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REGULATION OF HEMATOPOIESIS IN LONG TERM MARROW CULTURES: ROLE OF HUMORAL FACTORS IN THE PROLIFERATION AND DIFFERENTIATION OF STEM CELLS AND COMMITTED PROGENITORS

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Abstract

Established systems of long-term bone marrow culture (LTBMC) have contributed to our understanding of the interrelationships between the adherent stromal cells and the stem cells, committed progenitors and mature terminally differentiated cells. While cell-mediated or short range stromal interactions appear to be a major source of homeostatic control between the stromal microenvironment and the stem cells, positive and negative humoral influences or long-range mechanisms also regulate stem cell proliferation and differentiation. Adherent stromal conditioned medium generates factors which can trigger CFU-s into DNA synthesis within 18 hours or inhibit incorporation of tritiated thymidine into rapidly proliferating CFU-s. Other adherent stromal factors reduce proliferation and terminal erythroid differentiation of BFU-e. Stromal cells also produce a synergistic activity which stimulates formation of giant macrophage colonies in conjunction with CSF. Continued examination of these factors should lead to better understanding of the mechanisms involved in control of hematopoietic stem cell proliferation and differentiation.

Key Words: Hematopoiesis, humoral regulation, adherent cells, long-term bone marrow culture, growth factors, inhibitors, stem cells, CFU-s, stromal microenvironment, progenitor cells.

Introduction

Hematopoietic stem cells are defined as those cells which are capable of both extensive self-renewal and production of multiple lineages of fully differentiated cells. The development of clonal assay systems using semi-solid media has allowed quantitation of committed progenitor cells of the granulocytemacrophage (7,57), erythroid (30,35), lymphoid (22,10) and megakaryocytic lines (51,44). However, these assays do not measure pluripotent stem cells. In the mouse, there is a population of bone marrow cells that form colonies in the spleen of irradiated recipients. These spleen colony-forming cells (CFUs) have been shown to have extensive self-renewal capacity and can also produce progeny that give rise to all the differentiated cell types (67,39). Recently, Nakahata and Ogawa (50) reported the generation of small colonies of blast cells from murine bone marrow. These colonies possess extensive self-renewal capacity and can be replated with high efficiency. They are probably the in vitro eqiuvalent of the earliest hemopoietic stem cell which Hodgson and coworkers have termed the marrow repopulating cell (36).

While clonal analysis has contributed to our understanding of the responses of these committed progenitors to various regulatory factors it does not permit study of the interrelationships between the stem cells, their committed progenitors, and the stromal microenvironment. However, the development of long term bone marrow cultures (LTBMC) by Dexter and colleagues (13,14,16) enabled researchers to maintain both pluripotent stem cells (PSC) and committed progenitors in vitro for several months. This system also permits analysis of both cell-mediated and humoral interactions of these cells with the stromal microenvironment.

The purpose of this paper is to review some aspects of the humoral interrelationships between the adherent cells of LTBMC (which are representative of the medullary stroma), the stem cells, the committed precursors and the mature, terminally differentiated cells which are also generated in these cultures.

Established systems for the growth of bone marrow in long term culture

Since the first report of the successful

Table 1. Growth requirements and characteristics of long-term bone marrow cultures

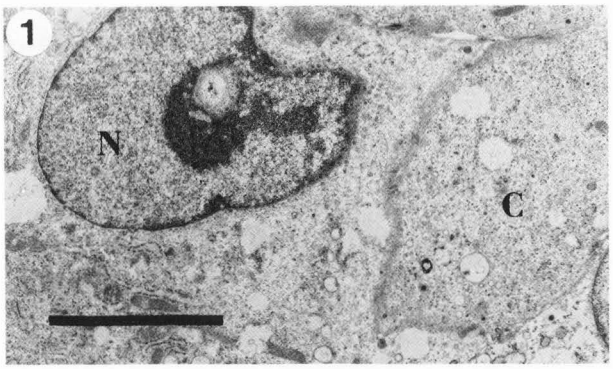
Species	Mouse	Hamster	Human
Optimal cell density per ml	10^6	10^6	$2 - 4 \times 10^6$
Media used for growth	Fischer's	RPMI-1640	MEM-alpha, IMDM RPMI-1640, McCoys
Serum type (%)	Horse or FCS (20) (20)	Horse (20)	Horse and FCS (12.5) (12.5)
Nutritional supplements	10^{-6} M hydrocortisone	None	10^{-6} M hydrocortisone 2-Mercaptoethanol Glutamine, folic acid, inositol, etc.
Optimal temperature (°C)	33	37	37 for establishment of adherent layer, then 33 for culture maintenance
Feeding schedule	Half refeed weekly	Half refeed weekly for adherent cultures Complete medium change weekly for suspension cultures	Half refeed for some, complete medium change for others, none appear optimal
Stem cell location	The most primitive stem cells are located in the adherent layer. Those with less proliferative capacity are found in suspension	Rapid decline of detectable stem cells in adherent layer Long-term survival of stem-cells in suspension	Almost exclusively found in adherent layer Rapid decline of stem cells in suspension
Special manipulations	Suspension cultures containing stem cells can be generated with addition of appropriate growth factors	Best long-term stem cell survival requires removal from the adherent layer	Removal of mature elements. Disruption of adherent layer to detect primitive stem cells

proliferation of both stem cells and committed progenitors in murine LTBMC (13) other investigators have reported similar results in the hamster (18,19) and the tree shrew (49). However, only limited success has been achieved in the long-term proliferation of human bone marrow (11,25,28) Table 1 summarizes the growth requirements for each system. While there are slight differences in the nutritional and temperature requirements for each system, on the whole they are quite similar. Unfortunately, investigators are still unable to duplicate the success of the rodent systems with human marrow despite myriad manipulations of nutritional and temperature requirements, variations in feeding schedules, and addition of growth factors to these cultures.

Perhaps the most significant difference among the LTBMC systems is the requirement for adherent layer formation. In all species, except the hamster, the maintenance of hemopoiesis seems to depend on the continued presence of the adherent layer (1,5,15). The hamster system appears to be unique in that the formation of the adherent layer is necessary for the initiation of hemopoiesis in these cultures but extensive proliferation and differentiation can continue for several months after the cells are removed from the adherent layer without addition of growth factors and in the absence of identifiable stromal elements (19,20). While epitheloid, fibroblastic and macrophage type cells, similar to those found in murine cultures, are present there is a distinct absence of the so-called fat cells probably due to the fact that hydrocortisone is not needed for culture maintenance. Transmission electron micrographs of typical hamster epitheliod and macrophage type cells are shown in figures 1 and 2. Other investigators have demonstrated continued maintenance of murine erythroid and multipotential stem cells from LTBMC in the absence of adherent stromal elements but by addition of a source of specific growth factors (29,70).

The in vitro LTBMC system provides an excellent model for examining the contributions of both humoral and cellular mechanisms to the maintenance of hematopoietic stem cell proliferation and differentiation. Homeostatic control demands some form of interaction between the stromal microenvironment and the stem cells. In general, humoral influences are considered long-range mechanisms while shorter range interactions are regarded as cell-mediated.

Because there have been several excellent papers that deal with the characterization of the stromal environment and its interactions with both



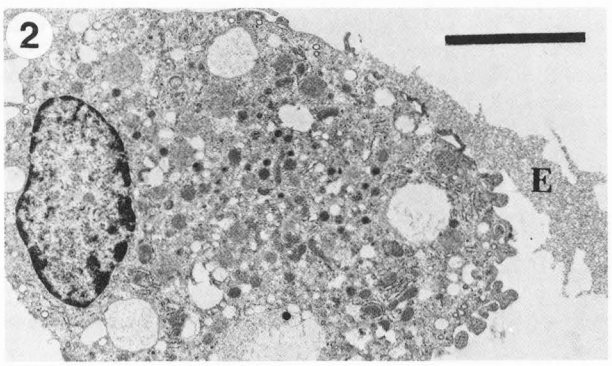


Figure 1. Two adherent epitheloid cells from a 3 week old adherent layer. Note that the left cell has an euchromatic nucleus (N). The right cell contains organelle-free cytoplasm (C) similar to that seen in murine long-term adherent cultures. Bar is 5 μm.

Figure 2. Adherent macrophage from the same 3 week old parent culture. Note the adjacent extracellular matrix material (E). Bar is 5 μm.

stem cells and committed precursors (2,27,65) the rest of this paper will examine the influence of both positive and negative humoral factors in the control of hematopoiesis.

Identification of humoral regulatory factors

Humoral growth factors for hemopoiesis were initially reported in the early 1960's with the first successful descriptions of in vitro techniques for the clonal growth of murine bone marrow cells in vitro (7,58). Since that time investigators have attempted to define those tissues that are involved in the production of stimulatory hemopoietic growth factors. Table 2 contains a list of the best characterized murine and human growth factors and their primary target cell population(s).

The murine granulocyte and macrophage colony stimulating factors (GM-CSF,G-CSF and M-CSF) are well characterized and were recently the subject of a detailed review by Metcalf (46). These CSFs have been purified tko homogeneity and the gene for GM-CSF has been cloned. These growth factors induce proliferation and differentiation of both granulocyte (G) and macrophage (M) progenitors and are named after the predominant type of colonies stimulated.

Table 2. Humoral hemopoietic growth factors

Activity	Source	Alternative Names	Target Cell Populations
GM-CSF	Murine	CSF-II MGI-1GM	GM-CFU CFU-eos CFU-meg BFU-E
M-CSF	Murine	MGI-1M CSF-1	CFU-M
G-CSF	Murine	MGI-1G	CFU-G
CSF-α	Human	-	GM-CFU CFU-eos CFU-GEMM BFU-E
CSF-1	Human	-	CFU-M
CSF-β	Human	-	CFU-G
IL-3	Murine	multi-CSF BPA PSF Mast cell growth factor	CFU-mix BFU-E Mast cell GM-CFU CFU-meg
Meg-CSF	Human Murine	-	CFU-meg
MMF	Human	-	CFU-meg
MK-potentiator	Murine	-	CFU-meg

At very high concentrations GM-CSF can also stimulate eosinophil, megakaryocyte, and erythroid colony formation (45,46,59) M-CSF, in combination with hemopoietin I which is not in itself a CSF, stimulates formation of very large macrophage colonies whose progenitors are known as high proliferative potential colony forming cells (HPP-CFC) (4). Hemopoietin I appears to be a synergistic factor which induces receptors for M-CSF on the appropriate bone marrow cells but lacks the capacity to stimulate colony formation in the absence of M-CSF.

Like the murine CSFs, there appear to be multiple forms of human CSF which are capable of stimulating granulocyte-macrophage colony formation. CSFα was purified to homogeneity from Mo conditioned medium (26) and preferentially stimulated 14 day colonies which are primarily composed of monocytes and macrophages. The recombinant form of human CSFα is also an effective stimulus for eosinophil proliferation and in the presence of epo is able to potentiate both erythroid and mixed or GEMM colony formation (47).

CSF-1 appears to be in the human equivalent of murine M-CSF. It is a 45,000-70,000 dalton glycoprotein dimer that appears to regulate only cells of the monocyte-macrophage lineage (66). In concert with other regulators, CSF-1 is able to influence multipotent stem cells and direct their commitment to the monocyte pathway (4). It also stimulates

proliferation and maturation of committed monocyte progenitors and its continued presence appears to be essential to the maintenance of cultural integrity (68).

CSFβ appears to be the human analogue of murine G-CSF and indeed can induce differentiation in the murine myelomonocytic cell line, WEHI-3B, and also competes with murine G-CSF for binding of ^{125}I G-CSF to murine or human cells (55).

Murine interleukin 3 (IL3) or multi-CSF has also been purified to homogeneity and the cDNA has been cloned (23). Like the other CSFs it is a glycoprotein with the ability to stimulate in vitro formation of mixed colonies containing granulocytes, macrophages, megakaryocytes, eosinophils, mast cells and erythroid cells (12). IL3 stimulates CFU-S self-generation (24), proliferation of primitive erythroid precursors (BFUe) and megakaryocyte progenitors (38,60) and promotes mast cell colony formation (75). In addition, IL3 has the ability to imitate the proliferative functions of GM-CSF, G-CSF and M-CSF. Recent experiments by Walker et al. have demonstrated that the binding of IL3 to its own receptor induces a concentration-dependent down-modulation (i.e., activation) of the other CSF receptors (69). It appears that GM-CSF, G-CSF and M-CSF also compete with each other in a similar fashion and the pattern of down-modulation for each of the CSFs parallels the range of observed biological activity.

Megakaryocyte colony stimulating factor (Meg-CSF) is found in human serum and plasma from patients with thrombopoietic diseases and the amount present appears to be inversely related to the number of megakaryocytes found in the bone marrow. A similar factor has been isolated from a number of sources in the murine system including WEHI-3 CM (48). Like the other CSFs it is a glycoprotein which greatly increases the efficiency of megakaryocyte colony formation in semi-solid media and also increases the mean number of cells per colony (43). Purified human Meg-CSF does not appear to stimulate early (BFU-E) or late (CFU-E) erythroid or granulocyte-macrophage colonies.

Megakaryocyte maturation factor (MMF) accelerates cytoplasmic maturation of morphologically recognizable megakaryocytes without affecting megakaryocyte proliferation or number. MMF is found in human plasma from aplastic anemia patients and appears to be similar to the MK-potentiator activity that Williams et al. described in the murine system (71). MK-potentiator is found in WEHI-3B CM and is also produced endogenously by the bone marrow (72).

Stromal stimulatory and inhibitory factors

The debate over the contribution of the stromal environment versus humoral factors in LTBMC has centered on the ability to detect various regulatory molecules in the supernatant of the long term culture system. However, these studies can be misleading. Original attempts to identify GM-CSF, a regulator of granulocyte and macrophage differentiation, in the supernatant of murine LTBMC were unsuccessful and led researchers to conclude that this growth factor was not generated in these cultures (17). Subsequently, a more sensitive radioimmunoassay system was able to detect GM-CSF in the culture system, although the levels were indeed low (63).

In recent years scientists have used several approaches to define more precisely the production of both positive and negative factors by the adherent stromal cells of LTBMC. These include the isolation of adherent stromal lines through sequential trypsinization of adherent cells from Dexter cultures, (65) in vitro irradiation of adherent layers several weeks after formation in culture (31,52-54) and, in the case of the hamster system, separation of adherent stromal cells from the supernatant cell population (18). Zipori et al. were the first to show that cloned murine stromal cell lines form the same source differed in morphology, collagen biosynthesis, hematopoietic factor production and their effects on leukemic cells (76). In addition, Harigaya and Handa showed that transfection of human bone marrow stromal cells from long term Dexter cultures with recombinant plasmid pSV3gpt generated lines that produced factors which stimulated proliferation of human GM-CFU, BFU-E and also produced a substantial number of mixed colonies in the presence of epo (33). Table 3 is a summary of the positive and negative humoral factors which have been detected in LTBMC.

In 1981 Harigaya et al. reported the isolation and characterization of a long term adherent cell line from a 14 week old Dexter culture (34). This line, termed H-1, produces large amounts of GM-CSF similar to that derived from mouse L-cell conditioned medium.

Regenerating bone marrow extract III (RBME III) is a murine stem cell proliferation factor which is produced by any hemopoietic tissue containing rapidly proliferating CFU-s, including supernatant conditioned medium form LTBMC one day after feeding. It is a protein with a molecular weight between 30,000 and 50,000 daltons (74). This protein triggers minimally proliferating CFU-s into DNA synthesis. When RBME III is added to LTBMC active cycling of CFU-s is induced within 18 h. There is no significant effect on total nucleated cell number nor is there any increase in the number of CFU-c or CFU-s.

Normal bone marrow extract IV (NBME IV) is a reversible, non-cytotoxic CFU-s proliferation inhibitor with a molecular weight between 50,000 and 1000,000 daltons. It inhibits the incorporation of tritiated thymidine into rapidly proliferating CFU-s but does not alter the capacity of the CFU-s to from spleen colonies (42). The factor is released from normal bone marrow when incubated in saline for 2-4 h and is also present in medium conditioned by spleen cells from phenylhydrazine treated mice. NBME IV can be detected in fractions obtained from day 7 supernatants of LTBMC. When added to other long term cultures NBME IV reduced CFU-s proliferation to less than 10% within 12-18 h while CFU-c proliferation remained unaffected.

Synergistic activity is a CSF-1 dependent factor produced by the TC-1 cells (64). TC-1 cells are an adherent cell line which was isolated from murine LTBMC by sequential trypsinization. It appears to be similar to the factor described by Bradley et al. (6) and in conjunction with CSF-1 stimulates formation of giant macrophage colonies that are more than 1mm in diameter, containing as many as 50,000 cells. Addition of anti-CSF-1 serum prevents formation of these giant colonies indicating that this

Table 3. Humoral regulatory factors secreted by the stromal cells of long-term bone marrow culture.

Activity	Target Cell	Mode of action
GM-CSF	GM-CFU	Induces granulocyte-macrophage colony formation
Regenerating BM extract III (RBME III)	CFU-S	Triggers "resting" CFU-S into DNA synthesis
Normal BM extract IV (NBME IV)	CFU-S	Reversible, non-cytotoxic CFU-S proliferation inhibitor
Inhibitory factor	BFU-E	Inhibits terminal erythroid differentiation and proliferation of BFU-E
Synergistic activity	CFU-M	In conjunction with CSF-1 stimulates formation of giant macrophage colonies
Secondary adherent activity	?	Induces adherent cell lines from fresh murine BM and non-adherent LTBM cells

activity is CSF-1 dependent. Synergistic activity can be separated from CSF-1 by immunoadsorbent affinity chromatography. It appears to be a glycoprotein with an apparent molecular weight of 60,000 daltons. While this protein shares some of the characteristics of another synergistic factor called hemopoietin I, the latter does not bind to Con A and appears to be yet another synergizing activity (59).

Secondary adherent activity is also produced by the TC-1 cell line (64). Incubation of either fresh bone marrow cells or nonadherent cells from murine LTBMC with TC-1 conditioned medium for 2-4 weeks induces adherent layer formation which morphologically resembles the parent cell line. These induced marrow cell lines then produce growth factors which permit continued self-maintenance suggesting the presence of an autocrine system. However, at this point there is no hard evidence for the existence of a true autocrine system.

Inhibitory factor is a protein isolated from the adherent cells of hamster LTBMC which reduces both erythropoietin-dependent terminal erythroid differentiation and the number of BFU-e without affecting the proliferation and differentiation of GM-CFU (21). The inhibitory effect is at least partially reversible and appears to be exerted early in the maturation process. Incubation of hamster LTBMC suspension cells with epo for 96 h prior to inhibitor exposure virtually eliminated the inhibition; suggesting that the more mature erythroid forms were not affected by the inhibitor. However, continued presence of the inhibitor for more than two weeks irreversibly affected the proliferation and differentiation of all cell types. It is not known whether this inhibitor is similar to the one reported by Axelrad et al. which acts as a negative regulator by putting BFU-e in a non-cycling state (3). Their inhibitor also appeared to be important in preventing the development of polycythemia following exposure of mice to Friend virus since the proliferative state of the host cells and their stage of differentiation are both necessary for the expression of the viral genes and subsequent viral infection.

Recent evidence from our laboratory suggests that the erythroid inhibitor produced by the adherent cells of hamster LTBMC also prevents proliferation of several leukemic cell lines including the murine MEL cells and human K562 cells (Eastment et al., unpublished observations) but does not appear to affect terminal induction of these cells in the presence of hemin and epo. If this inhibitor is found to be active in vivo its antiproliferative capacity could prove useful in the treatment of leukemia.

Possible role of humoral regulatory factors in the etiology of human disease

At the present time it is difficult to ascertain the physiological relevance of these in vitro proliferative and inhibitory factors. However, there are many reports in the literature concerned with the in vivo or in vitro effects of other so-called biological response modifiers. Other investigators have demonstrated that adherent cells from patients with specific diseases can inhibit erythroid colony formation (61,62) and Hanada et al. reported a T cell-mediated inhibition of late erythroid colony formation (CFU-e) in two patients with pure red cell aplasia (32). In recent years reports by Broxmeyer and colleagues point to a specific role for monocyte/macrophage acidic isoferritins as feedback regulators of myelopoiesis (8,9,40).

Recent work by several different groups has documented an homology between the receptor for CSF-1 and the human c-fms proto-oncogene, establishing a link between proto-oncogenes and growth regulation in hematopoiesis (56). LeBeau and co-workers showed that the human CSF gene is located within a chromosome region that is frequently deleted in patients with neoplastic myeloid disease (41). Other groups have shown that the receptor for CSF-1 is located on the terminal portion of the long arm of chromosome 5 (37). The significance of this observation correlates with the recognition that acquired partial deletions of this chromosome have been recognized in several hematopoietic disorders including acute myelogenous leukemia (73).

With rapid advances being made in purification and cloning of various growth factors the physiological relevance of these factors as normal mediators of hematopoiesis should be elucidated in the near future. Further experiments with these hematopoietic regulators could lead to a better understanding of the mechanisms involved in the control of proliferation and differentiation and could also generate advances in the treatment of hematopoietic disorders.

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Discussion with Reviewers

S.A. Bentley: Have you investigated the possibility that non-adherent stromal elements in hamster long term bone marrow cultures could provide a cellular microenvironment for hematopoiesis?

Author: The most plausible explanation for the ability to grow hamster bone marrow in the absence of an adherent stromal layer is the existence of non-adherent stromal elements in the suspension cultures which generate the factors essential for continued stem cell proliferation and differentiation. Indeed as the suspension cultures age they will form secondary adherent layers which closely resemble the original parent adherent layer. However, there are still important differences between the hamster and murine adherent layers. Although a inhibitory factor (NBME IV) has been detected in conditioned medium from the murine adherent stromal layer, the predominant function of this layer is stimulatory and there is no evidence that murine stem cells can proliferate in the absence of his layer without addition of humoral factors. The opposite is true of the hamster adherent layer. While the initial formation of this layer appears to be essential for the proliferation of stem cells continued incubation of stem cells in the presence of this layer eventually leads to a termination of hemopoiesis. This is also true if you add suspension cells to a secondary adherent layer. There appears to be some fundamental difference between the mouse and hamster adherent layers which determines whether a stimulatory on inhibitory influence predominates. In the human long term marrow culture system the adherent layer appears to be closer to that of the hamster in that stem cell proliferation is severely limited after initial establishment of the culture and the number of non-adherent stem cells rapidly declines in the suspension. Whether these differences are due to qualitative or quantitative variation in the adherent layer composition is still unknown.

S.A. Bentley: Would you care to speculate on the possible relationship between humoral and cellular factors in hematopoietic regulation?

Author: I feel that the role of humoral and cellular factors is similar to the coarse and fine adjustment controls on a piece of scientific equipment. The cellular factors are necessary for maintenance of hematopoiesis at a basal level much like the coarse adjustment is used to set a baseline in the desired area while the fine adjustment is necessary for precision tuning of the instrument. Humoral factors are probably the body's way of responding to alterations in hematopoietic homeostasis which may occur as a result of injury or disease.