EFFECTS OF HEMATOPOIETIC GROWTH FACTORS ON PURIFIED BONE MARROW PROGENITOR CELLS

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Fredrik Jan Bot
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PROMOTIECOMMISSIE:

Promotor: Prof. Dr. B. Löwenberg

Overige leden: Prof. Dr. W. van Ewijk
Prof. Dr. R. Willemze
Dr. R. Ploemacher

Gedrukt door: Drukkerij Haveka B.V., Alblasserdam

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Voor mijn ouders

Aan Loes,

Ingeborg en Daniel

Contents

	Abbreviations	6
1.	Introduction	
1.1	Hematopoiesis	
	1.1.1 Hematopoiesis in vivo	8
	1.1.2 Hematopoiesis in vitro	8
1.2	Growth factors and cytokines involved in the regulation of hematopoiesis	
	1.2.1 Interleukin-3	11
	1.2.2 Granulocyte-macrophage colony stimulating factor	12
	1.2.3 Granulocyte colony stimulating factor	13
	1.2.4 Macrophage colony stimulating factor	14
	1.2.5.1 Other cytokines that affect hematopoiesis	16
	1.2.5.2 Interleukin-6	17
	1.2.5.3 Interleukin-1	18
	1.2.5.4 Tumor necrosis factor	20
1.3	The objective of the study	22
2.	Stimulating spectrum of human recombinant multi-CSF (IL-3) on human marrow precursors: importance of accessory cells. Blood 71:1609-1614, 1988	23
3.	Human granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates immature marrow precursors but no CFU-GM, CFU-G or CFU-M. Experimental Hematology 17:292-295, 1990	33
4.	Effects of human interleukin-3 on granulocytic colony forming cells in human bone marrow. Blood 73:1157-1160, 1989	41
5.	Synergistic effects between GM-CSF and G-CSF or M-CSF on highly enriched human marrow progenitor cells. Leukemia 4:325-328, 1990	50
6.	Interleukin-6 (IL-6) synergizes with M-CSF in the formation of macrophage colonies from purified human marrow progenitor cells. Blood 73:435-437, 1989	60
7.	Interleukin-1α (IL-1) regulates the proliferation of immature normal bone marrow cells. Blood 76:307-311, 1990	66
8.	General discussion	
	8.1: Interleukin-3 and Granulocyte-Macrophage-CSF	78
	8.2: Granulocyte-CSF and Macrophage-CSF	82
	8.3: Interleukin-6, Interleukin-1 and Tumor Necrosis Factor	85
	Summary	88
	Samenvatting	90
	Appendix	92
	References	99
	Dankwoord	118
	Curriculum vitae	120

Abbreviations

BFU-E	burst forming unit-erythroid
BPA	burst promoting activity
CFU	colony forming unit
CFU-Baso	CFU-basophil
CFU-Blast	CFU-blast cells
CFU-Eo	CFU-eosinophil
CFU-G	CFU-(neutrophilic) granulocyte
CFU-GEMM	CFU-granulocyte-erythroid-macrophage-megakaryocyte
CFU-GM	CFU-granulocyte-macrophage
CFU-M	CFU-macrophage
CFU-Mega	CFU-megakaryocyte
CSA	colony stimulating activity
CSF	colony stimulating factor
DNA	deoxyribonucleic acid
Epo	erythropoietin
FMLP	N-formylmethionylleucylphenylalanine
G-CSF	granulocyte-CSF
GM-CSF	granulocyte macrophage-CSF
HTLV	human T-lymphotropic virus
IFN	interferon
IGF	insulin-like growth factor
IL-x	interleukin-x
LPS	lipopolysaccharide
M-CSF	macrophage-CSF
mRNA	messenger ribonucleic acid
NK-cells	natural killer-cells
PDGF	platelet derived growth factor
PHA	phytohemagglutinin
PHA-LCM	PHA-leucocyte conditioned medium
PMA	phorbol myristate acetate
SAC	Staphylococcus aureus strain Cowan I
SCF	stem cell factor
TGF	transforming growth factor
TNF	tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate

Chapter 1

INTRODUCTION

1.1 Hematopoiesis

1.1.1 Hematopoiesis in vivo

The maintenance of blood cell function requires the continuous production, differentiation and maturation of considerable numbers of cells with very diverse functions. These cells originate from common ancestors, i.e. pluripotent hematopoietic stem cells.[303,316] The stem cells, which reside in the bone marrow, are able to renew themselves and to produce daughter cells. These divide further into lineage committed cells and differentiate along separate pathways: the erythroid, myelo-monocytic, megakaryocytic and lymphoid lineages. Eventually the functional end-cells like erythrocytes, monocytes, granulocytes, megakaryocytes/platelets and lymphocytes are formed (Fig.1). The presence of a sufficient number of stem cells ensures the ability of the body to keep up a constant supply of blood cells. Various regulatory mechanisms permit this dynamic system to raise an adequate response to fluctuating needs, e.g. during infection or blood loss, and result in increased production of the desired cell types.

1.1.2. Hematopoiesis in vitro

Research on blood formation has traditionally been morphological, based on the recognition and quantification of the maturation-phases of the various blood cell lineages in a stained smear of blood or bone marrow. This approach gives no detailed information with respect to regulation of the production of blood cells. With the development of in vitro clonogenic assays tools for research regarding the function of the bone marrow as the blood cell producing organ came within reach.

Certain cells in the bone marrow demonstrated the ability to form colonies in vitro when incubated in a suitable culture medium for one or two weeks.[81,82,127,130,240,267] The progenitor cell able to form such a colony (a group of at least 50 daughter cells) was designated "colony-forming unit" (CFU), and eventually these observations led to the postulation of a hierarchical system of immature and mature CFUs (Fig.1).

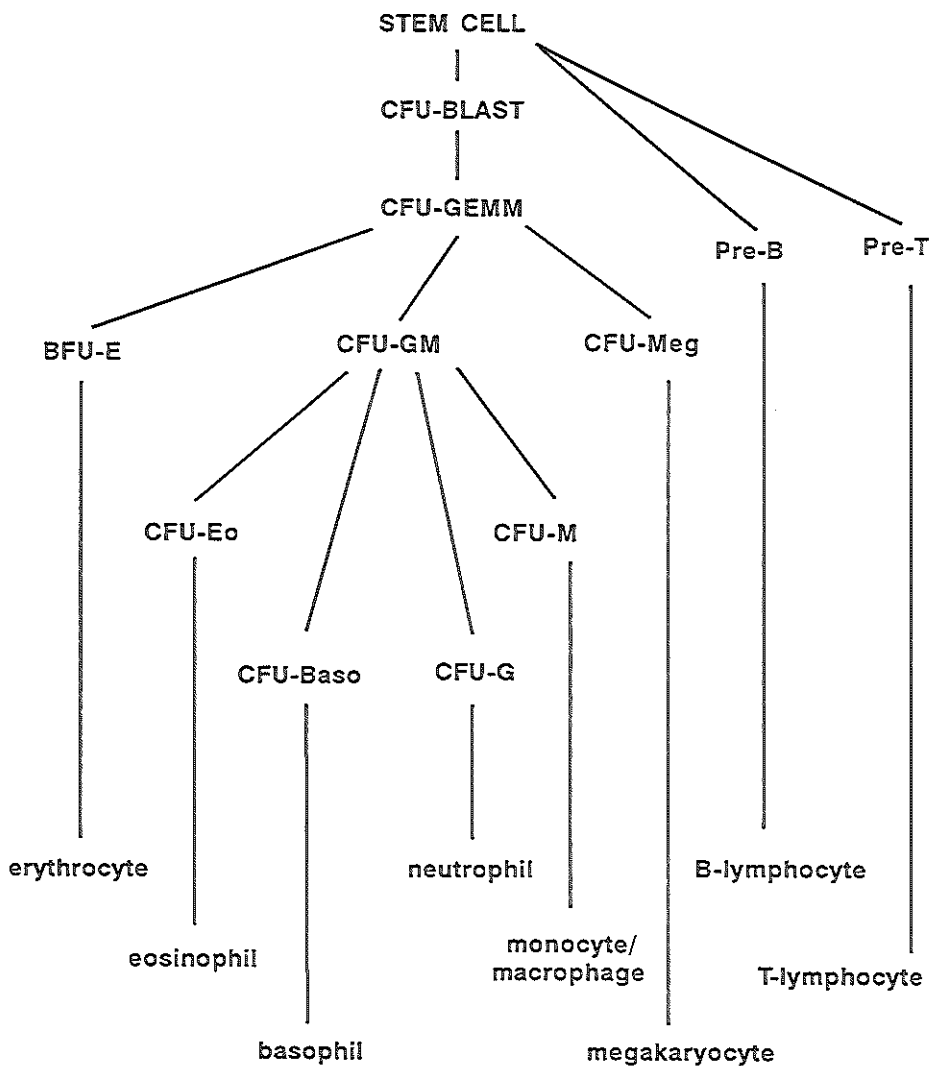

Figure 1. The hierarchical system of hematopoiesis from pluripotent stem cell to functional end cells.

The various types of CFUs or "hematopoietic progenitor cells" are listed in table 1.1.

table 1.1 Nomenclature of progenitor cells

CFU	Composition of colony
CFU-Blast	blast cells
CFU-GEMM	granulocytes, erythrocytes, monocytes and megakaryocytes
CFU-GM	granulocytes, monocytes
CFU-G	neutrophilic granulocytes
CFU-Eo	eosinophilic granulocytes
CFU-Baso	basophilic granulocytes
CFU-M	monocytes/macrophages
CFU-Mega	megakaryocytes
BFU-E	(nucleated) erythrocytic cells

Proliferation, differentiation and maturation of hematopoietic progenitor cells as well as reactions to stress are regulated by hematopoietic growth factors that are produced by a variety of cells. Initially, humoral factors were not available as pure factors and had to be added to in-vitro cultures as impure conditioned media. The advent of recombinant DNA-technology and the cloning of cDNA of several hematopoietic growth factors represented a major development in experimental hematology and created new possibilities to unravel the regulation of bone marrow progenitor cell growth.

In this chapter the current knowledge of the regulation of hematopoietic progenitor cell growth will be introduced. Chapters 2 to 7 describe the experimental work that forms the basis of this thesis. The last chapter is a general discussion of the presented data. An appendix describes the development and characteristics of the clonogenic culture system used for the investigations of this thesis.

1.2 Growth factors and cytokines involved in the regulation of hematopoiesis

The hematopoietic growth factors and cytokines that are subject of the experimental work of this thesis will be briefly introduced here. We shall first discuss the major hematopoietic growth factors IL-3, GM-CSF, G-CSF and M-CSF, followed by the modulating cytokines IL-6, IL-1 and TNF-α.

1.2.1 Interleukin-3

Most growth factors were initially discovered in the murine species. Interleukin-3 was the first murine growth factor to be molecularly cloned.[70,91,353] It took until 1986 before a gibbon IL-3 was cloned [351] which appeared almost identical to human IL-3. At about the same time human IL-3 was identified and cloned by virtue of its homology (93%) to certain A-T rich repetitive sequences in the 3' terminal region of murine IL-3.[71] The mature polypeptide has a length of 133 amino acids with a molecular weight of 15 kD. Others have also succeeded in cloning human IL-3.[229] The cellular source of IL-3 in humans is restricted to a small fraction of lectin-stimulated T-lymphocytes and NK-cells.[221,344]
The biologic activity of murine IL-3 is broad and encompasses erythroid, myeloid and mixed progenitor cells as targets.[70,105,122,251] A number of studies using the gibbon IL-3 preparation of Yang et al[349] demonstrated that this IL-3 induced colony formation from human progenitors of the granulocyte, macrophage, eosinophil and megakaryocyte lineages and, when combined with Epo, also from erythroid and mixed progenitor cells.[156,169,196,272] Human IL-3 also expressed a relatively broad spectrum of stimulation: progenitor cells from the erythroid, myeloid and mixed lineages were induced to colony formation in vitro,[71,231,255] as were megakaryocytic progenitors.[191] The activity of IL-3 on early progenitors is also illustrated by its effect on the blast-colony forming cell. This CFU-blast is responsive to IL-3 and IL-6.[156,157,222]
IL-3 has effects on mature cells as well: this especially applies to eosinophils. Functional properties of eosinophils are enhanced by IL-3. Thus IL-3 increases killing of antibody-coated target cells, enhances survival of eosinophils in vitro, augments proteoglycan synthesis and increases production of superoxide anions in eosinophils.[168,169,252,253] Segmented neutrophils are not activated by IL-3.[170] Basophilic granulocytes also express IL-3 receptors and acquire a differentiated phenotype following stimulation with IL-3. Histamine however is not released by basophils following stimulation with IL-3.[314,315] Monocytes express IL-3 receptors and following stimulation monocyte survival is enhanced.[75] IL-3 augments antibody dependent cytotoxicity of monocytes[356] and increases the expression of TNF mRNA.[39]

1.2.2 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

The murine GM-CSF gene was molecularly cloned shortly after murine IL-3.[96] The recombinant product stimulates preferentially granulocyte and macrophage progenitors and at high concentrations also eosinophil, megakaryocyte and erythroid progenitors (reviewed by Metcalf[197]).
The human GM-CSF gene was the first human hematopoietic growth factor gene that was molecularly cloned. The gene was first isolated from the GM-CSF-producing HTLV-transformed T-lymphoblast cell line Mo.[347] The purified recombinant protein had a molecular mass of 18 to 24 kD. Other groups subsequently reported the cloning of the GM-CSF gene.[35,41,136,159] Contrary to IL-3, GM-CSF is an ubiquitous growth factor produced by remarkably diverse cell types in various tissues, i.e. activated T-lymphocytes, endothelial cells, fibroblasts, keratinocytes and monocytes (table 1.2).
GM-CSF acts as a multipotential hematopoietic growth factor. It stimulates granulocyte-macrophage and eosinophil progenitors as well as megakaryocytic and mixed progenitors.[191,197,271,286,306] GM-CSF in fact expresses BPA (burst promoting activity) and stimulates erythroid colony formation in association with erythropoietin.[68,190,200,262,285] Initially though some groups had failed to show a significant stimulatory effect of GM-CSF on CFU-mix[35,256] and BFU-E.[78,203]

table 1.2 GM-CSF producing cells

cell type	stimulus for GM-CSF production	references
T-lymphocyte	lectins	227
	IL-1	111, 342
endothelium	IL-1	29, 88, 261, 262, 273-275
	TNF	28, 30, 261, 273, 275
fibroblasts	IL-1	86, 137, 275
	TNF	275
keratinocytes	IL-1	147
monocytes	IL-1	275
	TNF	275
	adherence + LPS	161

GM-CSF has effects on mature cells as well. It enhances the lysis of antibody coated target cells and phagocytosis of bacteria by neutrophils[89,168,198] as well as the N-formylmethionyl-leucylphenylalanine (FMLP) induced superoxide anion production by neutrophils concomitantly with an increase in FMLP-receptors.[6,35,168,217,293,331,332] Expression of the leucocyte-adhesion molecules Mo1, LeuM5 and LAM-1 on the surface of neutrophils increase following GM-CSF stimulation.[4,97,101] GM-CSF stimulated eosinophils exhibit enhanced cytotoxicity against opsonized target cells[35,168,198,277] and an increased rate of proteoglycan-synthesis.[253] IL-1 production and HLA-DR expression of macrophages are upregulated by GM-CSF.[282] GM-CSF enhances the antibody dependent cytotoxicity of monocytes against tumor cells[97,356] via a TNF-dependent mechanism.[39] The fungicide activity of macrophages against Candida albicans increases through superoxide anion production following stimulation with GM-CSF.[283] Adherence of monocytes to endothelium is also enhanced by GM-CSF. Endothelial cells themselves are induced to migrate and proliferate under the influence of GM-CSF.[36]

Finally GM-CSF acts as an inducer of the production of several cytokines by mature blood cells. Thus GM-CSF may increase the RNA-accumulation as well as the protein production of IL-1, TNF, M-CSF and G-CSF in monocytes[118,228,280,320] and IL-6, IL-1, TNF-α, G-CSF and M-CSF in neutrophils.[48,165,166] These abilities emphasize the important role of GM-CSF in the regulation of hematopoiesis. GM-CSF can initiate a range of regulatory cascades through the induction of other factors.

1.2.3 Granulocyte colony-stimulating factor (G-CSF)

Murine G-CSF was cloned in 1986.[311] The human G-CSF gene was cloned from a squamous cell carcinoma line (CHU-2)[213] and from the 5637 bladder carcinoma cell line[288] using a synthetic oligonucleotide probe. The molecular mass of G-CSF is 19,6 kD. G-CSF may be produced by monocytes, endothelial cells and fibroblasts (table 1.3).

Initially G-CSF was considered a growth factor with pluripotent activity, hence it was named pluripoietin.[339] Later it became apparent that pluripoietin and G-CSF were identical.[291] It is now clear that G-CSF is mainly a late-acting, lineage restricted growth factor, stimulating the outgrowth of neutrophilic colonies.[200,203,231,288,357] However there is evidence indicating that G-CSF may also influence immature cells in vitro. G-CSF for instance synergizes with IL-3 in the stimulation of blast cell colony formation in mice,[124] an observation which was later extended to humans.[223] These findings however await further confirmation.

Mature neutrophils express high-affinity G-CSF receptors[9,16] and show increased oxidative metabolism in response to agonists like FMLP.[9,217] Furthermore interferon-α is produced by neutrophils after stimulation with G-CSF.[269]

table 1.3 G-CSF producing cells

cell type	stimulus for G-CSF production	references
monocytes	LPS	161, 275, 320
	lectins	227
	IL-4	340
	GM-CSF or IL-3	228
endothelium	IL-1	29, 88, 261, 275
	TNF	30, 261, 275
fibroblasts	IL-1	86, 275
	TNF	144, 275

1.2.4 Macrophage colony-stimulating factor (M-CSF)

The cloning of the murine M-CSF gene[56] was accomplished somewhat later than that of the human equivalent.[138,246,346] Subsequent research revealed that M-CSF mRNA exists as multiple species ranging from 1.5-4 kilobases.[247] M-CSF is provided by monocytes/macrophages which are at the same time the prime targets for M-CSF. Other M-CSF producers are endothelial cells, fibroblasts and keratinocytes (table 1.4). Possibly M-CSF is produced constitutively, i.e. without external stimulation, by monocytes, endothelium and fibroblasts.[275]
Human M-CSF induces the formation of macrophage colonies from human hematopoietic progenitor cells[138,247] although not as efficiently as compared to the activity of murine M-CSF in mice.[52] In humans macrophage colonies are much more efficiently induced by M-CSF in the presence of small amounts of GM-CSF.[42]
The effects of human M-CSF on mature cells are confined to the monocyte-macrophage lineage. M-CSF induces macrophages to produce interferon, TNF and IL-1[327] and it enhances antibody dependent cytotoxicity by macrophages in mice[214] and man.[209,356] Furthermore M-CSF induces the migration of monocytes via increased chemotaxis,[324] enhances monocyte survival in vitro[15] and increases the tumoricidal activity of murine and human monocytes.[248,258,300]

table 1.4 M-CSF producing cells

cell type	stimulus for M-CSF production	references
monocytes	phorbolesters	117, 119, 227, 249
	LPS	161
	IFN-γ	249
	TNF-α	226
	IL-3	320
	GM-CSF	118, 320
	IL-4	340
endothelium	IL-1	88, 261, 275
	TNF	261, 275
fibroblasts	IL-1	86, 275
	"activators"	80
keratinocytes	LPS	48

1.2.5.1 Other cytokines that affect hematopoiesis

Apart from the four classical hematopoietic growth factors IL-3, GM-CSF, G-CSF and M-CSF, other cytokines may influence hematopoiesis. These cytokines are listed in table 1.5. IL-6, IL-1 and TNF, which are part of the experimental work described in this thesis, will be briefly discussed.

table 1.5 **Non-classical growth factors affecting hematopoiesis**

cytokine	effect	references
SCF	stimulation of primitive hematopoiesis	32, 183, 194, 359
IL-4	co-stimulation with G-CSF; inhibition of IL-3 effect; proliferation of B-lymphocytes	57, 287, 340, 321
IL-5	stimulation of CFU-Eo	37, 54, 286
IL-7	proliferation of B- and T-lymphocytes	47, 95, 337
IL-8	chemotactic cytokine for neutrophils and T-lymphocytes	148, 186
IL-9	enhancement of BFU-E formation	116, 176, 352
IL-10	enhancement of proliferation of T- and B-lymphocytes and mast cells	206
IL-11	enhancement of proliferation of CFU-blast and CFU-Mega	234, 299, 312
TGF-ß	inhibition of BFU-E and CFU-GEMM, enhancement of CFU-GM formation	65, 225, 230, 289
PDGF	enhancement of BFU-E and CFU-GEMM formation	135, 199
IGF	enhancement of BFU-E and CFU-G formation	56, 195
IL-6	see § 1.2.6.2	
IL-1	see § 1.2.6.3	
TNF	see § 1.2.6.4	

1.2.5.2 Interleukin-6

IL-6 is a pleiotropic cytokine that exerts important effects on hematopoiesis (reviewed by Le & Vilcek[149]). Before the name interleukin-6 was accepted[243] the cytokine had been variously labelled interferon-ß2, B-cell stimulating factor-2,[263,264,307] hybridoma growth factor[317] and hepatocyte stimulating factor.[94] Weissenbach et al[336] first succeeded in isolating and cloning an IL-6 cDNA. Later several groups independently cloned the same gene.[27,104,113] The molecular mass of the IL-6 protein is 20,8 kD. IL-6 may be produced by fibroblasts, monocytes, endothelial and epithelial cells (table 1.6).

table 1.6 IL-6 producing cells

cell type	stimulus for IL-6 production	references
monocytes	serum LPS IL-1	1, 14, 120, 188, 308
fibroblasts	IL-1 TNF PDGF LPS	55, 145, 146, 188
endothelium	IL-1 TNF LPS	129, 189, 279
neutrophils	GM-CSF TNF LPS PMA	49
T-lymphocytes	PHA TPA	120
B-lymphocytes	SAC IL-4	120 281

IL-6 has been reported not to stimulate colony growth from human bone marrow[292] but also to induce low numbers of CFU-G[43] and even to inhibit G-CSF induced colony formation.[132] These

studies were done with bone marrow cells at different levels of enrichment. Most likely the presence of various amounts of accessory cells has influenced these dissimilar results.
A positive effect on megakaryocytopoiesis has been noted and has initially been interpreted as an indirect effect of IL-6.[33] Later evidence however showed that megakaryocytes produce IL-6 and express IL-6 receptors as well, suggesting that terminal megakaryocyte differentiation may be regulated by autocrine IL-6 stimulation.[109] In-vitro experiments demonstrated a synergistic effect of IL-6 on IL-3 induced megakaryocytic colony formation.[139]
IL-6 indirectly induces proliferation of immature blast colony forming cells. IL-6 has been reported to synergize with IL-3 in shortening the G_0-phase of the cell cycle of the CFU-blast.[157,222,223] Thus IL-6 activates the earliest stages of hematopoiesis.
IL-6 has profound effects on immune-effector cells. It induces B-lymphocyte terminal differentiation[141] and it provides an important signal for human T-cell proliferation and differentiation.[121] Furthermore IL-6 induces the synthesis of acute phase proteins in hepatocytes.[44] These activities illustrate the key role of IL-6 in the acute phase and immune response[250] initiating simultaneously immune-competent cells as well as hematopoiesis.

1.2.5.3 Interleukin-1 (IL-1)

IL-1 is a cytokine with diverse effects that are part of the response of the organism to inflammatory stress. Two forms of IL-1 (α and ß) exist and their genes have been cloned.[8,92,181] Both IL-1 isoforms bind to the same receptor[185] and they express very similar biological activity. The molecular weight of both IL-1α and IL-1ß is 17.5 kD. Many cell types have the ability to produce IL-1 (table 1.7). Noteworthy is the autostimulatory capacity of IL-1, i.e. IL-1 may induce IL-1 in several cell types. The multiple effects of IL-1 include effects on hematopoiesis and hematopoietic functions. IL-1 may augment the production of effector cells like neutrophils and monocytes. These effects of IL-1 are presumably indirect: IL-1 generally serves as an mRNA stabilizing agent for hematopoietic growth factor genes[12] and thus increases the production of G-CSF, GM-CSF, M-CSF and IL-6 in monocytes,[85,275,308] endothelium,[29,261,262,274,279,358] and fibroblasts.[54,86,137,160,188] Two research groups have reported the production of TNF by monocytes following IL-1 stimulation.[125,238]

table 1.7 **IL-1 producing cells**

cell type	stimulus for IL-1 production	references
monocytes	adherence	83, 90
	LPS	112
	IFNγ+IL-2	282
	GM-CSF	
	LFA-3, CD44 CD45	330
endothelium	LPS TNF IL-1	162, 167, 202, 324, 327
fibroblasts	TNF	150
	IL-1	187
smooth muscle	LPS IL-1	163, 328
keratinocytes	PMA	20
neutrophils	GM-CSF	165

The hematopoietic response to infection is amplified by the interplay between monocytes, endothelial cells and fibroblasts involving several positive feedback loops. Activated monocytes produce IL-1 that induces endothelial cells and fibroblasts to produce IL-1, GM-CSF and M-CSF. These cytokines in turn further stimulate monocytes to produce IL-1, as well as TNF, GM-CSF and M-CSF (figure 2). Thus multiple pathways permit IL-1 to stimulate hematopoietic progenitor cells indirectly. A negative effect is provided by IL-4 that inhibits IL-1 production by monocytes.[69,319,335] Initially it was thought that IL-1 has a direct stimulative effect on the CFU-blast. From subsequent investigations however it became likely that this indirect effect is mediated by IL-6 produced by cells accessory to progenitor cells in a fraction of enriched bone marrow cells.[157] Another indirect effect of IL-1 includes the induction of G-CSF production in enriched bone marrow progenitors.[259] IL-1 also induces CSA in bone marrow stroma.[87] These indirect effects of IL-1 apparently may influence the level of activity of hematopoiesis.

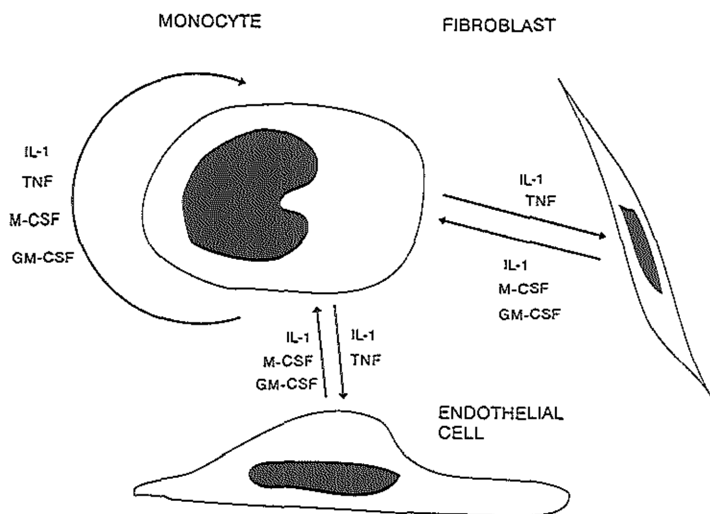

Figure 2. Interactions between monocytes, endothelium and fibroblasts.

1.2.5.4 Tumor necrosis factor (TNF)

TNF initially received interest because of its ability to induce necrosis in certain tumors (reviewed by Semenzato[266]). Afterwards the effects of TNF on hematopoiesis became apparent. TNF shows certain similarities to IL-1.[149] The major effects of TNF on hematopoiesis, alike those of IL-1, are achieved via the release of other cytokines that serve as intermediators of TNF activity (see below).

Two types of TNF have been described, TNF (formerly: cachectin), which is now called TNF-α, and lymphotoxin, now called TNF-ß. TNF-α and TNF-ß are structurally and functionally related.[236] There exact relationship has not been elucidated, however TNF-α and TNF-ß have differential activities in several systems.

Several groups have reported the cloning of the TNF-α cDNA[236,270,325] and TNF-ß c-DNA.[98] The non-glycosylated protein has a molecular weight of 17,300 Da. Cells that may produce TNF are listed in table 1.8. The monocyte is a prominent producer of TNF-α. TNF-ß is produced by activated T- and B-lymphocytes. TNF-α production in monocytes is inhibited by IL-6.[2,260]

Early experiments concerning the effects of TNF-α on hematopoiesis demonstrated a dose-

dependent inhibition of colony formation from erythroid, myeloid and mixed progenitor cells.[31,110,210,345] Most of this work has been done with unpurified bone marrow and also with crude conditioned media as the source of CSF. More recent work suggests that TNF-α and to a lesser extent TNF-ß preferentially inhibits CFU-G or the more mature myeloid progenitor cells.[211,212]

table 1.8 TNF producing cells

cell type	stimulus for TNF-production	references
monocytes	adherence LPS IL-3 IL-2 + IFNγ LFA-3, CD44 CD45	38, 39, 107, 112, 149 219, 329, 332
T-lymphocytes	PMA + αCD3 αCD3 + αCD28	294, 301, 360
B-lymphocytes	PMA SAC	295

Backx et al[11] and others[239] have demonstrated in detailed analysis that the effect of TNF-α in vitro depends on the type of colony stimulating factor used to generate growth. IL-3- and GM-CSF-induced BFU-E and CFU-Eo numbers are significantly enhanced when TNF-α is added to the cultures as well. On the other hand colony formation by Epo-induced BFU-E and G-CSF-induced CFU-G is inhibited by TNF-α. Comparable results were obtained by Caux et al[46] in cultures of CD34 positive cells. Thus it appears that TNF-α enhances the early stages of hematopoiesis and suppresses the mature stages.

TNF-α enhances monocyte-cytotoxicity,[39,238] enhances the neutrophil superoxide-anion production in response to FMLP[7,268] and augments eosinophil cytotoxicity.[277] TNF-α is a strong inducer of the release of M-CSF, G-CSF, IL-1 and TNF itself from monocytes,[173,226,238] GM-CSF, G-CSF, M-CSF and IL-1 from endothelial cells[28,30,162,167,208,218,261,273] and G-CSF, GM-CSF, IL-1 and IL-6 in fibroblasts.[144,145,150,208] TNF-ß, as TNF-α, induces growth factor production from fibroblasts.[360] PHA-activated T-lymphocytes produce GM- and G-CSF in response to TNF-α.[174] Generally TNF-α is a more active inducer of cytokines than is TNF-ß. This is apparent by the release of G-CSF by fibroblasts,[144] IL-1, IL-6 and CSF by endothelium[30,129,167] and IL-6 by

fibroblasts.[180] TNF-ß failed to induce M-CSF production from monocytes, where TNF-α did.[226] It has been suggested that TNF is produced constitutively in several tissues[310] and for this reason may have an important function in the steady-state regulation of hematopoiesis.

1.3 The objective of the study

We have used highly enriched hematopoietic progenitor cells and in-vitro culture to examine the following questions:

1. The effects of recombinant IL-3 and GM-CSF on proliferation and differentiation of enriched hematopoietic progenitor cells have not been clearly defined:
   - how do IL-3 and GM-CSF compare with respect to number and types of colonies induced?
   - to what extent do accessory cells influence colony formation induced by IL-3 and GM-CSF? (chapters 2 and 3)

2. The effects of recombinant G-CSF, M-CSF and IL-6 on enriched hematopoietic progenitor cells in connection with IL-3 and GM-CSF have not been fully elucidated:
   - what is the role of synergistic effects between G-CSF, M-CSF and IL-6 on the one hand and IL-3 and GM-CSF on the other hand, on the proliferation and differentiation of colony forming cells?
     (chapters 4, 5 and 6)

3. Consistent results on the effects of IL-1 on proliferation of immature bone marrow cells are lacking:
   - does IL-1 directly induce proliferation?
   - what is the role of GM-CSF and TNF as mediators of the IL-1 effect?
     (chapter 7)

Chapter 2

STIMULATING SPECTRUM OF HUMAN RECOMBINANT MULTI-CSF (IL-3) ON HUMAN MARROW PRECURSORS: IMPORTANCE OF ACCESSORY CELLS

Fredrik J Bot, Lambert Dorssers, Gerard Wagemaker

and Bob Löwenberg

Blood 71:1609-1614, 1988

Abstract

Recently, human multi-CSF was obtained by molecular cloning. In the present study, the effects of multi-CSF in vitro were investigated by comparative culture of whole marrow progenitor cells obtained by sorting the cell fraction that binds the monoclonal antibody (MoAb) BI3C5 (CD 34). Multi-CSF stimulated erythroid (BFU-E), multipotential (CFU-GEMM) and eosinophil (CFU-Eo) colonies in cultures of the progenitor cell enriched fraction, whereas (besides BFU-E, CFU-GEMM and CFU-Eo) granulocyte (CFU-G), granulocyte-macrophage (CFU-GM) and macrophage (CFU-M) colony-forming cells also were stimulated by multi-CSF when unfractionated bone marrow was cultured. Reconstitution of the progenitor cell fraction (BI3C5 positive) with the BI3C5 negative population restored the broad spectrum of progenitor cell stimulation. This suggested that accessory cells are required for expression of the full spectrum of progenitor cell stimulation by multi-CSF. Subsequently, specific marrow cell populations, including T-lymphocytes, granulocytic cells, and monocytes, were prepared by using selected MoAbs in complement-mediated lysis or cell sorting, added to cultures of hematopoietic progenitors and tested for accessory cell function. The results demonstrate that small numbers of monocytes permit the stimulation of CFU-G, CFU-GM and CFU-M by multi-CSF. These monocyte-dependent stimulating effects on CFU-G, CFU-GM and CFU-M could also be achieved by adding recombinant GM-CSF as a substitute for monocytes to the cultures. Therefore, multi-CSF most likely has direct stimulative effects on BFU-E, CFU-GEMM and CFU-Eo and indirect effects on CFU-G, CFU-GM and CFU-M in the presence of monocytes.

Introduction

The proliferation and differentiation of hematopoietic cells is regulated by specific growth factors.[197] The genes encoding several human hematopoietic growth factors have been molecularly cloned and expressed in suitable host systems to produce the recombinant growth factors.[35,138,213,288,347] The murine growth factor interleukin-3 (IL-3), has been described as a proliferative stimulus for early progenitors in the mouse, including the multipotential progenitors and those of granulocytes and macrophages, erythrocytes, eosinophilic granulocytes, megakaryocytes and mast cells.[70,105,122] Recently, a homologous human growth factor (multilineage colony-stimulating factor, multi-CSF) was discovered by cDNA cloning.[71,351] Human multi-CSF stimulates erythroid, myeloid and multipotential hematopoietic progenitor cells.[71] Hence, the spectrum of stimulation of this molecule resembles that of murine IL-3. In the experiments we present, we provide further insight into the stimulative effects of multi-CSF on human hematopoietic progenitor cells in vitro.

Materials and methods

Preparation of cell suspension. Bone marrow was obtained by posterior iliac crest puncture from hematologically normal adults who had given their informed consent. A separate donor was used for each experiment. The marrow was collected in Hanks' balanced salt solution (HBSS) with heparin, diluted in HBSS and layered over a Ficoll-gradient (1.077 g/cm^3; Nycomed, Oslo). After centrifugation, the mononuclear cells were harvested, washed twice in HBSS and resuspended in phosphate-buffered saline (PBS) with 2% heat-inactivated fetal calf serum (FCS).

Recombinant human CSFs. The preparation of recombinant human multi-CSF has been described in detail elsewhere.[71] In brief, mRNA was prepared from activated human lymphocytes and used for cDNA synthesis. The cDNA clone was identified by hybridization with mouse IL-3 cDNA. This cDNA was then inserted into a eukaryotic expression vector (pLB4) and transfected into monkey COS cells, which were then cultured for 48 to 72 hours. The resulting conditioned medium [COS(pLB4)CM} was used in bone marrow colony assay and is designated "multi-CSF". Medium conditioned by COS-cells transfected with the vector without the insert encoding multi-CSF did not stimulate colony formation in cultures of human bone marrow cells.[71] Recombinant human G-CSF[213,288] from Genetics Institute (Cambridge, MA) and recombinant GM-CSF[35,347] from Biogen SA (Geneva) were used at optimal concentrations of 1:1,000 and 1,000 U/mL, respectively.

Labeling and cell sorting. A cell sample was incubated with the monoclonal antibody (MoAb) BI3C5 (CD 34; Sera-lab, Crawley Down, England)[133] at a final dilution of 1:100 in PBS and 5% FCS for 30 minutes on ice. After being washed in PBS and 5% FCS, the cells were further incubated with goat-anti-mouse-FITC (GAM-FITC, Nordic, Tilburg, The Netherlands) at a dilution of 1:40 for another 30 minutes on ice. The cells were then washed twice and resuspended in PBS at a concentration of 10^6 nucleated cells/mL. Control cells were incubated with GAM-FITC alone. Analysis and cell sorting were performed under sterile conditions with a FACS 440 (Becton Dickinson, Sunnyvale, CA) at a maximum rate of 2,000 cells/second. The separation between positive and negative fractions was done so that the BI3C5 positive cell fraction regularly contained 3% to 4% of the total nucleated cell number. This resulted in a cell population enriched for blast cells and hematopoietic progenitors but depleted of mature erythroid and myeloid cells and T lymphocytes. in certain experiments, the BI3C5 negative fraction was incubated with VIM-2 (IgM, reactive with myelomonocytic cells; final dilution 1:50),[179] or T3 (IgG2, CD3, mature T lymphocytes; final dilution 1:10), or B4-3 (IgM, CD15, myeloid cells; final dilution 1:20)[237] and stained with GAM-FITC, after which the positive cell were sorted and added to the cultures. the number of these cells added to the enriched BI3C5 positive cells in culture reconstituted the original numerical proportions of the two cell populations in the Ficoll fractionated marrow specimen unless stated otherwise.

Complement mediated cytolysis. In several experiments, we used a different approach for the addition of subfractions from the BI3C5-negative cell fraction. After sorting, the BI3C5-negative cells were incubated with either MoAb VIM-2 or T3 at optimal concentrations (30 minutes on ice) and then incubated with rabbit complement at a final concentration of 40% (30 minutes at 25 °C) and washed twice. These VIM-2- or T3-depleted BI3C5-negative cells were then added to the appropriate cultures and tested for accessory abilities.

CFU-GEMM culture assay. Mixed colonies were grown as described before by Fauser and Messner with slight modifications.[5,81] Sorted or unsorted marrow cells were cultured in a 1 mL mixture of Iscove's modified Dulbecco's medium (IMDM), 1.1% methylcellulose, 30% autologous heparinized plasma, BSA, transferrin, lecithin, sodium-selenite and ß-mercaptoethanol. Cells were added at a concentration of $8x10^4$/mL in total marrow cultures and 0,2 to $0,5x10^4$/mL for the BI3C5-positive cell fractions. Exogenous growth stimuli were added in the form of various concentrations of multi-CSF and recombinant human erythropoietin (Epo) at a concentration of 1 U/mL (Kirin-Amgen, Thousand Oaks, CA). Cultures with 10% of a medium stimulated by leukocytes in the presence of 1% phytohemagglutinin (PHA-LCM),[10] as based on the original CFU-GEMM assay, were done for comparison. Dishes were incubated at 37°C and 100% humidity in an environment of 5% CO_2 in air. Colonies were scored at day 15 and identified by their distinct morphological appearance at 100x magnification. Numbers of colonies refer to the means of duplicate cultures. In selected cases, the nature of the colonies was verified cytologically after they had been plucked from the plates with a finely drawn Pasteur pipette, and stained with May-Grünwald-Giemsa. Mixed colonies were always verified cytologically. Megakaryocyte colonies were not assayed since CFU-Meg could not be detected reproducibly in every bone marrow sample. However, ± 15% of CFU-GEMM in the multi-CSF stimulated cultures contained megakaryocytes.

Results

Multi-CSF as a stimulator of purified hematopoietic progenitor cells. To minimize a possible interference of nonclonogenic accessory cells, we enriched progenitor cells from normal human bone marrow by sorting the cell fraction positive for MoAb BI3C5 (CD34), and cultured the cells at low cell concentrations (2 to $5x10^3$/mL) in the presence of multi-CSF. The results of a representative experiment are shown in Fig 1. When Epo was added as the only exogenous growth-stimulating factor some background erythroid bursts (BFU-E) were formed. BFU-E numbers rose markedly when graded concentrations of multi-CSF were added and reached a plateau at 1% to 3% (vol/vol) multi-CSF. In subsequent experiments, a concentration of 3% multi-CSF was used. Eosinophil (CFU-Eo) and multipotential (CFU-GEMM) colonies appeared in multi-CSF stimulated

cultures, and their numbers rose as a function of increasing concentrations of multi-CSF. When only multi-CSF but no Epo was added to the cultures, neither red nor mixed colonies appeared, whereas the number of eosinophil colonies remained constant. A remarkable number of eosinophil colonies was formed in the presence of multi-CSF with or without Epo. Multi-CSF did not stimulate significant numbers of granulocyte (CFU-G), granulocyte-macrophage (CFU-GM), or macrophage (CFU-M) colonies.

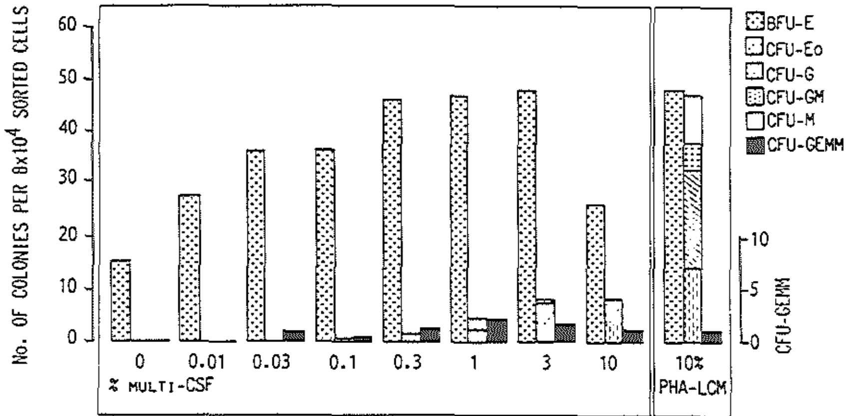

Fig 1. Colony formation in response to multi-CSF: culture of purified human hematopoietic progenitors. Numbers of erythroid, multipotential and four classes of myeloid colonies (Eo, G, GM, M) in vitro are plotted as a function of increasing concentrations of recombinant multi-CSF. The results from one representative experiment are shown. The BI3C5 (CD34)-positive fraction was obtained by cell sorting from 8×10^4 mononuclear marrow cells and represented 5% of the original cell population. Values represent mean colony counts of duplicate cultures. Epo (1 U/mL) was added to all cultures; in cultures without Epo and without multi-CSF no colonies appeared. Colony growth after stimulation with 10% PHA-LCM is shown for comparison. Data from one of duplicate experiments are shown.

The numbers of BFU-E obtained with multi-CSF were similar to those stimulated by a crude conditioned medium (PHA-LCM), but somewhat lower numbers of CFU-GEMM and CFU-Eo were obtained with multi-CSF than with PHA-LCM. In several experiments with a greater concentration of multi-CSF (10% vol/vol), the colony numbers, in particular those of BFU-E declined. This reduction suggests the presence of inhibitory factors in the multi-CSF preparation (COS-supernatant). Recently we used partially purified Escherichia Coli-derived multi-CSF in concentrations up to 30 times (expressed as activity) greater than the maximal concentration of COS-supernatant multi-CSF, and demonstrated no inhibitory effect. The pattern of stimulation, i.e. induction of colony formation from BFU-E, CFU-Eo and CFU-GEMM, was identical for both types of multi-CSF.

Role of accessory cells. To examine a possible role of BI3C5-negative accessory cells in the stimulatory effects of multi-CSF on: (a) a mock-sorted cell fraction, i.e., passed through the cell sorter without selecting for a specific marker (total marrow nucleated cells)(Fig 2A); (b) the sorted BI3C5-positive cell population (precursor cell fraction) (Fig 2B); and (c) the BI3C5-positive cell fraction supplemented with the number of BI3C5-negative cells that reconstituted the total marrow cell population (Fig 2C).

Multi-CSF appeared to stimulate not only BFU-E, CFU-GEMM and CFU-Eo but also CFU-G, CFU-GM, and CFU-M in unfractionated bone marrow cells (Fig 2A). This contrasts with the pattern of stimulation in the BI3C5-positive precursor cell fraction (Figs 1 and 2B): i.e., stimulation of BFU-E, CFU-GEMM and CFU-Eo only, but not the other myeloid colony-forming cells (CFU-G, CFU-GM, CFU-M) (Fig 2C).

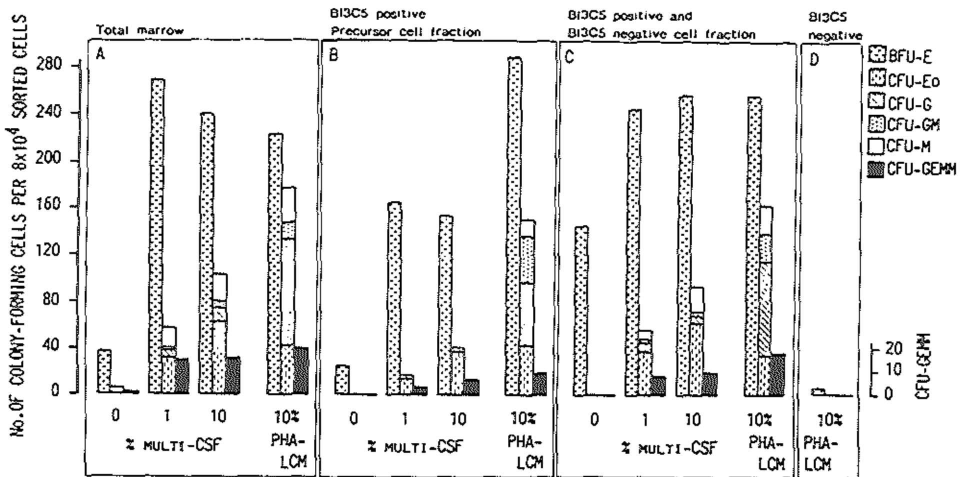

Fig 2. Colony formation in response to multi-CSF: direct comparison between total human bone marrow and purified progenitor cells. Different cell populations prepared from human marrow were cultured. (A) Total mononuclear marrow cells (separated on Ficoll-Isopaque) were mock-sorted and cultured at 8 x 10^4 cells/dish. (C) The sorted BI3C5-positive fraction was reconstituted with the original proportion of BI3C5-negative cells and then cultured. (D) The sorted BI3C5-negative fraction plated in culture with Epo plus PHA-LCM. Colony numbers are plotted as a function of stimulation with various concentrations of multi-CSF, i.e., 0%, 1% and 10% (vol/vol). Control cultures with 10% PHA-LCM were run in parallel; results are included in A through C. All cultures also contained Epo (1 U/mL). Cultures with no Epo and no multi-CSF did not support any colony growth (data not given). Data from one of duplicate experiments are shown.

The addition of BI3C5-negative cells to the BI3C5-positive cell fraction completely restored the stimulative effect of multi-CSF on CFU-G, CFU-GM and CFU-M. The BI3C5-negative cell fraction per se did not contain significant numbers of colony-forming cells (Fig 2D).

Because these experimental data suggested that CFU-G, CFU-GM and CFU-M colony formation in response to multi-CSF depended on the presence of BI3C5-negative cells or a subpopulation of these in culture, we subsequently examined the effect of the removal of specific subpopulations from this cell fraction using complement mediated cytolysis of VIM-2 (myelomonocytic) or T3 (mature T-lymphocytes) positive cells (Fig 3). VIM-2 lysis abrogated the augmentory effect of the BI3C5 negative cells whereas T3 lysis did not. These results suggested that the active accessory cells are VIM-2 positive and thus belong to the myelomonocytic lineage.

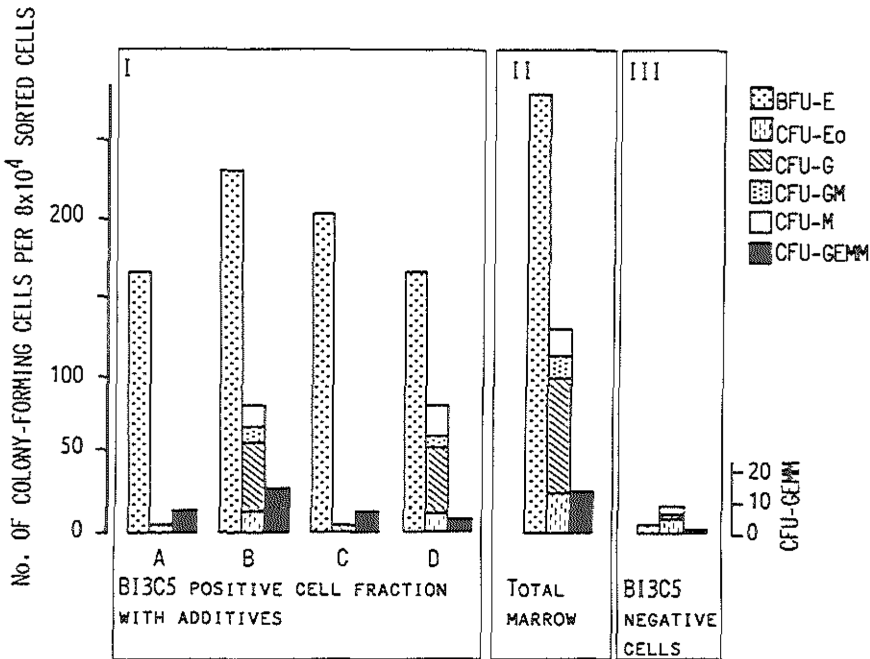

Fig 3. Colony formation in response to multi-CSF: addition of accessory cells to hematopoietic progenitor cells. Different subsets of cells were added in vitro and tested for accessory functions in cultures of the purified hematopoietic progenitor cell fraction (BI3C5 positive) that contained Epo and 3% multi-CSF. (I) Purified progenitor cell fraction (BI3C5 positive), 2.4×10^3 cells/dish with various cell supplements. (A) No accessory cells added. (B) Addition of BI3C5-negative cells (7.8×10^4 cells/dish). (C) Addition of BI3C5-negative cells depleted of VIM-2-positive cells following complement-mediated lysis. (D) Addition of BI3C%-negative cells depleted of T3-positive cells following complement-mediated lysis. Colony data from the total marrow population (II) and the BI3C5-negative fraction (III) are given for comparison. Data from one of duplicate experiments are plotted.

Following an alternative approach, specific VIM-2, T3 (mature T-lymphocytes), B44.1 (monocytes), and B4.3 (granulocytes) positive subsets were obtained from the BI3C5-negative cell fraction by cell sorting (Fig 4) and examined for their abilities to exert the accessory cell effects.

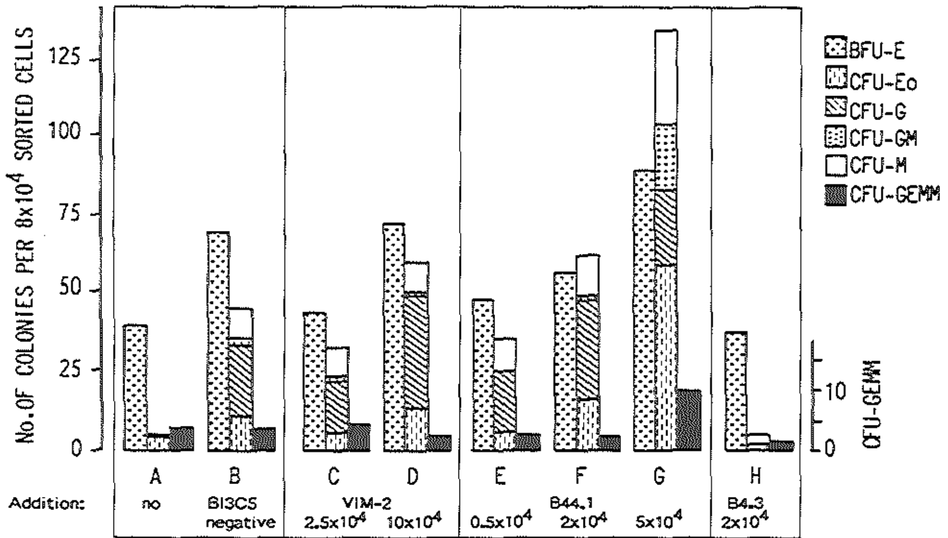

Fig 4. Colony formation in response to multi-CSF: effect of addition of different subpopulations of accessory cells. Purified hematopoietic progenitor cells (BI3C5 positive; 4 x 10^3 cells/dish) were plated with 3% multi-CSF and Epo. To these cultures VIM-2-positive, B44.1-positive, B4.3 positive or T3-positive cells were added and examined for enhancement of colony formation. The VIM-2-, B44.1-, B4.3- and T3-positive cells were separated by cell sorting from the BI3C5-negative cell fraction. (A) Progenitor cells with no addition of accessory cells. (B) Addition of BI3C5-negative cells (7.6 X 10^4 cells/dish). (C and D) Addition of VIM-2-positive cells (promyelocytes to granulocytes and monocytes) (C, 2.5 x 10^4 and D, 1 x 10^5 cells/dish). (E through G) Addition of the B44.1-positive cells (monocytes) in numbers that were originally present in the BI3C5 negative accessory cell population (E, 0.5 x 10^4 cells/dish) and increasing numbers (F, 2 x 10^4 and G, 5 x 10^4 cells/dish). (H) Addition of the B4.3-positive cells (metamyelocytes to granulocytes) in original numbers (2 x 10^4 cells/dish). Results of the supplemented T3-positive cells were identical to those of the B4.3-positive cells (not shown). Numbers of colonies grown from the separate VIM-2-positive, B44.1-positive, or B4.3 positive fractions were: BFU-E, 2-4-1; CFU-G, 0-1-0; CFU-Eo, 1-1-0; CFU-M, 0-1-0; CFU-GM, 1-0-0; CFU-GEMM, 0-0-0. Results of one of three repeat experiments are shown.

Supplementation of VIM-2 positive or B4.1-positive cells to the BI3C5-positive cell fraction restored the development of colonies originating from CFU-G, CFU-GM and CFU-M in cultures stimulated with multi-CSF. As few as 2.5×10^4 VIM-2 or 0.5×10^4 B44.1 surface marker positive cells were capable of enhancing colony growth. However 2×10^4 B4.3-positive granulocytes or T3-positive lymphocytes were ineffective. Indeed, CFU-GEMM, BFU-E and CFU-Eo were also slightly susceptible to the potentiating effect of monocytes, at least when large numbers of monocytes were added (Fig 4G).

We examined whether the effect of the addition of monocytes to cultures of the BI3C5-positive progenitor cell fraction to evoke CFU-G, CFU-GM, and CFU-M colony formation could be mimicked by supplementing exogenous GM-CSF or G-CSF (instead of monocytes) to the multi-CSF cultures (Fig 5).

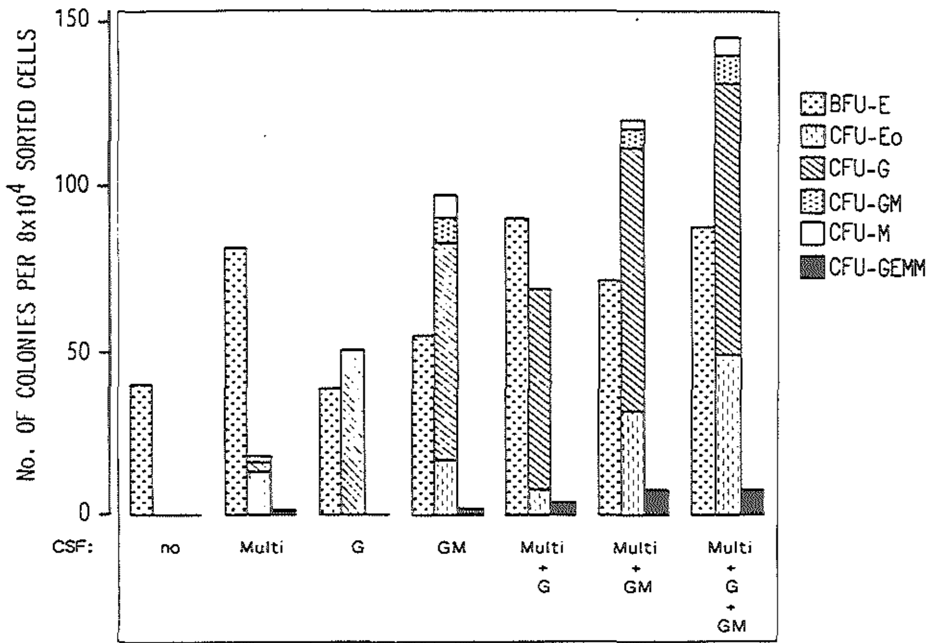

Fig 5. Colony formation from purified marrow cells in response to multi-CSF, G-CSF and GM-CSF. Hematopoietic progenitor cells (BI3C5 positive, 4.5×10^3 cells/dish) were plated with multi-CSF, G-CSF and/or GM-CSF; all cultures contained Epo. Results are those of one of two repeat experiments.

When G-CSF or GM-CSF were added to multi-CSF cultures of purified bone marrow progenitors, additional CFU-G or CFU-G, CFU-GM and CFU-M were induced to colony formation. There was no evidence for a synergistic effect between these factors. When all three factors (i.e., multi-CSF, GM-CSF and G-CSF) were included in culture, no significant further increase in colony formation

was seen above the level of multi-CSF plus GM-CSF stimulation. This indicates that multi-CSF and GM-CSF in conjunction provide optimal stimulation of CFU-G, CFU-GM and CFU-M from the BI3C5-positive progenitor cell fraction and that GM-CSF can substitute the monocyte accessory cell effect.

Discussion

We assessed the stimulating abilities of the recombinant human growth factor multi-CSF. In cultures of enriched human hematopoietic progenitor cells (based on BI3C5 reactivity), multi-CSF stimulated BFU-E, CFU-Eo and CFU-GEMM. In the presence of BI3C5-negative cells, the spectrum of multi-CSF stimulation was broader and also included CFU-G, CFU-GM and CFU-M. These data suggest a direct stimulative effect of multi-CSF on BFU-E, CFU-Eo and CFU-GEMM and an effect on CFU-G, CFU-GM, and CFU-M in the presence of a secondary cell. Experiments based on the addition of specific subsets of cells (selected by cell sorting) as well as elimination of these subsets from the accessory cell fraction (by complement mediated lysis) identified a VIM-2 and B44.1 surface marker-positive cell population as the active accessory cell. The accessory cell function could not be attributed to the subsets that expressed the T-lymphocyte T3 or granulocytic B4.3 markers. The VIM-2 and B44.1-positive surface phenotype of the accessory cells demonstrates the monocytic identity of the cells. It became apparent that the accessory cell phenomenon was cell dose dependent and that minimal numbers (0.5×10^4 per dish) of these monocytic cells were sufficient to allow the outgrowth of CFU-G, CFU-GM and CFU-M in multi-CSF stimulated cultures. The addition of four times the proportional number of VIM-2-positive cells to purified progenitors further elevated GM and M colony numbers, and also raised the number of BFU-E, CFU-Eo, and CFU-GEMM. Three possible mechanisms through which monocytes enhance growth of these colony types stimulated by multi-CSF can be proposed: (a) production of growth factors (e.g. GM-CSF or M-CSF) by monocytes in response to multi-CSF, (b) production of growth factors by monocytes independent of multi-CSF, and (c) cell-cell interactions between monocytes and colony-forming cells. The present experiments do not allow a distinction between the alternative mechanisms for the enhancement of CFU-G, CFU-GM and CFU-M colony growth. The possibility that monocytes produce growth factors that can induce CFU-G, CFU-GM, and CFU-M would at least be consistent with the observation that monocytes can be stimulated to produce colony-stimulating factors.[207] More recently, specific evidence was obtained demonstrating that monocytes may produce M-CSF, interferon (IFN), tumor necrosis factor (TNF) and GM-CSF.[118,329] This could explain our results showing that the addition of recombinant GM-CSF could fully substitute the monocyte effect and that G-CSF could partly replace the role of the monocytes in the multi-CSF cultures, thereby resulting in the induction of G, GM and M colony types from purified marrow progenitor cells.

Chapter 3

HUMAN GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR (GM-CSF) STIMULATES IMMATURE MARROW PRECURSORS BUT NO CFU-GM, CFU-G OR CFU-M.

Fredrik J Bot, Loes van Eijk, Pauline Schipper
and Bob Löwenberg

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Abstract

Human GM-CSF has been described as a multilineage growth factor that induces in vitro colony formation from BFU-E, CFU-Eo and CFU-GEMM as well as from CFU-GM, CFU-G and CFU-M. In this paper we provide evidence indicating that GM-CSF, when tested for its stimulating capacities expressed upon highly enriched hematopoietic progenitor cells (CD34+/monocyte depleted), is unable to induce colonies from CFU-GM, CFU-G or CFU-M. Only BFU-E, CFU-Eo and CFU-GEMM were stimulated, and thus GM-CSF induces a similarly restricted spectrum of progenitor cells as does recombinant human interleukin-3 (IL-3). We then compared the relative stimulating potencies of GM-CSF and IL-3 by measuring colony numbers of CFU-GEMM, BFU-E and CFU-Eo generated from CD34+ progenitor cells. IL-3 and GM-CSF as single factors were equally active in stimulating CFU-GEMM but the combination of both factors produced additive stimulative effects upon CFU-GEMM. IL-3 was a more potent stimulus of BFU-E and GM-CSF was the more active stimulating factor for CFU-Eo. We conclude that GM-CSF and IL-3 although stimulating the outgrowth of identical types of progenitor cells particularly differ as regards their comparative quantitative efficiency of stimulation.

Introduction

In recent years recombinant human GM-CSF has been defined as a growth factor that induces colony formation in vitro from hematopoietic progenitor cells of the erythroid (BFU-E), eosinophilic (CFU-Eo), granulocyte-macrophage (CFU-GM), granulocytic (CFU-G), macrophage (CFU-M) and multipotential (CFU-GEMM) lineages.[35,198,271,306] Consequently GM-CSF is regarded as a multilineage growth factor. It has also been shown that GM-CSF affects the function of mature granulocytic cells[168] and possibly also modifies the production of growth factors by these cells[166] as well as by monocytes.[118] Therefore it is possible that the reported stimulative effects of GM-CSF on hematopoietic colony formation depend upon a mixture of direct effects on the progenitor cells and indirect effects mediated through the action of GM-CSF on mature cells with auxiliary stimulating functions. We set out to define more precisely the stimulating abilities of GM-CSF, using populations of highly enriched human marrow progenitor cells. The progenitor cell targets of GM-CSF were defined and directly compared to those of the other human multilineage colony stimulating factor, i.e. IL-3.[21,71]

Materials and methods

Preparation of cell suspension: Bone marrow was obtained by posterior iliac crest puncture from hematologically normal adults, which had given their informed consent. The marrow was collected in Hanks balanced salt solution (HBSS) with heparin and the mononuclear cells were separated over Ficoll-Isopaque (1.077 g/cm^2; Nycomed, Oslo, Norway) as described.[21]

Enrichment of progenitor cells: In 7 experiments cells labeled with a monoclonal antibody (MoAb) against the CD34 antigen (BI3C5, IgG1; Sera-Lab Crawley Down, UK[133]) were obtained following fluorescence activated cell sorting. The CD34 antigen is expressed on less than 5% of bone marrow cells and this fraction contains virtually all, i.e. more than 90% of blasts and in vitro colony forming cells,[133] and is referred to as CD34+ cells. The labeling and cell sorting procedures have been described.[21]

In 14 experiments the progenitor cells were further enriched following an additional separation step: contaminating monocytes were removed by complement mediated cytolysis preceding CD34+ sorting. The cells (20x10^6/ml in PBS with 5% heat inactivated FCS) were incubated with the MoAb VIM-2 (IgM, reactive with myelo-monocytic cells;[179] final dilution 1:50) or B44.1 (IgM, CD14, monocytes;[237] final dilution 1:1000) for 30 min. on ice, then incubated with rabbit complement (40%) for another 30 min. at 25°C and washed twice. This procedure resulted in a recovery of 51±13% of nucleated cells. Following complement mediated cytolysis the CD34+ cells were sorted as described above. The final cell population, designated as CD34+/Mono-, comprised 2-5% of the original Ficoll-Isopaque separated cell fraction and contained less than 1% monocytes as checked by a differential count on a May-Grünwald-Giemsa stained cytospin preparation.

In four separate experiments we focussed on the recoveries of the myeloid colonies throughout the subsequent enrichment steps in order to ensure that no specific loss of myeloid progenitor cells occurred. This was done by culturing (A) ficoll fractionated bone marrow cells, (B) monocyte-depleted cells and (C) CD34+/Mono- cells in direct comparison, stimulated with either PHA-LCM, GM-CSF or IL-3. The colony numbers obtained from each fraction were related to the numbers obtained in the ficoll fraction, in order to assess the progenitor cell recoveries.

Recombinant human CSFs: Recombinant human GM-CSF was a highly purified E.Coli product and a kind gift from Dr JF DeLamarter (Biogen SA, Geneva, Switzerland[35]). A dose-response curve was made and the optimal concentration (1000 U/ml) was used. In one experiment that was designed to directly compare the stimulating abilities of two GM-CSF preparations we employed another GM-CSF product (expressed in CHO cell-line and used at 1000 U/ml; kindly provided by Dr. S. Clark, Genetics Institute, Cambridge, USA) and observed an identical spectrum of activity upon CD34+/Mono- cells (data not shown). Recombinant human IL-3, expressed in monkey COS-cells,

was generously donated by Drs L. Dorssers and G. Wagemaker (Radiobiological Institute TNO, Rijswijk, The Netherlands[21,71]) and used at 10 U/ml.[59] Recombinant human erythropoietin (Epo) was purchased from Amgen Biologicals (Thousand Oaks, California) and used at 1 U/ml.

Colony culture assay: The enriched progenitor cells ($0.5\text{-}1 \times 10^4$) were cultured in a 1 ml mixture of Iscove's modified Dulbecco's medium (IMDM), 1.1% methylcellulose, 30% autologous heparinized plasma, BSA, transferrin, lecithin, sodium-selenite and 2-mercapto-ethanol exactly as described before.[21] Epo and GM-CSF and/or IL-3 were supplemented to the cultures as growth stimuli. PHA-LCM was added to control cultures to apply an impure but rich source of CSFs as in the original CFU-GEMM assay.[81] A constant batch of PHA-LCM[21] was used throughout these studies and was added along with Epo at a concentration of 10%. Colonies of more than 50 cells were scored at day 15. Duplicate cultures were set up for each point. Colonies were identified at a 100x magnification. Regularly the colony morphology was checked by examining MGG stained cytospin preparations of plucked single colonies. In selected experiments we stained granulocytic colonies with α-naphthyl-esterase and found that they did not contain monocytes.

Results

Effect of GM-CSF on purified marrow progenitors: The numbers of colonies stimulated in vitro by GM-CSF from CD34+ enriched progenitor cells (n=7 experiments) and CD34+/Mono- cells (n=14 experiments) are presented in table 1. For comparison the results obtained after stimulation with PHA-LCM are also given. Apparently from highly enriched (CD34+/Mono-) progenitor cells GM-CSF stimulates BFU-E, CFU-Eo and CFU-GEMM selectively, but not CFU-GM, CFU-G nor CFU-M. The inability of GM-CSF to induce CFU-GM, CFU-G and CFU-M held up for supraoptimal concentrations of GM-CSF (up to 5000 U/ml). PHA-LCM stimulation confirmed that colonies from CFU-GM, CFU-G and CFU-M could be generated from the stem cell concentrates indicating that these progenitor cells had been retained following the one-step (CD34+) or two-step (CD34+/Mono-) cell separation procedures. None or only minimal numbers of CFU-G and CFU-M and no CFU-GM could be induced to colony formation by GM-CSF from the highly enriched (CD34+/Mono-) progenitor cells. GM-CSF stimulation of the CD34+ (one step separation) cells resulted in intermediate numbers of CFU-GM, CFU-G and CFU-M.

Table 1 GM-CSF compared to PHA-LCM stimulation of CD34+ or CD34+/Mono- enriched progenitor cells

	CD34+ marrow fraction		CD34+/Mono- marrow fraction	
	GM-CSF	PHA-LCM	GM-CSF	PHA-LCM
BFU-E	65 ± 42	99 ± 62	53 ± 38	104 ± 68
CFU-Eo	21 ± 11	19 ± 11	18 ± 14	19 ± 17
CFU-GEMM	5 ± 3	7 ± 5	5 ± 5	6 ± 5
CFU-G	23 ± 22	25 ± 9	2 ± 2	17 ± 12
CFU-M	6 ± 8	21 ± 15	2 ± 4	16 ± 15
CFU-GM	3 ± 4	7 ± 3	0	6 ± 6

CD34+ cells or monocyte depleted CD34+ cells (CD34+/Mono-) were cultured in the presence of Epo and GM-CSF or PHA-LCM. Colony numbers are expressed per 5000 plated cells and are mean values ± SD of 7 experiments (CD34+) or 14 experiments (CD34+/Mono-). In the absence of growth factors, no colony growth occurred.

Comparison between the stimulating spectrum of IL-3 and GM-CSF:

We next assessed the stimulating abilities of GM-CSF in direct comparison to those of IL-3 or GM-CSF plus IL-3 on purified progenitor cells (table 2).

The numbers of CFU-GEMM induced by IL-3 and GM-CSF were not significantly different. The effects of the two factors were additive in 6 of 7 experiments with CD34+ cells and in 12 of 14 experiments with CD34+/Mono- cells, i.e., the combination of GM-CSF and IL-3 stimulated more CFU-GEMM than did GM-CSF or IL-3 alone. As regards BFU-E: IL-3 gave rise to more BFU-E in all cultures of CD34+ cells or CD34+/Mono- cells than did GM-CSF. Stimulation with IL-3 plus GM-CSF did not elevate BFU-E numbers above the level obtained with IL-3 alone. Thus it appears that IL-3 is the most active stimulus of erythroid colony formation and can trigger all BFU-E, including those responsive to GM-CSF.

On the other hand, IL-3 was less potent in stimulating CFU-Eo, as greater numbers of CFU-Eo arose in response to GM-CSF. This was evident in all seven experiments with CD34+ cells and in 12 of the 14 experiments with CD34+/Mono- cells. However, IL-3 enhanced the CFU-Eo stimulating

effect of GM-CSF as the combination of GM-CSF plus IL-3 stimulated more CFU-Eo in 20 of the 21 cumulated experiments than did either factor alone.

Table 2 Comparison between stimulating efficiency of IL-3 and GM-CSF upon purified marrow progenitors.

	GM-CSF	IL-3		IL-3 + GM-CSF	
	(no.)	(no.)	(%)	(no.)	(%)
BFU-E	57 ± 39	91 ± 53	179 ± 40	84 ± 52	162 ± 34
CFU-Eo	19 ± 13	15 ± 12	81 ± 25	32 ± 20	181 ± 49
CFU-GEMM	5 ± 5	4 ± 2	98 ± 47	8 ± 6	220 ± 116

CD34+ cells or monocyte depleted CD34+ cells (CD34+/Mono-) were cultured in the presence of GM-CSF (first column), IL-3 (second column) or GM-CSF plus IL-3 (third column). All cultures contained Epo. Since the results from both populations of progenitor cells were identical, they were pooled. The results are presented as the mean±SD of the colony numbers from 21 experiments. Within each experiment the colony numbers obtained after stimulation with IL-3 or GM-CSF plus IL-3 were expressed as a percentage of the colony numbers generated by GM-CSF. The mean ± 99% confidence limits (Students t-test) of these percentile values are given ("%" columns).

Comparison of purification procedures: To exclude whether specific loss of myeloid progenitor cells had occurred following the subsequent steps of the enrichment procedure, we compared the growth stimulating abilities of PHA-LCM, GM-CSF and IL-3 on ficoll fractionated marrow cells, on monocyte depleted cells and on the CD34+/Mono- fraction (fig.1). These results confirm the inability of GM-CSF and IL-3 to induce most myeloid colony forming cells from CD34+/Mono- cells. The moderate decrease of CFU-Eo numbers suggests some aspecific loss. However PHA-LCM stimulation shows that CFU-G, CFU-Eo, CFU-M and CFU-GM had been retained in the highly enriched cell fractions.

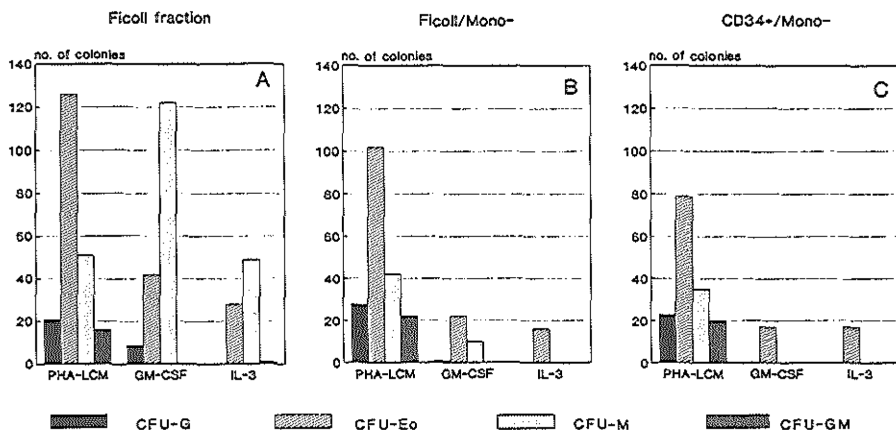


Figure 1. Recovery of clonogenic cells following enrichment: Increasingly enriched bone marrow cells were cultured in the presence of PHA-LCM, GM-CSF or IL-3. Ficoll fractionated cells (panel A) were cultured at a cell concentration of 0.75×10^5 per ml. Exactly the same cell portions were cultured following monocyte depletion (panel B) or monocyte depletion followed by CD34+ sorting (panel C) with no correction for removal of cells. Colony recoveries from CFU-G, CFU-Eo, CFU-M and CFU-GM in panels A, B and C are therefore directly comparable.

Discussion

In this paper we present evidence that human GM-CSF stimulates selectively BFU-E, CFU-Eo and CFU-GEMM and, to a very limited extent, CFU-G and CFU-M, when assayed with highly enriched progenitor cells, i.e. the CD34 positive fraction of monocyte depleted human bone marrow cells (CD34+/Mono-). When however progenitor cells are used which are concentrated to a lesser degree, i.e. the CD34 positive fraction without prior monocyte depletion, not only BFU-E, CFU-Eo and CFU-GEMM but also moderate numbers of CFU-GM, CFU-G and CFU-M were induced to colony formation by GM-CSF. Thus relatively minor alterations in the composition of the cell suspension plated may significantly influence the proliferative outcome in vitro. Although the name designates GM-CSF as a major growth factor for granulocyte-macrophage progenitors, these data support the idea that GM-CSF rather stimulates the outgrowth of multipotent, erythroid and eosinophil progenitors. In this sense the progenitor target cell spectrum of GM-CSF is strikingly similar to that of IL-3.

The culture results demonstrating the limited spectrum of stimulation of GM-CSF are not artifacts, since recovery experiments have shown that CFU-G, CFU-M and CFU-GM had not been eliminated from the highly enriched cell fractions. As a matter of fact, significant numbers of these progenitors can be demonstrated in the CD34+/Mono- cell fractions when PHA-LCM, a potent mixture of growth factors, is added to the cultures.

The lack of stimulating effects of GM-CSF exerted upon CFU-GM, CFU-G and CFU-M, as noted

here, is not at all in agreement with results concerning the in vitro stimulative activities of human GM-CSF that have been published until today.[35,198,271,306] Stimulation by GM-CSF of CFU-Eo, CFU-GM, CFU-G and CFU-M has been a common observation in previous studies. The target cell populations plated in culture in those studies varied and represented "unfractionated",[198] or "non adherent light-density" bone marrow cells,[35,306] or progenitor cells that were depleted of accessory cells using several MoAbs and adherence to Ig-coated plates.[271] Based upon the observations of this paper, indicating that CD34 positive cells may still be contaminated by accessory cells which significantly modify the stimulating spectrum, we believe that most likely the different conditions of enrichment and the variable stem cell purity resulting from these procedures can explain the different colony responses to GM-CSF in those and our reports. The mechanism by which GM-CSF can only stimulate significant numbers of CFU-GM, CFU-G and CFU-M in the presence of accessory cells was not analyzed. A similar contaminating cell phenomenon has been well established for urinary CSF[207] as well as for human IL-3.[21] In reconstitution experiments with different subpopulations the monocytes were identified as the major auxiliary cells of IL-3. Monocytes, when present in small numbers, may extend the stimulating spectrum of IL-3 to include also CFU-GM, CFU-G and CFU-M. In a similar experimental approach we noted that GM-CSF would also stimulate the latter types of progenitor cells when B44.1 positive monocytes or B4.3 positive myeloid cells,[317] but not T-lymphocytes were supplemented to the cultures (data not shown).

Two recent studies with highly enriched murine progenitor cells[339,340] show murine GM-CSF to be a potent stimulator of murine CFU-GM. Thus our data suggest an as yet not noted difference in the stimulating spectrum of GM-CSF in vitro between mouse and man. The basis of this difference cannot readily be explained.

As regards the colony stimulating abilities of GM-CSF and IL-3 it is evident that both factors act on CFU-GEMM, BFU-E and CFU-Eo. This similarity in stimulating capacities of GM-CSF and IL-3 led us to compare the relative stimulating potencies of these growth factors. CFU-GEMM numbers induced by IL-3 or GM-CSF from purified marrow cells were found to be equivalent but increased when both IL-3 and GM-CSF were supplemented to the cultures. Greater numbers of BFU-E were stimulated by IL-3 than by GM-CSF. Combined stimulation by IL-3 and GM-CSF did not increase the BFU-E numbers beyond those stimulated by IL-3 alone. This suggests that those BFU-E which are responsive to GM-CSF are stimulable by IL-3 as well and that IL-3 recruits an additional BFU-E population into colony growth. GM-CSF is more potent than IL-3 in stimulating eosinophil progenitor cells and the combination of the two factors further enhances CFU-Eo growth. This supports the notion that subpopulations of CFU-Eo exist that are susceptible to stimulation by either IL-3 or GM-CSF.

These studies emphasize the importance of very stringent cell purification when growth factor effects upon hematopoietic progenitor cells per se are to be defined, as major changes of the target cell spectrum may result from small admixtures of accessory cells.

Chapter 4

EFFECTS OF HUMAN INTERLEUKIN-3 ON GRANULOCYTIC COLONY FORMING CELLS IN HUMAN BONE MARROW

Fredrik J Bot, Loes van Eijk, Pauline Schipper and Bob Löwenberg

Blood 73:1157-1160, 1989

Abstract

Human multilineage colony stimulating factor (multi-CSF/IL-3) induces colony formation from CFU-GEMM, BFU-E and CFU-Eo when applied to in vitro cultures of highly enriched hematopoietic progenitor cells. No granulocytic colonies are formed in response to IL-3. However, using appropriate assays we demonstrate here that IL-3 increases the size of G-CSF induced granulocytic colonies; these colonies contain greater proportions of immature cells as compared to colonies stimulated by G-CSF alone. Furthermore, IL-3 promotes the survival of CFU-G in vitro, whereas in cultures not supplemented with IL-3 CFU-G extinguishes within 7 days. We conclude that IL-3, although by itself not stimulating granulocytic colony formation, regulates the survival and proliferative rate of granulocytic progenitors.

Introduction

We have recently reported that human recombinant interleukin-3 (IL-3) stimulates multilineage progenitor cells (CFU-GEMM), erythroid progenitor cells (BFU-E), and the eosinophilic progenitor cells (CFU-Eo) when added to enriched human hematopoietic progenitor cells in vitro.[21,71] No stimulatory effect of IL-3 was apparent on the granulocytic (CFU-G), the granulocyte-macrophage (CFU-GM) and the macrophage (CFU-M) progenitor cells, when colony numbers were taken into account.

Here we report experiments that demonstrate stimulative effects of IL-3 exerted on CFU-G. Using highly purified marrow progenitors we show that IL-3 promotes the survival of CFU-G in vitro, increases the size of colonies stimulable by G-CSF[213,288] (granulocytic colony stimulating factor) and modifies the composition of these granulocytic colonies.

Materials and methods

Preparation of cell suspension: Bone marrow was obtained by posterior iliac crest puncture from hematologically normal adults, which had given their informed consent. The marrow was collected in Hanks balanced salt solution (HBSS) with heparin and the mononuclear cells were separated over Ficoll-Isopaque (1.077 g/cm^2; Nycomed, Oslo, Norway) as described.[21]

Enrichment of progenitor cells: Since monocytes can significantly influence IL-3 stimulated colony formation from hematopoietic progenitor cells in vitro,[21] monocytes were rigorously removed from the cell suspension utilizing complement mediated cytolysis with the monoclonal antibody (MoAb)

VIM-2 (IgM, reactive with myelo-monocytic cells;[179] final dilution 1:50) or anti-CD14 (MoAb B44.1, IgM, monocytes;[237] final dilution 1:1000) exactly as described.[21] This procedure resulted in an average recovery of 58±18% of nucleated cells. Subsequent enrichment was achieved following fluorescence activated sorting of the cells expressing the CD34 antigen (MoAb BI3C5, IgG-1; Sera-Lab, Crawley Down, UK[133]). Labeling and cell sorting were performed as described before.[21] The final cell population (CD34+ Mono- progenitor cells) contained 2-3% of the original ficoll separated cells and consisted of immature blast cells with less than 1% monocytes as evaluated by a differential count on May-Grünwald-Giemsa stained cytospin preparations. The recoveries of clonogenic cells following the purification procedures were always assessed: in all experiments control cultures with an impure CSF source (PHA-LCM) were included in order to verify that the diverse progenitor cells had not been selectively eliminated during the cell separation procedures. This method of enrichment resulted in a recovery of all types of progenitor cells, including CFU-G, of 80 to 100% of the number of progenitors present in the ficoll fraction (based upon PHA-LCM stimulation). There was an average 40x enrichment of CFU-G.

Recombinant human CSFs: The preparation and biological effects of recombinant human IL-3 have been described.[21,71] It was generously donated by Drs L. Dorssers and G. Wagemaker (Radiobiological Institute TNO, Rijswijk, The Netherlands). Two preparations of IL-3 (at 10 U/ml) either expressed in COS-cells or E.Coli gave identical results. One U was defined as giving half-maximal stimulation in a clonogenic assay of a particular case of AML that was absolutely dependent on the exogenous supply of IL-3, as described.[59] Recombinant human G-CSF was a medium conditioned by a Chinese hamster ovary (CHO) cell line expressing the human G-CSF gene. It was a gift from Dr S. Clark (Genetics Institute, Cambridge, MA) and was used at a concentration of 1:1,000 or as indicated. Recombinant human erythropoietin was purchased from Amgen Biologicals (Thousand Oaks, California) and used at 1 U/ml.

Colony assay: The CD34+ fraction sorted from $8x10^4$ monocyte depleted cells, i.e. real cell number plated: $0.4\text{-}1x10^4$/ml, was cultured in a 1 ml mixture of Iscove's modified Dulbecco's medium (IMDM), 1.1% methylcellulose, 30% autologous heparinized plasma, BSA, transferrin, lecithin, sodium-selenite and 2-mercapto-ethanol as reported.[21] Exogenous growth stimuli were added as Epo plus G-CSF with or without IL-3. In selected cultures the addition of G-CSF was delayed for 7 or 10 days; G-CSF was then carefully dispersed over the culture layer in a volume of 0.050 ml and allowed to diffuse through the medium. Colonies of more than 50 cells were scored at day 15 following plating and identified according to their appearance at a magnification of 100x. In selected experiments the morphology of single granulocytic colonies was further determined (see below).

Determination of colony size and morphology: In certain experiments the colony size was determined as follows: six groups of 5 colonies were randomly plucked from the plates using a finely drawn Pasteur pipette and pooled in 0.050 ml PBS, the nucleated cells per group were then counted to give the average cell number per colony. Thus each value represents the mean estimate of 6 collections of five colonies. To determine the morphology these pooled colonies were put onto slides in a cytocentrifuge and stained using May-Grünwald-Giemsa (MGG) stain. A differential count was then performed on 200 cells. In selected experiments separate granulocytic colonies were picked from the plates, stained with non-specific esterase and found to be devoid of monocytes.

Results

IL-3: effects on monocyte depleted CD34+ progenitor cells. In table 1 we present the results from 34 experiments demonstrating that from monocyte depleted CD34+ enriched progenitor cells IL-3 stimulates BFU-E, CFU-Eo and CFU-GEMM. The other progenitor cells CFU-G, CFU-M and CFU-GM could only be stimulated by a mixture of growth factors as PHA-LCM, indicating that they had not been removed from the cell suspension during the enrichment procedure.

Table 1 Effect of IL-3 on enriched CD34+ progenitor cells.

	IL-3	PHA-LCM
BFU-E	169 ± 99	209 ± 130
CFU-Eo	20 ± 13	32 ± 18
CFU-GEMM	6 ± 3	9 ± 4
CFU-G	< 1	36 ± 21
CFU-M	< 1	30 ± 16
CFU-GM	< 1	9 ± 5

The CD34+ fraction of $8x10^4$ monocyte depleted cells (absolute number 0.4 - $1x10^4$) was cultured. IL-3 plus Epo or PHA-LCM plus Epo were used as colony stimulating factors. The mean number of colonies per dish (±SD) from 34 experiments is given.

IL-3: effect on numbers of G-CSF stimulated colonies. In 10 experiments using bone marrow samples from different normal subjects the numbers of granulocytic colonies induced from the

enriched progenitor cell fraction after stimulation with G-CSF alone (median 81 CFU-G per $8x10^4$ sorted cells; range 38-163) were directly compared to those following induction with G-CSF plus IL-3 (median 66 CFU-G per $8x10^4$ sorted cells; range 53-164). Although some variation in colony numbers was evident between different experiments, no significant difference appeared between CFU-G numbers induced by G-CSF and G-CSF plus IL-3.

IL-3: effect on size and cellular composition of G-CSF stimulated colonies. In 3 experiments the size and cellular composition of the granulocytic colonies induced by G-CSF at concentrations from 1:30,000 to 1:300 in the absence or presence of IL-3 (10 U/ml) were determined. The average numbers of cells per colony of 3 experiments are plotted in fig.1. The granulocytic colonies induced by G-CSF in the presence of IL-3 were significantly larger than those grown with G-CSF alone. The data of the morphological analysis of these colonies are plotted in Fig. 2. The colonies stimulated by G-CSF alone (fig.2 panels A1 and A2) mainly contain mature granulocytic cells (band forms and segmented cells), whereas the IL-3 plus G-CSF stimulated colonies (panels B1 and B2) show greater proportions of immature (promyelocytes) cells.

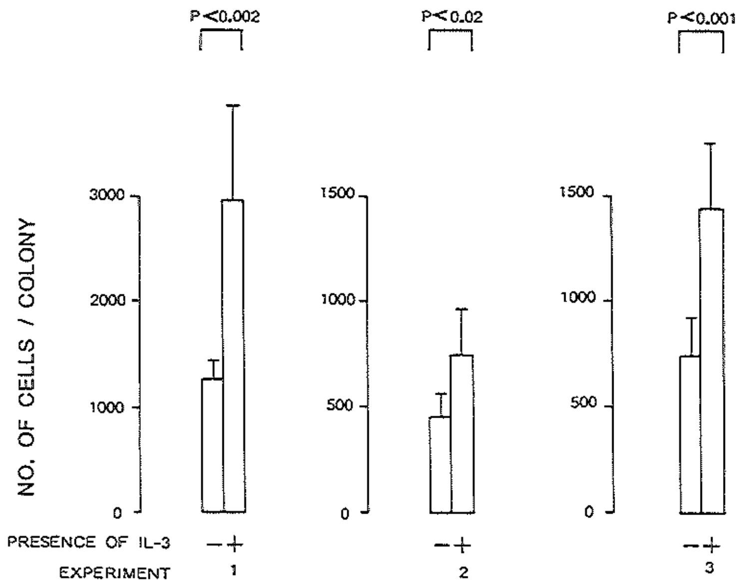


Fig 1. Granulocytic colony formation in response to G-CSF with and without IL-3: size of the colonies. The cell numbers per granulocytic colony produced from CD34+/Mono- progenitor cells in response to G-CSF with (+) or without (-) IL-3 (10 U/ml) are given. Data from 3 separate experiments are shown. G-CSF was added at concentrations of 1:3,000 (exp.#1) and 1:1,000 (exp.#2 and #3). Each value represents the mean ± SD of 6 pools of 5 randomly sampled colonies each. The data were compared according to Students t-test and the significance of differences is indicated.

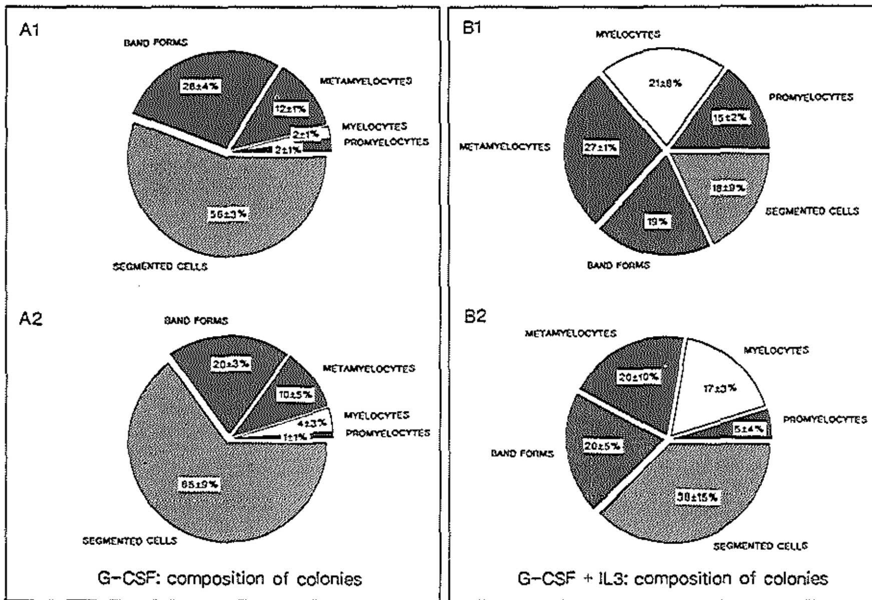


Fig 2. Granulocytic colony formation in response to G-CSF with and without IL-3: cellular composition of colonies. The cellular composition of granulocytic colonies grown from CD34+/Mono- progenitor cells in response to G-CSF in the absence (panels A) or presence (panels B) of IL-3 (10 U/ml) is plotted. Each chart presents the differential counts (mean ± SD) of three pools of five randomly sampled colonies (200 cells scored). A1 and B1 indicate the distribution of cell types in the colonies from experiment #1 (G-CSF 1:3,000), A2 and B2 the results related to experiment #3 (G-CSF 1:1,000)(see fig.1). In exp.#3 the colonies were not only examined morphologically for the 1:1,000 G-CSF level but also for other G-CSF concentrations (1:3,000 and 1:300). The results of the latter analyses were identical (not shown).

IL-3: enhances survival of CFU-G in vitro. Four experiments were done in which G-CSF was added to the cultures at day 0 or withheld for 7 or 10 days and then added. Cultures were run with and without IL-3 to evaluate the effect of IL-3 on the maintenance of G-CSF responsive progenitor cells in the cultures that were deprived of G-CSF for the initial 7-10 days. Epo was also added and all colonies induced were scored. A representative experiment is shown in fig.3. In response to G-CSF 64 granulocytic colonies were formed in culture. When the addition of G-CSF was postponed for 7-10 days, rare CFU-G survived to form colonies upon subsequent addition of G-CSF. When IL-3 was added the decline of CFU-G numbers resulting from the delayed addition of G-CSF could partly be prevented. Thus a significant proportion of CFU-G could be maintained in culture in the presence of IL-3 and were still able to form colonies when G-CSF was added following the 7-day interval.

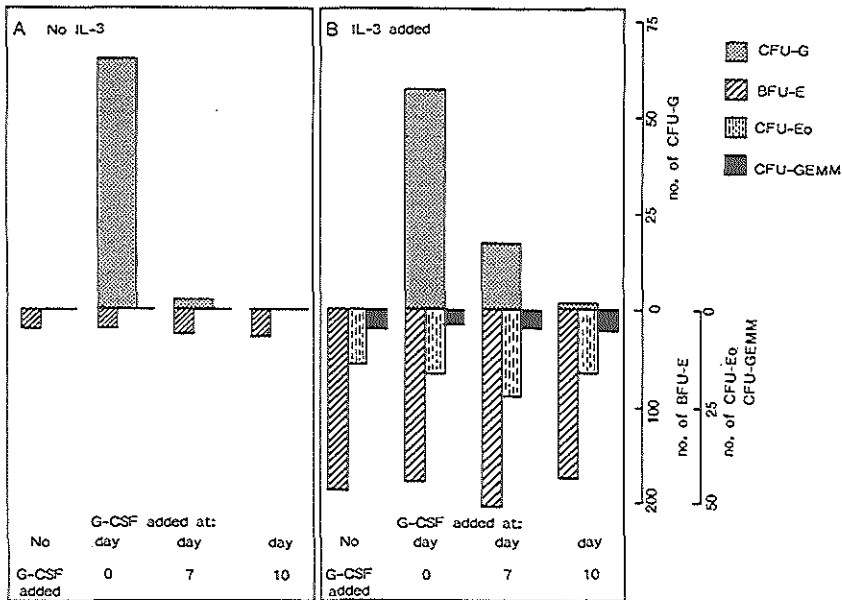


Fig 3. Effect of IL-3 on in vitro survival of CFU-G. Colony numbers grown in response to G-CSF (concentration 1:1,000) added at the beginning of culture (day 0) or after a delay of 7 or 10 days (upper section). When G-CSF was withheld from the cultures during the first 7 days and no IL-3 added, CFU-G numbers fell rapidly (panel A). When G-CSF was withheld but IL-3 supplemented, the drop of CFU-G numbers within 7 days was partially prevented (panel B). Data of control cultures with no G-CSF are also shown. Each point represents the mean value of duplicate cultures of the CD34 positive fraction of $8x10^4$ monocyte-depleted cells (CD34+/Mono-). BFU-E, CFU-Eo and CFU-GEMM numbers did not vary as a result of the delay in G-CSF addition (lower section). The figure presents the complete set of data of one experiment that is representative of a series of four experiments (see table 2).

Table 2 shows the granulocytic colony data compiled from 4 experiments. On the average less than 1% of granulocytic colony forming cells survived when the addition of G-CSF was delayed for 7 days. When, however, IL-3 was supplemented to the cultures at their initiation (day 0), the fraction of CFU-G that survived in the absence of G-CSF (for 7 days) was 29±4% (mean±SD). IL-3 could not prevent the decline of CFU-G in cultures without G-CSF beyond a 10-day interval. It is of note that the delayed supplementation of G-CSF did not at all modify the numbers of BFU-E, CFU-Eo and CFU-GEMM that were scored in parallel, indicating that G-CSF does not regulate their proliferation or survival (Fig.3).

Table 2 **Effect of IL-3 on in vitro survival of CFU-G.**

G-CSF added at:		No IL-3 day 0	No IL-3 day 7	No IL-3 day 10	With IL-3 day 0	With IL-3 day 7	With IL-3 day 10
Exp.	#1	42	0	nd	53	15	nd
	#2	135	0.5	nd	95	22	nd
	#3	64	2	0	57	18	0.5
	#4	38	1	0	63	20	0
Mean (%) ±	SD	100	0.9±0.9	-	100	29±4	-

The addition of G-CSF to the cultures was delayed for 7 or 10 days and the effect of IL-3 on the in vitro maintenance of CFU-G was then assessed. The results of the comparative cultures "without IL-3" (panel A) versus "with IL-3" (panel B) are shown (4 experiments). Each value represents the average number of colonies of duplicate cultures of the CD34+ fraction derived from 8×10^4 monocyte-depleted marrow cells. The mean CFU-G numbers (±SD) obtained following the delay of added G-CSF were expressed as percentage of the recoveries of CFU-G numbers of cultures to which G-CSF had been added at day 0 (= 100%). Nd denotes "not done".

Discussion

In cultures of purified hematopoietic progenitor cells from human marrow no granulocytic colonies are induced following stimulation with IL-3. In this paper we show that the granulocytic colony forming cells, however, are not really unresponsive to IL-3. When colony size is taken into account, a positive effect of IL-3 on G-CSF induced colony formation was apparent. The colonies generated in response to G-CSF became significantly greater when IL-3 was added to those cultures as well. The morphological analysis revealed an increase of immature cells in the IL-3 plus G-CSF stimulated colonies whereas mainly mature cells were found among colonies induced by G-CSF alone. Thus it appears that IL-3 enhances the rate of proliferation of more immature myeloid cells leading to larger colonies containing a greater proportion of early cell stages. It is presently unclear how this effect of IL-3 on the colony forming abilities of CFU-G is achieved. One could hypothesize that CFU-G do concomitantly express receptors for the two hematopoietic growth factors (G-CSF and IL-3) and that the exposure to G-CSF will preferentially stimulate maturation whereas IL-3 mainly

controls self-renewal of CFU-G, so that the simultaneous exposure to both G-CSF and IL-3 will result in colonies with a greater proportion of immature cells. Evidently IL-3 is not able to stimulate a sufficient number of cell divisions to permit the formation of a granulocytic colony. If IL-3 stimulates a few cell divisions, G-CSF could then evoke additional cell divisions and induce terminal differentiation. This interpretation would be consistent with the observations indicating that in cultures deprived of G-CSF, CFU-G disappeared within approximately one week, while this extinction of CFU-G could partially be counteracted by exposure of the cells to IL-3. Whether these G-CSF responsive cells are maintained in culture due to a self-renewal effect of IL-3 or promotion of survival needs further investigation. Another explanation for these results may be that IL-3 induces proliferation of a less mature, i.e. multi-potent, progenitor cell after which differentiation of this cell is directed by G-CSF into the granulocytic lineage. We consider this a less likely explanation since the absolute number of granulocytic colonies did not increase in G-CSF plus IL-3 stimulated cultures indicating that no additional progenitor cells were recruited as a result of IL-3 addition to G-CSF stimulated cultures.

In two recent studies, one employing gibbon IL-3[272] and one human IL-3,[229] positive effect of IL-3 on numbers of human CFU-G were reported. The discrepancy between those results and the data of this study is not immediately clear, but is most likely explained by differences in the progenitor cell enrichment procedures. We have utilized complement mediated cytolysis to remove accessory cells followed by a positive selection of progenitor cells using a MoAb against the CD34 antigen and cell sorting. Sieff et al.[236] used several MoAbs and adherence to Ig-coated plates as a method to deplete accessory cells. Otsuka et al.[229] also used immune panning to enrich for CD34+ cells, followed by overnight depletion of adherent cells. As it has been clearly demonstrated that few admixed monocytes can extend the stimulative spectrum of IL-3 to include CFU-G,[21] it is conceivable that relatively small differences in the quantitative admixtures of accessory cells modulating the effect of IL-3 on CFU-G are responsible for these dissimilarities.

Chapter 5

SYNERGISTIC EFFECTS BETWEEN GM-CSF AND G-CSF OR M-CSF ON HIGHLY ENRICHED HUMAN MARROW PROGENITOR CELLS

Fredrik J Bot, Loes van Eijk, Pauline Schipper, Bianca Backx
and Bob Löwenberg

Leukemia 4:325-328, 1990

Abstract

The human multilineage hematopoietic growth factor granulocyte-macrophage colony stimulating factor (GM-CSF) induces multipotent, erythroid and eosinophil colony formation from highly enriched normal bone marrow cells. We have examined the effects of GM-CSF combined with granulocyte-CSF (G-CSF) or macrophage-CSF (M-CSF) on the monolineage granulocytic, eosinophilic and macrophage progenitor cells (CFU-G, CFU-Eo and CFU-M) in accessory cell depleted marrow fractions. GM-CSF effects were assessed in direct comparison with those of interleukin-3 (IL-3) plus G-CSF or M-CSF. GM-CSF strongly synergized with G-CSF in the formation of granulocytic colonies with respect to number and size and enhanced the in-vitro survival of CFU-G. More immature cells were present in colonies induced by the mixture of GM-CSF and G-CSF than by G-CSF alone. GM-CSF also synergized with M-CSF in the formation of macrophage colonies (number and size). The addition of G-CSF and M-CSF did not influence eosinophil colony formation induced by GM-CSF or IL-3. Experiments directly comparing GM-CSF and IL-3 revealed that the effects of GM-CSF on G-and M- colony forming cells were significantly greater than those of IL-3. The potent positive effects between GM-CSF and G-CSF as well as between GM-CSF and M-CSF provide a powerful mechanism of amplification of granulopoiesis and monocytopoiesis.

Introduction

Human hematopoiesis is under the control of a number of hematopoietic growth factors, e.g. interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF) and erythropoietin (Epo). GM-CSF when added to cultures of highly enriched human hematopoietic progenitor cells, mainly stimulates erythroid (BFU-E), eosinophil (CFU-Eo) and multipotential (CFU-GEMM) precursors.[22] Direct comparisons between the proliferative stimulating activities of GM-CSF and IL-3 have shown that both factors have a very similar target cell spectrum.[21,22] Thus GM-CSF and IL-3 do not induce colonies from granulocyte and macrophage progenitors (CFU-G, CFU-M and CFU-GM) when accessory cells are rigorously depleted from the target marrow cell suspension. On the other hand G-CSF, M-CSF and Epo predominantly stimulate colony formation from committed progenitors. Interactions between the early (IL-3, GM-CSF) and late acting factors (G-CSF, M-CSF and Epo) may occur. These cooperative effects between CSFs are assumed to represent essential features of the physiologic regulation of hematopoiesis. Several murine studies on synergistic effects between CSFs have been published.[192,341] As regards human marrow, synergism between IL-3 and G-CSF or GM-CSF has been demonstrated in one study using unpurified marrow cells[338] and in another more recent study only with respect to GM-CFC.[193] We have recently demonstrated positive effects of IL-3 on G-CSF-

responsive precursors.[23] This work was extended and in the present study we have examined the cooperative effects between GM-CSF and G-CSF or M-CSF on several aspects of colony formation. Highly enriched marrow progenitor cell fractions were used and we have evaluated the stimulating effects on granulocytic, eosinophilic and macrophage progenitors separately.

Materials and methods

Preparation of cell suspension: Bone marrow was obtained by posterior iliac crest puncture from hematologically normal adults, after informed consent. The marrow was collected in Hanks balanced salt solution (HBSS) with heparin and the mononuclear cells were separated over Ficoll-Isopaque (1.077 g/cm^3; Nycomed, Oslo, Norway) as described.[21]

Enrichment of progenitor cells: A two-step progenitor cell enrichment procedure was employed as described.[24] First, non-clonogenic accessory cells were removed by complement mediated cytolysis with a mixture of monoclonal antibodies (MoAb) against the antigens CD14 (B44.1, IgM, monocytes;[237] final dilution 1:1000), CD15 (B4.3, IgM, granulocytic cells;[317] final dilution 1:500) and CD3 (T3, IgG2, final dilution 1:10). By FACS-analysis we had previously verified that the antigens CD14, CD15 and CD3 are not expressed on progenitor cells. The lysis-procedure resulted in an average recovery of 51% ± 7% of nucleated cells. Secondly, cells were positively selected following CD34 labeling (BI3C5, IgG1; Sera-Lab, Crawley Down, UK[133]) and cell sorting (FACS 440, Becton Dickinson, Mountain View, Cal.). The final cell population contained 4%-6% of the original ficoll separated cells and mainly represented morphologically immature blast cells. This method of enrichment has been shown to provide an efficient separation of clonogenic cells from accessory cells.[24] The complete recovery of clonogenic cells following cell separation was evaluated in individual experiments by running control cultures with PHA-LCM (phytohemagglutinin-leucocyte conditioned medium) as described.[21] In each experiment the discarded CD34- fraction was cultured with PHA-LCM as stimulus and found to contain only a minor number (<10%) of erythroid colonies, but never myeloid colonies. The purified fraction of accessory cell depleted, CD34+ bone marrow cells is also referred to as enriched (progenitor) cells in the text.

Recombinant human CSFs: Recombinant human IL-3, obtained from Drs L Dorssers and G Wagemaker (Daniel den Hoed Cancer Center, Rotterdam and Radiobiological Institute TNO, Rijswijk, The Netherlands)[21,71] was used at the optimal concentration of 10 U/ml.[21] Purified recombinant human G-CSF was a gift from Dr S Gillis (Immunex Corp., Seattle, WA), specific activity > 10,000,000 CFU/mg protein and was used in a concentration of 10 ng/ml. Purified recombinant M-CSF was donated by Dr P Ralph (Cetus Corp., Emeryville, CA), specific activity 5x10^7 U/mg and

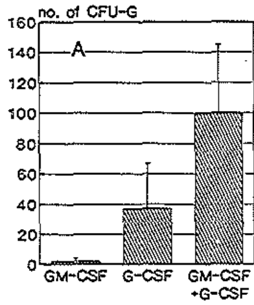
used in a concentration of 20 ng/ml. E.Coli derived purified recombinant human GM-CSF (from Dr JF DeLamarter; Biogen SA, Geneva, Switzerland[35]), specific activity 10^5 U/ml was used at a concentration of 1:1,000. The abovementioned optimal stimulatory concentrations were determined for each growth factor batch separately (data not shown) and used throughout the experiments unless stated otherwise.

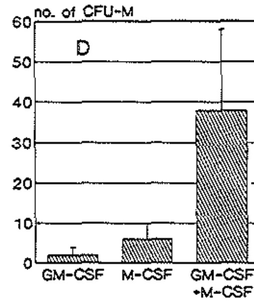
Colony culture assay: The CD34+ fraction of $8x10^4$ accessory cell depleted bone marrow cells (actual number plated: $0.5\text{-}1x10^4$ cells/ml) was cultured in a 1 ml mixture of Iscove's modified Dulbecco's medium (IMDM), 1.1% methylcellulose, 30% autologous heparinized plasma, BSA, transferrin, lecithin, sodium-selenite and 2-mercapto-ethanol as reported.[22] Cultures were stimulated by the addition of GM-CSF, IL-3, G-CSF, M-CSF or PHA-LCM. In selected cultures the addition of G-CSF or M-CSF was delayed for 2, 7 or 10 days following the initiation of culture as described.[23] In all experiments a control culture of enriched cells without added growth factors was done; colonies never formed in these cultures. Estimates are based upon duplicate cultures. Colonies of more than 50 cells were scored at day 15 following plating.

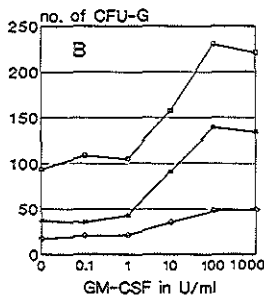
Determination of colony size and morphology: In three experiments the size of colonies stimulated by G-CSF alone and G-CSF plus IL-3 or GM-CSF was determined after picking-off colonies from the culture dishes as described.[23] In each experiment the mean cell number per colony was derived from 6 pools of five colonies each. To determine the morphology these pooled colonies were put onto slides in a cytocentrifuge and stained using May-Grünwald-Giemsa (MGG) stain. A differential count was then performed on 200 cells.

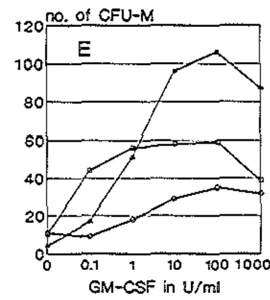
Results

Granulocytic and eosinophilic colony formation after costimulation with GM-CSF + G-CSF: Stimulation of enriched bone marrow cells with G-CSF resulted in the formation of granulocytic colonies. Significantly more CFU-G were induced by G-CSF when GM-CSF was added to cultures as well (100 ± 45 (SD) versus 37 ± 30/5000 cells; t-test, $p < 0.001$; $n = 17$) (Fig.1A). GM-CSF alone induced rare G-colonies from the enriched marrow fraction (2 ± 2) (Fig.1A). The dose-response curves for GM-CSF added to G-CSF stimulated cultures ($n = 3$ experiments) demonstrated that enhancement of G-colony formation by GM-CSF became apparent at GM-CSF concentrations between 1 to 10 U/ml, and was maximal at 100 U/ml of GM-CSF (Fig.1B).
Eosinophilic colony numbers in GM-CSF versus GM-CSF plus G-CSF stimulated cultures were identical (20 ± 13 and 21 ± 11/5000 cells, respectively), indicating that G-CSF does not influence eosinophilic colony growth.









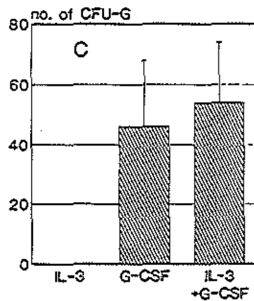


Fig 1. Synergy between hematopoietic growth factors on granulocytic and macrophage colony numbers. Highly enriched normal bone marrow progenitor cells were incubated with different combinations of CSFs. All colony numbers are expressed per 5000 plated cells. Panel A: numbers of G-colonies (mean ± SD; n = 17 experiments) are shown after culture with GM-CSF, G-CSF or GM-CSF + G-CSF. Panel B: Numbers of G-colonies induced by G-CSF plus titrated concentrations of GM-CSF (0.1 to 1,000 U/ml). The dose-response curves from three experiments are plotted. Panel C: Numbers of G-colonies (mean ± SD; n = 14 experiments) after culture with IL-3, G-CSF or IL-3 + G-CSF. Panel D: Numbers of CFU-M (mean ± SD; n = 11 experiments) after culture with GM-CSF, M-CSF or GM-CSF + M-CSF. Panel E: Numbers of CFU-M induced by M-CSF plus titrated concentrations of GM-CSF (data from three experiments).

By comparison, optimal concentrations of IL-3 did not affect G-CSF induced colony numbers (Fig.1C).[23] Equal numbers of eosinophilic colonies were formed in cultures stimulated by G-CSF+IL-3 versus IL-3 alone (13 ± 5 and 13 ± 7/5000 cells; n=14).

Macrophage colony formation after costimulation with GM-CSF + M-CSF: GM-CSF synergized strongly with M-CSF and induced a significant increase in macrophage colony formation (Fig.1D). Both GM-CSF and M-CSF alone produced few macrophage colonies from purified normal marrow progenitors (2 ± 2 and 6 ± 4/5000 cells, respectively). Considerably greater numbers of CFU-M (38 ± 20; $p < 0.001$; n=11) were induced in response to stimulation with GM-CSF plus M-CSF. This effect was clearly dose-dependent, beginning at a GM-CSF concentration of 0.1 U/ml (Fig.1E). Maximal synergy between GM-CSF and M-CSF was evident at approximately 10 - 100 U/ml GM-CSF. The numbers of eosinophilic colonies were identical in cultures stimulated with GM-CSF (22 ± 7) as compared to those in cultures stimulated with GM-CSF plus M-CSF (22 ± 6). The addition of IL-3 did not influence M-CSF induced colony formation (data not shown).

Effect of GM-CSF and IL-3 on granulocytic and eosinophilic colony size: The average numbers of cells per granulocytic colony induced by G-CSF alone, G-CSF plus IL-3 or G-CSF plus GM-CSF were determined (fig.2). The latter estimates were derived from three experiments i.e. from 18 series of 5 colonies. A progressive increase ($p<0.001$; t-test) of the average granulocytic colony size was apparent in the following order: G-CSF (1056 ± 276 cells/col.), G-CSF + IL-3 (1832 ± 586) and G-CSF + GM-CSF (3726 ± 1146).

Eosinophilic colonies induced by IL-3 (1011 ± 197 cells/col.) were significantly smaller than those induced by GM-CSF (4587 ± 1976 cells/col.; n=4). The size of the latter colonies did not change as a result of G-CSF addition, confirming the lack of activity of G-CSF on eosinophilic colony formation (data not shown).

The size of macrophage colonies could not be determined reliably, because it was difficult to pick them from the plates completely. An approximation of the relative size was made from the appearance of the colonies in the dishes. M-CSF induced colonies were generally small and were estimated to contain 50-100 cells. In contrast M-CSF plus GM-CSF-induced colonies appeared considerably larger, regularly containing more than 200 cells. IL-3 did not influence the size of M-CSF induced colonies.

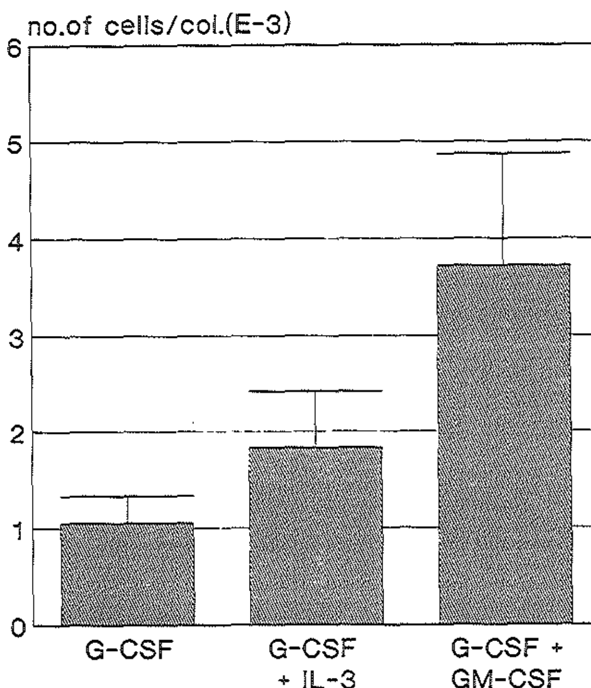


Fig 2. Size of granulocytic colonies formed in response to G-CSF, G-CSF + IL-3, or G-CSF + GM-CSF. The size of the colonies is expressed as average numbers of cells per colony ($x10^{-3}$). Results are derived from three experiments and each estimate represents the mean (±SD) of 18 pools of five colonies.

Effect of GM-CSF and IL-3 on cell maturation within colonies: In three experiments the cellular composition of granulocytic colonies induced by G-CSF, G-CSF plus GM-CSF or G-CSF plus IL-3 was assessed (Fig.3). G-CSF stimulated colonies consisted mainly of mature segmented granulocytes. The addition of GM-CSF to cultures as a costimulus of G-CSF enhanced the proportion of immature cells in the colonies: almost 50 % of the cells were promyelocytes and myelocytes while segmented granulocytes were virtually absent (see fig.3). The addition of IL-3 resulted also in colonies containing more immature cells although the effect was less prominent. Maturation of eosinophilic colonies induced by GM-CSF or IL-3 did not change as the consequence of G-CSF addition.

Effect of GM-CSF on survival of CFU-G and CFU-M: The effect of GM-CSF on the in vitro survival of CFU-G was examined in two experiments. This was done by postponing the addition of G-CSF to the cultures for 2, 7 or 10 days. The majority of CFU-G disappear within 2 - 7 days in non-supplemented cultures (Fig.4, panels A and B).

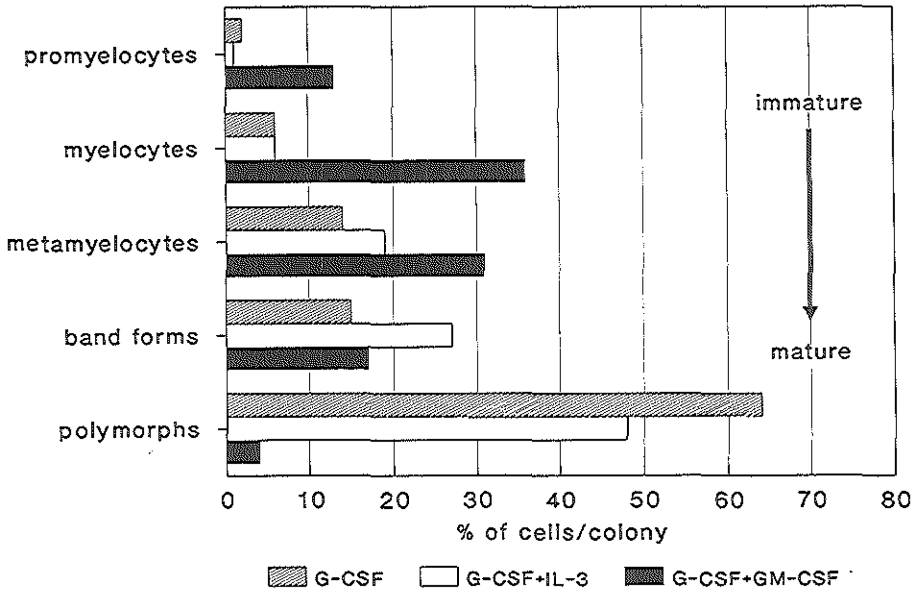


Fig 3. Comparison between the cellular composition of granulocytic colonies induced by G-CSF, G-CSF + IL-3 and G-CSF + GM-CSF. Differential counts (200 cells) were performed on cytospin slides of pooled granulocytic colonies. Estimates are the mean results of six slides. One representative of three experiments is shown. X-axis: proportions of cells in colonies expressed by length of bars; Y-axis: morphologically identified granulocytic cells.

Addition of GM-CSF to those cultures during G-CSF deprivation prevented the drop of CFU-G numbers at least partially. Thus the presence of GM-CSF permitted the survival of a considerable fraction of CFU-G in vitro until day 7.

Data from a similar pair of experiments for CFU-M are shown in panels C and D. M-CSF alone stimulates very few macrophage colonies, and a positive effect of GM-CSF on M-CFU numbers is apparent. We cannot draw conclusions on the survival of CFU-M in the presence of GM-CSF, since the numbers of macrophage colonies stimulated by M-CSF alone are too low to measure significant survival promoting effects of GM-CSF on CFU-M.

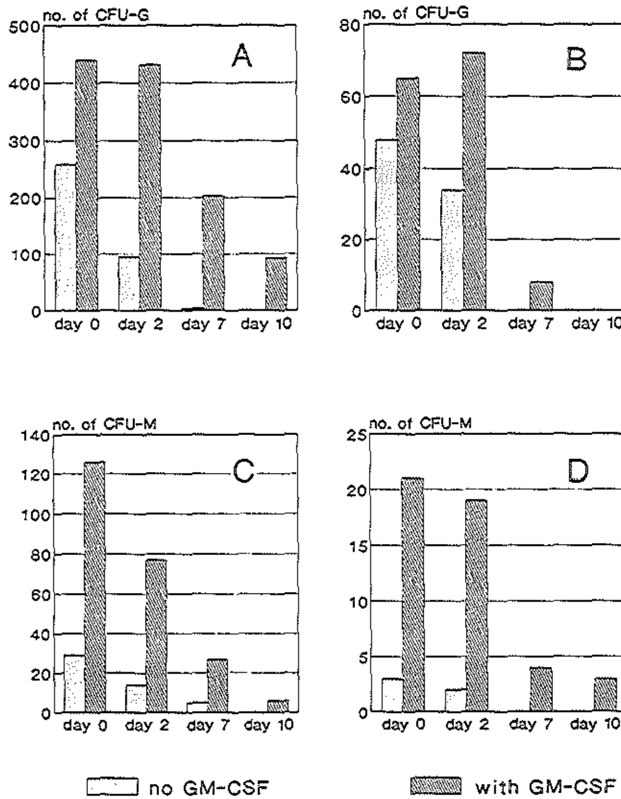


Fig 4. In vitro survival of CFU-G and CFU-M in the presence of GM-CSF. Cultures of enriched progenitor cells without GM-CSF (dotted bars) and with GM-CSF (hatched bars) to assess the survival of CFU-G (panels A and B) and CFU-M (panels C and D). The in vitro survival of CFU-G and CFU-M was determined at day 0, 2, 7 and 10. Colony numbers are expressed per 5000 plated cells.

Discussion

We have demonstrated that the multilineage hematopoietic growth factor GM-CSF, although by itself not stimulating CFU-G or CFU-M from highly enriched marrow blast cells, strongly synergizes in a dose-dependent way with the single lineage hematopoietic growth factors G-CSF and M-CSF. Thus small doses of GM-CSF may considerably elevate G-CSF and M-CSF stimulating activities. The positive effects of the GM-CSF plus G-CSF combination on size, maturation and survival of granulocytic progenitors have previously been reported for IL-3.[23] The experiments reported here

however show that the magnitude of the effect of GM-CSF on CFU-G is considerably greater than the effect of IL-3. Macrophage colony formation was enhanced similarly by adding GM-CSF to M-CSF. In contrast costimulation of M-CSF with IL-3 did not promote the formation of M-colonies. Synergism between GM-CSF and M-CSF was already apparent at low doses of GM-CSF and is consistent with a previously described synergistic effect between GM-CSF and M-CSF.[42] The latter investigations however were carried out with less enriched bone marrow progenitors and this may explain that GM-CSF alone could induce CFU-M proliferation in those studies accessory cells releasing endogenous hematopoietins may modify the growth factor response considerably.[21] A remarkable feature is the poor stimulative effect of M-CSF alone. Apparently macrophage-CFU generally need two factors, GM-CSF plus M-CSF, to express full colony forming potential.[42] IL-6 plus M-CSF have also been demonstrated to be a potent stimulatory combination for M-CFU.[24]
Eosinophilic colonies induced by IL-3 or GM-CSF did not change in number, size or maturational status after the addition of G-CSF, indicating that G-CSF does not modify proliferation and maturation of CFU-Eo.

From our studies it appears that G- and M-progenitors do respond to GM-CSF, however GM-CSF stimulation alone is insufficient to elicit colony formation. This can only be achieved when G-CSF and M-CSF are also present, either exogenously added to the cultures, or endogenously produced by accessory cells, e.g. monocytes.[22] The exact mechanism by which GM-CSF synergizes with both G-CSF and M-CSF cannot be deduced from these experiments; we have considered four different mechanisms: (a) GM-CSF may upregulate receptors for G-CSF and M-CSF respectively, thereby recruiting more immature cells to become responsive to these factors, (b) GM-CSF may induce only a few cell divisions but no maturation while G-CSF and M-CSF subsequently trigger further cell divisions and maturation so that full size colonies appear. This mechanism could very well explain the survival experiments: a few cell-divisions would suffice to keep the cells alive until the late acting factor is added, (c) different subpopulations of CFU-G and CFU-M may coexist, as has been suggested before,[193] e.g. one subset responding to G-CSF or M-CSF alone and another subset that requires the dual combination of GM-CSF and G-CSF or GM-CSF and M-CSF for effective stimulation, or (d) the requirement of CFU-M for multiple hematopoietins for optimal proliferation may be compatible with a "priming" function of GM-CSF or IL-6, rendering CFU-M responsive to the proliferation inducing factor M-CSF, as has been suggested before for GM-CSF.[42]
It is now clear that the multipoietins IL-3 and GM-CSF have a broad progenitor cell spectrum of stimulation[21,22,35,42] that depends on their combination with other factors. The diverse interactions between different factors and the strong synergistic effects between GM-CSF and G-CSF or between GM-CSF and M-CSF significantly enhance certain proliferative effects. This mechanism of cooperation may provide a powerful system of positive regulation e.g. in situations of stress hemopoiesis.

Chapter 6

INTERLEUKIN-6 (IL-6) SYNERGIZES WITH M-CSF IN THE FORMATION OF MACROPHAGE COLONIES FROM PURIFIED HUMAN MARROW PROGENITOR CELLS

Fredrik J Bot, Loes van Eijk, Lianne Broeders, Lucien A Aarden and Bob Löwenberg

Blood 73:435-437, 1989

Abstract

We have examined the in vitro stimulative effects of recombinant human interleukin-6 (IL-6, or interferon-β_2) on purified human bone marrow progenitor cells. IL-6 alone or in combination with Epo, IL-3, GM-CSF or G-CSF did not induce colony formation. However IL-6 strongly synergized with M-CSF in stimulating macrophage colony formation (colony numbers and size). The magnitude of IL-6 synergism with M-CSF was dose-dependent, maximal potentiation of M-colony formation being evident at approximately 100-1000 U/ml IL-6. When the addition of IL-6 to M-CSF supplemented cultures was delayed for more than one day after the beginning of culture, enhancement of macrophage colony formation was lost. IL-6 stimulation of M-CSF responsive colony formation was not apparent when nonpurified marrow cells were plated, most likely due to endogenous IL-6 release. These observations suggest that IL-6 besides its role in B lymphocyte proliferation can potentiate the human immune defence mechanism by stimulating monocyte-macrophage development as well.

Introduction

Interleukin 6 (IL-6, originally described as interferon ß2[263,264,336] and also known as B-cell stimulating factor-2) is produced by many cell types and was recently cloned from monocytes,[27] fibroblasts[104] and a T-cell line.[113] It has a regulatory function in the differentiation of B lymphocytes.[113] IL-6 also supports, in synergy with IL-3, the proliferation of granulocyte macrophage- and multilineage progenitors from murine bone marrow.[123] IL-6 and IL-3 showed synergistic activity in the human marrow blast cell colony assay as well.[157] IL-6, when added to IL-3 stimulated cultures, accelerated the appearance and increased the numbers of blast cell colonies. This suggested that IL-6 acts as a competence factor mediating the transition of cells from G_0 to a cycling state.[123,157] IL-6 did not induce colony formation from other hematopoietic progenitors neither as a single factor, nor in combination with IL-3 or IL-1α. We demonstrate here that IL-6 enhances M-CSF induced colony formation in cultures of highly enriched progenitor cells from human bone marrow.

Materials and methods

Enrichment of marrow progenitor cells: Bone marrow was obtained from hematologically normal adults, which had given their informed consent, and separated over Ficoll-Isopaque (1.077 g/cm^3; Nycomed, Oslo, Norway) according to standard procedures.[21] As a first step of enrichment marrow cells were depleted of non-clonogenic accessory cells following complement mediated cytolysis

with a cocktail of monoclonal antibodies (MoAb) against the antigens CD14 (B44.1, IgM, monocytes;[237] final dilution 1:1000), CD15 (B4.3, IgM, granulocytic cells;[317] final dilution 1:500) and CD3 (T3, IgG2, final dilution 1:10). This resulted in an average recovery of 51±7% of nucleated cells. Further enrichment (second step) was achieved following fluorescence activated sorting of the cells bearing the CD34 antigen (BI3C5, IgG1; Sera-Lab, Crawley Down, UK[133]). The labeling and cell sorting procedures have been described.[21] Thus 4-6% of the original ficoll separated cells were recovered as the final cell population and consisted mainly of immature blast cells. The latter fraction is designated as CD34+ fraction of accessory cell depleted marrow or enriched progenitor cell fraction.

Recombinant human CSFs: E. Coli derived recombinant human IL-6 was purified to apparent homogeneity and had a specific activity of 10^9 units/mg.[1] The concentration used in culture was 1000 U/ml or as indicated. Recombinant human IL-3 was generously donated by Drs L Dorssers and G Wagemaker (Radiobiological Institute TNO, Rijswijk, The Netherlands[21,71]), recombinant human G-CSF and M-CSF by Dr S Clark (Genetics Institute, Cambridge, MA) and recombinant human GM-CSF by Dr JF DeLamarter (Biogen SA, Geneva, Switzerland[35]) and used at optimal concentrations of 10 U/ml,[23] 1:1,000, 1:1,000 and 1000 U/ml respectively.

Colony culture assay: The CD34+ fraction of $8x10^4$ accessory cell depleted bone marrow cells (real number plated: $0.5\text{-}1x10^4$ cells/ml) was cultured in methylcellulose as reported[21]. Recombinant growth factors were added in several combinations, or an impure PHA-LCM (phytohaemagglutinin-leucocyte conditioned medium) was used as control. In selected cultures the addition of IL-6 was delayed for 1,3 or 6 days after which IL-6 was carefully dispersed over the culture layer in a volume of 0.050 ml and allowed to diffuse through the medium. Estimates are based upon the data from duplicate cultures. Colonies were scored at day 15 following plating. Macrophage colonies were defined as aggregates of more than 50 cells which were recognized as macrophages at a 100x magnification, presenting as large, often dark, vacuolated cells and verified using May-Grünwald-Giemsa stained cytospin preparations of single colonies.

Results

IL-6 did not induce colonies in vitro from enriched bone marrow progenitor cells (n=8 experiments). A synergistic effect was noted between IL-6 and M-CSF (fig. 1A). In response to costimulation with IL-6 and M-CSF significantly more CFU-M were induced to form colonies (at day 15) as compared to stimulation with M-CSF alone. Furthermore, M-CSF induced colonies were loosely arranged and contained 50-100 cells while M-CSF plus IL-6 stimulated colonies with more compact appearance

contained over 200 cells. For comparison, similarly elevated colony numbers were also obtained in cultures after stimulation with impure PHA-LCM.

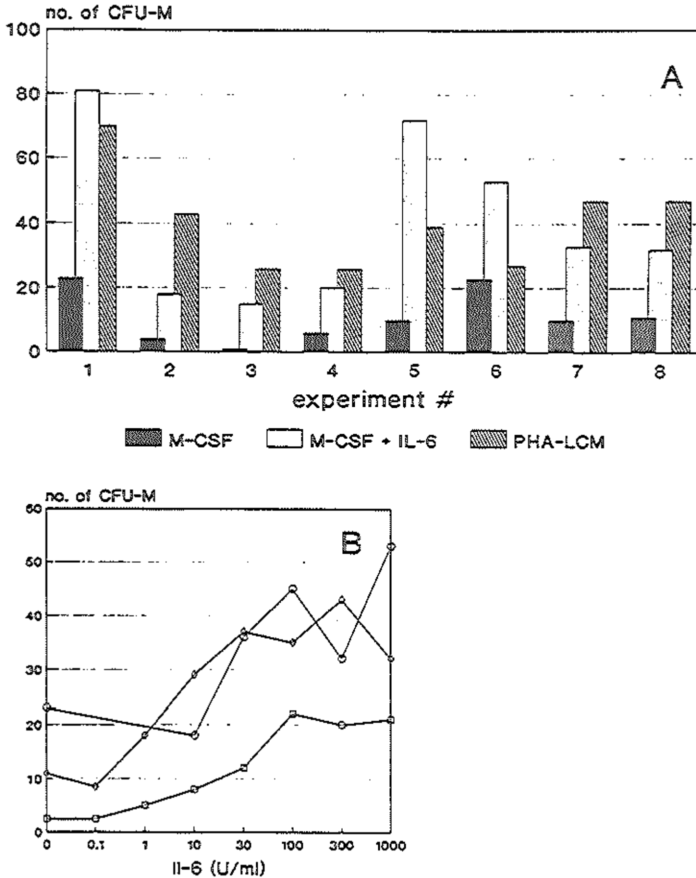


Fig 1. Synergism between Il-6 and M-CSF. The CD34+ fraction from $8x10^4$ accessory cell depleted bone marrow cells (= $0.5\text{-}1x10^4$) was plated per ml. Panel A shows the results from 8 experiments with bone marrow from 8 different donors. Numbers of CFU-M stimulated by M-CSF, M-CSF plus IL-6 or PHA-LCM are shown. In panel B the results are shown from three IL-6-dose titration experiments in which IL-6 at concentrations from 0 to 1000 U/ml was added to cultures containing M-CSF. Each point is the mean of duplicate cultures. In the presence of Il-6 alone or without any factor no CFU-M were stimulated to colony formation.

IL-6 did not modify the colony forming efficiency when added to cultures containing Epo, IL-3, GM-CSF or G-CSF (data not shown). The dose-effect relationship (figure 1B) reveals that the potentiating effect of IL-6 is concentration dependent and begins to be expressed at concentrations of 1-10 U/ml. Plateau values of IL-6 synergistic activity are reached at concentrations between 100-1000 U/ml.

When IL-6 was not supplemented at the initiation of cultures but with a delay of 1-3 days it appeared that the synergistic effect of IL-6 on M-CSF dependent colony formation was lost (figure 2).

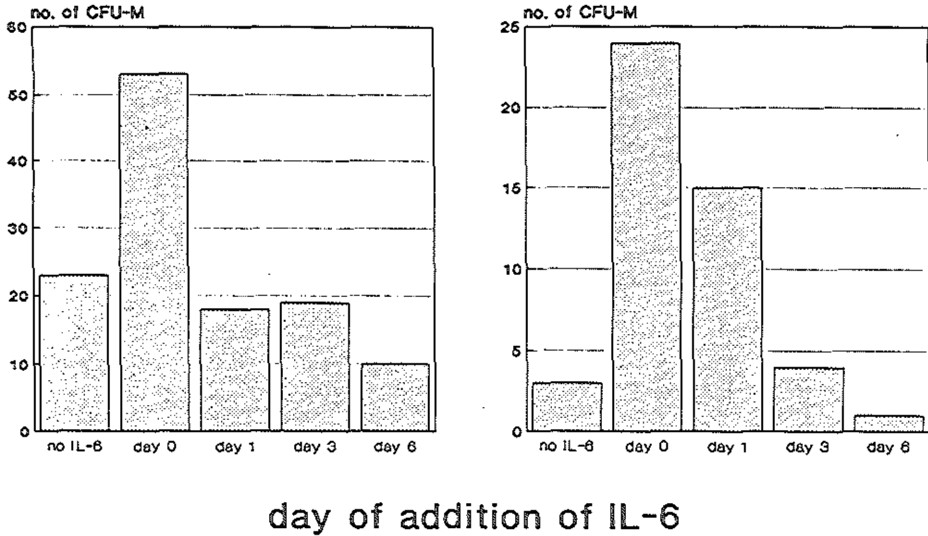


Fig 2. IL-6 enhances M-CSF induced colony formation: delayed addition of IL-6. The CD34+ fraction isolated from 8×10^4 accessory cell depleted bone marrow cells (= $0.5\text{-}1 \times 10^4$) was plated. Numbers of macrophage colonies stimulated by M-CSF are given. IL-6 (1000 U/ml) was added at the beginning of culture (day 0) or at day 1, 3 or 6 of culture. Data are from two separate experiments. Each point is the mean of duplicate cultures.

These experiments were conducted with highly enriched bone marrow progenitor cells in an attempt to exclude interference of accessory cells. In three experiments the effects of IL-6 on

M-CSF induced colony formation from enriched marrow progenitor cells were directly compared with those on non-enriched marrow cells, i.e. ficoll separated cells. It appeared that M-CSF stimulated greater numbers of CFU-M from ficoll separated marrow mononucleated cells (20, 16 and 48 macrophage colonies per 10^5; n=3 experiments) than from the purified fraction (5, 1 and 28 colonies per 10^5 cells, respectively). When IL-6 was added to M-CSF stimulated cultures of ficoll fractionated cells the synergistic activity of IL-6 could not be demonstrated (M-CSF + IL-6: 23, 20 and 31 colonies per 10^5 cells).

Discussion

We demonstrate that IL-6 has no colony stimulating effect on enriched human hematopoietic progenitor cells. However IL-6 synergizes with M-CSF in the stimulation of CFU-M with respect to both number and size of macrophage colonies. A delay in the addition of IL-6 to M-CSF stimulated cultures of more than one day abolishes the potentiating effect of IL-6. Thus there is an immediate requirement of the M-CFU target cell population for IL-6, suggesting that M-CFU rapidly lose their susceptibility to IL-6 in vitro in the absence of IL-6 or extinguish in culture when they are deprived of IL-6. One explanation for the synergism between IL-6 and M-CSF is that IL-6 upregulates the sensitivity of M-CFU for M-CSF or recruits additional subsets e.g. by upregulating M-CSF receptor expression. As an alternative explanation these data could fit the theory[123,157] that IL-6 acts as a competence factor that induces CFU-M from the resting G_0 state into G_1, after which M-CSF acts as a progression factor that induces proliferation of CFU-M. These and other possible mechanisms to explain the effects described here will require additional investigation.
The synergistic effect of IL-6 on M-CSF induced colony formation could not be demonstrated in ficoll-fractionated bone marrow. Furthermore M-CSF induced considerably more CFU-M from this impure population of marrow cells than from the enriched progenitor cell population. As monocytes are potent IL-6 producers[1] and IL-6 synergism is evident already at relatively low concentrations of IL-6 (1-10 U/ml), we believe that the release of endogenous IL-6 by monocytes retained in the ficoll fraction of bone marrow bypasses the dependence of CFU-M on exogenous IL-6.
IL-6 is a stimulator of B-cells and is thought to have an important role in the immune defence system. The observations presented here support a broader role of IL-6 in host defence, i.e. not only along the B-lineage but also through the generation of monocyte-macrophage cells from their progenitors.

Chapter 7

INTERLEUKIN-1α (IL-1) REGULATES THE PROLIFERATION OF IMMATURE NORMAL BONE MARROW CELLS

Fredrik J Bot, Pauline Schipper, Lianne Broeders,
Ruud Delwel, Kenneth Kaushansky and Bob Löwenberg

Blood 76:307-311, 1990

Abstract

The cytokine interleukin-1 (IL-1) plays a role in the regulation of normal as well as leukemic hematopoiesis. In acute myeloid leukemia (AML), IL-1 induces autocrine GM-CSF and TNF production and these factors may then synergistically induce proliferation in AML blast cells. Here we demonstrate that IL-1 stimulates DNA-synthesis of highly enriched normal bone marrow blast cells (CD34 positive, adherent cell depleted, CD3/CD14/CD15 negative). The stimulative effect of IL-1 can be blocked with neutralizing anti-TNFα and anti-GM-CSF antibodies and most efficiently by the combination of anti-TNFα and anti-GM-CSF but not with anti-G-CSF antibody, suggesting that IL-1 induced proliferation was initiated through TNF and GM-CSF release. Concentrations of TNF and GM-CSF increased in the culture medium of normal bone marrow blast cells after IL-1 induction. Twelve percent of those IL-1 induced cells were positive for GM-CSF mRNA by in-situ hybridization as opposed to 6% of non-induced cells. Thus IL-1 acts in addition to its effect on leukemic blast cells also on normal marrow blast cells. We propose a scheme where IL-1 stimulation of normal bone marrow blast cells leads to the induction of TNFα and GM-CSF which in association stimulate DNA-synthesis efficiently according to a paracrine or autocrine mechanism within the marrow blast cell compartment.

Introduction

The hematopoietic growth factors interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF) stimulate proliferation and differentiation of hematopoietic progenitor cells. The pleiotropic factors interleukin-1 (IL-1) and tumor necrosis factor (TNF) are also involved in the regulation of hematopoiesis, e.g., through the induction of CSF release. Interleukin-1 may exert positive effects on hematopoietic progenitor cells in vitro while TNF may exhibit both stimulatory and inhibitory effects.[149] Whether IL-1 and TNF act directly on the progenitor cells themselves in addition to indirect effects via accessory cells has not been settled.

IL-1 has recently been demonstrated to stimulate the proliferation in vitro of acute myeloid leukemia (AML) cells through the induction of GM-CSF production,[60] suggesting that the latter mechanism of autocrine GM-CSF mediated growth of AML blast cells[355] is under the control of the inducer IL-1. As yet it is not clear whether the phenomenon of autocrine stimulation of proliferation is related to the pathophysiology of AML growth. In some cases of AML IL-1 has the ability to induce the production of TNF as well,[61] furthermore, costimulation of TNF and GM-CSF may show strong synergism in stimulating AML cell proliferation in vitro.[113] Based on these observations it appears that IL-1 serves as an inducer of TNF and GM-CSF release followed by enhanced growth depending

on the TNF/GM-CSF ratio.

Here we present experiments to investigate whether similar cascades of stimulation involving IL-1, TNF and GM-CSF are operative in the in vitro proliferative activity of normal bone marrow blast cells.

Materials and methods

Enrichment of marrow progenitor cells: Bone marrow was obtained from hematologically normal adults, which had given their informed consent, and separated over Ficoll-Isopaque (1.077 g/cm^3; Nycomed, Oslo, Norway) according to standard procedures.[21] To ensure a thorough removal of accessory cells, progenitor cells were enriched in two steps. Initially marrow cells were depleted of adherent- and other accessory cells using a combined adherence/complement mediated cytolysis procedure. The marrow cell suspension (20x10^6/mL in phosphate buffered saline (PBS)/5% fetal calf serum (FCS)) was incubated with a cocktail of monoclonal antibodies (MoAb) against the antigens CD14 (B44.1, IgM, monocytes;[237] final dilution 1:1000), CD15 (B4.3, IgM, granulocytic cells;[317] final dilution 1:500) and CD3 (T3, IgG2, final dilution 1:10) for 15 minutes on ice. Next rabbit complement was added (40%), the cells were adjusted to a concentration of 10 x 10^6/mL, and allowed to adhere onto the surface of a 60 mm petri-dish at 37 °C for one hour. The nonadherent cells were harvested and washed in cold HBSS. This resulted in an average recovery of 26% ± 7% of nucleated cells. As a second step the cells expressing the CD34 antigen (BI3C5, IgG1; Sera-Lab, Crawley Down, UK[133]) were positively selected using a FACS 440 (Becton Dickinson, Mountain View, Cal.). The labeling and cell sorting procedures have been described.[21] Thus 3% ± 1% of the original ficoll separated cells were recovered as the final cell population. This fraction is designated as CD34+ fraction of accessory cell depleted marrow or enriched blast cell fraction and consists of more than 95% immature blast cells. Rare lymphocytes/plasma cells were present but monocytes and mature myeloid cells were not distinguishable in these cell preparations by cytochemical examinations.

Hematopoietic growth factors and antibodies: Human IL-1α and polyclonal rabbit anti-G-CSF were kindly donated by Dr S. Gillis (Immunex Corporations, Seattle, WA). IL-1 was used at a concentration of 100 U/ml,[60] based on maximal stimulating abilities as established in dose-response experiments (data not shown). Polyclonal sheep anti-GM-CSF was a gift from Dr. S Clark (Genetics Institute, Cambridge, MA) and was used in a concentration of 0.25%. Anti-G-CSF was also used in a concentration of 0.25%. The antibodies at these concentrations neutralized 2000 U/ml of GM-CSF and G-CSF in a dilution of 1:10,000, as described.[60] Monoclonal antibodies against TNF-α and TNF-ß were provided by Dr. GR Adolf (Ernst Boehringer Institute, Vienna, Austria). Anti-TNF

antibody at a concentration of 1:10,000 neutralized up to 10,000 U/mL of TNF in in-vitro proliferation assays of B-CLL cells[204] (data not shown). This concentration of anti-TNF was chosen to ensure a complete neutralization of all TNF present in the medium. Recombinant human erythropoietin was purchased from Amgen Biologicals (Thousand Oaks, California) and used at 1 U/ml.

Tritiated thymidine incorporation assay: Proliferation of the enriched normal bone marrow blast cells was measured by ^{3}H-TdR uptake. The cells were cultured for 48 hours in round-bottom 96-well microtiter plates (mean 8200 ± 1700 cells/well) in 0.1 mL of a serum-free medium[257] composed of BSA, transferrin, Na-selenite, linoleic acid, cholesterol, insulin, ß-mercapto-ethanol and Iscove's modified Dulbecco's medium (IMDM), supplemented with growth factors and antibodies, exactly as reported.[60]

Preparation of conditioned media: In three experiments conditioned media were collected following culture of enriched normal marrow cells with and without IL-1 in serum-free medium. In parallel a control medium with IL-1 but without cells was also prepared. The media were harvested after 64 hours of culture, centrifuged to remove cells and stored at -20°C. The cells from which these supernatants were derived were also examined cytologically following May-Grünwald-Giemsa staining.

TNF- and GM-CSF assays: Immunoreactive TNF was measured with the TNFα immunoradiometric assay (IRMA;IRE-Medgenix, Fleurus, Belgium), which makes use of several monoclonal antibodies directed against distinct epitopes of TNFα. The assay showed no cross-reactivity with TNFß, IL-1, IL-2 and interferon α, ß and γ. The detection limit was 2 pg/mL TNF and the inter-assay coefficient of variation at a level of 131 pg/mL was 7.2% (n = 10).
GM-CSF was measured by ELISA (Zymogenetics Inc, Seattle, Washington), with a sensitivity threshold of 8 ng/L. For positive release of GM-CSF and TNF in the culture medium, values were required to be 10 ng/L or more.

In situ hybridization with cDNA GM-CSF: In situ hybridization was performed as described,[131] with minor modifications. Enriched bone marrow blast cells (approximately $2x10^{5}$/group) were cultured with or without IL-1 in serum-free medium, harvested after 18 hours, washed twice with α-Minimal Essential Medium (Gibco) and resuspended in 0.050 mL. Then 0.010 mL of this cell suspension was applied to fibronectin-precoated (4μg/mL in PBS; 1 hour) glass slides and permitted to stick for 10 min. Subsequently, the slides were washed in PBS followed by fixation with formaldehyde (3.7% diluted in PBS) for 2 min, dehydration in 70% ethanol for 5 min and air-dried. The 0.8 Kbp cDNA GM-CSF probe (obtained from Genetics Institute[347]) and a control 1.8 Kb cDNA EGF-receptor probe[312] were nick-translated using ^{35}S-dATP resulting in a specific activity of 2-6x10^{8} cpm/μg,

with a fragment size of ±300 bp. Prehybridization (1 hour) and hybridization (18 hours) were done at 37 °C applying 1 ng of radiolabelled probe per slide. Following autoradiography (using NTB-2 Kodak photoemulsion) for 6 to 28 days the slides were stained with May-Grünwald-Giemsa and examined at 1000x magnification.

Results

IL-1 stimulation of DNA-synthesis of purified normal bone marrow blasts: The results of stimulation of highly enriched blast cells from 16 different normal bone marrow donors with IL-1 in the presence or absence of neutralizing antibodies against TNFα, GM-CSF and G-CSF are shown in table 1. IL-1 stimulates DNA-synthesis significantly above background ^{3}H-TdR incorporation in all cases (mean (±SD) stimulation index (SI): 3.5 ± 2.1). The addition of antibodies against TNFα to the cultures abrogated IL-1 dependent proliferation ("blocking") in 9/16 cases partially and in 2/16 cases completely.

Similarly, among those cases the addition of anti-GM-CSF antibody suppressed IL-1 induced proliferation in 4/16 instances partially and in 3/16 cases completely. In 5/16 cases DNA-synthesis was not reduced when either anti-GM-CSF or anti-TNFα were added to IL-1 stimulated cultures. The combination of anti-TNFα and anti-GM-CSF suppressed DNA-synthesis significantly in 13 of 14 cases and complete suppression was evident in 7 of those. In the 5 cases where the single antibodies had not reduced DNA-synthesis the combination of anti-TNF and anti-GM-CSF was clearly active and in two of the latter cases blocking was complete. In 11 of the 14 cases DNA-synthesis was suppressed more efficiently in the presence of both anti-TNFα and anti-GM-CSF antibodies than with either antibody alone, indicating that the effects of the antibodies were additive.

Anti-G-CSF antibody did not diminish ^{3}HTdR uptake in four cases tested. Anti-TNFß had no inhibitory effect on IL-1 induced DNA-synthesis in two experiments (#12 and #16 of table 1) with significant suppression by anti-TNFα (data not shown).

Finally in comparison with IL-1, the ability of LPS (lipopolysaccharide) to stimulate DNA-synthesis of normal bone marrow blast cells was also assessed. LPS (E.Coli serotype 0127:B8; Sigma, St.Louis,MO,USA) added to cultures in concentrations of 0.01 μg/ml up to 1 μg/ml did not induce any stimulation of DNA-synthesis, indicating that the IL-1 effect could not be the result of LPS addition.

Table 1

Stimulation of normal bone marrow progenitor cells by IL-1 and blocking of this effect by anti-TNF, anti-GM-CSF and anti-G-CSF

exp no.	no factor	IL-1 without antibody	SI	IL-1 plus antibody (α) αTNF	αGM-CSF	αTNF αGM-CSF	αG-CSF	αTNF αG-CSF
1	2269±699	5792±527	2.6	5708	6630	4877	-	-
2	3721±1589	8124±2047	2.2	6790	8601	3788	-	-
3	3503±673	7333±1584	2.1	6241	4857	4502	-	-
4	512±289	2488±197	4.9	1971	2260	1668	-	-
5	3305±175	5781±884	1.7	5077	5411	4125	-	-
6	687±115	1990±102	2.9	1087	1666	-	-	-
7	3404±157	4926±606	1.4	2985	3812	-	-	-
8	528±141	4684±445	8.9	3675	3336	4771	-	-
9	3304±192	8443±532	2.6	5925	3710	4128	-	-
10	378±138	1891±564	5.0	815	1019	504	-	-
11	722±173	2873±396	4.0	1916	2004	1840	2816	1646
12	618±298	4423±854	7.2	3164	2842	1914	4542	3286
13	3326±708	4970±584	1.5	3292	3260	2247	4871	3664
14	387±109	776±81	2.0	596	477	349	712	562
15	731±399	3010±593	4.1	1586	1827	856	-	-
16	1252±245	2877±256	2.3	2092	1721	1653	-	-

Triplicate cultures of accessory cell depleted/CD34+ normal bone marrow cells were set up with or without IL-1 (100 U/ml) and in the presence of anti-TNFα, anti-GM-CSF and anti-G-CSF antibodies in several combinations. After 48 hours of culture DNA-synthesis was assessed. Values are expressed as mean desintegrations per minute (DPM) ± standard deviations. The stimulation index (SI) is defined as the quotient of dpm of IL-1 stimulated cultures and dpm of noninduced cultures. Blocking is considered positive (figures in bold face) when dpm ± SD are significantly less ($P < 0.05$; Students t-test) in cultures with antibody as compared to cultures without antibody. Underlined values are not significantly different from the background incorporation (i.e. complete blocking).

TNFα and GM-CSF production by enriched bone marrow blast cells: Conditioned media were prepared from accessory cell depleted CD34 positive blast cells from three donors and the concentrations of immunoreactive TNFα and GM-CSF in those supernatants determined. No GM-CSF and low levels of TNFα were demonstrated in medium conditioned by those cells without IL-1. Both TNFα and GM-CSF concentrations increased significantly in the supernatants of marrow blasts that had been cultured with IL-1 (table 2).

When 10% IL-1 marrow blast derived conditioned medium was added to a standard culture system, no myeloid colonies were induced, however an increase in the number of Epo-induced BFU-E from 100% to 193 % ± 49% (n=3) was noted (data not shown). This suggested the presence of burst promoting activity, a well-known function of GM-CSF[22,68,262] in those supernatants.

Table 2

TNF- and GM-CSF-concentration in supernatants of enriched blast cells from normal marrow induced with IL-1

	TNF and GM-CSF (ng/l) in supernatants					
	blast cells incubated with IL-1		blast cells with no IL-1		cell-free medium with IL-1	
	TNF	GM-CSF	TNF	GM-CSF	TNF	GM-CSF
exp #1	373	nd	186	nd	-	nd
exp #2	638	61	400	-	-	-
exp #3	69	12	18	-	nd	-

Accessory cell depleted/CD34+ normal bone marrow cells were incubated for 64 hours with or without IL-1 (100 U/ml) and the supernatants collected. Control medium without cells but with IL-1 was incubated similarly. The TNF and GM-CSF concentrations are expressed as ng/L. - : negative, i.e., values of 10ng/L or less; nd = not done

In situ demonstration of GM-CSF messenger RNA (mRNA) in normal marrow blasts: Enriched normal bone marrow progenitor cells were incubated with and without IL-1 for 18 hours in serum-free medium. In-situ hybridization of the cells with a GM-CSF cDNA probe revealed GM-CSF mRNA in 6% of unstimulated and in 12 % of IL-1-stimulated cells (table 3). The percentage of strongly positive cells (> 20 grains/cell) was 3% in non-induced cultures, and 7% in IL-1 induced cultures. Figure 1 shows a cytospin slide of bone marrow blast cells after incubation with IL-1 and probed with GM-CSF cDNA.

Table 3

In situ hybridization of GM-CSF mRNA on normal bone marrow blast cells with and without IL-1 stimulation

no. of grains per cell	% positivity unstimulated	% positivity stimulated with IL-1
0 - 5	94.25	88
6 - 10	1.75	3.75
11 - 20	1	1.5
21 - 50	3	5
> 50	0	1.75

In-situ hybridization with a GM-CSF probe was performed on unstimulated and IL-1-stimulated (100 U/ml) enriched normal bone marrow blast cells. After autoradiography the number of grains were counted per cell (400 cells counted). The distribution of cells with 0-5, 6-10, 11-20, 21-50 and more than 50 grains per cell is given for noninduced normal marrow blasts (left column) and IL-1 induced blasts (right column). Control hybridization with an EGF-receptor cDNA probe was less than 0.5 grain/cell.

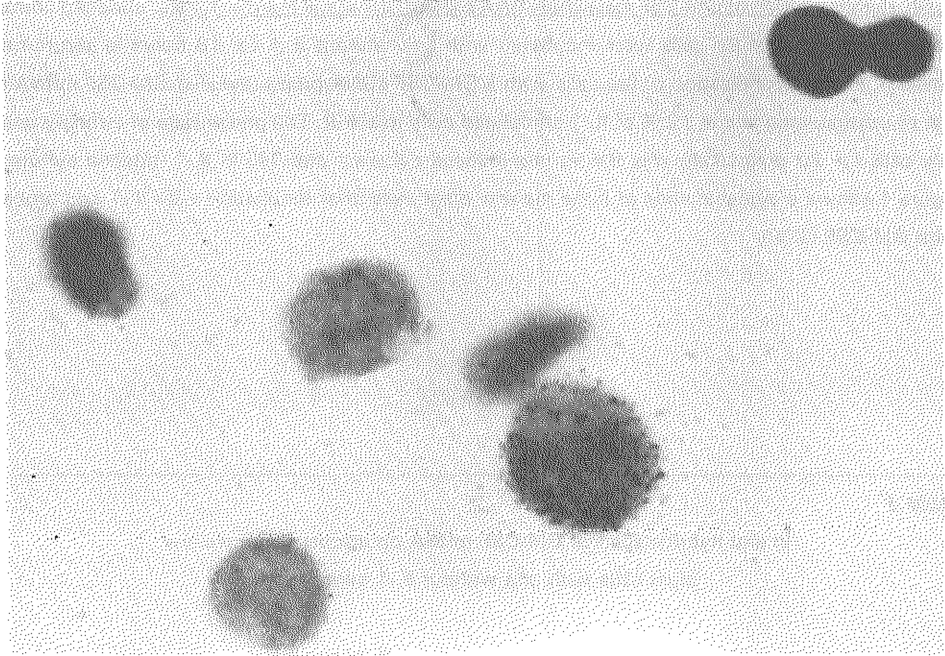


Fig 1. An autoradiogram of a cytospin slide of accessory cell depleted/CD34+ normal bone marrow cells, incubated with IL-1 (100 U/ml) and in-situ hybridized with ^{35}S-labeled GM-CSF cDNA. A cell with multiple grains indicating strong positivity for GM-CSF mRNA is visible among several negative cells.

Discussion

We demonstrate here that IL-1 may induce proliferation of highly enriched immature normal bone marrow cells. The stimulating effect of IL-1 is apparently the result of a two-stage process that includes the induction of TNFα and GM-CSF as a first step and a cellular response of DNA-synthesis as a second event. The notion that IL-1 induces cytokines in normal blast cells to elicit proliferation is supported by three sets of experimental findings: (a) the inhibition of IL-1 induced DNA-synthesis with specific antibodies against TNFα and GM-CSF; (b) the increased release of immunoreactive TNFα and GM-CSF by bone marrow blasts into the culture supernatant following IL-1 induction; (c) the increase of GM-CSF mRNA positive cells among normal marrow blasts and the appearance of cells strongly positive for GM-CSF mRNA ($>$ 50 grains/cell) in response to IL-1. The role of IL-1 in the regulation of human hematopoiesis is diverse. Hitherto direct effects of IL-1 upon blast cells have not been recognized. Indirect effects of IL-1 however are well established. For instance GM-CSF and G-CSF production is induced in fibroblasts and endothelial cells following stimulation

by IL-1.[29,137,262,275] The enriched blast cell population which was separated from normal marrow and used in the experiments described here was carefully depleted of accessory cells and consisted almost exclusively of cells with blast-morphology. After 64 hours in culture there was no cytological change in the cell population. Nevertheless we cannot absolutely exclude that minimal numbers of accessory cells were admixed with the immature cell fraction and responsible for the IL-1 effect. We however do not consider this a likely explanation since up to 12% of IL-1 induced cells expressed GM-CSF mRNA and this fraction does not correlate with the number of morphologically recognizable accessory cells. Our observations suggest that certain normal marrow blast cells may produce GM-CSF and TNFα. In analogy leukemic blast cells are able to produce GM-CSF in response to IL-1 and thus stimulate their own proliferation.[60,352] It follows that autocrine stimulation of blast cells may be a physiologic event rather than being leukemia specific (pathologic).

Observations that TNF may strongly enhance GM-CSF induced proliferation of human AML-cells[61,113] suggest an important role for TNF and its inducer IL-1 in the regulation of hematopoiesis. Even at low levels of GM-CSF significant proliferative activity can be induced in immature marrow cells when TNF is present as costimulus. Thus positive interactions between TNF and GM-CSF permit powerful stimulatory effects that appear under control of the common inducer IL-1. In addition it is conceivable that as an extension of the here described stepwise mechanism of positive regulation primed by IL-1, TNF may in turn trigger the release of GM-CSF and G-CSF in tertiary blast cells in analogy to its ability to induce GM-CSF and G-CSF in fibroblasts and endothelial cells[28,208] thereby further augmenting the hematopoietic response. Furthermore it has been shown that TNFα induces the production of IL-1 in endothelial cells and fibroblasts.[150,218] The evidence that IL-1 and TNF may reciprocally stimulate the production of each other in different target cells implies that both molecules may significantly amplify their positive effects on CSF production providing a very powerful mechanism for expansion of hematopoiesis in times of stress.

Chapter 8

GENERAL DISCUSSION

8.1 Interleukin-3 and Granulocyte-Macrophage-CSF

The colony-inducing abilities of the two multipotent growth factors IL-3 and GM-CSF were examined and compared. The experiments described in chapter 3 demonstrate that IL-3 and GM-CSF have partly overlapping targets in vitro. Both molecules induce colony formation from BFU-E, CFU-GEMM and CFU-Eo (chapters 2 and 3). The narrow spectrum of target cell stimulation contrasts to the stimulation of a broad array of progenitor cells that has been reported by other investigators.[156,168,196,198,271,272,306] Our findings suggest that the published wider range of target cells stimulated by IL-3 and GM-CSF is probably due to costimulation by admixed accessory cells.[71,168,191,196,198,231,271,272,286,306] The limited stimulative spectrum of IL-3 and GM-CSF becomes apparent only when the target progenitor cells are separated from accessory cells. The admixture of monocytes (for IL-3 and GM-CSF) or granulocytes (for GM-CSF) with progenitor cells results in the outgrowth of CFU-G, CFU-M and occasional CFU-GM in IL-3 or GM-CSF stimulated cultures. This most probably relates to the abilities of monocytes to produce G-CSF, M-CSF and possibly GM-CSF in response to IL-3 and GM-CSF (table 8.1), and of the abilities of granulocytic cells to produce G-CSF and M-CSF in response to GM-CSF (table 8.2). The cytokines with regulatory functions in hematopoiesis that are produced by monocytes are listed in table 8.1 Many other cytokines are also produced by monocytes (reviewed by Nathan[216]). The hematopoietic regulators that may be produced by neutrophilic granulocytes are listed in table 8.2.

The experiments described in chapter 3 demonstrate that even in the progenitor cell-enriched CD34-positive cell fraction a strong accessory cell effect may still be apparent. Additional depletion of myeloid cells and monocytes with immune cytolysis restricts the stimulating effects of IL-3 and GM-CSF significantly. The results from chapters 2 and 3 thus emphasize the need for a rigorous elimination of accessory cells from the target cell population in order to separate direct effects of growth factors from indirect effects. Recently the limited spectrum of stimulation of IL-3 and GM-CSF has been confirmed by others. The effect of IL-3 was shown to be confined to early progenitors[170] and eosinophil progenitor cells.[54,286] The IL-3 effects on more mature granulocytic progenitors have been demonstrated to be largely dependent on the presence of lineage-specific growth factors in culture, e.g. G-CSF and/or serum components.[284]

table 8.1 **CYTOKINES PRODUCED BY MONOCYTES**

	Following stimulation by		References
M-CSF	adherence	PHA	
	IL-1	IFN-γ	117, 118, 161, 173
	TNF	IL-3	226, 227, 248, 275
	LPS	GM-CSF	320, 340
	IL-4		
	phorbolesters		
G-CSF	adherence	PHA	
	IL-1	IFN-γ	161, 173, 227, 228
	TNF	IL-3	275, 320, 340
	LPS	GM-CSF	
	IL-4		
GM-CSF	adherence	LPS	161, 275
	TNF	IL-1	
TNF	adherence	LPS	
	IL-1	TNF	39, 107, 112, 125
	IL-3	GM-CSF	161, 219, 238
	M-CSF		329, 332
	LFA-3, CD44, CD45		
IL-1	adherence	LPS	
	TNF	M-CSF	83, 112, 282
	IFN-γ+IL-2	GM-CSF	329, 332
	LFA-3, CD44, CD45		
IL-6	adherence		1, 14
	LPS	IL-1	120, 308

With respect to GM-CSF several studies have shown that the stimulatory spectrum of GM-CSF depends largely on the level of enrichment of the bone marrow and the culture conditions.[13,84,200] GM-CSF is a multipotential hematopoietin with effects on early maturation stages.[284] Part of the GM-CSF production may be autocrine and IL-1 driven, with an enhancing role of unknown significance for TNF (chapter 7). The stroma is part of a positive feedback loop that includes IL-1 and TNF-α resulting in the production of GM-CSF. The colony stimulating effects of GM-CSF on CFU-G and CFU-M mainly depend on accessory cells and synergistic activity with other growth factors (Chapters 3 and 5[13,84,193]). CFU-Eo are stimulated strongly by GM-CSF.[54]

table 8.2	CYTOKINES PRODUCED BY NEUTROPHILS Following stimulation by	References
IFN-α	G-CSF	269
IL-6	GM-CSF TNF LPS + PMA	49
IL-1	GM-CSF	165, 184
TNF-α G-CSF M-CSF	GM-CSF	166

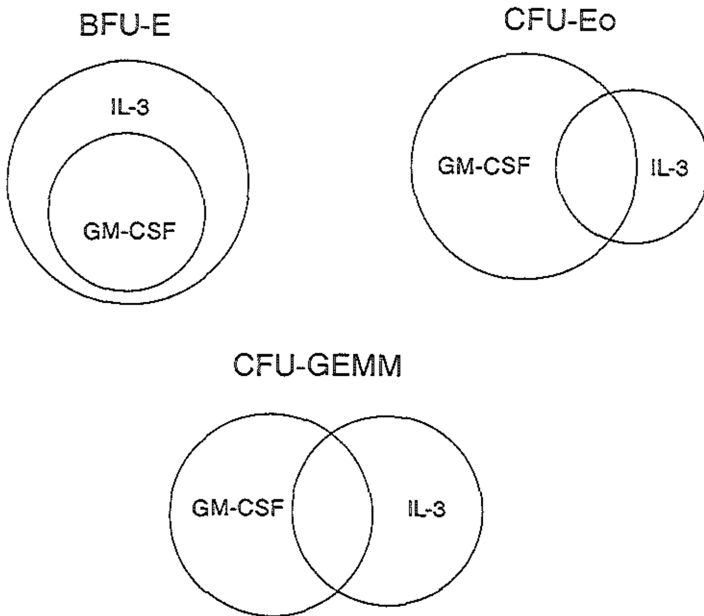


Figure 8.1. A comparison of the stimulatory effects of IL-3 and GM-CSF on BFU-E, CFU-Eo and CFU-GEMM.

Although IL-3 and GM-CSF stimulate largely similar types of progenitor cells, they differ in the magnitude of their response (figure 8.1). IL-3 is a more efficient stimulator of BFU-E. In cultures stimulated with optimal concentrations of IL-3, the addition of GM-CSF does not further enhance BFU-E numbers. This indicates that the population of IL-3 responsive progenitors includes the population of GM-CSF responsive BFU-E. Equivalent numbers of CFU-GEMM are induced in response to GM-CSF and IL-3. The colonies formed do not differ with respect to size and maturational status.

CFU-Eo are stimulated equally well by GM-CSF and IL-3 with respect to numbers. Furthermore the effects of GM-CSF and IL-3 on eosinophilic progenitor cells are partially additive indicating that incompletely overlapping populations of CFU-Eo are stimulated by GM-CSF and IL-3. Eosinophil colonies stimulated by GM-CSF tend to be larger than colonies stimulated by IL-3. For full expansion and maturation of eosinophils IL-5, produced by T-cells, is also required.[164,278]

The resemblance in activity between GM-CSF and IL-3 is supported by similarities in their receptor binding capacities. IL-3 and GM-CSF reciprocally inhibit binding of the other growth factor to its receptor on eosinophils[171] and on monocytes which has led to the speculation that a common receptor chain may be involved.[75] Park et al[233] have shown that a subclass of IL-3 receptors binds GM-CSF. Budel et al[34] demonstrated a common IL-3/GM-CSF binding site on monocytes and leukemic cells. These investigators also found low affinity GM-CSF binding sites that cannot bind IL-3 and high affinity IL-3 sites that cannot bind GM-CSF on monocytes. A structural explanation for these phenomena was at least partially provided by the demonstration of the existence of a common ß-chain for the GM-CSF and IL-3 receptors.[108,142] Later the common β-chain was also found to be associated with the IL-5 receptor.[297,298] The ß-chain together with the specific GM-CSF or IL-3 or IL-5 α-chain thus may create the high-affinity GM-CSF, IL-3 and IL-5 receptors respectively. The GM-CSF, IL-3 and IL-5 α-chain per se bind the corresponding ligand with low affinity (recently reviewed by Nicola & Metcalf, Cell 67:1, 1991).

The fact that IL-3 and GM-CSF seem to act largely on identical target cells may suggest redundancy within the system of growth factor regulation of hematopoiesis. The overlap in target cell spectrum between IL-3 and GM-CSF may however be incomplete with IL-3 stimulating the more immature progenitors and GM-CSF stimulating the more mature progenitors. This is supported by the finding that IL-3 responsive CFU-blast are not responsive to GM-CSF[151,224]. At the other end of the spectrum it appears that the synergistic effects between GM-CSF and G-CSF/M-CSF are greater than the synergistic effects between IL-3 and G-CSF/M-CSF (see below).

8.2 Granulocyte-CSF and Macrophage-CSF

As is evident from the experiments described in chapters 4 and 5, the unipotent CSFs G-CSF and M-CSF stimulate the formation of mature granulocytic colonies and small numbers of macrophage colonies, respectively. The stimulatory efficiency of these factors increases considerably in synergy with IL-3 or GM-CSF. GM-CSF synergizes strongly with G-CSF: G-CSF induced colonies are augmented in number and size with an increased proportion of immature cells when GM-CSF is present in culture as costimulus. Hematopoietic progenitor cells generally survive in the presence of hematopoietic growth factors. Following the withdrawal of these factors the cells undergo active cell death (apoptosis) and expire. Hematopoietic growth factors factors can prevent or postpone apoptosis, a mechanism that has recently been demonstrated in cell lines.[309,343] The survival of CFU-G in culture which depends on the presence of G-CSF improves when GM-CSF is also added to the cultures. IL-3 in combination with G-CSF has a positive effect on CFU-G colony size and CFU-G survival and inhibits maturation moderately. The magnitude of the response to IL-3 is however considerably smaller than that to GM-CSF and the numbers of G-CSF induced colonies are not elevated in the presence of IL-3.
The colony response to M-CSF alone, that is comparatively small, is also enhanced by GM-CSF. Costimulation with M-CSF and GM-CSF augments numbers and size of macrophage colonies. IL-3 however has no measurable effect on M-CSF induced colony formation.
It is thus apparent that IL-3 and GM-CSF show in addition to the similarities in action (see section 8.1) differences as regards their synergistic effects with G-CSF and M-CSF. It is apparent from the results of chapter 4 and 5 that GM-CSF has a great influence on colony numbers from CFU-G and CFU-M while IL-3 has not. Apparently with progressive maturation the cells remain dependent on GM-CSF but meanwhile loose responsiveness to IL-3.[224]
The newly characterized hematopoietic growth factor SCF also synergizes with G-CSF resulting in increased numbers and larger CFU-G colonies.[17,194] Broxmeyer et al[32] demonstrate enhancement of size only with respect to CFU-G colonies in response to G-CSF + SCF. Thus SCF has a role that adds to that of IL-3 and GM-CSF on CFU-G. SCF has no effect on M-CSF induced CFU-M colonies.

The three pluripotent stages with declining self-renewing abilities, stem cell, CFU-blast and CFU-GEMM are indicated in Fig.8.2. Following entry into cell cycle these progenitors become dependent on IL-3 for survival and proliferation.[157,223,224] The total number of cell divisions that the progeny of a particular stem cell undergoes may be genetically programmed.[224] The role of SCF[183,359] in stimulating CFU-blast is still unclear. SCF (in cooperation with Epo) stimulates more

and larger CFU-GEMM and BFU-E colonies than do IL-3 and GM-CSF.[17,32,194] Furthermore SCF synergizes with IL-3 and GM-CSF in the formation of CFU-GEMM and BFU-E colonies. Responsiveness to GM-CSF becomes apparent at the CFU-GEMM stages of the stem cell compartment. Cell division of CFU-GEMM marks the transition from immature pluripotent progenitor cells towards lineage restricted progenitor cells. This transition depends on IL-3 and GM-CSF, however it is not clear how the actual choice between the major myeloid lineages is made. It has been hypothesized[224] that the latter decision is the result of a process of sequential loss of differentiation options, proceeding in a stochastic fashion. In in-vitro experiments the majority of the colonies are single-lineage, either erythroid (from BFU-E), or myeloid (from CFU-G, CFU-M or CFU-Eo) or megakaryocytic (from CFU-Meg). A minority of the colonies represent more than one lineage and every combination of mixed colonies may appear.[154] This is considered as evidence that the loss of differentiation options does not proceed in a fixed order but randomly. The relative ratio of the end products is then determined by the relative concentrations of the growth factors involved. In addition the myeloid progenitors may become responsive to IL-5 in a stochastic manner.[153,154]

The progenitor cell termed "CFU-GM" may require further discussion. In most of the older literature on in-vitro bone marrow cultures the term "CFU-GM" is used as a collective name for progenitor cells for all types of myeloid colonies. The use of the term CFU-GM indicates the existence of a myeloid stem cell which can give rise to lineage specific progenitor cells like CFU-G, CFU-M, CFU-Eo and CFU-Baso. Analogous to the CFU-GEMM, CFU-GM should also be able to form mixed colonies with exclusively myeloid elements. In our experiments with highly enriched progenitor cells and pure recombinant growth factors, either as single factors or as combinations, these mixed myeloid colonies were very rare. Even with the combined stimulation of IL-3, GM-CSF, G-CSF and M-CSF scarce CFU-GM colonies were detected. Mixed granulocyte-macrophage colonies appear in appreciable numbers only when PHA-LCM is applied. This is perhaps best demonstrated by figure 1 and table 1 in chapter 3. With increasing level of enrichment of the target cell population GM-CSF loses its ability to stimulate CFU-GM (as well as CFU-G and CFU-M), while PHA-LCM continues to stimulate CFU-GM. This may indicate that PHA-LCM contains an additional substance needed for the outgrowth of GM-colonies.

Efficient stimulation of colony formation requires the cooperation of a variety of growth factors of which IL-3, GM-CSF, G-CSF and M-CSF and SCF have been identified. Not unlikely the existence of more molecules that regulate the proliferation and survival of committed myeloid progenitor cells may continue to be discovered This complex network of enhancing interactions suggest the functional importance of synergism for steady state and/or compensatory control of hematopoiesis.[42,84,193,259] In addition it most likely has important implications for the therapeutic use of these molecules in vivo for stimulating hematopoiesis optimally.

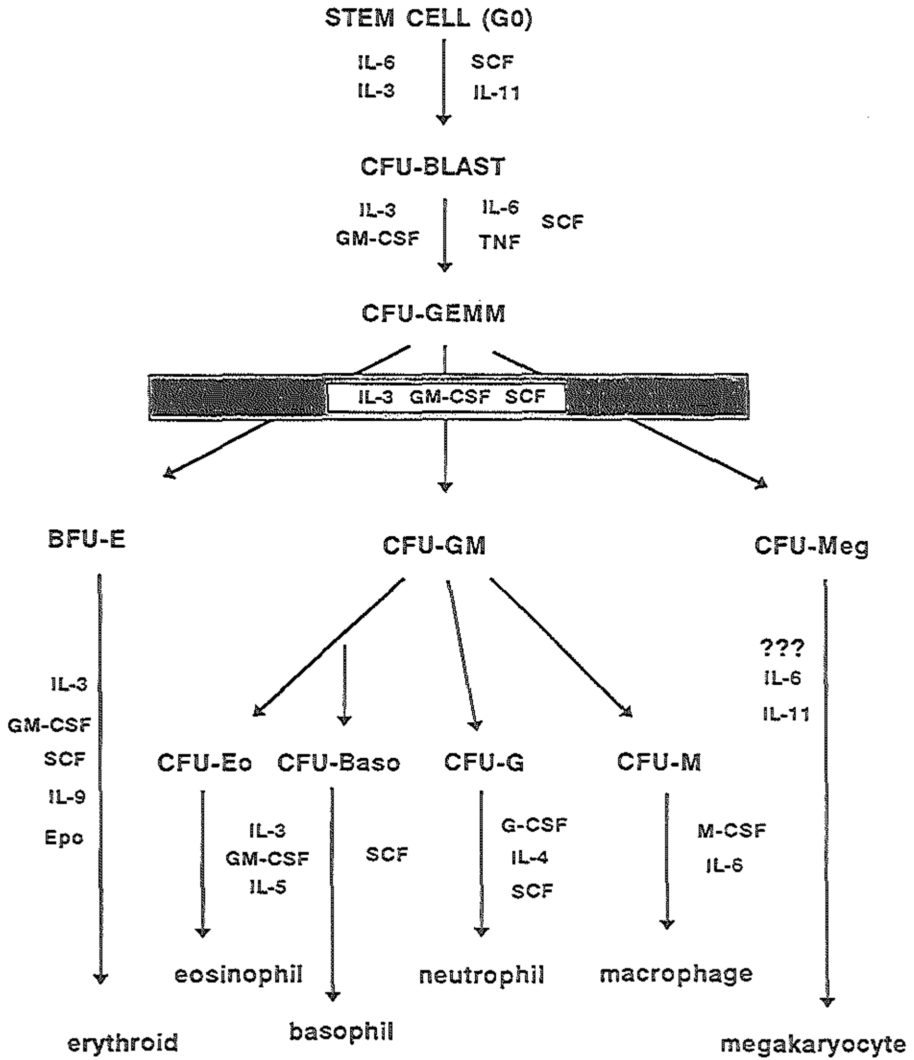


Figure 8.2. The hierarchical system of hematopoietic progenitor cells with the growth factors and cytokines that are active at the transitions between the various cell types.

8.3 Interleukin-6, Tumor Necrosis Factor and Interleukin-1

From the experiments described in chapter 6 it appears that IL-6 when applied in vitro to highly enriched progenitor cells, neither stimulates colony growth, nor affects colony growth induced by IL-3, GM-CSF or G-CSF. However when combined with M-CSF a strong synergistic effect appears with respect to number and size of macrophage colonies (chapter 6). Furthermore IL-6 has a positive effect on the primitive pluripotent progenitor cells. These cells are generally dormant (in G_0-phase) and enter active cell cycle after variable G_0 periods. IL-6 shortens the G_0 period and recruits more stem cells into the G_1-phase.[157,223,224] IL-6 has been shown to counteract wild-type p53 induced apoptosis in myeloid leukemia cells.[354] Furthermore IL-6 promotes the survival of an IL-3 dependent cell-line possibly by delaying apoptosis.[309,343] It may be speculated that IL-6 also can promote survival of stem cells by delaying apoptosis.
Thus IL-6 has an effect on at least one type of committed progenitor cell (CFU-M) as well as on immature progenitor cells. The effect on CFU-M was recently confirmed by Jansen et al.[128], who found IL-6 to be "a permissive factor for monocytic colony formation".
IL-6 is a prime mediator in the host response to infection and injury[349] and the IL-6 effects on hematopoiesis could be part of this response. IL-6 production is an early event in the response to infection and likely leads to stimulation of hematopoiesis as a whole and to an expansion of the monocyte/macrophage pool in addition to its positive action on B cells. The macrophages capable of producing IL-6 thus may establish an autocrine feedback loop.

The effects of interleukin-1 became evident in a proliferation assay of highly enriched bone marrow blast cells (chapter 7). IL-1 induced the production of TNF and GM-CSF from this population of very immature cells. These secondary factors in concert induced proliferation of the cells (chapter 7). Schaafsma et al[259] have shown that IL-1 also induces G-CSF from a population of CD34/HLA-DR enriched bone marrow cells. We did not demonstrate the latter effect. The discrepancy between these results could be due to a different method of progenitor enrichment and consequently different numbers of contaminant monocytes which may have been responsible for the IL-1 induced G-CSF production. IL-1 is an efficient inducer of diverse hematopoietic growth factors and other cytokines in monocytes, endothelial cells and fibroblasts (table 8.3).
Another indirect effect of IL-1 on hematopoiesis is its synergistic activity with IL-3 in the induction of CFU-blast. This effect has later been attributed to (IL-1 induced) IL-6 production from accessory cells[157], i.e., IL-6 acting as the actual synergistic factor. The observation that IL-1 can induce secondary growth factors in normal bone marrow blast cells as well as in accessory cells emphasizes its potentially important role in the regulation of hematopoiesis.

table 8.3 **CYTOKINES PRODUCED BY ENDOTHELIAL CELLS AND FIBROBLASTS**

	Following stimulation by	References
M-CSF	IL-1	88, 261, 273, 274, 275
	TNF	261, 275
	LPS	275
G-CSF	IL-1	29, 86, 88, 261, 273, 275, 358
	TNF	30, 144, 261, 275
	LPS	275
GM-CSF	IL-1	29, 86, 88, 137, 261, 262 274, 275, 358
	TNF	28, 30, 308, 261, 273, 275
IL-1	LPS	162, 167, 202, 324
	TNF	150, 162, 167, 218
	IL-1	187, 327
IL-6	IL-1	55, 129, 146, 188, 189, 279
	TNF	129, 145, 188, 189
	LPS	129, 188, 189

Apparently IL-1 occupies a central position in the cytokine network that directs the hematopoietic and immune systems as well as the response to infection and injury.

IL-1[87], and perhaps TNF and IL-6 as well, may provide continuous signals to the bone marrow stroma production of G-CSF and M-CSF, factors that are essential for the survival and proliferation of mature hematopoietic progenitor cells. Tovey et al[310] have demonstrated high-level expression of the genes for IL-1, TNF and IL-6 in tissues of normal individuals. The constitutive expression of IL-1 may promote hematopoiesis in three ways, at three different levels of maturity:

a. induction of IL-6 which, in synergy with IL-3, recruits stem cells from the G_0-phase into cell cycle,

b. induction of the production of GM-CSF (and TNF-α) by very immature progenitor cells for their own survival and proliferation,

c. induction of the production of GM-CSF, G-CSF and M-CSF by endothelial cells and fibroblasts, for survival and proliferation of the more mature types of progenitor cells.

In the response to infection and injury the elevated production of IL-1 may augment hematopoiesis according to the same mechanisms.

TNF-α clearly has indirect effects on the proliferation of immature progenitor cells. The release of TNF-α and GM-CSF as triggered by IL-1 may result in a proliferative response in bone marrow blast cells (see above).In addition TNF-α may further augment the release of GM-CSF. Whether TNF-α also exerts a direct stimulative effect on the blast cells remains a possibility, since in several experiments proliferation can be blocked only with antibodies to TNF-α and not with antibodies to GM-CSF. This can be explained by the intermediate production of a factor other than GM-CSF by TNF-α, or by a direct effect of TNF-α.
Our observations are in agreement with recent data,[11,239] indicative of a positive stimulatory effect of TNF-α on IL-3- or GM-CSF-induced progenitors.
TNF-α inhibits the proliferation of G-CSF induced progenitors. This may relate to the observed TNF-induced down-regulation of G-CSF receptors on myeloid cells.[73] Apparently TNF-α has dual effects on hematopoiesis: enhancement of the immature stages and suppression of the mature stages. At the same time TNF-α is a strong inducer of GM-CSF, G-CSF and M-CSF in monocytes, endothelial cells and fibroblasts.
These positive and negative actions may be part of the physiologic response to infection and injury: a central response directed at expansion of the early stages of myelopoiesis, combined with a peripheral response characterized by stimulation of monocytic and granulocytic effector cells.[40]

These investigations shed some light on the complex interactions in the humoral regulation of hematopoiesis. With the emergence of an increasing number of new growth factors, research will continue. On the one hand this will be done at the single cell level, eliminating all accessory cell effects. On the other hand research will focus on growth factor-receptor interactions and signal-transduction pathways. Eventually this may lead to a full description of the human hematopoietic stem cell in terms of growth factor requirements and possibly to the ability to propagate this stem cell in vitro.

Summary

Hematopoiesis is the process of cell division, differentiation and maturation that begins at the hematopoietic stem cell and terminates at the functional end cells. This process is controlled by a set of glycoprotein molecules termed hematopoietic growth factors. The cDNAs encoding for the major hematopoietic growth factors have been cloned and expressed and the pure recombinant growth factors became available for research. In this thesis in vitro experiments are described concerning the regulation of growth and differentiation of hematopoietic progenitor cells by recombinant hematopoietic growth factors.

In chapter 1 the growth factors and cytokines that were part of the experimental work, i.e. IL-3, GM-CSF, G-CSF, M-CSF, IL-1, IL-6 and TNF, are discussed briefly.

Chapter 2 describes the effects of IL-3 on in-vitro colony formation from enriched, BI3C5-(CD34)-positive, bone marrow progenitor cells. We demonstrate that IL-3 stimulates colony formation from BFU-E, CFU-Eo and CFU-GEMM. A broader spectrum of stimulation, i.e. including CFU-G, CFU-M and CFU-GM becomes evident when monocytes are added to the cultured cell suspension. T-lymphocytes and granulocytes do not show this effect. Apparently monocytes produce secondary growth factors, e.g. GM-CSF and/or G-CSF, which induce additional colony formation from CFU-G, CFU-M and CFU-GM.

The important role of accessory cells in the expression of the colony stimulating activity of hematopoietic growth factors is also demonstrated in chapter 3. GM-CSF is considered a multilineage colony stimulating factor, similar to IL-3. The experiments described in chapter 3 provide evidence that from a population of highly enriched progenitor cells GM-CSF stimulates colony formation from BFU-E, CFU-Eo and CFU-GEMM only. When unpurified marrow is cultured, GM-CSF also stimulates colony formation from CFU-G, CFU-M and CFU-GM. IL-3 and GM-CSF thus have a similar restricted spectrum of target cell stimulation, i.e. BFU-E, CFU-Eo and CFU-GEMM. They mainly differ in a quantitative sense. IL-3 is a more potent stimulus for BFU-E. GM-CSF is a more efficient stimulator of CFU-Eo.

When we take into account that IL-3 and GM-CSF have a limited spectrum of stimulation, full outgrowth of all types of colonies can only occur when these early acting hematopoietic growth factors cooperate with other factors e.g. G-CSF and M-CSF. In chapter 4 the cooperative effects of IL-3 and G-CSF are described. Although IL-3 does not induce colony formation from CFU-G, we demonstrate that IL-3 has significant effects on granulocytic colony size, composition and survival.

Specific interactions between certain early acting and late acting hematopoietic growth factors are discussed in chapter 5. GM-CSF, that does not stimulate colony formation from CFU-G, CFU-M or CFU-GM, exhibits a strong synergistic effect on G-CSF and M-CSF derived colony formation. Colonies stimulated by G-CSF plus GM-CSF are considerably larger and contain more

immature cells than colonies stimulated by G-CSF alone. Furthermore the in-vitro survival of CFU-G is enhanced by GM-CSF. M-CSF by itself stimulates scarce macrophage colonies from highly enriched progenitor cells. In combination with GM-CSF however many CFU-M are induced to colony formation. Eosinophil colony formation induced by GM-CSF or IL-3 is not affected by the supplementation of G-CSF or M-CSF to culture. The two multilineage growth factors, IL-3 and GM-CSF, have been compared directly. IL-3 only synergizes with G-CSF with respect to colony size and stage of maturation, not with respect to numbers. IL-3 has no effect on M-CSF induced colony formation.

Apart from the four classical hematopoietic growth factors many other cytokines influence hematopoiesis, directly or indirectly. Some of these cytokines have been listed and briefly described in chapter 1. In chapter 6 the effects of IL-6 on in-vitro cultures of highly enriched hematopoietic progenitor cells are discussed. Strong synergism is observed when IL-6 is combined with M-CSF with respect to macrophage colony number and size. IL-6 itself does not induce colony formation, nor does it affect colony formation induced by Epo, IL-3, GM-CSF or G-CSF. This effect of IL-6 on CFU-M is not observed in unpurified marrow cells, presumably because of endogenous IL-6 production by monocytes in the cultures. Monocytes are efficient producers of IL-6 and the synergistic effect of IL-6 and M-CSF on CFU-M can be regarded as a positive feedback mechanism.

Chapter 7 describes another approach to the assessment of effects of cytokines on immature bone marrow cells. IL-1 induces proliferation of highly enriched bone marrow cells (as tritiated thymidine incorporation) through the intermediate production of TNF-α and GM-CSF. G-CSF production is not induced by IL-1. Since the cultured cell population consists almost completely of blast cells, we suggest that the blast cells themselves can be induced to produce TNF-α and GM-CSF. This was confirmed for GM-CSF by in-situ hybridization experiments. An estimated proportion of 12 % of IL-1 induced bone marrow blast cells produce GM-CSF mRNA versus 6 % of noninduced cells. Furthermore TNF-α and GM-CSF are detected by immunologic assays in the supernatants of cultured IL-1 induced blast cells. In cooperation with accessory cells several positive feedback loops can be postulated encompassing IL-1, TNF, GM-CSF, G-CSF and M-CSF. This highlights the importance of the inflammatory mediators IL-1 and TNF in the stimulation of the early stages of hematopoiesis. IL-1 may thus have a function in the regulation of base-line hematopoiesis in three ways: induction of GM-CSF production by immature bone marrow cells and induction of the production of IL-6 and late acting growth factors by accessory cells. These experiments also demonstrate that blast cells can serve as paracrine accessory cells in the stimulation of other blast cells.

In chapter 8 the results are discussed in the context of current knowledge of the humoral regulation of hematopoiesis.

Samenvatting

Hematopoiese is het proces van celdeling, differentiatie en maturatie wat bij de hematopoietische stamcel begint en eindigt bij de uitvoerende cellen. Dit proces wordt gestuurd door een aantal glycoproteinen welke hematopoietische groeifactoren worden genoemd. De cDNA's welke voor de voornaamste hematopoietische groeifactoren coderen zijn gecloneerd en tot expressie gebracht, waarna de gezuiverde recombinant groeifactoren beschikbaar kwamen t.b.v. wetenschappelijk onderzoek. In dit proefschrift worden in-vitro experimenten beschreven aangaande de regulatie van de groei en differentiatie van hematopoietische stamcellen door recombinant hematopoietische groeifactoren.

In hoofdstuk 1 worden de groeifactoren die onderwerp waren van het onderzoek, te weten IL-3, GM-CSF, G-CSF, M-CSF, IL-1, IL-6 en TNF kort besproken.

Hoofdstuk 2 behandelt het effect van IL-3 op de in-vitro kolonievorming door verrijkte BI3C5-(CD34)-positieve beenmerg stamcellen. Wij laten zien dat BFU-E, CFU-Eo en CFU-GEMM tot kolonievorming worden gestimuleerd door IL-3. Een breder stimulatiespectrum, inclusief CFU-G, CFU-M en CFU-GM wordt bereikt wanneer monocyten aan de kweken worden toegevoegd. Met T-lymfocyten en granulocyten wordt dit effect niet bereikt. Klaarblijkelijk produceren monocyten secundair groeifactoren, bijvoorbeeld GM-CSF en/of G-CSF welke op hun beurt kolonievorming door CFU-G, CFU-M en CFU-GM induceren.

De belangrijke rol van accessoire cellen bij de expressie van kolonie-stimulerende activiteit van hematopoietische groeifactoren wordt ook aangetoond in hoofdstuk 3. GM-CSF wordt beschouwd als een multipotente kolonie-stimulerende factor, evenals IL-3. De experimenten beschreven in hoofdstuk 3 laten zien dat in een populatie van sterk verrijkte hematopoietische voorlopercellen GM-CSF alleen kolonievorming induceert door BFU-E, CFU-Eo en CFU-GEMM. Wanneer ongezuiverd beenmerg wordt gekweekt, stimuleert GM-CSF ook kolonievorming door CFU-G, CFU-M en CFU-GM. IL-3 en GM-CSF hebben dus een overeenkomend beperkt stimulatiespectrum, te weten BFU-E, CFU-Eo en CFU-GEMM. Zij verschillen voornamelijk in kwantitatieve zin. IL-3 is de sterkere stimulus voor BFU-E. GM-CSF is een efficiëntere stimulator van CFU-Eo.

Wanneer we er van uitgaan dat IL-3 en GM-CSF een beperkt stimulatiespectrum hebben, dan kan uitgroei van alle typen kolonies alleen plaatsvinden wanneer deze vroeg aangrijpende hematopoietische groeifactoren samenwerken met andere groeifactoren, zoals G-CSF en M-CSF. In hoofdstuk 4 worden de effecten van de gezamenlijke stimulatie door IL-3 en G-CSF beschreven. Hoewel IL-3 geen kolonievorming door CFU-G induceert, blijkt toch dat IL-3 significante effecten heeft op grootte, samenstelling en overleving van granulocytaire kolonies.

Specifieke interacties tussen vroeg aangrijpende en laat aangrijpende hematopoietische groeifactoren worden besproken in hoofdstuk 5. GM-CSF, dat zelf geen kolonievorming door CFU-G, CFU-M en CFU-GM induceert, vertoont een sterk synergistisch effect op kolonie vorming door CFU-G en CFU-M. Kolonies gestimuleerd door G-CSF plus GM-CSF zijn aanzienlijk groter en bevatten meer onrijpe cellen dan kolonies gestimuleerd door G-CSF alleen. Bovendien

wordt de in-vitro overleving van CFU-G door GM-CSF bevorderd. M-CSF zelf stimuleert geringe aantallen macrofagen-kolonies uit sterk verrijkte voorlopercellen. In combinatie met GM-CSF echter worden vele CFU-M aangezet tot kolonievorming. Eosinofiele kolonievorming geïnduceerd door IL-3 of GM-CSF wordt niet beïnvloed door G-CSF of M-CSF. De twee multipotente groeifactoren IL-3 en GM-CSF werden direct vergeleken. IL-3 vertoont alleen synergisme met G-CSF met betrekking tot grootte en maturatiestadium van de kolonies, niet met betrekking tot aantallen kolonies. IL-3 heeft geen effect op M-CSF geïnduceerde kolonievorming.

Behalve de vier klassieke hematopoietische groeifactoren hebben vele andere cytokines direct of indirect invloed op de hematopoiese. Enkele van deze cytokines worden kort beschreven in hoofdstuk 1. In hoofdstuk 6 worden de effecten van IL-6 op in-vitro kweken van hooggezuiverde hematopoietische voorlopercellen besproken. Sterk synergisme met betrekking tot aantal en grootte van macrofagen-kolonies wordt gezien wanneer IL-6 wordt gecombineerd met M-CSF. IL-6 zelf induceert geen kolonievorming, noch beïnvloedt het de kolonievorming geïnduceerd door Epo, IL-3, GM-CSF of G-CSF. Dit effect van IL-6 op CFU-M wordt niet gezien in ongezuiverde beenmergcellen, waarschijnlijk a.g.v. endogene IL-6 produktie door monocyten in de kweken. Monocyten zijn krachtige producenten van IL-6 en het synergistisch effect van IL-6 en M-CSF op CFU-M kan worden beschouwd als een positief terugkoppelingsmechanisme.

Hoofdstuk 7 beschrijft een andere benadering van het onderzoek naar de effecten van cytokines op onrijpe beenmergcellen. IL-1 induceert proliferatie van sterk verrijkte beenmergcellen (gemeten als tritium-thymidine incorporatie) via de intermediaire produktie van TNFα en GM-CSF. G-CSF produktie wordt niet geïnduceerd door IL-1. Aangezien de gekweekte celpopulatie vrijwel geheel uit blastaire cellen bestaat, veronderstellen wij dat de blastaire cellen zelf kunnen worden aangezet tot produktie van TNFα en GM-CSF. Ten aanzien van GM-CSF werd dit bevestigd door in-situ hybridizatie experimenten. 12 % van de IL-1 geïnduceerde beenmerg blast cellen bleken GM-CSF mRNA te produceren tegen 6 % van de niet-geïnduceerde cellen. Bovendien werden TNFα en GM-CSF door middel van immunologische bepalingen aangetoond in de supernatanten van de gekweekte IL-1 geïnduceerde blastaire cellen. IL-1, TNF, GM-CSF, G-CSF en M-CSF nemen deel aan positieve terugkoppelingsmechanismen in samenwerking met accessoire cellen. Dit onderschrijft het belang van de ontstekingsmediatoren IL-1 en TNF bij de stimulatie van de vroege stadia van de hematopoiese. IL-1 kan op drie manieren een plaats hebben bij de regulatie van de hematopoiese: inductie van GM-CSF produktie door onrijpe beenmergcellen en inductie van de produktie van IL-6 en laat aangrijpende groeifactoren door accessoire cellen. Deze experimenten tonen eveneens aan dat blastaire cellen kunnen dienen als paracriene accessoire cellen bij de stimulatie van andere blastaire cellen.

In hoofdstuk 8 worden de resultaten besproken in de context van de thans bekende gegevens over de humorale regulatie van de hematopoiese.

APPENDIX

A culture system for human bone marrow

A historical overview

The development of colony assays for hematopoietic progenitor cells started in the early sixties using inbred mouse-strains. Till & McCulloch[302] developed the in-vivo spleen colony assay in irradiated recipient mice. True in-vitro cell-culture techniques using agar gel or methylcellulose based culture media[240] (Bradley & Metcalf 1966) were initially developed for murine progenitors. Culture systems for human myeloid[240,267] and erythroid[127] progenitor cells followed. These cultures did not permit the formation of colonies from pluripotent stem cells, equivalent to the murine spleen colony forming cell. Based on an in vitro assay for murine cells[130] Fauser & Messner[81,82] then developed a culture system, later modified by Ash et al (5), that permitted the colony formation from both erythroid and myeloid progenitor cells. Among the single-lineage colonies a small fraction of colonies was recognized, composed of both erythroid and myeloid elements. These mixed lineage colonies were assumed to have originated from a more immature cell-type, possibly the pluripotent stem cell itself. This cell was named CFU-GEMM, i.e., colony-forming-unit granulocyte-erythroid-macrophage-megakaryocyte.

The development of an assay for CFU-GEMM.

After analysis of several variables a culture medium was prepared that was used for our experiments with normal bone marrow; it was based on the system originally described by Fauser & Messner.[81,82] The formula of the medium is listed in table A.

Table A

ingredient		add to medium
methylcellulose 2.8%	(a)	40 %
human plasma	(b)	30 %
mixture of nutrients	(c)	10 %
erythropoietin	(d)	1 U/ml
PHA-LCM	(e)	10 %
cells	(f)	0.5 - 1 x 10^5/ml
Iscove's modified Dulbecco's medium (IMDM)		

a. Methylcellulose: This substance makes the medium semi-solid, thus immobilizing the cells and allowing the formation of compact colonies. Throughout all experiments we have made use of Methocel A4M premium quality (DOW Chemical, Rotterdam) with a viscosity of 3500-5600 cps. The powdered methylcellulose is dissolved in Iscove's modified Dulbecco's medium (IMDM). First double strength IMDM is prepared by dissolving one package of IMDM (Gibco) in 500 ml water, adding 3.024 g $NaHCO_3$, 100,000 units penicillin and 100,000 μg streptomycin, followed by filter

sterilization using a .22 filter. Next, 14 g of methylcellulose is put into an erlenmeyer flask together with a magnetic stirring bar; the flask is capped and autoclaved. Then, while stirring, 235 ml boiling water is added and after the methocel has dissolved it is boiled again for 2 minutes. The solution is now allowed to cool (while stirring) to a temperature of 40°C followed by the addition of 235 ml double strength IMDM. This method is neccessary to avoid boiling IMDM which leads to deterioration. Stirring proceeds for two hours at room temperature and then overnight at 4°C. The material is divided in small aliquots while checking for the homogeneity of the solution, and frozen at -20°C.
A culture medium containing 40% of this methylcellulose will have a final methylcellulose-concentration of 1.12%. Many researchers employ methylcellulose in a concentration of 0.8 % to 0.9 %. The advantage of the higher concentration is that in the more viscous medium the colonies are more compact and separated from each other, thus diminishing the occurence of overlapping colonies. An extra advantage is that the medium remains in place when the culture dish is inverted, allowing the dish to be examined under an ordinary microscope, which is definitely superior to an inverted microscope. The possibility of inversion is however also dependent on the type of dish.

b. Plasma: For reasons of convenience we prefer to use autologous heparinized plasma. When this is not available pooled human plasma will also do, in order of preference AB-plasma or blood group compatible plasma. Citrated plasma can also be used, however EDTA-plasma has adverse efects on colony formation. The plasma is centrifuged at 1100g for 10 minutes, collected, centrifuged again and filtrated through a .22 filter.

c. Mixture: The mixture of nutrients was modified by Swart & Löwenberg[296] after Guilbert & Iscove.[103] It contains bovine serum albumin (BSA, 10% in IMDM; Sigma, fraction V, dialysed): 75%; human transferrin (9.62×10^{-4}M) in a solution of $FeCl_3$ (1.92×-10^{-3}M): 8%; egg lecithin (3.75×10^{-3}M): 8%; sodium-selenite (1.25×10^{-5}M): 8% and ß-mercaptoethanol (0.5×10^{-3}M): 0.5%. This mixture is added in a 10% final concentration to the culture medium.

d. Erythropoietin (Epo): Sheep Epo (Connaught, step III) was used until recombinant human Epo (Amgen) became available. In several experiments they were compared and found to be equally effective.

e. PHA-LCM[10]: This medium is used as a source of colony stimulating activity (CSA). For the preparation of PHA-LCM, human blood was separated over a Ficoll-gradient and the mononuclear cells were harvested and washed. To prevent proliferation they were irradiated (25 Gy) and suspended in a concentration of 2×10^6/ml in a medium of 10 % fetal calf serum (FCS) and 1% PHA (Wellcome) in α-MEM (Gibco) in 14 ml tubes (max. 4 ml). After 7 days incubation at 37°C the tubes were centrifuged and the supernatant was sterilized by membrane filtration (0.22). Each batch of PHA-LCM was tested for optimal colony stimulating abilities (number and diversity of colonies) and discarded when not satisfying.
In most experiments however pure, recombinant growth factors were used; PHA-LCM was then, of course, not added to the cultures.

f. Cells: The number of cells per dish depends on the level of enrichment of the cultured cell fraction. In general too high a cell concentration should be avoided as this may lead to inadvertantly low colony counts.[305] The cell number cultured must therefore always balance between the formation of a sufficient amount of colonies and possible negative effects due to crowding of cells.

During preparation of the culture medium the abovedescribed ingredients are added together in a tube of suitable size. Methylcellulose has to be added using a syringe. Due to the viscosity of the medium, one has to prepare more than the desired culture volume, e.g. for a 1 ml culture, 1.5 ml medium is prepared and for duplo 1 ml cultures 2.5 ml. After addition of all ingredients the medium is thoroughly mixed by shaking or vortexing to ensure homogeneity. It is then plated into the desired number of dishes using a syringe. The dishes are collectively positioned into a large glass petri-

dish including a water dish and then placed into the incubator (37°C, air with 5% CO_2, 100% humidity). The incubation period is ideally 15 days; a longer period will lead to increasing degeneration, while a shorter period leaves insufficient time for especially erythroid colonies to mature.

Colonies formed in cultures of human bone marrow

Introduction

The assay that was described in the previous paragraph allows for the recognition of cells by their ability to form colonies. A colony is defined as a group of 50 cells or more that have most likely originated from one parental cell. This cell is designated the "colony-forming-unit" (CFU) and several classes of these are distinguished according to the type of end cells they generate.

Ideally, one should be able to enumerate and classify the colonies in the culture dishes, using an inverted microscope. With the necessary skill and experience this is possible using criteria like color, size and shape of the colony and the nature of the cells that are lying in close proximity to the colony. For reasons of quality control the appearance of a colony has to be compared regularly with the morphology of the cells that make up the colony. To this extent a finely drawn glass Pasteur pipette is used to aspirate single colonies under direct vision through an inverted microscope taking care not to aspirate other colonies or single cells surrounding the colony to be examined. The aspirated colony is then put onto a glass slide using a cytocentrifuge, stained with May-Grünwald-Giemsa stain and examined under a microscope.

Erythroid colonies

In cultures containing erythropoietin (Epo) hemoglobinized erythroid colonies are easily identified by their red color after 15 days in culture. Erythroid colonies are named "Burst Forming Unit-Erythroid" (BFU-E) and the method to culture them in methylcellulose was first described by Iscove et al.[127] The name refers to the tendency of these colonies to form subcolonies which are lying together as "bursts".

The red hue, so typical of colonies containing erythroid cells, cannot be relied on for identification of all BFU-E. When erythroid colonies are relatively immature, hemoglobinization has not yet taken place and the colonies are transparant with a faint brownish tint and may resemble myeloid colonies. Furthermore in cultures which have degenerated to a certain extent the red color becomes brown.

The shape of the colonies is another clue to their identity. A typical feature of erythroid colonies is their compactness, it is often not possible to distinguish single cells along the rim of the colony. An alternative configuration can be seen when the colony lies on the bottom of the dish and a larger rim of single layered erythrocytes surrounds the red center, giving it more or less the appearence of a fried egg. When it is possible to discern single cells, they are relatively large, i.e. being a size between that of an eosinophil and a macrophage, and are not round but rather angular.

Mixed colonies

Mixed colonies, derived from CFU-GEMM, are generally defined as heterogeneous with areas of red cells and areas of colorless cells[81,82] or as aggregates with a compact hemoglobinized center and a peripheral "lawn" of colorless cells.[5] For the distinction of unilineage erythroid colonies from multilineage colonies two characteristics are important: the color of the colony and the appearance of the cells lying directly adjacent to the colony. As has been discussed above, the

color of the colony is not always helpful leaving as the most important criterium the appearence of the cells surrounding the colony. The presence of myeloid cells in close proximity of the red center is the hallmark of a mixed colony.
This directly relates to the problem of overlapping colonies and the percentage of non-erythroid cells that must be present to classify a colony as multilineage.
A major critique to the CFU-GEMM culture system has been that two single-lineage colony-forming cells, e.g. a BFU-E and a CFU-Eo, could develop close to each other in such a way that the result resembles one mixed colony.[243] However with careful observation it is possible to exclude a large part of these overlapping colonies. The use of highly concentrated methylcellulose is an advantage here as it results in a more viscous culture medium with more compact colonies and preventing the outgrowth of large spread-out overlapping colonies.[143] Furthermore, visualization through an ordinary microscope, which is superior to an inverted microscope, allows for a better distinction of separate colonies. Crowding of colonies has to be avoided by culturing low numbers of cells. Thus with critical colony scoring it is possible to give an accurate account of the number of mixed colonies, with a low number of "false-positives".
When candidate mixed colonies are picked off in order to examine the morphology of the cells, surrounding myeloid cells may be aspirated inadvertantly thus confounding the differential count. To control for this error we have calculated the percentage of myeloid cells in 60 carefully aspirated erythroid colonies. We found on average 0.5% ± 0.6% of myeloid cells (range 0 - 2.4%) and assumed that these gave background contamination levels. For a mixed colony we then required the minimal contribution of myeloid cells to be 5%. All in situ suspect mixed colonies, i.e. with a small number of myeloid cells around the red center, consistently contained more than 5% myeloid cells, which apparently were mixed with the red cells and were not visible.
Mixed colonies are composed of a mixture of erythroid and non-erythroid (myeloid) cells and are often named CFU-GEMM, i.e. derived from a cell that can form a colony incorporating granulocytes, erythrocytes, macrophages and megakaryocytes. In practice part of the mixed colonies do not incorporate all four lineages present in the name CFU-GEMM, however by definition at least two have to be present.

Eosinophil colonies

Eosinophil colonies have a distinct morphology and are easily recognized in culture dishes. They appear as small and compact "tight" colonies composed of round cells with a highly refractile border.[62,72,126] The roundness of the cells is a very distinct feature and together with the refractileness it seems as if the cells are surrounded by a thin, black circular line. When eosinophil colonies are stimulated with a combination of IL-3 and GM-CSF they can reach a considerable size. Nicola et al[220] mentioned that most eosinophil colonies contain only eosinophils. In a minority of cases we found also mixed granulocytic/eosinophilic colonies. The very large and compact eosinophil colonies can easily be mistaken for a somewhat degenerated BFU-E, the size of the cells then serves to classify the colony correctly.

Granulocytic colonies

The name "granulocytic colony" usually refers to colonies of the neutrophil lineage. Granulocytic colonies are not easily discribed; they may appear in many patterns from compact round to loosely arranged and dispersed. The colonies are composed of clear cells which are slightly smaller than eosinophils. Usually these cells are not round but angular. The small dispersed type of colony is often composed of small and round cells which are mature segmented granulocytes. The diversity in colony- and cell type reflects differences in maturation state and depends largely on the type of stimulus used.
Important in the classification of granulocytic colonies is the question whether other, non-granulocytic cells are admixed. This is not difficult in case of admixed eosinophils or mature macrophages, which are readily distinguished. However it may be particularly difficult to distinguish immature cells of the monocytic lineage. These cells are

somewhat larger than myeloid cells. A colony composed of cells of uneven size may be suggestive of a granulocyte-macrophage (GM) instead of a granulocytic colony. In many cases it will be necessary to identify the nature of the colony by microscopical examination of a stained cytospin preparation.

Macrophage colonies

Macrophages, together with megakaryocytes, are the largest cells that can be found in colony cultures of human marrow. The macrophages are apparent by their brown color and the abundance of internal structure. Megakaryocytes on the contrary appear empty and have a highly refractile border.

Most macrophage colonies are loosely arranged and dispersed. They contain a comparatively small number of cells, i.e. rarely more than 200 (in the mouse , macrophage colonies are the largest colonies, containing many cells). Under optimal stimulatory conditions however larger, compact colonies may arise as well. When immature monocytic cells are involved problems may arise in differentiating the colonies from granulocytic colonies.

Granulocyte-macrophage colonies

This appears to be a very rare type of colony (again, in the mouse this is a very common type of colony). Their occurrence depending largely on the type of stimulus used. They are composed of a mixture of myeloid cells and macrophages and are usually easily recognized. Identification may be more difficult when the macrophage part consists of immature monocytes or when a dispersed macrophage colony overlaps with a granulocytic colony.

Megakaryocytic colonies

In rare cases megakaryocytes can be encountered in the cultures. They are very easily recognized being large clear cells with a very peculiar refractile border. Because their appearence is not very reproducible, depending amongst other things on the plasma batch used in the cultures, we have elected not to evaluate them. In some cases mention is made of their appearence, however not in a quantitative way.

Blast cell colonies

Ogawa et al. have described a colony type which contains blast cells and has self-renewal capacity.[156] These colony forming cells are more primitive than the CFU-GEMM and as yet they represent the most immature progenitor cells that can be cultured routinely. The blast-cell colonies develop in cultures of enriched bone marrow cells that have been left without growth factors for 14 days, allowing the more mature progenitor cells to die due to lack of growth factors. Next IL-3 and IL-6 are added[157] and after another 7 days colonies start to appear which consist mainly of immature blast-like cells and have a very high replating capacity. Since these blast-CFC are present at the initiation of the culture, it is possible that they develop during the 14 to 16 days of an ordinary bone marrow culture, however they are difficult to distinguish between the multitude of much larger mature colonies.

Basophilic colonies

Basophilic colonies are potentially very interesting, since the multilineage growth factor IL-3 was originally described in the mouse as mast cell growth factor.[351] Furthermore human basophils have IL-3 receptors and acquire a differentiated phenotype following stimulation with IL-3.[313,314] It has been suggested that eosinophils and basophils share a common progenitor cell[62] and many CFU-GEMM have been shown to contain histamine.[63] Colonies consisting of basophilic

granulocytes however are not regularly seen in the normal bone marrow cultures described above. They are described as rather small loosely arranged colonies of cells that resemble eosinophils.[152]

Enrichment of progenitor cells

Clonogenic versus accessory cells

The clonogenic hematopoietic progenitor cells comprise only a small fraction of the total cell content of the bone marrow; depending on the definition of a progenitor cell this fraction is estimated to be 0.05% - 0.1%.[100] The other cells present in bone marrow and blood do not form colonies themselves. However these accessory cells may greatly influence colony formation by the clonogenic cells. The group of accessory cells comprises lymphocytes, monocytes and mature myeloid cells, and also stromal cells, e.g. endothelial cells and fibroblasts. In order to determine direct effects of growth factors on progenitor cells, it is necessary to use a cell population which is depleted of all possibly interfering accessory cells. Or, in other words, ideally a cell population consisting solely of progenitor cells.

Methods of enrichment

Instrumental in enrichment procedures is the availability of a useful characteristic of the cell to be enriched. Initially physical characteristics like size and density were employed in often very ingenious procedures. A variety of enrichment methods has recently been reviewed by Visser & Van Bekkum.[323]

Velocity sedimentation is one such method. Its cell separating capacity depends on the size of the cells; when used as a single method however high enrichment factors cannot be expected.[338] Counterflow centrifugation[244,346] is a method that has the same physical basis as velocity sedimentation, i.e. it separates on the basis of size[182] and can process large quantities of cells in relatively short time.

Separation based on the density of cells has shown more promise. Dicke et al[66] reached a 70-100x enrichment of human CFU-C with a two-step discontinuous albumin density centrifugation technique. Moore et al[205] enriched monkey CFU-C 100x using buoyant density gradients. A simplified variant of density centrifugation is the density-cut; this consists of layering the unseparated cells on top of a fluid with a fixed, specific density. After centrifugation the cells with a higher density are pelleted under the separation fluid, while the cells with a lower density are accumulated on top of the separation fluid. The classic and most widely used example is Ficoll-Hypaque with a density of 1.077 g/cm^3.[26] It separates erythrocytes and granulocytes (pellet) from mononuclear cells which remain in the interphase above the Ficoll layer.

The methods listed above do not take advantage of specific phenotypic differences between populations of cells. The use of immunological markers to distinguish cells can serve for both positive or negative selection.

Antibody-labeled cell can be lysed following the addition of complement (usually rabbit-serum).[251]

T-lymphocytes are removed efficiently from the marrow specimen using the sheep-erythrocyte rosette technique followed by Ficoll-separation[177,178,201] A variant of this technique is the method of immune-rosetting[100,115]: cells, labeled with antibodies of murine origin form rosettes with sheep erythrocytes coupled to rabbit anti-mouse antibodies.

In the method of immune-panning[236,350] labeled cells are allowed to attach to petri-dishes coated with rabbit-anti-mouse Ig and separated from the supernatant.[76,77,99]

A final method for selecting cells using several parameters at the same time employs the fluorescense-activated cell sorter (FACS).[18] This method was selected for the work presented in this thesis.

Selection of the method for progenitor cell enrichment

Our finally adopted method for progenitor cell enrichment is a combination of negative selection, i.e. depletion, and positive selection, combining the advantages of a crude method, i.e. fast, simple and able to process large quantities of cells, with a highly selective method. Most authors rely on these combinations of methods.

First, using a nylon filter, bone particles and debris are separated from the marrow cells, which are suspended in HBSS. Second, a density-cut (Ficoll-Hypaque) serves to isolate the mononuclear cells from the erythrocytes and most of the granulocytes. Third, the mononuclear cell suspension is incubated with monoclonal antibodies (MoAbs) against non-progenitor cells. We have made use of several MoAbs like VIM-2[179] directed against a broad range of myelo-monocytic cells, B44.1 (CD14)[237] against monocytes, B4.3 (CD15)[317] against granulocytic cells and OKT-3 against T-lymphocytes. Subsequently the labeled cells were lysed using rabbit complement. In later experiments this labeling-lysis step was combined with an adherence-step. The fourth and final step consisted of selecting the cells positive for the CD34 antigen using the FACS. CD34 is expressed on immature hematopoietic cells and a small subpopulation of monocytes[50,51,133,290,304,330,331]

In summary, the combination of methods we have used consists of two preparative followed by a very selective enrichment step.

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Ik besluit met de volgende stelling: "het is goed dat er geen bijl aanwezig is in de FACS-kamer".

Curriculum Vitae

Freek (Jan) Bot werd geboren op 29 maart 1956 te Wormerveer. Het gymnasium-ß werd doorlopen aan het Zaanlands Lyceum te Zaandam, waarna in 1974 werd begonnen met de studie geneeskunde aan de Vrije Universiteit te Amsterdam. In het derde en vierde studiejaar was hij tevens student-assistent in de anatomie onder leiding van Prof. Dr. F. van Faassen, in welke periode de belangstelling voor wetenschappelijk onderzoek wortel heeft geschoten. Het kandidaatsexamen (cum laude) werd behaald in 1977 en het doctoraalexamen in 1980. Direct aansluitend was hij van april tot oktober 1980 vakantie-assistent bij de afdeling pathologische anatomie van het Academisch Ziekenhuis der Vrije Universiteit (hoofd destijds: Prof. Dr. R. Donner). Het artsexamen werd behaald in 1983 waarna gedurende negen maanden als assistent in een huisartsenpraktijk een zeer praktische leerschool werd doorlopen. In deze periode was hij ook docent chirugie aan de opleiding voor verpleegkundigen SOVAZ te Amsterdam. Vanaf 1984 tot 1989 werd het in dit proefschrift beschreven onderzoek verricht in het celkweeklaboratorium van de Dr. Daniel den Hoedkliniek te Rotterdam (hoofd: Prof. Dr. B. Löwenberg). In deze periode werd geparticipeerd in de lopende beenmergtransplantatie- en thrombocytensupportprogrammas. Tevens is hij sinds 1984 bakenarts bij het Integraal Kankercentrum Rotterdam. Vanaf juni 1989 is hij in opleiding tot patholoog op de afdeling pathologie van het Dijkzigt ziekenhuis/Erasmus Universiteit te Rotterdam (hoofd destijds: Prof.Dr. R. van der Heul, thans: Prof.Dr. F.T. Bosman).