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Hemopoietic Stem Cells: Stochastic Differentiation and Humoral Control of Proliferation

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The central feature of hemopoiesis is life-long, stable cell renewal. This process is supported by hemopoietic stem cells which, in the steady state, appear to be dormant in cell cycling. The entry into cell cycle of the dormant stem cells may be promoted by such factors as interleukin-1, interleukin-6 (IL-6), and granulocyte colony-stimulating factor (G-CSF). Once the stem cells leave G_0 and begin proliferation, the subsequent process is characterized by continued proliferation and differentiation. While several models of stem cell differentiation have been proposed, micromanipulation studies of individual progenitors suggest that the commitment of multipotential progenitors to single lineages is a random (stochastic) process. The proliferation of early hemopoietic progenitors requires the presence of interleukin-3 (IL-3), and the intermediate process appears to be supported by granulocyte/macrophage colony-stimulating factor (GM-CSF). Once the progenitors are committed to individual lineages, the subsequent maturation process appears to be supported by late-acting, lineage-specific factors such as erythropoietin and G-CSF. Synthesis of a hemopoietic factor may take place in different cell types and is regulated by multiple factors. The physiological regulator of erythropoiesis is erythropoietin, which, by a feedback mechanism, provides fine control of erythrocyte production. Feedback mechanisms for leukocyte production have not been identified. It is possible that there is no feedback regulator of leukopoiesis. In this model, leukocyte production in the steady state is maintained at a genetically determined level. When an infection occurs, the bacterial lipopolysaccharides may augment the production of interleukin 1α and β, tumor necrosis factor, macrophage colony-stimulating factor, etc. These factors circulate and stimulate the production in the marrow of G-CSF, GM-CSF, IL-3, IL-6, etc., with resultant leukocytosis. Once the infection subsides, the production of the factors and leukopoiesis return to the steady-state levels.

Introduction

The central feature of the hemopoietic system is its continuous process of cell turnover. It is estimated that approximately 200 billion erythrocytes (*1*) and 60 billion neutrophilic leukocytes (*2*) are produced and destroyed daily in a man weighing 70 kg. This life-long cell renewal process is supported by stem cells that possess the ability to self-renew and to produce progenitors that are committed to differentiation in single lineages. The mechanisms regulating stem cell functions have intrigued not only hematologists but also biologists interested in cellular differentiation and have been the object of active investigations and model building. During the last three decades, studies using functional assays for hemopoietic progenitors, i.e., the *in vivo* spleen colony assay for murine stem cells (*3*) and clonal cell culture assays for progenitors at various stages of development (*4*), have resulted in significant elucidation of the mechanisms of hemopoiesis. In addition, these assays allowed identification of several types of hemopoietic factors. Recent clinical trials indicate that some of the recombinant proteins promise to be effective in the treatment with patients with hemopoietic disorders. In this review, I will summarize first the current understanding of the stem cell kinetics and differentiation and second the mechanisms of the hemopoietic factors regulating the dynamics of cell turnover.

Progenitors

General Consideration

Available evidence suggests that at a given time, hemopoietic cells occupying the entire hemopoietic tissue may be supplied by a small number of stem cell clones. Mulligan and his associates (*5,6*) reconstituted the hemopoietic system of mice by transferring a retrovirally labeled clone of stem cells, thereby demonstrating that the descendants of a single stem cell can generate enough

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cells to occupy the entire lympho-hemopoietic system. Follow-up studies of such animals indicated that hemopoiesis may be supported by sequential activation of different stem cell clones (*6*). This clonal succession of hemopoietic stem cells *in vivo* had also been evinced by Mintz et al. (*7*) who injected suspensions of liver cells from 13-day mouse fetuses into the placental circulation of *W/W* or W^f/W^f mice and obtained mice whose hemopoiesis was supported by more than one lone of stem cells. Serial observations of the erythrocyte types up to 60 weeks after transplantation revealed a complementary rise and fall in the proportion of the cells of different genotypes. These observations clearly demonstrated major proliferative potentials of a single stem cell and the exponential increase in the incidences of the progenitors as they progress through the developmental process.

Cell-Cycle Dormancy of Stem Cells

It is generally held that in the steady state, most hemopoietic stem cells are not engaged in active proliferation. Experimental evidence for this concept has been provided by investigators in several laboratories. Becker et al. (*8*) demonstrated that short-term exposure to ^{3}H-thymidine with high specific activity does not kill colony-forming units in spleen (CFU-S). Similarly, multilineage colony formation in culture was not affected by the brief exposure to ^{3}H-thymidine with high specific activity (*9*). Hodgson and Bradley (*10*) reported that high-dose 5-fluorouracil (5-FU), which preferentially kills cells in the cell cycle, does not affect cells that form late-appearing spleen colonies termed pre-CFU-S. In our laboratory, sequential observations of the growth of individual multipotential progenitors in culture also suggested that most primitive multipotential progenitors are dormant in cell proliferation (*11*). The concept of a true resting state was originally proposed by Lajtha (*12*) and was based on studies of liver cell regeneration. He envisioned that this state is metabolically distinct from the other phases of the cell cycle, and coined the term G_0 state. Lajtha proposed that hemopoietic stem cells are in G_0 since cell-cycle dormancy confers the stem cells time to repair DNA damage and thus allows maintenance of the genetic integrity of the stem cell populations (*13*). The mechanisms that initiate the cell division of the stem cells are not known. Lajtha proposed that this is a random process (*13*). As will be discussed later in this review, there is evidence that interleukin-1, interleukin-6, and granulocyte colony-stimulating factors appear to shorten the G_0 period and may induce the stem cells to begin active proliferation. It is possible that these factors, like the other hemopoietic factors, act as survival and/or growth factors on the most primitive hemopoietic stem cells and that the survival of stem cells and the duration of G_0 state may depend on the ambient levels of these factors.

Self-Renewal of Stem Cells

When a stem cell divides, its progeny can self-renew or differentiate. Following the development of the spleen colony assay, Till and his associates (*14*) transplanted single-cell suspensions of individual spleen colonies into secondary recipient mice and observed a very heterogeneous distribution of the incidences of CFU-S in individual spleen colonies. The frequency distribution could not be fitted by a Poisson distribution but could be approximated by a γ distribution (*14*). Simultaneously, they carried out a computer simulation of a birth and death model in which they envisioned that self-renewal of CFU-S is a birth process and that loss of colony-forming ability associated with differentiation is a death process. This simulation of the model based on generation of random numbers also resulted in a γ distribution of CFU-S incidences. Based on these data, they proposed that self-renewal or differentiation is a stochastic (random) process.

In our laboratory, we analyzed distributions of colony-forming cells for blast cell colonies and those of multilineage colonies by replating individual blast cell colonies. We assumed that the blast cell colony formation and multilineage colony formation represent the culture equivalent of the birth and death processes, respectively (*15*). The frequency distributions of the secondary blast cell colonies and multilineage colonies could be approximated by variates of γ distributions. Therefore, the studies *in vivo* and in culture mutually corroborated and were consistent with the concept that self-renewal and commitment to differentiation of an individual stem cell is a stochastic process.

We also carried out serial observations (mapping) of the development multi-potential blast cell colonies (*11*) from spleen cells of 5-FU-treated mice (*10*). Blast cell colonies emerged after variable lag times and once identified, grew with relatively fixed cell doubling times and developed into multilineage colonies. These observations were consistent with the notion that in the steady-state the dormant stem cells enter cell-cycle randomly and that once committed to differentiation, their progeny proliferate at a relatively constant cell doubling time.

Differentiation of Stem Cells

Several models for stem cell differentiation have been proposed (*16*). Two deterministic models propose that external factors instruct individual stem cells regarding their directions of differentiation. The hemopoietic inductive microenvironment (HIM) model of Trentin and his associates (*17,18*) features small anatomical niches that are specific for individual lineages and determine the commitment of stem cells. The stem cell competition model envisions control of stem cell differentiation by lineage-specific humoral factors. For example, VanZant and Goldwasser (*19,20*) proposed that exposure of a stem cell in a certain cell cycle phase to erythropoietin or colony-stimulating factors determines the commitment to the erythroid or granulocytic lineage. In addition, a model of predetermined, sequential loss of lineage potentials has been proposed (*21*). As discussed previously in our review (*16*), specific criticisms may be raised against the data supporting these models. Most importantly, these models are based on studies of populations of colony-forming cells

rather than on individual progenitors. Identification of murine blast cell colonies with high replating efficiencies (*22*) provided us with a unique opportunity to micromanipulate single progenitors and study differentiation potentials of single stem cells.

When we carried out cytological analysis of multilineage colonies that were derived from single progenitor cells and were cultured under identical conditions, we observed varying lineage combinations expressed in individual colonies (*23*). Subsequently, studies of paired progenitors revealed heterologous pairs of single and multilineage colonies expressing diverse lineage combinations (*24*). These results were consistent with the concept that the lineage selection by the progenitors at the time of cell division is a random process. Sequential manipulation of paired progenitors and analysis of the differentials of colonies derived from these progenitors indicated that the apparent random commitment takes place sequentially during stem cell differentiation (*25*). Analysis of single and paired human progenitors also revealed similar results and were consistent with the notion that the principle of human stem cell differentiation is a random restriction in the types and number of lineage potentials (*26,27*).

In the analysis of human multilineage colonies of single cell origin, we observed that individual lineages were represented by a variable number of cells. For example, a mixed colony consisted of 1340 erythrocytes and 4 eosinophilic leukocytes (*26*). This observation suggested that the number of times that a progenitor divides after commitment to a single lineage is variable. This observation is also in agreement with earlier observations in replating analysis of single lineage colonies. Investigators had observed variable replating efficiencies and heterogeneous size distributions of secondary colonies. For example, Wu (*28*) reported that the cumulative frequency distribution of the numbers of secondary T-lymphocyte colonies per primary T-lymphocyte colony could be approximated by a γ distribution. Analysis of the size of secondary mast cell colonies and computer simulation of a modification of the birth and death model of Till et al. (*14*) also suggested that mast cell proliferation in culture is a stochastic process (*29*). Together these data are consistent with the notion that both differentiation and proliferation of hemopoietic progenitors are separate stochastic processes.

Humoral Factors

General Considerations

While differentiation may be a stochastic process, proliferation and survival of progenitors appear to be under the control of humoral factors. Recently, a number of hemopoietic factors have been identified, purified, and molecularly cloned. The major hemopoietic factors include erythropoietin (Ep) (*30–32*), a physiological regulator of erythropoiesis; granulocyte/macrophage colony-stimulating factor (GM-CSF) (*33–36*); granulocyte (G)-CSF (*37–39*); interleukin-3 (IL-3) (*40–43*); and macrophage/monocyte (M)-CSF (*44–46*). Except for Ep, the physiological roles of these factors have not yet been clearly established. However, recent clinical trials of some of the factors indicate that they may possess significant therapeutic potential in the treatment of some of the hemopoietic disorders. Readers are referred to recent reviews (*47–49*) for detailed information on the biosynthesis, biochemistry and molecular biology of these proteins.

G-CSF was initially identified as a differentiation-inducing factor for leukemic cell lines (*37*). Some investigators have proposed that hemopoietic factors induce differentiation of progenitors in normal hemopoiesis. As reviewed earlier, our micromanipulation studies of differentiation of single and paired progenitors suggested that stem cell differentiation is not a directed process. The primary function of the humoral factors is to support the survival and/or proliferation of hemopoietic progenitors. In this model, the apparent induction of differentiation is a mere reflection of survival/proliferation of a population of progenitors that are supported by a humoral factor and the death of populations of progenitors that are not supported by the factor. Based on these concepts, I have summarized the recent evidence on the targets of the individual hemopoietic progenitors. The factors are arbitrarily divided into late-acting, lineage-specific factors, early-acting, lineage-nonspecific factors and those affecting dormant stem cells. The proliferative kinetics of the stem cells and the targets of the major hemopoietic factors are schematically presented in Figure 1.

Late-Acting, Lineage-Specific Factors

Ep is a physiological regulator of erythropoiesis and is secreted by the kidney. The recombinant protein has been given to patients with chronic renal failure and intractable anemia with remarkable therapeutic success (*50*). While some controversy still exists, the cells that are the most sensitive to Ep appear to be late erythroid progenitors that include erythroid colony-forming units (CFU-E) (*51*), erythroid cluster-forming units (*52*), and pronormoblasts. Since these cells are present in large numbers, are close to terminal maturation and are actively engaged

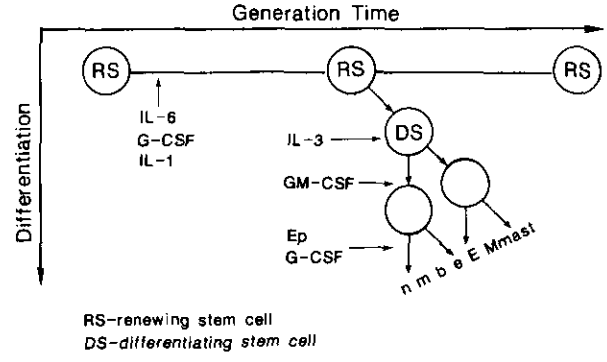

FIGURE 1. The model depicts the cell cycle dormancy of the stem cells and active proliferation of their progenies once they begin differentiation. It also illustrates the principle targets of the major hemopoietic factors.

in cell proliferation, a slight fluctuation in the ambient Ep levels would result in rapid changes in the production and release of reticulocytes. Experimental data suggest that multipotential stem cells (*53*) and early erythroid progenitors (*54–56*) are not under the control of Ep.

G-CSF appears to be a late-acting, neutrophil-specific factor. It has been demonstrated that in serum-containing cultures G-CSF supports the formation of neutrophil/macrophage colonies and pure neutrophil colonies (*57*), whereas in serum-free culture, it supports formation of only neutrophil colonies (*58*). In our laboratory, we studied the effects of G-CSF alone and in combination with other factors on colony formation by purified human progenitors in serum-free culture (*59*). G-CSF revealed significant synergism with earlier-acting factors such as IL-3 and GM-CSF in support of the formation of neutrophil colonies. When G-CSF was injected into animals, prompt and dose-dependent neutrophilic granulocytosis was observed (*60–62*). In addition, the differential revealed predominance of mature neutrophils (*61*). Together, these observations support the concept that G-CSF is a late-acting, neutrophil-specific factor. Interestingly, G-CSF appears also to play a role in stimulation of active proliferation of dormant stem cells. This will be elaborated in more detail later.

M-CSF has long been considered as a late-acting macrophage/monocyte-specific factor. Recently, CSF-HU that is abundant in human urine (*63*) was proved to be human M-CSF (*46*). A clinical trial of this material produced modest elevation in the granulocyte and macrophage counts in patients (*63*). It was also reported that during pregnancy, there is concomitant increase in the serum levels of M-CSF and blood monocyte counts (*64*). In serum-free culture of purified human progenitors, M-CSF alone or in combination with GM-CSF, G-CSF, IL-3, and Ep was an ineffective factor for macrophage colony formation (*59*). It is possible that M-CSF needs to interact with other factors in serum in macrophage colony fornation.

Early-Acting, Lineage-Nonspecific Factors

IL-3 appears to be an important factor for proliferation of early hemopoietic progenitors. Purified murine IL-3 supports the growth of various types of colonies, including multilineage colonies (*65–67*) and blast cell colonies (*66,68*). Gibbon IL-3, which is 93% homologous with human IL-3, supported the formation of human multilineage and blast cell colonies (*69*). We also observed that delayed addition of murine IL-3 to cultures 7 days after cell plating decreases the number of multipotential blast cell colonies to one-half the number in cultures with IL-3 added on day 0. It did not, however, alter the proliferative and differentiative characteristics of late-emerging, multipotential blast cell colonies (*66*).

Based on these observations, we proposed that IL-3 does not trigger stem cells into active proliferation but is required for the continued proliferation of early multipotential progenitors. Subsequently, we observed that the development of multipotential blast cell colonies requires less IL-3 than the process of multilineage colony formation from blast cell colonies (*68*). These observations suggested that during stem cell development, the early multipotential progenitors are sensitive to IL-3, whereas as they gradually differentiate, the sensitivity to IL-3 slowly declines.

More recently, we examined the effects of human IL-3 on colony formation by purified hemopoietic progenitors in serum-free culture. IL-3 alone was not effective in support of colony formation except for a few small eosinophil colonies (*59*). Serial observation of the serum-free culture dishes containing human IL-3 revealed that blast cell clusters appear after varying lag time, reach approximately the 50 cell stage, and degenerate in the absence of late-acting, lineage-specific factors. IL-3 revealed significant synergism with Ep and G-CSF in support of erythroid bursts and neutrophil colonies, respectively. These observations indicated that IL-3 supports progenitors that are in the early stages of hemopoietic development, but it does not support the terminal maturation process (*59*).

The primary function of GM-CSF may also be to support the intermediate stages of hemopoietic development. It may not support the terminal neutrophil/macrophage maturation process effectively. Although it was originally identified as the factor that supported neutrophil/macrophage colonies in culture, Metcalf et al. (*70*) in 1980 observed that the few cell divisions of multipotential progenitors are supported by murine GM-CSF. Using serial transfer of multipotential blast cell colonies, we demonstrated that a subpopulation of the multipotential progenitors that respond to IL-3 also respond to GM-CSF (*71*). Investigators in several laboratories (*72–74*) have reported that human GM-CSF supports multilineage colony formation in serum-containing culture and possesses significant burst-promoting activity (BPA). In the studies of human enriched progenitors in serum-free culture, we observed that GM-CSF alone did not support colony formation except for a few small eosinophil colonies (*59*). However, in combination with G-CSF and Ep, GM-CSF effectively supported a large number of neutrophil colonies and erythroid bursts. Recently, administration of the recombinant GM-CSF to primates (*75,76*) and patients with acquired immunodeficiency syndrome (*77*) raised the levels of circulating neutrophils, eosinophils, and monocytes. These observations indicate that the primary target of GM-CSF is a population of multipotential progenitors that are intermediate between those responding to IL-3 and those sensitive to late-acting, lineage-specific factors.

Factors That Affect Proliferation of Dormant Hemopoietic Stem Cells

Very recently, several factors have been identified that appear to induce proliferation of hemopoietic stem cells in G_0. Stanley and his co-workers (*78*) reported that hemopoietin-1 (H-1), which was purified from human bladder carcinoma cell line, 5637 (*79*), acts synergistically with IL-3 in support of proliferation of murine hemopoietic stem cells. The recent reports by Mochizuki and her as-

sociates (*80*) and Moore and Warren (*81*) suggest that IL-1, which is abundant in the supernatant of this cell line, may account for the H-1 activity.

In our laboratory, we have found IL-6 [also called interferon β2 (*82*) and B-cell stimulatory factor-2 (83)] to possess synergistic activity with IL-3 in the support of the active proliferation of murine hemopoietic stem cells (*84*). In the presence of IL-3, multipotential blast cell colonies emerged after varying lag times from spleen cells of 5-FU-treated mice (*66,84*). When IL-6 is also present, the appearance of multipotential blast cell colonies is significantly hastened, although the speed of the development of the blast cell colonies was not affected (*84*). These data suggested that part of the H-1 effect of IL-6 is the apparent shortening of the G_0 period of hemopoietic stem cells.

Human IL-6 also works synergistically with human IL-3 in stimulation of the proliferation of early human progenitors (*85*). Most recently, we have identified human G-CSF as an additional synergistic factor for the IL-3-dependent proliferation of murine hemopoietic stem cells (*86*). Both G-CSF and IL-6 appear to possess stronger synergistic effects than IL-1. It is of interest that there appears to be structural similarity between IL-6 and G-CSF including the positions of the cysteine residues, indicating similarity in three-dimensional structures of the two proteins. It is possible that the genes for the two proteins may share a common ancestral gene.

There are experimental data to indicate that production of IL-1, IL-6, and G-CSF may be significantly augmented immediately after invasion by microorganisms. For example, bacterial lipopolysaccharides induce synthesis of IL-1 in macrophages (*87*). IL-1 then circulates in the body and may augment synthesis of G-CSF (*88,89*) and IL-6 (*90,91*) by fibroblasts and other cell types. G-CSF stimulates the production of neutrophils, and IL-6 enhances maturation of B-cells and thereby the synthesis of immunoglobulins. The additional effects of these factors on hemopoietic stem cells in G_0 may assure an uninterrupted supply of the hemopoietic and possibly lymphoid progenitors for an integrated and effective defense against bacterial infections.

Factors Regulating Other Lineages

Regulation of megakaryopoiesis and thrombopoiesis remains unclear. While several factors such as megakaryocyte-CSF (*92*) and megakaryocyte stimulatory factor, which enhances synthesis of platelet-specific proteins by a rat megakaryoblast cell line (*93*), have been purified, their roles in the physiology of megakaryopoiesis and thrombopoiesis *in vivo* have not been established.

The proliferation of mast cells appears to be regulated by IL-3 and IL-4. Several years ago, investigators in a number of laboratories succeeded in growing murine mast cells in suspension culture in the presence of medium conditioned by pokeweed mitogen-stimulated spleen cells (PWM-SCM) (*94–97*). IL-3 was identified to be the main factor in PWM-SCM. However, IL-3 could not support the continued proliferation of mast cells in suspension cultures. In addition, it was found that IL-3 supports primarily proliferation of mucosal-type mast cells but not connective tissue-type mast cells. More recently, IL-4 (*98*) [also known as B-cell stimulatory factor-1 (*99*)] was found to be an additional mast cell stimulating factor and was identified as the factor that is needed for the proliferation of connective tissue-type mast cells (*100*). The roles of human IL-3 and IL-4 in the proliferation of human mast cells have not been established.

Several factors have been shown to stimulate proliferation of eosinophils recently. Previously, several investigators documented that the human and murine GM-CSF and IL-3 support eosinophil proliferation in methylcellulose culture (*66,68,69,72–74*). In addition, *in vivo* administration of human GM-CSF in monkeys and men (*75–77*) revealed significant augmentation of the number of circulating eosinophils. Recently, Sanderson and his colleagues (*101*) identified eosinophil differentiation factor in the supernatant of cultures of alloreactive T-cell clones. This factor proved to be identical to T-cell replating factor (*102*) and is generally referred to as interleukin-5 (IL-5). The exact relationship between IL-5, GM-CSF, and IL-3 in eosinophilopoiesis remains yet to be determined.

Control of Hemopoiesis—A Model of Granulopoiesis

In addition to the factors already described, there are a number of factors known to affect hemopoiesis indirectly. Foremost, IL-1α and β appear to play major roles in granulocyte/macrophage production. The synthesis of IL-1 by macrophages may be augmented by bacterial lipopolysaccharides (*87*). IL-1 then augments the production and the release of G-CSF (*88,89*) and GM-CSF (*88,103–106*) by a variety of cells, including fibroblasts, endothelial cells, and smooth muscle cells. Tumor necrosis factors (TNF α and TNF β) and interferons are known to inhibit myeloid proliferation in culture. A confusing aspect is that TNFα can stimulate the mesenchymal cells to synthesize and release G-CSF (*107*) and GM-CSF (*108,109*). It was reported that GM-CSF may in turn augment the synthesis and release of TNFα by monocytic cells (*110*). IL-4 may indirectly influence proliferation of progenitors supported by other cytokines (*111,112*). These observations indicate that a complex network of the cellular and molecular interactions regulate the continuous cellular production in hemopoiesis.

It is generally held that the feedback mechanisms exist for the control of individual lineages. In erythropoiesis, tissue oxygenation and Ep provide the feedback fine control of cell production. Regarding other lineages, no feedback system has been identified. It is possible that there is no such feedback regulation existing for granulocyte production. In this model, the production of granulocyte/macrophages in the steady state is maintained at genetically determined levels and is augmented only by external stimuli such as infections. In the presence of infections, bacterial lipopolysaccharides, IL-1 and TNFα will be involved in the augmentation of the synthesis and release of G-CSF and GM-CSF by various types of mesenchymal cells as outlined above. These factors, produced lo-

cally in the bone marrow, in turn augment the production of neutrophils, macrophages, and eosinophils. Once the external stimuli are removed, the production of the leukocytes reverts back to the steady-state level. Since the lifespans of leukocytes are brief, only coarse regulation of the cellular proliferation may be necessary. The wide normal ranges of granulocyte counts may attest to this hypothesis.

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