4**

Table 6.7 Effect of anti-CD44 mAb on CFU-GM adhesion to FN.

Exp no			Actual cells	CFU-GM/400 cells	Actual no CFU-GM	% CFU-GM adherent
1	original cell fraction		1.4×10^5	41.3	14,455	100
	Nonadherent fraction	C	0.83×10^5	16.7	3465.2	24
		CD44	0.88×10^5	19.5	4290	29.7
		CD29	1.2×10^5	22.7	6810	47.1
	adherent fraction	C				**76**
		CD44				**70.3**
		CD29				**52.9**
2	original cell fraction		6.7×10^4	30.2	5058.5	100
	Nonadherent fraction	C	4.3×10^4	19.2	2064	40.8
		CD44	4.4×10^4	19.5	2145	42.4
		CD29	5.8×10^4	27.2	3944	78
	adherent fraction	C				**59.2**
		CD44				**57.6**
		CD29				**22**
3	original cell fraction		2.22×10^5	42	23,310	100
	Nonadherent fraction	C	1.65×10^5	16.7	68888.7	29.5
		CD44	1.55×10^5	15.2	5890	25.3
		CD29	1.93×10^5	29.2	14,089	60.4
	adherent fraction	C				**70.5**
		CD44				**74.7**
		CD29				**39.6**

Table 6.8 Adhesion of CFU-GM to HA and FN.

	Percentage adhesion					
	HA			FN		
	CNTRL	CD44	CD29	CNTRL	CD44	CD29
1	76.70	52.80	78.40	76	70.3	52.9
2	66.30	32.10	50.00	59.2	57.6	22
3	63.10	42.40	66.40	70.5	74.7	29.6
	68.7 ± 7.1	42.4 ± 10.4	64.9 ± 14.3	68.6 ± 8.6	67.5 ± 8.9	38.2 ± 15.5

Table 6.9 Clonogenic output generated by HA and FN nonadherent cell fractions with or without antibody pre-treatment.

Exp no	CFU-GM output per 200 cells							
	HA				FN			
	no Ab	C	CD44	CD29	no Ab	C	CD44	CD29
1	14.1	12	20.7	13.7	17.2	16.2	19.5	22.7
2	15.2	14.5	25	21.5	20.1	19.2	19.5	27.2
3	17.8	19	28	18.2	17.8	16.7	15.2	29.2
M ± SD	15.7 ± 1.9	15.2 ± 3.5	24.6 ± 3.7	17.8 ± 3.9	18.4 ± 1.5	17.5 ± 1.4	18.1 ± 2.5	26.4 ± 3.3

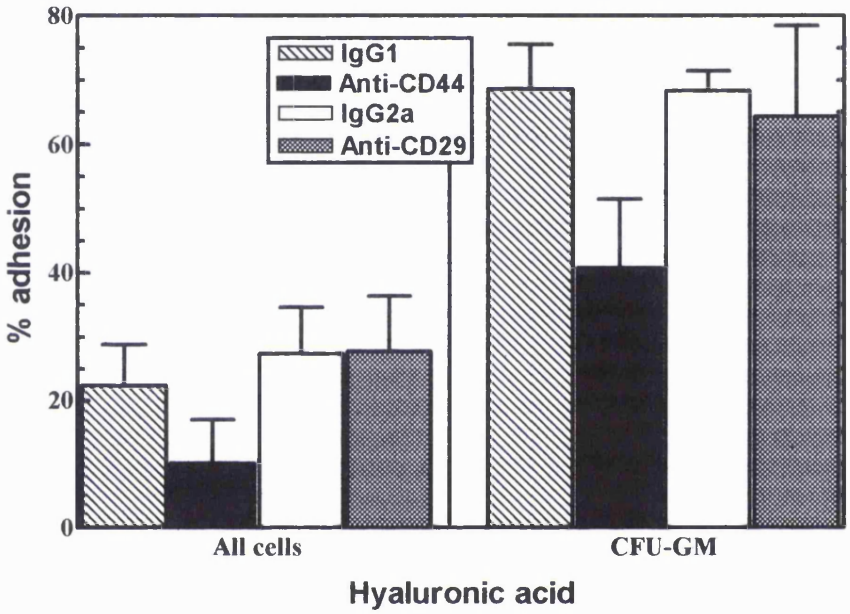


Figure 6.3 Effect of Anti-CD44 and anti-CD29 on adhesion of PBPC and assayed CFU-GM to hyaluronic acid. The two antibodies were incubated with aliquots of the selected $CD34^+$ cells and then incubated on the substratum, nonadherent cells were then assayed for CFU-GM and percentage adherence calculated. Corresponding isotype controls were used with each antibody. Results as mean ± SD of three experiments

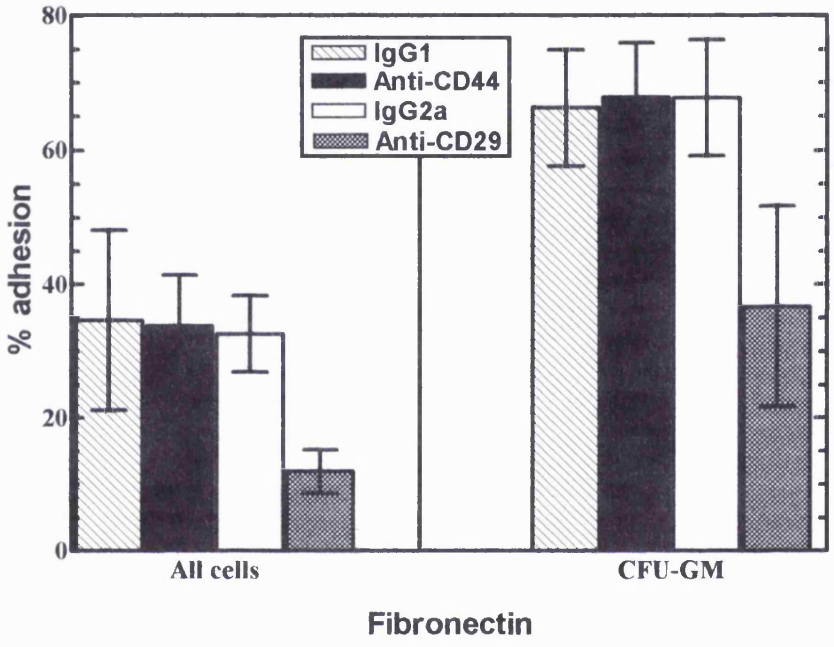


Figure 6.4 Effect of anti-CD29 and anti-CD44 moAbs on adhesion of CFU-GM to fibronectin. Cell aliquots were treated with the two antibodies for 30 minutes at 37^0C and then added to fibronectin coated surfaces and further incubated for 1 hour. Nonadherent cells were then assayed for CFU-GM. Results are mean ± SD of three experiments to compare percentage adhesion control and antibody, All cells $P< 0.01$; CFU-GM, $P<0.05$

6.3 DISCUSSION

The results presented here have shown that PBPC adhere to both HA and FN. Incubation of PBPC on ligand coated surfaces resulted in depletion of CFU-GM in the nonadherent fraction, suggesting specific binding of these progenitor cells to HA and FN.

One of the surface molecules which might play an additional role in adhesive progenitor/bone marrow stromal cell interactions is CD44 (Gunji et al, 1992). The receptor-ligand interaction in this study was further elucidated by pre-incubation of the PBPCs with an anti-CD44 moAb, which in previous studies has been shown to have a blocking effect on CD44 mediated adhesion, of the primitive myeloid cell line KG1a cells, chapter 4. The results following this line of investigation showed a reduction in the PBPC adhesion to HA following pre-incubation with the specific anti-CD44 moAb, this effect was not however seen with the use of an anti-CD29 moAb, an Ab with specificity for binding to the β_1 integrin (Oostendorp R A J, 1995).

There was no direct effect of any of the mAbs on CFU-GM output in clonogenic assays (fig 6.2)

Similarly, adhesion of CFU-GM to HA was inhibited by the anti-CD44 moAb but not by anti-CD29 moAb.

The contrasting effects of the anti-CD44 and anti-CD29 moAbs therefore demonstrated that anti-CD44 moAb partially inhibited granulocytic progeny-Hyaluronic acid binding in much the same way as anti-CD29 moAb blocked the CFU-GM-Fibronectin adhesion. CD44 therefore seems to play a role in mediating progenitor cell-HA interactions in peripheral blood derived progenitor cells.

Other studies have also recently shown that up to 15% of $CD34^+$ cells spontaneously bound to immobilised hyaluronan (F. Smadja-joffe, 1995)and that flow cytometric analysis showed that CD44 expression on adherent cells was brighter than on the nonadherent ones. However there remains continued speculation as to whether HPCs express both low and high affinity molecules which would explain the low binding to the ligand observed in most other studies. Yet other studies have hypothesised that adhesion of committed myeloid progenitor cells to a heparin-binding domain of fibronectin requires a co-ordinated action of both CD44 and an $\alpha_4\beta_1$ integrin (Vefaillie et al, 1994).

Difficulties arose when I attempted to use a synthetic peptide, with very low background adhesion obtained with PBPC. Five heparin-binding amino acid sequences have been identified in the 31-KDa heparin-binding fibronectin domain (Woods et al, 1993). The proteoglycan form of CD44 binds to FN C/H-1 and FN C/H-II, heparin binding peptides present in the carboxy-terminal domain of FN.

Peptides used in assays		
	Name	Sequence
1	FN C/H-1	YEKPGSPPREVVPRPRPGV
2	FN C/H II	KNNQKSEPLIGRKKT
3	FN C/H-III	YRVRVTPKEKTGPMKE
4	FN C/H-IV	SPPRRARVT
5	FN C/H-V	WQPPRARI
6	CS1	DELPQLVTLPHPNLHGPEILDVPST

The peptide used in my experiments was number five which is much smaller than the other peptides (FN C/H-1, FN C/H II). This could thus explain the low background adhesion to this peptide when used as the adhesive substrate.

RESULTS: ADHESION OF UMBILICAL CORD BLOOD MONONUCLEAR AND CD34$^+$ SELECTED PROGENITOR CELLS AND ASSAYED CFU-C TO HA AND FN ROLE OF CD44.

7.1 INTRODUCTION

The interest in progenitor/stem cells from human umbilical cord blood has arisen due to the potential of these cells as a source of haemopoietic stem cells for use in transplantation. Several studies have evaluated the clonogenic capacity of human umbilical cord (UCB), and many of these found high numbers of both primitive and committed haemopoietic progenitor cells than in adult marrow (Broxmeyer et al, 1992).

UCB progenitor cells have further been demonstrated to have an advantage over bone marrow (BM) in terms of their proliferative capacity (Traycoff et al, 1994).
Other notable observations included an increased generation of clonogenic cells as well as a longer life span of cultures, when UCB was used as the inoculum in long-term cultures compared to normal bone marrow (Hows et al, 1992).

The expression of CD44 and other cell adhesion molecules on umbilical cord blood primitive cells has so far not been studied extensively and thus information on the adhesive properties of the UCB primitive cells is limited.
The adhesive interactions of UCB progenitor cells were studied by examining the adhesion of mononuclear and CD34+ selected cells derived from human umbilical cord blood to HA and the blocking effect of mAb against the extracellular domain of the CD44 receptor. Assayed progenitor colony forming unit cells (CFU-C) as well as actual cell numbers were enumerated.

7.2 RESULTS.

7.2.1 Progenitor cell output in umbilical cord blood cells.

To evaluate the numbers of progenitor colony-forming unit cells generated from the mononuclear and UCB $CD34^+$ cell fractions, 10^4 mononuclear and 200 purified cells respectively were cultured for 14 - 21 days in the presence of SCF, IL-3, GM-CSF and EPO in methylcellulose cultures, and colonies of granulocytic and erythroid progenitors generated were counted and expressed as progenitor colony forming unit cells in culture (CFU-C).

Figures 7.1 and 7.2 shows CFU-GM and BFU-E colonies generated by an aliquot of UCB $CD34^+$ selected cells.

The mean numbers of CFU-C generated in three experiments of UCBMNC was 99.9 ± 11.4 per 10^4 cells, whereas 93.6 ± 32.1 per 200 cells CFU-C were generated in three samples of umbilical cord blood $CD34^+$ selected cells, Tables 7.1.

The clonogenic output in human umbilical cord blood mononuclear cells was higher than those in the corresponding adult bone marrow cell populations under similar culture conditions (99.9 ± 11.4/10^4 cells for UCB and 23.7 ± 3.7/10^4 cells for adult BM respectively, n=3, $P<0.05$) Table 7.2.

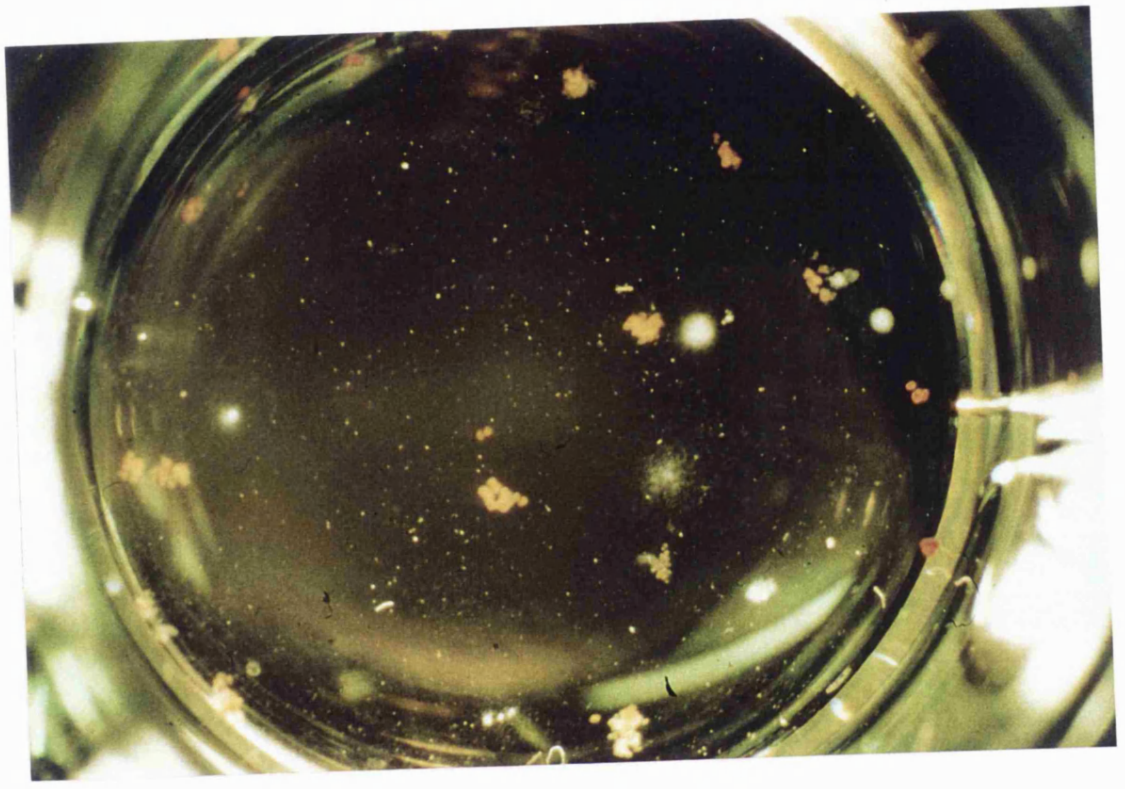


Figure 7.1 Photomicrograph of CFU-GM and BFU-E colonies generated from human umbilical cord blood $CD34^+$ selected cells. Colonies were counted after 14 days in culture. Magnification x 20

Figure 7.2 Photomicrograph of CFU-GM AND BFU-E under high power.

Table 7.1 Numbers of CFU-C generated by UCBMNC cell fraction nonadherent to either HA or Fn. Cell fractions harvested from adhesion assay were assayed for CFU-C in methylcellulose cultures

I. UCB MNC

Exp No	Numbers of CFU-C produced / 10^4 cells		
	Original	HA-nonadherent	FN-nonadherent
1	102.3	69.7	49.3
2	110	16	56
3	87.5	25.2	42.5
Mean ± SD	99.9 ± 11.4	37 ± 28.7	49.3 ± 6.7

II. UCB $CD34^+$ selected cells.

Exp No	Numbers of CFU-C produced /200 cells		
	Original	HA-nonadherent	FN-nonadherent
1	114.2	51.7	-
2	81.7	65.7	-
3	54	9.3	11.3
4	124.5	20.2	15.7
Mean ± SD	93.6 ± 32.1	36.7 ± 26.4	

Table 7.2 Clonogenic output from mononuclear cell samples of Adult bone marrow and umbilical cord blood

Exp number	Clonogenic output per 10^4 MNC	
	BM	UCB
1	28	102.3
2	21.7	110
3	21.4	87.5
mean ± SD	23.7 ± 3.7	99.9 ± 11.4

7.2.2 Progenitor CFU-C adhesion to HA and FN:

To examine whether the ligands HA and Fn were able to select an enriched population of the assayed progenitor colony forming unit cell population from samples of both UCBMNC and UCB $CD34^+$ selected cells, aliquots of these cell suspensions were incubated on the respective ligand coated surfaces, nonadherent cells were then washed off, pooled and counted in order to determine percentage of adherence.

The nonadherent cells were then set-up in progenitor colony forming assays, in parallel with aliquots of the original cell population.

The percentage of the actual UCBMN cells adherent to HA and Fn, determined from the counting of the nonadherent cell fractions in the adhesion assays, were 25.9 ± 11.7% and 28.7 ± 10.5%, n=3 respectively. Using $CD34^+$ selected CB cells, the proportion of cells adherent to the ligand was 31.1 ± 6.1%, n=3 to HA, and in two experiments percentage adherence to FN were 24.5% and 37.5%.

The proportion of the CFU-C assayed in both mononuclear and UCB $CD34^+$ cells adherent to either HA or Fn was determined as previously detailed in chapter 6. The calculations are shown in Tables 7.3 and 7.4. Figure 7.3 shows that using UCBMNC there was a two fold enrichment of CFU-C in the HA-adherent fractions (25.9 ± 11.7% adhesion of actual cells compared with 71.2 ± 22.9%, n=3 adhesion of CFU-C, see Table 7.5)

Similarly, the FN-adherent fraction was also enriched for CFU-C (28.7 ± 10.5% for actual cell adhesion and 65 ± 4.2% CFU-C adhesion, Table 7.5). In four assays (Table 7.5) using UCB $CD34^+$ cells the HA-adherent fraction were enriched for CFU-C by more than two fold (31.1 ± 6.1% adhesion of actual cells compared with 71.5 ± 23.4% adhesion of CFU-C, P=0.0156, n=3, Table 7.5, Fig 7.4).

In two experiments in which Fn was used as the substrate, cell adhesion was 24.5% and 37.5%, while CFU-C adhesion was 92.6% and 92.1%). Table 7.5 summarises these findings

TABLE 7.3 Adhesion of I. UCBMNC, II. UCB $CD34^+$ and CFU-C to HA. Percentage adhesion of CFU-C to HA calculated from original cell numbers seeded in the adhesion assay as well as the number of nonadherent cells and the CFU-C output of each fraction.

I. UCBMNC

		Actual no of cells	CFU-C/10^4 cells	Actual no CFU-C	% CFU-C Adherent
1	Original	3.8×10^6	102.3	19,437	100
	nonadherent	3.0×10^6	69.7	10,455	53.8
	adherent			8982	**46.2**
2	Original	1.5×10^6	110	8250	100
	nonadherent	9.1×10^5	16	728	8.8
	adherent			7522	**91.2**
3	Original	10^6	87.5	4375	100
	nonadherent	8.26×10^5	25.2	10408	23.8
	adherent			3334.2	**76.2**

II. UCB $CD34^+$

		Actual no of cells	CFU-C//200 cells	Actual no CFU-C	% CFU-C Adherent
1	Original	2×10^5	114.2	114,200	100
	nonadherent	1.4×10^5	51.7	36190	31.7
	adherent			78010	**68.3**
2	Original	2×10^5	81.7	81700	100
	nonadherent	1.5×10^5	65.7	49275	60.3
	adherent				**39.7**
3	Original	2×10^5	54	54000	100
	nonadherent	1.4×10^5	9.3	6510	12.1
	adherent			47490	**87.9**
4	Original	5.15×10^4	124.5	32058.5	100
	nonadherent	3.11×10^4	20.2	3141.1	9.7
	adherent				**90.3**

Table 7.4 Adhesion of CFU-C to FN. Percentage adhesion of CFU-C to FN was calculated as in Table 7.2

I. UCBMNC

		Actual no of cells	CFU-C/10^4 cells	Actual no CFU-C	% CFU-C Adherent
1	Original	3.8×10^6	102.3	19,437	100
	nonadherent	3.08×10^6	49.3	7592.2	39.1
	adherent			11,844.8	**60.9**
2	Original	4.1×10^5	110	2255	100
	nonadherent	2.47×10^5	56	691.6	30.7
	adherent			1563.4	**69.3**
3	Original	10^6	87.5	4375	100
	nonadherent	7.27×10^5	42.5	1544.9	35.3
	adherent			2830.1	**64.7**

II. UCB $CD34^+$ selected cells

		Actual no of cells	CFU-C//200 cells	Actual no CFU-C	% CFU-C Adherent
1	Original	2×10^5	114.2	114,200	100
	nonadherent	1.5×10^5	11.3	8475	7.4
	adherent				92.6
2	Original	5.15×10^4	124.5	32,058.5	100
	nonadherent	3.22×10^4	15.7	2527.7	7.9
	adherent				92.1

Table 7.5 Data from 3 experiments showing Adhesion of actual UCBMNC and UCBPC as well as their assayed CFU-C to HA and FN

I. **UCBMNC**

	HA		FN	
	Actual cells	CFU-C	Actual cells	CFU-C
1	21.1	46.2	19	60.9
2	39.3	91.2	39.8	69.3
3	17.4	76.2	27.3	64.7
M ± SD	25.9 ± 11.7	71.2 ± 22.9	28.7 ± 10.5	65 ± 4.2

II. **UCB $CD34^+$ selected cells**

	HA		FN	
	Actual cells	CFU-C	Actual cells	CFU-C
1	30	68.3	-	-
2	25	39.7	-	-
3	30	87.9	24.5	92.6
4	39.6	90.3	37.5	92.1
M ± SD	31.1 ± 6.1	71.55 ± 23.4		

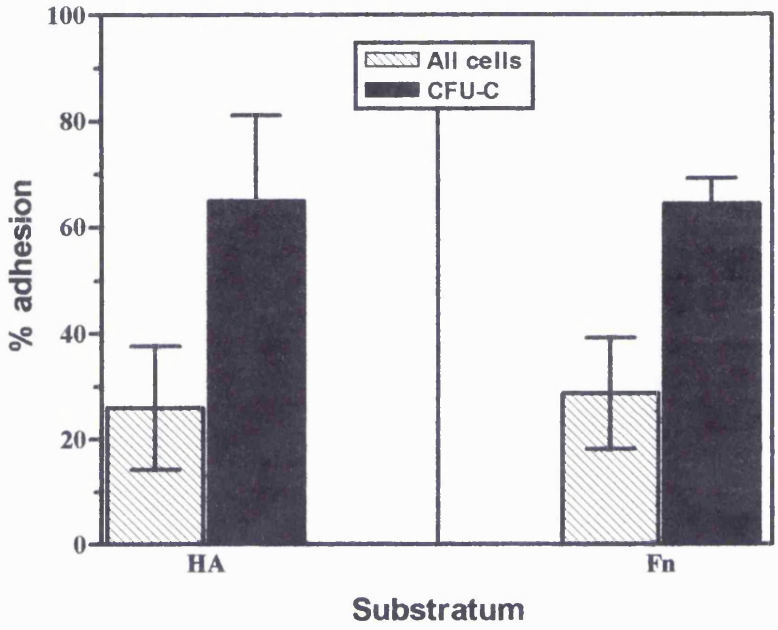


Figure 7.3: Adhesion of UCBMNC as well as the assayed colony forming unit cells to both HA and Fn. The data from 3 experiments are expressed as the mean ± SD. The percentage adhesion of CFU-C to HA and Fn was determined by calculations outlined above. Comparison between all cells and CFU-C adhesion: $P<0.05$ to both HA and Fn

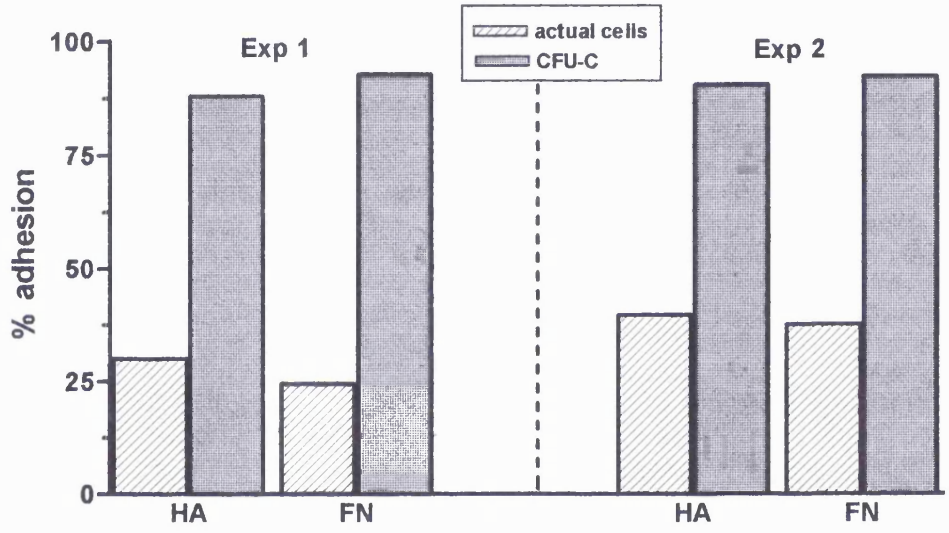


Figure 7.4 Adhesion to HA and Fn by UCB $CD34^+$ selected cells in two experiments. Cells selected were placed on ligand-coated plates, incubated for 1 hour and nonadherent cells then re-plated on methylcellulose. Percentage of adherent CFU-C was then calculated.

7.2.3 Effect of anti-CD44 on adhesion of assayed progenitor CFU-C to HA:

The effect of the specific anti-CD44 moAb on the adhesion of the assayed progenitor CFU-C to both HA and Fn, was examined in both UCBMNC and UCB $CD34^+$ selected cells.

As in preceding experiments the clonogenic output, was not affected by the treatment of the cells with the monoclonal antibody as was demonstrated by an insignificant difference in the numbers of CFU-C generated when aliquots of these cells were pre-incubated with the various mAbs.

Following the adhesion assay, the numbers of CFU-C generated by the nonadherent cell fraction treated with anti-CD44 mAb ($80.2 \pm 26.8/10^4$) were higher than the numbers produced by the corresponding fraction treated with control mAb ($37.0 \pm 28.7/10^4$, $p<0.05$, $n=3$)

Similarly in two experiments using UCB $CD34^+$ cells, the clonogenic output was higher in nonadherent cells treated with anti-CD44 than those treated with control mAb (Table 7.6).

Anti-CD44 mAb reduced the adhesion of UCB CFC to HA, from $71.2 \pm 22.9\%$ to $32.8 \pm 19.8\%$, Table 7.11 and Figures 7.5 and 7.6. The same mAb did not however have any blocking effect on the adhesion of UCB CFU-C to Fn ($64.5 \pm 0.6\%$ with anti-CD44 compared with $65 \pm 4.2\%$ adhesion with control mAb,), Table 7.11 and fig 7.7 thus confirming the specificity of inhibition of CD44 (Table 7.10 and 7.11).

Adhesion assays were undertaken using the heparin binding fragment peptide of fibronectin to assess adhesiveness of both MNC and selected $CD34^+$ cells to the synthetic peptide substratum. However the background adhesion obtained, as in BMMNC was low (<10%) and thus further experiments to evaluate this adhesion pathway were not undertaken.

Table 7.6 Clonogenic output generated by HA and FN nonadherent cell fractions following addition of mAbs .

UCBMNC

Exp No	CLONOGENIC OUTPUT (/ 10^4 cells)			
	HA-nonadherent cells		FN-nonadherent cells	
	CNTRL	anti-CD44	CNTRL	anti-CD44
1	69.7	96.3	49.3	49
2	16	95	56	66
3	25.2	49.2	42.5	45.2
M ± SD	37.0 ± 28.7	80.2 ±26.8	49.3± 6.7	53.40 ± 11.1

UCB $CD34^+$ cells

Exp No	CLONOGENIC OUTPUT (200 cells)			
	HA-nonadherent cells		FN-nonadherent cells	
	control	anti-CD44	control	anti-CD29
1	9.3	22	11.3	7.6
2	20.2	47.2	15.7	17

Table 7.7 Effect of anti-CD44 mAb on the adhesion of UCB CFU-C to HA.

UCBMNC

Exp no			Actual cells	CFU-C/10^4 cells	Actual no CFU-GM	% CFU-C adherent
1	original cell fraction		3.8×10^6	102.3	19,437	100
	Nonadherent fraction	C	3.0×10^6	69.7	10,455	53.8
		CD44	3.57×10^6	96.3	17,189.5	88.4
	adherent fraction	C				**46.2**
		CD44				**11.6**
2	original cell fraction		1.5×10^6	110	8250	100
	Nonadherent fraction	C	9.1×10^5	16	728	8.8
		CD44	1.11×10^6	95	5272.5	63.9
	adherent fraction	C			7522	**91.2**
		CD44			2977.5	**36.1**
3	original cell fraction		10^6	87.5	4375	100
	Nonadherent fraction	C	8.26×10^5	25.2	1040.8	23.8
		CD44	8.77×10^5	49.2	2157.4	49.3
	adherent fraction	C				**76.2**
		CD44				**50.7**

Table 7.8 Two experiments showing the Effect of anti-CD44 on adhesion of CFU-C derived from UCB. $CD34^+$cells to HA and FN.

UCB. $CD34^+$cells

HYALURONIC ACID			Actual no of cells	CFU-C//200 cells	Actual no CFU-C	% CFU-C Adherent
1	Original cell fraction		2×10^5	54	54000	100
	nonadherent fraction	C	1.4×10^5	9.3	6510	12.1
		CD44	1.8×10^5	22	19800	36.1
	adherent fraction	C				**87.9**
		CD44				**63.3**
2	Original cell fraction		5.15×10^4	124.5	32058.5	100
	nonadherent fraction	C	3.11×10^4	20.2	3141.1	97
		CD44	4.77×10^4	47.2	11257.2	35.1
	adherent fraction	C				**90.3**
		CD44				**64.9**
FIBRONECTIN						
1	Original cell fraction		2×10^5	114.2	114,200	100
	nonadherent fraction	C	1.5×10^5	11.3	8475	7.4
		CD29	1.6×10^5	7.6	6080	5.3
	adherent fraction	C				**92.6**
		CD29				**94.7**
2	Original cell fraction		5.15×10^4	124.5	32,058.5	100
	nonadherent fraction	C	3.22×10^4	15.7	2527.7	7.9
		CD29	3.67×10^4	17	3119.5	9.7
	adherent fraction	C				**92.1**
		CD29				**90.3**

Table 7.9 Three experiments showing the Effect of anti-CD29 mAb on the output of CFU-C assayed from UCBMNC after adherence to FN. Percentage Adhesion of CFU-C is calculated as above.

		Actual cells	CFU-C/10^4 cells	Actual no CFU-C	% CFU-C adherent
original cell fraction		3.8×10^6	102.3	19437	100
Nonadherent fraction	C	3.08×10^6	49.3	7592.2	39.1
	CD29	2.84×10^6	49	6958	35.8
adherent fraction	C				**60.9**
	CD29				**64.2**
original cell fraction		4.1×10^5	110	2255	100
Nonadherent fraction	C	2.47×10^5	56	691.6	30.7
	CD29	2.37×10^5	66	782.1	34.8
adherent	C			1558.4	**69.3**
	CD29			1467.9	**65.2**
original cell fraction		10^6	87.5	4375	100
Nonadherent	C	7.27×10^5	42.5	1544.9	35.3
	CD29	6.96×10^5	45.2	1573	36
adherent	C				**64.7**
	CD29				**64**

Table 7.10 Effect of anti-CD44 on the adhesion of actual UCBMNC to HA and FN. Determined from harvested nonadherent cell fractions.

UCBMNC

Exp no	Percent adhesion			
	HA		FN	
	CNTRL	anti-CD44	CNTRL	anti-CD44
1	21.1	5.9	19	25.3
2	39.3	26	39.8	42.2
3	17.4	12.3	27.3	30.4
M ± SD	25.9 ± 11.7	14.7 ± 10.3	28.7 ± 10.5	32.6 ± 8.7

Table 7.11 Effect of anti-CD44 on the adhesion of UCB CFU-C to HA and FN. Percentage adhesion was calculated as shown in table 7.7

UCBMNC

Exp no	Percent adhesion			
	HA		FN	
	CNTRL	anti-CD44	CNTRL	anti-CD44
1	46.2	11.6	60.9	64.2
2	91.2	36.1	69.3	65.2
3	76.2	50.7	64.7	64
M ± SD	71.2 ± 22.9	32.8 ± 19.8	65 ± 4.2	64.5 ± 0.6

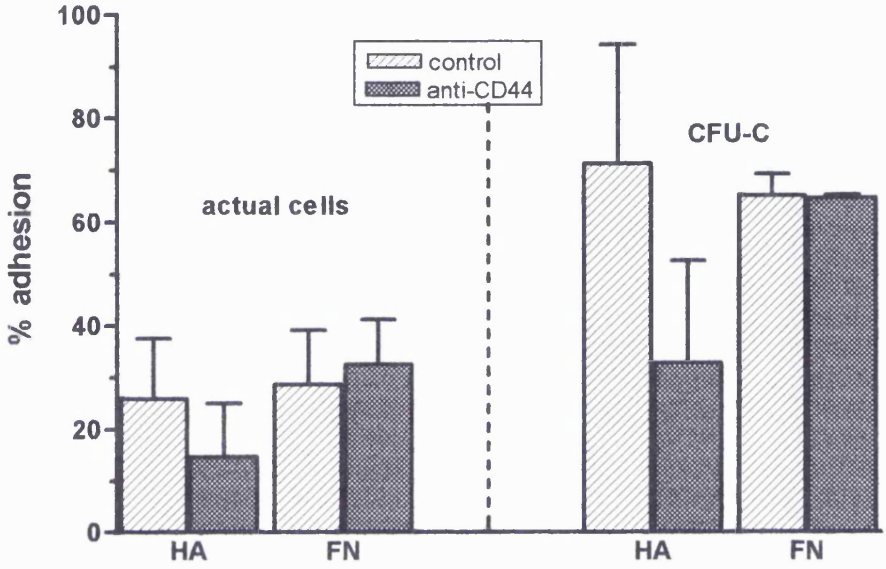


Figure 7.5 Effect of anti-CD44 moAb on adhesion of UCBMNC derived CFU-C to HA and Fn. UCBMNC were incubated with anti-CD44 mAb or IgG as control and then plated onto substrate for 1hour. Nonadherent cells were then re-plated in methylcellulose progenitor cultures. Results from three experiment are expressed as the mean ± SD.

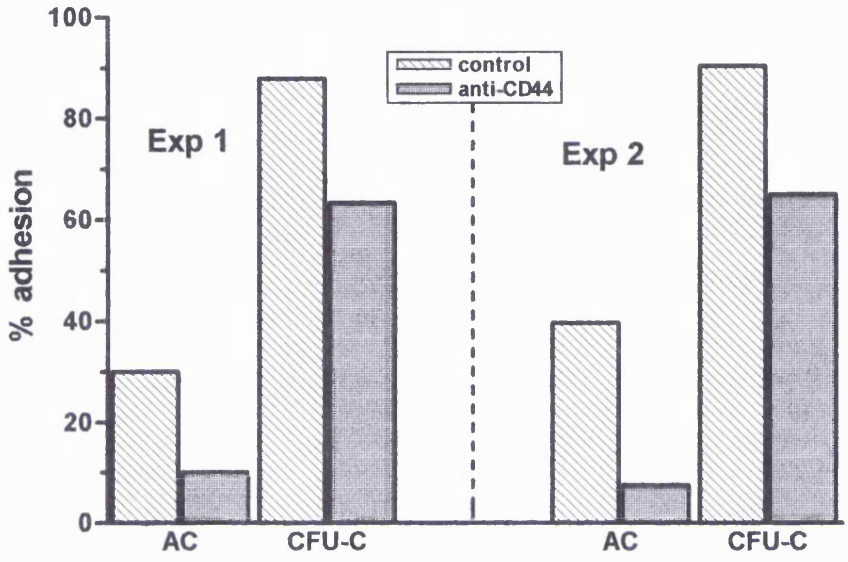


Figure 7.6 Effect of anti-CD44 moAb on adhesion of $CD34^+$ selected CB cells to HA. $CD34^+$ selected CB cells were pre-incubated with the anti-CD44 moAb for 30 minutes at 37^0C and following incubation on substrata, nonadherent cells then re-plated in methylcellulose progenitor cultures. Data from two experiments (AC = Actual cells).

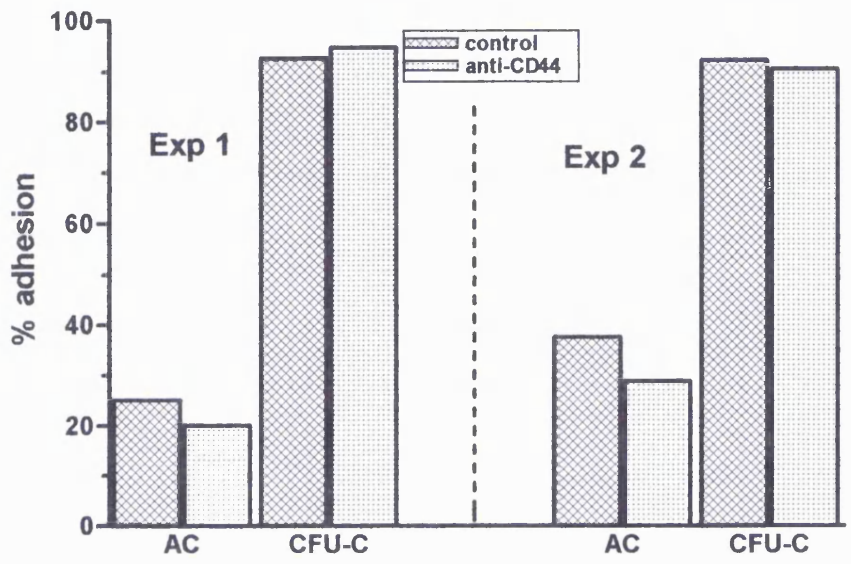


Figure 7.7 Effect of anti-CD44 moAb on adhesion of UCB $CD34^+$ selected cells to Fn, in which following pre-incubation with the anti-CD44 moAb was plated on FN substrata and after incubation, nonadherent cells harvested then re-plated onto methylcellulose cultures. The data is from two separate experiments using different samples of UCB (AC = Actual cells).

7.3 DISCUSSION

The results presented in this chapter show that CB cells adhere to the ligand HA and that this adhesion is mediated in part by the adhesion molecule CD44. Furthermore it has been demonstrated that the clonogenic output of the two CB cell populations is much higher than in bone marrow.

The clonogenic output in mononuclear cells derived from UCB ($99.9 \pm 11.4/10^4$, n=3, $p<0.05$) in all the experiments were much higher than those in adult BM ($23.7 \pm 3.7/10^4$, n=3, $p<0.05$), Table 7.2. In my studies therefore it does appear that UCB contains a higher proportion of committed progenitor cells than adult BM. This confirms previous studies (Steen et al, 1994) in which significantly higher number of CFU-GM and BFU-E were detected in MNC and $CD34^+$ cells derived from UCB and mobilised PB compared with BM.

The adhesion of haemopoietic progenitor cells in this study was investigated in an assay in which CFU-C was used as the end point.
The data in my experiments show that CFU-C progeny generated from UCB adhere to both HA and Fn. While 20-30% of all cells adhered to either HA or FN, the adhesion of CFU-C was much higher (60-90%), suggesting that the committed progeny were enriched in the adherent cell population.

Pre-incubating with a blocking mAb against CD44 produced partial inhibition of both MNC and $CD34^+$ CB cells adhesion to HA but not to FN. However unlike in G-CSF mobilised PB $CD34^+$ cells in which pre-incubation of anti-CD29 mAb produced inhibition of the cells to FN, this effect is not reproduced in UCB derived cells. It is difficult to speculate why the anti-β_1 integrin mAb did not block the adhesion of UCB derived cells to FN.

These results suggest a role for the CD44 receptor on UCB cells in mediating adhesion of these progenitor cells to HA. The observed inhibitory effect is only partial, suggesting involvement of other adhesion pathways in mediating adhesion of CB derived HPCs to HA.

Although there are studies suggesting a role for CD44 in adhesive interactions involving fibronectin, the data presented in my experiments using CB cells and the generated HPCs did not demonstrate any link between the CD44 receptor and fibronectin. However further studies using the fibronectin peptide fragment would have provided more conclusive information, however as in previous experiments use of the commercial peptide as substratum did not result in enough adhesion by the CB cells in order to permit further evaluation of this adhesion pathway.

CHAPTER 8 FINAL DISCUSSION

8.1 INTRODUCTION

It is speculated that homing represents a step wise process which ultimately results in permanent lodgement of transplanted haemopoietic progenitors introduced into circulation and subsequently establishment of long-term haemopoiesis (Hardy et al, 1995). Furthermore studies of interaction of HPC's with components of haemopoietic microenvironment has defined numerous cytoadhesion molecules which mediate adhesion of progenitors to the BM stroma in vitro (Gordon et al, 1990). The relevance of this in vitro observation to the in vivo homing process remains unexplained.

The studies presented in this thesis therefore aim to investigate as well as outline some of these adhesive interactions of HSC with ECM components and stroma with particular emphasis on the CD44 dependent pathways.

8.2 Adhesion of haemopoietic cell line, KG1a, to HA , BMS and BMEC

The initial studies in this thesis focused on evaluating the adhesion of the human haemopoietic cell line KG1a cell, to the ECM components HA and FN and to bone marrow stroma (BMS) and endothelial cells (BMEC).

The functional binding behaviour of KG1a cells was assessed using ^{51}chromium labelling assay. Adhesion studies showed strong binding of the cell line to both BMS and BMEC and less so to the ECM ligands HA and FN. Adhesion blockade studies showed partial inhibition by both anti-CD44 and anti-CD29 mAbs to HA and FN, respectively.

The complexity underlining the haemopoietic cell line-stromal interaction leading to adhesion of these cells has been demonstrated in the thesis (Chapter 4), where a blocking strategy using multiple antibodies, principally, against CD44 and β_1 integrin did not result in blockade of the adhesion of the primitive myeloid cell, KG1a to stromal monolayers. Similar findings were obtained when the adhesion surface (stromal monolayer) was ligand depleted by enzymatic treatment with hyaluronidase. These findings would also suggest that, at least, in vitro adhesion of the two assayed stem cell populations does require participation of a number of other adhesion pathways.

Some of these adhesion pathways have been extensively evaluated and their role in mediating stem cell adhesion is now much more understood and defined. Foremost of these adhesion pathways is the VLA-4/VCAM interaction which mediates HPC binding to stroma (Oostendorp et al, 1997). The critical role of the other integrins such as VLA-5 and β_2 integrin as mediators of critical stem cell-stromal interaction in normal haemopoiesis has also been underscored (Kerst et al, 1993). Detailed discussion of the interaction of these adhesion receptors are covered in the general introduction.

8.3 Adhesion of LTC-IC and PΔ cells to HA

It has been shown in this thesis that a modified limiting dilution culture using both standard long-term culture initiating cell assays as well as assays of plastic adherent progenitors in delta type cultures can provide qualitative and quantitative information about stem cell numbers and identity. It has further been demonstrated that both the plastic-adherent cells as well as LTC-initiating cells in human bone marrow and peripheral blood are capable of sustained haemopoiesis for at least 5 weeks in vitro and producing clonogenic progeny.

They thus have been used to study the adhesive characteristic of early human haemopoietic stem cells. The absolute numbers or frequencies of LTC-IC and PΔ stem cells enumerated in BMMNC by limiting dilution analysis were 2.8 ± 0.2 and 5.3 ± 2.2 respectively. The mean numbers of stem cells obtained using the two different assay systems didn't differ significantly, $P > 0.05$.

Since only one type of stromal feeder was used in these LTC assays, it was not possible to explore whether the lack of success observed in assaying of LTC-IC in $CD34^+$ cells population was in fact dependent on the stromal feeder as suggested in an abstract by Punzel M et al (Abstract, ASH 1997). On the other hand PΔ stem cells were successfully assayed in peripheral blood derived progenitor cells.

Assays of HA-nonadherent cells in both human bone marrow and peripheral blood derived progenitor cells produced lower absolute numbers of both LTC-IC and adherent PΔ cells relative to the numbers obtained in the original cell population in respective samples. The numbers of LTC-IC and PΔ stem cells present in the adherent cell fraction, calculated as outlined in chapter 5 showed enrichment of each of the two stem cell populations in the HA-adherent fraction, giving a percentage adhesion of 47.8% for LTC-IC and 42.1% for PΔ cells.

The specificity of the binding to HA of the two candidate stem cells was confirmed by the finding that anti-CD44 moAb partially blocked the adhesion of these cells to HA. On the other hand anti-CD29 mAb (β_1 integrin) did not have any effect on the adhesion of the 'stem cells' to HA.

8.4 Adhesion of Peripheral and Umbilical cord blood derived progenitor cells to HA.

Umbilical cord blood of full-term new-borns contains high concentrations of lineage-restricted, as well as primitive pluripotential haemopoietic progenitor cells (Traycoff et al, 1989). In addition, cord blood of term new-borns contains sufficient numbers of reconstituting haemopoietic stem cells to achieve haemopoietic engraftment in children (Kohli-Kumer et al, 1993).

Interest in the underlying mechanisms involved in the adhesion and homing of these cord blood derived progenitor cells has been generated by the increasing use of the cord blood derived progenitors and stem cells in transplantation.

In this thesis the clonogenic potential as well as the adhesiveness of human umbilical cord blood progenitor cells in mononuclear cell and $CD34^+$ selected cell fractions as well as PB $CD34^+$ selected cell, to specified purified ligands, were evaluated.
The clonogenic output generated by human UCBMNC was more than four fold higher than that in the adult BMMNC population (99.9 ± 11.4 and 23.7 ± 3.7 $/10^4$ cells, respectively).
The higher clonogenic output generated in UCB MNC as well as $CD34^+$ selected cells compared with adult BM indicate the presence of a higher number of haemopoietic progenitor cells in UCB compared to adult BM, thus confirming the findings obtained in similar studies (Traycoff et al, 1996)

The adhesion of granulocyte and macrophage (CFU-GM) progeny assayed from PB $CD34^+$ selected cell, to HA (68.2 ± 6.6) was similar to that of the clonogenic cells (CFU-C) assayed in UCB mononuclear (71.2 ± 22.9) as well as $CD34^+$ selected cells (71.55 ± 23.4).

The CFU-C progeny were thus enriched in both the HA and FN-adherent fractions of UCB mononuclear and $CD34^+$ selected cell populations. The adhesion of both CFU-C generated from UCB as well as CFU-GM generated from PB $CD34^+$ selected cell was partially blocked by the anti-CD44 monoclonal antibody. Similarly, the anti-integrin β_1 antibody blocked the adhesion of CFU-C to Fn with the anti-CD44 mAb showing no inhibition of the adhesion of these cells to FN.

8.5 Interaction of CD44 with adhesion receptors

Although the CD44 adhesion receptor is thought to be involved mainly in cell-extracellular matrix interactions, it is increasingly being shown that the receptor could also be involved in cell-cell interactions.

Haemopoietic progenitor-microenvironment interactions are probably required to regulate proliferation and differentiation of the Haemopoietic stem cells and their more committed progeny resulting in an ordered progression of haemopoiesis.

Cell-cell interactions involving the CD44 receptor was shown in various studies. In one study the receptors involved in the adhesion of CFC to peptides FN-C/H-1, FN-C/H-II and CS-1 (Verfaillieet al, 1994) were studied and results suggested that adhesion of committed myeloid progenitor to the FN-C/H-1 peptide (McCarthy et al, 1990) in the C-terminal heparin-binding domain of fibronectin required a co-ordinated action of both CD44/CS proteoglycan and $\alpha_4\beta_1$ integrin. It was shown in this study that the two anti-CD44 antibodies, hermes III (Jalkanen et al, 1987) and 50B4 (Letarte et al, 1985) inhibited adhesion to FN-C/H-1, as well as anti-$\alpha_4\beta_1$ antibodies.

In this study the anti-CD44 antibody 50B4 potentiated the anti-$\alpha_4\beta_1$ integrin-mediated inhibition of progenitor adhesion to the CS-1 peptide, to which the $\alpha_4\beta_1$ integrin bound, this despite the fact the anti-CD44 antibody did not directly inhibit progenitor adhesion to CS1 and CS1 did not bind the CD44 CS proteoglycan. This receptor-receptor co-operation in the cellular adhesion events may play a crucial role in Haemopoietic progenitor cell proliferation and differentiation

8.6 CD44 and Signalling

The growth and differentiation of hematopoietic stem cells are highly dependent on regulatory molecules produced by stromal cells of the marrow environment. Evidence has accumulated over the past years which shows that adhesive receptors on hematopoietic cells and their ligands on stromal cells and extracellular matrix play a crucial role in these interactions.

Adhesion has an important role in regulating cellular positioning, but there is also compelling evidence that information transduced via adhesion molecules may alter cell survival, proliferation and differentiation.

Adhesion receptors can serve as primary signal transduction molecules that convey information into cells that can affect cell proliferation and differentiation.
Many of these adhesion receptors, including integrin and non-integrin receptors such as CD44, PECAM-1 and ICAM-1 involved in adhesive interactions are expressed on hematopoietic progenitor cells and tightly regulated during differentiation but their function is still controversial. The interaction of integrins with their ligands can invoke intracellular signals inducing many of these changes (Hynes, 1992).

Since hematopoietic progenitors adhere to marrow stroma and fibronectin via the alpha 4 beta 1 integrin and CD44, various groups have examined the role of these receptors in the transfer of proliferation-regulatory signals to progenitors following interaction with their specific ligands.

In one of these studies the group examined whether HA-CD44 binding promoted the proliferation of eosinophil precursor cells. Using UCB $CD34^+$ cells, they demonstrated a concentration-related increase in proliferation of $CD34^+$ progenitors stimulated initially with IL-3 and IL-5, this proliferative effect was blocked by pre-treatment of HA-coated plates with hyaluronidase. The augmented production of eosinophil precursors caused by HA was CD44 receptor mediated, as shown by the complete attenuation of proliferation following pre-treatment with anti-CD44 mAbs (Hamann et al, 1995)

Indeed, evidence is emerging suggesting the role of CD44 and other adhesion receptors in signal transduction cascades that impinge on the regulation of cell growth and differentiation (Lesley et al, 1993).

In B-Lymphopoiesis bone marrow microenvironment influences whether a given B cell proliferates, differentiates or undergoes apoptosis. The adhesive ligands which are expressed on the membrane of stromal and other endothelial cells in the marrow may contribute to lymphopoietic regulation.

Transmembrane proteins such as VCAM-1 and CD44 present on the surface serve not only to retain maturing B cells in the marrow (Jacosen et al, 1996) but may also provide survival signals.

This is evidenced by the fact that addition of anti-CD44 to LTBMC abrogated lymphopoiesis (Miyake et al, 1990), and disruption of VCAM-1/VLA-4 or ICAM-1/LFA-1 interaction increased levels of apoptosis in tonsillar germinal centre B cells (Koopman et al, 1994).

8.7 CD44 as a 'homing receptor'

The evidence for the role of CD44 as a 'homing receptor' comes from the demonstration that anti-CD44 mAb (e.g. Hermes-1) inhibits binding of human lymphocytes to lymph node HEV (Jalkanen et al, 1987; Pals et al, 1989a). In murine studies, depletion of CD44 positive cells from the bone marrow by anti-CD44 mAb prevented their ability to reconstitute the thymus of irradiated mice (O'neill, 1989). In contrast lymphocyte migration into lymph nodes and other organs was normal, despite the removal of the CD44 receptor containing cells, by anti-CD44 mAb treatment (Camp et al, 1993).

CD44 may also be involved in the interaction of lymphocytes with inflamed endothelium, and in the subsequent locomotion into the ECM after lymphocytes have traversed to vascular endothelium (Camp et al, 1993).

The role of integrins as a homing receptors has been shown in a number of experiments. In one of these experiments, it was shown that pre-treatment of animals with anti-VCAM-1 antibodies produced a reduction in the lodgement of transplanted progenitor cells, similarly anti-VLA-4 antibodies blocked this lodgement, thus demonstrating the importance of the ligand VCAM-1 in VLA-4-mediated progenitor homing (Papayannopoulou et al, 1995).

8.8 Other ligands for CD44

Recently it has been reported that chondroitin sulfate-type serglycin is a novel ligand for CD44 (Toyama-sorimachi et al, 1995). Serglycin is a secretory granule proteoglycan whose mRNA is transcribed in haemopoietic lineage cells (Stevens rl, et al, 1988).

The function of serglycin remains to be fully determined. However among other things it has been suggested that it is involved in myeloid cell differentiation (Matsumoto R et al, 1990). Studies of the extent of expression of CD44-binding serglycin in haemopoietic cells (Toyama-sorimachi et al, 1997) and characterisation of their chain showed that the chondroitin sulfate-type serglycin capable of binding CD44 is secreted by a wide range of haemopoietic cells.
The production of the CD44-binding serglycin by a variety of haemopoietic cells implies that serglycin may serve as a major ligand for CD44 in the haemopoietic system.

It has also been reported that an anti-CD44mAb that binds to the common region of CD44 (Peach et al, 1993), but does not inhibit HA-binding (Culty et al, 1990), can inhibit stromal cell-dependent haemopoiesis in vitro (Gunji et al, 1992). Some of the anti-CD44 mAbs recognising the HA-binding site of CD44 have also been found to block serglycin binding (Toyama-sorimachi et al, 1995).

Antibody mediated interference with CD44 binding to any one of several ECM components may result in either promotion or prevention of the cross-linking of multiple CD44 molecules on the cell surface, thereby resulting in the blocking (or initiation) of important intracellular signalling events. It is known that CD44 is capable of homotypic interactions (Droll et al, 1995) and that CD44 binding may lead to intracellular signalling following as yet undefined interactions with components of the cytoskeleton (Lesley et al, 1993)

Therefore evidence regarding involvement of the CD44 receptor in various biological processes such as homing, proliferation, differentiation and survival is now accumulating. However insight into the mode in which CD44 communicates with intracellular signalling pathways is still lacking.

8.9 CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis therefore it has been established that both LTC-Initiating cell and delta assay for plastic adherent PΔ progenitor cell assays can be used to quantify HSC in different cell populations and that a large proportion the LTC-IC, in bone marrow, and plastic adherent PΔ progenitor cell population in both the BM and PB $CD34^+$ selected cell populations are adherent to HA and that this is dependent at least in part on the CD44 receptor. CD44 seems to also play a role in mediating progenitor cell-HA interactions in both UCB and PB $CD34^+$ selected cells.

The KG1a cell line can be used effectively to screen both blocking as well as activating monoclonal antibodies directed against the CD44 molecule.

However the use of blocking antibodies or other reagents against single adhesion molecules resulted in only partial inhibition of adhesion. This therefore confirms the assertion that several adhesion pathways may be involved in HPC-stromal interactions even in an in vitro model of adhesion.

The role of the HA-CD44 interaction as well as the interaction of the CD44 adhesion receptor with other non-ligands such as serglycin in mediating various haemopoietic processes remains largely presumptive and further studies are needed to determine how CD44 partcipates with intracellular signalling pathways to influence the survival, proliferation and differentiation of haemopoietic cells.

9. BIBLIOGRAPHY/APPENDICES AND PRESENTATION RELATED TO THE THESIS.

9.1 BIBLIOGRAPHY

Albelda SM. Buck CA (1990) Integrins and other cell adhesion molecules. FASEB Journal. 4(11):2868-80.

Albelda SM, Muller WA, Buck CA and Newman PJ (1991) Molecular and cellular properties of PECAM-l (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. Journal of cell biology 114:1059-1068.

Anklesaria P, Greenberger JS, Fitzgerald TJ, Sullen-berger B, Wicha M, and Campbell A (1991) Hemonectin mediates adhesion of engrafted murine progenitors to a clonal bone marrow stromal cell line from SI/Sld mice. Blood 77: 1691-1698.

Arnaout AM (1990) Structure and function of the leukocyte adhesion molecules CD11 /CD18. Blood. 75: 1037-50.

Arkin S. Ferrone S. Lipton JM (1995) Modulation of adhesion molecule CD54 expression on CD34+ hematopoietic progenitors. Experimental Haematology. 23(7):588-96.

Arkin S. Ferrone S. Lipton JM, Naprstek B, Guarini L (1991) Expression of intracellular adhesion molecule-l (CD54) on haemopoietic progenitors. Blood 77(5):948-953.

Aruffo A. Stamenkovic I. Melnick M. Underhill CB (1990) Seed B. CD44 is the principal cell surface receptor for hyaluronate. Cell. 61(7):1303-13.

Asa D, Raycroft L, Ma L, Aeed PA, Kaytes PS, Elhammer AP, and Geng JG (1995) The P-selectin protein ligand function as common human leukocyte ligand for P- and E-selectin. Journal of biological chemistry 270, 11662-11670.

Bentley SA (1984) The role and composition of the adherent layer in long-term bone marrow culture. Kroc Foundation Series. 18: 141-56.

Bentley SA (1984) Studies of bone marrow stromal cells in vitro. Progress in Clinical & Biological Research. 154: 179-207.

Bentley SA, Tralka TS (1983) Fibronectin-mediated attachment of hematopoietic cells to stromal elements in continuous bone marrow culture. Experimental Hematology. 11 (2): 129-38.

Bentley SA, Alabaster O, Foidart JM (1981) Collagen heterogeneity in normal human bone marrow. British Journal of Haematology. 48(2):287-91.

Bentley SA (1981) Close range cell:cell interaction required for stem cell maintenance in continuous bone marrow culture. Experimental Hematology. 9(3):308-12.

Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD Bernstein ID (1988) Antigen $CD34^+$ marrow cells engraft lethally irradiated baboons.Journal of Clinical Investigation. 81(3):951-5.

Bevilacqua M, Butcher E, Furie B (1991) Selectins: A family of adhesion receptors. Cells 67: 233.

Bevilacqua M and Nelson RM (1993) Selectins. Journal of clinical investigation. 91: 379-387.

Bhatia R, Wayner EA, McGlave PB, Verfaillie CM (1994) Interferon-a restores normal adhesion of chronic myelogenous leukaemia hematopoietic progenitors to bone marrow stroma by correcting impaired ,tl 1 integrin receptor function. Journal of Clinical Investigation. 94:384-91.

Blackett NM. Gordon MY (1996) Optimising limiting dilution assays: frequency and 'ability' measurements of haemopoietic progenitor cells. British Journal of Haematology. 92(3):507-13.

Brandt J. Srour EF. van Besien K. Briddell RA (1990) Hoffman R. Cytokine-dependent long-term culture of highly enriched precursors of hematopoietic progenitor cells from human bone marrow. Journal of Clinical Investigation. 86(3):932-41.

Brown TA. Bouchard T. St. John T. Wayner E. Carter WG (1991) Human keratinocytes express a new CD44 core protein (CD44E) as a heparan-sulfate intrinsic membrane proteoglycan with additional exons. Journal of Cell Biology. 113(1):207-21.

Broxmeyer HE, Hangoc G, Cooper S, Ribeiro RC, Graves V, Yoder M, Wagner J, Vadhan-Raj S, Benninger L, Rubinstein P (1992) Growth characteristics and expansion of human umbilical cord blood and estimation of its potential for transplantation in adults. Proceedings of the National Academy of Sciences of the United States of America. 89(9):4109-13.

Camp RL. Scheynius A. Johansson C. Pure E (1993) CD44 is necessary for optimal contact allergic responses but is not required for normal leukocyte extravasation. Journal of Experimental Medicine. 178(2):497-507.

Campbell AD, Long MW, Wicha MS (1990) Developmental regulation of granulocytic cell binding to hemonectin. Blood. 76(9):1758-64.

Campbell AD, Wicha MS (1988) Extracellular matrix and the hematopoietic microenvironment. Journal of Laboratory & Clinical Medicine. 112(2):140-6.

Campbell AD, Long MW, Wicha MS (1987) Haemonectin, a bone marrow adhesion protein specific for cells of granulocyte lineage. Nature 329(6141):744-6.

Cashman JD, Lapidot T, Wang JC, Doedens M, Shultz LD, Lansdorp P, Dick JE, Eaves CJ (1997) Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. Blood. 89(12):4307-16.

Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-la cells (1984) Journal of Immunology. 133(1): 157-65.

Clark BR, Gallagher JT, Dexter TM (1992) cell adhesion in the stromal regulation of haemopoiesis. Baillière's clinical haematology.

Culty M. Miyake K. Kincade PW. Sikorski E. Butcher EC. Underhill C. Sikorski E (1990) The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins. Journal of Cell Biology. 111(6 Pt 1):2765-74.

Craig W, Kay R, Cutler RL, Lansdorp PM (1993) Expression of Thy-1 on human hematopoietic progenitor cells. Journal of Experimental Medicine. 177(5):1331-42.

Dexter TM. Allen TD. Lajtha LG (1978) Factors controlling the proliferation of haemopoietic stem cells in vitro. Symposium of the Society for Developmental Biology. (35):149-60.

Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling the proliferation of haemopoietic stem cells in vitro. Journal of Cellular Physiology. 91(3):335-44.

Dexter TM, Wright EG, Krizsa F, Lajtha LG (1977) Regulation of haemopoietic stem cell proliferation in long term bone marrow cultures. Biomedicine. 27(9-10):344-9.

Dexter TM (1979) Haemopoiesis in long-term bone marrow cultures. A review. Acta Haematologica. 62(5-6):299-305.

Dexter TM, Heyworth C, Spooncer E (1985) Studies on the self renewal and differentiation of stem cells in Long-term marrow cultures. Progress in clinical and biological research. 235-46.

Dougherty G J, Cooper D L, Memory J F, Chiu R K (1994) Ligand binding specificity of alternatively spliced CD44 isoforms. Recognition and binding of hyaluronan by CD44Rl . Journal of biological Chemistry 269: 9074-9078.

Droll A. Dougherty ST. Chiu RK. Dirks JF. McBride WH. Cooper DL. Dougherty GJ (1995) Adhesive interactions between alternatively spliced CD44 isoforms. Journal of Biological Chemistry. 270(19):11567-73.

Eaves CJ, Sutherland HJ, Udomsakdi C, Lansdorp PM, Szilvassy SJ, Fraser CC. Humphries RK, Barnett MJ, Phillips GL, Eaves AC (1992) The human hematopoietic stem cell in vitro and in vivo. Blood Cells. 18(2):301-7.

Eaves CJ, Cashman JD, Eaves CA (1991) Methodology of long-term culture of human hemopoietic cells. Journal of tissue culture methods. 13 :55-62.

Elenius K and Jalkanen M (1994) Function of the syndecans: a family of cell surface proteoglycans. Journal of Cell Science. 107: 2975-2982.

Frenette PS, Mayadas TN, Rayburn H, Hynes RO and Wagner DD (1996) Susceptibility to infection and altered haemopoiesis in mice deficient in both P- and E-selectin. Cell 84:563-574.

Gallagher JT. Morris AJ. Dexter TM (1987) Developmentally-related changes in surface membrane glycopeptides of murine haemopoietic cells. Biochemical Journal. 242(3):857-65.

Gallagher JT, Spooncer E, Dexter TM (1983) Role of the extracellular matrix in haemopoiesis. 1. Synthesis of glycosammnoglycans by mouse bone marrow cell cultures. Journal of Cell Science 63: 155.

Gartner S, Kaplan HS (1980) Long-term culture of human bone marrow cells. Proceedings of the National Academy of Sciences of the United States of America. 77(8):4756-9.

Ghaffari GJ, Dougherty PM, Lansdorp, Eaves AC, Eaves CJ (1995) Differentiation-associated changes in CD44 isoform expression during normal hematopoiesis and their alteration in chronic myeloid leukaemia. blood 86(8): 2976-2985.

Ghaffari GJ, Dougherty PM, Lansdorp, Eaves AC, Eaves CJ (1997) Diverse effects of anti-CD44 antibodies on the stromal cell-mediated support of normal but not leukaemic (CML) haemopoiesis in vitro. British journal of haematology 97:22-28.

Goldstein LA. Zhou DF. Picker LJ. Minty CN. Bargatze RF. Ding JF. Butcher EC (1989) A human lymphocyte homing receptor, the hermes antigen, is related to cartilage proteoglycan core and link proteins. Cell. 56(6): 1063-72.

Gordon MY. Lewis JL. Scott MA. Roberts IA. Goldman JM (1995) Probability of progenitor renewal (PPR) and production of clonogenic progeny (CFU-GM) by

primitive adherent progenitor cells in adult human bone marrow and umbilical cord blood. British Journal of Haematology. 90(4):744-9.

Gordon MY. Blackett NM. Lewis JL. Goldman JM (1995) Evidence for a mechanism that can provide both short-term and long-term haemopoietic repopulation by a seemingly uniform population of primitive human haemopoietic precursor cells. Leukemia. 9(7): 1252-6.

Gordon MY (1994) Plastic-adherent cells in human bone marrow generate long-term hematopoiesis in vitro. Leukemia. 8(5):865-70.

Gordon MY, Blackett NM (1994) Routes to repopulation--a unification of the stochastic model and separation of stem-cell subpopulations. Leukemia. 8(6): 1068-72; discussion 1072-3.

Gordon MY, Amos TA (1994) Stochastic effects in hemopoiesis. Stem Cells. 12(2): 175-9.

Gordon MY, Lewis JL, Grand FH, Marley SB, Goldman JM (1996) Phenotype and progeny of primitive adherent human hematopoietic progenitors. Leukemia. 10(8):1347-53.

Gordon MY, Riley GP, Watt SM, Greaves MF (1987) Compartmentalisation of a haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature. 326(6111):403-5.

Gordon MY (1993) Human haemopoietic stem cell assays. Blood Reviews. 7(3): 190-7.

Gordon, M.Y, Dowding C.R, Riley G.P. & Greaves M.F (1987) Characterisation of stroma-dependent blast colony-forming cells in human marrow. Journal of cellular physiology, 130, 150-156.

Gordon MY. Riley GP. Clarke D (1988) Heparan sulfate is necessary for adhesive interactions between human early hemopoietic progenitor cells and the extracellular matrix of the marrow microenvironment. Leukemia. 2(12):804-9.

Gordon MY (1988) Extracellular matrix of the marrow microenvironment. British Journal of Haematology. 70(1):1-4.

Gordon MY, Dowding CR, Riley GP, Goldman JM. & Greaves MF (1987) Altered adhesive interactions with marrow stroma of haemopoietic progenitor cells in chronic myeloid leukaemia. Nature. 328, 342-344.

Gordon MY, Dowding CR, Riley GP. & Greaves MF (1987) Characterisation of stroma-dependent blast colony-forming cells in human marrow. Journal of Cellular Physiology, 130, 150-156.

Gordon MY (1988) The origin of stromal cells in patients treated by bone marrow transplantation. Bone Marrow Transplantation. 3(4):247-51.

Gordon MY (1988) Adhesive properties of haemopoietic stem cells. British Journal of Haematology. 68(2):149-51.

Gordon MY Clarke D Atkinson J Greaves MF (1990) Hemopoietic progenitor cell binding to the stromal microenvironment in vitro. Experimental Hematology. 18(7):837-42.

Greenberger JS, Sakakeeny M, Parker LM (1979) In vitro proliferation of hemopoietic stem cells in long-term marrow cultures: principles in mouse applied to man. Experimental Hematology. 7 Suppl 5: 135-48.

Griffin JD, Spertini O, Ernst TJ, Belvin MP, Levine HB, Kanakura Y, Tedder TF (1990) Granulocyte-macrophage colony-stimulating factor and other cytokines regulate surface expression of the leukocyte adhesion molecule LAM-I on human neutrophils, monocytes and their precursors. Journal of Immunology 145: 576-584.

Guan JL, Trevithick JE, Hynes RO (1991) Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. Cell Regulation. 2(11):951-64.

Guan JL, Hynes RO (1990) Lymphoid cells recognise an alternatively spliced segment of fibronectin via the integrin receptor alpha 4 beta 1. Cell. 60(1):53-61.

Gunji Y, Nakamura M, Hagiwara T, Hayakawa K, Matsuhita H, Osawa H Nagayoshi K, Nakauchi H, Yanagisawa M, Miura Y (1992) Expression and function of adhesion molecules on human hematopoietic stem cells: CD34+ LFA-l cells are more primitive than CD34+ LFA-l+ cells. Blood. 80(2):429-36.

Hamann JK, Dowling TL, Neeley SP, Grant JA, Leff RA (1995) Hyaluronic acid Enhances cell proliferation during Eosinopoiesis through the CD44 surface Antigen. Journal of immunology 154 (8):4073-80.

Hardy CL. Minguell JJ (1995) Modulation of the adhesion of hemopoietic progenitor cells to the RGD site of fibronectin by interleukin 3. Journal of Cellular Physiology. 164(2):315-23.

Hardy CL (1995) The homing of hematopoietic stem cells to the bone marrow. [Review]. American Journal of the Medical Sciences. 309(5):260-6.

Haugen PK, McCarthy JB, Skubitz AP, Furcht LT, Letourneau PC (1990) Recognition of the A chain carboxy-terminal heparin binding region of fibronectin involves multiple sites: two contiguous sequences act independently to promote neural cell adhesion. Journal of Cell Biology. 111(6 Pt 1):2733-45.

Heider KH, Hofmann E, Horst F, van den Berg H, Ponta, P. Herrlich. and S. T. Pals (1993) A human homologue of the rat metastasis-associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. Journal of cell biology. 120: 227-233.

Hofmann MW, Rudy M, Zoller C, To H, Ponta P, Herrlich U, Gunthert (1991) CD44 splice variants Confer rnetastatic behaviour in rats: homologous sequences are expressed in human tumour cell lines. Cancer research. 51:5292-5297.

Hows JM, Bradley BA, Marsh JC, Luft T, Coutinho L, Testa NG, Dexter TM (1992) Growth of human umbilical-cord blood in long-term haemopoietic cultures. Lancet. 340(8811):73-6.

Huang S, Terstappen LW (1994) Lymphoid and myeloid differentiation of single human $CD34^+$, $HLA\text{-}DR^+$, $CD38^-$ hematopoietic stem cells. Blood. 83(6):1515-26.

Hunter T. Cooper JA (1985) Protein-tyrosine kinases [Review]. Annual Review of Biochemistry. 54:897-930.

Hynes RO (1992) Integrins: versatility, modulation, and signalling in cell adhesion. Cell. 69(1): 11 -25.

Hynes RO. Fibronectin. New York: springer-Verlag, 1990

Jalkanen S. Bargatze RF. de los Toyos J. Butcher EC (1987) Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85-95-kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal, or synovial endothelial cells. Journal of Cell Biology. 105(2):983-90.

Katoh S, Zheng Z, Oritani K, Shimozato T, KincadePW (1995) Glycosylation of CD44 negatively regulates its recognition of hyaluronan. Journal of Experimental Medicine. 182(2):419-29.

Kawashima I, Zanjani ED, Almaida-Porada G, Flake AW, Zeng H, Ogawa M (1996) $CD34^+$ human marrow cells that express low levels of Kit protein are enriched for long-term marrow-engrafting cells. Blood. 87(10):4136-42.

Keating A, Gordon MY (1988) Hierarchical organisation of hematopoietic microenvironment: role of proteoglycans. Leukemia. 2(11):766-9.

Kerk DK, Henry EA, Eaves AC, Eaves CJ (1985) Two classes of primitive pluripotent hemopoietic progenitor cells: separation by adherence. Journal of Cellular Physiology. 125(1): 127-34.

Kerst JM, Sanders JB, Slaper-Cortenbach IC, Doorakkers MC, Hooibrink B, van Oers RH, von dem Bome AE, van der Schoot CE (1993) Alpha 4 beta 1 and alpha 5 beta 1 are differentially expressed during myelopoiesis and mediate the adherence of human CD34+ cells to fibronectin in an activation-dependent way. Blood. 81(2):344-51.

Kim CH, Pelus LM, White JR, Broxmeyer HE (1998) Macrophage-inflammatory protein-3 beta/EBI1-ligand chemokine/CK beta-11, a CC chemokine, is a chemoattractant with a specificity for macrophage progenitors among myeloid progenitor cells. Journal of Immunology. 161(5):2580-5.

Kincade PW, Lee G, Pietrangeli CE, Hayashi S, Gimble JM (1989) Cells and molecules that regulate B lymphopoiesis in bone marrow. Annual Review of Immunology. 7:111-43.

Kirby SL, Bentley SA (1991) Xyloside effects on in vitro hematopoiesis: functional and biochemical studies. Journal of Cellular Physiology. 148(1): 116-23.

Klein G (1995)The extracellular matrix of the hematopoietic microenvironment. Experientia. 51(9-10):914-26.

Klein G, Muller CA, Tillet E, Chu ML, Timple R (1995) Collagen type VI in the human bone marrow microenvironment: a strong cytoadhesive component. Blood. 86(5): 1740-8.

Klein G, Muller C, Beck S (1993) Tenascin is a cytoadhesive extracellular matrix component of the human hematopoietic microenvironment. Journal of cell biology 123: 1027.

Koeffler HP, Billing R, Lusis AJ, Sparkes R, Golde DW (1980) An undifferentiated variant derived from the human acute myelogenous leukaemia cell line (KG-1). Blood 56:265.

Koenig L, Baron S, Berenson R, Heimfeld S, Deisseroth AB (1994) L-selectin is associated with higher plating efficiencies of clonogenic progenitor cells, and is present at higher levels in CD34+ cord blood cells and chronic myelogenous leukaemia cells than in normal marrow or peripheral blood cell. Blood 84:569.

Koenigsmann M. Griffin JD. DiCarlo J. Cannistra SA (1992) Myeloid and erythroid progenitor cells from normal bone marrow adhere to collagen type I. Blood. 79(3):657-65.

Kohli-Kumar M, Shahidi NT, Broxmeyer HE, Masterson M, Delaat C, Sambrano J Morris C, Auerbach AD, Harris RE (1993) Haemopoietic stem/progenitor cell transplant in Fanconi anaemia using HLA-matched sibling umbilical cord blood cells. British Journal of Haematology. 85(2):419-22.

Koopman G. Keehnen RM. Lindhout E. Newman W. Shimizu Y. van Seventer GA. de Groot C. Pals ST (1994) Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. Journal of Immunology. 152(8):3760-7, 1994.

Korbling M, Fliedner TM (1994) History of blood stem cell transplants. Blood stem cell transplants. in Gale RP, Juttner CA, Henon P (eds): Peripheral blood stem cell autografts. New york, NY, Cambridge university press, p9.

Lajtha LG (1979) Stem cell concepts. [Review] Differentiation. 14(1-2):23-34.

Lansdorp PM, Schmitt C, Sutherland HJ, Craig WH, Dragowska W, Thomas TE. Eaves CJ (1992) Hemopoietic stem cell characterisation. Progress in Clinical & Biological Research. 377:475-84; discussion 485-6.

Lapidot T, Fajerman Y, Kollet O (1997) Immune-deficient SCID and NOD/SCID mice models as functional assays for studying normal and malignant human hematopoiesis. [Review] Journal of Molecular Medicine. 75(9):664-73.

Larochelle A, Vormoor J, Hanenberg H, Wang JC, Bhatia M, Lapidot T, Moritz T. Murdoch B, Xiao XL, Kato I, Williams DA, Dick JE (1996) Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. Nature Medicine. 2(12):1329-37.

Larsen E, Palabrica T, Sajer S, Gilbert GE, Wagner DD, Furie BC, Furie B (1990) PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). Cell. 63(3):467-74.

Lasky LA (1994) Sialomucin ligands for selectins. a new family of cell adhesion molecules. Princess-Takamatsu symposium, 24: 81-90.

Leavesley DI, Oliver JM, Swart BW, Berndt MC, Haylock DN, Simmons PJ (1994) Signals from platelet/endothelial cell adhesion molecule enhance the adhesive activity of the very late antigen-4 integrin of human $CD34^+$ hemopoietic progenitor cells. Journal of Immunology. 153(10):4673-83.

Lerner C, Harrison DE (1990) 5-Fluorouracil spares hemopoietic stem cells responsible for long-term repopulation. Experimental Haematology. 18(2):114-8.

Lesley J, Hyman R and Kincade PW (1993) CD44 and its interaction with extracellular matrix. Advances in immunology, 54:271-335.

Letarte M. Iturbe S. Quackenbush EJ (1985) A glycoprotein of molecular weight 85,000 on human cells of B-lineage: detection with a family of monoclonal antibodies. Molecular Immunology. 22(2):113-24.

Lewis JL, Blackett NM, Gordon MY (1994) The kinetics of colony formation by CFU-GM in vitro. British Journal of Haematology. 88(2):440-2.

Liesveld JL, Winslow JM, Frediani KE, Ryan DH, Abboud CN (1993) Expression of integrins and examination of their adhesive function in normal and leukemic hematopoietic cells. Blood. 81(1):112-21.

Liesveld JL, Dipersio JF, Abboud CN (1994) Integrins and adhesive receptors in normal and leukemic CD34+ progenitor cells: potential regulatory checkpoints for cellular traffic. Leukemia & Lymphoma. 14(1-2):19-28.

Long MW and Vishva MD (1990) Thrombospondin functions as a cytoadhesion molecule for human hematopoietic progenitor cells. blood.75(12): 2311 -2318.

Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark H, Gunthert U (1994) Expression and modulation of CD44 variant isoforms in humans. Journal of Cell Biology. 124(1-2):71-82.

Mauch P, Greenberger JS, Botnick L, Hannon E, Hellman S (1980) Evidence for structured variation in self-renewal capacity within long-term bone marrow cultures. Proceedings of the National Academy of Sciences of the United States of America. 77(5):2927-30.

Mayani H, Lansdorp PM (1994) Thy-1 expression is linked to functional properties of primitive hematopoietic progenitor cells from human umbilical cord blood. Blood. 83(9):2410-7.

Mayne R, Berwyn RG (1993) New members of the collagen superfamily. Current Opinion in Cell Biology. 5(5):883-90.

McCarthy JB. Skubitz AP. Qi Z. Yi XY. Mickelson DJ. Klein DJ. Furcht LT (1990) RGD-independent cell adhesion to the carboxy-terminal heparin-binding fragment of fibronectin involves heparin-dependent and -independent activities. Journal of Cell Biology. 110(3):777-87.

McCredie KB, Hersh EM, Freireich EJ (1971) Cells capable of colony formation in the peripheral blood of man. Science. 171(968):293-4.

Minguell JJ, Hardy CL, Tavassoli M (1993) Adhesive interaction of haemopoietic progenitor cell membrane with RGD domain of fibronectin. Biochim Biophys Acta. 105:7-14.

Minguell JJ and Tavassoli M (1989) Proteoglycan synthesis by hemopoietic progenitor cells Blood 73 :1821-1827

Miyake K, Underhill CB, Lesley J, Kincade PW (1990) Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. Journal of Experimental Medicine. 172(1):69-75.

Miyake K, Medina KL, Hayashi S, Ono S, HarnaokaT, KincadePW (1990) Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. Journal of Experimental Medicine. 171 (2):477-88.

Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L (1998) The chemokine receptor CXCR-4 is expressed on $CD34^+$ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood. 91(12):4523-30.

Moore MA. Dexter TM (1978) Stem cell regulation in continuous hematopoietic cell culture. Transplantation Proceedings. 10(1):83-90.

Morimoto K, Robin E, Le Bousse-Kerdiles MC, Li Y, Clay D, Jasmin C, Smadja-Joffe F (1994) CD44 mediates hyaluronan binding by human myeloid KG1a and KG1 cells. Blood. 83(3):657-62.

Muller WA, Berman ME, Newman PJ, DeLisser HM and Albelda SM (1992) A heterophilic adhesion mechanism for platelet/endothelial cell adhesion molecule-1 (CD31). Journal of experimental medicine 175: 1401-1404.

O'neill HC (1989) Antibody which defines a subset of bone marrow cells that can migrate to thymus. Immunology. 68(1):59-65.

Oostendorp RA. Reisbach G. Spitzer E. Thalmeier K. Dienemann H. Mergenthaler HG. Dormer P (1995) VLA-4 and VCAM-1 are the principal adhesion molecules involved in the interaction between blast colony-forming cells and bone marrow stromal cells. British Journal of Haematology. 91(2):275-84.

Oostendorp RA, Dormer P (1997) VLA-4-mediated interactions between normal human hematopoietic progenitors and stromal cells. [Review]. Leukemia & Lymphoma. 24(5-6):423-35.

Orlic D, Fischer R, Nishikawa S, Nienhuis AW, Bodine DM (1993) Purification and characterisation of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of c-kit receptor. Blood. 82(3):762-70.

Osawa M, Hanada K, Hamada H, Nakauchi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science. 273(5272):242-5.

Pals ST. Horst E. Ossekoppele GJ. Figdor CG. Scheper RJ. Meijer CJ (1989) Expression of lymphocyte homing receptor as a mechanism of dissemination in non-Hodgkin's lymphoma. Blood. 73(4):885-8.

Papayannopoulou T. Brice M (1992) Integrin expression profiles during erythroid differentiation. Blood. 79(7):1686-94.

Papayannopoulou T, Craddock C (1997) Homing and trafficking of hemopoietic progenitor cells. Acta Haematologica. 97(1 -2):97-104.

Papayannopoulou T, Nakamoto B (1993) Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. Proceedings of the National Academy of Sciences of the United States of America. 90(20):9374-8.

Patel VP, Lodish HF (1986) The fibronectin receptor on mammalian erythroid precursor cells: characterisation and developmental regulation. Journal of Cell Biology. 102(2):449-56.

Patel VP, Ciechanover A, Platt O, Lodish HF (1985) Loss of adhesion of erythrocyte precursors to fibronectin during erythroid differentiation. Progress in Clinical & Biological Research. 184:355-68.

Peach R J, Hollenbaugh D, Stamenkovic 1. and Aruffo A (1993) Identification of hyaluronic acid binding sites in the extra-cellular domain of CD44. Journal of Cell biology 122: 257-264.

Peault B, Weissman IL, Buckle AM, Tsukamoto A, Baum C (1993) Thy-1-expressing CD34+ human cells express multiple hematopoietic potentialities in vitro and in SCID-hu mice. Nouvelle Revue Francaise d Hematologie. 35(1):91-3.

Peled A, Petit I, Kollet O, Magid M, Ponomaryov T, Byk T, Nagler A, Ben-hur H, Many A, Shultz L, Lder O, Alon R, Zipor D, Lapidot T (1999) Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science. 283; 845-848.

Ridley RC, Xao H, Hata H, Woodliff J, Epstein J and Sanderson RD (1993) Expression of syndecan regulates human myeloma plasma cell adhesion to type 1 collagen. Blood 81(3):767-774.

Roberts R, Gallagher J, Spooncer E, Allen TD, Bloomfield F, Dexter TM (1988) Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. Nature. 332(6162):376-8.

Sage H and Bornstein P (1991) Extracellular proteins that modulate cell-matrix interactions. SPARC, tenascin, and thrombospondin. Journal of biological Chemistry. 266: 14831 - 14834.

Saeland S, Duvert V, Caux C, Pandrau D, Favre C, Valle A, Durand I, Charbord P de Vries J, Banchereau J (1992) Distribution of surface-membrane molecules on bone marrow and cord blood $CD34^+$ hematopoietic cells. Experimental Hematology. 20(1):24-33.

Sackstein R (1997) Expression of an L-selectin ligand on hematopoietic progenitor cells.[Review]. Acta Haematologica. 97(1-2):22-8.

Salmivirta M and Jalkanen M (1995) Syndecan family of cell surface proteoglycans: developmentally regulated receptors for extracellular effector molecules. Experientia 51: 863-872.

Scott MA, Gordon MY (1995) In search of the haemopoietic stem cell. British Journal of Haematology. 90(4):738-43.

Simmons PJ, Masinovsky B, Longenecker BM, Berenson R, Torok-Storb B. Gallatin WM (1992) Vascular cell adhesion molecule-l expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. Blood. 80(2):388-95.

Simmons PJ, Niutta S, Ashman LK, Berndl MC and Juttner CA (1992) Primitive human bone marrow cells express L-selectin and PECAM- 1 (abstract.). Experimental Hematology 20: 329.

Simmons PJ, Zannettino A, Gronthos S, Leavesley D (1994) Potential adhesion mechanisms for localisation of haemopoietic progenitors to bone marrow stroma. Leukemia & Lymphoma. 12(5-6):353-63.

Siczkowski M, Clarke D, Gordon MY (1992) Binding of primitive hematopoietic progenitor cells to marrow stromal cells involves heparan sulfate. Blood. 80(4):912-9.

Smadja-Joffe F, Legras S, Girard N, Li Y, Delpech B, Bloget F, Morimoto K, Le Bousse-Kerdiles C, Clay D, Jasmin C. & Levesque JP (1996) CD44 and hyaluronan binding by human myeloid cells. Leukemia and Lymphoma 21 :407-420.

Socinski MA, Elias A, Schnipper L, Cannistra SA, Antman KH, Griffin JD (1988) Granulocyte-macrophage stimulating factor expands the circulating haemopoietic progenitor cell compartment in man. Lancet i: 1194-1198.

Spooncer E, Gallagher JT, Krizsa F, and Dexter T M (1983) Regulation of haemopoiesis in long-term bone marrow cultures. IV. Glycosaminoglycan synthesis and the stimulation of haemopoiesis by beta-D-xylosides. Journal of Cell Biology. 96: 510-514.

Springer TA, Lasky LA (1991) Cell adhesion. Sticky sugars for selectins. Nature. 349(6306):196-7.

Srour EF, Brandt JE, Briddell RA, Grigsby S, Leemhuis T, Hoffman R (1993) Long-term generation and expansion of human primitive hematopoietic progenitor cells in vitro. Blood. 81 (3): 661-9.

Steen R, Tjonnfjord GE, Egeland T (1994) Comparison of the phenotype and clonogenicity of normal CD34+ cells from umbilical cord blood, granulocyte colony-stimulating factor-mobilized peripheral blood, and adult human bone marrow. Journal of Hematotherapy. 3(4):253-62

Stevens RL, Avraham S, Gartner MC, Austen KF, Lalley PA, Weis JH (1988) Isolation of a cDNA that encodes the peptide core of the secretory granule proteoglycan of rat basophilic leukemia-1 cells and assessment of its homology to the human analogue. Journal of Biological Chemistry. 263(15):7292-6.

Sullenbarger BA, Petitt MS, Chong P, Long MW, Wicha MS (1995) Murine granulocytic cell adhesion to bone marrow hemonectin is mediated by mannose and galactose. Blood 86(1): 135-140.

Sutherland HJ, Lansdorp PM, Henkelman DH, Eaves AC, Eaves CJ (1990) Functional characterisation of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. Proceedings of the National Academy of Sciences of the United States of America. 87(9):3584-8.

Sutherland HJ, Eaves CJ, Eaves AC, Dragowska W, Lansdorp PM (1989) Characterisation and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood. 74(5): 1563-70.

Tanaka Y, Albelda SM, Horgan KJ, van Seventer GA, Shimizu Y, Newman W. Hallam J, Newman PJ, Buck CA, Shaw S (1992) CD31 expressed on distinctive T cell

subsets is a preferential amplifier of beta 1 integrin-mediated adhesion. Journal of Experimental Medicine. 176(1):245-53.

Taswell C (1984) Limiting dilution assays for the determination of immunocompetent cell frequencies. III. Validity tests for the single-hit Poisson model. Journal of Immunological Methods. 72(1):29-40.

Taswell C (1981) Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. Journal of Immunology. 126(4):1614-9.

Tavassoli M, Friedenstein A (1983) Hemopoietic stromal microenvironment. American Journal of Hematology. 15(2): 195-203.

Tavassoli M (1983) Effect of the substratum on the growth of CFU-c in continuous marrow culture. Experientia. 39(4):411-2.

Tedder TF, Penta AC, Levine HB, Freedman AS (1990) Expression of the human leukocyte adhesion molecule, LAMl . Identity with the TQl and Leu-8 differentiation antigens. Journal of Immunology. 144(2):532-40.

Teixido J, Hemler ME, Greenberger JS, Anklesaria P (1992) Role of beta 1 and beta 2 integrins in the adhesion of human CD34hi stem cells to bone marrow stroma. Journal of Clinical Investigation. 90(2):358-67.

Terstappen LW, Huang S, Safford M, Lansdorp PM, Loken MR (1991) Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. Blood. 77(6):1218-27.

Terstappen LW, Lund-Johansen F (1993) Differential surface expression of cell adhesion molecules during granulocyte maturation. Journal of Leukocyte Biology. 54(1):47-55.

Toyama-Sorimachi N. Kitamura F. Habuchi H. Tobita Y. Kimata K. Miyasaka M (1997) Widespread expression of chondroitin sulfate-type serglycins with CD44 binding ability in hematopoietic cells. Journal of Biological Chemistry. 272(42):26714-9.

Toyama-Sorimachi N, Sorimachi H, Tobita Y, Kitamura F, Yagita H, Suzuki K Miyasaka M (1995) A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. Journal of Biological Chemistry. 270(13):7437-44.

Traycoff CM. Abboud MR. Laver J. Clapp DW. Hoffman R. Srour EF (1994) Ex vivo expansion of CD34+ cells from purified adult human bone marrow and umbilical cord blood hematopoietic progenitor cells. Progress in Clinical & Biological Research. 389:385-91.

Turner ML. Masek LC. Hardy CL. Parker AC. Sweetenham JW (1998) Comparative adhesion of human haemopoietic cell lines to extracellular matrix components, bone marrow stromal and endothelial cultures. British Journal of Haematology. 100(1):112-22.

Turner ML, McIlwaine K, Anthony RS, Parker AC (1995) Differential expression of cell adhesion molecules by human hematopoietic progenitor cells from bone marrow and mobilised adult peripheral blood. Stem Cells. 13(3):311-6.

Underhill C (1992) CD44: the hyaluronan receptor. Journal of Cell Science. 103 (2):293-8.

Verfaillie CM, Benis A, Iida J, McGlave PB, McCarthy JB (1994) Adhesion of committed human hematopoietic progenitors to synthetic peptides from the C-terminal heparin-binding domain of fibronectin: co-operation between the integrin alpha 4 beta 1 and the CD44 adhesion receptor. Blood. 84(6): 1802- 11.

Verfaillie CM, McCarthy JB, McGlave PB (1991) Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interaction with fibronectin Journal of Experimental Medicine. 174(3):693-703.

Verfaillie CM, McCarthy JB, McGlave PB (1992) Mechanisms underlying abnormal trafficking of malignant progenitors in chronic myelogenous leukaemia. Decreased adhesion to stroma and fibronectin but increased adhesion to the basement membrane components laminin and collagen type IV. Journal of Clinical Investigation. 90(4):1232-41.

Watt S.M, Williamson J, Genevier H, Fawcett J, Simmons DL, Hatzfeld A, Nesbitt S A and Coombe DR (1993) The heparin binding PECAM- I adhesion molecule is expressed by $CD34^{+}$ hematopoietic precursor cells with early myeloid and B-lymphoid phenotypes. Blood 82, 2649-2663.

Watt SM, Gschmeissner SE, Bates PA (1995) PECAM- 1: Its expression and function as a cell adhesion molecule on haemopoietic and endothelial cells. Leukaemia and Lymphoma. 17: 229-244

Watts MJ, Jones HM, Sullivan AM, Langabeer SE, Jamieson E, Fielding A, Williams C, Berenson RJ, Goldstone AH, Linch DC (1995) Accessory cells do not contribute to G-CSF or IL-6 production nor to rapid haematological recovery following peripheral blood stem cell transplantation. British Journal of Haematology. 91 (3):767-72.

Watt SM, Visser JW (1992) Recent advances in the growth and isolation of primitive human haemopoietic progenitor cells. Cell Proliferation. 25(4):263-97.

Weyrich AS, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA (1995) Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1and tumor necrosis factor-alpha secretion. Signal integration and NF-kappaB translocation. Journal of Clinical Investigation. 95(5):2297-303.

Wight TN, Kinsella MG, Keafing A, and Singer JW (1986) Proteoglycans in human long-term bone marrow cultures: biochemical and ultrastructural analyses. Blood 67: 1333-1343.

Williams DA (1991) In search of the self-renewing hematopoietic stem cell. Blood Cells. 17(2):296-300.

Williams DA, Rios M, Stephens C, Patel VP (1991) Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. Nature 352(6334):438-41.

Wolf NS (1979) The haemopoietic microenvironment. Clinics in Haematology. 8(2):469-500.

Woods A. McCarthy JB. Furcht LT. Couchman JR (1993) A synthetic peptide from the COOH-terminal heparin-binding domain of fibronectin promotes focal adhesion formation. Molecular Biology of the Cell. 4(6):605-13.

Yoder MC, Williams DA (1995) Matrix molecules interactions with hematopoietic stem cells. Experimental hematology 23:961-967.

Zannettino AC, Bemdt MC, Butcher C, Butcher EC, Vadas MA, Simmons PJ (1995) Primitive human hematopoietic progenitors adhere to P-selectin (CD62P). Blood. 85(12):3466-77.

Zhou D F, Ding J F, Pieker L F, Bargatze R, Butcher E C and Goeddel DV (1989) Molecular cloning and expression of Pgp- I -the mouse homolog of the human H-CAM (Hermes) Iymphocyte homing receptor. Journal of immunology, 143: 3390-3395.

Zuckerman KS, Rhodes RK (1985) The hematopoietic extracellular matrix. Progress in Clinical and Biological research 184:257-66.

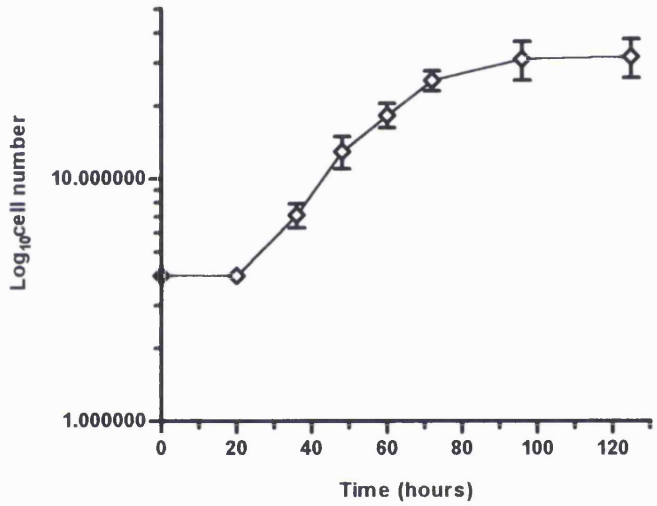
Zuckerman KS, Rhodes RK, Goodrum DD, Patel VR, Sparks B, Wells J, Wicha MS, Mayo LA (1985) Inhibition of collagen deposition in the extracellular matrix prevents the establishment of a stroma supportive of hematopoiesis in long-term murine bone marrow cultures. Journal of Clinical Investigation. 75(3):970-5.

Zuckerman KS (1984) Composition and function of the extracellular matrix in the stroma of long-term bone marrow cell cultures. Kroc Foundation Series. 18:157-70.

9.2 APPENDICES

APPENDIX ONE

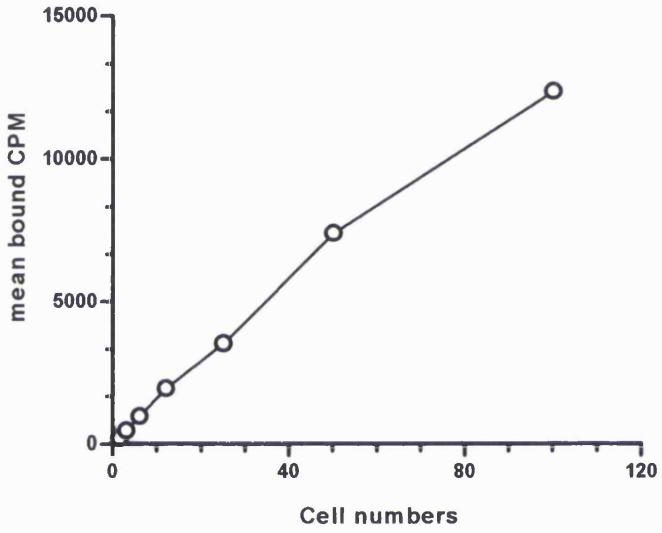
SETTING UP A GROWTH CURVE



The growth characteristics of KG1a were assessed through the growth curve. A cell suspension was diluted to 1 x 10^5/ml and subcultured in flasks. The cultures were counted daily for at least 5 days and a growth curve then constructed. Graph shows results from three cultures (mean ± S.D)

APPENDIX TWO

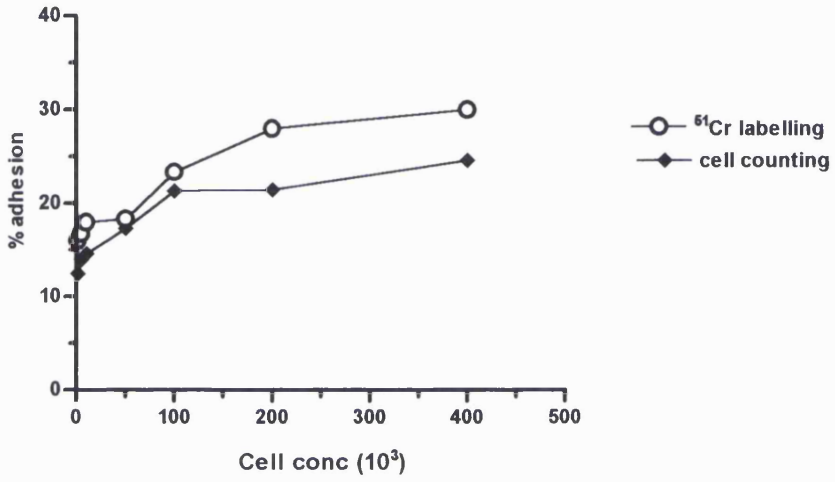
TITRATION OF ^{51}CHROMIUM LABELLED KG1a CELL BY DOUBLING DILUTION



Cell suspensions are labelled witrh ^{51}Cr by incubating the cells with the chromium and then washing out the unbound reagent. The cell suspension is then aliquoted into multiple tubes setup in doubling dilution and counted with gamma counter. The binding capacity of ^{51}Cr to KG1a cells increased with increasing cell numbers.

APPENDIX THREE

OPTIMISITION OF CELL QUANTITATION IN THE ADHESION ASSAY



Percentage adhesion of KG1a cells to HA-coated surface estimated using two methods; ^{51}Cr labelling of KG1a and counting of harvested nonadherent cells in the adhesion assay. Adhesion assays were set-up as described in general methods (chapter 2)

9.3 PRESENTATION

USE OF THE P DELTA STEM CELL ASSAY TO INVESTIGATE THE ADHESION OF HAEMOPOIETIC STEM CELLS TO HYALURONIC ACID.

M.M.Musami, M.Y.Gordon, K.L.Yong. Department of haematology, Royal Free Hospital Medical School, and LRF Centre for Adult Leukaemia, Royal Postgraduate Medical School, London, UK. British society for Haematology. April 1997.