THE BIOLOGY OF HEMATOPOIETIC STEM CELLS

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ABSTRACT

Hematopoietic stem cells (HSC) are the only cells in the blood-forming tissues that can give rise to all blood cell types and that can self-renew to produce

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more HSC. In mouse and human, HSC represent up to 0.05% of cells in the bone marrow. HSC are almost entirely responsible for the radioprotective and short- and long-term reconstituting effects observed after bone marrow transplantation. The subsets of HSC that give rise to short-term vs long-term multilineage reconstitution can be separated by phenotype, demonstrating that the fates of HSC are intrinsically determined. Here we review the ontogeny and biology of HSC, their expression of fate-determining genes, and the clinical importance of HSC for transplantation and gene therapy.

INTRODUCTION

The path to the identification of hematopoietic (blood forming) stem cells (HSC) began in response to the clinical need for cells capable of protecting humans exposed to minimum lethal doses of irradiation or chemotherapy (reviewed by Thomas 1991). Although death due to irradiation was associated with bleeding and infection, the precise cause was initially unknown. The first insight came with the observation that lead shielding of hematopoietic tissues prevented death from otherwise lethal doses of radiation (Jacobson et al 1949). Intravenous infusion of syngeneic marrow after irradiation also prevented death (Lorenz et al 1951), and it was shown that lymphohematopoietic cells arising from infused bone marrow repopulated hematopoietic tissues (Ford et al 1956, Makinodan 1956, Nowell et al 1956).

In a series of seminal experiments it was discovered that the bone marrow contained highly proliferative progenitor cells capable of giving rise to individual colonies of myeloid, erythroid, and megakaryocytic cells within the spleens of irradiated hosts (Till & McCulloch 1961, Wu et al 1967). The same cell type gave rise to lymphocytes (Wu et al 1968); furthermore, each spleen colony-forming cell could produce more spleen colony-forming cells (Siminovitch et al 1963). It was proposed that a population of radioprotective cells, called hematopoietic stem cells, must exist within the bone marrow, capable of multilineage differentiation as well as self-renewal (Siminovitch et al 1963) (Figure 1).

Extensive efforts to purify and characterize this population ensued. Initially, physical means were used to enrich the progenitor fraction (reviewed by Visser & van Bekkum 1990). With the invention of fluorescence-activated cell sorting (FACS), rare populations could be purified based on the expression of increasingly sophisticated patterns of cell surface markers. Concomitantly, means
were developed for studying the characteristics of progenitors in unseparated hematopoietic tissues. These efforts resulted in the ability to isolate various populations of stem cells that account for the blood-forming capacity of bone marrow. The understanding of HSC biology is now sufficiently advanced that it is often used as the model for predicting the properties of stem cells in other tissues.

THE ONTOGENY OF HEMATOPOIETIC STEM CELLS

During fetal development, hematopoietic activity sequentially progresses from the yolk sac to the liver, to the spleen, and to bone marrow (reviewed in Tavassoli 1991, Dieterlen-Lievre et al 1994). During adult life, hematopoiesis occurs in the bone marrow, spleen, and thymus. The developmental relationship between HSC in different tissues, and at different times during development, is fundamental to their biology.

The Formation of the Hematopoietic System

THE EARTH EMBRYO  Hematopoiesis begins in the mouse on day 7 of gestation in the yolk sac (Metcalf & Moore 1971) (Figure 2). This event, coupled with the observed migration of cells from the yolk sac to the embryo body (Barker 1968, Russell 1979), gave rise to the suggestion that the yolk sac is the only site of de novo stem cell formation and that all other hematopoietic tissues are

![Figure 2](Annu. Rev. Cell Dev. Biol. 1995.11:35-71. Downloaded from arjournals.annualreviews.org)

It remained to be determined whether the observed progenitor activity in the yolk sac comes from self-renewing multipotent progenitors or from more committed progenitors. Weissman et al (1978) injected donor-marked day 9 yolk sac cells into the yolk sac cavities of recipient mice, which resulted in the localization of the donor cells to the blood islands. Myelo-erythroid and lymphoid progenitors in the bone marrow of the recipient mice derived from the donor-type yolk sac cells. Huang & Auerbach (1993) identified a putative yolk sac HSC population as AA4.1+WGA (wheat germ agglutinin) bright, which was enriched for long-term multilineage reconstitution (LTMR). It remains to be demonstrated at a clonal level that individual yolk sac progenitors can give rise to LTMR.

Researchers studying birds and amphibians discovered a second early source of hematopoietic progenitors—the dorsal anterior mesoderm. Chimeric embryos, made by engrafting tissues from allogeneic sources, showed that the representation of yolk sac–derived hematopoietic cells declined during development and that hematopoietic progenitors in the adult are derived from an intra-embryonic source (Dieterlen-Lievre 1975, Carpenter & Turpen 1979). This source of hematopoietic cells and progenitors was localized to the dorsal aorta region in the splanchnopleural mesoderm (Turpen et al 1981, Dieterlen-Lievre & Martin 1981). This region is highly enriched for progenitors of the myeloid and erythroid lineages and appears to be the only intraembryonic source of hematopoietic progenitors in the early avian embryo (Dieterlen-Lievre et al 1988, Cormier & Dieterlen-Lievre 1988).

Progenitor activity in the mouse para-aortic mesoderm was simultaneously reported by Godin et al (1993) and Medvinsky et al (1993). At 10 days post-conception, progenitors capable of LTMR of adult recipients were observed only in the para-aortic mesoderm (Muller et al 1994). Most recently, Godin et al (1995) reported that clones capable of giving rise to B and multiple myeloid lineages in culture can be isolated from both the yolk sac and the para-aortic (splanchnopleural) mesoderm at 8.5 days post-conception in the mouse. Thus multipotent progenitors may arise simultaneously in the para-aortic mesoderm and in the yolk sac. It remains unknown whether either tissue, or both, provide the HSC that colonize the mammalian fetal liver and bone marrow.

ES CELL DIFFERENTIATION Efforts to understand the formation of hematopoietic progenitors in the embryo have included studies of embryonic stem (ES) cell differentiation. ES cells are perpetually self-renewing, totipotent progeni-
itors derived from the inner cell mass of blastocysts. ES cell lines can contribute to the formation of all tissues thus far examined, including germ and hematopoietic cells (Bradley et al 1984, Beddington & Robertson 1989, Forrester et al 1991). In culture, ES cells can form embryoid bodies containing endodermal, mesodermal, and ectodermal tissues, with structures that include blood islands and erythroid cells (Doetschman et al 1985). In vitro, ES cells can differentiate into embryonic and adult erythrocytes, macrophages, granulocytes, and mast cells (Schmitt et al 1991, Wiles & Keller 1991, Keller et al 1993). Recently, Nakano et al (1994) reported that ES cells can differentiate into B cells when cultured on a stromal cell line.

Given the ability of ES cells to give rise to hematopoietic structures and multiple lineages of hematopoietic cells in vitro, it is presumed that they also give rise to HSC. However, efforts to document HSC activity have been unsuccessful so far (Snodgrass et al 1992, Muller & Dzierzak 1993). Although Chen et al (1992) reported lymphoid and myeloid reconstitution of several mice by retrovirally immortalized ES-derived cells, most mice were killed by tumors. Under the conditions thus far used for ES cell differentiation, either HSC are not produced, the HSC are not capable of engrafting upon transplantation in vivo, or the conditions inside embryoid bodies do not promote sufficient self-renewal for HSC to be detected. The extent to which the pathways of hematopoietic cell differentiation in embryoid bodies recapitulate normal hematopoiesis remains to be determined.

The Transition to Adult Hematopoiesis

The fetal liver is the principal site of mid- and late-gestation hematopoiesis in mammals, beginning at 10 days post-conception in mice. The adult hematopoietic system begins to take shape in the fetal liver and includes the emergence of a large and expanding pool of multipotent progenitors (Ikuta et al 1990). Multipotent progenitors in the fetal liver are in a small population of Lineage\textsuperscript{lo}AA4.1\textsuperscript{+} cells; individual cells within that population are capable of LTMR (Jordan et al 1990). Fetal liver HSC have been more precisely characterized as Thy-1\textsuperscript{lo} Lineage\textsuperscript{−} Sca-1\textsuperscript{+} (Ikuta et al 1990) or AA4.1\textsuperscript{+} Lin\textsuperscript{lo} Sca-1\textsuperscript{+} (Jordan et al 1995), each representing ~0.05% of day 14 fetal liver.

To the extent that the relationship between early embryo and fetal liver stems cells is not understood, it is also unknown whether embryonic and adult-type erythrocytes are produced by the same lineage of stem cells in small mammals. More information is available on the switch from fetal to adult hemoglobin synthesis in larger mammals. When adult sheep (Wood et al 1985) or adult human patients (Papayannopoulou et al 1986) are reconstituted by fetal liver cells, fetal hemoglobin continues to be expressed for some time before the graft makes the transition to adult hemoglobin production. Indeed, the period over which fetal hemoglobin is expressed in the adult recipient is
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correlated with the gestational time remaining in the donor organ (Wood et al 1985). The transition to adult hemoglobin expression does not appear to be irreversible because a number of acquired conditions in the adult, such as pregnancy, are associated with an increase in fetal hemoglobin expression (reviewed by Wood et al 1977). Together these data implicate a developmental clock in HSC that coordinates reversible transitions in the type of hemoglobin produced (Wood et al 1987); however, it should be cautioned that all in vivo analyses have been done at the population level, and definitive conclusions will require clonal, in vivo analyses.

The development of the fetal thymus, beginning in the mouse 10 days post-conception, and the fetal spleen, beginning 15 days post-conception, mark the emergence of lymphoid cells. Subpopulations of γδ TCR+ T cells arise in waves in early ontogeny, as the expression of T cell receptor (TCR) variable region exons appears developmentally regulated (Coltey et al 1989). Vγ3 cells are the first wave of T cells detectable in the mouse thymus (Havran & Allison 1988), followed by Vγ4, Vγ2, and Vγ5 (reviewed by Ikuta & Weissman 1993). Vγ2 and TCR αβ T cells are predominant in the adult thymus. Ikuta et al (1990) demonstrated that fetal liver, but not adult HSC, could give rise to Vγ3 T cells in the fetal thymus. Thus there appears to be a developmental restriction in V region gene usage as HSC progress from the fetal to adult stages. The fetal microenvironment was also found to be required for the generation of Vγ3 T cells (Ikuta et al 1990), thus implicating microenvironmental changes in developmental restrictions throughout ontogeny.

The development of B cell subsets is also regulated. Three distinct subsets of B cells have been recognized in mice: B1a, B1b, and B2 (conventional) B cells. B1a and B1b subsets appear earlier in ontogeny, are enriched in the peritoneum, have greater self-renewal capacity, and express different antibody repertoires relative to conventional B cells (reviewed by Hardy & Hayakawa 1994). Fetal liver cells can reconstitute both B1 and B2 subsets in adult recipients (Solvason et al 1991, Kantor et al 1992). In contrast, adult bone marrow has very little potential to produce B1a cells but produces normal numbers of B1b and B2 cells (Hayakawa et al 1985, Kantor et al 1992). When the two tissues were cotransferred into adult recipients (Kantor et al 1992), fetal liver cells gave rise to B1a cells, and bone marrow remained deficient in B1a progenitor activity. Adult HSC are deficient in B1a cell production relative to newborn liver HSC (Hardy & Hayakawa 1992). Together these data suggest a restriction in the B lineage progenitor potential of adult HSC relative to fetal liver HSC.

It remains to be determined whether the bone marrow HSC pool arises from fetal liver HSC during normal development. The engraftment of mouse fetuses with fetal liver cells resulted in LTMR throughout adult life (Fleischman et al 1982). Zanjani et al (1993) infused fetal sheep with fetal liver cells and found
that the relative representation of the donor cells increased in bone marrow and decreased in the liver with increasing gestational age of the recipient. Although these observations may be indicative of developmentally regulated migration, a lineage relationship between fetal liver and adult stem cells can only be proven by observing adult bone marrow HSC, which clonally derive from marked fetal liver HSC in vivo.

Aging of HSC

Hayflick founded the study of cellular aging with his description of the "Hayflick limit," which asserts that all normal somatic cells have an intrinsic limit to the number of times they can divide (Hayflick 1965). Chronologically older cells, which tend to have undergone more divisions, tend to exhibit reduced potential for proliferation (Martin et al 1970). The hypothesis that the divisions of HSC are intrinsically limited is supported by the observation that if hematopoietic progenitors are serially transplanted through multiple recipients, then stem cell activity declines after the second transfer and can no longer be detected by the fifth transfer (Ogden & Micklem 1976). It is thought that serial transfer is tantamount to accelerated aging because upon each transfer, HSC must undergo many rounds of division to reconstitute the bone marrow of the recipient (Harrison et al 1978). Fetal liver HSC exhibit more self-renewal and proliferation potential than bone marrow HSC (Micklem et al 1972, Lansdorp et al 1993). However, the proliferation potentials of old and young bone marrow are indistinguishable when compared over the life span of a mouse (Harrison 1973, Harrison et al 1978, Harrison & Astle 1982). This seemingly antithetical result is reconciled by the observation that stem cell activity from old bone marrow declines faster than that of young bone marrow upon serial transfer (Harrison 1973, Harrison et al 1989). Whereas HSC proliferation and self-renewal are limited, the proliferation normally required of a bone marrow HSC clone over the course of a mouse's life may not approach its intrinsic limit. Unfortunately, all of the foregoing observations were made at the population level. Given the lack of understanding of HSC population dynamics, analyses of clonally marked HSC would be a more definitive means of analyzing HSC self-renewal potential.

THE CHARACTERIZATION OF HEMATOPOIETIC STEM CELLS

Properties of HSC

RADIOPROTECTION Radioprotection measures the ability of a population of progenitors to rapidly give rise to sufficient hematopoietic activity to rescue a lethally irradiated recipient from hematopoietic failure. To our knowledge,
all studies that have examined the radioprotective capacity of highly enriched multipotent progenitors have found them highly enriched for radioprotective capacity (Spangrude et al 1988, 1995; Ploemacher & Brons 1988, Li & Johnson 1992, Wolf et al 1993, Morrison & Weissman 1994). Transient and long-term multipotent progenitors appear to have very similar radioprotective capacities (Li & Johnson 1992, Morrison & Weissman 1994). The difference in the onset of reconstitution between long-term and transient progenitors is only about a week.

CFU-S The original assay for HSC was the colony-forming unit-spleen (CFU-S) assay (Till & McCulloch 1961). Primitive bone marrow progenitors give rise to nodules of hematopoietic cells on the spleens of lethally irradiated mice between 8 and 13 days after injection. For many years CFU-S, and especially late (day 12 or 13) CFU-S (Magli et al 1982), were considered equivalent to HSC: That is, HSC were thought to form late spleen colonies. This presumed equivalence has since been questioned (Jones et al 1990). Recently published papers demonstrate that while both long-term and transiently reconstituting multipotent progenitors appear highly enriched for late CFU-S activity, the majority of day 12 CFU-S are transient progenitors (Morrison & Weissman 1994, Van der Loo et al 1994). The most primitive, long-term reconstituting HSC usually have "pre-CFU-S" activity (Hodgson & Bradley 1979, Van Zant 1984, Ploemacher & Brons 1989, Spangrude & Johnson 1990). They are too primitive to form a spleen colony within 12 days, but they give rise to progenitors that can do so. Strain differences may also influence the CFU-S content of different populations of stem cells (Van Zant et al 1990).

HSC CELL CYCLE DISTRIBUTION To understand the dynamics of the primitive progenitor pool, the distribution of multipotent progenitors between the various phases of the cell cycle should be examined. Long-term reconstituting HSC appear to be refractory to retroviral infection (Williams et al 1984) and to cell cycle–specific chemotherapeutic agents (Bruce & Meeker 1967). These observations indicate that long-term reconstituting HSC are generally not mitotically active (Varmus et al 1977). Fifteen to twenty percent of Thy-1lo Sca-1+ Lin−/lo cells are in the S/G2/M phases of cell cycle, as assayed by Hoechst analysis (Fleming et al 1993a, Morrison & Weissman 1994); however, this population includes both long-term and transiently reconstituting progenitors. Fleming et al (1993a) observed that G0/G1 Thy-1lo Sca-1+ Lin−/lo cells are enriched for long-term reconstituting stem cells. Indeed, direct examination of the cell cycle distribution of Thy-1lo Sca-1+ Lin−/lo subpopulations indicated that only 4% of long-term cells, but up to 18% of transient cells, are in S/G2/M phases (Morrison & Weissman 1994). Forty percent of fetal liver HSC are in S/G2/M phases (Fleming et al 1993a).
IN VITRO CULTURE OF HSC  The most physiological means of in vitro culture of HSC is on hematopoietic stroma (Dexter et al 1977). Stroma are layers of adherent cells that can be grown in vitro from hematopoietic tissues and that produce the factors required to maintain hematopoiesis. Stromal cell cultures are capable of supporting the differentiation and sometimes limited self-renewal of hematopoietic progenitors (reviewed by Deryugina & Muller-Sieburg 1993). Both transient and long-term multipotent progenitors form colonies at high frequency in stromal cell culture systems (SJ Morrison et al, unpublished data). The “cobble-stone area forming cell” (CAFC), having a microscopically detectable pattern of cell growth in stromal cell cultures, is a phenotype that may develop from multipotent progenitor cells in coculture with stroma (Ploemacher et al 1991). Recently, a high correlation between the assays for CAFC and the long-term culture initiating cell (LTC-IC) was shown (Reading et al 1994). The LTC-IC assay tests for the presence of progenitors capable of initiating long-term bone marrow cultures and giving rise to progenitors that can be detected by replating into semi-solid cultures after 5 to 8 weeks (Eaves et al 1986). The limitations of CAFC/LTC-IC assays are that the HSC do not achieve the degree of self-renewal or range of differentiation in culture that they do in vivo.

HSC can also be cultured in liquid or semi-solid cultures, in the absence of supporting stroma. Various combinations of cytokines are used in such cultures to promote the proliferation or maintenance of progenitor cells (reviewed by Ogawa 1993). Unfortunately, no combination of cytokines has yet been identified that promotes a significant expansion or long-term maintenance of multipotent progenitors in such cultures. Only myeloid and erythroid differentiation are usually observed, although a means of achieving limited B lineage differentiation has been reported (Hirayama et al 1992). The self-renewal, and perhaps the differentiation, of HSC in liquid and semi-solid cultures does not match what is observed physiologically.

Ogawa and colleagues have described “blast-cell colonies,” which have secondary replating potential, as a feature of HSC (Nakahata & Ogawa 1982). Indeed, such colonies appear to uniquely derive from long-term reconstituting multipotent progenitors (K Akashi et al, unpublished data); however, only a minority of long-term HSC appear to form blast colonies. Therefore, blast-cell colony formation probably detects only a subset of HSC. Similarly, high proliferative potential colony-forming cells (HPP-CFC) represent an in vitro progenitor phenotype correlated with the enrichment of multipotent progenitors (Bradley & Hodgson 1979). The frequency of HPP-CFC in bone marrow is much higher than that of multipotent progenitors, suggesting this phenotype might include a number of early progenitors at different stages of differentiation (reviewed by Bertoncello 1992).
Isolation of Mouse HSC

Visser & van Bekkum (1990) have reviewed early efforts to enrich HSC. The most powerful approaches have been based on the purification of rare cell fractions by FACS. Visser et al (1984) showed that mouse HSC were highly enriched among H-2K<sup>high</sup> WGA<sup>+</sup> cells. Spangrude et al (1988) isolated mouse multipotent progenitors by defining the Thy-1<sup>lo</sup> Sca-1<sup>+</sup> Lin<sup>-</sup> population, which represented 0.05% of C57BL/Ka-Thyl.1 bone marrow. The Sca-1 marker is a member of the Ly6 family and is also known as the Ly6A/E antigen (van de Rijn et al 1989, Spangrude & Brooks 1993). Out of every 20 intravenously (iv) injected cells from the Thy-1<sup>lo</sup> Sca-1<sup>+</sup> Lin<sup>-</sup> population, on average 1 engrafted and gave rise to multipotent progenitor activity (Smith et al 1991, Uchida et al 1993) (Table 1). Given that most iv-injected cells are nonspecifically trapped in tissues like the liver and the lungs, where they are unable to express progenitor activity, this population was considered very highly enriched for HSC. Uchida & Weissman (1992) also demonstrated that the Thy-1<sup>lo</sup> Sca-1<sup>+</sup> Lin<sup>-</sup> population included all the detectable multipotent progenitors in the C57BL/Ka-Thyl.1 strain. Operationally, the Thy-1<sup>lo</sup> Sca-1<sup>+</sup> Lin<sup>-</sup> population is described as being multipotent because mice reconstituted by individual clones from this population are routinely shown to contain donor-type cells of the monocyte, granulocyte, B, and T lineages; however, the population is also

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<th>Table 1</th>
<th>The expression of hematopoietic cell surface markers by mouse multipotent progenitors</th>
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<td>Cell surface marker</td>
<td>Major lineages of expression</td>
</tr>
<tr>
<td>Thy-1</td>
<td>T cells</td>
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<tr>
<td>Sca-1</td>
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<tr>
<td>c-kit</td>
<td>Progenitors</td>
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<tr>
<td>Mac-1</td>
<td>Granulocytes, monocytes</td>
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<td>CD4</td>
<td>T cells</td>
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<td>T cells, B1a B cells</td>
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<td>CD8</td>
<td>T cells</td>
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<td>B220</td>
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<td>Gr-1</td>
<td>Granulocytes</td>
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<td>Ter19</td>
<td>Erythroid progenitors</td>
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<td>CD34</td>
<td>Progenitors</td>
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<tr>
<td>WGA</td>
<td>Cells expressing sialic acid residues</td>
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<tr>
<td>H-2K</td>
<td>All hematopoietic cells</td>
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<tr>
<td>Ly5</td>
<td>Most hematopoietic cells</td>
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known to completely reconstitute the natural killer (NK) (H Aguila et al, in preparation), erythrocyte, and platelet lineages (Uchida et al 1994a), thus all progenitors within the population are believed to be pluripotent.

Although this population was initially described as being Lin−, the lineage marker staining profile was consistent with the population containing Lin− as well as Linlo cells (Spangrude et al 1988, Ikuta et al 1990, Uchida & Weissman 1992). Later references to this population described it as Lin−lo (Fleming et al 1993a). Upon closer examination, CD4 (Fredrickson & Basch 1989, Wineman et al 1992) and Mac-1 (Morrison et al 1994) were found to be expressed at low levels on at least some multipotent progenitors.

Despite being a nearly pure population of multipotent progenitors, the Thy-1lo Sca-1+ Lin−lo population is heterogeneous with respect to functional activity. The majority of clones within this population give rise to transient reconstitution patterns, whereas about a quarter of clones yield LTMR (Uchida et al 1993). We have recently found that the Thy-1lo Sca-1+ fraction can be divided into three subpopulations based on heterogeneity in low level Mac-1 and CD4 expression: Lin− Mac-1− CD4− c-kit+, Lin− Mac-1lo CD4+, and Mac-1lo CD4lo (where Lin is used to refer to all lineage markers other than Mac-1 and CD4) (Morrison & Weissman 1994). Each of these populations is highly enriched for progenitor activity: 1 in 10 iv-injected cells show progenitor activity. However, clonal analysis indicates that only the Mac-1−CD4− population is highly enriched for long-term reconstituting cells. The Mac-1loCD4− population is highly enriched for transient multipotent progenitors and progenitors that only give rise to detectable B lineage reconstitution. These data demonstrate that the longevity of stem cell reconstitution is deterministic and depends principally on factors intrinsic to the cell.

Rhodamine123 (Rh123) has also proven useful for the purification and subfractionation of HSC. Several studies have demonstrated that enriched progenitor fractions can be further subdivided with respect to Rh123 uptake and that the Rh123lo fraction is more enriched for long-term multipotent progenitors than the Rh123med/hi fraction (Spangrude & Johnson 1990, Li & Johnson 1992, Wolf et al 1993). Spangrude et al (1995) has recently found that Thy-1lo Lin− Sca-1+ Rh123lo cells are an ostensibly pure population of LTMR progenitors. Injection of only 5 cells from this population into irradiated mice resulted in limit dilution LTMR. This establishes the benchmark for purity among long-term reconstituting progenitor fractions; furthermore, long-term and transiently reconstituting multipotent progenitors appear to be separable based on Rh123 uptake (Li et al 1994; JM Zijlmans et al, unpublished results). This observation is consistent with the conclusions of Morrison & Weissman (1994).

Several other markers have also proven useful for significantly enriching
HSC. The most interesting recent addition to these is murine CD34, against which antibodies have only recently become available (Zeigler et al 1994, Krause et al 1994). The selection of cells that bind high levels of WGA provides a substantial enrichment of HSC (Visser et al 1984, Jurecic et al 1993). Despite efforts to identify primitive c-kit\(^{-}\) progenitor populations, evidence continues to mount that HSC from normal bone marrow are c-kit\(^{+}\) and not c-kit\(^{-}\) (Ikuta & Weissman 1992, Orlic et al 1993, Morrison & Weissman 1994). Recent reports have emphasized the need for caution when describing c-kit expression profiles because several different antibodies, with different staining profiles, are available against c-kit. Populations may appear uniformly bright for c-kit using one antibody and appear dull or heterogeneous with other antibodies. In contrast to Thy-1 (Spangrude & Brooks 1992) and Sca-1 (Spangrude & Brooks 1993), no strain dependence has yet been detected in the expression of CD34, c-kit, and WGA reactivity by normal bone marrow HSC from the adult mouse.

Some differences in surface marker expression appear between fetal and adult hematopoietic tissues. AA4, which is expressed by HSC in the yolk sac and fetal liver, appears not to be expressed by HSC from adult bone marrow (Spangrude 1994, Trevisan & Iscove 1995). In contrast to bone marrow, Sca-1 has been reported not to be expressed on at least some primitive yolk sac progenitors (Cumano et al 1993, Huang & Auerbach 1993).

**Isolation of Human HSC**

Xenogeneic reconstitution systems have proven to be useful in vivo models for human hematopoiesis. McCune et al (1988) and colleagues (Namikawa et al 1990) developed the SCID-hu model in which human hematopoietic organs are engrafted into SCID mice. HLA-mismatched hematopoietic progenitors can be used to engraft these SCID-hu mice. Their developmental potential can then be monitored based on the representation of donor-type cells over time and the ability to transfer progenitor activity to human fetal bone or thymus in secondary SCID-hu recipients (Peault et al 1991, Chen et al 1994). Dick and colleagues have used immunodeficient mouse models such as nude/xid (Kamel-Reid & Dick 1988), and SCID (Lapidot et al 1994) to study the engraftment of human hematopoietic cells. The ability to engraft human HSC in xenogeneic hosts demonstrates the considerable conservation of the hematopoietic environment between species. Nonetheless, human hematopoiesis in SCID-recipient mice can be improved by the infusion of human cytokines (Lapidot et al 1992). Although mouse models of human hematopoiesis have provided important in vivo assays, significant barriers remain to be resolved before long-term multilineage reconstitution from human HSC can readily be observed.

Zanjani and colleagues (1991) developed the use of in utero transfer of xenogeneic HSC to pre-immune fetal sheep. Transfers of either human bone
marrow (Srouer et al 1992) or fetal liver (Zanjani et al 1992) resulted in LTMR of the sheep with human cells; furthermore, human HSC could be recovered from sheep bone marrow more than 3 years after engraftment and used to engraft secondary recipients (Zanjani et al 1994). CD34+Thy-1+Lin- cells from both adult bone marrow and mobilized peripheral blood are able to give rise to long-term human hematopoiesis when transplanted into fetal sheep (Uchida et al 1994c). Xenogeneic models of human HSC engraftment provide the ability to study LTMR, and complement the use of in vitro assays.

Primitive human hematopoietic progenitors are enriched in the CD34+ population but not in the CD34- population in adult and fetal bone marrow (Andrews et al 1986, 1989; Strauss et al 1986, Baum et al 1992, DiGiusto et al 1994). HSC from different hematopoietic tissues have been further enriched by various cell-surface marker combinations within CD34+ populations. HSC from fetal and adult bone marrow express low or undetectable levels of CD38 (Terstappen et al 1991), CD45RA (Lansdorp et al 1990), and CD71 (Lansdorp & Dragowska 1992). LTC-IC activity is enriched in CD34+ c-kitlo but not c-kithigh adult bone marrow cells (Gunji et al 1993). Just as in the mouse, a candidate human HSC population can be defined by Thy-1 expression as CD34+ Thy-1+ Lin- from fetal bone marrow (Baum et al 1992), adult bone marrow (Murray et al 1995a), and mobilized peripheral blood (Murray et al 1995b). Cord blood and fetal liver HSC have also been characterized as CD34+ Thy-1+ (Craig et al 1993, Mayani & Lansdorp 1994). Virtually all the LTC-IC activity from adult bone marrow is contained in the CD34+ Rh123dull subset, whereas clonogenic cells assayed in standard methylcellulose assays are distributed in both Rh123dull and Rh123bright fractions (Udomaskdi et al 1991). The Rh123lo, but not the Rh123hi, subset of CD34+ Thy-1+ Lin- cells from human fetal and adult bone marrow are highly enriched for CAFC activity (Baum et al 1992, Uchida et al 1994b). Human HSC from adult bone marrow have been described as CD34+ HLA-DR- (Srouer et al 1991), whereas HSC from fetal bone marrow (Huang & Terstappen 1994) and cord blood (Traycoff et al 1994) are CD34+ HLA-DR+.

Summary

Data on the expression of cell-surface markers by multipotent progenitors demonstrate that the correlation between surface marker expression and cell function is specific to the tissue and conditions under which the correlation is observed. For example, the cell-surface markers expressed by multipotent progenitors appear to change upon 5-fluorouracil treatment (compare Szilvassy & Cory 1993 to Morrison & Weissman 1994) and in mice reconstituted after lethal irradiation (Bertoncello et al 1989, Spangrude et al 1995). Correlations known to exist in one tissue or under one set of conditions should not be presumed to apply otherwise.
Siminovitch et al (1963) provided the functional criteria for defining HSC: progenitors that, at the single cell level, are capable of self-renewal as well as giving rise to greatly expanded numbers of blood cells of all lineages. Self-renewal, while often the subject of semantic arguments, should be considered as the ability of a pluripotent progenitor to give rise to pluripotent daughter cells. A good test of self-renewal potential is the ability to transfer multipotent progenitor activity to secondary recipients. Long-term reconstituting progenitors, as well as at least some transiently reconstituting multipotent progenitors, meet the definition for HSC. It is critical that HSC populations be pure, or nearly pure, if population level characteristics such as cell cycle distribution and radioprotective capacity are to be interpretable.

Recent studies present a relatively coherent view of the properties of normal bone marrow HSC. Long-term reconstituting multipotent progenitors can be purified and represent 0.005–0.01% of bone marrow. At least two discrete populations of transiently reconstituting multipotent progenitors can also be essentially purified. These populations represent 0.01–0.03% of mouse bone marrow. They provide somewhat faster (by about one week), although lower level, engraftment than long-term reconstituting progenitors and are equally radioprotective. In general, transient progenitor populations appear to contain fewer quiescent cells and/or more rapidly cycling cells.

DIFFERENTIATION AND SELF-RENEWAL

Multipotent progenitors are responsible for the great majority of early white blood cell, erythrocyte, and platelet reconstitution after bone marrow transplantation (Uchida et al 1994a). The differentiation of multipotent progenitors is tightly linked to the production of the large number of differentiated cells that must be produced by the bone marrow on a daily basis. Combined with the fact that HSC are responsible for the long-term perpetuation of hematopoiesis, this makes regulating the balance between the competing interests of HSC differentiation and self-renewal critical.

Models of Self-Renewal

A number of seminal papers present models for the perpetuation of hematopoiesis. These models start from the assumption that the self-renewal potential of HSC is finite (Reincke et al 1982), as was suggested by the serial transfer experiments reviewed above, and the Hayflick limit (Hayflick 1965). Till et al (1963) proposed that the self-renewal or differentiation of HSC is associated with non-equal probabilities that are determined by unknown variables. They further proposed that within the limits of such probabilities, the heterogeneity in the CFU-S content of spleen colonies could be explained by stochastic commitment of HSC to either differentiate or self-renew. This notion of sto-
stochastic commitment to differentiation or self-renewal was predominant in early models. Kay (1965) formally proposed clonal succession, which envisaged various models for the clonal relationship between self-renewal and differentiation, all of which were characterized by one lineage of HSC dominating hematopoiesis until its proliferative potential and that of its progeny were exhausted. This was suggested with the proviso that the probability of HSC self-renewal decreased with increasing divisions.

As techniques for studying HSC became more precise, models relied less on random events. Metcalf & Moore (1971) suggested that multiple lineages of HSC might be involved in hematopoiesis, that intrinsic differences between CFU-S in self-renewal potential might exist, and that microenvironmental differences might influence self-renewal and differentiation. The generation-age hypothesis was formally stated by Rosendaal et al (1979), who expanded the idea that multiple lineages of HSC might be required for the maintenance of hematopoiesis. Micklem & Ogden (1976) suggested that the stem cell pool was organized into at least three distinct populations, with discrete self-renewal potentials. In contrast, Helman et al (1978) posited a continuum of self-renewal potentials within the stem cell pool, in which the self-renewal potential of any clone would be inversely related to the number of divisions already undergone. Although there are data supporting all aspects of the models described above, none can be considered conclusive. Nonetheless, the models described above continue to provide the foundations for current conceptions of HSC differentiation and self-renewal.

A series of mainly retroviral marking experiments have provided important data in support of the generation-age and clonal-succession models of HSC differentiation. Retroviral marking of HSC makes it possible to uniquely mark and simultaneously follow multiple progenitor clones, based on retroviral integration sites (Dick et al 1985). Upon reconstitution, many clones of progenitors initially contribute to hematopoiesis, but over the long term, a small number of pluripotent HSC clones tend to dominate (Lemischka et al 1986, Snodgrass & Keller 1987, Capel et al 1989, Jordan & Lemischka 1990, Harrison & Zhong 1992). In general, it appears that most clones of long-term reconstituting stem cells are quiescent, most of the time.

**Stochastic and Deterministic Models**

We have presented a deterministic model of multipotent progenitor self-renewal based on the purification of three multipotent progenitor populations, with distinct self-renewal potentials (Morrison & Weissman 1994) (Figure 3). Our hypothesis that these populations represent a lineage of multipotent progenitors from the most primitive to the most differentiated is consistent with the results of extensive secondary transfer experiments (SJ Morrison et al, in preparation). Clones from the most primitive population exhibit long-term
Figure 3 A model of the differentiation of multipotent progenitors in adult mouse bone marrow. Three subpopulations of Thy-1.1^lo Sca-1^+ Lin^−/lo multipotent progenitors can be independently purified based on differences in Mac-1 and CD4 expression. These populations appear to form a lineage from the most primitive, isolatable, long-term reconstituting stem cell to the most differentiated multipotent progenitor prior to lineage commitment.

self-renewal, whereas clones from the most differentiated population exhibit no detectable self-renewal. The fact that we can predict the self-renewal potential of particular multipotent progenitors based on surface marker expression indicates that self-renewal is deterministic. Although self-renewal is determined by intrinsic factors, microenvironmental factors may still play a role. It is formally possible that different subpopulations of progenitors express different receptors that cause homing to microenvironments that regulate self-renewal or differentiation (Trentin 1970).
Our model contrasts with Ogawa’s stochastic model (1993) of HSC self-renewal, based on the formation of colonies with replating potential in methylcellulose culture. In such cultures, there is no HSC self-renewal in the way that it is generally considered in vivo; however, the formation of blast-cell colonies, with secondary replating potential, has been described as a form of limited self-renewal (Nakahata et al 1982). The pattern of formation of colonies with secondary replating potential has been interpreted as consistent with a stochastic model (Nakahata et al 1982). The fact that physiological self-renewal cannot be observed in methylcellulose culture makes it difficult to compare these data to in vivo results. Why does the formation of replatable colonies appear stochastic, whereas the productive life-span of multipotent clones in vivo appears deterministic?

The ability to identify three discrete populations of multipotent progenitors with different functional properties is reminiscent of the predictions of Micklem & Ogden (1976). Our data suggest that the stem cell pool is organized into a limited number of discrete differentiation states, rather than a continuum of self-renewal potentials. Multipotent progenitors within each subpopulation appear to exhibit highly reproducible self-renewal potentials, rather than a continuum of potentials within a certain range. Additionally, a number of studies have suggested that stem cells from embryonic and fetal sources have increased proliferative and self-renewal potentials relative to adult HSC (Metcalf & Moore 1971, Micklem et al 1972, Lansdorp et al 1993). Thus multipotent progenitors throughout ontogeny may be organized into a hierarchy composed of a small number of discrete differentiation states, with only a limited number of self-renewing divisions permissible within each differentiation state.

Models of HSC regulation that rely on a mitotic clock to count cell divisions all predict a continuum of self-renewal potentials. If multipotent progenitors are divided into a small number of discrete, self-renewal potentials, could a mitotic clock based on cell divisions still regulate self-renewal? As we have suggested (Morrison & Weissman 1995), the mitotic clock may be environmentally responsive. Within a constant environment, it may regulate the timing of transitions from one differentiation state to another by counting divisions, but upon transfer to irradiated bone, the clock could be reset to the maximum number of divisions within a particular differentiation state. This would cause clones of HSC to exhibit discrete, reproducible self-renewal potentials by competitive reconstitution, while enabling a clock-based mechanism to maintain homeostasis.

**Environmental or Intrinsic Influences on Differentiation**

Models have been advanced suggesting that cytokines are capable of instructing the differentiation of progenitors (Van Zant et al 1979; reviewed by Metcalf
HEMATOPOIETIC STEM CELLS

1989) and that the stromal microenvironment may thus determine their fate (Trentin 1970). Although cytokines may instruct the differentiation of some progenitors, there is no convincing evidence that cytokines can instruct the differentiation of HSC. Ogawa (1993) has argued that cytokines may influence the survival and proliferation of progenitors after commitment, but they do not determine the commitment of HSC. Indeed, many authors have recently examined the effects of cytokines on multipotent progenitors and found that the self-renewal or differentiation of the progenitors was regulated by intrinsic factors, and not by growth factors (Mayani et al 1993, Fairbairn et al 1993, Li & Johnson 1994).

Most growth factor studies have observed target cells at the population level. Observations at the population level cannot rigorously establish an instructive effect of a cytokine on differentiation. Alternate explanations such as the promotion of survival or proliferation of a progenitor after commitment, or the suppression of other progenitors must be ruled out at the clonal level. This process has been elegantly employed to demonstrate an instructive effect of glial growth factor on the differentiation of multipotent neural progenitors (Shah et al 1994), but there is no convincing evidence of a deterministic role of cytokines on the differentiation of HSC.

Lineage Commitment by HSC

Many experiments have been done in methylcellulose culture to examine the composition of colonies formed by single progenitors. It appears that lineage commitment by multipotent progenitors in this culture system is progressive and stochastic (Suda et al 1983, Leary et al 1985). Based on these observations, Ogawa has proposed a model suggesting the identities of the lineages into which HSC differentiate are stochastically determined (Ogawa 1993). It should be noted that the timing of HSC differentiation could be deterministic, while the choice of lineage is stochastic. Interpretations based on methylcellulose culture must be made with caution because the in vivo self-renewal and differentiation potentials of HSC have not been reproduced in such cultures.

In an effort to give form to the differentiation pathways followed by HSC, a number of authors have described putative oligopotential progenitors and early, lineage-committed progenitors. However, we have been unable to reproduce the functional activities of several of these populations when isolated from normal bone marrow (Morrison et al 1994). Many putative oligopotential progenitors share surface markers with HSC. Given that low-level contamination by HSC will impart the appearance of progenitor activity to a population, it seems that eliminating multipotent progenitors has been a general problem in the characterization of downstream progenitors. Careful controls must eliminate the possibility of low-level contamination by HSC, and the population
must be sufficiently purified for the functional activity to be characterized at the single-cell level.

A Candidate Mitotic Clock

Telomeres are long sequences of repetitive DNA found at the ends of all chromosomes. DNA can only be synthesized 5' to 3', and RNA primers are required to initiate synthesis. As a result, human somatic cells lose 50- to 100-base pairs of telomeric sequence at the end of each chromosome during each round of replication (Harley et al 1990). The loss or dramatic shortening of telomeres is associated with chromosomal instability, cell cycle arrest, and cell death (Lundblad & Szostak 1989, Sandell & Zakian 1993). Thus the length of telomeres and the rate at which telomeric sequence is lost may limit the number of divisions that somatic cells can undergo. Allsopp et al (1992) have shown that the replicative capacity of human fibroblasts correlates better with telomere length than with donor age. Immortal cell lines and germ cells express telomerase activity, which replaces the telomere sequence that would otherwise be lost upon chromosome replication (Counter et al 1992). The regulation of cell division potential by telomere length may be a general mechanism underlying the Hayflick limit (Hayflick 1965).

Changes in telomere length are correlated with HSC proliferation potential. Indeed, highly enriched human HSC populations show a decline in telomere length with age (Vaziri et al 1994). Consistent with what would be predicted based on differences in proliferative potential, fetal HSC have significantly longer telomeres than adult HSC, but differences between young and old adult donors are subtle. The progeny of human HSC in culture also show a division-dependent loss of telomere sequence. Although the loss of telomeres is known to cause cell cycle arrest, there is currently no evidence that quantum changes in telomere length may play a causative role in the timing of differentiation events. Unfortunately, elucidating the relationship between telomere length and the regulation of HSC self-renewal is complicated by the fact that, in mice, telomerase appears to be regulated differently (Prowse & Greider 1995), and telomeres themselves appear to be much longer and more variable (Kipling & Cooke 1990, Starling et al 1990).

Summary

Historically there has been a tendency to consider the pool of multipotent progenitors monolithic. As a result, stochastic models were invoked to explain variation in the proliferation or self-renewal of multipotent progenitors. We now know that the pool is not homogeneous and includes both long-term and transient multipotent progenitors. Both classes of progenitors include CFU-S, are capable of mixed colony formation in vitro, and form similar colonies on stromal feeder layers. Nonetheless, the two classes of progenitors are known
to vary dramatically in their self-renewal and proliferative potentials when
tested in vivo. Extreme caution must be used in interpreting limited measures
of HSC phenotype, such as CFU-S, or in vitro proliferation. Indeed, when
multipotent progenitors are separated into subpopulations with more homoge­
neous self-renewal potentials, and their actual in vivo reconstitution is exam­
ined, the results appear far less stochastic. In our view, the weight of current
evidence suggests that HSC differentiation and self-renewal are deterministic
and controlled principally by intrinsic factors.

MOBILIZATION OF HEMATOPOIETIC STEM CELLS

HSC in the adult mouse are found primarily in the bone marrow, although
they can be found in the spleen, and in the blood at much lower frequencies
(Metcalf & Moore 1971). During the regeneration phase following myelo-sup­
pressive chemotherapy, the blood stream also contains cells capable of saving
lethally irradiated hosts (Appelbaum et al 1986). Infusion of cytokines such
as granulocyte-colony stimulating factor (G-CSF) (Molineux et al 1990), gran­
ulocyte macrophage-colony stimulating factor (GM-CSF) (Siena et al 1989),
and steel factor (SLF) (Fleming et al 1993b) alone, or in combination with
cytoreductive agents (Siena et al 1989), leads to higher levels of primitive
progenitors in the blood. Upon cessation of treatment with cytokines, the levels
of progenitors in the blood decline to normal.

Despite the belief that SLF could increase HSC numbers, the mice whose
HSC were mobilized by SLF treatment did not show significant increases in
HSC when total HSC numbers in the blood, spleen, and bone marrow were
considered (Fleming et al 1993b). In fact, these mice showed a reduction of
up to 30% in bone marrow Thy^lo Lin^- Sca-1^ HSC, implying that the increase
in the numbers of these cells in the blood stream resulted from their movement
out of the bone marrow (Fleming et al 1993b). Others have reported evidence
for increased absolute numbers of HSC (Bodine et al 1993). In humans, as
well, treatment with cytoreductive chemotherapeutic agents such as cyclo­
phosphamide or VP16, followed by infusion of cytokines such as G-CSF or
GM-CSF, resulted in a substantial elevation of Thy-^ Lin^- CD34^ HSC levels
in the blood (Murray et al 1995b). Indeed, in humans, the majority of the
CAFC and SCID-hu bone reconstituting activity is present in the Thy^ subset
of the CD34^- Lin^- peripheral blood cells (Murray et al 1995b, Tsukamoto et
al 1995). Thus, from a clinical standpoint, it is not the number of CD34^ cells,
but the number of CD34^- Thy^- Lin^- cells that are likely to characterize a
transplant inoculum containing stem cell activity. The CD34^- Thy^- cells
mobilized into peripheral blood include both Rh123^ and Rh123^ subsets
(Uchida et al 1994b).

Unfortunately, most mobilization regimens lead to the mobilization not only
of HSC and CD34+ progenitors but also of tumor cells (Brugger et al 1994). A variety of tumors have been observed to express CD34 (Kohler et al 1994). It is not known whether the mobilization of tumor cells is from metastases in the bone marrow or from other sites. Nonetheless, rigorous selection of HSC must be considered to minimize the chances of tumor contamination. Efficient isolation of Thy+ Lin- CD34+ cells by FACS from the peripheral blood of multiple myeloma patients reduced the tumor load in a proposed engrafting inoculum (2–3 \times 10^7 HSC/70 kg patient) to undetectable levels (Gazitt et al 1994).

The biological basis for the mobilization of CD34+ cells is still unknown. Presumably cells must de-adhere from the bone marrow stroma, enter the bone marrow venous sinuses, and then the blood stream. Most adhesion studies have focused on enriched progenitor fractions, not isolated HSC. As a result, the observed interactions between stroma and progenitor cells do not necessarily involve HSC. Nonetheless, a number of molecules have been implicated in the adhesive interactions between primitive hematopoietic progenitors and stroma. In both the human (Gunji et al 1992) and mouse (Morrison & Weissman 1994) systems, heterodimers of CD18 and CD11 (LFA-1 or Mac-1) have been observed on transiently proliferating early progenitors. Agonist antibodies against CD34 appeared to increase CD18-mediated adhesion in a cell line (Traore & Hirn 1994). Antibodies against LFA-1 decreased colony formation in stromal cell culture (Gunji et al 1992), but did not mobilize progenitor cells in monkeys (Papayannopoulou & Nakamoto 1993). Teixido et al (1992) found integrin α4β1 (VLA4), integrin α5β1 (VLA5), and β2 integrin expressed on CD34^hi human cells, and their cognate receptors VCAM-1, fibronectin, and ICAM-1 expressed on bone marrow stroma. Antibodies that blocked any of these interactions decreased the number of CD34^hi cells bound to the stroma (Teixido et al 1992). Thy-1\textsuperscript{lo}Lin\textsuperscript{-}Sca-1\textsuperscript{+} mouse HSC are integrin α4 positive, β7 negative, and are presumably β1 positive (X Liao et al, unpublished observation). Most interestingly, blocking antibodies against α 4β1 or VCAM-1 impaired hematopoiesis in culture (Miyake et al 1991), mobilized hematopoietic progenitors (Papayannopoulou & Nakamoto 1993, Nakamoto & Papayannopoulou 1994), and impeded the homing of infused progenitors to bone marrow (Zanjani & Papayannopoulou 1994, Papayannopoulou et al 1994). Antibodies against CD44 (Pgp-1) impair hematopoiesis in long-term bone marrow cultures (Miyake et al 1990), and CD44 appears to be expressed by mouse and human HSC (Spangrude & Scollay 1990, Reuss-Borst et al 1992). Other extracellular matrix molecules have also been implicated in hematopoietic progenitor adhesion including thrombospondin on stromal cells (Long & Dixit 1990) and chondroitin sulfate proteoglycans on progenitor cells (Conget & Minguell 1994). Despite the possibilities suggested by these studies, no experiment has yet directly addressed the mechanisms by which hematopoietic progenitors are released from the bone marrow during mobilization.
It is interesting that mobilization of stem cells is evolutionarily conserved in all species tested thus far, including mouse, dog, monkey, and human. The selective forces for the conservation of this phenomenon are not immediately apparent. Naturally occurring events could cause the production of cytokines known to cause HSC mobilization. For example, massive bleeding or certain types of infections may be common events in the natural history of populations that could lead to the production of mobilizing cytokines. The biological significance of mobilization could lie in the very low level of steady-state mobilization and homing of HSC, which probably occurs constantly in the dynamic equilibrium of hematopoiesis. The massive mobilization that can be obtained clinically or experimentally might represent an unphysiological potentiation of a phenomenon that is much more subtle as it occurs naturally.

It remains unclear what selective advantage would be conferred upon an individual by the mobilization of HSC. Mobilization of HSC followed by homing to bone marrow microenvironments might augment blood cell production. Alternatively, there may be stimuli for the activation of HSC that are critical for recovery from acute cytoreductive events, but which are only locally produced or active. If this were true, it would be necessary to mobilize locally activated hematopoietic progenitors in order to maximize hematopoietic output. HSC mobilization could also play an important role in the homogenization of hematopoietic progenitors in different bone marrow compartments. If there were no exchange of HSC between different bones, populations of hematopoietic progenitors in different bones could diverge. Indeed, the homing of mobilized HSC could represent a defense against neoplastic growth if normal progenitors were able to out-compete neoplastic progenitors for bone marrow niches. Such a mechanism would be analogous to that recently described for the exclusion of autoreactive B cells from germinal centers (Cyster et al 1994).

BARRIERS TO ALLOTRANSPLANTATION

HSC express high levels of class I major histocompatibility complex (MHC) molecules (Visser et al 1984). Their transplantation into allogeneic hosts that retain immune responsiveness results in their rapid rejection. Recipient T cells have been demonstrated to play a role in the rejection of donor bone marrow cells, which express non-self major (Dennert et al 1985, Murphy et al 1987b) or minor (Voogt et al 1990) histocompatibility antigens. However, allogeneic bone marrow injection into immunosuppressed (Vallera et al 1982) or lethally irradiated (Cudkowicz 1965, Cudkowicz & Bennett 1971a) mammals reveals a powerful transplantation barrier as well. The barrier to bone marrow transplants in lethally irradiated mice extends to a phenomenon called hybrid resistance, in which F1 mice reject inbred parental bone marrow grafts, but not non-hematolymphoid grafts such as skin (Cudkowicz & Bennett 1971b).
Hosts that are genetically incapable of producing normal T cells retain a significant transplantation barrier believed to be mediated by NK cells (Murphy et al 1987a,b). Isolated NK cells have been demonstrated to inhibit in vitro myeloid colony formation (Bellone et al 1993) and bone marrow engraftment in vivo (Murphy et al 1990). NK cells effect early rejection, while T cells require days to be primed to participate in the rejection reaction (Dennert et al 1985, Murphy et al 1987b).

The barriers that apply to the transplantation of whole bone marrow or T cell-depleted bone marrow also apply to the engraftment of purified HSC into lethally irradiated mice. The ability of HSC to engraft lethally irradiated hosts can be measured in terms of the PD95: the protective dose of HSC required to save 95–100% of mice after lethal irradiation. The PD95 of HSC given to Ly5 congenic mice is 100 cells. In mice that share MHC haplotypes but differ at minor histocompatibility loci, the PD95 is on the order of 1000 cells. Finally, in fully allogeneic mice (major and minor histocompatibility differences) PD95 is on the order of 3000–6000 cells (JA Shizuru et al, unpublished data). Fully allogeneic grafts of 1000 HSC can usually be accomplished if the irradiated hosts are treated with antibodies to NK determinants, but not if they are treated with anti-CD4 (T cell-associated) antibody (J Shizuru et al, unpublished data).

In contrast, non-obese diabetic (NOD) mice that develop autoimmune diabetes, a T cell-mediated disease, can be reconstituted with 1000 HSC only if the host is treated with antibodies that lead to the death or inactivation of T cells and NK antigen-positive cells.

The importance of NK cells in HSC rejection is suggested by several observations. NK cells are relatively radioresistant and represent up to 30% of the viable cells remaining in the bone marrow after lethal irradiation (H Aguila et al, unpublished data). In the absence of prior immunization, Dennert et al (1985) claimed that only NK cells participated in bone marrow graft rejection. Indeed, early phase rejection reactions have been observed to be inhibitable by anti-NK1.1 antibodies but not by anti-CD8 antibodies (Murphy et al 1987b). Antibodies specific for asialo GM1, an NK cell-associated marker, improved the engraftment of both syngeneic and allogeneic bone marrow (Tiberghien et al 1990). Despite the considerable evidence that inactivation of cells expressing NK cell markers enhances early engraftment, it should be noted that neither asialo-GM1 nor other NK cell markers are restricted to NK cells. These observations could also implicate other subsets such as T cells that express NK cell markers (Yankelevich et al 1989). It will be important to elucidate the receptors used by NK cells to recognize target cells and the means by which NK antigen-expressing cells inhibit hematopoietic engraftment. It must also be determined whether NK cells play a role in regulating endogenous hematopoiesis under steady-state conditions.
GENES AFFECTING THE FORMATION OR DIFFERENTIATION OF HSC

The immediate precursors of HSC are not yet known. Presumably a genetic program is activated during embryogenesis that causes the precursor cell to differentiate into HSC. Homozygous mutations to any essential genes in that pathway would result in total hematopoietic failure, as would the loss of genes necessary for HSC self-renewal. Thus far, most mutations that affect early hematopoiesis impair the generation of cells in particular combinations of lineages. Whether the wild-type genes direct HSC commitment to the affected lineages, or affect the survival of cells of the affected lineages, is not clear.

GATA-2 and Tal-1/SCL have functions critical to the formation or function of HSC. GATA-2 is one of a large family of zinc-finger proteins that binds to GATA transcriptional control elements, which are important to the regulation of gene expression in hematopoietic cells (Orkin 1992). GATA-2 knockout mice show profound but not complete defects in the development of lymphoid as well as myeloid cells (Tsai et al 1994). The GATA-2 defect appears to be autonomous to hematopoietic cells because GATA-2-/- ES cells are unable to contribute to hematopoietic tissues in wild-type chimeras, or rag-2/-/- mice (Tsai et al 1994). Deletion of the Tal-1/SCL gene, which encodes a helix-loop-helix protein, gives a similar result—embryonic lethality with a profound defect in the development of erythroid, and to a lesser extent, myeloid cells (Shivdasani et al 1995). Tal-1/SCL is thought to be a hematopoietic regulator, but its locus of action is unknown. The Tal-1-deficient mouse embryos have not been thoroughly tested for the presence of hematopoietic progenitors by transplantation.

Two of the first classes of allelic mutations that were found to affect hematopoiesis were the W mutations (dominant white spotting), and the SL (steel color) mutations (reviewed by Russell 1979). Mutations of either type impair erythropoiesis, mast cell production, and neural crest and germline formation, which result in fetal lethality in the severe cases. Expression of the W mutation in hematopoiesis is intrinsic to hematopoietic progenitors, whereas the SL mutation is associated with the stromal cells that support hematopoiesis. The W gene encodes c-kit, a receptor tyrosine kinase (Chabot et al 1988, Geissler et al 1988) that is normally expressed by HSC. The SL gene encodes the ligand for c-kit, steel factor (SLF), a membrane-bound or soluble growth factor (Williams et al 1990, Copeland et al 1990, Flanagan & Leder 1990, Zsebo et al 1990). SLF is not necessary for the maintenance or self-renewal of HSC (Ikuta & Weissman 1992, Wineman et al 1993), but it synergizes with other early-acting cytokines to promote the formation of all lineages of cells (reviewed by McNiece & Briddell 1995). Similar to the c-kit/SLF interaction is the recently identified interaction of the flk-2/flt-3 receptor (Rosnet et al
1991, Matthews et al 1991) with its ligand (FL) (Lyman et al 1993, Hannum et al 1994). HSC respond little to FL alone, but FL synergizes with several other growth factors to promote proliferation (Lyman et al 1993, Hannum et al 1994). Flk-2 receptor-deficient mice have been produced, but such mice exhibit only a relatively mild defect in B lymphopoiesis (I Lemischka, unpublished data).

Ikaros is a zinc-finger protein that appears to be necessary for lymphoid cell formation in mice. Deletion of the Ikaros gene leads to a defect in the appearance of T, B, and NK cells, as well as all committed progenitors of those lineages that have been examined (Georgopoulos et al 1994). The actual locus of action of this gene is not clear insofar as transplant studies have not defined whether the mutation is cell autonomous or expressed in stromal cells. Even if Ikaros is cell autonomous, it is not known if it is active in the HSC or in committed progenitors of the lymphoid lineages. Similarly, mutation of the ets family transcription factor PU.1 severely impairs the generation of myeloid, B, and T cells and leads to fetal lethality (Scott et al 1994). Again, the locus of action of this gene has not been confirmed.

Understanding of the biology of HSC at a molecular level is in its infancy. Although several genes crucial to early hematopoiesis have been identified, their functions and the pathways through which they act remain completely unknown. Doubtless, many more genes with critical functions in early hematopoiesis remain to be identified. Studying the molecular biology of HSC has been limited by the difficulty associated with obtaining sufficient cells for most techniques. The ability to prepare representative cDNA libraries from a limited number of cells would circumvent this limitation (Brady et al 1990). The recent description of an immortalized cell line that recapitulates at least some properties of HSC might also greatly facilitate molecular biology (Wong et al 1994). The BL3 cell line was prepared by infecting mouse fetal liver cells with a retrovirus, followed by selecting immortalized bone marrow progenitors from a mouse reconstituted by retrovirally infected cells. This cell line may or may not prove to be functionally equivalent to HSC. Nonetheless, gene expression in immortalized cell lines must be interpreted with caution because the regulation of self-renewal, and perhaps differentiation, are no longer physiological.

FUTURE DIRECTIONS

Stem Cell Biology

The fundamental question of how stem cell differentiation and self-renewal are regulated pertains to stem cells in all tissues. What is the genetic program that leads to the alternative developmental choices of renewal vs differentiation, and what are the factors that induce or repress elements necessary for
that program to be read-out? With HSC, the approaches to this problem seem
to be within reach. Multipotent progenitors with long-term self-renewal po­
tential can now be separated from those with transient or no self-renewal
potential (Morrison & Weissman 1994). Unfortunately, the factors required
for the long-term maintenance or self-renewal of HSC have not yet been found.
It is reasonable to assume that soon they will be and open a door to under­
standing the molecular genetics of self-renewal and differentiation. The isola­
tion of self-renewal factors would also facilitate other scientific and clinical
objectives including the genetic manipulation and ex vivo expansion of HSC.

The study of stem cell models that are more amenable to genetic manipu­
lation than mammals may play an important role in the elucidation of the
environmental and intrinsic factors that regulate self-renewal and differentia­
tion. For example, the distal tip cell in C. elegans (Lambie & Kimble 1991),
and the germ cells in Drosophila (Fuller 1993) offer systems in which genetic
screens can be performed to identify the genes that regulate self-renewal and
differentiation. The relative simplicity of these systems might facilitate efforts
to understand the roles of the gene products. The beauty of these systems is
that, despite their simplicity, they recapitulate many of the phenomena asso­
ciated with mammalian stem cells including self-renewal, differentiation, in­
teraction with support cells, and migration. These systems could be studied in
concert with mice, in which knock-outs of homologous genes could be per­
formed.

Transplantation

Gene targeting could lead to the preparation of a universal donor MHC− cell
line; subsequently, it might be possible to genetically "customize" the universal
donor for individual recipients by causing it to express genes for host-specific
MHC complex antigens. Transplantation of antigenically modified HSC might
also offer a means to induce tolerance to histocompatibility antigens expressed
on a second organ to be engrafted (LeGuern et al 1993, Sykes et al 1993). It
is even conceivable that engineered HSC could tolerize recipients to receive
xenogeneic organ transplants, if such an approach were found to be ethical
and safe.

Gene Therapy

General progress in gene therapy (Morgan & Anderson 1993), as well as
prospects for gene therapies involving the immune and hematopoietic systems
(Cournoyer & Caskey 1993), have been recently reviewed. As our understand­
ing of the biology of hematopoietic progenitors improves, it will be possible
to increase the precision with which cells are targeted and the sophistication
with which therapies are designed. For the most part, the implications of the
heterogeneity among early progenitors for gene therapy have not been consid­
ered. As the ability to purify functionally homogeneous populations of progenitors improves, it will become possible to customize the choice of progenitors for genetic modification based on the specifics of individual therapies. That is, there may be advantages in some cases to genetically modifying only transient multipotent progenitors or particular lineage-committed progenitors. We call this approach differentiation state-specific gene therapy (Morrison & Weissman 1995). This contrasts with the current approach of always targeting long-term reconstituting HSC or a mixture of long-term and transient HSC.

Gene therapy is not limited to the replacement of diseased genes with healthy genes. Gene therapy can be extended to the expression of intracellular molecules, cytokines, and receptors that will benefit the host when expressed in any hematopoietic cell. Imparting drug- or radiation-resistance to hematopoietic cells might help patients tolerate cancer therapy (Corey et al 1990). The increased production of cytokines (Kuhr et al 1994) or the frequent expression of particular antigen receptors by immune system cells might increase the tumor-killing potential of the immune system. Similarly, the expression of other molecules could be increased (e.g. receptors specific to pathogen epitopes) or decreased (e.g. viral receptors) to increase resistance to pathogens. Blood cells could be engineered to produce cytokines such as erythropoietin in renal failure patients. Currently, our technical ability to pursue such approaches is limited by the inefficient infection of human HSC by retroviruses and by the inability to precisely control the expression of transgenes in vivo. As more effective transduction methods become available, and our understanding of how to predict and control transgene activity in vivo improves, the horizons for gene therapy will be dramatically expanded.

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