

Hematopoietic Growth Factors and *In Vitro* Growth of Human Acute Myeloblastic Leukemia

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I. HEMATOPOIETIC STEM CELLS: *IN VITRO* ASSAYS AND REGULATORY FACTORS

The production of mature blood cells is finely regulated, so that the homeostasis can be maintained and cell production be adapted to the specific needs of particular blood cell compartments (e.g., granulocytes during infection). A complex regulatory network of positive and negative signals and cellular interactions serves the hematopoietic system to fulfill this role. *In vitro* colony assays are powerful tools for studies that aim at the recognition of hematopoietic progenitor cells of distinct differentiation pathways and at different levels of maturation. These assays have also been used to define the complex role of hematopoietic growth factors in normal hematopoiesis. The same technology is currently being applied for elucidating growth factor responses of neoplastic clonogenic cells in hematopoietic disease.

An *in vitro* colony assay for primitive multilineage hematopoietic precursor cells of both rodents and humans with the capacity to form granulocytic cells, macrophages, erythrocytes, megakaryocytes, collectively designated as CFU-GEMM, has been developed.¹⁻³ Somewhat more mature progenitor cells that are committed to one hematopoietic lineage can be assayed *in vitro* as erythroid burst-forming units (BFU-E),^{4,5} erythroid colony-forming units (CFU-E),⁶ granulocyte/monocyte CFU (CFU-GM),^{7,8} granulocyte CFU (CFU-G), monocyte CFU (CFU-M), eosinophil CFU (CFU-Eo), or megakaryocyte CFU (CFU-Mega).⁹ Several regulatory molecules that induce colony formation from those precursor cells have been isolated following molecular cloning.

In acute myeloid leukemia (AML), a transformed malignant precursor cell arises that is unable to generate terminally differentiated cells. This neoplastic cell population apparently escapes normal maturation impulses and expands a proportionally in marrow and blood.

The technology of *in vitro* colony formation has been applied to examine the proliferation and differentiation abilities of leukemic progenitor cells. In this review we discuss our understanding of leukemic blood cell formation in human AML, mainly based on the study of *in vitro* growth.

II. RECOMBINANT HEMATOPOIETIC COLONY STIMULATING FACTORS

The hematopoietic colony stimulating factors and their target precursor cells are listed in Table 1. These glycoproteins

regulate the proliferation and outgrowth of different hematopoietic progenitors and frequently have a role in stimulating the metabolic and functional activities of the mature cell types as well.

A. Human Multilineage Colony Stimulating Factor (Multi-CSF) or Interleukin-3 (IL-3)

Murine IL-3,³⁸⁻⁴⁰ also termed multi-CSF,⁴¹ mast cell growth factor,⁴¹ or stem cell activating factor,⁴² is a stimulator of the proliferation of mouse *in vivo* multipotential stem cells (CFU-s)^{39,41} as well as the *in vitro* CFU-GEMM.⁴¹ It has been suggested that murine IL-3 is also involved in the self-replication of pluripotent stem cells.^{40,43,44} The human homologue of multi-CSF/IL-3 has been cloned,^{10,11} and was shown to stimulate the *in vitro* proliferation of normal bone marrow CFU-GEMM, BFU-E, and CFU-Eo.¹² A direct stimulating effect of IL-3 upon myeloid progenitors was not apparent *in vitro* when auxiliary cells had been removed from hematopoietic progenitors before culture.¹² IL-3 improves the *in vitro* survival of CFU-G.³¹

B. GM-CSF

Granulocyte macrophage colony stimulating factor (GM-CSF) has effects on the function of granulocytes^{22,45-47} and stimulates the proliferation of CFU-GEMM, BFU-E, and CFU-Eo in normal marrow.^{18-22,24,48} Human GM-CSF cDNA transferred into COS cells produces a glycoprotein with a molecular weight of 19,000 to 22,000^{22,24} and into *Escherichia coli* a nonglycosylated molecule with a molecular weight of 16,000.²³ Radioiodinated GM-CSF has been used to characterize membrane receptors on normal cells.⁴⁹⁻⁵¹ High-affinity (kDa 20 pM to 1 nM) receptors for GM-CSF are abundantly expressed on normal neutrophils and eosinophils (500 to 4000 sites per cell).

C. G-CSF and M-CSF

Complementary DNA of the gene encoding for human G-CSF has been cloned and expressed in *E. coli*.^{29,30} G-CSF (recombinant) has a molecular weight of 18,800 Da. G-CSF stimulates granulocytic colonies from normal human marrow. It does not induce eosinophilic colony formation *in vitro*.

cDNA encoding for human M-CSF, following expression, yields a mature protein of mol wt 26,000 to 45,000 Da depending on differences of posttranscriptional processing.^{32,33}

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Table 1
Human Hematopoietic Growth Factors

Acronym	Protein size (kDa)	Cellular sources		Hematopoietic progenitor cell targets			
		Cell type	Release activated by	Normal	Ref.	Leukemia	Ref.
IL-3	14—28	T cells	Mitogens	CFU-GEMM BFU-e CFU-Eo	10—12	AML-CFU	13—17
GM-CSF	14—35	T cells Endothelial cells Fibroblasts	Mitogens TNF, IL-1 TNF, IL-1	CFU-GEMM BFU-e CFU-Eo	18—24	AML-CFU	14—17,25—28
G-CSF	18—22	Monocytes Fibroblasts Endothelial cells	TNF, IL-1 TNF, IL-1	CFU-G	29—31	AML-CFU	14—17,28
M-CSF	35—45 (×2) 18—26	Monocytes Fibroblasts Endothelial cells	TPA, γIFN	CFU-M	32,33	AML-CFU (rare)	14,16,34
EPO	36	Kidney Liver	Hypoxia Low oxygen	BFU-e CFU-e	35,36	AML-CFU (M-6 type)	14,37

The biological activity on normal human hematopoietic cells is only scarcely defined. On the other hand, human M-CSF is a potent inducer of murine macrophage colonies *in vitro*.

D. Erythropoietin

Erythropoietin was purified following molecular cloning and expression in mammalian cells.^{35,36} Recombinant erythropoietin (mol wt 36,000 glycosylated) stimulates BFU-E and CFU-E *in vitro*.

III. HEMATOPOIESIS IN AML

The malignantly transformed cell population expands progressively when clinical AML evolves. In the marrow of AML patients immature cells, i.e., myeloblasts, predominate. Concomitantly, normal hematopoiesis is suppressed, presumably by physical displacement of the normal stem cells by leukemic cells or inhibitory humoral factors (such as acidic isoferritins)⁵² produced by the leukemic cells, leading to the characteristic anemia, thrombocytopenia, and leukopenia.

Although AML is the result of clonal expansion of a single cell,⁵³ the resulting leukemic cell population is biologically heterogeneous as is evident from morphological, ³H-thymidine labeling,⁵⁴ immunological, cytogenetic, and colony culture studies. For instance, a small population of proliferating cells expressing self-renewal capacities can be identified among the AML cell mass in colony assays.⁵⁵

A. Techniques for AML Colony Growth

In the initial attempts to grow leukemic colonies, normal leukocyte feeder cells were added to an agar underlayer as a

source of stimulating factors,^{56,57} and AML blast cells were plated in a second layer on top of the underlayer. The appearance of leukemia-derived clones in these cultures has been confirmed by cytogenetic analysis.⁵⁸ Other colony stimulating materials that were applied for *in vitro* growth of normal CFU-GM have also been employed for growing AML clonogenic cells, e.g., conditioned media from cell lines,^{59,60} human placental-conditioned medium (HPCM),⁶¹ or phytohemagglutinin (PHA)-leukocyte-conditioned medium.^{62,63}

While these methods efficiently induce normal myeloid colony formation, they are often insufficient to permit the outgrowth of AML colonies. In some cases, no or only single cells survive in the dish at the end of culture, indicating that no proliferation had occurred at all.⁶⁴⁻⁶⁶ Frequently, small (less than 20 cells) or somewhat larger clusters (less than 40 cells) are produced during culture. Using standard culture techniques, the nongrowing and small cluster-forming groups of patients account for the majority (60 to 70%) of the newly diagnosed cases of AML.^{66,67} Seven-day colonies are composed of morphologically identifiable blast cells carrying cytogenetic AML markers.

Attempts have been made to develop more efficient AML culture methods. Exposure of the cells to PHA during a 15-h preincubation in suspension and subsequent culturing in a soft agar dish with a leukocyte feeder allowed for the formation of significant numbers of AML colonies of more than 50 cells in the majority of patients with AML.⁷⁰ In subsequent modifications of the technique, PHA was added directly to one-stage cultures that contained irradiated leukocytes in the agar underlayer and the AML target cells in a liquid overlayer,^{68,69} or PHA and HPCM in combination have been used as additions

to agar cultures.⁷¹ Others have employed PHA-leukocyte-conditioned medium as a stimulus in methylcellulose.^{62,72,73} These modified cultures are permissive to AML colony formation in 80 to 90% of cases.⁵⁵ However, the disadvantage of this type of assay is that the quantitative analysis of colony formation of leukemic cells can be hampered to a certain extent due to agglutination by PHA.⁷¹

Thus the original colony culture techniques were based on the use of crude stimulating materials for growing AML-clonogenic cells, e.g., leukocyte feeder cells or conditioned media from cell lines, human placenta, or PHA-activated peripheral blood mononuclear cells.⁵⁵ It has been shown that particularly T-lymphocytes and monocytes are the active subsets among the leukocyte feeder cells.⁷⁴

Since the availability of pure growth factors, it has recently become possible to (1) understand the exact role of these hormones, either as individual or as combined activities in controlling the proliferation and differentiation of human AML cells and to identify possible abnormalities of AML growth *in vitro* and (2) standardize culture techniques for reproducible application in human AML, that is, with optimal concentrations of stimulators and without inhibitors. Therefore, *in vitro* studies are now undertaken to elucidate the complex system of growth factor regulation of human AML precursors. In this respect, the completely serum-free culture method for human AML cells, which was designed recently, appears a useful addition to the experimental arsenal.⁷⁵

B. Cell-Cell and Stroma-Cell Contact

Several investigators^{70,71} have noted that the application of a liquid phase to the culture of AML cells results in more efficient cell growth and, conversely, that growth was inhibited in increasing concentrations of immobilizing methylcellulose.^{68,69} The fact that the cells could easily form small aggregates from which colony formation was initiated suggested that cell to cell contact may provide signals essential to AML cell growth *in vitro*.⁷⁰ The importance of cell-cell interactions is further supported by enhanced DNA synthesis that was seen when cell proliferative activity of AML blasts was evaluated in direct cell contact facilitating conditions in comparison to cell contact prohibitive circumstances.⁷⁶ These comparisons were made under identical conditions, and an enhanced proliferative rate was positively related to the intensity of cell contact. Increased CSF concentrations in culture could not circumvent the dependence on cell-cell contact. Which membrane structures are involved in enhancement of AML proliferation *in vitro* is presently unknown. An analysis dealing with a possible role of the leukocyte function antigens (LFA), i.e., LFA1, Mac1, p150-95, did not provide evidence to indicate that these surface molecules are directly involved in the up-regulation of AML growth.⁷⁶

The importance of the marrow microenvironment for hematopoietic function is evident from *in vitro* and *in vivo* studies

in rodents, but the role of the marrow stroma with respect to growth of human leukemia is largely elusive at the present time. It has been found that loss of leukemic proliferating cells occurs following cultivation upon stromal layers and that this loss is selective in comparison to normal hematopoietic stem cells.⁷⁷ In CML, blast precursor cells were defective in their capacity to adhere to cultured stromal cells when compared with their normal marrow counterparts, and it was argued that alterations in cell surface properties of the cells may determine modified migration and maturation.⁷⁸ The adhesive properties of the progenitor cells of human acute leukemia have not yet been examined in any reasonable detail.

C. Cellular Heterogeneity

The heterogeneous cellular composition of human AML may be regarded as a hierarchy of subpopulations of cells of different maturation stages.^{55,79} This concept is supported by immunological data that indicate that among AML blasts the *in vitro* colony forming cells (AML-CFU) carry immature surface phenotypes, while the immunophenotypically more mature cells among the leukemia lack proliferative (i.e., colony forming) abilities.⁸⁰ Upon proliferation *in vitro*, colony forming cells give rise to maturing progeny, but maturation is incomplete.^{63,81} Apparently the dynamics and negative relationships between proliferation and differentiation events characteristic of the normal hematopoietic system have to a certain extent been retained in human AML. It is difficult to determine the *in vivo* significance of AML-CFU. *In vitro* observations support the idea that they function to maintain and expand the malignancy and indeed act as progenitor cells: (1) they have capacities for self-renewal,⁸²⁻⁸⁴ and (2) as normal hematopoietic precursors, they have the capacity not only to proliferate, but also to undergo some further differentiation *in vitro*.^{63,81,85,86}

Immunologic analysis has shown that the phenotypes of AML-CFU vary markedly among patients with AML. In certain cases, AML-CFU share immunologic features with CFU-GEMM cells, while in other instances AML-CFU express surface antigen combinations typical of committed granulocyte/macrophage progenitors in normal bone marrow (CFU-GM).⁵⁵ Thus it has been appreciated that the immunological phenotypes of AML progenitors and particular types of normal hematopoietic precursors are largely similar. However, detailed surface marker studies have revealed that discrepancies between the immunologic surface phenotypes of AML-CFU and the majority normal marrow progenitors may exist. In one study that considered not only the presence or absence of antigenic determinants, but also marker density at the cell surface, differences between the immunophenotypes of AML-CFU from most of the normal hematopoietic progenitor cells were recognized.⁸⁷⁻⁸⁹ Thus, frequently, the surface immunophenotypes of AML-CFU and the normal hematopoietic precursors are dissimilar. Crucial in these studies was that a combination of surface markers was selected that identified a constant surface

profile for CFU-GEMM, BFU-e, as well as day 7 CFU-GM and 14 CFU-GM and thus permitted the recognition of phenotypes of AML-CFU at variance with these normal progenitors. Probably, these dominant progenitor phenotypes in AML reflect those of infrequent, although essentially normal, hematopoietic precursors that have expanded selectively in AML as a consequence of the abnormality of maturation.

D. The Role of Colony Stimulating Factors in AML

Have AML cells become independent of CSFs, so that they will expand in a truly autonomous fashion? The answer to this question is probably no. In most cases of clinical AML, the cells show normal susceptibility to growth factor stimulation. IL-3 and GM-CSF, at usual concentrations, each as individual factors, induce colony formation *in vitro*,^{15,27,28} stimulate DNA synthesis,^{14,16} and enhance the survival of AML progenitors *in vitro*.¹⁵ GM-CSF has also been shown to support the self-renewal of AML precursors.²⁶ In addition to IL-3 and GM-CSF, G-CSF is successful in inducing AML cell growth in culture at approximately similar frequency. Often, the same leukemias are susceptible to multiple CSFs. Each of these three CSFs as single factors is able to generate significant proliferation *in vitro*. Infrequently, M-CSF induces AML growth in culture, and erythropoietin has occasionally been reported to act as a growth factor in human AML, i.e., in cases of erythroleukemia (M6).^{14,37} The fact that in certain cases of AML proliferation can be maximally stimulated in the presence of an impure PHA-leukocyte-conditioned medium leaves the possibility open that yet other soluble growth factors operate in AML.

The CSFs affect overlapping stages of the hematopoietic pathways and thus, to a significant extent, exert stimulating effects upon the same target cells. As yet, it remains unclear why it would be useful for the progenitor cells to be stimutable by more than one factor. Obviously, insight into the cooperative interactions between these molecules in controlling human AML growth is of particular interest.

In tritiated thymidine uptake assays it appeared that the responsiveness of AML cells to IL-3 parallels the response to GM-CSF.^{14,16} This suggests that IL-3- and GM-CSF-responsive subpopulations of AML precursors to a certain extent overlap. Also it has become evident that usually IL-3 and GM-CSF do not produce additive proliferative effects, again suggesting that the two CSFs act mainly upon the same AML precursor cell subpopulation. In contrast, G-CSF produces additive growth-potentiating effects in certain cases of AML, when supplemented to cultures with IL-3 or GM-CSF. In colony culture, IL-3 has been shown to elevate colony numbers in a majority of AML cases when supplemented along with G-CSF or GM-CSF to the cultures,¹⁵ but the total colony numbers formed in response to the combinations of CSFs were less than the sum of the individual effects.²⁸ Therefore, most likely, the AML cell mass contains a spectrum of subsets of precursors

with variable growth factor reactivities that can be recruited into cell proliferation, depending on the exact combination of growth factors supplied. These AML cells may represent the neoplastic counterparts of normal marrow progenitors at more or less primitive stages of maturation exhibiting specific CSF requirements.

From dose-effect studies with CSFs, a marked patient-to-patient variation with respect to growth factor responsiveness of AML cell proliferation has become apparent (Table 2). Thus different patterns of response can be distinguished among clinical cases, of AML. For instance, AML cell cycle activation can be achieved with any one of the four CSFs in certain cases of AML, whereas the cells of other patients respond to three or two factors only, or sometimes even to only one of the CSFs. These variations may provide indicators of classifying patients with biologically different disease and may prove to be of prognostic value. Generally, the categories of growth factor reactivity do not correlate with morphological classification of human AML, except in the rare cases of erythroleukemia that appeared especially sensitive to erythropoietin. AML progenitor immunophenotypes established as a parameter of precursor cell maturation did not show a relationship with *in vitro* growth factor dependence either.¹⁴

Table 2
Frequency of Recombinant Hematopoietic Growth Factors Acting As Stimuli of Human AML Growth *In Vitro*

IL-3	GM-CSF	G-CSF	M-CSF	Assay	Ref.
—	32/48	28/48	0/48	Colony formation	28
—	16/20	—	—	Colony formation	27
8/9	9/9	5/9	—	Colony formation	15
19/25	16/25	13/25	4/25	DNA synthesis (serum-free)	14
7/20	9/20	6/20	2/20	Colony formation (serum-free)	14

Note: Ratios indicate quotients of positive responses and total number of cases examined.

The biological basis of the heterogeneity of growth factor responsiveness in clinical AML is not clear. The abilities of the AML blasts to react to certain CSFs and not to others could reflect the physiological diversity of normal hematopoietic progenitors with respect to their susceptibility to the hematopoietic growth factors. In this sense, the diversity of response could indicate the selective increase of certain precursors that have accumulated as a result of the differentiation arrest. An alternative hypothesis to explain these phenomena of growth factor responses is that variations of cell growth are expressions of the transformed status of the cells and that asynchronous maturation is associated with abnormalities (e.g., a loss) of growth factor regulation. As our understanding of specific differences

of growth factor regulation between discrete precursor cell stages even in normal hematopoietis is only beginning to emerge, it is impossible to distinguish between these two explanations at the present time.

Which features of AML growth determine the imbalance of growth between AML and the normal hematopoietic tissue, and why does AML at a certain stage outgrow the normal tissue? Evidence indicating that the overgrowth of AML in normal marrow is caused by specific regulatory abnormalities is still lacking. Preferential growth of AML cells could result from an extraordinary susceptibility to one of the hematopoietic growth factors, for example, due to an overexpression of receptors or their permanent activation by structural receptor abnormalities. Generally, the susceptibility of human AML to IL-3, GM-CSF, or G-CSF^{22,24,26} and the density of GM-CSF receptors upon AML blasts are in the same order as for normal cells.⁴⁹⁻⁵¹ For instance, GM-CSF acts optimally at concentrations of 1 to 5 ng/ml (40 to 200 U/ml, 60 to 300 pM).^{22,24} AML blasts express comparatively low levels (8 to 100 sites per cell) of GM-CSF receptors.^{49,51}

Another possible way of conferring a growth advantage upon AML cells would be the creation of an autocrine loop of stimulation. RNA message of GM-CSF has been demonstrated in the blasts of certain cases of AML.^{90,91} Colony stimulating activity was demonstrated in supernatants conditioned by AML cells. This activity could be neutralized by an anti-GM-CSF antibody.⁹² These data provided evidence for the autocrine production and secretion of GM-CSF in human AML. Indeed, in certain cases, AML cells will form colonies *in vitro* in the absence of any exogenously supplemented growth stimulus. It remains questionable as to whether or not GM-CSF production and GM-CSF response truly reside in one and the same cell that both belong to the leukemic clone.

Release of CSFs from monocytes,⁹³ fibroblasts,⁹⁴ and endothelial cells⁹⁵⁻⁹⁷ is normally induced by specific stimuli, e.g., tumor necrosis factor (TNF)⁹⁷⁻⁹⁹ or interleukin-1 (IL-1).^{94,95,100} A question that is not definitely settled is whether the synthesis and release of CSFs by AML cells is controlled by physiological regulators or if it is constitutive. It has become apparent that synthesis of GM-CSF by AML blasts can be induced following exposure of the cells to interleukin-1 alpha (IL-1 alpha) or IL-1 beta,¹⁰¹ and that in certain cases spontaneous AML cell proliferation *in vitro* can be switched off not only by anti-GM-CSF antibody, but also with neutralizing anti-IL-1 antibody. As a matter of fact, GM-CSF elaboration from AML blasts and the resulting proliferative activity may be governed by similar inducers (e.g., IL-1) as that in normal cell types. Thus, CSF release from AML blasts may be under regulatory control rather than constitutive.

One could argue that a CSF-mediated autocrine process of stimulation of AML blasts is not a malignant feature of the cells, i.e., associated with their transformed status. Autocrine-mediated growth in hematopoietis is not unusual and does not

necessarily represent neoplastic alteration. For example, the production of IL-2 by T-lymphocytes and the stimulability of the same cells by this factor, or the synthesis of M-CSF by monocytes are physiological phenomena.⁹³ Normal hematopoietic blast cells have not been critically examined for this particular feature.

Taken together, no experimental evidence has been obtained to explain why AML growth gains a selective advantage over normal hematopoietis. Obviously, aspects of cell-cell interactions and the susceptibility of progenitor cells to recombinant inhibiting factors have not yet been critically evaluated in the context of the recombinant hematopoietic growth factors.

E. Maturation

Whereas the hematopoietic growth factors are highly active in triggering cell proliferation, they appear usually incapable of inducing maturation in human AML blasts. Surface markers indicative of progressive myeloid maturation did not appear as the result of incubation of the cells with IL-3, GM-CSF, or G-CSF.^{15,16,26} Only in rare instances do IL-3, GM-CSF, G-CSF, or M-CSF bring about morphological alterations that are indicative of monocytic or granulocytic maturation.^{16,34} Thus, although the cells express surface receptors for these factors and their cell cycle status can be activated by these molecules, they are unable to proceed along the maturation pathway. These observations suggest an abnormality in the intracellular machinery that follows growth factor receptor ligand binding. The inefficiency of these factors to induce maturation in AML cells was only slightly improved when the factors were presented to the cells *in vitro* as complete cocktails of IL-3, GM-CSF, G-CSF, M-CSF, and EPO.

Why are AML cells incompetent to mature to terminally differentiated granulocytic cells when exposed to the appropriate growth factors? Obviously, the answer to this question is fundamental with respect to our understanding of neoplastic growth. As the cells are intact at the level of ligand receptor binding, one may assume that the defect preventing proper maturation resides further downstream in the cell and involves the signal transduction route. Why a normal hematopoietic progenitor cell that responds to growth factor activation is capable of mounting a sequential series of maturation events and why the leukemic cell is incapable of eliciting a similar response are unresolved and remain a main target of future research.

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