

The hematopoietic stroma

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Abstract

All blood cells are derived from a small common pool of totipotent cells, called hematopoietic stem cells. The process is strictly regulated by the hematopoietic microenvironment, which includes stromal cells, extracellular matrix molecules and soluble regulatory factors. Several experimental *in vitro* assays have been developed for the study of hematopoietic differentiation, and have provided valuable information on the stroma, which includes, among other cell types, macrophages, fibroblasts, adipocytes, and endothelial cells. The composition, ontogeny, and function in physiological as well as pathological conditions of stroma are discussed.

Key words

- Hematopoiesis
- Microenvironment
- Stroma

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The hematopoietic system

Blood cells composing the hematopoietic system can be classified into two main classes, i.e., lymphoid (B, T and natural killer lymphocytes) and myeloid (erythrocytes, megakaryocytes, granulocytes and monocytes). The life span of the fully differentiated mature forms of blood cells may vary considerably, being of the order of several hours for some cells (granulocytes), and several weeks (erythrocytes) to several years (memory cells) for others. This system is extraordinarily complex, since not only huge numbers of new mature cells are produced per day (about 1 trillion cells, including 200 billion erythrocytes and 70 billion neutrophilic leukocytes, in a 70-kg man), but there is also a need for the maintenance of a pool of undifferentiated cells and for a rapid response to situations of acute stress (1).

Increased production of cells is largely restricted to the specific cell type that is required in the particular stress situation: hemolysis, for example, induces erythroid

hyperplasia, while granulocyte hyperplasia is observed in response to bacterial infections. Alterations in the balance between self-renewal and differentiation can lead to the emergence of cells that survive and grow in situations unfavorable to the growth of normal cells and hence to the establishment of leukemias.

During ontogeny, there are a number of hematopoietic sites. Studies in mice and birds have shown that an early intra-embryonic site of hematopoiesis is found in the para-aortic splanchnopleura (2) and the aorta, gonads, and mesonephros or AGM (3). It is believed that hematopoietic stem cells arise in these two structures and in blood islands of the yolk sac, enter the embryonic circulation and colonize the newly formed liver rudiment. The fetal liver is the site of definitive hematopoiesis early during embryonal development. At birth, and throughout adult life, the bone marrow with its intersinusoidal spaces is the site responsible for the generation of blood cells. The mechanisms responsible for these ontogenic shifts are not well

known, and may involve changes in the developing hematopoietic cells (4), or in the hematopoietic microenvironment (5,6).

Differentiation in the hematopoietic system

All different types of blood cells are derived, through a series of maturational cell divisions regulated by the hematopoietic microenvironment, from a small common pool of totipotent cells called hematopoietic stem cells. The stem cell concept originated from the work of Till and McCulloch in 1961 (7), showing the formation of nodules of hematopoietic cells in the spleens of lethally irradiated mice after reconstitution with bone marrow from normal syngeneic donors. In spite of much effort since then, stem cells are still elusive entities, as illustrated by some of the reports available in the literature (“What defines a pluripotent hematopoietic stem cell (PHSC): will the real PHSC please stand up!” (8), “In search of the hemopoietic stem cell” (9) or “Which are the hematopoietic stem cells? (or: Don’t debunk the history!)” (10)).

These cells are of interest not only because of their developmental capacity but also because of their potential usefulness for the treatment of hematological disorders and as vectors for gene therapy. The most widely used criterion for their identification, that of a multipotential cell capable of self-renewal as well as of reconstituting long-term hematopoiesis after marrow ablation, presents theoretical as well as practical problems (11). Besides these two main characteristics, other well-established features of human stem cells include (12): a) They constitute a very small compartment, with estimates varying from less than 0.05% to up to 0.5% of cells in the bone marrow. b) The majority of stem cells are normally quiescent, as shown by their resistance to treatment with 5-fluorouracil or 4-hydroperoxycyclophosphamide, which spare them and eliminate dividing cells with-

out adversely affecting the long-term repopulating ability of bone marrow. Estimates of periodicity of mitosis vary widely (once a month to once in a few years), and the direct examination of the cell cycle of long-term cells indicates that at any moment only 4% of them are in the S/G₂/M phases. c) Their surface phenotype is poorly known, and includes the presence of CD34 and the absence of CD38, HLA-DR and other lineage markers. d) Besides bone marrow, they can be found in umbilical cord blood and in peripheral blood, particularly after “mobilization” treatments.

When stem cells divide, they may return to the G₀ phase of the cell cycle generating more stem cells; alternatively, they may generate large numbers of committed progenitors with a progressively restricted differentiation potential. The mechanisms involved in the process of self-renewal versus differentiation of the stem cell at each division are poorly known (1,11). Recent progress has been made with the identification of homeotic genes that appear to be of fundamental importance in these and other cellular processes (13).

Most models assume that the self-renewal potential of stem cells is finite, as evidenced by serial transfer experiments (14) and supported by the “Hayflick limit” concept (15). Stochastic and deterministic models (reviewed in 12) attribute different weights to the roles played by intrinsic and microenvironmental factors in the decision of self-renewal or differentiation.

The transition from stem cells to mature hematopoietic cells occurs through several intermediate steps characterized by the progressive loss of the self-renewal capacity and the commitment to a specific cell lineage. The progenitor cell compartment, which can be identified by the expression of specific lineage markers, comprises cell types which are determined to differentiate into any of the hematopoietic lineages. The developmental potential of these cells is gener-

ally limited to only one or two of the hematopoietic lineages, and these cells progressively display the antigenic, biochemical, and morphological features characteristic of the mature cells of the appropriate lineages, losing their capacity for self-renewal. Their proliferation is normally tightly controlled and coupled to development, so that cells leaving the bone marrow usually possess little or no proliferative potential.

The expression of different receptors on the surface of hematopoietic progenitors permits the interaction with various regulatory elements present in their environment, which includes stromal cells, extracellular matrix molecules (ECM) and soluble regulatory factors (cytokines and growth/differentiation factors). Although the role played by the microenvironment in the determination of the stem cell fate is still unclear, its pivotal role in the regulation of the amplification of the progenitor cell compartment is well established. Further differentiation of cells into one of several lineages critically depends on the nature of factors acting on these cells at a particular time and at a particular concentration.

The experimental analysis of hematopoietic differentiation

Although histologic observations of the hematopoietic system continue to be the primary source of information on the process of differentiation (16), experimental assays have been fruitfully employed in the study of this system. As different assays detect and analyze hematopoietic cell types specifically stimulated by the experimental conditions employed, and the correspondence among the assays is not always easily established, many different names are given to the cell types observed, as detailed below.

Till and McCulloch (7), as already mentioned, established in 1961 the first quantitative assay for cells with a radioprotective effect. Although it is now clear that these

cells - denominated spleen colony-forming units or CFU-S - do not represent the more primitive stem cells, the assay is useful for the investigation of early events in hematopoiesis. *In vivo* assays developed more recently, in which human hematopoietic cells are engrafted in immunodeficient mice, have demonstrated the existence of human pluripotent cells either by limiting dilution analysis or by clonal integration of a retroviral marker gene (17-19). The term marrow repopulating ability, derived from *in vivo* studies, refers to primitive totipotent hematopoietic stem cells with self-renewal capacity that are capable of repopulating the bone marrow of lethally irradiated mice (20). Two different types of cells with marrow repopulating ability have been distinguished in the mouse. Initial engraftment (short-term repopulation) is due to CFU-S. Long-term engraftment is attributed to a different cell type but is possible only if the animals also receive short-term repopulating cells. A cell type which is more primitive than CFU-S (pre-CFU-S) is considered to be responsible for long-term marrow repopulating ability.

However, it was the development of *in vitro* cultivation systems for the study of hematopoiesis (reviewed in 21), which had its heyday between 1965 and the late 1970s, that allowed the identification and quantification of several different types of precursor cells. The colony formation assay (22) allows the enumeration of early progenitors capable to form colonies when cultured under appropriate conditions in semisolid medium. In this test, cells are grown *in vitro* in soft agar or other highly viscous media, containing, for example, methylcellulose, plasma gel or fibrin clots. These semisolid media reduce cell movement and allow individual cells to develop into cell clones that are identified as single clusters (<50 cells) or colonies (>50 cells) of differentiated cells after a culture period of 7 to 14 days. These colonies are the progeny of single cells called colony-forming cells (CFC) or colony-form-

ing units (CFU), and the composition of the colonies determines which CFU is being assessed. Thus, CFU-blasts give rise to colonies composed of cells with blast-like morphology, CFU-GEMM corresponds to pluripotent progenitor cells identified by the production of multilineage colonies (granulocytes, erythrocytes, monocytes and megakaryocytes), CFU-meg is a more restricted progenitor giving origin to megakaryocyte colonies, and so on (21). This system has also allowed the identification of the high proliferative potential colony-forming cells (HPP-CFC), defined by their ability to form very large colonies (>5 mm in diameter) containing approximately 50,000 cells and including progenitor and mature hematopoietic cells of the granulocyte, macrophage, and megakaryocyte lineages. Cells that give rise to colonies smaller than 1 mm, on the other hand, are usually referred to as low proliferative potential colony-forming cells (LPP-CFC). These primitive hematopoietic stem cells are considered to comprise cell types such as BFU-E (erythrocyte blast-forming unit) and CFU granulocytes and monocytes (CFU-GM) (20).

Another culture system currently used for the study of hematopoietic progenitors is the delta assay (23). This assay uses a short-term suspension culture in which potentially colony-forming cells are grown first in liquid culture for one week and then replated onto semisolid medium. Numbers of colonies observed are considered as an indicator of the number of hematopoietic progenitors of an earlier stage than those normally obtained after 14 days of growth in semisolid medium.

The generation of hematopoietic colonies is absolutely dependent on the continuous presence of so-called colony-stimulating factors (CSF), and this system was the key to the original discovery and characterization of many of the hematopoietic growth factors (24). Colony formation assays, therefore, allow the study of the influences of

given growth factors or cytokines on the determination of the lineage along which colony-forming cells differentiate. Growth factors are denominated according to the colonies originating under their influence - M-CSF or GM-CSF, for instance, for factors inducing colonies composed of monocytes or granulocytes and monocytes, respectively.

Sustained production or self-renewal of clonogenic cells, however, has not been possible with standard semisolid culture systems. The long-term culture system (LTC), originally described by Dexter for murine cells (25) and later adapted for human cells (26,27), makes use of a rich culture medium containing high concentrations of horse serum and hydrocortisone and lower incubation temperatures, which allows the self-renewal of stem cells over a period of several months in the presence of a supportive microenvironment. The long-term culture of bone marrow cells employs primary adherent layers of stromal cells as an important source of cytokines and low molecular weight substances required for the controlled differentiation and proliferation of hematopoietic progenitor cells. Stromal cells provide a complex functional ECM allowing direct cell-to-cell contacts between different cell types. These, either alone or in synergy with defined cytokines, can conserve primitive stem cells, induce early differentiation of a fraction of the primitive progenitors, and prevent their terminal differentiation.

A second type of widely used LTC is the Whitlock-Witte long-term bone marrow culture (28), initially developed for murine bone marrow to obtain stromal layers devoid of hematopoietic cells. It is a lymphoid culture system which uses a "poor" culture medium containing 5% fetal calf serum without cortisone and permits the growth of freshly isolated bone marrow cells that form confluent adherent stromal cell layers within 2-3 weeks. Whitlock-Witte cultures can reconstitute the B-lymphocyte compartment in immune-compromised mice, but do not main-

tain primitive multilineage hematopoietic precursors such as CFU-S (20).

In addition to CFU-S and HPP-CFC, pluripotent stem cells and early precursors can be identified by the LTC assays as cobblestone area-forming cells (CAFC; 29) and long-term culture-initiating cells (LTC-IC). Sophisticated analyses such as limiting dilution analysis are used for the quantification of these cells (30). However, even these culture systems are unable to maintain hematopoiesis indefinitely due to limiting culture conditions or, alternatively, to a natural process of senescence of the hematopoietic cells. More recent evidence involving the transduction of CFU cells and LTC-IC using retroviral vectors, indicate that the *in vitro* progenitor assays currently available measure functionally different, and presumably less quiescent, populations than the long-term repopulating stem cell (31).

The hematopoietic stroma

To obtain sustained hematopoiesis, primitive hematopoietic cells must interact with an adequate microenvironment, which includes, as already mentioned, stromal cells, ECM components and soluble regulatory factors (32). The experimental analysis of hematopoiesis, as described above, has provided much of the present knowledge on the role played by the stroma in the process.

The term "stromal cells" is used rather loosely and the true histogenic origin of these mesenchyme-derived cells is still uncertain. Stromal cells, which mechanically support the differentiating hematopoietic cells, include among others macrophages, fibroblasts, adipocytes, and endothelial cells and are frequently defined as non-hematopoietic cells (1,33). Adventitial reticular cells reside on the adluminal surface of venous endothelial cells, which branch through the medullary cavity, and appear to provide a reticular network that supports hematopoietic cells. Marrow adipocytes possess the

mechanical function of controlling hematopoietic volume: impaired hematopoiesis is associated with increased accumulation of fat inclusions, whereas accelerated hematopoiesis is associated with loss of fat vacuoles and the provision of increased space for hematopoietic cells (34). Adipocytes may play an additional role in blood cell production as a reservoir for lipids needed in cell metabolism during proliferation. Macrophages and osteoclasts, cells derived from hematopoietic precursor cells and osteoblasts, may also play important roles in the hematopoietic microenvironment. Macrophages are important in the clean-up of ineffective erythropoiesis and in the removal of the nuclear pole, produced during the process. Stromal cells represent a highly dynamic structure which plays an active role in hematopoiesis by producing ECM components and both soluble and membrane-associated growth factors (35).

Stromal cells are rare in the marrow (approximately 0.125% of the marrow cellularity), and since in bone marrow suspensions they are mostly contained in tight aggregates, their analysis in freshly isolated material is difficult (36). Although adherent stromal cell layers in LTC, established by growing bone marrow cells over a period of several weeks, are considered to mimic many characteristics of the marrow microenvironment, it has not yet been established conclusively that these cultures encompass all types of stromal cells identified *in vivo* and that they retain all of their functional properties *in vitro*.

As the heterogeneous composition of the stroma makes it extremely difficult to analyze the role of individual cell types in hematopoietic development, numerous stroma-derived cell lines have been established from bone marrow and a variety of other tissues, including spleen, thymus, and non-hematopoietic organs such as kidney, lung, skin, or mammary tumors. These studies have shown *in vitro* the heterogeneity and compartment-

talization of cell types already observed during the *in situ* study of bone marrow. In the analysis of 33 bone marrow stromal cell lines, for instance, lineage-restricted stimulatory activity and a reciprocal relationship between granulocyte and macrophage formation were observed (37). A more detailed characterization of stromal lines generally shows differences with respect to growth potential, cell surface markers and cytokine transcripts, secondary to the varying capacity to expand bone marrow or cord blood stem/progenitor cells (38,39). The cell types which compose these heterogeneous populations are also being separately analyzed. In one study, three cell types could be grown separately *in vitro*, and were identified as macrophages, endothelial-like cells and myofibroblasts, with different potential for the support of hematopoietic cell growth (40).

Hematopoietic stromal precursors have been described, besides adult bone marrow, in fetal liver and fetal bone marrow, with reported differences in the anatomic and ultrastructural characteristics which, however, have not been clearly correlated to functional differences (6,41,42). Several studies suggest that hematopoietic progenitors collected at one stage of ontogenetic development may not be able to interact with a microenvironment originating from cells at a different ontogenetic stage. The observation that fetal liver progenitors may not be capable of differentiating in an adult bone marrow microenvironment, for instance (43), may have important implications for fetal liver transplantation into postnatal recipients.

The ontogeny of stromal cells is currently very poorly understood. Based on the well-established generation of multiple mesenchymal cell types from bone marrow cells, the existence of stromal stem cells has been proposed (44). Besides gaps in our knowledge of the biology of these cell populations, much information at the molecular level is also lacking. At least 16 *Hox* genes and 5

genes with homeobox domains have already been identified, although their temporal expression has not yet been determined (45).

The transplantation capability of stromal cells is also a controversial subject. Some studies have indicated that the stroma of bone marrow receptors is developed from the host's precursor cells (46-48), whereas others show that stromal cells in LTC of transplanted patients progressively originate from the donor marrow (49), a situation also reported for human/sheep chimeric cultures (50). In an *in vivo* system, it has been reported that murine bone marrow mesenchymal precursor cells, expanded in culture, were detected in the bone, cartilage and lungs of recipient mice at 1 to 5 months after transplantation (51). Recently, the migration of bone marrow-derived cells to areas of induced muscular degeneration, followed by differentiation along the myogenic pathway and participation in the regeneration of damaged fibers, has been reported in mice (52).

Differently from hematopoietic cells, the existence of stromal precursors outside the bone marrow in adults is highly controversial, so that while some studies have detected their presence in peripheral blood (53-55), others have reported negative results (56,57). The existence of stromal precursors in cord blood under normal conditions is the subject of intense discussion (58-62). A representative recent study (58), for instance, reports deficient myeloid progenitor cell growth in LTC of umbilical cord blood, suggesting that this is due to the impaired development of an adherent layer. Under special conditions, Ye et al. (59) and Nieda et al. (60) reported the establishment of an adherent stromal layer (using a special substratum) and of an endothelial layer (in the presence of IL-2 and conditioned medium from the 5637 carcinoma cell line), respectively. We have recently described the establishment, under normal long-term culture conditions, of an adherent layer from human umbilical cord blood capable to support the

proliferation of hematopoietic clonogenic cells (63; Alfonso ZC, Forneck ED and Nardi NB, unpublished results). Different results probably reflect the varying experimental conditions employed.

The hematopoietic stroma is operationally defined by its ability to support hematopoiesis. This functional characteristic is more probably a result of interactions between the adherent cells and hematopoietic cells they make contact with, but although stromal layers can be developed by *in vitro* cultivation of different organs, only those derived from bone marrow were shown to support the proliferation of more primitive hematopoietic cells (64). Although stem cells adhere to stroma developed from other organs, their hematopoietic potential is not maintained. Additional factors - ECM interactions and cytokine stimulation - are important for the interaction of the hematopoietic cells with the stroma. Actually, the need for contact between elements of these two compartments is being questioned, and in culture systems in which progenitors are physically separated from the stroma layer by a microporous membrane (stroma noncontact culture), the LTC-IC has been shown to be maintained

(65). In LTC established under conditions favoring lymphoid development, however, contact between stem and stromal cells is required for maturation of hematopoietic cells (66). A more detailed study in which the composition of the stem cell compartment was analyzed suggested that different sets of stem cells may or may not require contact with stroma to proliferate and differentiate (67).

The investigation of the stromal compartment is also important for direct applications in human health. Abnormalities in the stromal compartment have been implicated as one of the possible mechanisms of aplastic anemia (68,69), and may also be involved in the abnormal behavior of Ph⁺ cells in chronic myeloid leukemia (68). The implications of a compatibility between transplanted hematopoietic cells and the receptor stroma has already been mentioned; the adequate *in vitro* expansion of hematopoietic cells for transplant or gene therapy purposes requires the establishment of stromal layers in various systems; and transduction of hematopoietic-supportive stromal cells with genes of interest is already being reported (70).

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