

**CYTOKINE-DEPENDENT REGULATION OF HUMAN HEMATOPOIETIC CELL
SELF-RENEWAL AND DIFFERENTIATION IN SUSPENSION CULTURES**

by

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ABSTRACT

This study investigated the processes limiting hematopoietic progenitor proliferation and differentiation in expansion cultures of human hematopoietic cells. A significant net expansion of many types of progenitors, including direct colony-forming cells (CFC) and their more primitive precursors (long-term culture-initiating cells, LTC-IC) could be reproducibly obtained in cytokine-supplemented, stirred suspension cultures. Due to its homogeneous nature, this culture system represents a simple and effective way to approach clinical scale hematopoietic bioreactor design.

Factorial design experiments, undertaken to optimize cytokine combinations for the expansion of primitive cell populations, showed that Flt-3 Ligand (FL) was the only factor that, alone, increased marrow-derived LTC-IC above input values. The effects of FL, Steel Factor (SF) and Interleukin-3 (IL-3) on expansion of these LTC-IC were shown to be additive and greater than or equal to those obtained in the presence of combinations of 13 other factors. A dose response analysis undertaken to define the cytokine concentration requirements for marrow LTC-IC self-renewal revealed that the greatest LTC-IC amplification (62-fold) was obtained in cultures containing significantly elevated levels of cytokines (FL and SF at 300 ng/mL plus IL-3 at 60 ng/mL). Subsequent experiments showed that the LTC-IC expansion was primarily and significantly dependent on a high concentration of FL and, as the frequency of clonal growth from individual cells did not change with cytokine concentration, the decrease in LTC-IC expansion observed at lower cytokine concentrations could be attributed to differentiation rather than selective death.

Analysis of the average cell specific cytokine depletion rates from both stirred suspension and purified cell cultures revealed proportional increases in these rates at increased cytokine concentrations. In similar investigations analyzing the responses of specific phenotypically defined cell populations, the greatest factor depletion rates were observed in cultures of the most primitive cells. Examination of the kinetics of SF receptor (*c-kit*) internalization in response to SF stimulation showed that concentration-dependent SF depletion and *c-kit* internalization were closely associated, suggesting that the extent and/or duration of cytokine signaling may be an important factor in determining cellular responses.

Taken together, these results demonstrate the ability to expand hematopoietic cells in a scaleable stirred suspension culture system. Cytokine type, concentration and depletion rates were shown to be parameters important for the development of bioreactor technology. The observations that the hematopoietic cell types whose maximum proliferation *in vitro* depends on the highest concentration of cytokines also exhibit the greatest capacity to deplete the same cytokines from the medium may explain why the identification of conditions that support the expansion of hematopoietic stem cells has been so elusive. Additionally, these results provide evidence that extrinsically acting cytokines can alter the self-renewal behavior of primary human hematopoietic cells independent of effects on their viability and proliferation.

TABLE OF CONTENTS

ABSTRACT	ii
TABLE OF CONTENTS.....	iv
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF ABBREVIATIONS.....	xiii
ACKNOWLEDGMENTS	xv
DEDICATION	xvi

CHAPTER 1: INTRODUCTION..... 1

1. Hematopoiesis..... 1

A. Blood Cell Hierarchy	1
B. Identification of Primitive Human Hematopoietic Cells.....	4
a) <i>In vitro</i> assays	4
b) <i>Phenotypic markers</i>	5
c) <i>In vitro</i> assays and phenotypic markers for the detection of in vivo repopulating cells.....	8
C. Cell Proliferation and Differentiation Control	9
a) <i>A permissive versus an inductive environment?</i>	9
b) <i>Hematopoietic stem cell stimulation by cytokines</i>	12
D. Hematopoietic Growth Factor Receptors	16
a) <i>Differential responses mediated by receptor type</i>	17
b) <i>Differential responses mediated by receptor stimulation</i>	18
E. Steel Factor Receptor (<i>c-kit</i>)	21

2. *In Vitro* Hematopoietic Cell Culture..... 22

A. Long-Term (Dexter-type) Culture System.....	22
B. Alternate Hematopoietic Culture systems	23
a) <i>Cultures providing a surface for stromal cell growth</i>	23
b) <i>Suspension or stromal cell-free cultures</i>	25
C. Culture Modifications	28
a) <i>Perfusion/medium exchange rate</i>	28
b) <i>Culture inoculum, cell density and accessory cells of stromal origin</i>	30

c) <i>Culture oxygen condition</i>	33
d) <i>Cytokine-supplemented expansion systems</i>	36
3. Requirements, Limitations and Applications of <i>Ex Vivo</i> Cultured Hematopoietic Cells	41
A. Applications and Requirements for <i>Ex Vivo</i> Cultured Hematopoietic Progenitor Cells.....	41
B. Clinical Cell Production Requirements.....	42
C. Potential Problems and Limitations of <i>Ex Vivo</i> Cultured Hematopoietic Cells.....	43
4. Thesis Objectives	45
 CHAPTER 2: MATERIALS AND METHODS	48
A. Bone marrow cells	48
B. Cytokines.....	49
C. Stirred Suspension Cultures	49
D. Static Cultures	51
E. Stroma-free Liquid Suspension Cultures.....	51
F. Mo7e Cell Cultures	53
G. Progenitor Assays.....	53
a) <i>CFC</i>	53
b) <i>CFU-F</i>	54
c) <i>LTC-IC</i>	54
H. Flow Cytometric Purification and Analysis of Bone Marrow Cells.....	56
I. Flow Cytometric Analysis of Mo7e Cells.....	57
a) <i>Cell surface c-kit expression</i>	57
b) <i>Intracellular analysis of SF</i>	58
J. Statistics	58
a) <i>General</i>	58
b) <i>Factorial design analysis experiments</i>	59
K. Medium analysis	60

**CHAPTER 3: EXPANSION OF HEMATOPOIETIC PROGENITOR CELL
POPULATIONS IN STIRRED SUSPENSION BIOREACTORS OF
NORMAL HUMAN BONE MARROW CELLS62**

1. Introduction.....	63
2. Results.....	65
A. Preliminary Studies With Stirred Suspension Cultures and the Effect of Adding Porous Microcarrier Beads	65
B. Net Expansion of Hematopoietic Cells in Stirred Suspension Cultures Containing Growth Factor-Supplemented Medium	66
C. Changes in the Marrow Fibroblast Colony-Forming Unit (CFU-F) Population in Stirred Suspension Cultures	71
3. Discussion	72

**CHAPTER 4: CELLULAR DETERMINANTS AFFECTING THE RATE OF
CYTOKINE DEPLETION IN CULTURES OF HUMAN
HEMATOPOIETIC CELLS.....75**

1. Introduction.....	76
2. Results.....	76
A. CFC Output in Stirred Suspension Cultures can be Increased Without Affecting LTC-IC Maintenance.....	76
B. Dependence of Cytokine Depletion Rates on the Concentration of Cytokines Present in the Medium	78
C. Different Types of Hematopoietic Cells are Characterized by Different Rates of Cytokine Depletion.....	84
3. Discussion	88

**CHAPTER 5: DIFFERENTIAL CYTOKINE EFFECTS ON PRIMITIVE
(CD34⁺CD38⁻) HUMAN HEMATOPOIETIC CELLS: NOVEL
RESPONSES TO FLT3-LIGAND AND THROMBOPOIETIN92**

1. Introduction.....	93
2. Results.....	94
A. Multifactorial Analysis of the Relative Contributions of 6 Growth Factors on LTC-IC Amplification.....	94
B. Effects of Different Growth Factors on CFC Production by CD34 ⁺ CD38 ⁻ Cells. ..	100
C. Ability of Other Factors to Influence LTC-IC Amplification.	103

**CHAPTER 6: CYTOKINE MANIPULATION OF PRIMITIVE HUMAN
HEMATOPOIETIC CELL SELF-RENEWAL..... 111**

1. Introduction.....	112
2. Results.....	113
A. Differential Cytokine Dose Dependence of LTC-IC and CFC Expansion.....	113
B. Cytokine Concentration-Dependence of Colony Formation by Different Subpopulations of CFC in Methylcellulose Cultures.....	120
C. Analysis of the Effect of Independently Varying the Concentrations of FL, SF and IL-3 on LTC-IC and CFC Expansion.....	122
D. Characterization of the Phenotypes of the Cytokine-Expanded Populations.....	125
3. Discussion	127

**CHAPTER 7: ANALYSIS OF *C-KIT*/STEEL FACTOR RECEPTOR
INTERNALIZATION IN FACTOR DEPENDENT
HEMATOPOIETIC CELLS..... 131**

1. Introduction.....	132
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2. Results.....	134
A. Kinetics of SF dose-dependent reduction of cell surface <i>c-kit</i> expression on Mo7e cells:	134
B. Cytokine dose- and time-dependent kinetics of SF depletion by Mo7e cells.	138
C. Flow cytometric analysis of dose dependent SF internalization.	139
3. Discussion	142
 CHAPTER 8: SUMMARY AND FUTURE DIRECTIONS	146
1. Summary	146
2. Future directions	149
 REFERENCES.....	153

LIST OF TABLES

Table 1.1: Phenotypically defined cell populations in uncultured adult bone marrow	7
Table 1.2: Cytokines acting on hematopoietic progenitors	13
Table 1.3: Effect of differences in oxygen tension on progenitor cell expansion.....	35
Table 1.4: Summary of mononuclear and CD34 ⁺ cell cytokine mediated expansion studies...	37
Table 3.1. Total viable cells, CFC and LTC-IC numbers in stirred suspension cultures with and without the addition of porous microcarrier beads as compared to static cultures.....	66
Table 3.2: Total CFC per 10 ⁶ light density marrow input cells at days 0, 14 and 28 in stirred or static cultures containing cytokine supplemented and non-supplemented medium.....	70
Table 4.1: Changes in the numbers of different types of progenitors after maintenance of different subpopulations of normal human marrow cells in serum-free medium supplemented with 100 ng/mL of SF and FL, and 20 ng/mL of IL-3, IL-6 and G-CSF for 7 days.....	87
Table 4.2: Ratio of depletion rate constants for IL-3 SF and FL in cytokine-supplemented cultures of human marrow and Mo7e cells.....	89
Table 5.1: Effects of selected culture conditions on the LTC-IC and CFC detected in serum-free cultures initiated with 200 CD34 ⁺ CD38 ⁻ cells	106
Table 6.1: Differential effects of altering the relative concentrations of FL, SF and IL-3 on LTC-IC and CFC expansion.....	123
Table 7.1: Cell specific dose- and time-dependent SF depletion in Mo7e cell cultures	139
Table 7.2: Estimated cell surface concentration based on the maximal SF flux to Mo7e cells in a surface monolayer	144

LIST OF FIGURES

Figure 1.1: The hematopoietic hierarchy	2
Figure 3.1: Calculated changes as a function of time in total viable cell and CFC numbers relative to input in stirred suspension cultures.....	67
Figure 3.2: Total viable cell and CFC numbers relative to input after maintenance of the cells for 14 and 28 days in SF-and IL-3-supplemented medium in stirred suspension or static, non-fed, or static, fed cultures, and in non-supplemented medium in stirred suspension or static, non-fed cultures.....	69
Figure 3.3: Calculated changes in CFU-F numbers in SF and IL-3 supplemented stirred suspension cultures	71
Figure 4.1: Calculated numbers of total viable cells, CFC and LTC-IC relative to the input in stirred suspension cultures initiated with light density human marrow cells in 10 ng/mL SF and FL, and 2 ng/mL IL-3, IL-6 and IL-11 or 50 ng/mL SF and FL, and 10 ng/mL IL-3, IL-6 and IL-11	78
Figure 4.2: Measured glucose concentrations in the supernatant media from cytokine supplemented stirred suspension cultures (Figure 4.1)	79
Figure 4.3: Measured SF, IL-3 and FL concentrations in the supernatant medium from cytokine supplemented stirred suspension cultures (Figure 4.1)	80
Figure 4.4: Calculated average cell-specific SF, IL-3 and FL depletion rates vs the average measured cytokine concentrations for cytokine supplemented stirred suspension cultures.....	82
Figure 4.5: Calculated cell-specific rates of IL-3 depletion from cultures of Mo7e cells.....	83
Figure 4.6: Calculated cell-specific rates of IL-3 and SF depletion for CD34 ⁺ CD38 ⁻ , CD34 ⁺ CD45RA ⁺ CD71 ⁻ , CD34 ⁺ CD45RA ⁻ CD71 ⁺ or light density marrow cells in cytokine supplemented serum-free medium	85

Figure 5.1: Variable LTC-IC expansion from CD34 ⁺ CD38 ⁻ cells incubated for 10 days in serum-free cultures containing different cytokine combinations of FL, SF, IL-3, IL-6, G-CSF and NGF- β	95
Figure 5.2: Linearized normal probability distribution of the normalized effect of different combinations of cytokines on LTC-IC expansion in 10 day serum-free cultures of CD34 ⁺ CD38 ⁻ cells.....	97
Figure 5.3: Variable CFC expansion from CD34 ⁺ CD38 ⁻ cells incubated for 10 days in serum-free cultures containing different cytokines	100
Figure 5.4: Effect of 10 specific cytokines and HC on LTC-IC expansion from CD34 ⁺ CD38 ⁻ cells incubated for 10 days in serum-free cultures either alone or in combination with a 6 growth factor cocktail.....	102
Figure 5.5: Effect of TPO alone or together with a 6 growth factor cocktail on the relative proportions (ratio) of primitive erythroid (BFU-E) and granulopoietic progenitors (CFU-GM) produced after 6 weeks from LTC-IC	105
Figure 6.1: Cytokine dose dependent LTC-IC and CFC expansion.....	115
Figure 6.2: Photomicrographs of cytokine expanded CD34 ⁺ CD38 ⁻ cells.....	116
Figure 6.3: Limiting dilution analysis of cytokine expanded cells.....	117
Figure 6.4: Size distribution and cumulative frequencies of clones generated from single PI ⁻ AnnexinV ⁻ CD34 ⁺ CD38 ⁻ cells in serum-free cytokine-supplemented medium.....	119
Figure 6.5: Cytokine concentration-dependent generation of colonies from CD34 ⁺ CD38 ⁻ , CD34 ⁺ CD45RA ⁺ CD71 ⁻ , or CD34 ⁺ CD45RA ⁻ CD71 ⁺ cells.....	121
Figure 6.6: Flow cytometric analyses of side scatter, and CD34, CD38, CD71, CD45RA and CD33 expression on cytokine stimulated CD34 ⁺ CD38 ⁻ Lin ⁻ cells.....	126
Figure 7.1: Time-dependent change in <i>c-kit</i> expression on Mo7e cells.....	135

Figure 7.2: Mean YB5.B8 fluorescence expressed as percent of levels measured on input Mo7e cells after stimulation for 30 min by different doses of SF at 4 and 37°C.....	136
Figure 7.3: Dose- and time-dependent changes in calculated <i>c-kit</i> expression on Mo7e cells	138
Figure 7.4: Internal SF staining of cells.....	141
Figure 7.5: A representative experiment of the detection of internal SF staining on cells exposed to 1, 10 or 100 ng/mL input SF in serum-free medium for 30 min at 37°C	141

LIST OF ABBREVIATIONS

bFGF	basic Fibroblast growth factor
BFU-E	Burst forming unit-erythroid
BM	Bone marrow
CB	Cord blood
CD	Cluster determinants
CFC	Colony forming cell
CFU	Colony forming unit
CSF	Colony stimulating factor
E	Erythroid
F	Fibroblast
FACS	Fluorescent activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FL	Flt-3 ligand
G	Granulocyte
GEMM	Granulocyte, erythroid, macrophage, megakaryocyte
GM	Granulocyte Macrophage
HFN	Hank's buffered saline with 2 % FCS and 0.1% sodium azide
HPP	High proliferative potential
IL	Interleukin
LIF	Leukemia inhibitory factor
LTC	Long term culture
LTC-IC	Longterm culture-initiating cell
ME	Mercaptoethanol
MFI	Mean fluorescence intensity
MIP-1 α	Macrophage inflammatory protein-1 α
MK	Megakaryocyte
MNC	Mononuclear cell
NGF	Nerve growth factor
PB	Peripheral blood

PBS	Phosphate buffered saline
PE	Phycoerythrin
PI	Propidium iodide
SEM	Standard error of the mean
SF	Steel factor
TGF- β	Transforming growth factor- β
U	Units

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DEDICATION

For Ilse and Hubert Zandstra

CHAPTER 1: INTRODUCTION

1. Hematopoiesis

A. Blood Cell Hierarchy

Hematopoiesis is a complex process in which a variety of specialized blood cell types are generated continuously from a relatively small population of pluripotent stem cells. During embryonic development, hematopoietic cells are thought to originate independently from primitive mesenchymal cells— first in the yolk sac blood islands and shortly thereafter within the embryo in the paraaortic splanchnopleura. Hematopoiesis then shifts via the circulation to the fetal liver and later to the spleen and bone marrow (BM). At birth, large numbers of progenitors can still be found in the umbilical cord blood (CB). In adults, hematopoietic progenitor cells are found predominantly in the BM, although they are also found at low concentrations in the peripheral blood (PB) (Godin et al., 1995; Dzierzak and Medvinsky, 1996; Tavain et al., 1996).

The production of mature blood cells from their precursors involves a series of differentiation events that take place over a large number of cell generations. This results in a recognizable hierarchy of hematopoietic progenitor cells of decreasing proliferative and differentiative potential (Figure 1.1). Maturing blood cell populations can be divided into two major groups: those belonging either to the myeloid or the lymphoid lineages. The myeloid lineages lead to the output of erythrocytes, monocytes, neutrophils, eosinophils, basophils/mast cells, and platelets (derived from megakaryocytes). Thymus (T) and bone marrow (B) derived lymphocytes as well as natural killer (NK) cells are produced by differentiation of cells of the lymphoid lineages.

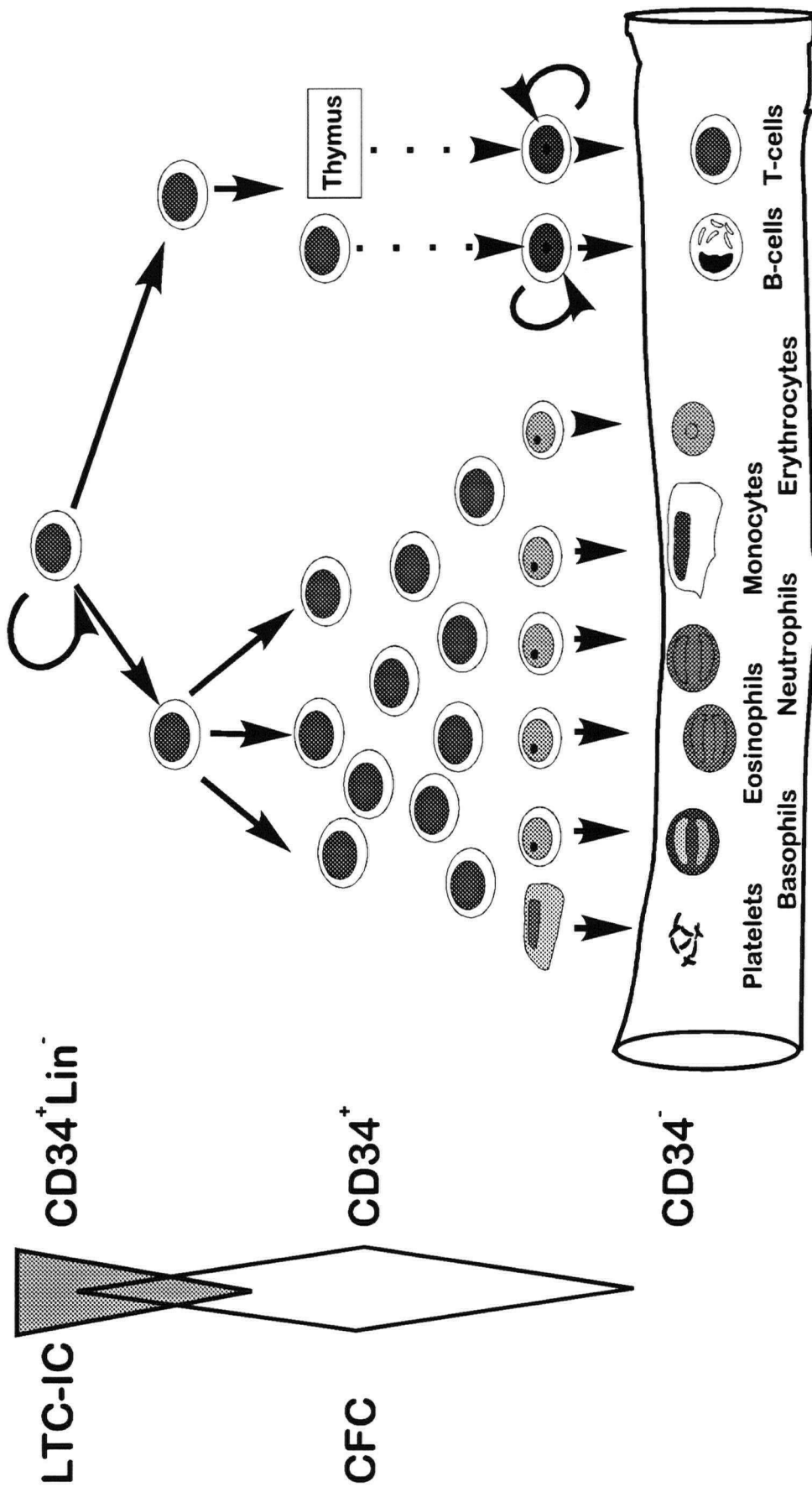


Figure 1.1: The hematopoietic hierarchy. Primitive stem cells can be functionally assayed as longterm culture-initiating cells and are generally CD34⁺Lin⁻ while committed progenitors can be assayed as colony forming cells (CFC) and are generally CD34⁺Lin⁺. Mature effector cells are generally CD34⁻.

Most mature blood cells exhibit a limited life span *in vivo* (e.g. erythrocytes and neutrophils have half-lives of 120 days and 8 h, respectively) (Cronkite, 1988). Thus, a continuous process of regeneration, resulting in the production of $\sim 4 \times 10^{11}$ blood cells/day (Koller and Palsson, 1993), is required to maintain cellular homeostasis. These numbers of mature blood cells are constantly being generated from dividing, lineage-restricted progenitors, which themselves are maintained by an influx of new progenitors ultimately derived from the hematopoietic stem cell compartment.

Hematopoietic stem cells are defined functionally by expression of their ability to generate and sustain multi-lineage hematopoiesis after transplantation into a hematologically compromised host. Experimental evidence supporting the existence of such cells was first provided by Till, McCulloch and coworkers (reviewed in Till and McCulloch, 1980) who injected lethally irradiated mice with BM cells from healthy syngeneic (genetically identical) donors and observed the formation of multi-lineage colonies in the spleen that could be shown to be derived from single injected cells. These multi-lineage colonies included pluripotent cells capable of generating additional multi-lineage colonies when transplanted into irradiated secondary recipients. The hematopoietic system of mice receiving donor cells was reconstituted, whereas control mice died within a week. Subsequent experiments in mice utilizing genetic marking have demonstrated that long-term engraftment of both the lymphoid and myeloid lineages can be achieved by the progeny of a single cell (Wu et al., 1968; Dick et al., 1985; Szilvassy et al., 1989). In addition, the ability to further repopulate multiple secondary recipients with the progeny of a single clone (Lemischka et al., 1986; Keller and Snodgrass, 1990), along with their extensive amplification when limiting numbers are transplanted (Pawliuk et al., 1996), has demonstrated that these very primitive murine cells

are capable of *in vivo* expansion. Subsequent studies have shown that retrovirally marked cells grown in culture are also able to repopulate multiple secondary recipients from the progeny of a single clone, indicating that individual stem cells are able to undergo self-renewal *in vitro* (Fraser et al., 1992; Pawliuk et al., 1996).

These results, obtained in the murine system, are more extensive than those realized in the human system where slower progress in the isolation and identification of the cells of interest, as well as limitations in their *in vivo* and *in vitro* analysis, has made expansion of functionally defined human stem cells difficult to demonstrate. Nonetheless, indications that *in vivo* repopulating cells continue to proliferate throughout adult life *in vivo* (Morrison et al., 1996; Bradford et al., 1997), as well as recent evidence of their cytokine mediated expansion *in vitro* (Conneally et al., 1997), supports a parallelism between the murine and human systems (Eaves et al., 1993a).

B. Identification of Primitive Human Hematopoietic Cells

a) *In vitro* assays

Various criteria are available to define sub-populations of human hematopoietic cells thought to contain pluripotent stem cells. One approach has been to consider certain functional properties measured by *in vitro* biological endpoints; for example, by assessment of the numbers and types of daughter cells they generate and the periods of time over which these continue to be generated. Such assays allow the detection *in vitro* of a variety of colony-forming cell (CFC) types as well as of a more primitive class of CFC precursors referred to as long-term culture-initiating cells (LTC-IC) (Figure 1.1). Myeloid, erythroid and

mixed CFC are identified by their ability to form colonies of mature cells after 2-3 weeks of culture in semi-solid media. LTC-IC, are retrospectively enumerated by their ability to quantitatively give rise to CFC after >5 weeks of liquid culture with stromal cells (Sutherland et al., 1991; 1993a) or genetically engineered murine fibroblast cell lines (Sauvageau et al., 1994; Hogge et al., 1996). The BM stromal cell population, which include cells of fibroblast, adipocyte, endothelial and osteogenic lineages (Herbertson and Aubin, 1995) produces a variety of soluble and membrane bound factor(s) that support the proliferation of repopulating stem cells (reviewed in Charbord, 1995) and the production for many weeks *in vitro* of derivative hematopoietic progenitors (Sutherland et al., 1990). LTC-IC do not, in general, give rise to detectable colonies in the CFC assay (Petzer et al., 1996a). Recently, differences in both the frequency and proliferative potential of LTC-IC detected on human marrow feeders and engineered murine fibroblast cell lines have been demonstrated (Hogge et al., 1996). These results provide evidence of biologic heterogeneity between different cell populations that are operationally identified as LTC-IC and emphasize the importance of limiting dilution assays to verify quantitative effects on populations of these cells.

b) *Phenotypic markers*

Another set of parameters used to define sub-populations of primitive hematopoietic cells is based on their expression of particular cell surface antigens (Table 1.1). CD34 is a marker expressed on the majority of CFC and LTC-IC but not on more differentiated cells in normal human BM (Civin et al., 1989). This antigen, which has recently been shown to bind L-selectin, is a 110 kD transmembrane glycoprophosphoprotein and is thought to play a role in stem/progenitor cell localization/adhesion in the BM (reviewed in Krause et al., 1996). Cell

populations isolated from light density human marrow on the basis of their expression of the CD34 antigen and light scatter characteristics have been shown to contain 10-20% colony forming cells (Lansdorp and Thomas, 1992). In the mouse, stem cells do not express cell surface markers associated with myeloid or lymphoid differentiation commitment and therefore are termed lineage negative (Lin⁻). In humans, LTC-IC are enriched in the fractions of cells that similarly do not express CD33, HLA-DR, and other mature cell antigens (Brashem-Stein et al., 1993; Mayani et al., 1993a; Verfaillie et al., 1990). Characterization of CD34⁺/Lin⁻ cells has led to observations that primitive human hematopoietic cells can be further subdivided according to their expression of Thy-1, CD38 (reviewed in Metha et al., 1996) and CD45RA (a high molecular weight isoform of a leukocyte specific tyrosine phosphatase) antigens (Clement, 1992) and also by their ability to retain the supra-vital dye Rhodamine 123 (Udomsakdi et al., 1991). These populations have been found to represent approximately 0.01-0.1% of normal human marrow mononuclear cells (MNC) and can be enriched 500 to 1000-fold in LTC-IC [Table 1.1 (Lansdorp and Dragowska, 1992; Sauvageau et al., 1994)]. Lansdorp and Dragowska (1992) also demonstrated the existence of functionally distinct sub-populations of CD34⁺ BM cells based on their expression of CD45RA and CD71 (transferrin receptor). Similar results were obtained by Mayani et al. (1993a) for the same CD34⁺ cell populations isolated from CB samples where up to 42% of the CD34⁺CD45RA⁻CD71⁻ cells were shown to be multipotent progenitors whereas CD34⁺CD45RA⁺CD71⁻ cells were shown to be 90% myeloid progenitors and CD34⁺CD45RA⁻CD71⁺ cells were 70% erythroid progenitors. Recently, using a purification strategy based on the isolation of CD34⁺CD45RA⁻CD71⁻CD38⁻ populations, LTC-IC purities of between 10-30% have been achieved (Sauvageau et al., 1994; Petzer et al., 1996a).

Table 1.1: Phenotypically defined cell populations in uncultured adult bone marrow.

Cell Population	Frequency in light density marrow	CFC Cloning Efficiency (%)	Colony Type (%)		LTC-IC (%)	References
			CFU-GM	BFU-E/ CFU-GEMM		
Unseparated (light density)	1	0.6-1.5	60-80	20-40	0.002-0.005	reviewed in Moore, 1995
CD34 ⁺	0.03-0.08	30-50	50-70	30-50	0.02-0.1	Civin et al. 1989 Sutherland et al. 1989 Lansdorp and Thomas, 1992
CD34 ⁺ CD45RA ⁺ CD71 ⁻	0.015-0.02	20-40	85-95	5-15	0.05-0.2	Lansdorp and Dragowska, 1992
CD34 ⁺ CD45RA ⁺ CD71 ⁺	0.01-0.015	30-50	20-40	60-80	0.2-1	Mayani et al., 1993a Lansdorp et al., 1993
CD34 ⁺ CD45RA ⁺ CD71 ⁻	0.005-0.01	20-30	10-20	80-90	5-10	Sauvageau et al., 1994
CD34 ⁺ CD38 ⁺ Lin ⁻	0.003-0.006	20-30	40-60	40-60	2-8	Terstappen et al., 1991
CD34 ⁺ CD38 ⁺ Lin ⁻	0.001-0.004	5-10	20-40	60-80	15-30	Issaad et al., 1993 Sauvageau et al., 1994 Petzer et al., 1996a Petzer et al., 1996b
CD34 ⁺ Thy1 ⁺ Lin ⁻	0.001-0.004	10-20	35-50	50-65	3-8	Baum et al., 1992 Craig et al., 1993 Mayani et al., 1994 Petzer et al., 1996c
CD34 ⁺ HLA-DR ⁺ Lin ⁻	0.002-0.006	3-8	25-40	60-75	1-3	Verfaillie et al., 1990 Brandt et al., 1990 Verfaillie, 1992

c) *In vitro* assays and phenotypic markers for the detection of *in vivo* repopulating cells

Murine LTC-IC have been shown to have surface markers and other cellular properties that are shared by long-term *in vivo* repopulating cells and not by most CFC suggesting that LTC-IC and long-term *in vivo* repopulating cells may be similar populations (reviewed in Eaves et al., 1993a). This concept was originally supported by the demonstration that some murine *in vivo* repopulating cells can undergo self-renewal divisions under the same culture conditions as are used to stimulate LTC-IC proliferation and differentiation (Fraser et al., 1992). Correlated increases in LTC-IC and *in vivo* repopulating cell numbers from both murine (Miller and Eaves, 1997) and human (Conneally et al., 1997) systems suggests the existence of a close relationship between these functionally defined cell types (reviewed in Eaves et al., 1992a). Assays involving repopulation of human tissue implants (Kyoizumi et al., 1992) or of murine hematopoietic tissues (Dick et al., 1991) in SCID-hu mice using cell populations that are highly enriched in LTC-IC further support these observations.

It is important to note that although *in vivo* repopulating cells and LTC-IC share overlapping properties, recent data from a variety of sources suggest that LTC-IC are a heterogeneous population (Hogge et al., 1996; Hao et al., 1996; Shah et al., 1996), a fraction of which are *in vivo* repopulating cells. Although LTC-IC can be found in both the CD34⁺CD38⁻ and CD34⁺CD38⁺ compartments (Sauvageau et al., 1994), a majority of cells capable of *in vivo* repopulation are CD34⁺CD38⁻ (Civin et al., 1996, Conneally et al., 1997). Additionally, limiting dilution experiments extending the LTC-IC assay to >6 weeks (Petzer et al., 1996a) increased the number of cells capable of reading out in this assay. These extended

LTC-IC have subsequently been shown to be located primarily in the CD34⁺CD38⁻ compartment (Hao et al., 1996).

Differences between short-term (6 week survival after lethal irradiation) and long-term (multilineage engraftment after >6 months) bone marrow repopulating ability have also been isolatable by phenotype (Morrison and Weissman, 1994; Zijlmans et al., 1995). Interestingly in these studies, cells capable of long-term repopulation were generally also able to enhance short-term survival after lethal irradiation suggesting that this property is not uniquely associated with more differentiated cells (Morrison et al., 1997b). It is important to note that the detection of *in vivo* repopulating cells is dependent upon their homing and engraftment properties. Recent evidence suggesting that both the cell cycle status and/or the expression cell surface molecules may regulate these properties (Spangrude et al., 1995; Morrison et al., 1997c) suggest that further experimentation is required in order to understand the limitations of this assay, especially after *in vitro* culture. Differences in the functional properties of cells exhibiting the same cell surface antigens before and after *in vitro* culture (Rebel et al., 1994; Spangrude et al., 1995) suggests that this criterion may also be independently regulated.

C. Cell Proliferation and Differentiation Control

a) *A permissive versus an inductive environment?*

Regulation of hematopoietic cell proliferation and differentiation *in vivo*, allows the continuous and controlled production of large populations of mature and maturing cells from a small population of stem cells as required during development and later in response to physiological demands. This process, which implies the existence of direct or indirect

feedback control mechanisms (Potten and Loeffler, 1990), involves the production and action of a large group of cytokines, many of which have been characterized and produced in recombinant form. Since hematopoietic stem cells are segregated among different bones and organs throughout the body, at least some of these controlling factors must act independently of direct cellular contact for the stem cell pool to be regulated in a coordinated fashion (Morrison et al., 1997a). These molecules can exert some of their effects as single agents, but can also act in synergistic or additive combinations (reviewed in Metcalf, 1993).

There is still considerable controversy as to whether the effects of these factors on the self-renewal and differentiation of primary normal hematopoietic cells are permissive or directive. In permissive models of differentiation, exposure to cytokines provides an environment in which the phenotypes of the daughter cells are stochastically determined and the role of extrinsic factors is simply to vary the survival or proliferation of specific populations. In directive models of differentiation, cytokines direct the responsive cell to differentiate along a defined pathway and clonal variations in the frequencies of particular lineages are thus largely attributable to the particular cytokines to which the cells are exposed. The distinction between these two theories is important as it may constrain the ability of a particular culture system to control the production of particular cell types. Evidence by Mayani et al. (1993b) supports previously documented work showing stochastic commitment patterns in spleen-colony forming unit (CFU-S) production (Till et al., 1964; Humphries et al., 1981) and suggests that the addition of cytokines to culture systems does not influence the lineage commitment of multipotent cells. This conclusion is supported by observations in the multipotent hematopoietic cell line, FDCP-Mix, where transfection of *bcl-2* (which blocks apoptotic cell death) yielded multilineage differentiation in the absence of cell division and/or

cytokines (Fairbairn et al., 1993). These data suggest that exposure to growth factors may not be obligatory for the differentiation of primitive cells and that, at least under certain conditions, differentiation may be intrinsically determined.

In contrast to these results, the same group has shown (also using FDCP-Mix cells), a differential response to stimulation by different concentrations of the same cytokine such the multipotent IL-3-dependent murine cell line can proliferate in the presence of lower concentrations of IL-3 than are necessary to support the concomitant maintenance of their pluripotent state (Dexter et al., 1990). Additionally, changes in the *in vivo* environment with ontogeny have been thought to be responsible for the increased cycling (possibly affecting homing in repopulation assays) and numbers of *in vivo* repopulating cells throughout adult life (Morrison et al., 1996). Furthermore, exposure of primitive progenitor cells with lymphomyeloid potential to IL-3 and IL-1 has been shown to block their cytokine-stimulated generation of lymphoid precursors and to impair their self-renewal *in vitro* (Hirayama et al., 1994; Yonemura et al., 1996). Each of these results support, at least in part, a cytokine directive mode of self-renewal and differentiation.

It is noteworthy that investigations of hematopoietic mechanisms may be limited by both the nature of the culture system in which a majority of these experiments are performed and the heterogeneity that exists among primitive cells, even within highly purified populations (Uchida et al., 1993). High density static tissue culture systems expose cells to physical and chemical gradients [e.g. oxygen (Randers-Eichhorn et al., 1996)] which may result in confounded observations. Furthermore, *in vivo* studies will have been affected by differences in hematopoietic cell homing pre- vs post- cytokine stimulation (Spangrude et al., 1995).

Some of these issues can be addressed by the use of: 1) homogeneous culture systems, 2) single cell experiments, and 3) purification strategies which obtain uniformly responsive cell populations. As each cell may be differentially affected by its developmental history, differences in the actions of individual vs populations of cells may be very difficult to distinguish, even using the most modern tools.

b) *Hematopoietic stem cell stimulation by cytokines*

The most primitive hematopoietic cells in adult marrow are in a state of slow (Lajtha, 1979) but finite (Ponchio et al., 1995; Bradford et al., 1997) turnover. The recruitment of at least some of these into the cell cycle can be influenced by several so-called “early-acting” growth factors (Leary et al., 1992). These factors, whose major properties are outlined in Table 1.2 include, IL-6, granulocyte-colony stimulating factor (G-CSF), IL-3, IL-11, SF, granulocyte-macrophage-colony stimulating factor (GM-CSF), thrombopoietin (TPO) and flt-3 ligand (FL) (Toksoz et al., 1992; Lyman et al., 1993; Dosil et al., 1993; Hangoc et al., 1993; Miyajima et al., 1993; Brandt et al., 1994).

Table 1.2: Cytokines acting on hematopoietic progenitors

Cytokine	Molecular Weight (kD) ^a	Cell Population (hematopoietic)		References
		Source	Target	
IL-1 α	18	Osteoblasts, monocytes, macrophages, SAC ^b	Myeloid, lymphoid and primitive progenitors,	March et al., 1985 Muench et al., 1993
G-CSF	19	Macrophages, monocytes and SAC	Myeloid and primitive progenitors	Souza et al., 1986 Nagata et al., 1986
IL-6	21	T- and B- cells, monocytes, macrophages, SAC	Megakaryocytes, myeloid, erythroid and primitive progenitors	Hirano et al., 1986 Koike et al., 1988
IL-3	17	Activated T- and NK cells	Myeloid, erythroid and primitive progenitors	Yang et al., 1986
SF	18.5 ^c	Fetal liver cells, SAC	Mast cells, primitive progenitors	Martin et al., 1990
IL-11	19 (19-23)	Macrophages, monocytes and SAC	Myeloid, erythroid primitive progenitors	Keller et al., 1993 Hangoc et al., 1993
FL	17.5 (17-30)	Primitive hematopoietic cells, germ line cells, SAC	Myeloid and primitive progenitors	Lyman et al., 1993 Hannum et al., 1994 Small et al., 1994
TPO	35 (35-47)	Megakaryocytes, SAC	Megakaryocytes, erythroid and primitive progenitors	Sohma et al. 1994 Kaushansky, 1995 Young et al., 1996

^a Molecular weight range given for non-*E.coli* produced factors (due to differences in glycosylation or alternative isoforms).

^b SAS, stromal associated cells, i.e. fibroblasts, adipocytes, endothelial cells.

^c Exists in solution as a non-covalently associated dimer.

These growth factors can be subdivided into groupings that share multiple and frequently overlapping functions. For example GM-CSF and IL-3 transduce signals through the β -common receptor and IL-6 and IL-11 through the gp130 receptor (reviewed in Gearing and Ziegler, 1993). Neither of these groups of cytokine receptors have intrinsic tyrosine kinase activity. This is in contrast to SF and FL whose receptors possess an intrinsic tyrosine

kinase domain (Lyman et al., 1993; Dosil et al., 1993). Using serial observations of blast colony formation, Ogawa and co-workers (reviewed in Ogawa, 1993) have divided cytokines into groups that *a*) initiate primitive progenitor cell cycling (e.g. SF and FL), *b*) expand the committed progenitor cell pool (e.g. IL-3 and GM-CSF) and *c*) promote maturation of committed progenitors (e.g. EPO, G-CSF).

The actions of some of these cytokines (i.e. groups *a* and *b*) when used individually has been found to result in the rescue of the cells from apoptotic cell death. For example, both SF and TPO alone can promote survival but not proliferation of primitive hematopoietic cells (Keller et al., 1995; Borge et al., 1997), whereas when used with other factors (e.g. IL-3), proliferation and differentiation are enhanced (Ikuta et al., 1991; Resnick et al., 1992; Kobayashi et al., 1996). It is important to note, as shown by Dexter et al. (1990) with FDCP-Mix cells, that the survival vs proliferation promoting effect of individual cytokines also may be related to the concentrations at which they are used.

In contrast to the results for SF and TPO, FL, which although first shown to have effects on the proliferation of high proliferative potential (HPP) CFC in combination with other cytokines (Small et al., 1994) or on LTC-IC stimulation by marrow feeder layers (Muench et al., 1995), has more recently been shown to have direct proliferation inducing effects on primitive cells when used alone (Shah et al., 1996; Petzer et al., 1996b; Nordon et al., 1997). It is important to note that in the above studies, cytokine supplementation has occurred in the presence of transferrin and insulin. Investigations of the interactions of these factors and the above cytokines will have to be performed to fully understand the individual

growth factor requirements for the self-renewal and differentiation of primitive hematopoietic cells.

In addition to "early-acting" factors, the effect(s) on primitive cells of other cytokines of both hematopoietic and non-hematopoietic origin has been investigated. Transforming growth factor- β (TGF- β) and macrophage inflammatory protein-1 α (MIP-1 α) have been shown to inhibit primitive hematopoietic cell cycling in long-term culture (Cashman et al., 1990). Neutralization of endogenous MIP-1 α [by the addition of excess MIP-1 β , (Eaves et al., 1993c)] and TGF- β [by specific antibody addition, (Eaves et al., 1991a; Bonewald, 1992)] prevents the return of normal primitive progenitors (HPP-CFC) to a non-cycling state. Fibroblast growth factor (FGF) has been shown to have a supportive function on primitive hematopoietic cells in culture by indirect stimulation when used in combination with other cytokines. Addition of FGF to cultures of CD34⁺CD33⁻ cells resulted in an increase in both colony size and number (Gabbianelli et al., 1990). Molecules that enhance the binding of cytokines to their receptors, [e.g. sIL-6R, (Sui et al., 1996)] or act during embryonic development [e.g. bone morphogenic proteins (BMP's), IL-12, and ligands to the frizzled receptor family (Jacobsen et al., 1993; Luo et al., 1995; Wang et al., 1996)] have also been reported to have stimulatory actions on primitive adult hematopoietic cells.

All of these stimuli indicate the potential of a complex set of extracellular influences that may affect primitive cell behavior both *in vivo* and *in vitro*. A systematic investigation of the balances of factor types, concentrations and methods of cytokine presentation to stem cells is required to identify key elements which govern, influence or promote changes in the self-renewal and lineage commitment of primitive hematopoietic cells.

D. Hematopoietic Growth Factor Receptors

Cell growth, death and differentiation are controlled by soluble and membrane-bound polypeptides that bind to specific cell surface receptors, transmitting biological signals across the cell membrane resulting in the induction of specific genes. Receptor molecules are highly selective for their ligands and, upon binding, transmit signals into the cell activating multiple metabolic pathways, including those resulting in mitogenesis (the induction of cell division). This signal transduction across biological membranes ubiquitously involves a receptor oligomerization step. There are several ways in which ligand binding can cause this phenomenon: 1) A monomeric ligand causes a conformational change in the receptor that enhances the affinity of one receptor molecule for another receptor molecule [e.g. epithelial growth factor (EGF), IL-3, GM-CSF]. 2) A dimeric ligand binds to two receptor chains [e.g. SF, platelet-derived growth factor, ciliary stimulatory factor-1 (Lev et al., 1992)]. 3) The receptor is a covalently linked dimer and its cytoplasmic domain undergoes a conformational change upon ligand binding (e.g. insulin, insulin-like growth factor). 4) A monomeric ligand has two binding sites for monovalent receptor chains (e.g. human growth hormone). 5) A monovalent ligand binds to a heterodimeric receptor promoting the noncovalent association of two distinct cytoplasmic domains, both of which are necessary for signaling (e.g. IL-2, IL-4, IL-7). 6) A monomeric ligand binds to a single chain that induces its association with a separate signaling chain, which then associates with another ligand-binding chain-signaling pair (e.g. IL-6, IL-11, CNTF).

Oligomerization, as a result of ligand/receptor interaction is the initial step that results in the initiation of an intracellular signaling cascade. For example, after stimulation of

homodimerization of gp130 in response to IL-6/IL6R binding, tyrosine specific phosphorylation of gp130 is observed (Murakami et al 1993). Unlike other cytokine receptor systems (e.g. *c-kit*, EGFR), gp130 does not possess an intrinsic tyrosine kinase in the cytoplasmic domain, but interacts with the JAK kinases, a family of cytoplasmic kinases in order to initiate phosphoprotein signaling (Lutticken et al., 1994). The activation of these molecules then leads to a series of protein phosphorylation steps, often mediated by Src-homology 2 (SH2) containing cytoplasmic messengers (Koch et al., 1991), leading to the activation of DNA binding proteins which determine the nature of the cellular response to the stimulus.

a) *Differential responses mediated by receptor type*

The ability of a cell to respond to a soluble or membrane bound stimulus is dependent on the array of receptors expressed on its surface. Changes in cell surface receptor expression occur with development (Ikuta et al., 1990; Huang and Terstappen, 1994; Rebel et al., 1996), differentiation (Fantoni et al., 1981; Tajima et al., 1996; McKinstry et al., 1997) and cytokine stimulation (Murthy et al., 1989; Welham and Schrader, 1991; Korpelainen et al., 1993). The stimulatory effects of combinations of cytokines have been attributed to a requirement for cell stimulation through multiple signaling pathways (Nathan and Sporn, 1991; Muench et al., 1992a; Ogawa, 1993; Whetton and Dexter, 1993) and to cytokine-mediated potentiation of cell surface receptor expression (Sato et al., 1993a).

An interesting example of changes in the cytokine responsiveness with receptor expression has recently been shown by Tajima et al. (1996) who found that large expansions of CFU-GEMM could be obtained in cultures of CD34⁺ CB cells negative for the IL-6

receptor (IL-6R⁻) when these cells were stimulated by IL-6, SF and the soluble IL-6 receptor (sIL-6R). If the sIL-6R was not supplemented, no CFU-GEMM production was detected. These studies also showed that the IL-6R⁻ population included cells initially detectable as LTC-IC, BFU-E and CFU MK, whereas most CB CFU-GM were IL-6R⁺. Interestingly, the IL-6R⁺ CFU-GM did not require the addition of sIL-6R for their proliferation.

b) *Differential responses mediated by receptor stimulation*

Based on observations of IL-2-mediated T-cell growth, Smith (1995) has formulated a "ligand-receptor model" which accounts for the observed effects of changes in IL-2 concentration, receptor density, and the duration of receptor activation on T-cell cycle progression. Particularly noteworthy was the finding that Gaussian distributions in cycle progression times closely correlated with differences in IL-2 receptor expression, even within otherwise identical clonal cell populations, suggesting that the rate-limiting step in IL-2-stimulated T-cell growth is the interaction of IL-2 with its receptor (Cantrell and Smith, 1984). By separating synchronized T-cell populations on the basis of IL-2 receptor densities, Smith (1995) suggested that there is some finite number of ligand/receptor interactions that must occur before the cell replicates its DNA. This threshold was reached earlier in the high receptor expressing cells relative to low receptor expressing cells. Similar data showing that overexpression of receptors potentiates a mitogenic response have more recently been shown for insulin (de Meyts et al., 1994) and EGF (Wells et al., 1990).

The kinetics of EGF binding to its receptor have been extensively investigated by Wiley and Lauffenburger (Wiley and Cunningham, 1982; Knauer et al. 1984; Starbuck et al., 1990; Reddy et al., 1994; 1996a, b; French et al., 1995). Their results suggest that the

information essential for cell regulation can be found in both the magnitude and persistence of the cytokine signal (instead of simply the presence of the signal per se) (Marshall, 1995). Examples of this phenomenon have been reported for the IL-3-dependent survival, proliferation and degranulation of mast cells (Welham et al., 1994), and the proliferation and differentiation of murine FDCP-mix cells (Dexter et al., 1990). In the PC12 neuronal cell line, high concentrations of NGF resulted in proliferation whereas low concentrations result in the activation of a terminal differentiation pathway (Traverse et al., 1994).

To investigate mechanisms for the concentration-dependent stimulation of cytokine-dependent cells, the human EGF-receptor (EGFR) system was used by Lauffenburger and co-workers to compare the kinetics of the wild-type (WT) receptor with that of a carboxy-terminal truncated mutant deficient in receptor mediated internalization (Chen et al., 1989). Cell-specific proliferation rate constants and EGF depletion kinetics were measured for both WT and mutant transfected cells (Reddy et al., 1994). When EGF depletion was minimized by medium replenishment, the EGF concentration dependencies of the proliferation responses of the two cell types were similar, whereas when EGF depletion was not prevented, maximal proliferation of WT cells required initial EGF concentrations approximately 10-fold higher than those required by the mutant cells. This suggested that diminished depletion of EGF from the extracellular media was the major reason for the increased mitogenic activity of EGF by cells possessing internalization-deficient receptors. Similar results have been shown by the same group comparing the mitogenicity of TGF α and EGF (French et al., 1995). In this case, although receptor binding and internalization rates through the EGFR were similar for the two cytokines, due to differences in the rates of ligand-receptor dissociation at intracellular pH (Korc and Finman, 1989; French et al., 1995), EGF was depleted less rapidly from the

extracellular media and thus exhibited a higher mitogenic potency at a given initial concentration.

Based on these observations, Reddy et al. (1996b) have proposed that the successful use of growth factors in bioprocessing applications requires the control of two attenuation mechanisms, growth factor depletion and receptor down-regulation. To test this hypothesis, the mitogenic potency and factor depletion rates for EGF were compared to a low EGFR binding affinity human EGF variant (EGF_{Y13G}) (Tadaki and Niyogi, 1993). In this system, when factor-containing media was not continuously replenished, EGF_{Y13G} acted as a more potent mitogen, whereas if the factor containing media was replenished, EGF_{Y13G} was a less potent mitogen (Reddy et al., 1996a). These differences were attributed to a 50-fold lower binding affinity of EGF_{Y13G} vs EGF to the EGFR which resulted in lower factor depletion and receptor down-regulation rates for the mutant cytokine. When cytokine containing media is continuously replaced, due to its higher binding affinity, EGF provides the most potent stimulus. In contrast, when the cytokine containing media is not replaced, EGF_{Y13G} is depleted less rapidly and thus provides the most potent stimulus.

Taken together, these studies support a model of factor-dependent cell stimulation where differences in factor concentration may result in distinct cellular fates due to differences in threshold levels of stimulation and factor depletion rates. Further, these differences in factor depletion rates can be attributed to ligand receptor association and dissociation constants, cell surface receptor expression densities and receptor internalization rates. It is of note that most of the previous studies have focused on cell proliferation as their endpoint; further investigations are now required to investigate these phenomena in primary cells where

growth factor depletion and stimulation control not only proliferation but also cell survival and differentiation.

E. Steel Factor Receptor (*c-kit*)

First identified as the normal cellular counterpart of *v-kit*, the transforming gene of a feline sarcoma virus (Besmer et al., 1986), the *c-kit* proto-oncogene has been shown to encode a 145-155 kD tyrosine kinase receptor that is differentially expressed on multiple hematopoietic cell populations. Upon binding by its ligand, SF, *c-kit* undergoes dimerization and tyrosine phosphorylation (Blechman et al., 1995). Soluble SF exists mainly as a noncovalently associated bivalent molecule (Lev et al., 1993), although at physiological concentrations, SF may be predominantly monomeric (Hsu et al., 1997) suggesting an equilibrium between these two forms. Two lines of evidence indicate that dimer formation by *c-kit* is independent of ligand bivalency: first, no transition to monomeric receptors has been observed at high SF concentrations (Lev et al., 1992) and second, heterodimers between human and murine *c-kit* molecules have been observed in the presence of rat SF, which is not recognized by human *c-kit* (Lev et al., 1993).

Receptor dimerization, activation of the intrinsic tyrosine kinase domain and the consequent receptor transphosphorylation (reviewed in Ullrich and Schlessinger, 1990) is followed by ligand induced down-regulation (Yee et al., 1993). Studies with mutated forms of *c-kit* show that although internalization requires an active tyrosine kinase domain (Yee et al., 1994), internalization is not required for a mitogenic response (Miyazawa et al., 1995). In fact, studies with (predominantly) membrane bound SF (Avraham et al., 1992; Miyazawa et al., 1995) or immobilized monoclonal antibodies (MAb) to SF (Kurosawa et al., 1996) have

suggested that prevention of internalization prolongs tyrosine kinase activation and *c-kit* cell surface half-life. Similar studies using MAb to the extracellular domain of *c-kit* have shown that down regulation occurs in a rapid, dose-dependent manner (Miyazawa et al 1994; Shimizu et al., 1996; Baghestanian et al., 1996) resulting in degradation of internalized SF and *c-kit* as well as the transient desensitization of cells to further stimulus.

An understanding of the extracellular requirements for stimulation of specific responses from primitive hematopoietic cells requires both knowledge of the particular factor requirements and the responses of the cells to those factors. This information can then be used in the design of efficient and productive systems for the *ex vivo* culture of hematopoietic cells.

2. *In Vitro* Hematopoietic Cell Culture

A. Long-Term (Dexter-type) Culture System

The first culture system in which the production of hematopoietic cells could be reproducibly sustained for many weeks was described for murine cells by Dexter and colleagues in 1977. Following the recognition of the importance of adding corticosteroids to the medium (Greenberger, 1978), this system was also shown to be applicable to human BM cells by Gartner and Kaplan (1980). A critical feature for the establishment of durable hematopoiesis was the rapid formation or provision of a supportive adherent feeder layer containing cells of the fibroblast, endothelial and adipocyte lineages. When primitive hematopoietic cells are co-cultured with such cells they are selectively retained in the adherent layer (Mauch et al., 1980; Coulombel et al., 1983) while their maturing granulopoietic progeny are continuously released into the non-adherent fraction (Slovick et al., 1984). Many

aspects of this system, including some of the cellular interactions that take place between the hematopoietic cells and the stroma, have been shown to mimic *in vivo* hematopoiesis [Dexter et al., 1980; Eaves et al., 1992b (for review of human systems)]. However, due to the complex nature of the system, many of the molecular mediators responsible for hematopoietic regulation are only beginning to be defined. Additionally, it has been shown that these cultures expose cells to growth environments that fluctuate greatly outside physiological ranges of pH, nutrient supply, and metabolic by-product concentrations (i.e. lactic acid, Koller et al., 1993a). These fluctuations may be responsible, at least in part, for the decrease over time in the numbers of total and progenitor cells observed in such cultures (Cashman et al., 1985).

B. Alternate Hematopoietic Culture systems

Adaptations of the original long-term culture system can be divided into two main groups; those that further attempt to mimic the *in vivo* BM microenvironment (including the use of stromal cells, either in the absence or presence of exogenous cytokines), and those that utilize stroma-free or suspension-culture systems (relying on conditioned media and/or exogenously added cytokines for cell stimulation). This section describes the development of these systems, and outlines the major results obtained in their use. Results specific to culture parameters affecting hematopoietic cell expansion are discussed in the subsequent section.

a) Cultures providing a surface for stromal cell growth

Increases in the medium addition rates have significantly increased the productivity of hematopoietic culture systems (e.g. Schwartz et al., 1991a, b; Koller et al., 1993a). This

finding, along with evidence that intermittent medium feeding causes artificial cycling of the cell growth pattern (Toksoz et al., 1980; Cashman et al., 1985), led to the development of several perfusion culture systems. The first of these were either flat-plate bioreactors consisting of 25-cm² rectangular glass inserts that served for adherent cell attachment (Koller et al., 1992a) or membrane bioreactors in which the hematopoietic cells were inoculated into a collagen porous microcarrier matrix in a compartment enclosed by ultrafiltration membranes (Wang and Wu, 1992). In the flat-plate bioreactors, medium was continuously re-circulated from a pH- and O₂-controlled reservoir with weekly exchange of half the bioreactor medium (Koller et al., 1992a). In the porous microcarrier bioreactor non-serum containing medium (replaced daily) was continuously re-circulated unidirectionally above and below the unperfused serum- and collagen bead-containing center chamber (Wang and Wu, 1992). The center chamber received serum containing media intermittently with cell sampling. Each of these systems reported significant increases in total cell and CFU-GM production from murine BM over that achieved in Dexter-type T-flask cultures (Wang and Wu, 1992; Koller et al., 1992a). The collagen-chamber bioreactor has subsequently been shown to support multi-lineage (lymphoid, erythroid, granulocyte, macrophage and megakaryocyte) murine hematopoiesis in the absence of cytokines at 37 °C for periods of up to 60 days (Wang et al., 1995) whereas the flat-plate bioreactor achieved significant LTC-IC expansions (~3-fold over input) from human BM in cytokine supplemented cultures (Koller et al., 1993a). In both of these systems, oxygenation occurred in the medium reservoir and thus oxygen gradients could develop in the direction of medium flow (Piret and Cooney, 1991). Since the medium in the reservoir was replaced intermittently, the cells were also subjected to temporal changes in the concentrations of nutrients, cytokines and inhibitory metabolites.

Palsson et al. (1993) developed a modified bioreactor system to oxygenate the cells via a gas permeable membrane along the length of the culture bed to reduce oxygen gradients in the direction of medium flow. Their system also provided continuous single pass fluid flow, which minimized temporal gradients, but would maintain spatial gradients in soluble nutrients and inhibitor metabolites. This system was subsequently scaled-up 10-fold to enable inoculation with $\sim 3 \times 10^7$ human BM MNC per reactor, and they obtained 10-, 21- and 7.5-fold average expansions of total cells, CFU-GM and LTC-IC, respectively (Koller et al., 1993b). Furthermore, 2- to 3-fold increased overall cell and progenitor expansions could be achieved by intermittent harvesting (Oh et al., 1994; Palsson et al., 1995) or replating of the expanded cells, indicating that cell proliferation in this system may be limited by surface area.

Highfill et al. (1996) investigated the use of a packed glass fiber-matrix bed system for the expansion of murine BM cells. The long-term (> 10 weeks) cell generation achieved in this system (in the absence of exogenously added cytokines) was dependent on the BM inoculum density, the pre-establishment of a stromal cell layer on the matrix, and re-circulation of conditioned medium. Further experimentation with more biocompatible packing material using this system is ongoing.

b) *Suspension or stromal cell-free cultures*

The use of an adherent stromal cell layer may be undesirable for clinical scale expansion cultures due to their poorly defined composition, variability, requirement for enzymatic cell harvesting, absence in CB or PB and/or surface area-limited growth (Oh et al.,

1994). Consequently, there has been a significant effort to develop culture systems that do not rely on the formation of such a layer.

Sardonini and Wu (1993) first reported the use of stirred suspension cultures with and without porous microcarriers as well as air-lift and hollow fibre bioreactor systems for the culture of human hematopoietic cells. In two experiments using human BM MNC, total cell expansions of 10 to 20-fold over input were obtained both in the stirred suspension cultures (regardless of the presence of microcarriers) and the air-lift bioreactor. In the static, stirred suspension and airlift bioreactors, CFU-GM and BFU-E expansions were maintained at input levels for the 28 day duration of the cultures. No expansion was obtained using the hollow-fibre bioreactor, possibly due to difficulties in harvesting the cells from the extracapillary space (Sardonini and Wu, 1993). Toxic by-products of the hollow-fiber manufacturing process may also have affected the cultures (C. Eaves, personal communication). Stirred suspension cultures have also been investigated by Zandstra et al. (1994; 1997a; see Chapters 3 and 4), Collins et al. (1995) and Kogler et al. (1996a, b). Significant CFC expansions have been achieved in both serum-containing and serum-free cultures of BM, PB and CB MNC. These expansions have been shown to be dependent on input cell density, vessel configuration (specifically, the ratio of impeller diameter to vessel diameter) and medium composition (Zandstra et al., 1994, 1997a; Collins et al., 1995). Significant CFC (50- to 100-fold over input) and LTC-IC (5- to 15-fold over input) expansions also have been recently reported from CD34⁺ (44-72% purity) CB cells in stirred suspension cultures supplemented with elevated concentrations of FL (Kogler et al., 1996a, b).

A modification of the flat-plate culture system has also allowed the development of stroma-free hematopoietic perfusion cultures (Sandstrom et al., 1996). In this system, the chamber contains multiple micro-grooves (perpendicular to the direction of medium flow) designed to allow the diffusion of nutrients to multiple layers of cells while retaining the cells in the culture chamber (Sandstrom et al., 1996). The dimensions of the grooves (200 X 200 μm) were chosen based on an estimate of oxygen diffusivity ($2.5 \times 10^{-5} \text{ cm}^2/\text{s}$) and the calculated constraint of providing oxygen above 60% of air saturation at the bottom of the last groove. Using this system, significant expansions of total cells (10- to 15-fold) and CFU-GM (15- to 25-fold), have been obtained [Table 1.4, (Sandstrom et al., 1996)].

Gas-permeable tissue culture bags, in which the cells generally grow undisturbed until harvested, have also been used successfully for the stroma-free culture of hematopoietic cells. CD34⁺ cells from the BM and PB of normal patients have been expanded 30- to 120-fold in the presence of recombinant growth factors in these cultures (Purdy et al., 1995; Williams et al., 1996). The non-adherent nature of this system, although limited in terms of the ease of media manipulation, has an advantage in terms of the ease of harvesting the expanded progenitor cells under aseptic conditions. Nonetheless, due to the static nature of these cultures, cells settling on the bottom surface may be exposed to gradients in oxygen and medium components.

The development of the above bioreactor configurations address the design requirements of particular culture modification that have been shown to be important in the growth of hematopoietic progenitors *ex vivo*. The following section discusses these

modifications and their specific effects on the output of functionally and phenotypically defined populations of hematopoietic cells.

C. Culture Modifications

Modifications to traditional static cultures have been developed with the goal of obtaining systems capable of supporting the continued growth of hematopoietic cells. Most of the work in this area has focused on defining the influence of controllable design parameters on functionally or phenotypically defined cell populations from a variety of hematopoietic cell sources. The design parameters investigated in these studies have been: 1) the medium exchange interval/perfusion rate, 2) the composition, density, and presence of accessory cells of stromal origin in the input population, 3) the oxygen tension, and 4) the addition of exogenous growth factors to the culture medium. Although these parameters have, in general, been treated individually, it is clear that they are often interrelated. In the following sections, results related to each of the above four design parameters are reviewed individually, followed by a discussion of the results in relation to other parameters.

a) Perfusion/medium exchange rate

Traditional semi-continuous static cultures, where part of the culture medium is exchanged with fresh medium at periodic intervals, appear to be limited by nutrient depletion, cytokine stimulation, and/or the local build-up of inhibitory compounds (Cashman et al., 1985; Caldwell et al., 1991a). Medium exchanges have been shown to cause primitive progenitor cells in the adherent layer of Dexter cultures to proliferate (Toksoz et al., 1980; Cashman et al., 1985) as a result of increased growth factor supplementation and increased cytokine

secretion rates (Caldwell et al., 1991b; Eaves et al., 1993b). Cell-specific glucose consumption and lactate production increased with greater medium exchange rates, due primarily to increased serum supplementation (Caldwell et al., 1991b). Increasing medium exchange rates in static cultures (from 0.5 to 3.5 volumes/week) resulted in an increased production of CFC (Schwartz et al., 1991a). In several intermittently-fed perfusion culture systems, increased medium supplementation increased both total cell and progenitor cell output by comparison to controls (Wong and Wu, 1992; Koller et al., 1992a; Sandstrom et al., 1995, 1996). Direct comparisons between semi-continuously (i.e. static Dexter cultures and intermittently-fed perfusion cultures) and continuously fed cultures are difficult to make as comparisons of systems receiving equivalent average cell-specific media supplementation rates have not been reported. One interesting result that may be attributed to continuous medium supplementation is the increased growth of PB CFU-GM progenitors (15- to 22-fold over input) from two samples that, in the intermittently fed cultures grew poorly (5- to 10-fold over input) or not at all, leading to significantly less inter-sample variability in continuously fed cultures (Sandstrom et al., 1996). Surprisingly, although significant total cell and progenitor expansions (~ 10-, 21- and 7.5-fold expansions of total cells, CFC and LTC-IC, respectively) have been measured in one-pass continuous bioreactor systems, the effect of varying the feed rates has not been reported (Koller et al., 1993a,b; Palsson et al., 1993; Oh et al., 1994) and, consequently, it is unclear how the performance of this system is dependent on the rates of medium addition, continuous medium addition, or both. The medium perfusion rates used in at least two of these systems were based on estimated *in vivo* perfusion rates of 0.1 mL per cm³ bone marrow tissue per min (Martait et al., 1987). Assuming *in vivo* cell densities on the order of 5x10⁸ cells/cm³, this perfusion rate corresponds to ~0.2 mL serum

per 10^6 cells per day, or, for 20% serum-containing medium, one medium exchange per day (Koller and Palsson, 1993). It should be noted that although the medium exchange rate used in one-pass perfusion systems was based on physiological rates, its applicability to *in vitro* expansion systems, where culture systems and media formulations are very different, is open to question.

The positive effects of increases in the rate of medium addition may be attributable to several mechanisms. The introduction of fresh media both replenishes depleted nutrients and cytokines, and dilutes metabolic by-products and inhibitory factors (Koller et al., 1995a; Lauffenburger et al., 1996; Zandstra et al., 1997a). Increased medium exchange frequencies may also reduce the amount of time that cells are exposed to anoxic conditions and, by increasing stromal cell metabolic activity, thereby increase cytokine secretion rates by these cells (Caldwell et al., 1991b). Each of these factors may act together or independently to enhance *ex vivo* hematopoietic progenitor production.

b) *Culture inoculum, cell density and accessory cells of stromal origin*

The need for the provision to expansion cultures of both mature hematopoietic and non-hematopoietic cells (i.e. endothelial, adventitial reticular and adipocyte cells) from the BM environment has been a source of controversy. Although several different hematopoietic growth factors can substitute for the supportive function of the adherent layer (Brandt et al., 1990; Sutherland et al., 1992; Sutherland et al., 1993b), cultures performed on a "feeder" layer of BM stroma have generally reported greater culture longevity and progenitor cell expansion than those performed in the absence of a feeder layer (Koller et al., 1992b; Knospe et al., 1993; Wineman et al., 1993; Rowley et al., 1993) and/or with purified cells (Koller et

al., 1995b, 1996). The role of the BM stroma in these studies has been attributed to the creation of a microenvironment that is suitable for promoting hematopoietic cell growth. Stromal cells condition the media with a variety of soluble cytokines and extracellular matrix components as well as presenting cellular adhesion molecules and membrane-bound cytokines to nearby cells (Simmons and Torok-Storb, 1991; Long et al., 1992; Quesenberry et al., 1993; Verfaillie et al., 1994a; Gupta et al., 1996). Consequently, hematopoietic cells cultured on this substratum will receive highly localized signals, both positive and negative, to which they respond (Cashman et al., 1985; 1990; Hogge et al., 1995). The relative contributions of the presence of stromal cells, direct stromal cell contact, and medium inoculum density on hematopoietic cell expansion are examined below.

Studies showing enhanced primitive progenitor cell production when hematopoietic cells are separated from the stromal layer suggest that direct contact between LTC-IC and the supportive cells of the adherent layer is not required for LTC-IC maintenance (Verfaillie, 1992). Using a culture system where hematopoietic cells are separated from the stromal cell layer by a 0.4- μ m microporous filter membrane insert, Verfaillie et al. (1994b) have demonstrated that input LTC-IC numbers are maintained for a longer period of time in "stroma-non-contact" conditions than in traditional LTC (with stromal cell contact), regardless of cytokine supplementation (Verfaillie et al., 1993; Gupta et al., 1996). These results, like those of Simmons and coworkers (Simmons et al., 1994; Levesque et al., 1996) indicate that the identity or nature of the soluble factors necessary for the culture of primitive hematopoietic cells is produced by the stromal cell layer and that the role of these soluble factors in this maintenance has yet to be elucidated.

As discussed above, increased medium supplementation rates have yielded significant LTC-IC expansions from BM MNC (Koller et al., 1993a; Palsson et al., 1993) when cells were either inoculated at a high density (Zandstra et al., 1994; Koller et al., 1996) or onto a pre-formed stroma (Koller et al., 1993a; 1996). These LTC-IC expansions are in contrast to cultures initiated with purified $CD34^{+}lin^{-}$ cells where, even in the presence of pre-formed stroma and with rapid medium exchange rates, only a maintenance of LTC-IC numbers was obtained (Koller et al., 1996). In the absence of stroma, LTC-IC numbers from highly purified cells decreased regardless of the rate of medium exchange (Koller et al., 1995) further supporting the hypothesis that increased medium exchange rates stimulate factor secretion by the stromal cells (Caldwell et al., 1991; Schwartz et al., 1992). In experiments where purified cells were re-mixed with increasing numbers of $CD34^{-}$ cells, LTC-IC expansion ratios were found to be inversely correlated with input $CD34^{+}lin^{-}$ purity (regardless of the presence of stroma), such that maximum LTC-IC expansions were obtained at $CD34^{+}lin^{-}$ purities typical of BM MNC (Koller et al., 1995). Incrementally higher overall LTC-IC expansions have been measured between $CD34^{+}$ enriched (50% of input), $CD34^{+}$ enriched on pre-formed stroma (maintenance of input numbers), and MNC (4-fold over input) cultures, suggesting the presence of increasing stimulatory effects from each of these cell sources (Koller et al 1995).

Taken together, these results support a model whereby factors secreted by both stromal and more differentiated hematopoietic (i.e. $CD34^{-}$) cells can affect the maintenance and proliferation of primitive hematopoietic cells. As the frequency of media exchanges and the presence of non-progenitor cells affects both the secretion rates of these factors and their concentrations in the growth media [i.e. for IL-6 and LIF production (Koller et al., 1995)] it is

likely that these manipulations also impact on LTC-IC expansion. It is important at this point to emphasize that none of the above expansion studies was performed in the presence of FL or TPO, two recently isolated factors shown to have stimulatory effects on primitive hematopoietic cells (see section 1.C.b; Kobayashi et al., 1996; Gabbianelli et al., 1995). It remains to be determined if the positive effects of stroma secreted factors can be attributed to these or other recently isolated molecules, i.e. IL-6sr, BMPs, IL-12 and ligands to the frizzled receptor family (Jacobsen et al., 1993; Sui et al., 1996; Luo et al., 1995; Wang et al., 1996), or to additional factors that remain to be identified. The observations that TPO and FL are secreted by stromal cells, along with the additive contributions of both stromal and accessory cells suggest that each of these possibilities may be important.

c) *Culture oxygen condition*

There have been multiple studies of the effects of different oxygen levels on the proliferation of hematopoietic cells both in semi-solid and liquid cultures. Most of these studies have been motivated by the observation that *in vivo* BM oxygen tensions are on the order of 2-7% by volume (Lindop and Rotblat, 1960). Under oxygen conditions ranging from 1 to 10% (v/v), the size and number of hematopoietic colonies in semi-solid media were significantly enhanced (Bradley et al., 1978; Rich and Kubanek, 1982; Broxmeyer et al., 1990). Increased growth of hematopoietic cells at low oxygen concentrations has been, in part, attributed to the increased responsiveness of cells to growth factors (Rich and Kubanek, 1982), increased fibroblast cell proliferation (Bradley et al., 1978), and a decrease in H₂O₂, oxygen radical generation as well as other oxygen intermediates derived from mature cells (Pick and Mizell, 1981; Darfler and Insel, 1983; Meagher et al., 1988). The oxygen

environment in these cultures has been controlled by either direct manipulation of the gas phase concentration, or by the addition of oxygen radical scavengers such as catalase or mannitol, molecules which promote the reduction of H_2O_2 to H_2O and O_2 .

Probably due to difficulties in measuring the oxygen tension in the culture environment, attempts to extend these results to liquid culture systems have given conflicting results. In investigations carried out in static cultures or in perfused bioreactor systems, the oxygen tension in the bulk medium was measured and controlled. In all cases, the concentration of oxygen in the cellular microenvironment was not directly determined and may have varied widely due to different cell concentrations, oxygen gradients (Randers-Eichhorn et al., 1996) and/or medium delivery schedules. Inconsistencies in reported results may be due to 1.5- to 3-fold differences in culture cell densities; i.e. between the work reported by Palsson et al. (1993) (20% v/v O_2 optimal) and those of Koller et al. (1992a, b, c) (5% v/v O_2 optimal; Table 1.3). The effects of oxygen tension reported in these studies may be further confounded by the particular cytokines used. For example, differences in both cell proliferation (Table 1.3) and in cell specific oxygen radical production [e.g. for M-CSF-supplemented cultures, (Broxmeyer et al., 1990)] where different cytokines may lead to condition-dependent "optimal" oxygen tensions have been shown.

More recently, Muench et al. (1992c) has shown that reduced oxygen tensions do not enhance colony formation by primitive murine progenitors (HPP-CFC), suggesting that proliferation of these progenitors may be differently regulated by oxygen. On the other hand, the most mature cells also appear less sensitive to changes in their oxygen environment than their immediate precursors (Table 1.3, Cipolleschi et al., 1993). These results indicate that the

definition of different "optimal" oxygen tensions for hematopoietic cell culture may reflect the particular cells being stimulated. Under conditions where there is little or no expansion of very primitive (human) cells, low oxygen conditions resulted in the maintenance of higher numbers of LTC-IC (Koller et al., 1992b), whereas higher oxygen concentrations were optimal for significant LTC-IC expansions [Palsson et al., 1993, (Table 1.3)]. Overall, these results suggest that oxygen tension may be an important determinant of hematopoietic cell production and additional investigations of the effects of manipulations in oxygen tension in parallel with measurements taken in the cellular environment should be informative.

Table 1.3: Effect of Differences in Oxygen Tension on Progenitor Cell Expansion

Oxygen Levels Tested (% v/v)	Result	Culture System ^a	Reference
5 % 20 %	greater CFU-GM expansion at 5 % v/v O ₂	Static	Koller et al., 1992a
5 % 20 %	greater CFU-GM, BFU-E, and CFU-Mix expansion at 5 % v/v O ₂	Static	Koller et al., 1992b
5 % 20 %	greater CFC expansion at 5 % v/v O ₂	Perfusion Bioreactor	Koller et al., 1992c
5 % 20 % 40 % 60 %	greater CFU-GM and BFU-E expansion at 20 % v/v O ₂	Perfusion Bioreactor	Palsson et al., 1993
1 % 20%	greater total and CFC maintenance at 20 % v/v O ₂ , where as marrow repopulating ability (MRA) was greater at 1 % v/v O ₂	Static	Cipolleschi et al., 1993

^a Static= static tissue culture flasks

d) *Cytokine-supplemented expansion systems*

The ability of growth factor supplements to expand primitive cell populations *in vitro* has been extensively investigated. The availability of an increasing number of growth factors that can stimulate primitive hematopoietic cells either alone or in combination has made possible the identification of culture conditions that support a significant amplification of a variety of phenotypically or functionally defined cells (Table 1.4). For example, Haylock et al. (1992) showed that significant CFU-GM expansions (26-fold) could be obtained from CD34⁺ mobilized PB in the presence of IL-1, IL-3, IL-6, G-CSF and SF. Using similar protocols, including multiple cytokines, CD34 selection and dilute culture conditions, these results have subsequently been reproduced by many other groups (e.g. Brugger et al., 1993; 1995; Srour et al., 1993; Sato et al., 1993b; Henschler et al., 1994; Fel et al., 1994). Significant progenitor expansion in these static cultures is dependent on the initial purification of CD34 cells, suggesting that the presence of more mature cells, or their secreted metabolites, may exert a strong negative effect on hematopoietic cell proliferation under these conditions (Emerson, 1996). Progenitor expansion in such static cultures could also be extended by the adoption of regular medium dilution protocols (Moore and Hoskins, 1994; Van Zant et al., 1994; Piacibello et al., 1997). The continuous or semi-continuous addition of media also reduced the need for CD34 cell purification (Koller et al., 1993b; Palsson et al., 1993) presumably by the dilution of inhibitory cells or metabolites.

Although multiple combinations of cytokines were tested in the studies presented in Table 1.4, few systematic analyses of the effects of individual or groups of cytokines under defined conditions have been reported. Nonetheless, several observations can be made. The

Table 1.4: Summary of mononuclear and CD34⁺ cell cytokine mediated expansion studies

Study	Cell Type ^a	Culture Parameters		Maximum expansions					Growth factors (ng/mL)										
		Size (mL)	Inoculum density (mL ⁻¹)	Vessel	TC	CFC		LTC-IC	Epo	G-CSF	GM-CSF	IL-1	IL-3	IL-6	IL-11	SF	Tpo	FL	
						E	GM												IL-1
Lemoli et al., 1992	BM MNC	100	3 x 10 ⁶	Bag	10	9	6	n.r.				50*	5						
Haylock et al., 1992	PB CD34 ⁺	50	5 x 10 ³	T-Flask	28	n.r	26	n.r.		10	10	10	10	10		10			
Brugger et al., 1993	PB CD34 ⁺	100	1.5 x 10 ⁴	T-flask	205		170	n.r.	1*			100	100*	100*		100			
Koller et al., 1993b	BM MNC	360	3 x 10 ⁷ / vessel	Flat-bed bioreactor	10	12	21	7.5	0.1*	5			2			10			
Palsson et al., 1993	BM MNC	n.r.	10 ⁵ / cm ²	Flat-bed bioreactor	25	5	23	n.r.	0.1*	5			5			10			
Sardonini and Wu, 1993	BM MNC	250	5 x 10 ⁵	Spinner	21		3	n.r.		0.3			0.5			100			
Van Zant et al., 1994	PB MNC	n.r	1 x 10 ⁶	Flat-bed bioreactor	22	n.r	25	16	0.1*	5			2			10			
Zandstra et al., 1994	BM MNC	40	10 ⁶	Spinner	16		35	7					2			10			
Henschler et al., 1994	PB CD34 ⁺	100	3 x 10 ⁴	T-flask	64		50	1.5	1			3	100	100		10			

Table 1.4: Continued...

Study	Cell Type	Size	Inoculum density	Vessel	TC	CFC		LTC-IC	Epo	G-CSF	GM-CSF	IL-1	IL-3	IL-6	IL-11	SF	Tpo	FL
						E	GM											
Moore and Hoskins, 1994	CB CD34 ⁺	300	4 x 10 ⁴	Bag	75	60		17	5*	10 ³ *			50	20		20		
Brugger et al., 1995	CD34 ⁺ PB	100	3 x 10 ⁴	T-Flask	62	50		n.r.										
Purdy et al., 1995	BM CD34 ⁺	250	2 x 10 ⁴	Bag	n.r	120		3		100			100	100		100		
Sandstrom et al., 1996	PB CD34 ⁺	120	5 x 10 ³	Grooved bioreactor	113	18		0.76		150*			150*	40		50		
Williams et al., 1996	PB CD34 ⁺	100	0.5-1 x 10 ⁵	Bag	26	5		n.r.		100 ^b			100 ^b					
Kogler et al., 1996a	CB CD34 ⁺	40	1 x 10 ⁴	Spinner	150	60		8					100		100	100		100
Ohmizono et al., 1997	CD CD34 ⁺	1	1-3 x 10 ³	24-well plate	n.r.	197	60	n.r.					20		40	10	100	
Piacibello, et al., 1997	CD CD34 ⁺	1	0.2-1 x 10 ⁴	24-well plate	>10 ⁵	>10 ⁵		>10 ⁴									10*	50
Zandstra et al., 1997a	BM MNC	40	1 x 10 ⁶	Spinner	14	66		9					10	10	10	50		50

^a BM (bone marrow), PB (peripheral blood) and CB (cord blood)^b PLY321 (the fusion product of IL-3 and GM-CSF) was used in this study

*indicates U/mL

n.r. = not reported

CD34⁺ mobilized PB cell expansion studies repeatedly have shown SF, IL-6, IL-3, IL-1 and G-CSF to be important. Interestingly, the extent and longevity of both total cell and CFC expansion in these cultures was directly related to the concentration at which the cytokines were supplemented. Haylock et al. (1992) who added cytokine supplements at 10 ng/mL achieved 28- and 26-fold total cell and CFU-GM expansions whereas Brugger et al. (1993), who used 100 ng/mL concentrations, achieved 205- and 170-fold total cell and CFC expansions. Henschler et al. (1994) and Sandstrom et al. (1996) added similar cytokines at intermediate concentrations (10-100 ng/mL) and achieved intermediate total cell (64- to 100-fold) and CFC (20- to 50-fold) expansions. In contrast to the CD34⁺ mobilized PB cell expansion cultures, greater expansions of total cells, CFC and LTC-IC have been achieved when BM MNC were cultured in medium supplemented with only SF and IL-3 [and in some studies GM-CSF, (Koller et al., 1993b; Palsson et al., 1993; Zandstra et al., 1994)]. The lack of a dependence on IL-1, IL-6 and GM-CSF supplementation in these cultures may be attributed to the substantial quantities of these factors that are endogenously produced by stromal and accessory cells (Zsebo et al., 1988; Eaves et al., 1991a; Guba et al., 1992), although more primitive progenitors have also been shown to produce some of these cytokines (Watari et al., 1996).

It should be noted that although qualitatively similar results have been obtained with BM and CB expansion cultures, some interesting differences have been reported. When tested under the same culture conditions, 31- to 250-fold higher CD34⁺ cell expansions were achieved over a 5 week period when CB vs BM cells were used as the input, even when the same number of CD34⁺CD45RA^{lo}CD71^{lo} cells were initially present (Lansdorp et al., 1993). Elevated expansions of total cell and CFC populations in CB vs BM initiated cultures were

also found in these and similar (Moore, 1993) studies. Although some of this differential expansion can be attributed to a higher frequency of primitive cells in CB (Conneally et al., 1997), higher rates of cell generation in the CB cultures (Lansdorp et al., 1993) indicate ontogeny-related changes in proliferative responses. Differences in the cytokine responsiveness of phenotypically similar cell populations isolated from CB and BM have been reported. CB progenitors have produced synergistically greater numbers of total and CD34⁺ cells in response to SF, IL-11 and G- or GM-CSF (van de Ven et al., 1995). Under similar conditions, only additive (at best) differences in the proliferative responses were seen for BM initiated cultures. Conversely, Tsujino et al. (1993) have shown significant increases in the short term generation of clonogenic cells from CD34⁺Lin⁻DR⁻ BM cells with the addition of IL-3 to SF containing cultures whereas no differences were reported between SF alone and SF and IL-3 containing cultures for similarly maintained CB cultures. Due to the potential of impact of successful *ex vivo* expansion of primitive progenitors from CB, a greater understanding of the cytokine requirements of CB progenitors would be particularly valuable.

The survival and proliferation of defined populations of hematopoietic progenitors is also likely to be influenced by the cytokines that are added to expansion cultures. Addition of TPO to cultures of CD34⁺ CB cells significantly elevated erythroid progenitor expansion (Ohmizono et al., 1997). The addition of FL to both BM and CB cultures has resulted in significant LTC-IC expansions from each of these progenitor sources (Gabbianelli et al., 1995; Kogler et al., 1996a; Zandstra et al., 1997b). Very recently, it has been reported that addition of both of these cytokines to CB cultures can result in a sustained (>20-week) and marked expansion of many primitive cells, including LTC-IC [$>10,000$ -fold over input (Piacibello et al., 1997)]. The complexity and number of cytokines which can stimulate hematopoietic

expansion cultures supports the need for the use of systematic techniques such as factorial analysis (Box et al., 1979) for the optimization of cytokine cocktails for a variety of clinically relevant applications.

3. Requirements, Limitations and Applications of *Ex Vivo* Cultured Hematopoietic Cells

A. Applications and Requirements for *Ex Vivo* Cultured Hematopoietic Progenitor Cells

There are several potential applications for *ex vivo* expanded hematopoietic cells. Interest in this area has been focused on the potential use of such cells to abrogate the early phase of neutropenia and thrombocytopenia associated with recovery from high dose therapy even with marrow transplantation (To et al., 1992; Williams et al., 1996). Evidence showing that specific growth factors (e.g. tumor necrosis factor- α (TNF- α), IL-12, EPO) favor the selective *in vitro* amplification of mature cells (Miller et al., 1992; Pierson et al., 1994) and may allow the production of clinically useful large numbers of erythrocytes, platelets, natural killer (NK) cells, or other lymphoid or myeloid cells. Dendritic cells, which are efficient antigen-presenting cells able to prime naïve helper T-cells, have been generated in significant numbers from CD34⁺ cells (Szabolcs et al., 1995). A significant potential exists for the use of these cells in *in vivo* immune therapy and *ex vivo* stimulation of cytotoxic T lymphocytes as specific effectors against foreign antigens.

The BM and PB of patients with chronic myeloid leukemia (CML) contain both normal and leukemic (Philadelphia positive, Ph⁺) primitive progenitors (Coulombel et al., 1983; Petzer et al., 1996c). One approach to the treatment of this disease is based on the rapid

loss of Ph⁺ LTC-IC when CML blood or marrow cells are cocultured on stroma under conditions that maintain normal LTC-IC (Udomsakdi et al., 1992). The ability to selectively expand normal progenitor cell populations without changing the leukemic progenitor culture kinetics would lead to an increase in the effective log depletion of the transformed cells (Petzer et al., 1997).

Finally, hematopoietic cells make an excellent choice for gene therapy because they should persist for the lifetime of the individual and continue to produce large numbers of progeny all of which would carry the gene of interest. As retroviral infection rates are demonstrably increased if the target cells are stimulated to divide (Miller et al., 1990), the design of a culture system in which hematopoietic stem cell numbers are maintained or expanded following their mitogenic activation could lead to higher efficiencies of their genetic modification. Recent developments in the use of adenovirus-based or non-viral vectors, which allow genetic modification without the requirement for cell cycling, may also be feasible.

Current BM transplantation protocols require large numbers of cells in order to ensure adequate long-term re-engraftment. Each of the above applications would benefit from an understanding of the *ex vivo* culture requirements of primitive hematopoietic cells. *Ex vivo* expansion of this population may provide for both more accessible and less invasive therapies. Additionally, the ability to expand primitive progenitor cells from small initial populations may allow cryopreservation of these cells to ensure an autologous source of transplantable cells.

B. Clinical Cell Production Requirements

The cellular requirements for *ex vivo* expanded hematopoietic cells are dependent on the particular clinical application. One area of current investigation is the use of expanded

cells in the reduction of transplant associated neutropenia. Based on a model of granulopoiesis where at least 5 stages of terminal CFU-GM differentiation occur over 7-8 days, Schmitz et al. (1993) have predicted that infusion of $\geq 4.5 \times 10^8$ myelocytes/kg patient body weight at day zero of the transplant and $\geq 1.5 \times 10^8$ myelocytes/kg a week following transplantation may be sufficient to reduce transplant-associated neutropenia. While these estimates have yet to be confirmed, the small reductions in the neutrophil nadir reported in clinical trials where ~ 10 -fold higher numbers of culture expanded cells were reinfused (Williams et al., 1996) suggest that inefficiencies in progenitor cell homing may affect the cell numbers required.

The numbers of repopulating cells required for long-term engraftment is not accurately known. The most widely accepted clinical correlate suggests that $1-2 \times 10^5$ CFU-GM are required per kilogram of patient body weight. Based on inefficiencies in progenitor cell homing, treatment survival and extrapolation of data from mice to humans, Emerson (1996) suggests that 1.5×10^4 repopulating cells may be required for autologous transplants. As significant differences exist in the size of hematopoietic clones between mice and larger mammals, these correlations, generally established for freshly isolated cell populations, will have to be carefully re-derived for cytokine expanded cell populations.

C. Potential Problems and Limitations of *Ex Vivo* Cultured Hematopoietic Cells

Although cultured human hematopoietic cells have been used in a variety of clinical settings since Dexter and coworkers first returned cultured bone marrow to patients in 1983 (Chang et al., 1986), an important concern has been whether culture manipulated cells, expanded according to functional or phenotypic criteria *in vitro*, retain the capacity required for their medical effectiveness *in vivo*. There are several lines of evidence supporting the use

of culture expanded hematopoietic cells in a clinical setting. Fraser et al. (1990) showed that retrovirally marked totipotent hematopoietic stem cells could be maintained and amplified in *ex vivo* cultures. Muench et al. (1992b) have shown that mouse bone marrow, harvested 5 days after treatment of the donor with 150 mg/kg of fluorouracil (FU) and then cultured with IL-1 and IL-3 for 5 days, accelerated the recovery of PB leukocytes, neutrophils, platelets and erythrocytes as compared with input day 5-FU BM, without affecting the long-term survival of the animals. Similar results have been reported in humans using hematopoietic cells cultured with GM-CSF and IL-3 (Naparstek et al., 1992). More recently, reduced hospitalization lengths and more predictable neutrophil recovery times for cytokine expanded cells in either flat-bed bioreactors (Champlin, 1995) or stroma-free tissue culture bags (Brugger et al., 1995) have been reported. These results, along with those obtained from CML culture purging protocols (Barnett et al., 1994), suggest the long-term repopulating potential of hematopoietic cells can be at least maintained in vitro under conditions that allow terminal cell expansion.

Comparing populations of highly purified primitive hematopoietic cells (CD34⁺ CD45RA⁻CD71⁻) from fetal liver, CB and adult BM, Lansdorp et al. (1993) have shown that a decrease in the proliferative capacity of these cells may occur with ontogeny. These results, along with decreasing lengths of the telomeric repeats in these populations (Vaziri et al., 1994), may indicate an ultimately limited proliferation capacity in certain primitive cell populations. It should be noted that even if these observations are consistent throughout very primitive hematopoietic cell populations, the types of cell expansions required in clinical scale culture systems are small compared to the replicative capacity of primitive cells (reviewed in

Lansdorp, 1995). These issues, however, may become more relevant in attempts to produce much larger quantities of mature blood cells *in vitro*.

4. Thesis Objectives

The overall goal of this project was to develop an understanding of the parameters important in the design of a scalable bioprocess for the expansion of primitive adult human hematopoietic cells. When this project was initiated in 1992, it had already been shown that some murine cells with long-term *in vivo* hematopoietic repopulating ability were amplified in LTC, although not sufficiently to allow a net expansion of the entire input population (Fraser et al., 1990). Using the same culture system, Sutherland et al. (1992) had shown that maintenance of the closely related human marrow LTC-IC population could be enhanced by supplementation of the cultures with IL-3, SF and G-CSF. These results suggest that in standard static LTC, primitive hematopoietic cells may be exposed to suboptimal levels of stimulatory cytokines as well as to other cytokines that may selectively inhibit primitive cell proliferation (Eaves et al., 1991). Indeed the first successful system reported to support a net increase in LTC-IC *in vitro* utilized a flat-bed bioreactor modified from the design of traditional experimental LTC to allow for medium and cytokine supplementation and oxygen tension control (Koller et al., 1993a, b). In addition to these earlier studies, it had also recently been shown that direct contact between human primitive hematopoietic cells and bone marrow stroma was not required for the maintenance of LTC-IC observed in LTC (Verfaillie, 1992; Sutherland et al., 1993a) and preliminary studies by Eaves and Piret (Chapter 3, Table 3.1) had suggested that it was possible to maintain human LTC-IC at the same level in stirred suspension cultures as in static LTC. Because stirred suspension cultures represent a relatively

homogeneous system in which the cellular environment can be readily analyzed, it was decided to explore this system as a basis for developing a scalable bioreactor. This required characterizing the parameters that limit the growth of primitive hematopoietic progenitors (including LTC-IC) to select the conditions that optimize LTC-IC expansion. An analysis of various parameters that influence the expansion of primitive human bone marrow cells as determined by different manipulations of the extracellular environment is presented in Chapters 3 and 4. The results of these studies revealed that both the types and concentrations of cytokines to which the cells were exposed critically influenced LTC-IC expansion.

A detailed analysis of the cytokine dependence of LTC-IC and CFC generation was performed using a subpopulation of cells defined by its $CD34^+CD38^-$ phenotype and believed to contain most of the cells from which all other hematopoietic cells ultimately derive. In order to also reduce potentially confounding influences of serum, these investigations were done in serum-free, short term suspension cultures. The results of these experiments are presented in Chapters 5 and 6.

Since initial experiments in stirred suspension cultures had shown cytokine depletion to be a major factor, it was hypothesized that the optimization of the bioreactor expansion of primitive cells on a continuing basis would require alternative strategies for maintaining higher cytokine concentrations in the medium. Accordingly a third objective of this project was to define the rates of cytokine depletion from hematopoietic cells, to analyze the mechanisms involved and to determine the potential effects on the rate of cytokine depletion of cell type, extracellular cytokine concentration and time of exposure. To facilitate analysis of the internalization kinetics of receptor-bound SF under different conditions, the SF-responsive

human Mo7e cell line was used as a model. The results of these experiments are described in Chapter 7.

CHAPTER 2: MATERIALS AND METHODS

A. Bone marrow cells

Most experiments were performed with thawed samples of previously frozen bone marrow originally removed with prior consent by the Northwest Tissue Center (Seattle, WA) from the vertebral bodies of normal cadaveric organ donors. Vials of frozen cells were rapidly thawed and slowly diluted with Iscove's medium (StemCell Technologies, Vancouver, BC) containing 20% FCS (StemCell) and 0.1 mg/mL DNase (D4513, Sigma Chemicals, St. Louis, MO) and washed by centrifugation. Light density ($<1.077 \text{ g/cm}^3$) cells were isolated from thawed cells by centrifugation of the initial suspension on Ficoll-Paque (Pharmacia, Uppsala, Sweden). The cells were then washed twice in Iscove's medium with 2% FCS. To initiate stirred suspension cultures, the cells were diluted to $10^6/\text{mL}$ in alpha medium containing 12.5% FCS, 12.5% horse serum, 0.1 mM 2-ME, 2 mM glutamine, 0.6 mM l-inositol and 0.016 mM folic acid (Myelocult, StemCell) and, just prior to use, supplemented with freshly dissolved hydrocortisone sodium hemisuccinate (Solucortef, Sigma) to give a medium concentration of 10^{-6} M . When more purified subpopulations of starting cells were required, the light density cells were washed again in PBS containing 2% FCS prior to antibody staining and sorting as outlined below. Some experiments were performed with freshly aspirated human bone marrow cells obtained as leftover material with informed consent from individuals donating their marrow for allogeneic or autologous transplantation. These cells were either used directly after washing (without further separation) or after isolation of the light density fraction as outlined above. Viable cell numbers were determined at this point by staining for nigrosin dye exclusion and using a hemacytometer.

B. Cytokines

All of the cytokines used were purified human recombinant proteins either prepared in the Terry Fox Laboratory by transient expression of the appropriate protein cDNAs in COS cells (SF, IL-6, LIF) or obtained as gifts or purchased from the following companies: FL and IL-7, Immunex, Seattle, WA; SF and G-CSF, Amgen, Thousand Oaks, CA; IL-1, Biogen, Cambridge, MA; IL-3 and GM-CSF, Sandoz Pharmaceutical, Basel, Switzerland; IL-11 and IL-12, Genetics Institute, Cambridge, MA; IL-6, Cingene, Mississauga, ON; MIP-1 α and NGF- β , R&D Systems., Minneapolis, MN; Epo, StemCell; TNF- α , Genentech, San Francisco, CA; and TPO, ZymoGenetics, Seattle, WA.

C. Stirred Suspension Cultures

The suspension culture system utilized 50 or 100 mL stirred suspension spinner flasks (Bellco, Vineland, NJ) as indicated. The flasks were siliconized (Sigma) prior to each use to prevent the attachment of adherent cells. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air with constant stirring at 40 rpm. In the first experiments (Chapter 3, Table 3.1), each suspension culture was initiated with 1.8×10^8 whole aspirate marrow cells in 60 mL of Myelocult medium with either 0, 1, or 3 g/L of Cultisphere G porous microcarrier beads (Hyclone, Logan, UT). The microcarrier beads had been previously washed in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS, StemCell), allowed to hydrate overnight, then sterilized and washed twice in fresh Myelocult medium before addition to the cell suspension.

In the subsequent experiments, the stirred suspension cultures were initiated with 20 mL volumes containing 2×10^7 light density marrow cells suspended in hydrocortisone-supplemented Myelocult and cytokines as indicated. For the experiments presented in Chapter 3, a further 20 mL of fresh LTC medium (with or without cytokine addition, as at the start) were added after 7 days and this was followed by replacement of 1/2 (Figure 3.1) or 1/3 (Figures 3.2 and 3.3) of the medium with fresh medium every 7 (Figure 3.1) or 3.5 days (Figures 3.2 and 3.3) unless otherwise indicated. For the experiments presented in Chapter 4, at the end of the first 7 days, 20 mL of fresh complete medium (supplemented with fresh hydrocortisone and cytokines) were added and this was followed by replacement of 1/3 of the medium with fresh medium every 3.5 days. These protocols allowed samples of cells and progenitors to be obtained on a regular basis for analysis and at the same time allowed the volume of the cultures to be maintained at ~ 40 mL. Progenitor and total (viable and nucleated) cell expansions were calculated assuming that the cells removed from the cultures during the course of the experiment would have performed in the same way as the cells remaining in the cultures. This assumption is based on the homogeneous nature of these cultures and the finding that there was no reservoir of adherent progenitors in the stirred cultures. To determine whether progenitor cells were adhering to the spinner flasks, these were trypsinized (Eaves et al., 1991b) after removing all of the suspended cells, and any cells obtained (typically $2-6 \times 10^5$ cells) then plated in both CFC and LTC-IC assays. Since neither CFC nor LTC-IC were detected for up to 4 weeks in initial trypsinized washings of the spinner flask surface, this procedure was eventually discontinued.

D. Static Cultures

This section describes the procedure used to initiate and maintain the static cultures referred to in Chapter 3. In the first of these experiments (Chapter 3, Table 3.1), static cultures were initiated with 3×10^6 light density cells/mL in 35 mm tissue culture dishes (Corning) to provide a basis for comparison of the stirred cultures with historical static control cultures (Coulombel et al., 1983; Eaves et al., 1991b). In the later experiments described in Chapter 3 (Figures 3.1, 3.2 and 3.4), static cultures were initiated at 10^6 light density cells/mL in 35 mm petri dishes (Greiner, Germany) which do not allow the formation of an adherent layer. This was done to make them more analogous to the spinner flasks, yet provide a control for the stirring. Cytokines were added as indicated. Static cultures were maintained in the same incubator as the stirred suspension cultures either without any medium changes ("non-fed"), or with weekly 1/2 medium changes and removal of 1/2 of the nonadherent cells ("fed"), as indicated. In tissue culture dishes (Chapter 3, Table 3.1), an adherent layer formed which contained at least half of all the cells in the culture by two weeks. In cultures established in petri dishes, few, if any, adherent cells (or progenitors) were found even after 4 weeks of incubation.

E. Stroma-free Liquid Suspension Cultures

Sorted cells were cultured at 37°C for 7 (Chapter 3, Figure 3.6) or 10 (Chapters 5 and 6) days in Iscove's medium supplemented with 20 mg/mL of BSA, 10 µg/mL of human insulin and 200 µg/mL of human transferrin (BIT, StemCell) plus 40 µg/mL low density lipoproteins (LDL, Sigma), 10^{-4} M 2-ME ('serum-free medium') and various growth factors

plus or minus 10^{-6} M HC (as indicated). The use of this medium was based on previous evidence that it might be an effective substitute for fetal calf and horse serum in this system (Migliaccio and Visser, 1986; Lansdorp and Dragowska, 1992; Petzer et al., 1996a). In one case (Chapter 4, Figure 4.4), Myelocult was used instead of the serum-free medium. For the experiments presented in Chapter 4, Figure 4.6, the cultures were initiated with 5000 sorted cells of a given phenotype in 24 well plates, each well containing 1 mL of serum-free media and cytokines as indicated. In some experiments (Chapter 4, Figure 6), 10^5 light density cells from the initial starting population were placed in additional wells. For the experiments presented in Chapters 5 and 6, 1-200 purified cells were incubated in 100 μ L of serum-free medium in round bottomed wells of 96 well plates. Suspension cultures were typically incubated unperturbed for 10 days at 37°C (unless otherwise indicated), and then all cells in each type of culture were harvested and assayed for LTC-IC and/or CFC and the medium supernatants frozen at -20°C for subsequent analysis of their cytokine and/or glucose contents (see below).

For the single cell cultures, individual cells were deposited into wells preloaded with the desired medium using the FACS and the wells were then examined 1-2 days later to identify those that contained a single viable (refractile) cell. After 10 days, the number of viable cells in each positive well (single cell cultures) was then recounted for clone size determinations and then pooled and assayed for CFC and LTC-IC as described above.

F. Mo7e Cell Cultures

Mo7e cells (Avanzi et al., 1988) were maintained in 10% FCS plus 10^{-5} M 2-ME and 5 ng/mL G-CSF. Exponentially growing cells were washed twice in Iscove's before being put into either 10% FCS plus 10^{-5} M 2-ME (for the 72 h cell surface SF receptor and SF and IL-3 depletion studies) or serum free media (BIT, for the 30 min SF internalization studies). 1 mL aliquots containing $3-10 \times 10^5$ cells were placed into individual wells of 24 well plates (Nunc) and incubated with IL-3 or SF at concentrations ranging from 1-100 ng/mL at 37°C for 0.5 to 72 h as indicated. In some control experiments, SF stimulated cells were maintained at 4°C for 30 min. At the end of each experiment, cell counts and flow cytometric analysis were performed and supernatants frozen.

G. Progenitor Assays

a) CFC

Cell suspensions to be assayed for erythropoietic (CFU-E and BFU-E), granulopoietic (CFU-GM) and multi-lineage (CFU-GEMM) colony-forming cells (CFC) were plated at suitable concentrations (to give < 100 colonies per 1 mL culture) in Iscove's medium containing 0.9% methylcellulose, 30% FCS, 1% bovine serum albumin (BSA), 10^{-4} M 2-ME (Methocult, H4430, StemCell) supplemented with 3 U/mL of highly purified human Epo, 50 ng/mL of SF, and 20 ng/mL each of IL-3, IL-6, G-CSF and GM-CSF. SF and IL-6 were purified from the supernatants of COS cells transiently transfected in the Terry Fox Laboratory with corresponding human cDNAs. Methylcellulose cultures were aliquotted in 1.1 mL volumes in 35 mm petri dishes and then incubated at 37°C for 2 to 3 weeks. At the

end of this time, colonies containing at least 3 clusters of erythroblasts (from BFU-E) or 20 or more granulocytes and macrophages (from CFU-GM) or both (from CFU-GEMM) were distinguished and sized by direct visualization *in situ* using well established criteria (Eaves et al., 1991b). Colonies derived from BFU-E were further classified as of primitive or mature BFU-E origin according to their content of more or less than 8 erythroblast clusters, respectively (Cashman et al., 1985). Primitive and mature CFU-GM were similarly distinguished by their ability to have generated colonies containing more or less than 500-1000 granulocytes and macrophages.

b) *CFU-F*

CFU-F numbers were determined using a slightly modified version of the method described by Castro-Malaspina et al. (1990). Briefly, aliquots of between 3×10^5 and 10^6 washed cells were suspended in 8 mL of Iscove's medium containing 20% FCS and placed in 60 mm tissue culture dishes which were then incubated without being perturbed for 14 days. The cultures were then removed, the medium decanted, and the dishes fixed and stained with Giemsa stain (Sigma). Readily visible adherent fibroblast colonies were counted on a light box and checked microscopically.

c) *LTC-IC*

These were initially performed (Chapter 3, Table 3.1) using the original procedure described by Sutherland et al. (1990) in which cells to be tested for their LTC-IC content are seeded onto pre-established irradiated (15 Gy) human marrow LTC adherent layers and maintained in myeloid LTC medium at 33°C for 5-8 weeks with weekly half-medium changes

and simultaneous removal of half of the nonadherent cells. The LTC-IC measurements performed using this procedure were derived from assessment of the total CFC (BFU-E plus CFU-GM plus CFU-GEMM) content of 8 week-old cultures, divided by 4. This calculation is based on previous findings indicating that the progeny of each LTC-IC present after 8 weeks will contain, on average, 4 CFC (Sutherland et al., 1990, Sutherland et al., 1992). In the subsequent experiments, the LTC-IC assay was performed on a 1:1 mixture of two murine fibroblast cell lines that had been genetically engineered to produce human IL-3, G-CSF and SF (Hogge et al., 1996). Aliquots of test cells were cultured for 6 weeks at 37°C on feeder layers of these cells after they had been irradiated with 400 cGy and at weekly intervals thereafter, half of the nonadherent cells and spent medium were removed and replaced with fresh hydrocortisone-supplemented Myelocult medium. LTC-IC assays were initiated with $10^6 - 3 \times 10^6$ cells from unseparated samples of light density marrow cells and 100-500 input $CD34^+CD38^-$ cells (or their 10 day culture-derived progeny) per 35 mm long-term culture (LTC). Multiple LTC were set up with different numbers of cells in each culture for the LTC-IC measurements to ensure that the total number of CFC generated would be sufficient to provide an estimate of the number of LTC-IC in the input sample. The entire content of BFU-E, CFU-GM and CFU-GEMM in these cultures was then determined by plating appropriate aliquots of the harvested, suspended and combined nonadherent and adherent cells in methylcellulose assays as described above. The total CFC content thus obtained provides a relative measure of the input LTC-IC since these two parameters are linearly related under the assay conditions used (as long as the input is not excessive) and the CFC output per LTC-IC after 6 weeks does not change even when the LTC-IC are purified or cultured (Petzer et al., 1996; Sutherland et al., 1990). For the LTC-IC assay conditions used

here, the yield of CFC after 6 weeks from BM LTC-IC has been found to be 18 ± 6 (Hogge et al., 1996)

H. Flow Cytometric Purification and Analysis of Bone Marrow Cells

The procedure and reagents used were based on previously published work from the Terry Fox Laboratory (Lansdorp and Dragowska, 1992; Petzer et al., 1996a). Light density cells ($<1.077 \text{ g/cm}^3$) were isolated as described above and washed once in phosphate-buffered saline (PBS) containing 2% FCS, resuspended in Hank's hepes-buffered salt solution containing 2% FCS and 0.1% sodium azide (HFN) and then incubated simultaneously at $\leq 10^7$ cells/mL for 30 min at 4°C with monoclonal antibodies specific for either CD34 (10 $\mu\text{g/mL}$; 8G12-Cy5), CD45RA (4 $\mu\text{g/mL}$; 8d2-R-phycoerythrin [PE]), CD71 (1 $\mu\text{g/mL}$; OKT9-fluorescein isothiocyanate [FITC]) or CD34 (10 $\mu\text{g/mL}$; 8G12-FITC) and CD38 (2.5 $\mu\text{g/mL}$; Leu17-PE). Cells were then washed once in HFN and once in HFN containing 2 $\mu\text{g/mL}$ propidium iodide (PI, p-5264; Sigma) prior to resuspension in Hank's hepes-buffered salt solution containing 2% FCS for isolation of $\text{CD34}^+\text{CD38}^-$, $\text{CD34}^+\text{CD45RA}^+\text{CD71}^-$ or $\text{CD34}^+\text{CD45RA}^-\text{CD71}^+$ populations. In the experiments where the phenotype of cultured cells was to be investigated, anti-CD45RA-FITC and anti-CD33 (4 $\mu\text{g/mL}$; D3HL60.251)-FITC (Immunotech, Westbrook, ME) were used in addition to anti-CD34 (8G12)-Cy5, anti-CD71-FITC and anti-CD38-PE for the isolation of $\text{CD34}^+\text{CD38}^-\text{CD45RA}^-\text{CD71}^-\text{CD33}^-$ cells. For analyses of cultured cells, the cells were washed as described above and then stained using anti-CD34 (8G12)-Cy5, anti-CD38-PE, and one of anti-CD45RA-FITC, anti-CD71-FITC or anti-CD33-FITC. For the single cell cloning experiments, recombinant human

AnnexinV-FITC (50 $\mu\text{g/mL}$; Biowittaker, Walkersville, MD) was used in addition to anti-CD34 (8G12)-Cy5 and anti-CD38-PE for the isolation of AnnexinV⁻ CD34⁺CD38⁻ cells. Cells were sorted on a FACStar Plus[®] (Becton Dickinson, San Jose, CA) equipped with a 5 W argon laser and a 30 mW helium laser. Viable (PI⁻) cells with low-medium forward scattering, low side scattering characteristics and the desired phenotype were collected in Myelocult rinsed Eppendorf tubes containing serum-free Iscove's medium. Cells regarded as positive were those showing greater fluorescence than $\geq 99.9\%$ of all cells stained with a matched fluorochrome-labelled irrelevant isotype control antibody.

I. Flow Cytometric Analysis of Mo7e Cells

a) Cell surface c-kit expression

SF stimulated Mo7e cells ($0.5\text{-}20 \times 10^5/\text{point}$) or the appropriate controls were washed once in 4°C PBS with 2% FBS and resuspended in Hank's hepes-buffered salt solution containing 2% FCS, 0.1% sodium azide (HFN) and 10% human serum and incubated on ice for 10 min, washed again in HFN and then incubated at $\leq 10^7$ cells/mL for 30 min at 4°C with Phycoerythrin (PE) labeled anti-*c-kit* mAb YB5.B8 (10 $\mu\text{g/mL}$, Cedarlane, Ontario, Canada) or with an isotype matched murine IgG (Biorad). In order to compare the relative expression of *c-kit* between time points and experiments, cells were analyzed in parallel with 3 μm calibration beads (Cedarlane) conjugated to a discrete range (5 groups of beads containing 506082, 83915, 30094, 8857 and 0 binding sites) of anti-IgG binding sites. Stained cells and beads were washed twice in HFN (2 $\mu\text{g/mL}$ of PI in the second wash) and finally resuspended in HFN for analysis. The relative *c-kit* binding capacity of the cells was determined from a

calibration curve of the mAb binding capacity and the mean channel number for the cells and beads and calculated using the QuickCal (Version 1.0) software package.

b) *Intracellular analysis of SF*

For the analysis of SF internalization, surface bound SF was first stripped from SF stimulated cells by washing once in ice cold PBS and then resuspending in the same buffer adjusted to pH 4.0 with acetic acid (Bowen-Pope and Ross, 1985). Surface stripped cells were then washed with PBS, and fixed and permeabilized using a kit (Fix and Perm, Caltag, San Francisco, CA) as recommended by the manufacturer. Briefly, cells were resuspended in 5 μ L PBS to which 100 μ L of Reagent A was added for 15 min. Cells were the washed once in PBS and incubated for 30 min in 100 μ L Reagent B. Fixed and permeabilized cells were then incubated with biotin labeled anti-SF (R&D) for 90 min, washed twice in 2% PBS and incubated for 60 min with streptavidin-PE, washed twice and resuspended in HF for analysis. Non-permeabilized acid stripped cells, permeabilized cells treated with only streptavidin-PE, and permeabilized cells stained in the presence of 1 μ g/ mL SF served as negative controls.

J. Statistics

a) *General*

Differences between test populations were determined by either a one (Figure 6) or two-tailed Student t-test. Values derived from replicate measurements are reported as the mean \pm SEM.

b) *Factorial design analysis experiments*

Factorial design experiments were performed based on the methods described by Box et al. (1979). In Chapter 5, a 2^5 factorial analysis design was used to investigate the relative roles of 6 growth factors (2 of which, NGF- β and G-CSF, were considered as a fixed pair) in stimulating LTC-IC amplification. Each of the 32 possible combinations of growth factors was tested in duplicate (each with a different source of CD34⁺CD38⁻ cells). The actual testing of each of these sets was broken into 2 subexperiments which were performed on different occasions (16 combinations each time) with the same cells. Each of these subexperiments also included 4 replicate cultures containing all 6 factors at half the concentrations used in the other combinations. The LTC-IC expansion obtained with each growth factor or combination of growth factors in individual subexperiments was normalized as follows. In each subexperiment the difference between the logarithm (log) of each LTC-IC expansion measured and the mean of all the log LTC-IC expansions was first determined. This difference was then divided by the standard deviation of all the log LTC-IC expansions. This transformation allowed the results from the 4 subexperiments to be analyzed as a single data set, using the Jass Software program (version 2.0, Joiner Associate, Madison, WI).

In Chapter 6, a 2^3 orthogonal factorial analysis was used to investigate the individual cytokine concentration dependence of LTC-IC expansion (in bulk cultures of CD34⁺CD38⁻ cells) on FL, SF and IL-3. These cytokines were tested at 10 and 300 ng/mL for SF and FL, and 2 and 60 ng/mL for IL-3. Each of the 8 different cytokine combinations possible was tested with 3 different sources of CD34⁺CD38⁻ cells. In addition, each separate experiment included 4 replicate cultures containing FL, SF and IL-3 at 60, 60 and 10 ng/mL, respectively.

The logarithm (log) of the LTC-IC expansion measured with each cocktail was then normalized for each separate marrow by subtracting the mean of all the log LTC-IC expansions and dividing the resultant value by the standard deviation of all the log LTC-IC expansions. This transformation allowed the results from the 3 separate marrows to be analyzed as a single data set, as described above, using the Jass Software program.

K. Medium analysis

Cytokine concentrations were determined on batches of thawed supernate samples of medium from an entire experiment. Commercial ELISA kits were used for IL-3 and SF measurements according to the instructions recommended by the supplier (R&D). Recombinant standards supplied with the kits were used to establish reference dilution curves. Reagents and the ELISA procedure used for determining FL levels were provided by Immunex. The sensitivities of these assays for IL-3, SF and FL were 30, 30 and 15 pg/mL, respectively. Glucose concentrations were measured using a calorimetric glucose oxidase-based assay (Diagnostic Chemical, Vancouver, BC) with a sensitivity of 0.1 mM. Cell-specific cytokine depletion rates were calculated using the following equation:

$$q = \frac{1}{\bar{X}} \left[\frac{\Delta C}{\Delta t} - k\bar{C} \right] \quad 2.1$$

where q is the cell-specific cytokine depletion rate (ng/10⁶ cells/day), Δt is the time interval between measurements (days), ΔC is the change in the measured cytokine concentration (ng/mL) over the time interval, \bar{C} is the average cytokine concentration (ng/mL), \bar{X} is the average viable cell concentration (expressed as 10⁶ cells/mL) and k is the cytokine degradation constant (day⁻¹). The cytokine degradation constants (k) were determined in

cell-free cytokine supplemented medium or culture supernatants obtained from medium previously exposed for 72 h to exponentially growing Mo7e cells (up to $\sim 1.5 \times 10^6$ cells/mL). The cytokine depletion results assume that bio-active cytokine is measured by ELISA. This assumption was tested and confirmed in preliminary experiments using IL-6 (data not shown).

CHAPTER 3: EXPANSION OF HEMATOPOIETIC PROGENITOR CELL POPULATIONS IN STIRRED SUSPENSION BIOREACTORS OF NORMAL HUMAN BONE MARROW CELLS*

* The contents of this chapter are essentially as published in Zandstra et al., 1994. G. Cameron is gratefully acknowledged for her expert technical assistance in the microcarrier cultures and in the establishment of assay protocols for CFC and LTC-IC from cytokine expanded hematopoietic cell populations.

1. Introduction

As reviewed in Chapter 1, the development of a bone marrow culture technology capable of generating large numbers of transplantable human hematopoietic cells would be useful for a wide range of clinical conditions. The first culture system in which the production of hematopoietic cells could be reproducibly sustained for many weeks was described by Dexter and colleagues (Dexter et al., 1977). A critical feature of this system was the rapid formation or provision of a supportive adherent feeder layer containing cells of the fibroblast-endothelial-adipocyte lineages (Dexter et al., 1980; Sutherland et al., 1991). When very primitive hematopoietic cells are cocultured with such cells they are selectively retained in the adherent layer (Mauch et al., 1980; Coulombel et al., 1983), but will continuously release their maturing granulopoietic progeny into the nonadherent fraction (Slovick et al., 1984). Although many aspects of this system, including some of the cellular interactions that occur in it, have been shown to mimic the regulatory mechanisms operative in the marrow *in vivo*, molecular mediators responsible for these mechanisms are just beginning to be defined.

At the time this work was initiated (1992), the number of growth factors able to stimulate primitive hematopoietic cells, either alone or in combination, had made possible the identification of culture conditions that would allow extensive CFC production in the absence of stromal cells (Brandt et al., 1991; Haylock et al., 1991; Bernstein et al., 1991; Migliaccio et al., 1992), but LTC-IC numbers were not similarly expanded under these conditions (Sutherland et al., 1993a). However, for some therapeutic applications, it was thought that this might not be the most important requirement. For example, in many patients given myeloablative treatments, substantial numbers of endogenous stem cells, able to contribute to

the stable maintenance of blood cell production at later times, survive (Petz et al., 1987) and the primary need is to hasten short-term blood count recovery potentially by the transplantation of large numbers of intermediate types of progenitors.

Regardless of the type(s) of hematopoietic cells desired, large scale cultures suitable for clinical applications required new systems to circumvent anticipated limitations in nutrient supply, oxygen delivery and/or cytokine concentrations. Previous studies indicated that each of these parameters was likely to be important (Schwartz et al., 1991a, b; Caldwell et al., 1991b; Koller et al., 1993a, c; Palsson et al., 1993). The most promising results reported involved the use of a medium-perfused flat-plate bioreactor, which gave a 10-fold expansion of total cells, a 21-fold expansion of CFC and a 7.5-fold expansion of LTC-IC from light density human bone marrow cells cultured for a 2-week period (Koller et al., 1993b). Studies of alternative approaches, including the use of a stirred suspension system, were recently described by Sardonini and Wu (1993). Although some expansion of both total cells and CFC were demonstrated in this latter system, the extent of these were less than observed by Koller et al. (1993b) and did not continue beyond 2 to 3 weeks. The experiments described in this chapter were therefore undertaken to determine the behavior of CFC and LTC-IC of primitive human bone marrow cells maintained in stirred suspension cultures with and without supplementation of the medium with growth factors.

2. Results

A. Preliminary Studies With Stirred Suspension Cultures and the Effect of Adding Porous Microcarrier Beads

Table 3.1 shows the results of an initial series of experiments in which aliquots of washed, fresh human marrow cells were suspended in long-term culture (LTC) medium and maintained at 37°C for 8 days without any medium exchange as either conventional (static) LTC in plastic tissue culture dishes or in siliconized spinner flasks with continuous stirring. At the end of the 8-day incubation period, the numbers of viable cells, CFC and LTC-IC present in the spinner flask (where there were no adherent progenitors detectable), were similar to the total yields of each of these populations in the conventional LTC (non-adherent and adherent combined). In two experiments, an extra spinner culture was first set up in which the cells were first allowed to adhere to porous gelatin microcarrier beads for 1 to 2 h prior to initiation of the stirring. This was done to provide a surface for adherent cell attachment and growth, anticipating that this might enhance or be a requirement for primitive progenitor production and/or maintenance of viability in stirred cultures. After 8 days, 5% and 8% of all of the cells were attached to the beads in stirred cultures containing 1 and 3 g/L of microcarrier beads, respectively. However, as shown in Table 3.1, the presence of the beads in the stirred suspension cultures for 8 days had no apparent effect, either inhibitory or stimulatory, on total cell or CFC or LTC-IC numbers. Addition of the same amounts of microcarrier beads to static LTC also had no effect on these progenitor output parameters. Overall, the results for CFC and LTC-IC numbers from stirred suspension cultures after

8 days were $113 \pm 18\%$ and $93 \pm 14\%$, respectively, of values measured in parallel conventional LTC (n=5).

Table 3.1. Total viable cells, CFC, and LTC-IC numbers in stirred suspension cultures with and without the addition of porous microcarrier beads as compared to static cultures.

Experiment Number	Concentration of Microcarrier Beads Added	Percent Recovery		
		Cells	CFC	LTC-IC
1	0	78	94	81
2	0	125	84	52
3	0	43	112	85
4	3 g/L	57	73	97
	0	65	72	142
5	1 g/L	110	180	n.d.
	0	102	181	103

*Values shown are expressed as a percentage of results obtained in parallel static cultures (set-up in tissue culture dishes). All cultures in each experiment were initiated at the same time with 3×10^6 unseparated washed marrow aspirate cells/mL of long-term myeloid culture media and maintained without feeding at 37°C for 8 days prior to viable cell, CFC and LTC-IC counts. Mean recoveries of total viable cells (63 ± 16), CFC (91 ± 40) and LTC-IC (150 ± 86) in static cultures (n=5) expressed as a percentage of input were similar to previously published results (Eaves et al., 1991b; Udomsakdi et al., 1992). n.d.=not determined.

B. Net Expansion of Hematopoietic Cells in Stirred Suspension Cultures Containing Growth Factor-Supplemented Medium

Previous studies with static long-term cultures had demonstrated that progenitor output could be influenced by the initial concentration of bone marrow cells present, the addition of various hematopoietic growth factors, and the rate of medium exchange (Schwartz et al., 1991b; Koller et al., 1993a; Eaves et al., 1991b; Otsuka et al., 1991a, b). Experiments

were therefore undertaken to begin to explore these variables using more prolonged incubation times.

Figure 3.1 shows a comparison of stirred suspension culture expansions in total cell and CFC numbers that occurred over a 21-day period. This pilot experiment was initiated with starting cell concentrations of previously frozen cadaveric (normal) marrow ranging from 10^5 to 10^6 light density cells per mL using a medium which was supplemented with 2 ng/mL of IL-3 and 10 ng/mL of SF. It can be seen that there was a net increase in both CFC and total

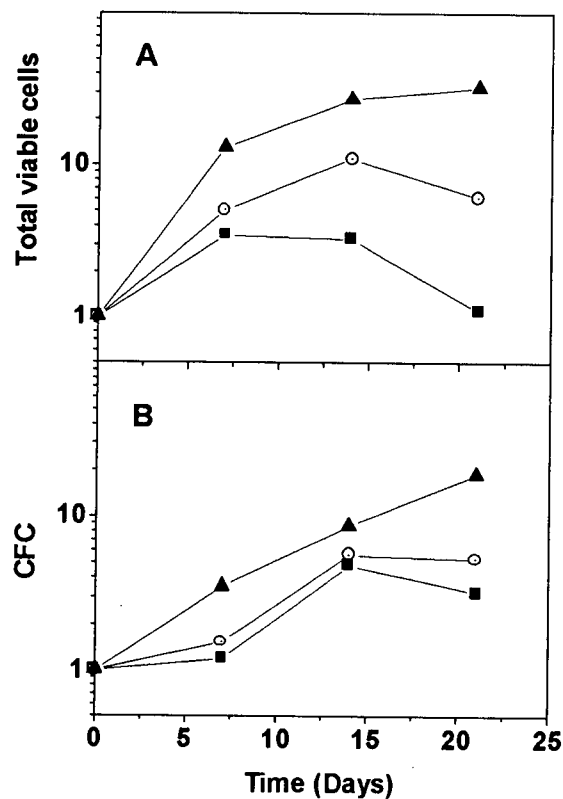


Figure 3.1: Calculated changes as a function of time in total viable cell (A) and CFC (B) numbers relative to input in stirred suspension cultures. These were initiated with previously frozen normal cadaveric, light density marrow cells at (■) 10^5 cells/mL, (○) 3×10^5 cells/mL, and (▲) 10^6 cells/mL in IL-3 plus SF-supplemented medium. Input values for CFC were 6142 per 10^6 cells.

cells within 14 days in all of these cultures. However, both the rate and duration of these expansions were dependent on the initial cell concentration suggesting an important additional contribution from endogenously produced stimulators.

Further experiments were then carried out with both previously frozen normal cadaveric marrow (6 cases) and freshly obtained normal marrow aspirate cells (1 case) using the same starting cell concentration (i.e. 10^6 cells/mL) and growth factor supplements (i.e. 2 ng/mL of IL-3 and 10 ng/mL of SF) that had given maximal progenitor yields in the pilot experiments. As shown in Figure 3.2, these demonstrated the reproducibility of the total viable cell and CFC expansions achievable in stirred suspension cultures under these conditions, with significant ($p < 0.05$) net increases in these populations of 5- and 14-fold after 2 weeks and 16- and 22-fold after 4 weeks. Assessment of LTC-IC in these experiments showed a 7-fold expansion in their numbers at both time points. In addition, these experiments showed that when no IL-3 or SF was added to the stirred suspension cultures, there was a rapid decline in the number of total cells, CFC and LTC-IC during the first 14 days. Results for total cells and LTC-IC for similarly maintained, but static, cultures were equivalent to those in stirred cultures, although by 4 weeks CFC yields from the static cultures were significantly lower (at 1.5-fold above input versus 22-fold above input in the stirred cultures, $p < 0.05$). In the static cultures that were not intermittently fed, the initial modest growth-factor dependent increases in all cell types measured after 14 days gave way to a rapid decline during the second 14-day period, regardless of whether SF and IL-3 had initially been added to the medium (Figure 3.2).

The representation of different types of primitive CFC (as indicated by their relatively high proliferative or multilineage differentiation potential) in the variously expanded total CFC populations measured in the above experiments is shown in Table 3.2. It can be seen that under those conditions which supported maximal expansion of the total CFC population (Figure 3.2), the advantage gained using the stirred suspension system (by comparison to

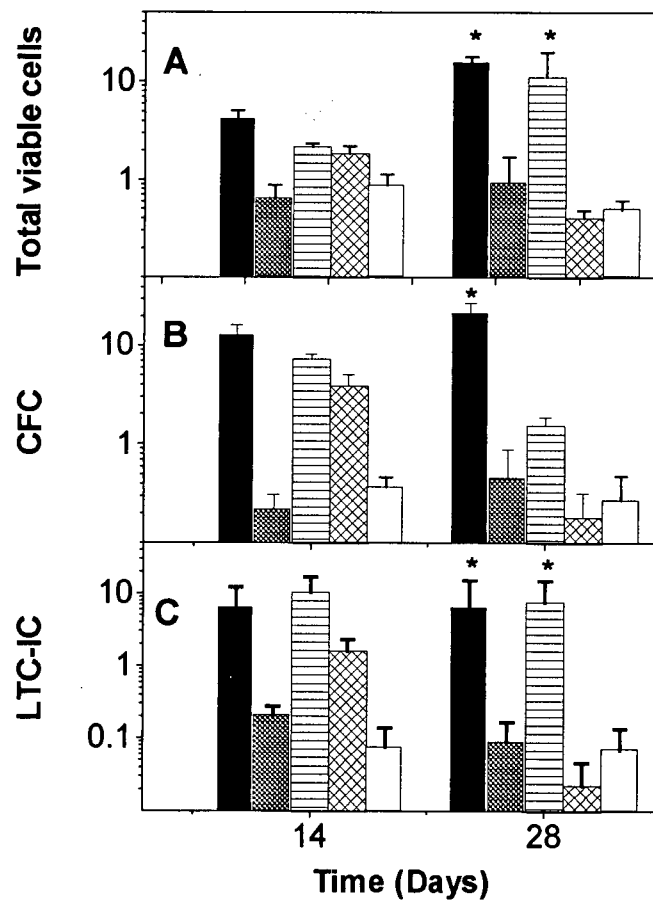


Figure 3.2: Calculated total viable cell (A) and CFC (B) numbers relative to input after maintenance of light density marrow cells (initially at 10^6 cells/mL) for 14 and 28 days in SF- and IL-3-supplemented medium in stirred suspension (■, n=5) or static, fed (▨, n=2), or static, non-fed (▩, n=2) cultures, and in nonsupplemented medium in stirred suspension (□, n=4) or static, non-fed (□, n=4) cultures. Input values for CFC and LTC-IC were 7000 ± 900 and 300 ± 120 per 10^6 cells respectively. Each bar represents the mean \pm SEM of values from n different experiments. Significant ($p < 0.10$) increases, relative to input determined using a one-tailed Student t-test, are marked by an asterisk.

Table 3.2: Total CFC per 10^6 light density marrow input cells at days 0, 14 and 28 in stirred or static cultures containing cytokine supplemented and non-supplemented medium.

	Feeding	Mature BFU-E	Primitive BFU-E	Mature CFU-GM	Primitive CFU-GM	CFU-GEMM	Number of experiments
Day 0							
Start		898 \pm 117	1880 \pm 214	2447 \pm 932	1680 \pm 422	98 \pm 41	7
Day 14							
Stirred culture + cytokines	+	1236 \pm 573	2063 \pm 855	70830 \pm 17519	20873 \pm 6543	287 \pm 135	7
Stirred culture - cytokines	+	0 \pm 0	58 \pm 29	1180 \pm 583	905 \pm 512	23 \pm 15	4
Static culture + cytokines	+	398 \pm 320	598 \pm 598	31866 \pm 1850	3116 \pm 724	0 \pm 0	2
Static culture + cytokines	-	626 \pm 527	1260 \pm 970	25209 \pm 13406	2372 \pm 665	10 \pm 10	4
Static culture + cytokines	-	0 \pm 0	104 \pm 52	3042 \pm 626	2347 \pm 288	43 \pm 18	4
Day 28							
Stirred culture + cytokines	+	2255 \pm 761	1971 \pm 731	156246 \pm 41365	44633 \pm 12949	476 \pm 283	7
Stirred culture - cytokines	+	3 \pm 2	45 \pm 35	4767 \pm 3820	598 \pm 299	2 \pm 2	4
Static culture + cytokines	+	90 \pm 90	54 \pm 54	6277 \pm 696	1523 \pm 1523	18 \pm 18	2
Static culture + cytokines	-	0 \pm 0	13 \pm 13	704 \pm 602	220 \pm 138	5 \pm 3	4
Static culture + cytokines	-	23 \pm 20	23 \pm 23	1031 \pm 845	375 \pm 298	0 \pm 0	4

Values are the means \pm SEM from the number of experiments shown. These are the same experiments for which the total CFC values are shown in Figure 3.2

static cultures) was greater for the most primitive types of CFC, although their differentiation into later types of CFC was also enhanced.

C. Changes in the Marrow Fibroblast Colony-Forming Unit (CFU-F) Population in Stirred Suspension Cultures

To evaluate the number of marrow stromal cell precursors in cultures maintained in suspension by continuous stirring and the possible effect of added IL-3 and SF, we used the colony-forming unit-fibroblast (CFU-F) assay. The results of all such assays (on cells from some of the same experiments shown in Figure 3.2) are shown in Figure 3.3. In all types of cultures, CFU-F numbers increased. This expansion was initially the greatest in static

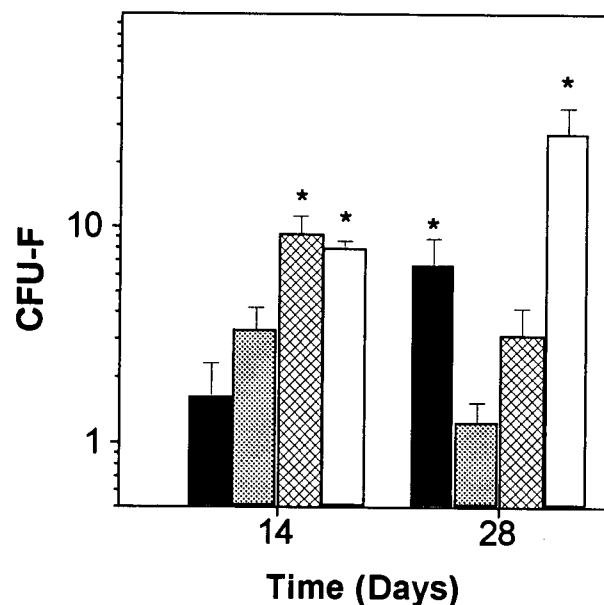


Figure 3.3: Calculated changes in CFU-F numbers relative to input in the same experiments shown in Figure 3.2. Input CFU-F numbers were 47 ± 26 per 10^6 light density bone marrow cells. Legend as in Figure 3.2.

cultures, regardless of whether the medium was supplemented with IL-3 and SF. Unexpectedly, a continuing expansion ($p < 0.05$) of the initial CFU-F population was also seen in the stirred suspension cultures supplemented with IL-3 and SF.

3. Discussion

The experiments presented in this chapter demonstrate the potential of stirred suspension cultures to support the continuous and significant net expansion of primitive normal human hematopoietic progenitor cells and stromal cell precursors over extensive periods of time from starting populations of light density bone marrow cell preparations. This was true of both CFC and LTC-IC hematopoietic progenitors, neither of which showed any evidence of deleterious effects from stirring on their survival or functional integrity. In the presence of IL-3 (2 ng/mL) and SF (10 ng/mL), total CFC numbers were expanded 22-fold over input values over a 28-day period. This amplification in the total CFC population was significant ($p < 0.05$) and could not be attributed simply to an accumulation of more terminally differentiated progenitors at the expense of more primitive cells. The latter, which are distinguished by their ability to express a higher proliferative capacity and/or multilineage differentiation potential in routine colony assays (Fauser and Messner, 1979; Cashman et al., 1985), were similarly and significantly amplified. Moreover, the number of LTC-IC, which represent the most primitive hematopoietic cells detectable *in vitro*, were also significantly increased (7-fold over input numbers, $p < 0.10$) in the same cultures.

These results extend those of Verfaillie (1992) who demonstrated an enhanced progenitor output of hematopoietic progenitors in cultures containing supportive fibroblasts when both cell types were separated from one another so that the hematopoietic cells could respond only

to soluble factors released by the fibroblasts. They also confirm and extend those of Sardonini and Wu (1993) who obtained less extensive expansion of total cells and CFC numbers in similarly maintained stirred suspension cultures initiated at what we have found to be a suboptimal starting cell concentration ($1-5 \times 10^5$ cells/mL). From the present studies, it is clear that the addition of IL-3 and SF were important contributors to the progenitor expansion achieved. However, the fact that obtaining progenitor expansions greater than those previously reported in a similar system (Sardonini and Wu, 1993) was also cell-concentration dependent suggests that other endogenously produced factor(s) play a stimulatory role. Bioassays (Lansdorp et al., 1986) of the spent medium from both stirred and static LTC-IC revealed levels of IL-6 of 1-10 ng/mL to be present after one week (data not shown). The finding that CFU-F numbers were also markedly amplified in stirred suspension cultures was not anticipated. However, stimulation of CFU-F by hematopoietic growth factors has been previously described (Dedhar et al., 1988) and the present finding is consistent with the possibility that appropriately stimulated marrow fibroblasts can serve as a source of multiple growth factors important to the maintenance and proliferation of very primitive hematopoietic cells (Anderson et al., 1990; Paul et al., 1990; Hogge et al., 1994).

The homogeneous nature of stirred suspension cultures makes them uniquely suited for further investigations of various parameters (e.g. O_2 tension, cytokine concentrations, serum components, medium exchange rates, etc.) that may influence the viability and turnover of specific stages and types of hematopoietic cells and hence lead to new insights into mechanisms governing (or limiting) a variety of hematopoietic cell proliferation and differentiation responses.

The relative simplicity of stirred suspension cultures also makes them attractive candidates for applications to clinical transplant protocols. Our results suggest it may be possible to produce the same number of CFC present in a "typical" transplant of 6×10^9 light density normal marrow cells (or $\sim 1-2 \times 10^{10}$ total marrow cells) from a starting inoculum of 4×10^8 light density marrow cells, somewhat larger starting inocula being required to match the LTC-IC content. It is important to recognize, however, the assumptions inherent in extrapolating from small scale experiments to the large numbers of cells that must be generated for clinical purposes. From a practical point of view, this poses additional challenges to the development of a large volume stirred suspension bioreactor system that will allow the progenitor expansions demonstrated here.

CHAPTER 4: CELLULAR DETERMINANTS AFFECTING THE RATE OF CYTOKINE DEPLETION IN CULTURES OF HUMAN HEMATOPOIETIC CELLS[†]

[†] The contents of this chapter are essentially as published in Zandstra et al., 1997a. A.L. Petzer is gratefully acknowledged for his assistance in the isolation and analysis of the purified cell populations.

1. Introduction

To develop a bioreactor technology that will allow the consistent production of clinically useful numbers of primitive hematopoietic cells, it is critical to identify culture variables that regulate the attributes required to sustain hematopoietic stem cell competence. Stirred suspension cultures have a number of advantages over static systems in this regard. Constant agitation of the suspension maintains the cells in a homogeneous environment. In addition, representative samples of these cultures can be readily removed (without altering the composition of the culture) so that numerous parameters can be defined and controlled. As shown in Chapter 3, inoculum cell density, medium addition and cytokine supplementation all affect the degree of LTC-IC amplification achievable in stirred suspension cultures of adult human marrow cells. The initial purpose of the present study was to determine whether the concentrations as well as the types of cytokines added might also be limiting variables. During the course of the investigation, it was discovered that primitive hematopoietic cells are able to deplete cytokines from the medium much more rapidly than their more mature progeny using a mechanism that is strongly dependent on the concentration of cytokines to which the cells are exposed.

2. Results

A. CFC Output in Stirred Suspension Cultures can be Increased Without Affecting LTC-IC Maintenance

Preliminary experiments suggested that the addition of 10 ng/mL of FL and 2 ng/mL of IL-6 and IL-11 to the previously used SF plus IL-3 cytokine supplement (Chapter 3) in

stirred suspension cultures initiated with light density normal human marrow cells would support production of larger numbers of progenitors and/or total cells. As shown in Figure 4.1, this 5-factor cocktail caused a rapid expansion of the CFC population (to 24 ± 8 -fold above input numbers within the first 7 days and increasing further to 45 ± 9 -fold above the input after a second week). However, in these experiments, the maximum net expansion of LTC-IC was only 2.5 ± 0.8 -fold after 14 days which is lower than what we had observed previously using SF and IL-3 (Chapter 3, shown for comparison as the dotted lines in Figure 4.1).

To determine whether improved expansions might be obtained with higher cytokine concentrations, 5-fold higher levels of the same 5-factor cocktail were added to another set of cultures in the same experiment. This resulted in a 1.5-fold greater production of CFC (to 66 ± 19 -fold above the input value after 14 days) as well as a significant enhancement ($p < 0.07$) in the expansion of the LTC-IC population (9 ± 4 -fold above input after 14 days) compared to cultures containing the same 5 cytokines at the lower concentration. Interestingly, none of these changes in the cytokine supplements had any significant effect on the total number of nucleated cells produced relative to the original SF plus IL-3 combination. The rate of glucose consumption also did not vary significantly with the cytokine concentration used and by day 21 the levels of glucose present (< 2 mM) (Figure 4.2) were at levels which can be growth limiting for transformed mammalian cells (Xie and Wang, 1993).

B. Dependence of Cytokine Depletion Rates on the Concentration of Cytokines Present in the Medium

Since the rate of progenitor expansion appeared to be influenced by the concentrations of cytokines added to the medium, the levels of IL-3, SF and FL during the course of the

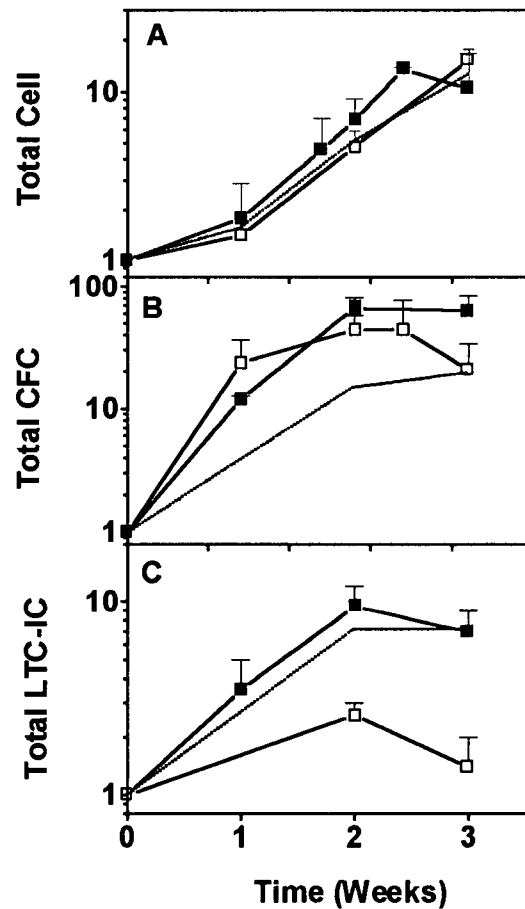


Figure 4.1: Calculated numbers of total viable cells (A), CFC (B) and LTC-IC (C) relative to the input in stirred suspension cultures initiated with light density human marrow cells (initially 10^6 cells/mL) in 10 ng/mL SF and FL, and 2 ng/mL IL-3, IL-6 and IL-11 (□) or 50 ng/mL SF and FL, and 10 ng/mL IL-3, IL-6 and IL-11 (■). Input values for CFC and LTC-IC were 11100 ± 6300 and 57 ± 23 per 10^6 cells, respectively. The dotted lines represent Figure 3.2 data where similarly maintained cultures were grown in a medium containing only 10 ng/mL SF and 2 ng/mL IL-3. Values shown are the mean \pm SEM for data from 3 independent experiments.

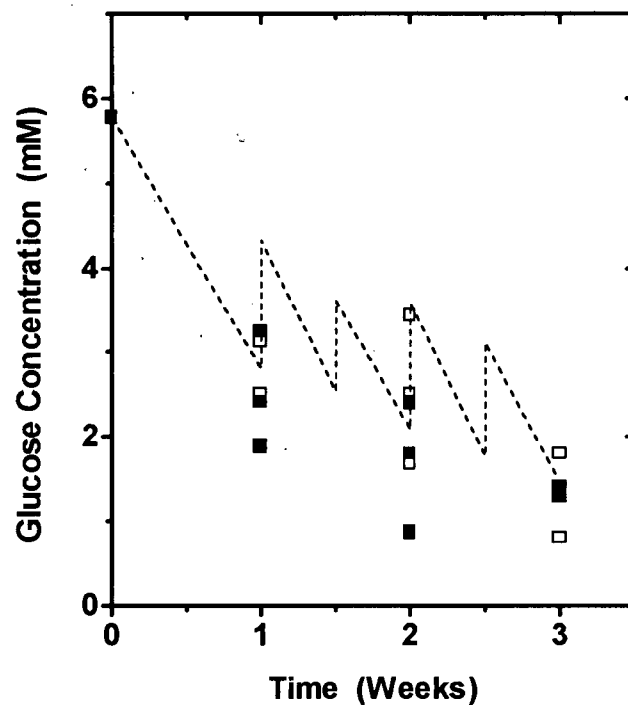


Figure 4.2: Measured glucose concentrations in the supernatant media from the same cultures whose progenitor and cell number changes are shown in Figure 4.1. Dashed lines represent the calculated concentration of glucose based on the amount of fresh media and cytokines added to the cultures and the average measured concentration at each time point.

above experiments were also measured. As shown in Figure 4.3, the concentrations of each of these cytokines declined over 3 weeks by a factor of 2- to 20-fold. Thus part of the failure of CFC and LTC-IC populations to continue expanding during the latter part of the cultures (Figure 4.2) might be explained by a failure to have maintained adequate cytokine levels.

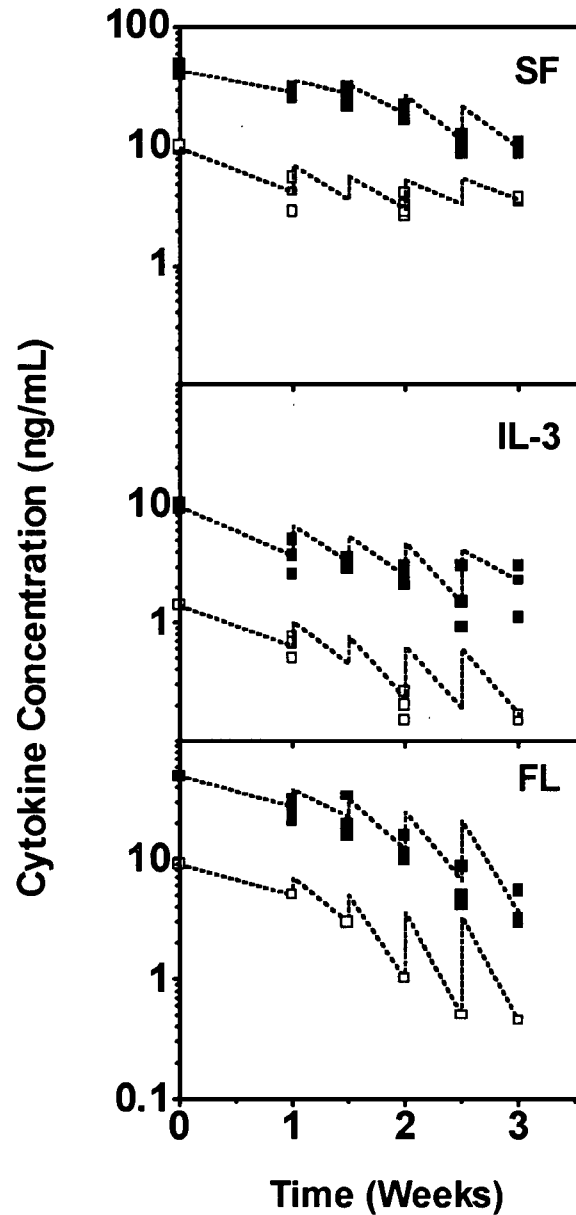


Figure 4.3: Measured SF, IL-3 and FL concentrations in the supernatant media from the same experiments whose progenitor and cell number changes are shown in Figure 4.1. Dashed lines represent the calculated concentrations of these cytokines based on the average measured concentrations and the amount of fresh medium and cytokines added to the cultures at each time point. Solid symbols refer to assays of cytokine levels in the supernatants obtained from cultures to which 50 ng/mL of SF and FL were added plus 10 ng/mL of IL-3 (n=3). Open symbols refer to assays of cytokine levels in supernatants obtained from cultures to which 10 ng/mL of SF and FL were added plus 2 ng/mL of IL-3 (n=3 for SF and IL-3 measurements, n=1 for FL measurements).

To obtain a better understanding of the relationship between the concentration of cytokines present and the rate of cytokine depletion, these rates were normalized by the average total number of cells present during each interval (Chapter 2, Equation 2.1) and then plotted as a function of the average concentration of the corresponding cytokine (Figure 4.4). It should be noted that these calculated cell-specific cytokine depletion rates are also average values for the cell populations present and that rates for specific subpopulations of cells can vary markedly (see below). The considerable scatter in the data may thus reflect some variability between experiments in the relative proportions of different cell types as well as other variations in the levels of nutrients, metabolites and cytokines present at different times in these periodically fed cultures. Nonetheless, a proportional relationship between the calculated average cell-specific rate of depletion of a given cytokine and its concentration in the medium was observed ($r = 0.89$, 0.89 and 0.79 for SF, IL-3 and FL, respectively) indicating that higher cytokine concentrations are associated with increased rates of cytokine depletion. In the absence of cells, all of these cytokines were relatively stable at 37°C for periods of up to at least 21 days in either fresh medium or in a medium previously conditioned for 3 days with Mo7e cells at concentrations that ranged from 3×10^5 to 1.5×10^6 cells/mL. The cytokine half-lives in these media were 46 ± 14 , 99 ± 30 , and 350 ± 170 days for SF, IL-3 and FL, respectively. Thus, even for the most labile cytokine (SF), less than 12% of the measured cytokine depletion can be attributed to degradation that would occur in the absence of viable cells.

To determine whether the observed association between cytokine depletion rates and cytokine concentration might be a general feature of factor-responsive hematopoietic cells, Mo7e cells were investigated as a test population. Mo7e cells are an immortal cell line that

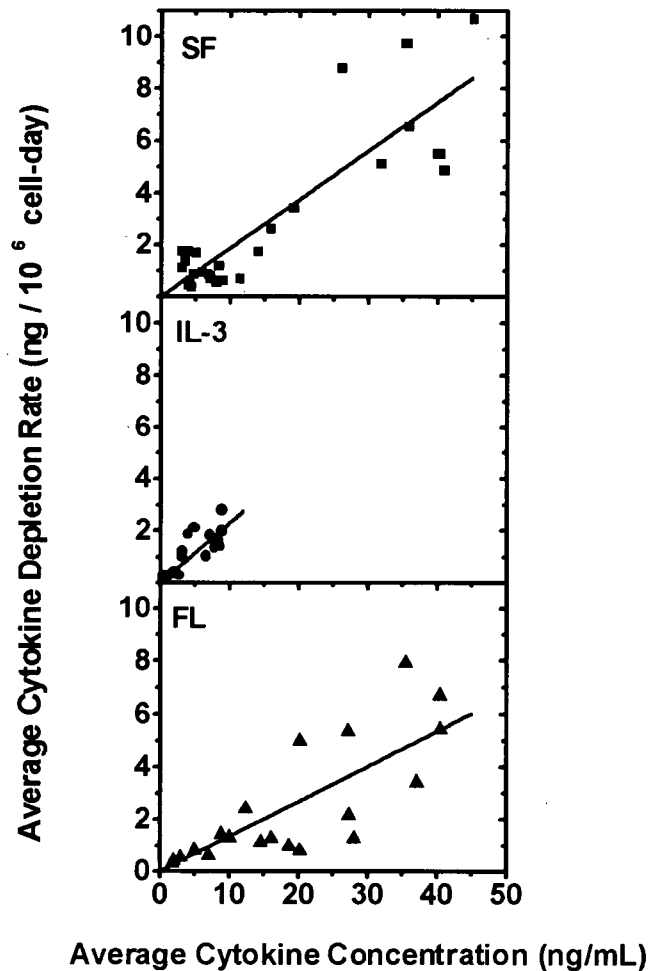


Figure 4.4: Calculated average cell-specific SF (■), IL-3 (●) and FL(▲) depletion rates vs the average measured cytokine concentrations for the same experiments shown in Figure 4.1. The solid line represents a linear fit of the data.

originated in a patient with leukemia and require, for their viability and proliferation, factors such as IL-3 or SF in addition to serum (Avanzi et al., 1988). In this study, IL-3 was the only

cytokine added and the cultures were terminated after 72 h. The maximum decrease in IL-3 concentration measured in these experiments was 30% of the input value. Figure 4.5 shows the calculated Mo7e cell-specific IL-3 depletion rates. These show much clearer trends than is evident from the marrow data shown in Figure 4.4. The IL-3 depletion rates (per 10^6 Mo7e cells) increased linearly up to 3 ng/ 10^6 cells/day at 7 ng/mL IL-3. At higher IL-3 concentrations, the rate of IL-3 depletion by Mo7e cells appears to approach an asymptote.

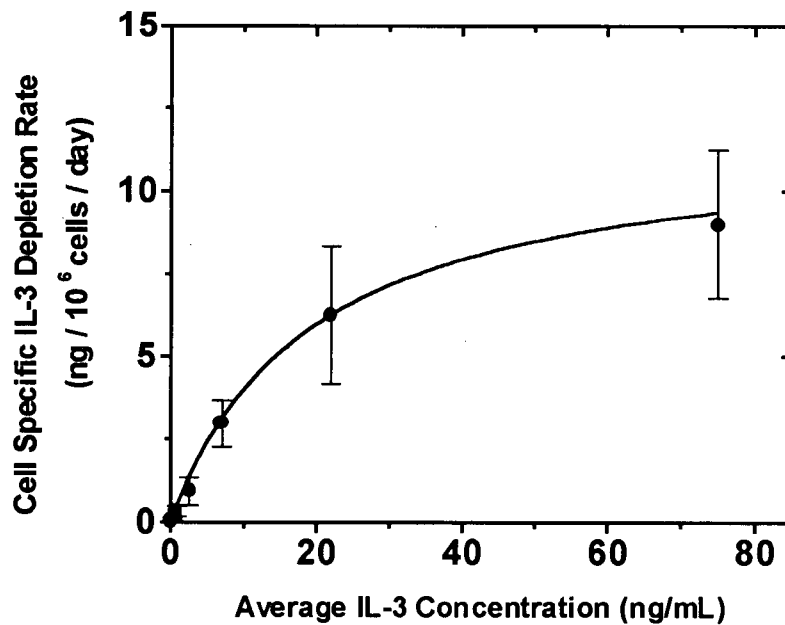


Figure 4.5: Calculated cell-specific rates of IL-3 depletion from cultures of Mo7e cells initiated at 3×10^5 cells/mL and maintained for 72 h in media supplemented with varying concentrations of IL-3. Values shown are the mean \pm SEM for data from 3 independent experiments. The solid line represents the best fit of the data by Equation 4.1.

C. Different Types of Hematopoietic Cells are Characterized by Different Rates of Cytokine Depletion

An additional variable in cultures of hematopoietic cells is the heterogeneity of cell types present since the relative proportions of these can alter dramatically depending on the culture conditions (e.g. see Figure 4.1). Therefore, another series of experiments was performed to determine whether the rate of cytokine depletion might vary for different types of hematopoietic cells. 5×10^3 highly purified (>99%) $CD34^+CD38^-$, $CD34^+CD45RA^+CD71^-$ or $CD34^+CD45RA^-CD71^+$ cells were used to initiate 1 mL cultures. These phenotypes were chosen because they represent highly enriched populations of LTC-IC, CFU-GM and BFU-E/CFU-E, respectively (Lansdorp and Dragowska, 1992). Another set of 1 mL cultures were established with 10^5 cells taken directly from the initial light density cell suspension from which the $CD34^+$ subpopulations were isolated. Each of these cell populations were then cultured for 7 days in a serum-free medium containing FL and SF at 100 ng/mL and IL-3, IL-6 and G-CSF at 20 ng/mL. These conditions had previously been shown to stimulate a 20- to 30-fold expansion of LTC-IC and a 500-fold expansion of CFC in cultures of $CD34^+CD38^-$ cells (Petzer et al., 1996b). In parallel cultures, to which no cytokines were added, SF and IL-3 levels were indistinguishable from background (<30 pg/mL) at day 7. The numbers of cells and the levels of SF and IL-3 at the end of the 7-day period were measured and then used to calculate average cell-specific SF and IL-3 depletion rates (Equation 2.1).

As shown in Figure 4.6, the highest factor depletion rates were seen in cultures initiated with CD34⁺CD38⁻ cells and these were at least 5-fold higher ($p<0.08$) than the rates measured for any of the other populations evaluated and ~35-fold higher ($p<0.05$) than the rates measured for the original, unseparated, low density marrow cells (only $8 \pm 4\%$ of which were CD34⁺). The factor depletion rates calculated for cultures initiated with the other types

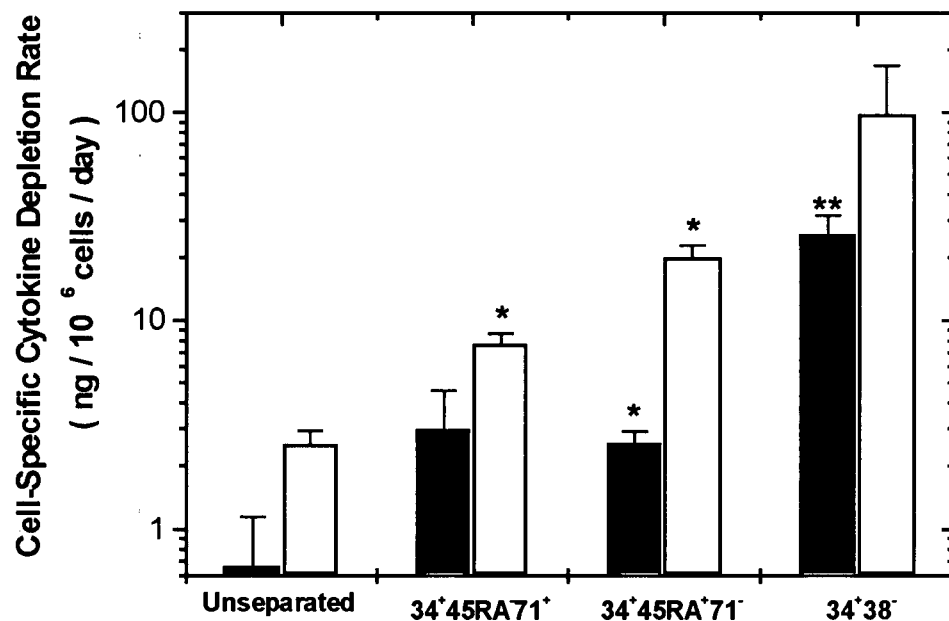


Figure 4.6: Calculated cell-specific rates of IL-3 (solid bar) and SF (open bar) depletion for 5×10^3 CD34⁺CD38⁻, CD34⁺CD45RA⁺CD71⁺ or CD34⁺CD45RA⁻CD71⁺ cells or 10^5 light density marrow cells placed separately in 1 mL serum-free medium supplemented with 100 ng/mL SF and FL, and 20 ng/mL IL-3, IL-6 and G-CSF. Significant differences ($p<0.05$) between the cytokine depletion rates measured for the CD34⁺CD38⁻ cells and the CD34⁺CD45RA⁺CD71⁺ or CD34⁺CD45RA⁻CD71⁺ populations are indicated by two asterisks. Significant differences ($p<0.05$) between the cytokine depletion rates of the CD34⁺CD45RA⁺CD71⁺ and CD34⁺CD45RA⁻CD71⁺ cells and light density marrow are indicated by a single asterisk. Bars represent the mean \pm SEM for 3 independent experiments.

of CD34⁺ cells tested, although lower, were all still greater by a factor of approximately 4 than those measured for the unseparated cells ($p < 0.05$). It should be noted that, in all cases, the composition of the hematopoietic populations changed during the 7-day culture period. As, in general, the frequency of more primitive cells decreased with cell culture, the measured population specific depletion rates may represent a conservative estimate of their actual rates. Although a significant ($p < 0.05$) absolute expansion of LTC-IC and/or CFC occurred in each of the cultures (Table 4.1), their frequencies were not equivalently increased, presumably due to the greater expansion of more differentiated cell types. For example, it can be seen in Table 4.1 that an absolute increase in LTC-IC numbers from the CD34⁺CD38⁻ population of 17-fold was accompanied by a 6-fold decrease in their frequency.

Table 4.1: Changes in the numbers of different types of progenitors after maintenance of different subpopulations of normal human marrow cells in serum-free medium supplemented with 100 ng/mL of SF and FL, and 20 ng/mL of IL-3, IL-6 and G-CSF for 7 days. Values shown are the mean \pm SEM for 3 independent experiments.

Input Cell Phenotype	Progenitor Analyzed	Input (% of total cells)	Day 7	
			per 100 input cells	% of total cells
Light density marrow	CFU-E/BFU-E	0.6 \pm 0.04	15 \pm 9	1 \pm 1
	CFU-GM	0.9 \pm 1.0	24 \pm 13	2 \pm 1
	CFU-GEMM	0.01 \pm 0.01	1.2 \pm 0.9	0.08 \pm 0.08
	LTC-IC	0.006 \pm 0.003	0.011 \pm 0.003	0.001 \pm 0.0002
CD34 ⁺ CD45RA ⁻ CD71 ⁺	CFU-E/BFU-E	55 \pm 15	4000 \pm 440	8 \pm 2
	CFU-GM	9 \pm 3	750 \pm 260	1.3 \pm 2.0
	CFU-GEMM	2 \pm 1	160 \pm 90	<1
	LTC-IC	n.d.	n.d.	n.d.
CD34 ⁺ CD45RA ⁺ CD71 ⁻	CFU-E/BFU-E	1 \pm 0.6	<10	<1
	CFU-GM	11 \pm 3	1300 \pm 450	5 \pm 2
	CFU-GEMM	<1	<10	<1
	LTC-IC	n.d.	n.d.	n.d.
CD34 ⁺ CD38 ⁻	CFU-E/BFU-E	0.6 \pm 0.3	290 \pm 5	2 \pm 1
	CFU-GM	4 \pm 2	920 \pm 210	6 \pm 2
	CFU-GEMM	2 \pm 1	7 \pm 5	<1
	LTC-IC	24 \pm 10	410 \pm 23	4 \pm 1

n.d. = not determined

3. Discussion

This Chapter continues from Chapter 3 in exploring the use of stirred suspension cultures to obtain expansion of the most primitive elements from such a starting population and specifically to determine whether manipulating extracellular cytokine levels might increase primitive progenitor outputs in this system. Since the first of these experiments showed that both the type and concentration of cytokines added might be rate limiting, even with the biweekly addition of 50 ng/mL of FL and SF and 10 ng/mL of IL-3, IL-6 and IL-11, further experiments were performed which showed the rate of cytokine depletion from the medium to be a function both of the concentration of the cytokine(s) and the types of cells present.

For investigations of the cytokine concentration effect, we found the Mo7e factor-dependent human leukemic cell line to be a useful model. The rate of cytokine depletion (q) by Mo7e cells can be related to the concentration of cytokine(s) in the medium by the following equation:

$$q = \frac{q^{\max} C}{Kc + C} \quad (4.1)$$

where C is the cytokine concentration (ng/mL), q^{\max} is the maximum cell-specific rate of cytokine depletion (ng/10⁶ cells/day), and Kc is the cytokine depletion rate constant (ng/mL). Values of q^{\max} and Kc for IL-3 in cultures of Mo7e cells were calculated by fitting the data in Figure 4.5 to Equation 4.1. The resultant constants were 12 ± 3 ng/10⁶ cells/day and 20 ± 1 ng/mL, for q^{\max} and Kc , respectively. Although there was greater variability in the data obtained from the primary marrow cell cultures (Figure 4.4), for $C \ll Kc$, the concentration

dependence of the calculated cytokine depletion rates for marrow (Table 4.2) can be compared with the linear region of the Mo7e data whose slope $\left(q^{\max} / K_c \right)$ was 0.61 mL/10⁶cells/day. The approximately 3-fold larger value obtained for Mo7e cells may be due to their transformed nature. In addition, the rates of cytokine depletion measured in the cultures of marrow cells may have been influenced by other variables (e.g. glucose levels, Figure 4.2).

Table 4.2: Ratio of depletion rate constants for IL-3 SF and FL in cytokine-supplemented cultures of human marrow and Mo7e cells.

Cell type	Cytokine	q^{\max} / K_c *
		$\left(\frac{10^6 \text{ cells}}{\text{mL}} \text{ day} \right)^{-1}$
Human Marrow	SF	0.19 ± 0.04
	IL-3	0.22 ± 0.06
	FL	0.14 ± 0.03
Mo7e	IL-3	0.61 ± 0.03

*Values expressed as the ratio ± the 95% confidence interval as calculated by Equation 4.1 from Figures 4.4 (human marrow) and 4.5 (Mo7e).

Relatively few systematic studies of the rate of cytokine depletion in liquid cultures of hematopoietic cells have been reported. A 2-fold decrease in the concentrations of IL-1, IL-3 and IL-6 in the fluid phase of continuously perfused flat-bed cultures of hematopoietic cells maintained on irradiated stromal cell feeder layers was recently reported (Koller et al., 1993b, 1995) and this group has suggested that both the types and amounts of factor consumed may be correlated with the production of specific lineages of progenitors (Koller et al., 1995). However, calculation of cell-specific rates of cytokine depletion in static bioreactors is

understandably limited by the difficulty of recovering cell samples and accurately measuring cytokine levels within the layers of adherent cells in which the most primitive hematopoietic cells are concentrated. On the other hand, effects of cytokine exposure on cytokine receptor expression and internalization by factor-dependent cell lines and terminally differentiated primary cells have been extensively investigated (Korpelainen et al., 1995; Miyazawa et al., 1994; Murthy et al., 1989; Pietsch et al., 1992; Shimizu et al., 1996).

In these studies, exposure of the cells to higher cytokine concentrations has generally been found to lead to a decreased expression of the corresponding receptor (Murthy et al., 1989). The most likely mechanism is the stimulated internalization of ligand/receptor complexes as, for example, has been demonstrated for insulin (Martial et al., 1994). Moreover, it has been suggested that receptor internalization may be an important mechanism in regulating factor-dependent proliferative responses since cells bearing internalization-deficient receptors demonstrated diminished factor depletion and consequently, increased proliferation relative to wild-type controls (Reddy et al., 1994). Interestingly, for Mo7e cells, the rate of intracellular SF receptor (*c-kit*)/SF complex degradation was also reported to be proportional to the extracellular concentration of SF (Miyazawa et al., 1994). The extent of *c-kit* internalization has also recently been described to be proportional to extracellular SF concentration (Shimizu et al., 1996). This suggests that receptor/ligand internalization and degradation may be linked (sequential) events indicating a mechanism for the permanent removal of soluble stimulatory factors from the environment by responsive cells.

In addition to being cytokine concentration-dependent, cytokine depletion rates were found to decrease markedly as hematopoietic cells progress from a very primitive stage of

differentiation ($CD34^+CD38^-$) to later types of $CD34^+$ cells and finally to mature cells which no longer express the CD34 antigen (Figure 4.6). Cell-specific rates of cytokine depletion by $CD34^+CD38^-$, $CD34^+CD45RA^+CD71^-$, $CD34^+CD45RA^-CD71^+$ and unseparated light density marrow cells in culture had not been previously defined. The trend identified here is, however, consistent with previous reports of higher densities of receptors for cytokines on more primitive cell types (Metcalf and Nicola, 1991; Wagner et al., 1995).

Taken together, these results suggest the existence of a self-limiting mechanism that may regulate primitive hematopoietic cell stimulation by cytokines in a transport-limited environment. More primitive cells (i.e. LTC-IC) appear to have both higher cytokine requirements for their maximal amplification than later types of hematopoietic cells (Figure 4.1 and Petzer et al. (1996b)) and greater capacities to decrease the extracellular concentration of cytokines to which they are responsive (Figure 4.6).

In the diffusion-limited microenvironment thought to prevail in the adult marrow (Naeim et al., 1996) the operation of these two principles may have a profound influence on constraining the recruitment of very primitive cells into self-renewal divisions. However, regardless of the relevance of the present findings to physiological control mechanisms, they have clear implications for the design of clinical scale hematopoietic cell culture systems. In such systems, optimization of cytokine delivery will need to take account of changing rates at which these cytokines may be depleted. Because of the homogeneity of the microenvironment to which each cell is exposed in stirred suspension cultures, such bioreactors should be particularly well suited for the development of culture systems in which the continuous control of that environment is an important parameter.

CHAPTER 5: DIFFERENTIAL CYTOKINE EFFECTS ON PRIMITIVE (CD34⁺CD38⁻) HUMAN HEMATOPOIETIC CELLS: NOVEL RESPONSES TO FLT3-LIGAND AND THROMBOPOIETIN[†]

[†] The contents of this chapter are essentially as published in Petzer et al., 1996b. A. Petzer made a significant contribution in the development of the purified cell expansion protocol, in the isolation of the purified cells and in the maintenance and assays of some of the cultures.

1. Introduction

Recently, several studies have shown that significant expansions of input LTC-IC populations can be obtained in vitro when various cytokine supplements are added and other alterations to the culture conditions are made (Chapter 3; Petzer et al., 1996a; Koller et al., 1993; Moore and Hoskins, 1994). For example, in cultures initiated with single CD34⁺CD38⁻ cells isolated from normal adult human marrow, 30-fold amplifications of LTC-IC were seen within 10 days followed by a further 2-fold expansion of this primitive progenitor population after an additional 2 to 3 weeks (Petzer et al., 1996a). In these cultures, the cells were maintained in the absence of stroma in a serum-free medium containing 100 ng/mL each of Flt3-ligand (FL) and Steel factor (SF), 20 ng/mL each of interleukin-3 (IL-3), IL-6 and granulocyte colony-stimulating factor (G-CSF) and 5 ng/mL of nerve growth factor- β (NGF- β). This combination of cytokines was originally chosen on the basis of previous evidence that each component included might contribute to the proliferation or maintenance of various types of primitive hematopoietic cells (Otsuka et al., 1991; Lansdorp and Dragowska, 1992; Sutherland et al., 1993b; Ponchio et al., 1995) or was produced by fibroblast feeders that had these properties (L. Coulombel, personal communication; Hattori et al., 1993). The purpose of the studies described here was to identify the specific factors or combination(s) of factors that can stimulate an expansion of LTC-IC in serum-free cultures initiated with CD34⁺CD38⁻ cells. In addition, experiments were undertaken to determine how these factor requirements might compare with those needed to maximize CFC production from the same starting population.

2. Results

A. Multifactorial Analysis of the Relative Contributions of 6 Growth Factors on LTC-IC Amplification.

In a first series of experiments with 4 separately isolated CD34⁺CD38⁻ cell populations (obtained from 2 marrow sources), aliquots of 200 CD34⁺CD38⁻ cells were incubated for 10 days in 100 μ L liquid suspension cultures containing different combinations of 6 growth factors. LTC-IC assays were performed both on the initial CD34⁺CD38⁻ cells and on the cells harvested from the 10 day cultures to determine the extent of the expansion obtained with each condition (Figure 5.1). The 6 factors evaluated in these experiments were FL (100 ng/mL), SF (100 ng/mL), IL-3 (20 ng/mL), IL-6 (20 ng/mL) and G-CSF (20 ng/mL) plus NGF- β (5 ng/mL) since previous experiments had shown that a cocktail of all of these factors would stimulate a marked amplification of LTC-IC in cultures of single CD34⁺CD38⁻ cells (Petzer et al., 1996a). G-CSF and NGF- β were evaluated together because preliminary data had suggested that NGF- β may not stimulate the proliferation of CD34⁺CD38⁻ cells and use of this combination reduced considerably the magnitude of the experiments.

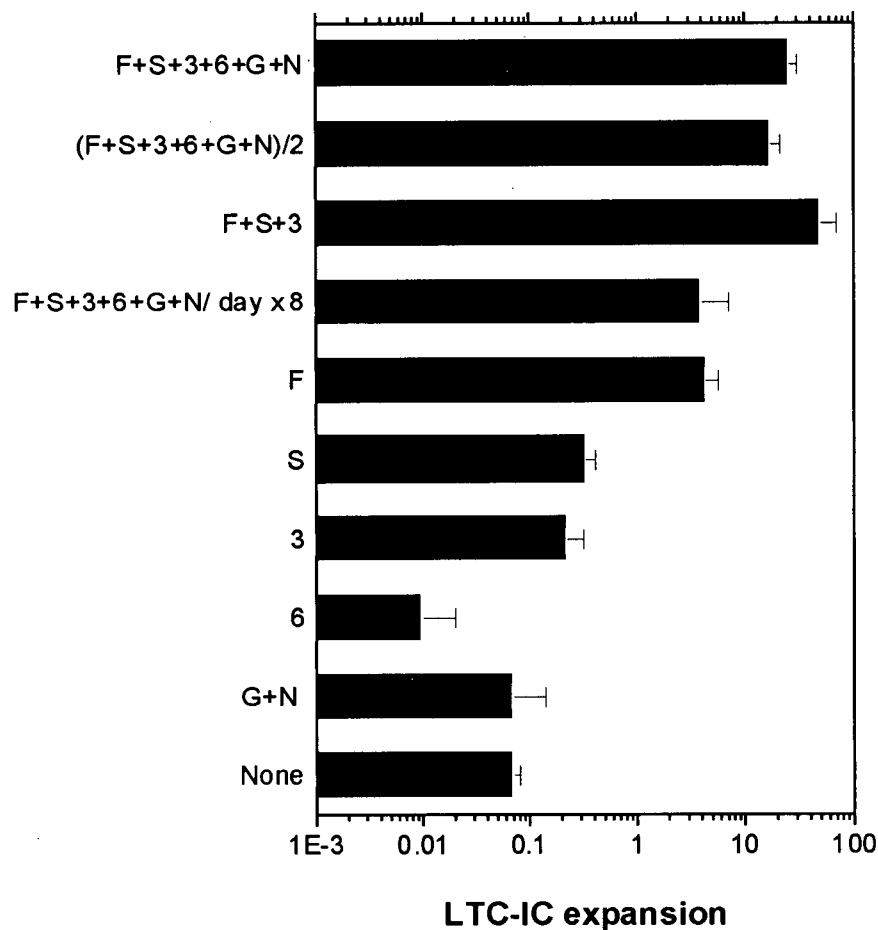


Figure 5.1: Variable LTC-IC expansion from $CD34^+CD38^-$ cells incubated for 10 days in serum-free cultures containing different cytokine combinations of FL (F), SF (S), IL-3 (3), IL-6 (6), G-CSF (G) and NGF- β (N). 168 ± 48 CFC per 100 input cells were generated in the 6 week LTC-IC assays of the original $CD34^+CD38^-$ cells used to initiate the serum-free expansion cultures. Bars indicate the mean \pm SEM of the increases measured relative to the starting LTC-IC-derived CFC output value of the same experiment for data pooled from 2 to 7 experiments.

A measure of the LTC-IC content of each of the 4 input $CD34^+CD38^-$ cell suspensions as well as for the cells harvested from each of the 80 different 10-day liquid suspension cultures (16 different combinations of growth factors plus the 4 replicates in each

of 4 subexperiments) was obtained by assessing the total CFC output for a fixed number of original CD34⁺CD38⁻ cells. The validity of this approach (rather than the use of limiting dilution analyses for quantitating LTC-IC in each suspension) is based on previous data showing that the numbers and types of CFC generated from individual LTC-IC, although highly variable, remain on average the same when freshly isolated and cultured LTC-IC are compared (Sutherland et al., 1991; Petzer et al., 1996a). More recently these relationships have been confirmed by formal limiting dilution analysis experiments (Chapter 6), although as noted below, one circumstance where at least the type of CFC produced can be altered was also discovered in the present studies. However, this change appeared to be uniquely related to the exposure of LTC-IC to a single factor. The extent of LTC-IC amplification when all 6 growth factors were present was 24 ± 5 -fold (n=7, Figure 5.1). This magnitude of LTC-IC expansion is similar to that measured previously when single CD34⁺CD38⁻ cells were cultured in the same cocktail (Petzer et al., 1996a), suggesting that the extent of LTC-IC amplification obtained is relatively independent of the initial concentration of CD34⁺CD38⁻ cells up to at least 200 per 100 μ l (i.e. 2×10^3 CD34⁺CD38⁻ cells per mL).

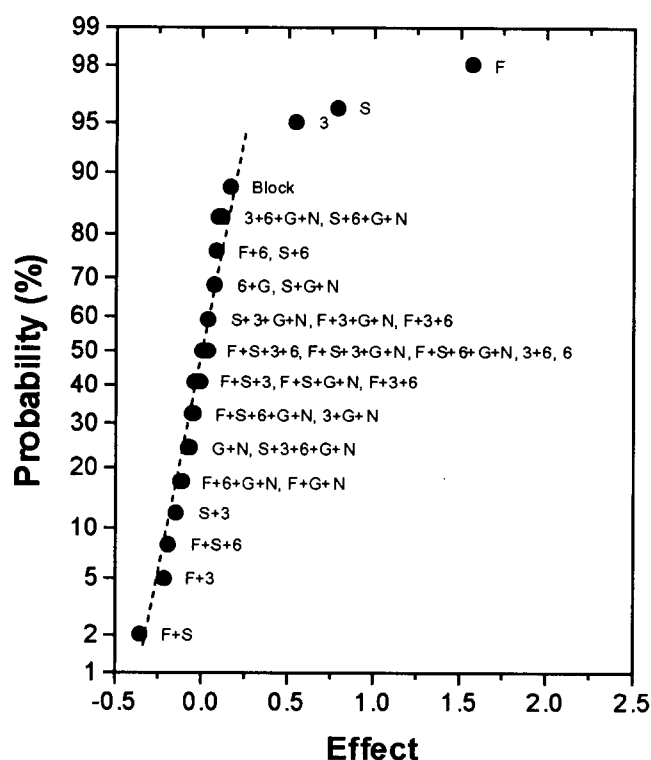


Figure 5.2: Linearized normal probability distribution of the normalized effect of different combinations of cytokines on LTC-IC expansion in 10 day serum-free cultures of CD34⁺CD38⁻ cells. Significant effects do not fall on the dashed line representing the normal probability distribution. In this plot, an effect is defined as the average change in LTC-IC expansion that occurred in cultures containing the growth factor(s) shown relative to cultures not containing these factor(s). Abbreviations are as indicated in the legend of Figure 5.1.

The ability of the 6 factors tested (4 individually and G-CSF and NGF- β as a pair) to support the amplification of LTC-IC was then evaluated by multi-parameter analysis of variance of the normalized data from all 4 experiments. Such an analysis identifies individual or interactive effects as significant when the result of any particular factor or combination of factors is significantly different from the variation accounted for by the normal probability distribution resulting from the combined data from all groups (Box et al., 1978). For the experiments reported in Figure 5.2, effects indistinguishable from the normalized mean at a

level of $p > 0.05$ were deemed to be insignificant. A comparison of the standard deviation of the 4 sets of replicate center points (i.e. data from cultures containing all factors at half the concentration used in all other groups) established that the interexperimental variability was not significant (block effect in Figure 5.2). Figure 5.2 shows that the results of all of the possible combinations of growth factors tested, with the exception of FL, SF and IL-3 alone, lie within the variance of the normal probability distribution. It is important to note that this experimental design detects significant effects for combinations of up to 3 factors, higher order interactions are convoluted with the main and 2-factor effects. An indication of the unique and relative activities of these 3 factors to support the amplification of LTC-IC from $CD34^+CD38^-$ cells can also be seen in Figure 5.1. Only FL alone was able to increase the number of LTC-IC significantly ($p < 0.05$) above the input value. SF and IL-3, alone, each maintained LTC-IC numbers at a significantly higher level ($p < 0.05$) than was seen when no factors were added, although neither SF nor IL-3 alone was able to stimulate a net increase in LTC-IC numbers. Interestingly, even in a completely defined medium, which contained insulin but no other growth factors, the rate of LTC-IC decline was relatively slow. Accordingly, under these conditions, many LTC-IC ($7 \pm 1\%$ of input values) could still be detected after 10 days (Figure 5.1). This is similar to results obtained previously for a similar starting population of $CD34^+HLA-DR^+$ cells incubated without growth factors but in the presence of serum (Sutherland et al., 1993a). Also evident in both Figures 5.1 and 5.2 is the finding that the minimum combination of FL plus SF plus IL-3 was sufficient to obtain the greatest LTC-IC amplification observed for any combination of the 6 factors tested. Moreover, there were no detectable effects ($p > 0.05$, Figure 5.2) attributable to interactions between multiple factors (Box et al., 1978). These higher order effects, along with any effects

of the other individual factors were indistinguishable from the experimental error. It remains possible that further effects would be identified with more replicates. When the concentration of each of the 6 growth factors in the cocktail was reduced two-fold, the extent of LTC-IC amplification obtained was also reduced (by 25%, Figure 5.1). On the other hand, repeated daily addition of all 6 growth factors (in the same amounts as when added only at the beginning of the culture at full strength) resulted in a decrease in LTC-IC yields (by 84%, Figure 5.1). Taken together, these results provide some indication of the existence of a dose-effect relationship for the stimulation of LTC-IC amplification by the combination of FL, SF and IL-3.

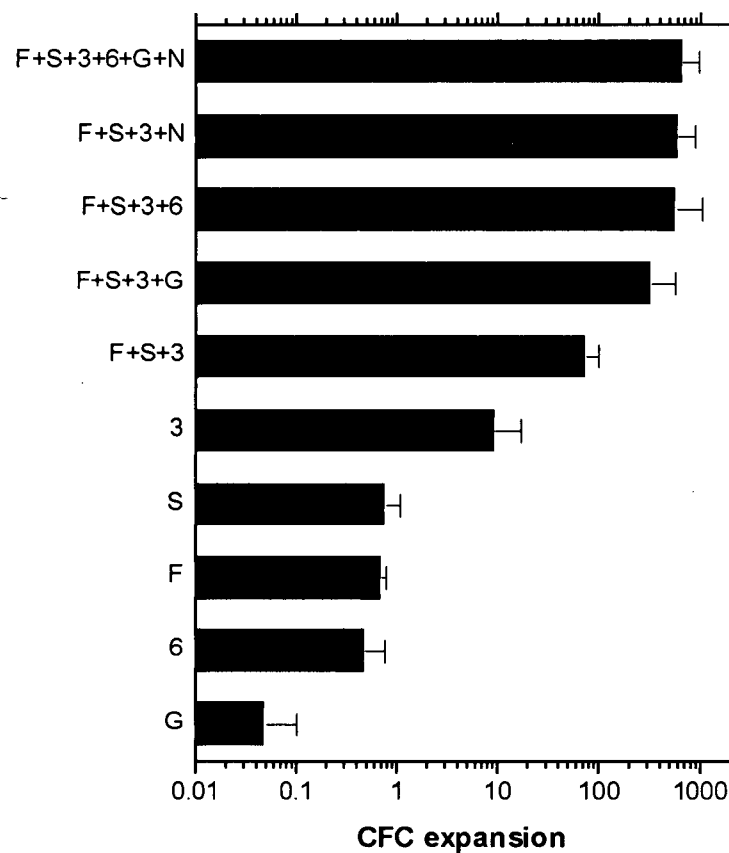


Figure 5.3: Variable CFC expansion from $CD34^+CD38^-$ cells incubated for 10 days in serum-free cultures containing different cytokines as shown. 7 ± 3 colonies per 100 $CD34^+CD38^-$ cells were generated in CFC assays performed on the original cells used to initiate the serum-free expansion cultures. Bars indicate the mean \pm SEM of the increases measured relative to the starting CFC value in the same experiment ($n \geq 3$).

B. Effects of Different Growth Factors on CFC Production by $CD34^+CD38^-$ Cells.

It has previously been shown that LTC-IC maintenance in serum-containing cultures was not affected by several variations in the growth factor composition of the medium that, nevertheless, had marked effects on the number of CFC detectable after 5 weeks (Sutherland et al., 1993c). It was, therefore, of interest to determine whether LTC-IC amplification and the production of CFC from $CD34^+CD38^-$ cells would show a similar or different growth

factor dependence. The CFC outputs measured for the various growth factor combinations tested in at least 3 experiments are shown in Figure 5.3. As noted previously (Terstappen et al., 1991; Petzer et al., 1996a), the frequency of CFC in the initial CD34⁺CD38⁻ populations was low ($7 \pm 3\%$). Of the single factors tested, IL-3 was the most potent stimulator of CFC production over the 10-day period (~10-fold increase), and neither FL nor SF alone supported a net expansion of the CFC population. On the other hand, when combined, these 3 factors stimulated an ~80-fold increase in CFC numbers and this was further increased (another 4 to 8-fold) when IL-6, G-CSF, NGF- β or all 3 of these factors were also present.

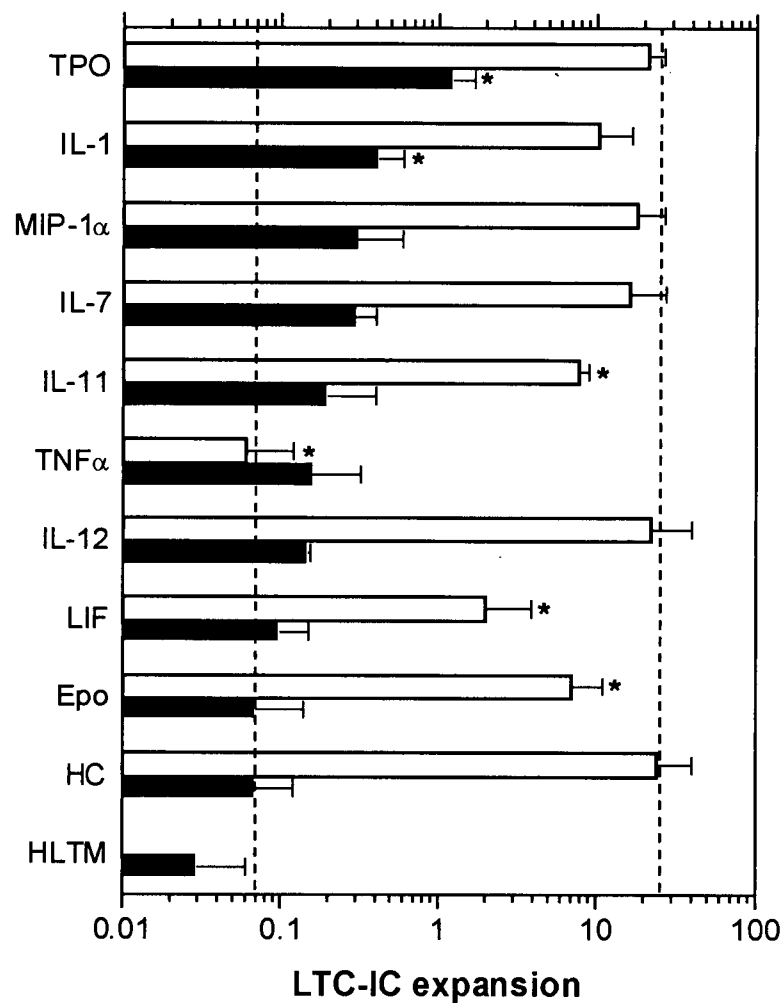


Figure 5.4: Effect of 10 specific cytokines and HC on LTC-IC expansion from CD34⁺CD38⁻ cells incubated for 10 days in serum-free cultures either alone (solid bars) or in combination with the 6-factor cocktail analyzed in Figure 5.1 (open bars). The effect of culture in serum containing-medium (HLTM, Myelocult) alone was also tested. The dotted lines indicate the fold change in numbers of LTC-IC detectable in the absence of factor addition or in the presence of the 6-factor combination alone (as indicated in Figure 5.1). Significant differences versus no growth factor addition (TPO and IL-1, $p < 0.05$) or versus the 6-factor combination (TNF- α , $p < 0.005$; LIF and IL-11, $p < 0.05$; Epo, $p < 0.10$) as determined by a two-tailed t-test are marked by asterisks. Bars represent the mean \pm SEM for data pooled from 2 to 7 experiments.

C. Ability of Other Factors to Influence LTC-IC Amplification.

A final set of experiments was undertaken to determine whether any of a number of other cytokines previously shown to have direct effects on primitive hematopoietic cells could further enhance LTC-IC yields from CD34⁺CD38⁻ cells cultured under serum-free conditions in the absence of stroma. In addition, the effects on LTC-IC amplification of hydrocortisone (HC) and HLTM (human long-term medium containing both horse and fetal calf serum) were examined. Each condition was tested either alone or in combination with the original 6-factor cocktail analyzed above. The results of these experiments are shown in Figure 5.4. Of the 10 additional cytokines tested, only TPO and IL-1 on their own (at 40 U/mL and 1700 U/mL, respectively) were able to significantly increase ($p < 0.05$) the number of LTC-IC detectable after 10 days by comparison to the numbers measured in the absence of any factor supplement. Moreover, aside from FL (Figure 5.1), TPO was also the only factor that, on its own, may have been able to stimulate LTC-IC numbers to increase above the input value ($p < 0.18$, see also Table 5.1). In addition, none of these 10 cytokines (including TPO) when added as a supplement (at the same concentration) to the original 6-factor combination was able to further enhance the LTC-IC expansion already stimulated (Figure 5.4). Many, (e.g. IL-12 at 50 ng/mL, IL-7 at 100 ng/mL, MIP-1 α at 100 ng/mL, HC at 10⁻⁶ M and TPO at 40 U/mL) in fact, appeared to be neutral in terms of having no modifying effect on LTC-IC output (like IL-6, G-CSF and NGF- β). Others clearly had a significant negative effect (e.g. TNF- α at 500 U/mL, $p < 0.005$; IL-11 at 100 ng/mL, $p < 0.05$; LIF at 100 ng/mL, $p < 0.05$ and Epo at 3 U/mL, $p < 0.10$). TNF- α in combination with the 6 factor cocktail was particularly potent in this regard resulting in a decrease in LTC-IC numbers after 10 days to

below both the input value and the number of LTC-IC detectable in cultures to which TNF- α alone was added. The dramatic decline in LTC-IC numbers that occurred when TNF- α was present together with the 6-factor cocktail was not, however, associated with a general loss of CD34⁺CD38⁻ cell viability. In fact, these conditions stimulated a large amplification (~350-fold) in the total number of cells present after 10 days which was also accompanied by the generation of a large population of progenitors of small macrophage-like colonies.

On evaluating the LTC-IC produced in cultures that contained TPO alone or TPO plus the 6-factor cocktail, a change in the type of CFC present in the 6 week-old LTC-IC assays became apparent. In both of these instances, a significantly higher proportion of the progeny CFC were erythroid progenitors ($p < 0.05$) by comparison to those generated either from the initial (CD34⁺CD38⁻) LTC-IC, or from the LTC-IC produced in parallel cultures containing the 6-factor cocktail but no TPO (Figure 5.5). It is particularly interesting to note that although this effect of TPO was most pronounced for the LTC-IC produced in the presence of TPO alone, a significant shift in favor of erythropoietic progenitors was also evident in the assays of LTC-IC obtained from cultures where TPO was added to the 6-factor cocktail in which case there was no apparent effect of the TPO on LTC-IC amplification. To examine whether an alteration of LTC-IC differentiation behavior had occurred in cultures containing any of the other cytokines tested, the relative numbers of CFU-GM, BFU-E and CFU-GEMM from all LTC-IC assays were compared. As is illustrated by the examples shown in Table 5.1, these values were not markedly altered by any other factor or factor combination tested.

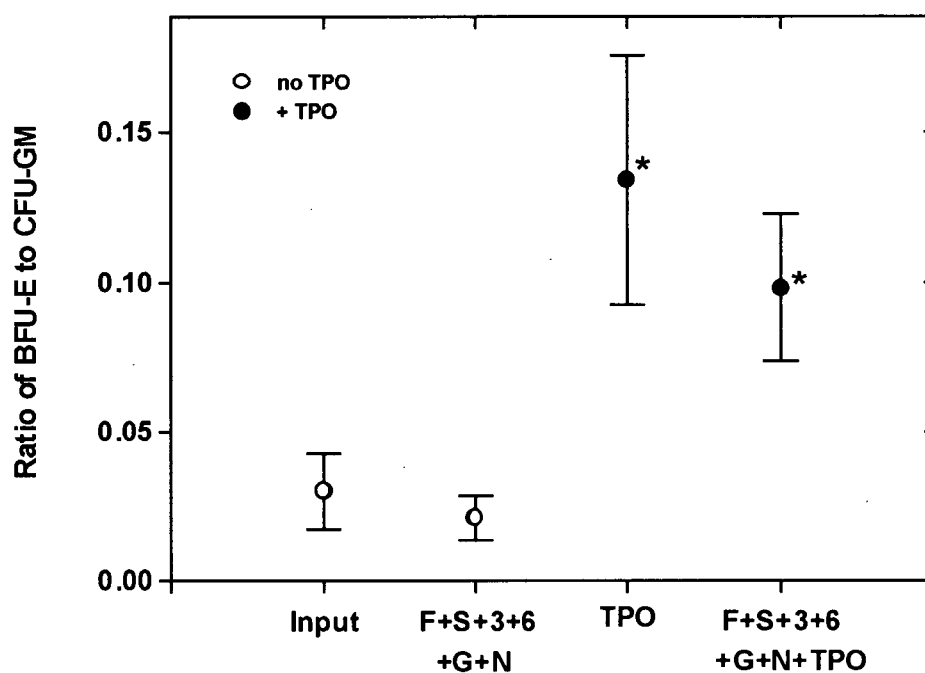


Figure 5.5: Effect of TPO alone or together with a 6 growth factor cocktail on the relative proportions (ratio) of primitive erythroid (BFU-E) and granulopoietic progenitors (CFU-GM) produced after 6 weeks from the LTC-IC generated in 10-day cultures containing these factors. Abbreviations are as indicated in the caption of Figure 5.1. Values shown are the mean \pm SEM of ratios calculated from 5 separate experiments. Asterisks indicate significant differences ($p < 0.05$) relative to the ratios measured for both the starting LTC-IC and the LTC-IC present after 10 days in cultures containing the 6-factor cocktail.

Table 5.1: Effects of selected culture conditions on the LTC-IC and CFC detected in 10 day-old serum-free cultures initiated with 200 CD34⁺CD38⁻ cells.

Cytokine(s)	LTC-IC Expansion*	No. of Experiments	LTC-IC-derived CFC GM/B/GEMM [†]	CFC Expansion*	No. of Experiments
TPO	1.3 ± 0.4	5	86:12:2	ND [‡]	
FL	4.3 ± 1.4	3	100:0:0	0.7 ± 0.1	4
SF	0.3 ± 0.1	2	100:0:0	0.8 ± 0.3	4
IL-3	0.2 ± 0.1	2	99:1:0	9.6 ± 2.5	4
IL-6	0.01 ± 0.01	2	98:2:0	0.5 ± 0.3	4
G-CSF/NGF-β	0.07 ± 0.07	2	100:0:0	0.05 ± 0.05 ^a	3
None	0.07 ± 0.01	6	100:0:0	ND	
FL/SF/IL-3/IL-6/G-CSF/NGF-β	24 ± 5	7	97:2:1	670 ± 300	4
FL/SF/IL-3	49 ± 20	2	97:3:0	76 ± 25	3
Input CD34 ⁺ CD38 ⁻ cells ^b		7	95:3:2		

^a CFC expansion determined for G-CSF alone; i.e. without the addition of NGF-β.

^b 168 ± 48 CFC were measured in the 6 week harvest of the LTC-IC assays from 100 input CD34⁺CD38⁻ cells. The same input cells contained 7 ± 3 CFC per 100 cells.

ND = not determined

DISCUSSION

This chapter presents a comprehensive analysis of the effects of various cytokines on the generation within a 10-day period of two functionally distinguished but closely related populations of primitive human hematopoietic cells. To minimize potentially confounding effects of serum or accessory cells (or their products), the cultures were initiated at a low cell density with a highly purified population of $CD34^+CD38^-$ cells isolated from normal marrow and incubated in a completely defined medium. The results (summarized in Table 5.1) show that the number of cells detectable as LTC-IC could be increased in 10-day cultures that contained only FL or TPO, indicating unique roles for these factors either in triggering the activation of properties required for cells to be detected as LTC-IC (within 6 weeks) or for their self-renewal, as has been confirmed for FL by LTC-IC 3H -thymidine suicide experiments (Ponchio et al., 1994). The action of TPO on LTC-IC amplification has not been reported previously. It has been reported that FL in combination with SF, Pixy-123, IL-3, GM-CSF and Epo can enhance the plating efficiency of high-proliferative potential CFC (Broxmeyer et al., 1995; Muench et al., 1995; McKenna et al., 1995) and a possible role of FL in LTC-IC stimulation by marrow feeder layers has been inferred from the results of anti-sense FL experiments (Small et al., 1994) and experiments in which FL has been added repeatedly to LTC-IC assay cultures (Gabbianelli et al., 1995). More recently we reported that FL in combination with SF, IL-3, G-CSF and NGF- β could amplify LTC-IC in cultures of single $CD34^+CD38^-$ cells although the specific role of FL in eliciting this response was not examined in those studies (Petzer et al., 1996a).

The results of the multifactorial analysis studies presented here show that within 10 days, FL alone can stimulate a significant net increase in the number of LTC-IC present in cultures initiated with CD34⁺CD38⁻ cells and that the addition of SF and IL-3 to FL further enhances this increase. The multifactorial analysis also revealed no other significant interactions between any of the 6 factors tested in terms of their effects on LTC-IC amplification. In contrast, IL-3 was the only factor that alone was able to expand the CFC population. Although, the combination of FL, SF and IL-3 significantly stimulated the production of CFC, this could be further enhanced by the addition of factors like G-CSF or IL-6 that had no effect on LTC-IC amplification. These latter findings are consistent with those reported for the effects of FL on primitive murine lymphomyeloid, myeloid and lymphoid CFC (Hirayama et al., 1995). Taken together, these results extend previous evidence of differences in the mechanisms that allow LTC-IC function to be maintained (with or without cell division) as compared to those that may stimulate loss of this function by the acquisition of properties that allow direct colony formation in semi-solid medium (Sutherland et al., 1993b).

Of the additional factors tested in combination with FL, SF, IL-3, IL-6, G-CSF and NGF- β , the negative effect of TNF- α on LTC-IC numbers was the most striking, particularly in view of the rapid cell proliferation and concomitant acquisition of restricted macrophage differentiation potential observed. This latter response could reflect the selective stimulation of a previously undefined unique cell type also present in the CD34⁺CD38⁻ population as has been suggested for less purified populations responding to SF, GM-CSF and TNF- α (Young et al., 1995). Alternatively, TNF- α may exert a direct deterministic action on CD34⁺CD38⁻

LTC-IC (or CFC) when these are simultaneously stimulated by certain other growth factors. Single cell experiments will be required to resolve between these possibilities. Additionally, the observation that LTC-IC generated in the presence of TPO subsequently showed a uniquely increased production of erythropoietic progenitors was also unanticipated. Occasional examples of apparently deterministic effects on lineage restriction or switching have been previously reported in various immortalized hematopoietic cell lines (Klinken et al., 1988; Borzillo et al., 1990; Elefanty et al., 1992); however, the ability of such lines to yield further information about mechanisms of normal hematopoietic differentiation processes are questionable because of their transformed state. The apparent ability of TPO to re-program the differentiation behavior of primary LTC-IC as suggested by the present findings would circumvent this type of criticism. This experimental model may therefore offer new opportunities for future studies of how commitment events are normally regulated.

Characterization of culture conditions that allow human hematopoietic stem cell expansion is an important requirement for the successful implementation of many clinical transplantation and gene therapy protocols currently under development. The present studies may represent a significant step towards the practical realization of such approaches by beginning to define the growth factor conditions that most effectively stimulate LTC-IC and CFC amplification. This information is also likely to be crucial for the design of larger scale systems for culturing human hematopoietic cells. Finally, these findings provide new distinctions between the biological effects of specific growth factors on a phenotypically defined ($CD34^{+}CD38^{-}$) human hematopoietic cell target population. Further analysis of the cellular and molecular basis of these different responses should provide additional clues about

early events in hematopoietic cell differentiation and how they are activated by interactions with the extracellular milieu.

CHAPTER 6: CYTOKINE MANIPULATION OF PRIMITIVE HUMAN HEMATOPOIETIC CELL SELF-RENEWAL[§]

[§] The contents of this chapter are essentially as published in Zandstra et al., 1997b. E. Conneally made a significant contribution in both the isolation and the flow cytometric analysis of some of the purified cell populations. A. Petzer also made significant contribution in the development of the purified cell expansion protocol and in the isolation of some of the purified cells.

1. Introduction

The recent development of methods for the purification of very primitive human hematopoietic cell populations to near functional homogeneity (Lansdorp and Dragowska, 1992; Sauvageau et al., 1994; Petzer et al., 1996a) has facilitated the acquisition of information about their responses to a wide variety of manipulations. For example, Chapter 5 showed that the addition of a fourth cytokine (i.e. either IL-6 or G-CSF or NGF- β) to a combination of FL, SF and IL-3 enhances the production of CFC (to values >500-fold above input numbers) in short-term (10 day) cultures of CD34⁺CD38⁻ human marrow cells but does not further amplify the concomitant production of LTC-IC. Previous experiments suggested that the production of cells with these functional properties might be differentially dependent on the concentration as well as the types of cytokines to which they were exposed (Chapter 4). In addition, it had been shown that LTC-IC and CFC in freshly isolated CD34⁺CD38⁻ human marrow populations differ from CFC in their ability to proliferate in methylcellulose-containing medium (LTC-IC being completely inhibited), even when stimulated by the same factors that stimulate LTC-IC expansion in liquid media (Petzer et al., 1996a). The present studies were therefore undertaken to examine independently the cytokine concentration-dependence of four distinct responses of human marrow CD34⁺CD38⁻ cells, i.e. LTC-IC amplification, CFC production, and cloning efficiency in liquid or semi-solid medium. The results obtained support a role for cytokines in influencing LTC-IC self-renewal decisions.

2. Results

A. Differential Cytokine Dose Dependence of LTC-IC and CFC Expansion

In a first series of experiments, aliquots of CD34⁺CD38⁻ marrow cells were incubated in serum-free medium supplemented with various concentrations of FL, SF, IL-3, IL-6 and G-CSF (5:5:1:1:1 (wt/wt)). After 10 days of culture, the numbers of LTC-IC, CFC and total nucleated cells were determined and compared with corresponding input values for that experiment. The results pooled from seven such experiments are shown in Figure 6.1. Maximum numbers of LTC-IC were produced in the cultures that contained 300 ng/mL of FL and SF, plus 60 ng/mL of IL-3, IL-6 and G-CSF. Under these conditions, LTC-IC expansions of 62 ± 28 -fold were obtained within 10 days. In contrast, near maximally increased CFC numbers (235-fold) were seen in cultures that contained 30-fold lower concentrations of the same cytokine cocktail. Interestingly, the total number of cells produced increased progressively as the concentration of the cytokine cocktail added was increased with the greatest expansion (78 ± 9 -fold over input) being obtained in the cultures containing the highest concentration of cytokines tested (1 μ g/mL of FL and SF, plus 200 ng/mL of IL-3, IL-6 and G-CSF) (Figure 6.2). Although ~15% of the initial CD34⁺CD38⁻ cells were either CFC or LTC-IC, after 10 days in 10-100 ng/mL of FL and SF plus 2-20 ng/mL of IL-3, IL-6 and G-CSF, all of the cells present were detectable as CFC. For example, in one experiment 100 input CD34⁺CD38⁻ cells generated ~4500 cells, 4400 ± 300 CFC and 200 ± 30 LTC-IC after 10 days in 100 ng/mL of FL and SF and 20 ng/mL of IL-3, IL-6 and G-CSF. Thus, some of the cultured cells could have had the capacity to be detected both as CFC and LTC-IC.

The magnitude of the LTC-IC expansions calculated in cultures supplemented with FL and SF at 300 ng/mL plus IL-3, IL-6 and G-CSF at 30 ng/mL (Figure 6.1) were based on measurements of CFC output values from "bulk" LTC-IC assays. To determine whether the LTC-IC generated in these cultures had the same proliferative potential as the input LTC-IC, their average CFC outputs (at 6 weeks) were compared. These were obtained from LTC-IC assays in which the test cells were seeded at concentrations that produced $<22 \pm 6\%$ positive cultures; i.e. $<4 \pm 2\%$ of these would have been seeded with >1 LTC-IC, $p = 0.05$). The resultant average CFC per LTC-IC values proved to be the same: 19 ± 27 CFC per LTC-IC (mean \pm SD; range, of 1-94) for the cultured LTC-IC vs 14 ± 24 (range, 1-108) for the LTC-IC in the starting population of $CD34^+CD38^-$ marrow cells (Figure 6.3). These values are also consistent with those previously reported for the LTC-IC population in unseparated normal marrow (Hogge et al., 1996).

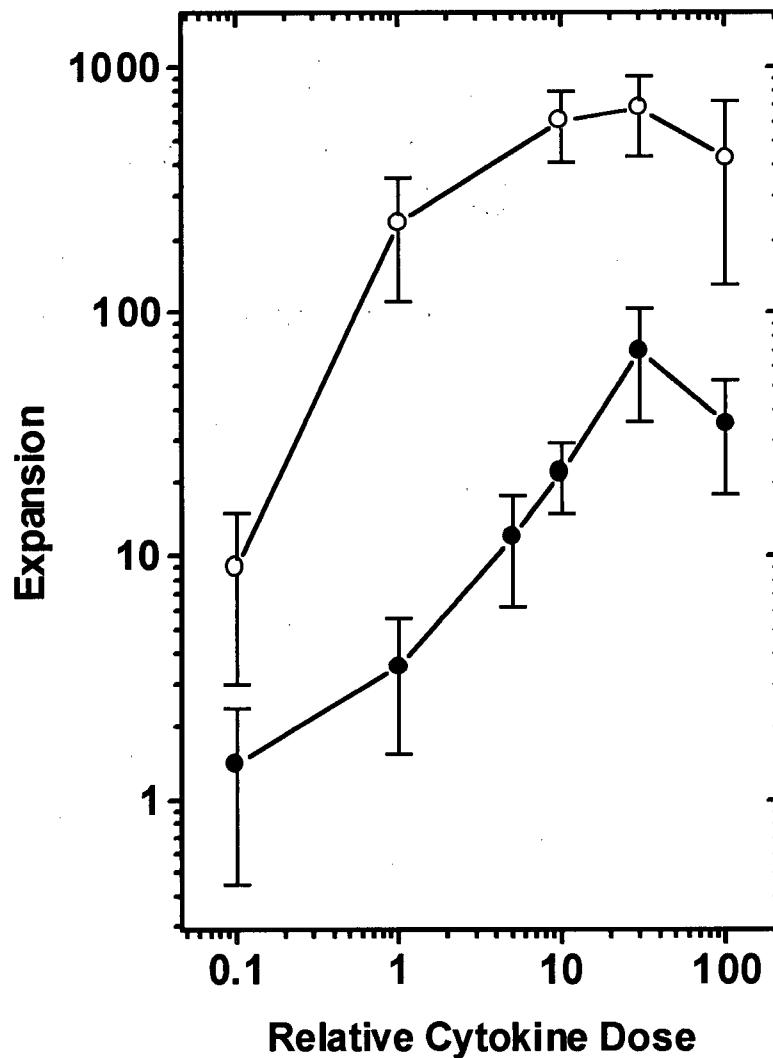


Figure 6.1: Expansion of LTC-IC (●) and CFC (○) numbers (relative to input) in 10-day 100 μ L serum-free cultures initiated with 200 CD34⁺CD38⁻ cells. Input LTC-IC and CFC numbers were 8.2 ± 3.3 and 7.3 ± 2.6 per 100 CD34⁺CD38⁻ cells, respectively. A relative cytokine dose of 1 represents 10 ng/mL SF and FL and 2 ng/mL of IL-3, IL-6 and G-CSF. Points represent the mean \pm SEM of data from 3 to 7 independent experiments which were performed with 3 to 5 different bone marrow samples.

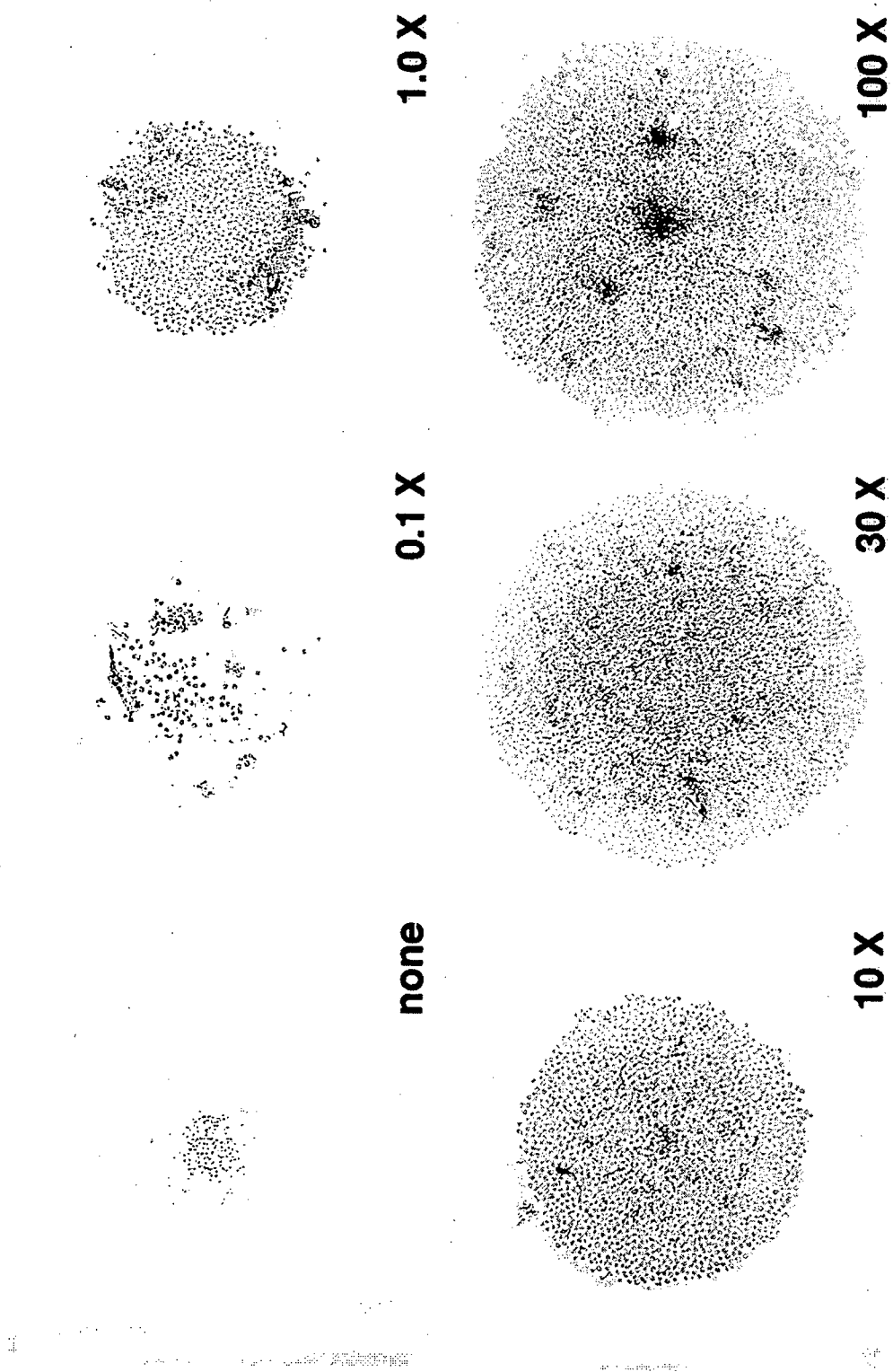


Figure 6.2: Photomicrograph of 10-day 100 μ L serum-free cultures initiated with 200 CD34⁺CD38⁻ cells. A relative cytokine dose of 1 represents SF and FL at 10 ng/mL and IL-3, IL-6 and G-CSF at 2 ng/mL.

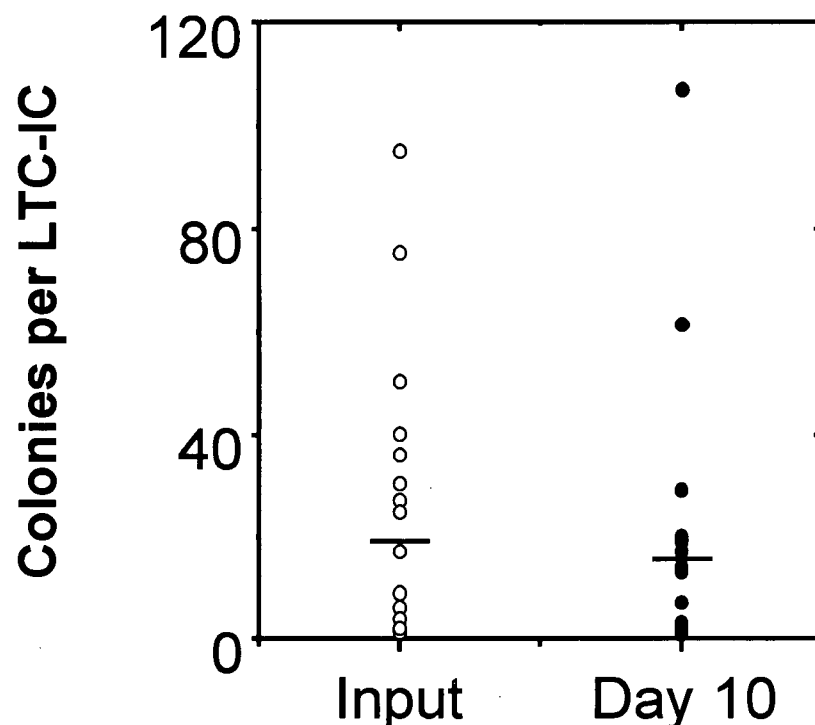


Figure 6.3: Limiting dilution analysis of CD34⁺CD38⁻ bone marrow cells prior to culture (○) or after 10 days in 300 ng/mL SF and FL plus 60 ng/mL IL-3, IL-6 and G-CSF (●). Each point represents the number of LTC-IC derived colonies from a single LTC-IC (see text).

As noted previously (Chapter 5, Sutherland et al., 1993b), a readily detectable proportion of the input LTC-IC population ($11 \pm 4\%$) was able to survive for a period of 10 days in the absence of any added cytokines and, even quite low levels of cytokines (1 ng/mL of FL and SF plus 0.2 ng/mL of IL-3, IL-6 and G-CSF) were sufficient to maintain LTC-IC numbers at input levels for 10 days (1.5 ± 1.2 -fold expansion over input). When the concentration of the 5-cytokine cocktail used in these experiments was increased above 300 ng/mL FL and SF, plus 60 ng/mL IL-3, IL-6 and G-CSF, the yield of LTC-IC and CFC both decreased slightly, in spite of the fact that there was a further increase in the total number of cells produced (Figure 6.2).

The marked difference in the cytokine dose-dependence of LTC-IC and CFC production revealed in these experiments could be due to differences in the viability or mitogenic responsiveness of different subsets of CD34⁺CD38⁻ cells or, alternatively, to a difference in the cytokine-stimulation requirement for the same cells to maintain or acquire the functions necessary for detection as LTC-IC or CFC. To discriminate between these alternatives, analyses of single cell cultures were undertaken. In these, the viability (total number of clones obtained) and proliferative activity (clone size distribution) of single PI⁻ AnnexinV⁻ CD34⁺CD38⁻ cells cultured in the presence of either (i) FL and SF at 300 ng/mL plus IL-3, IL-6 and G-CSF at 60 ng/mL or (ii) a 10-fold lower concentration of the same cytokine combination were compared. The results obtained in two experiments were similar. As can be seen in the example shown in Figure 6.4A, the yield of clones was similar for the two culture conditions compared ($p=0.4$), in spite of the fact that the production of LTC-IC was selectively reduced (~50-fold) with only a minimal decrease in the CFC population (~25%) in the cultures maintained at the lower cytokine concentration. (In these experiments the LTC-IC expansions were 57 ± 18 vs 1.2 ± 0.5 -fold, $p<0.1$, in the high vs the low cytokine concentration mixtures, respectively, with corresponding CFC expansions of 570 ± 50 - and 430 ± 90 -fold, $p=0.5$).

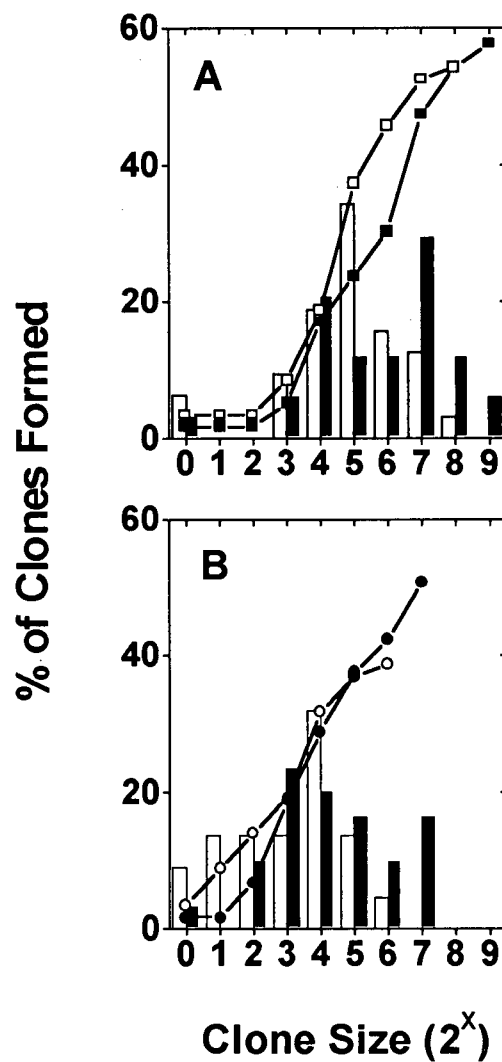


Figure 6.4: Size distribution (bars) and cumulative frequencies (symbols) of clones generated from a representative experiment in which single PI⁺ AnnexinV⁻ CD34⁺CD38⁻ cells were cultured for 10 days in serum-free medium supplemented with (i) FL and SF at 300 ng/mL plus IL-3, IL-6 and G-CSF at 60 ng/mL (solid bars, ■, n=59 wells) or (ii) FL and SF at 30 ng/mL plus IL-3, IL-6 and G-CSF at 6 ng/mL (open bars and □, n=59 wells)(A); or (iii) FL and SF at 300 ng/mL plus IL-3 at 60 ng/mL (solid bars, ●, n=59 wells), or (iv) FL and SF at 10 ng/mL plus IL-3 at 60 ng/mL (open bars, ○, n=57 wells)(B). At the end of the 10 days, all cells from all clones generated under a given set of conditions were pooled and assayed for their content of CFC and LTC-IC. The results of these assays confirmed the results shown in Figure 6.1 (for the comparison undertaken in A) and in Table 6.1 (for the comparison undertaken in B).

B. Cytokine Concentration-Dependence of Colony Formation by Different Subpopulations of CFC in Methylcellulose Cultures

Given the difference seen in the cytokine concentration requirements for LTC-IC and CFC expansion by $CD34^+CD38^-$ marrow cells in liquid suspension cultures (Figure 6.1), as well as previous evidence that the freshly isolated LTC-IC are not detectable as CFC in methylcellulose (Petzer et al., 1996a), it was of interest to determine the cytokine concentration-dependence of colony formation (in methylcellulose) by $CD34^+CD38^-$ marrow cells. However, because the majority of CFC in the marrow are not found within the $CD34^+CD38^-$ fraction, these studies were extended to include an examination of the CFC in other fractions. Therefore, $CD34^+CD45RA^+CD71^-$ cells (in which the CFC are predominantly granulopoietic) and the subset of $CD34^+CD45RA^-CD71^+$ cells (in which most of the CFC are erythroid) (Lansdorp and Dragowska, 1992) were also isolated and cultured in methylcellulose cultures containing the same range of concentrations of the 5-factor cocktail used in the experiments described in Figure 6.1. From all three cell fractions, maximum or close to maximum numbers of most types of colonies were generated in all cultures independent of the concentration of cytokines added over a 300-fold range starting at 1 ng/mL of FL and SF plus 0.2 ng/mL of IL-3, IL-6 and G-CSF (Figure 6.5). The only major exceptions to this were the granulopoietic CFC and the multilineage CFC present in the $CD34^+CD45RA^-CD71^+$ fraction which required higher cytokine concentrations to be optimally stimulated. Peak CFC plating efficiencies (measured in cultures containing 100 ng/mL FL and SF, plus 20 ng/mL IL-3, IL-6 and G-CSF) were 7 ± 4 , 14 ± 5 and $22 \pm 6\%$ for the $CD38^-$, $CD45RA^+CD71^-$, and $CD45RA^-CD71^+$ subpopulations of $CD34^+$

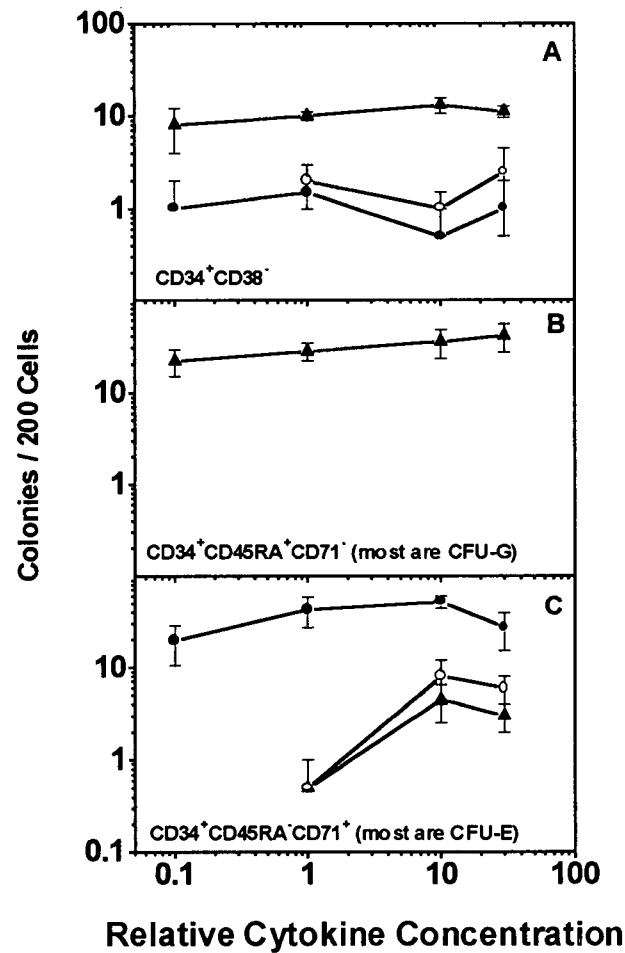


Figure 6.3: Number of colonies generated from 200 CD34⁺CD38⁻ (A), CD34⁺CD45RA⁺CD71⁻ (B), or CD34⁺CD45RA⁻CD71⁺ cells (C) plated directly in methylcellulose containing different concentrations of the same cocktail of 5 cytokines used in the experiments shown in Figure 6.1. Points for erythroid (●, from CFU-E + BFU-E), mixed (○, from CFU-GEMM) and myeloid (▲, from CFU-GM) colonies represent the mean \pm SEM of values obtained from 4 independent experiments.

cells, respectively. In the assays of the latter two subpopulations, colony formation by the more mature types of CFC (identified by their reduced proliferative potential) was also favored at the lower cytokine concentrations (data not shown).

C. Analysis of the Effect of Independently Varying the Concentrations of FL, SF and IL-3 on LTC-IC and CFC Expansion

The potential effects of independently altering the concentrations of the individual cytokines (FL, SF and IL-3) that were previously shown (Chapter 5) to stimulate LTC-IC expansion in 10-day liquid suspension cultures of CD34⁺CD38⁻ cells were investigated next. For this, a 2³ factorial design analysis was used. The combined results of the three experiments performed are shown in Table 6.1. As anticipated from the dose response studies shown in Figure 6.1, the greatest expansion of LTC-IC occurred when all three cytokines were present at the higher of the two concentrations tested (FL and SF at 300 ng/mL plus IL-3 at 60 ng/mL). Analysis of the normalized effect of each combination of different cytokine concentrations tested showed that for the three cytokines considered (FL, SF and IL-3), LTC-IC expansion was primarily and significantly ($p < 0.05$) dependent on the presence of a high concentration of FL. Thus high numbers of LTC-IC could be obtained when the concentration(s) of SF, IL-3 or both were reduced, as long as the concentration of FL was high. Conversely, when the concentration of FL was low, elevating the concentration of SF only, or even of both SF and IL-3, did not significantly enhance LTC-IC expansion over that observed when all three factors were present at a low level. Interestingly, when only the concentration of IL-3 was elevated, the yield of LTC-IC was reduced even further ($p < 0.1$, compared directly by the one-tailed student t-test ($n=3$) to that observed when all three cytokines were present at the lower levels). This markedly negative effect of a relatively high concentration of IL-3 was, however, not observed when a high concentration of either FL or SF was present. Of note, CFC expansion in the same cultures (Table 6.1) was not significantly affected by the presence of an elevated concentration of FL ($p=0.4$), although an elevated

Table 6.1: Differential effects of altering the relative concentrations of FL, SF and IL-3 on LTC-IC and CFC expansion.

Cytokine concentration (ng/mL)			Progenitor expansion ^a		Analysis of effect on LTC-IC expansion	
FL	SF	IL-3	LTC-IC	CFC	Normalized effect ^b	Significance of normalized effect ^c
300	300	60	45 ± 15	76 ± 19	-0.29	n.s.
300	300	2	33 ± 12	31 ± 5	0.85	n.s.
300	10	60	22 ± 6	76 ± 28	-0.26	n.s.
300	10	2	18 ± 9	41 ± 30	1.2	p<0.05
10	300	60	4 ± 2	62 ± 33	0.73	n.s.
10	300	2	4 ± 3	39 ± 11	0.49	n.s.
10	10	2	4 ± 1	28 ± 15	n/a	n/a
10	10	60	0.3 ± 0.1	68 ± 37	-0.83	p<0.10

^a Values shown are the mean ± SEM of data from 3 experiments.

^b Effects of individual cytokines (main effects) within the mixture were calculated from the sum of differences between the normalized LTC-IC expansion values obtained with that cytokine at the higher vs the lower concentration tested [the levels of the other cytokines being constant, (Box et al., 1978)]. Effects of elevated levels of multiple cytokines (interaction effects) were determined by analysis of variance (Box et al., 1978). All effects were determined relative to the cultures where all three cytokines were present at the lower level such that a change in cytokine concentration with no overall effect would have a value of 0. The SEM of the normalized effect on LTC-IC expansion was 0.24.

^c Significance was determined from a comparison of the normalized effect and the SEM of the normalized effect on LTC-IC expansion. n.s., not significant (i.e. $p > 0.1$); n/a, not applicable.

level of IL-3 did consistently allow significantly more ($p<0.01$) CFC to be produced, in keeping with the possibility that exposure of LTC-IC to high concentrations of IL-3 may, under certain circumstances, promote their differentiation to CFC.

To determine whether the negative effect of high concentrations of IL-3 on the production of LTC-IC in 10-day suspension cultures of $CD34^+CD38^-$ cells was associated with a failure of a subset of these to remain viable or to divide, additional experiments with single cell cultures were performed. In these, individual PI⁻ AnnexinV⁻ $CD34^+CD38^-$ cells were cultured in either the maximal (for LTC-IC expansion; i.e. FL and SF at 300 ng/mL plus IL-3 at 60 ng/mL), or the suppressive (FL and SF at 10 ng/mL plus IL-3 at 60 ng/mL) media. At the end of this time, the number of clones and their sizes were determined by visual inspection. The results of two such experiments (one of which is shown in Figure 6.4B), indicated that the mixture favoring LTC-IC expansion stimulated the formation of slightly more ($p<0.10$) and larger ($p<0.01$) clones than the mixture that suppressed LTC-IC. This suggests that an excess of IL-3 can be directly toxic for some of the $CD34^+CD38^-$ cells that have the potential to generate LTC-IC progeny. Measurements of the number of LTC-IC and CFC produced in parallel bulk cultures showed the same effects as those previously obtained in the factorial design experiments (i.e. LTC-IC expansions of 10.5 ± 0.6 - vs 1.1 ± 0.4 -fold, $p<0.05$, and CFC expansions of 420 ± 210 - vs 230 ± 100 -fold, $p=0.12$, in the high IL-3, SF and FL vs the IL-3 high, plus low SF and FL media, respectively). The proportion of $CD34^+CD38^-$ cells that formed clones was also significantly higher ($p<0.05$) in cultures containing FL and SF at 300 ng/mL plus IL-3 at 60 ng/mL when G-CSF and IL-6 at 60 ng/mL were also present. In addition, for each pair of conditions assessed, the average size of the clones was always higher when the cytokine concentration was higher ($p<0.01$).

D. Characterization of the Phenotypes of the Cytokine-Expanded Populations

Figure 6.6 shows the surface antigen profiles of the cells in 10-day serum-free cultures in the presence of either (i) FL and SF at 100 ng/mL plus IL-3, IL-6 and G-CSF at 20 ng/mL or (ii) FL and SF at 300 ng/mL plus IL-3 at 60 ng/mL. The first of these media was chosen because it had been shown to stimulate maximal expansion of CFC and near maximal expansion of LTC-IC (Chapter 5, Figure 5.1). The second was chosen because it was found to support maximal expansion of LTC-IC and significant expansion of CFC (Table 6.1). Cultures were initiated with CD34⁺CD38⁻CD45RA⁻CD71⁻CD33⁻ cells and the cells present after 10 days were analyzed for their surface expression of CD34, CD38, CD33, CD45RA and CD71. The total cell number had increased 74 ± 19 -fold in the culture containing the five-cytokine combination and 42 ± 11 -fold in the cultures containing the three-cytokine media (n=3). Of these, $85 \pm 16\%$ and $62 \pm 15\%$, respectively, were CD34⁺. Within the CD34⁺ cell populations, $45 \pm 23\%$ (five cytokines) and $88 \pm 9\%$ (three cytokines) of the cells were still CD38⁻. Thus, the total number of CD34⁺CD38⁻ cells present in the five- and three-factor supplemented cultures must have increased by 21- and 32-fold, respectively. Interestingly, very few of these expanded CD34⁺CD38⁻ cells (<1% for the five-factor combination and 6% for the three-factor combination) were still CD33⁻, CD71⁻ or CD45RA⁻. In fact, most of the CD34⁺ cells present after 10 days were positive for all of these antigens.

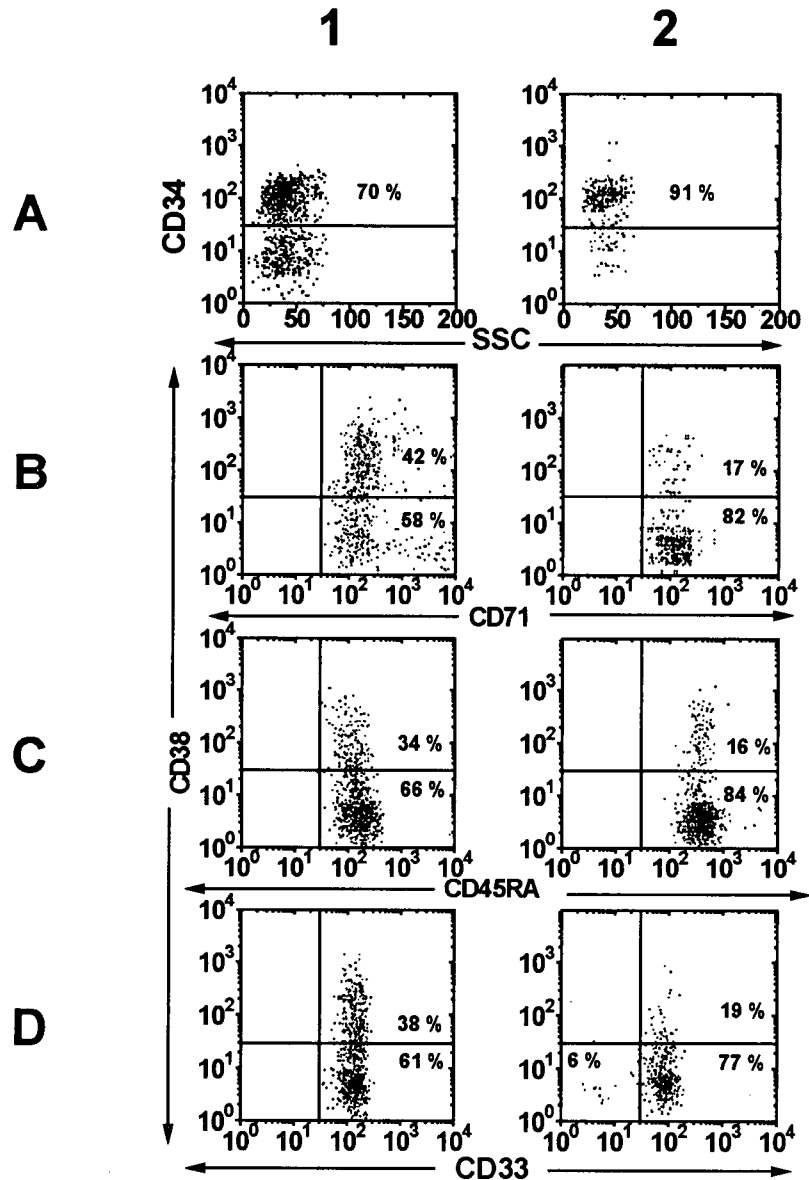


Figure 6.4: Representative flow cytometric analyses of CD34 expression vs side scatter (SSC) (row A), and within the CD34⁺ cell population, expression of CD38 and CD71 (row B), CD45RA (row C), or CD33 (row D) on subpopulations of viable (PI⁻) cells generated from 200 input CD34⁺CD38⁻Lin⁻ cells after 10 days in serum-free cultures supplemented with either 100 ng/mL FL and SF plus 20 ng/mL of IL-3, IL-6 and G-CSF (column 1) or 300 ng/mL FL and SF plus 60 ng/mL IL-3 (column 2). The lower left-hand quadrant in each plot has boundaries set by 99.9% of the unstained and isotype-labeled antibody controls.

3. Discussion

Four important and novel findings are reported in this chapter. The first is to documents a higher rate of LTC-IC amplification (≥ 50 -fold in 10 days) in cultures of CD34⁺CD38⁻ human marrow cells than has been previously described. Moreover, the LTC-IC thus generated display the same average, albeit highly variable, proliferative behavior as the LTC-IC in the original CD34⁺CD38⁻ marrow population. Interestingly, the *in vitro* amplified LTC-IC expressed phenotypes that are not expressed by LTC-IC in normal marrow (Lansdorp et al., 1990; Terstappen et al., 1991; Sauvageau et al., 1994). In addition, some of the amplified LTC-IC may have acquired an ability to proliferate in semi-solid medium (since under certain conditions, a majority of the cultured cells could also be detected as CFC), a situation that also contrasts with the LTC-IC present in the original marrow cell suspensions (Petzer et al., 1996a). These findings indicate that the combination of attributes associated with very primitive cells generated during normal hematopoiesis *in vivo* are not necessarily coordinately regulated, as noted for primitive murine hematopoietic cells expanding either *in vitro* (Rebel et al., 1994) or *in vivo* (Spangrude et al., 1995). The possible biological consequences of a primitive hematopoietic cell undergoing some phenotypic changes normally associated with its differentiation while, at the same time, retaining certain functional properties indicative of its original state await further investigation.

The second major finding is the demonstrated importance of the *relative* concentration of the cytokines in a given cocktail to support LTC-IC amplification in serum-free cultures of CD34⁺CD38⁻ marrow cells. In particular, these studies show that this response is most sensitive to the concentration of FL present in a combination of cytokines that consists of FL,

SF and IL-3. This is reminiscent of the findings presented in Chapter 5 where, of a large number of cytokines tested, FL is on its own the most potent stimulator of LTC-IC expansion.

The third observation of note is that high levels of IL-3 can have a direct and negative impact on LTC-IC expansion when both FL and SF levels are relatively low, and that this negative effect can be overcome if either the FL or SF concentration is sufficiently increased. An ability of IL-3 to block the cytokine-stimulated generation of lymphoid precursors from primitive murine hematopoietic cells with lympho-myeloid potential and to impair the self-renewal of the latter *in vitro* has recently been reported by Ogawa and co-workers (Hirayama et al., 1994; Yonemura et al., 1996). The present demonstration of a similar action of IL-3 on human LTC-IC amplification suggests the likelihood of a parallelism in signal transducing events in analogous types of IL-3-stimulated populations of primitive murine and human hematopoietic cells. The present observations also underscore the possibility of detrimental consequences of including IL-3 in cytokine cocktails used for the activation or expansion of stem cell transplants in clinical protocols. On the other hand, although factors that could override the negative effects of IL-3 on murine cells have been identified (Neben et al., 1996), these did not include either FL or SF. In contrast, we found both of these factors to have this salvaging activity on primitive human cells (LTC-IC). This latter discrepancy, together with our finding that human CFC expansion is enhanced in cytokine combinations containing a high concentration of IL-3 (some of which can reduce or suppress human LTC-IC expansion), suggests the possibility of some interesting differences between the two species.

The fourth point of interest is the evidence of a unique cytokine concentration-dependence of LTC-IC amplification from CD34⁺CD38⁻ cells. This dose-effect was found to

be independent of the ability of these cells to remain viable or to concomitantly generate CFC, as none of these latter responses were significantly affected when the cytokine concentration was decreased to the point that the LTC-IC expansion was reduced 25- to 50-fold. These findings argue that the retention or loss of LTC-IC potential by the progeny of freshly isolated $CD34^+CD38^-$ marrow cells may be influenced by the concentration of cytokines to which the cells are exposed. Thus, the different cytokine dose-effect relationships characterizing LTC-IC and CFC expansion (Figure 6.1) appear to reflect the differential cytokine concentration requirements for these two outcomes from a common precursor population rather than the requirements of two distinct types of $CD34^+CD38^-$ cells characterized by different cytokine sensitivities [as has been shown for other classes of marrow progenitors (Eaves and Eaves, 1985)]. Another example of cells showing a differential response to stimulation by different concentrations of cytokines was reported by Dexter et al. (1990). They showed that IL-3-dependent murine FDCP-mix cells can proliferate in the presence of lower concentrations of IL-3 than are necessary to support the concomitant maintenance of their pluripotent state. These findings, like those of Dexter et al. (1990) are thus supportive of a model of cytokine-induced signaling in which the biological response may be modified not only by the type(s) of factors to which the cell is exposed, but also by the duration of such interactions and the number of receptors bound per unit time (Marshall, 1995). The present studies provide the first evidence that this model is relevant to the control of primary human hematopoietic stem cells and focuses interest on the intracellular signaling pathways that must be able to direct (or block) primitive hematopoietic cell differentiation when these cells are exposed to cytokine cocktails that elicit different biological outcomes.

The extent of human LTC-IC expansion now shown to be possible also has important practical implications in terms of clinical stem cell transplants. The ability to achieve ≥ 50 -fold expansions of LTC-IC and, at the same time, >500 -fold amplifications of CFC, reinforces the future potential of *in vitro* technology to manipulate these cells for a variety of clinical applications. It is now clear that an important consideration in the design of any *in vitro* procedure or bioreactor system will be the provision of a mechanism that can maintain high extracellular levels of stem cell-stimulatory cytokines and also remove factors that may be selectively inhibitory. Interestingly, these results complement those presented in Chapter 4 where it was shown that the very hematopoietic cell types whose maximum proliferation *in vitro* depends on their stimulation by the highest concentration(s) of cytokines, also exhibit the greatest capacity to deplete the same cytokines from the medium. Taken together, these findings may explain why the identification of conditions that support the expansion of hematopoietic stem cells has been so elusive.

**CHAPTER 7: ANALYSIS OF *C-KIT*/STEEL FACTOR RECEPTOR
INTERNALIZATION IN FACTOR DEPENDENT HEMATOPOIETIC CELLS**

1. Introduction

Steel factor (SF) interacts with *c-kit*, a tyrosine kinase receptor, to modulate hematopoiesis, gametogenesis and melanogenesis (Arakawa et al., 1991). First identified as the normal cellular counterpart of *v-kit*, the transforming gene of a feline sarcoma virus (Charbot et al., 1988; Geissler et al., 1988), the *c-kit* proto-oncogene encodes a 145-155 kD tyrosine kinase receptor that is expressed on many types of primitive hematopoietic cells. Upon exposure to SF, *c-kit* undergoes dimerization and activation of its catalytic function (Blechman et al., 1995). Under physiological conditions, soluble SF exists as a noncovalently associated bivalent dimer (Arakawa et al., 1991; Lev et al., 1993) although it is also produced and believed to exert its major hematopoietic activity as a membrane bound entity (Lev et al., 1992). Dimerization of *c-kit*, activation of its intracellular tyrosine kinase domain and the subsequent transphosphorylation [reviewed in Ullrich and Schlessinger (1990)] is followed by *c-kit* down regulation (Yee et al., 1993; Shimizu et al., 1996; Miyazawa et al., 1994). Studies with mutated forms of *c-kit* have shown that internalization is not required for a mitogenic response (Miyazawa et al., 1995). In fact, cellular exposure to either membrane-bound (Avraham et al., 1992) or otherwise immobilized (Miyazawa et al., 1995) SF, as well as immobilized activating mAbs to SF (Kurosawa et al., 1996) reduce internalization of *c-kit* and prolong tyrosine kinase activation. Studies on mast cells using mAbs to the extracellular domain of *c-kit* have shown that down regulation occurs in a rapid, dose-dependent manner (Shimizu et al., 1996; Baghestanian et al., 1996) resulting in internalization and degradation of the SF/*c-kit* receptor complex (Miyazawa et al. 1994). Re-expression of *c-kit* has been shown to require novel protein synthesis (Yee et al., 1994). Several studies have postulated that SF induced internalization of the SF/*c-kit* complex serves both to remove receptor molecules

from the cell surface (Murthy et al., 1989; Yee et al. 1993; Shimizu et al. 1996) and to remove SF from the cellular environment (Reddy et al., 1994; Miyazawa et al 1994; Marshall, 1995). Both of these mechanisms have been suggested to result in a reduction in cell stimulation (Reddy et al., 1996b).

Soluble SF is a potent co-stimulating cytokine that, in combination with a number of other cytokines, stimulates the growth of hematopoietic progenitors *in vitro* (reviewed in Chapter 1). Although SF has been used extensively for the *in vitro* expansion of primitive hematopoietic progenitors, little is known about the effects of different exposure schedules on the responses of these cells. In Chapter 4 it was shown that SF depletion by primary human bone marrow occurs in a concentration-dependent manner (Figure 4.4). In addition, maintenance of primitive hematopoietic cell function (i.e. stimulation of LTC-IC self-renewal) appears to require exposure to higher concentrations of certain cytokines, including SF, than support of their viability and differentiation (Chapter 6). It was therefore of interest to investigate further the mechanisms underlying the concentration-dependent depletion of cytokines by hematopoietic cells. As a first step in this direction, the internalization kinetics of receptor-bound SF of hematopoietic cells exposed to different extracellular concentrations of SF were examined using the SF-responsive human Mo7e cells as a model. From parallel measurements of the extracellular SF concentration, *c-kit* expression on the cell surface and the appearance of SF inside the cells, two main regimes of SF depletion were shown to occur. One is associated with rapid SF depletion and receptor internalization. The second is characterized by a slower rate of SF depletion that was associated with a gradual recovery of cell surface receptor expression. An analysis of the calculated cell-surface SF concentration

was performed in order to ensure that the rapid cytokine depletion rate would not significantly deplete the cell microenvironment compared to the concentrations measured in the bulk fluid.

2. Results

A. Kinetics of SF dose-dependent reduction of cell surface *c-kit* expression on Mo7e cells:

Log phase Mo7e cells were resuspended in Iscove's media containing 10% FCS and 10 ng/mL SF and the cells subsequently incubated at 37°C. At varying intervals the level of *c-kit* surface expression was analyzed by flow cytometry using the YB5.B8 anti-*c-kit* antibody as described in Chapter 2. As shown in Figure 7.1, a significant reduction in the level of *c-kit* expression (YB5.B8 reactivity) occurred in the first hour after SF addition. This reduction was followed by a gradual recovery over the next 48 h.

YB5.B8 was chosen for these studies because Ashman et al. (1994) and Miyazawa et al. (1994) had found that SF binding did not affect its reactivity with *c-kit*. However, more recently, Baghestanian et al. (1996) reported significant blocking of YB5.B8 by SF. Additionally, it was recently shown that the washing procedures used to prepare cells for labeling with YB5.B8 can remove SF from the cell surface and thus eliminate YB5.B8 epitope blocking by SF (Shimizu et al., 1996).

In order to determine the relevance of these issues for the cells and procedures used in this investigation, a series of experiments were performed comparing *c-kit* binding at 4°C and 37°C. As receptor internalization does not occur at 4°C, any SF-dependent change in receptor expression that is measured at this temperature can be attributed to YB5.B8 epitope blocking.

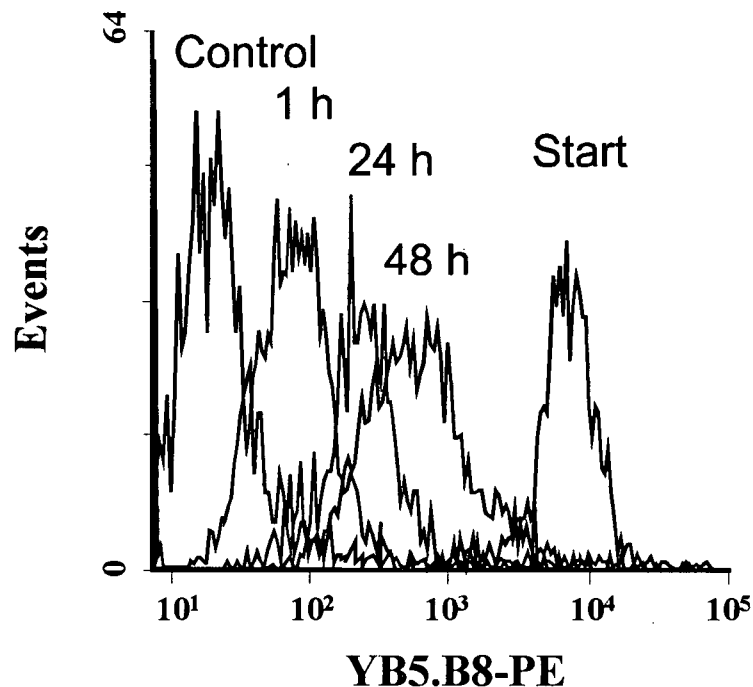


Figure 7.1: Time-dependent change in *c-kit* expression as evaluated by flow cytometry on Mo7e cells after incubation with media containing 10 ng/mL of input SF concentration.

As shown in Figure 7.2, YB5.B8 epitope blocking by SF was responsible for between 20-40% of the observed changes in receptor binding, an effect that is saturated at low SF concentrations (1-10 ng/mL). This epitope blocking was measured even when the washing procedures reported in Shimizu et al. (1996) were used (data not shown). In order to determine the net effect of different concentrations of SF on cell surface *c-kit* expression, the change in *c-kit* fluorescence that was measured for a specific SF concentration at 4°C was used to normalize the corresponding *c-kit* fluorescence measured at 37°C. This epitope blocking normalization technique is an adaptation of that reported by Baghestanian et al. (1996) and assumes that the epitope blocking by SF of *c-kit* receptors does not vary between

4 and 37°C. Using this procedure, the calculated *c-kit* expression of Mo7e cells exposed to different input concentrations of SF was:

$$R_{SF,37} = \alpha F_{SF,37} \left(1 + \frac{F_{0,4} - F_{SF,4}}{F_{0,4}} \right) \quad 7.1$$

where $R_{SF,37}$ is the calculated *c-kit* expression at 37°C (YB5.B8 molecules), α is the slope of the calibration curve (YB5.B8 molecules/ mean channel number) determined using the mAb binding calibration beads as discussed in Chapter 2, $F_{SF,37}$ is the fluorescence (mean channel number) determined by YB5.B8 binding to *c-kit* after exposure of the cells to 1, 10, or 100 ng/mL SF for 30 min at 37°C (as in Figure 7.1), $F_{0,4}$ is the fluorescence determined by

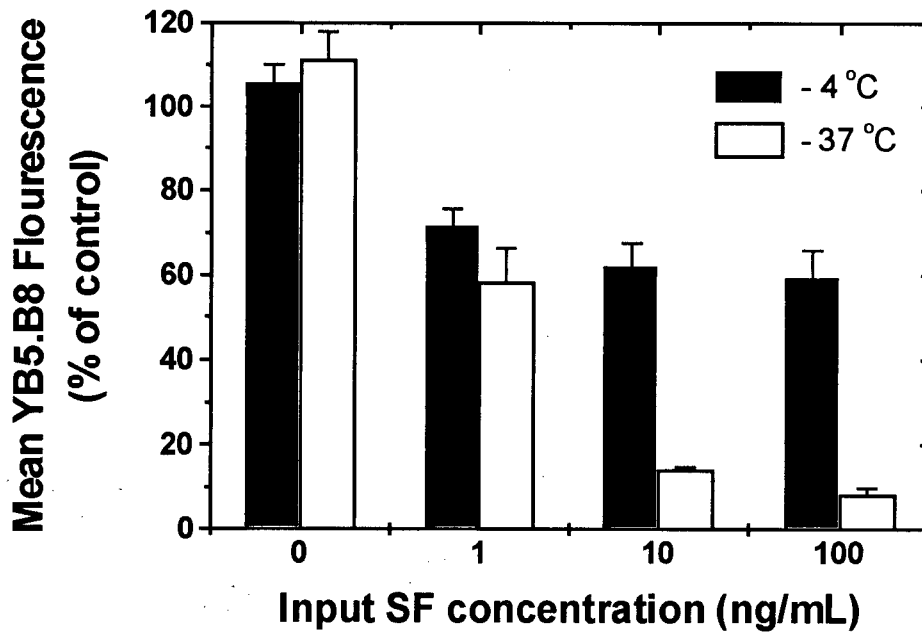


Figure 7.2: Mean YB5.B8 fluorescence expressed as percent of levels measured on input Mo7e cells after stimulation for 30 min by different doses of SF at 4 and 37°C. Bars represent the mean \pm SEM YB5.B8 fluorescence from 3 replicate cultures.

YB5.B8 binding to *c-kit* after exposure to 0 ng/mL SF for 30 min at 4°C and $F_{SF,4}$ is the fluorescence determined by YB5.B8 binding to *c-kit* after exposure to 1, 10; or 100 ng/mL SF for 30 min at 4°C (Figure 7.2). The first term in Equation 7.1 is a direct measure of the *c-kit* binding and the second term accounts for the extent of epitope blocking at each SF concentration.

This normalization procedure allowed SF dose- and time-dependent changes in cell surface *c-kit* expression to be determined (Figure 7.3). In order to examine the effect of changes in SF concentration on cell surface *c-kit* expression, Mo7e cells maintained in media containing ~1 ng/mL SF for 48-72 h were transferred to media containing different concentrations of SF. This induced a rapid (< 30 min) reduction in the detection of cell surface *c-kit* expression which occurred in a dose-dependent manner such that the transfer of washed cells from 0.8 ± 0.2 ng/mL SF medium to that containing to 1.4 ± 0.3 , 13 ± 3 and 98 ± 5 ng/mL SF resulted in decreases in the calculated *c-kit* expression of 18, 78 and 86%, respectively. The initially rapid decrease in cell surface *c-kit* expression was followed by a gradual increase over the next 71.5 h, regardless of the concentration of SF that the cells were originally exposed to. Interestingly, input levels of *c-kit* expression were detected at 72 h in cells where, due to the depletion of SF from 1.4 ± 0.3 to 0.7 ± 0.4 ng/mL SF, the SF concentration in the medium returned to levels similar to those measured on the input cells (~0.8 ng/mL).

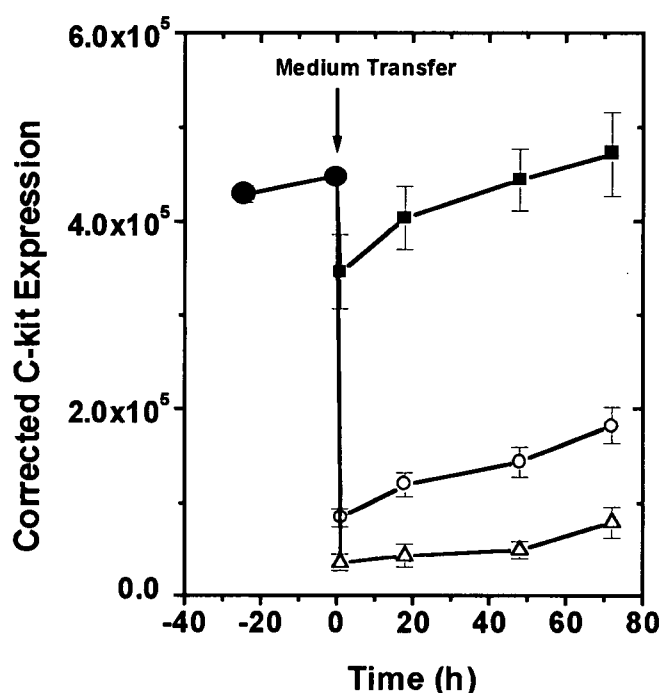


Figure 7.3: Dose- and time-dependent changes in calculated *c-kit* expression (Equation 7.1) of Mo7e cells transferred at 0 h from 0.8 ± 0.2 (●) ng/mL SF medium to 1.4 ± 0.3 (■), 13 ± 3 (○), or 98 ± 5 (Δ) ng/mL of SF containing medium. Cells were maintained in medium containing ~ 1 ng/mL SF for 48-72 h prior to the transfer to fresh media containing different concentrations of SF for analysis of *c-kit* expression. Points are the mean \pm SEM from 3 experiments.

B. Cytokine dose- and time-dependent kinetics of SF depletion by Mo7e cells.

Parallel measurements of the extracellular SF concentration of the medium in which the cells were incubated in the experiments presented in Figure 7.3 revealed a rapid cell associated depletion of SF in the first 30 min after the medium transfer (Table 7.1). This concentration-dependent depletion was directly associated with the presence of Mo7e cells as non-specific absorption or degradation of SF was not significant in Mo7e conditioned medium over the time scales investigated in these experiments (Chapter 4).

The rapid decrease in the concentration of extracellular SF that occurred during the first 30 min was followed by an approximately 100-fold lower cell-specific depletion rate that, nonetheless, resulted in the losses of 56, 79 and 82 % of the overall SF depleted (after 72 h) in the cultures initiated with 1.4 ± 0.3 , 13 ± 3 and 98 ± 5 ng/mL of SF, respectively (Table 7.1). Thus, although the cell-specific depletion rates were highest during the first 30 min of exposure of the cells to SF, most of the loss of SF occurred over the subsequent 71.5 h and the overall rate of SF depletion (0-72 h) was similar to that calculated for 0.5-72 h period.

Table 7.1: Cell specific dose- and time-dependent SF depletion in cultures initiated with 5×10^5 Mo7e cells^a.

Input	SF Concentration ^b		Average Cell Specific SF Depletion Rate (ng/ 10^6 cells/day) ^c		
	30 min	72 h	0-0.5 h	0.5-72 h	0-72 h
1.4 ± 0.3	1.1 ± 0.3	0.7 ± 0.4	26	0.2	0.27
13 ± 3	11 ± 2	6.8 ± 3	130	2.3	2.6
98 ± 5	96 ± 4	84 ± 5	280	6.4	6.8

^a In some 30 min wells 10^6 cells were added to ensure an accurate determination of the cell-specific rate of SF depletion over short periods of time.

^b Mo7e cell cultures initiated from cultures containing 0.8 ± 0.2 ng/mL SF and transferred to fresh media containing varying amounts of SF as indicated.

^c Average cell specific SF depletion rates calculated using Equation 2.1

C. Flow cytometric analysis of dose dependent SF internalization.

To confirm that the decrease in the level of *c-kit* expression on SF treated Mo7e cells and the concurrent decrease in the extracellular concentration of SF were, at least in part,

associated with an increase in cell-associated SF, flow cytometry was used to examine changes in internalized SF. To ensure that the cell-associated SF detected in these experiments was due to SF supplementation during the experiment, these short-term studies were performed in serum-free media using cells previously cultured in 10% FCS and 5 ng/mL G-CSF. As shown in Figure 7.4, a significant SF internalization occurred in the first 30 min after exposure of the cells to 100 ng/mL SF. Background levels in unexposed input cells could not be distinguished from results obtained with the control samples treated only with streptavidin-PE. To test the dose-dependence of this phenomenon, cells were exposed to 1, 10 and 100 ng/mL input SF concentration for 30 min, after which they were fixed, permeabilized and stained as described in Chapter 2. As shown in Figure 7.5 for a representative experiment (of 3 performed), increasing amounts of fluorescence were detected with increasing doses of SF. Interestingly, consistent with the cytokine depletion data, the greatest changes in the amounts of internalized SF detected occurred between 1 and 10 ng/mL SF. This suggests that the ability of the cells to internalize SF approaches saturation at the higher SF concentrations tested.

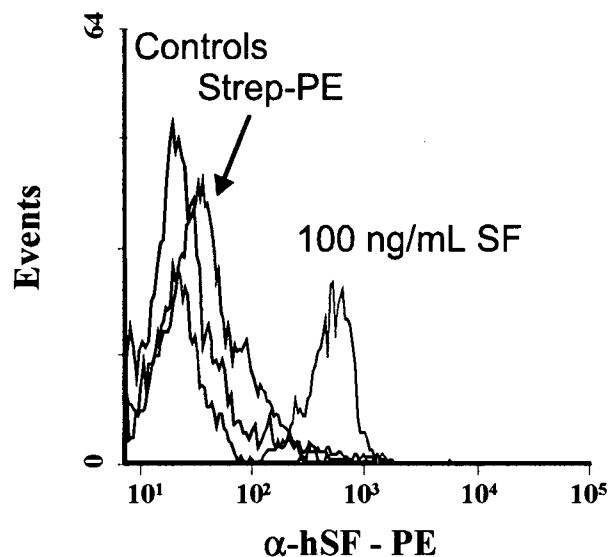


Figure 7.4: Internal SF staining of cells exposed to 100 ng/mL input SF concentration in serum-free medium for 30 min at 37°C. Controls are unexposed input cells, cells incubated the in presence of 1 μ g/mL SF during the staining procedure and 100 ng/mL stimulated cells stained with Streptavidin (Strep)-PE alone.

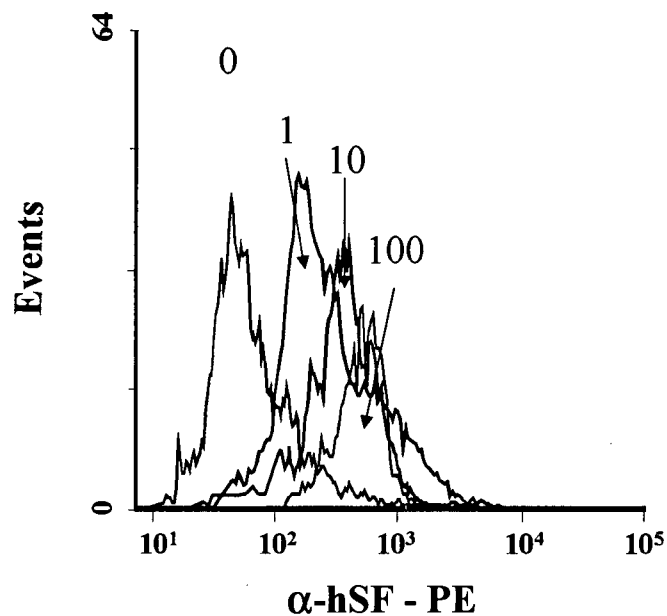


Figure 7.5: A representative experiment (of three performed) of the detection of internal SF staining on cells exposed to 1, 10 or 100 ng/mL input SF in serum-free medium for 30 min at 37°C.

3. Discussion

In Chapter 4, a significant dose-dependent depletion of the cytokines initially present in cultures of primitive hematopoietic cells was described. These studies were replicated in 72 h cultures of an immortalized factor-dependent and SF-responsive human leukemic cell line. Here it is shown that a least 2 distinct rates of depletion may be responsible for the average rate of depletion previously documented with the Mo7e model. In the first 30 min after the addition of SF, high cell-specific rates were observed (Table 7.1). These rapid cytokine depletion rates were accompanied by a rapid internalization of the corresponding receptor, *c-kit* (Figure 7.3), and the appearance of SF inside the cell (Figure 7.5). The initial rate of SF depletion was followed by a more gradual one which was closely associated with the average rate of SF depletion measured over the 72 h period. During this period there was a dose-dependent recovery of receptors on the cell surface. Continued depletion of extracellular cytokines when the numbers of receptors on the cell surface remains constant or is increasing has been attributed to receptor synthesis and recycling (Wiley et al., 1989; Countaway et al., 1992; Reddy et al., 1996a). Secretion of a variety of soluble cytokine receptors (including *c-kit*), has also been shown to occur (reviewed in Heaney and Gold, 1996). This latter mechanism might contribute to both the potentiation (Murakami et al., 1993) and attenuation (Heaney et al., 1995) of soluble protein-induced signaling.

The rapid *c-kit* mediated depletion of SF that occurs within the first 30 min after exposure to SF could significantly reduce the local concentration of the cytokine and hence the availability of soluble SF to responsive target cells. A mathematical model was used to determine whether the measured bulk concentrations are representative of the concentrations

of SF at the cell surface under the conditions of the Mo7e cell cultures. As the cells are initially in suspension (post-inoculation) and the rapid SF depletion occurs within the first 30 min, the rate of cytokine transfer was modeled assuming a cell in an infinite space. For this case (Berg and von Hippel, 1985) derived:

$$Q = 4\pi rD(C_b - C_s) \quad 7.2$$

where Q is the transfer rate to the cell in ng/s, r is the cell radius (7.5×10^{-4} cm), D is the diffusivity (cm^2/s), and C_b and C_s are the bulk and cell surface SF concentrations (ng/cm^3) respectively. Equation 7.2 assumes that the cells are in suspension during the initial 30 min. For cells that do settle to the surface (maximum < 10% surface coverage), the diffusive can be estimated to occur over $1/\pi$ of the total cellular area (Berg and von Hippel, 1985) and thus the concentration gradient ($C_b - C_s$) of cell which do settle would only be increased by up to π -fold compared to cells in suspension.

The diffusivity of SF ($8.7 \times 10^{-11} \text{ m}^2/\text{s}$ or $8.7 \times 10^{-7} \text{ cm}^2/\text{s}$) was estimated using the Polson (1950) equation:

$$D = \frac{9.4 \times 10^{-15} T}{\eta(M)^{1/3}} \quad 7.3$$

where T is the temperature (310 K), M is the dimer molecular weight (37,000 Da), and η is the viscosity ($1 \times 10^{-3} \text{ kg}/\text{m}\cdot\text{s}$). This equation is valid for globular proteins of molecular weight greater than 1,000 Da. As can be seen in Table 7.2, even using the rapid 30 min cell-specific rates of SF depletion as an estimate of the transfer rate to the cells, the concentrations of SF at the cell surface is not greatly reduced. These results suggest that the bulk SF measurements provided a sufficiently accurate estimate the SF concentration at the cell surface.

Table 7.2: Estimated cell surface concentration based on the SF flux to Mo7e cells in suspension. Maximal flux determined from Table 7.1 for 30-min exposure to cells suspended in media containing a range of concentrations of SF^a.

Average Bulk SF Concentration, C_b^b (ng/mL)	Calculated Average Cell Surface SF Concentration (ng/mL)	Calculated Cell Surface SF Concentration as % of Bulk SF Concentration
1.25	1.15	96
12.3	12	98
96.8	96	99

^a This estimate of the maximal flux to the cell assumes constant cytokine depletion rates over the first 30 min of cytokine exposure.

^b C_b is the average of the input SF concentration and the SF concentration after 30 min (Table 7.1).

In contrast to the diffusivity of proteins in solution, their diffusivity through packed layers of cells or extracellular matrix has been estimated to be 5- to 10-fold lower (Lightfoot, 1995; Chu et al., 1996). These lower diffusivities, along with longer diffusive path lengths, may significantly reduce the concentration of cytokines around packed cells, especially under conditions of rapid cytokine depletion. Endocytic relay (Chu et al., 1996) and enhanced diffusion along extracellular matrix molecules may increase diffusivities under particular circumstances. Detailed investigations of these issues using protein tracking methodologies would be required to establish more precisely how rates of cytokine depletion may affect the concentrations to which packed cells are actually exposed over time.

In addition to the above observations, the measurements of SF depletion rates underscore the importance of monitoring and appropriately regulating the concentrations of cytokines considered necessary for stimulating the expansion of primary hematopoietic cells in culture. Cytokine concentrations may be particularly important for cultures where the cells are

exposed to relatively low levels of soluble cytokines (i.e. <10 ng/mL, Table 7.1) such that a significant fraction of the molecules added may be rapidly consumed, thus potentially resulting in the subsequent exposure of the cells to sub-optimal cytokine levels.

These results may also have implications for the differential responses of multipotent cells to different cytokine concentrations and supplementation regimes (Chapter 6, Cantwell and Smith, 1984; Dexter et al., 1990; Traverse et al., 1994). Further studies are required to determine if modulation of the strength of induced signaling, possibly caused by differences in the timing, amount and/or duration of receptor stimulation, can be linked to distinct cellular genetic programs.

CHAPTER 8: SUMMARY AND FUTURE DIRECTIONS

1. Summary

The use of cytokine supplemented suspension cultures to study the self-renewal and differentiation of primitive hematopoietic cells has provided both significant insight into the regulation of the hematopoietic system and a basis for the development of clinical-scale expansion systems for the culture of these cells. The first objective of my research was to develop and characterize a homogeneous (stirred suspension) system for the growth of various populations of adult human hematopoietic cells. As shown in Chapters 3 and 4, these investigations identified several parameters important in achieving this goal. The finding that, in cultures supplemented with low concentrations of a minimal cytokine mixture, the extent of progenitor expansion was dependent on inoculum cell density, suggested that additional soluble factors were playing a role in the stimulation of hematopoietic cell proliferation. The accompanying expansion of fibroblast progenitors in those cultures suggested that at least some of these factors may be derived from this cell source. When additional cytokines were supplemented to the cultures, the expansion of primitive progenitors was shown to be dependent on a periodic medium and cytokine addition regimen which reduced cellular exposure to sub-optimal levels of cytokine due to the cell growth-associated depletion of supplemented cytokines and glucose (Chapter 4). These studies identified cytokine depletion as an important parameter in the maintenance of hematopoietic cell cultures. The cell-specific rates of cytokine depletion were subsequently shown to be dependent on both the concentration of cytokines that the cells were exposed to and the identity of the cells; more

primitive cells exhibiting higher cell-specific cytokine depletion rates than their more differentiated progeny.

The stirred suspension culture studies, due to their relatively homogeneous and controllable environment, allowed the examination of the effects of changes in the cellular environment on the growth of specific populations of cells within a mixed cell culture. They also revealed that elevated levels of the same cocktail of cytokines could preferentially affect the expansion of the more primitive cells (i.e. LTC-IC), without significantly changing the growth of more mature cell populations. In Chapter 6, this observation was extended to purified cells where it was suggested that the cytokine concentration-dependent retention or loss of LTC-IC activity was independent of the ability of the same cytokines to maintain the viability or stimulate the proliferation of CD34⁺CD38⁻ cells. These observations have a number of important implications. The responsiveness of primitive cells to supra-biological levels of cytokines supports a role for direct cell-cell interactions, cell surface-bound cytokines and/or cytokine presentation by extracellular matrix in the maintenance of primitive cells *in vivo*. This requirement is consistent with the well documented importance of accessory cells on hematopoietic progenitor behavior. The results presented in Chapter 6 additionally support a role for exogenously added factors in the determination of cellular fate outcomes. As outlined in the "Future Directions" section below, further investigation is required to identify the molecular mechanisms of this response.

A second objective of this study was to investigate the role of individual and combinations of cytokines on the proliferation and differentiation of highly purified primitive cells. Although, as reviewed in Chapter 1, there have been multiple studies of the effects of

cytokines on various populations of hematopoietic cells, a systematic analysis of the influence of candidate cytokines on the generation of LTC-IC from highly purified cells had not been performed. Using factorial design analysis, significant individual and combinatory effects of FL, SF and IL-3 on LTC-IC proliferation were identified. It was further shown in Chapter 6 that, of these three cytokines, only an elevated level of FL (in the presence of SF and IL-3) is required to achieve a similar expansion of LTC-IC. Interestingly, it has subsequently been shown that a distinct but overlapping group of factors promotes the differentiation of these cells to CFC (see "Future Directions"). These studies, which provide clues to the importance of different signaling pathways on the stimulation of primitive cells, now require further investigation to identify the molecular mediators responsible for these differential responses.

Finally, as reviewed in Chapter 1, it has been suggested that specific cellular responses may have distinct cytokine concentration thresholds. Additionally, cytokine supplementation of responsive cells modulates the expression of the corresponding receptor on the cell surface. As the interaction between the ligand and its receptor on the cell surface is the first level by which these differences may be sensed by the cell, it was of interest to determine the responses of cytokine receptor expression to changes in extracellular cytokine concentration on human factor-dependent hematopoietic cells. Using a human leukemia cell line (Mo7e) it was shown that SF induced a rapid (<30 min) dose-dependent increase in c-kit internalization that correlated with the depletion of SF from the extracellular media. After the initial internalization, a second more constant cell-specific cytokine depletion was observed. Although this rapid depletion does not impact significantly on the cell surface cytokine concentration in suspension cultures (stirred or not), this mechanism may play an important

role in regulating cytokine exposure to cells in the diffusion limited environment that occurs in static, high cell density cultures or *in vivo*.

2. Future directions

The studies in this thesis have identified two major areas where continuing research could significantly impact both the understanding of hematopoietic stem cell biology in general and, more specifically, the development of *ex vivo* culture systems for the growth of primitive human hematopoietic cells. The first is the need for further investigations of the medium supplementation strategies for suspension cultures. For example, although it is clear that medium supplementation plays an important role in the maintenance and expansion of primitive cells in these cultures, it is not clear whether intermittent or continuous medium addition may be superior. Furthermore, as suggested by the effects of cytokine supplementation on cytokine receptor expression, each of these approaches may elicit distinct cellular responses [i.e. in terms of medium and cytokine utilization, as has been found in recent experiments by J. Audet (personal communication)]. If it is found that continuous medium addition results in enhanced primitive cell production, this will require the development of continuous perfusion bioreactor systems to provide nutrients and growth factors while removing inhibitory factors and metabolic waste. Preliminary studies using a modified cross-flow and acoustic filtration technology have suggested the potential of this approach. In addition to these design requirements, optimization of the cell environment including medium development and the role of oxygen supplementation (see Chapter 1) on the generation of primitive hematopoietic cells merits further investigation.

Several studies have suggested that the behavior of primary stem cell populations, following their stimulation either *in vivo* or *in vitro*, appears to reflect the operation of a stochastic process (reviewed in Chapter 1). The results presented in Chapter 6 indicated, at least under the conditions investigated, that the proportion of primitive progenitors undergoing either differentiation (to or along defined lineages) or self-renewal (proliferation without alteration of their functional state) can be influenced by extracellular factors. The outcome of the presentation of a mitogenic signal to a pluripotent hematopoietic stem cell may thus be dependent, at least in part, on: (1) the type of signal presented (i.e. the types of cytokines and the physical environment of the cell), (2) the magnitude of the signal (i.e. the concentration at which cytokines are presented) and (3) the method of signal presentation (i.e. surface-bound vs soluble, as indicated by associated differences in mitogenic responses** and in the duration of tyrosine phosphoralization††). Each of these parameters presents a unique opportunity to investigate the role of environmental control on cellular survival, and mitogenic and differentiation responses.

As summarized above, the studies presented in Chapters 6 and 7 suggest that the information necessary for the generation of distinct cellular responses can be found, at least in part, in the interaction between the cell and the ligand. An extension of the concentration-dependent receptor internalization studies to multipotent factor-dependent cells, (e.g. FDCP-mix cells) would allow potential effects on differentiation responses to be examined. Furthermore, an analysis of the molecular mediators in these responses may identify unique

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signaling pathways and the molecular intermediates involved. An understanding of the molecular mechanisms that control cellular fate processes through the modeling, design and analysis of novel growth factor delivery strategies should facilitate the rational development of bioreactors capable of culturing primary human tissues. Information gathered from each of these approaches can then be used to develop predictive models of stem cell proliferation and differentiation which may help to elucidate general mechanisms governing the role of environmental cues in determining hematopoietic cell fate *in vivo* and *in vitro*.

The studies presented in Chapter 5 support a role for FL, SF and IL-3 in the “maximal” stimulation of LTC-IC from adult BM. Recent results (unpublished findings) have shown that these factors are significantly less potent in stimulating LTC-IC from CB under similar conditions (in spite of the expected increases in CFC numbers). This marked selective difference in the expansion of LTC-IC from similarly stimulated CB and BM suggests that the cytokine requirements for primitive cell expansion may change with ontogeny. Further exploitation of these models may provide important clues about the intracellular mechanisms regulating proliferation and differentiation of very primitive hematopoietic cells from a variety of sources.

In summary, these studies have provided clues about how the modulation of the extracellular environment may result in differential responses from primitive adult human bone marrow progenitors detected *in vitro* as LTC-IC and CFC. Further studies are now required to determine if the measured increases in LTC-IC numbers are indicative of increases in the *in vivo* repopulating ability of the input cell population (as recently shown by Conneally et al.,

^{††} M.M. Guarna, personal communication.

1997) or if, as has been shown here for LTC-IC and CFC, the proliferation and differentiation of these stem cells is also subject to independent control.

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