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Long-Term Marrow Cultures: Human and Murine Systems

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Abstract The intramedullary control of marrow cell production has been a difficult area to approach experimentally. The introduction by Dr. Dexter and colleagues of long-term stromal dependent culture systems for murine marrow and the adaptation of these systems to human marrow growth have allowed for in-vitro studies of stromal dependent hemopoiesis. Despite some controversy in this area, most studies appear to show that adherent murine or human stromal cells are capable of producing a relatively large number of hemopoietic growth factors including G-CSF, GM-CSF, CSF-1, IL-6 and, at least by PCR analysis, IL-3. Other work indicates that the most primitive hemopoietic cells which appear to be multifactor responsive adhere directly to these stromal cells presumably through mediation of various adherence proteins.

An early acting, multilineage factor termed hemolymphopoietic growth factor-1 (HLGF-1) has been isolated from a murine stromal cell line and may be identical to the recently described ligand for the c-kit receptor. This may represent an important early survival/maintenance factor for stem cells in this system.

Studies on primitive stem cells, especially the high proliferative potential colony forming cell (HPP-CFC), indicate that they are responsive to varying combinations of growth factors and that with increasing numbers of growth factors, as studied in serum-free systems, decreasing concentrations of the factors may be biologically active.

These observations altogether suggest that intramedullary hemopoiesis may be regulated by the positioning of early multifactor responsive stem cells via adherent proteins in juxtaposition to synergistically acting combinations of growth factors attached to stromal cell surfaces or the extracellular matrix. In addition, selective production of different growth factors from different subsets of cells may create growth factor gradients and explain the spacial distribution of different cell types within the marrow cavity.

Key words: stromal cells, cytokines, synergy, high proliferative potential stem cell, Dexter culture

Dexter and colleagues [1] introduced a system for the long-term growth of murine marrow, which Greenberger et al. [2] modified, by the inclusion of hydrocortisone, permitting widespread application of the system. A prerequisite for sustained hemopoietic cell maintenance in Dexter cultures is the formation of an adherent layer of cells on the plastic flask bottom [3]. These adherent cells have been felt to represent a paradigm for the in-vivo marrow microenvironment and allow for the prolonged maintenance of primitive stem cells with ongoing hematopoietic cell proliferation and differentiation.

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The establishment of a human Dexter system equivalent to that of the murine system has been an elusive goal of many investigators [4–6]. A number of culture modifications have been assessed, and stromal function and growth obtained. However, murine Dexter cultures routinely show plateau growth phases of months, while human do not; in general, human cultures show a steady decline in total numbers of progenitor and differentiated cells. In addition, recent observations have suggested that the human stroma may be composed predominantly of vascular smooth muscle, while murine stroma consists mainly of macrophages and preadipocytic fibroblasts [7,8]. Despite these differences, both marrow stromal culture systems appear 1) to support early hemopoietic progenitor/stem cells and 2) produce many growth factors.

A general area of controversy has been whether murine or human stromal culture systems are growth factor regulated. Early difficulties in detecting growth factors actually led several investigators to suggest that the growth factors might not be relevant for in-vivo hemopoiesis [9]. These problems were explained in part by rapid utilization of growth factors in the cultures.

As we will outline in more detail below, we feel the other part of the explanation may be that growth factors act in combination synergistically at low concentrations sequestered or compartmentalized in stromal extracellular matrix or membranes. While most investigators have detected the presence of CSF-1 (protein or mRNA) in human and murine stroma, most have not been able to detect constituitive production of the critical cytokines G-CSF, GM-CSF, IL-3, and IL-6 [10–17]. These cytokines can be detected after exposure of stroma to such inducers as TNF, IL-1, endotoxin, or pokeweed mitogen, but the failure to detect constituitive production, along with studies indicating that addition of anti-growth factor antibodies or growth factors themselves had relatively minor effects on in-vitro Dexter culture hemopoiesis, led Dr. Dexter to recently conclude "together the results indicated that although many of the myeloid cell growth factors so far characterized do play a role in the development of hemopoietic cells, they may not be involved in stromal cell mediated hemopoiesis" [18].

A number of groups, including our own, have also studied human or murine derived cell lines as models for stroma and sources of growth factors. In fact, the biotech companies have used marrow stromal cell lines as hunting grounds for new cytokines, and it is from this source that IL-7 was first cloned [19]. Even given the well-recognized problem of extrapolating cell line studies to normal explant tissue, much less in-vivo physiology, the observation that stromal lines were relatively rich sources of a number of growth factors suggested that these growth factors might be important in in-vivo physiology.

We have concentrated upon the study of a pre-B-myeloid synergistic activity termed hemolymphopoietic growth factor-1 (HLGF-1) which was derived from a murine marrow stromal line termed TC-1 [20–22]. This line was isolated under conditions approaching those permissive for lymphoid growth in the Whitlock-Witte culture system [23]. The latter represents an adaptation of the Dexter culture in which hydrocortisone is omitted, fetal calf sera substituted for horse sera, 2-mercaptoethanol added, and the temperature raised from 33°C to 37°C. Under these conditions pre-B cell formation is favored. Conditioned media from the TC-1 cell line produced CSF-1 and another activity which interacted with CSF-1 to give giant macrophage colonies termed high proliferative potential colony forming cells (HPP-CFC). It also interacted with IL-3 to give giant mixed colonies of macrophages, granulocytes, and megakaryocytes, and with sources of GM-CSF to give giant mixed colonies. A pre-B inducing activity was also seen, which copurified with the myeloid synergizing activity through DEAE, Conconavalin A, and Sephadex G100 column chromatography (molecular weight approximately 110 kd). This partially characterized biologic activity was a protease resistant glycoprotein. HLGF-1 also appeared to synergize with erythropoietin to stimulate human BFU-e [24], and was a potent mitogen for CFU-S [20]. HPP-CFC stimulated by HLGF-1 and then subcloned in HLGF-1 showed extensive renewal capacity with production of HPP-CFC, low proliferative potential colony forming cells (LPP-CFC), macrophage colony forming cells (M-CFC), and macrophages [25]. Synergistic combinations of growth factors have been reported to stimulate murine and human HPP-CFC, and in all cases there was a very significant concentration of these multifactor HPP-CFC in marrow harvested after 5-FU [26–28]; these combinations stimulated very few HPP-CFC from normal marrow. This was in contrast to HLGF-1 which stimulated a higher (or at least equal) concentration of HPP-CFC in normal marrow as compared to post 5-FU marrow [29,30]. This potent mitogen for both lymphoid and myeloid stem cells is an excellent candidate for a cytokine which stimulates the earliest stem cells in Dexter culture, and we speculate that it might function to support maintenance of such stem cells, and act in concert with other B cell or myeloid growth factors to then promote proliferation and differentiation along a number of different lineages. This is also consistent with the existence of a separate B cell factor in TC-1 conditioned media which may act preferentially on more differentiated B cell lineages [31].

Recent studies have characterized a cytokine which is the ligand for the receptor protein coded for by the kit oncogene and the absence of

which may be responsible for the stromal deficiency seen in SL/SL^d mice [31–33]. Studies on a growth factor which appears to be identical with the c-kit ligand (derived from buffalo-rat cells) have indicated that it synergizes with CSF-1 to stimulate murine HPP-CFC [31]. Further studies will be necessary to establish whether HLGF-1 is identical with the ligand for the kit oncogene receptor, but this seems likely. A unifying interpretation of these data is that stromal cells may make a critical membrane or extracellular matrix (ECM) associated multilineage growth factor (HLGF-1, c-kit ligand, mast cell growth factor, stem cell factor) which may act on stem cells common to myeloid and lymphoid lineages [34].

Dexter Stromal Cytokine Production

The difficulty in detecting CSF in Dexter culture was partially explained by growth factor utilization. Heard and colleagues [35] showed CSF activity using a stromal overlay system, and our group showed that ablating active hemopoiesis with in-vitro or in-vivo irradiation allowed for the detection of biologic granulocyte-macrophage and megakaryocyte colony stimulating activity, along with CSF-1. In further studies, we established that exposure of stroma to the lectin pokeweed mitogen or to lithium induced increased colony stimulating activity, and that at least a major part of this was GM-CSF (by antibody blocking of factor dependent cell line proliferation). Further studies by our group using standard Northern blot analysis have now established that irradiated or normal stroma constituitively express mRNA for CSF-1, GM-CSF, G-CSF, and IL-6, but not for IL-4, IL-5, or IL-3. Exposure of these stroma to lectin, lithium, or IL-1 augmented all the constituitively produced cytokine mRNAs, but did not induce those not expressed constituitively. Further, studies utilizing PCR amplification of cDNA derived from reverse transcribed stromal RNA have also demonstrated the existence of mRNA for IL-3. In addition, selective support of the factor dependent cell lines, FDC-P1 and 32D, directly on the stroma have indicated that biologically active protein is secreted, but may be localized to stromal surfaces. Although these data are in contradistinction to many published results, the detection of IL-3 by PCR techniques has been reported in human stroma by Barge et al. [36] and in human monocytes by Ernst et al. [37]. These studies suggest that whole stroma makes a variety of growth factors, possibly localized to their surface, and probably at relatively low concentrations.

The latter does not imply that these stromal associated growth factors are biologically inert. Observations of 2-6 factor synergy on early stem cells, especially HPP-CFC, suggest that synergy is the rule, and that with increasing numbers of growth factors the biologically effective concentrations of each may be lower, possibly below concentrations which can be detected in their primary assays. Work by Caracciolo et al. [38] further suggested that one growth factor at a subliminal level might exert major effects on another factor present at its optimal level. All of these data suggest the possibility that multiple factors act synergistically in concert, on stromal surfaces, many at subliminal levels. Further, it may be that the specific growth factor(s) in highest concentration then determines the pathway of differentiation.

Cellular Composition of Stroma and Distribution of Growth Factor Production

A variety of cell types have been reported in murine Dexter stroma, including macrophages, preadipocytic fibroblasts, adipocytes, "blanket cells," and endothelial cells [1,39]. The predominant cell types in murine Dexter stroma are alkaline phosphatase positive, factor VIII negative, preadipocytes, and nonspecific esterase and acid phosphatase, phagocytic macrophages, the latter constituting 60–85% of stromal cells. Approximately 5–10% of the cells are not clearly identifiable as either of the above, and recent data suggests that a significant percentage of these cells may be of endothelial origin [40]. Human stroma, on the other hand, may contain predominantly vascular smooth muscle cells [41].

A major question relates to whether these stromal cells selectively produce different cytokines. We have used the CSF-1 dependent explant macrophage culture system described by Tushinski et al. [42] as a model for the Dexter adherent marrow cells. This cell population did not show constituitive production of G-CSF, GM-CSF, CSF-1, or IL-3, but with reexposure of CSF-1 depleted cultures to CSF-1 plus the lectin, pokeweed mitogen, or the lectin alone, mRNA's for CSF-1, G-CSF, and IL-6 were detectable. As with whole stroma, utilization of PCR to detect RNA species showed evidence of IL-3 bands, although all were different in size from the size

predicted by the cDNA. Recent data by Ozawa and colleagues in Japan [43], using in situ hybridization for G-CSF, has indicated that G-CSF may be produced by a small minority of stromal cells, and Rich et al. [44], in a similar vein, have indicated that marrow macrophages may be a source of erythropoietin (Epo). The capacity for modulation here is increased many fold by the number of specific agents which can induce cytokine (either stimulatory or inhibitory) production, including the cytokines themselves.

Stromal Regulation Theory

These observations suggest a general theory for the stromal regulation of intramedullary hemopoiesis (Table 1).

TABLE I. Theory of Stromal Regulation-Experimental Observations

1) Stromal cells make a large number of individual cytokines probably including those acting on very early stem cells such as HLGF-1 or the ligand for the c-kit receptor protein.
2) Early stem cells (HPP-CFC as a model) are multifactor responsive, and most factors act synergistically on these cells.
3) Data indicate the existence of subliminal synergy, i.e., one factor at a conventional concentration may be synergized by another factor at very low concentrations.
4) As the number of factors increases, the effective concentration of each may decrease.
5) Cytokines may be expressed bound to extracellular matrix proteins, such as proteoglycans, or in stromal cell membranes.
6) Early progenitors appear to need to be directly attached to marrow stromal cells in order to survive and proliferate.
7) Specific growth factors are probably made by specific stromal cell subpopulations.

These observations indicate that early hemolymphopoietic marrow stem cells are physically attached to specific marrow stromal cells, and then exposed to synergistic combinations of growth factors. The c-kit ligand or HLGF-1 may play a central survival/maintenance role, while the degree of proliferation and the specific pathway of differentiation may then be determined by the mix of synergistically acting cytokines, their physical configuration, and/or the cytokine(s) in highest concentration (Figure 1). The actual attachment process may also provide either negative or positive regulatory signals for cytokine modulation. The cytokines themselves may act in one of three phases: 1) membrane associated, 2) bound to extracellular matrix, or 3) soluble in interstitial spaces.

A final and intriguing component of the experimental observations, which could explain the spatial localization of cells within the marrow, is the probable localization of growth factor production to cellular subsets. This could lead to areas of growth factor predominance thus creating niches favoring certain pathways, and explaining the discrete regional localization of different cell populations in whole marrow tissue. In effect, growth factor gradients would exist, which would result in the functional compartmentaliza-

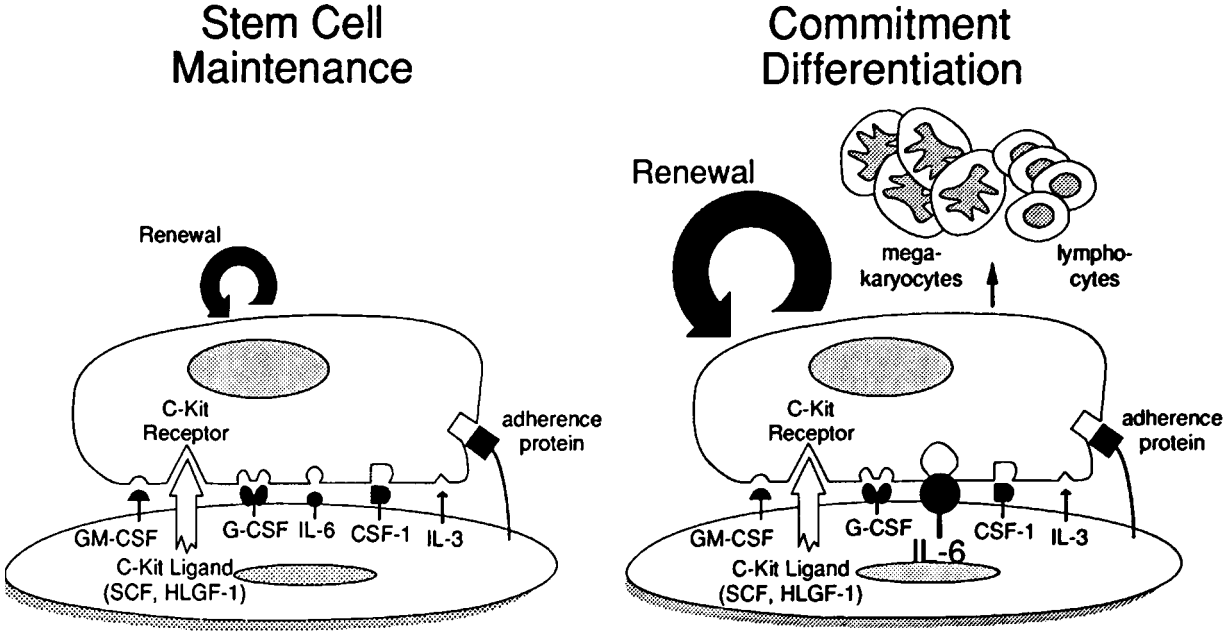

Fig. 1. Model of stromal growth factor regulation of hemolymphopoiesis.

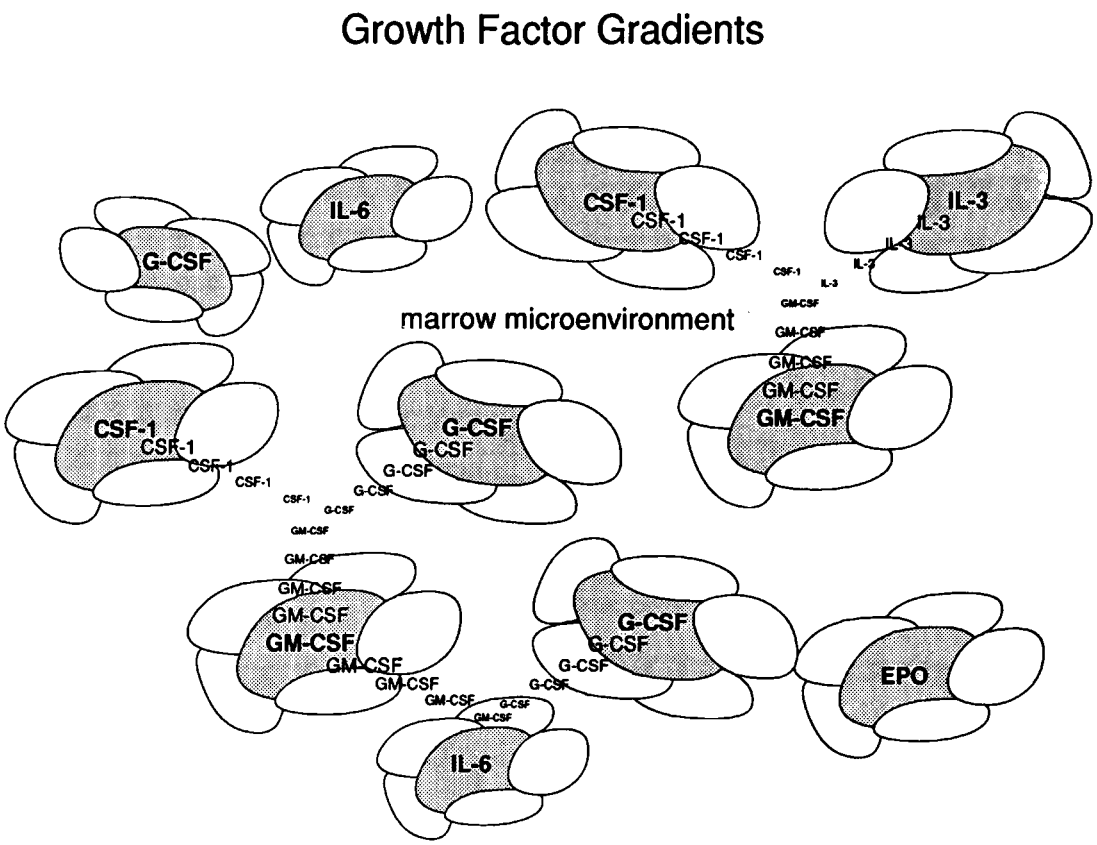

Fig. 2. Model for establishment of marrow growth factor gradients.

tion of hemopoiesis. This model is conceptualized in Figure 2.

The potential for in-vitro blood cell production will probably rest on recreating critical features of this model; stem cell maintenance factors, multiple differentiation/proliferation factors, adherence proteins, ECM or stromal cell surfaces, and, of course, the appropriate stem cells. The differentiation pathway for selective production of different cell populations could then be determined by predominant expression of specific cytokines such as G-CSF for granulocytes or pre-B cells, and erythropoietin for red cells.

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