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Embryonic Stem Cells and In Vitro Hematopoiesis

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Abstract To study hematopoietic differentiation a variety of in vitro systems have been established using hematopoietic precursors derived from various explanted adult and fetal tissues. In this prospective we describe and discuss the potential of a novel system for studying the earliest stages of hematopoietic development. In addition, some of the applications of this system as a unique in vitro model for studying other developmental systems are discussed. Murine embryonic stem cells (ESC), which are totipotent and can be maintained undifferentiated indefinitely *in vitro*, have the capacity to differentiate in vitro into hematopoietic precursors of most, if not all, of the colony forming cells found in normal bone marrow. This potential can be exploited to study the control of the early stages of hematopoietic induction and differentiation. Recent results have indicated that there is a strong transcriptional activation, in a well defined temporal order, of many of the hematopoietically relevant genes. Examples of the genes expressed early during the induction of hematopoiesis include erythropoietin (Epo) and its receptor as well as the Steel (Sl) factor (SLF) and its receptor (c-kit). Several other genes, including CSF-1, IL-1, and G-CSF were expressed during the later stages of hematopoietic differentiation. Contrasting with these observations, IL-3 and GM-CSF were not expressed during the first 24 days of ES cell differentiation suggesting that neither factor is necessary for the induction of hematopoietic precursors. Although these studies are just beginning, this system is easily manipulated and gives us an approach to understanding the control of the induction and differentiation of the hematopoietic system in ways not previously possible. © 1992 Wiley-Liss, Inc.

Key words: embryonic stem cells, hematopoiesis, development, stem cells

Embryonic stem cells (ESC) are pluripotent cell lines that are isolated from blastocysts and maintained in an undifferentiated state by the presence of leukemia inhibitory factor (LIF) [1–5]. These cells are truly pluripotent since they will participate in the normal development of all tissues of an animal if implanted back in a developing blastocyst [6,7]. It was noted several years ago that if ESC were cultured without LIF they would begin to spontaneously differentiate. This differentiation was characterized by the aggregation of the cells and the production of fluid filled cystic structures composed of many different cell and tissue types [8]. One of the striking findings was that under appropriate conditions the differentiating ESC would produce structures similar to yolk sac blood islands that contained immature erythrocytes [8]. This suggested that this was an in vitro system in which one could investigate the earliest stages of hematopoietic development. We will discuss recent studies using this new approach, some of the current problems, and will try to highlight some of the applications of this model system.

THE HEMATOPOIETIC SYSTEM

All cells within the hematopoietic system are derived from a common pluripotent hematopoietic stem cell (HSC) [9–11]. In our terminology, HSC is synonymous with a cell that can differentiate into all members of the hematopoietic system, has the ability to self-renew, and—most importantly—is capable of long-term in vivo repopulation of all lineages of the hematopoietic system. The HSC has a pivotal role in the hematopoietic system, and significant effort has been devoted to its characterization. Its surface phenotype and physical properties, although still controversial [12–15], have received significant attention. Little is known about the growth factor requirements or the immediate non-hematopoietic precursor of the HSC. This is due, in part, to the fact that this cell is present at a frequency of only approximately 10^{-5} in normal bone marrow [16], and true HSCs can only be reliably assayed by long-term in vivo repopulation. Several laboratories have studied the ex-

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pression of various markers on bone marrow cells to characterize HSCs [12,17,18]. The data suggest that a primitive hematopoietic precursor expresses low levels of Thy-1 [19–21] and significant levels of Ly-6 (Sca-1) [12], and lacks other markers associated with mature myeloid or lymphoid cells [22]. As indicated above, the HSC content and the purity of cell populations prepared by these methods has been debated. The monoclonal antibody AA4.1 purportedly defines a very small subpopulation of fetal liver cells that contains all the hematopoietic progenitors including the cell with long-term reconstituting potential [23]. Whether this antibody will be useful, alone or in combination with antibodies to the surface markers described above, for preparing HSCs from adult bone marrow is not known. Physical parameters also have been used to characterize HSCs [13,14,24]. Recently the life span and temporal clonal activity of hematopoietic stem cells have been addressed by several labs. These experiments suggest that besides the long-term expression of a few stem cell derived clones, dramatic fluctuations in the clonal makeup of the hematopoietic system occur following reconstitution [16,25,26]. It was also demonstrated that the HSC could clonally expand and still maintain its stem cell potential [27].

Hematopoietic development is controlled by a large number of cytokines [reviewed in 28,29]. Examples of such cytokines are erythropoietin (Epo), granulocyte-colony stimulating factor (G-CSF), macrophage-CSF (M-CSF or CSF-1), granulocyte/macrophage-CSF (GM-CSF), interleukin-1 (IL-1), IL-3, IL-4, and IL-6. These factors range from being lineage specific, such as Epo and CSF-1, to acting on multiple lineages, such as IL-3 and GM-CSF. There is significant synergy between various growth factors and many of them have overlapping activities [30,31]. Recently a new growth factor was identified which acts on both very immature and mature hematopoietic cells. This protein, which has been referred to as mast cell growth factor [32] or stem cell factor [33], is a product of the *Steel* (*Sl*) gene and we will refer to it as the Steel factor (SLF). The appropriate expression of the SLF is required for the production of normal numbers of HSCs [34], and it has been shown that SLF can act in synergy with other hematopoietic factors to expand the growth of a variety of colony forming precursors [32,35,36]. In contrast to the large body of information about the effects of cytokines on the hematopoietic precursors of in vitro colony forming cells, less information exists on their effects upon the pluripotent hematopoietic stem cell, and almost no information exists about their role during the induction and development of the embryonic hematopoietic system.

IN VITRO HEMATOPOIETIC DIFFERENTIATION OF ES CELLS

Culturing ES cells without differentiation inhibitory factors or feeder cells induces spontaneous differentiation that results in the formation of fluid filled cystic embryoid bodies with endodermal, mesodermal, and ectodermal layers [8]. While cells within these embryoid bodies can differentiate into a variety of tissues including muscle, hematopoietic, and neuronal cells, the regulation of this process remains poorly understood.

Several laboratories have taken advantage of this model system to study the early development of hematopoietic cells [37–39]. The two methods used to promote in vitro differentiation of ESC are a liquid culture system [38], and a methylcellulose differentiation system [37,39]. Both systems lead to similar observations, but there are some differences. In our hands we have not found an ES cell line that did not differentiate into significant numbers of hematopoietic cells in liquid culture, whereas this is not the case with the methylcellulose system [Keller, personal communication]. On the other hand the methylcellulose assay may be a more synchronous differentiation system, perhaps due to the more uniform colony size that develops. This may be related to the fact that the induction of hematopoietic development in this system requires intercellular inductive events. This conclusion is suggested by two observations. In our normal cultures, essentially all the hematopoietic development is associated with the large fluid filled cysts [38] and if the cellular aggregation is disrupted to inhibit embryoid body formation, or if large aberrant aggregation products are formed, little if any hematopoietic development ensues.

Clearly the optimal generation of hematopoietic cells is dependent upon the presence of exogenously supplied factors. Human cord serum has a strong promoting activity for hematopoietic development in ESC cultures that is present in relatively few batches of fetal calf serum (FCS) [8,38, and unpublished observa-

tions]. Of the few batches of FCS that we examined, the lack of hematopoietic promoting ability was not due to inhibitory factors as determined by mixing experiments [unpublished observations]. The hematopoietic development of ES cells is also sensitive to exogenously added factors such as IL-3 and Epo (see below).

All three of the above studies documented that essentially all in vitro myeloid colony forming cells are produced during the differentiation of ESC. This includes single lineage colonies of erythrocytes, neutrophils, mast cells, and macrophages, as wells as bi- (CFC-granulocyte/macrophage) and multipotent (CFC-MIXED) precursors [37–39]. The production of nucleated RBCs and fetal hemoglobin (see below), as well as the frequency of the various colony types, suggests that the hematopoietic development in these cultures is more similar to fetal hematopoiesis than adult [38]. ES cells give rise to a higher percentage of mixed and erythroid (BFU-E) colonies, but fewer pure monocytic and pure granulocytic colonies than normal bone marrow. Using the expression of CD45 as an indicator, as many as 2% of the total cells in liquid differentiation cultures are hematopoietic. Based on the frequency of hematopoietic cells, differentiating ESC produce 35 times more primitive precursors of mixed and erythroid colonies than normal bone marrow, approximately equal numbers of precursors for dual lineage CFC-GM, and nearly eightfold fewer precursors for single lineage myeloid CFC [38]. All the published data so far have documented the production of myeloid cells during ESC differentiation. An area of active interest is whether these cultures produce lymphoid precursors. Several groups are studying the growth and differentiation of ESC on established stromal layers, which are critical for promoting the growth and development of lymphoid precursors [40–42], but a consistent conclusion has not yet emerged.

In all of these reports, the precursors that give rise to in vivo spleen colonies and the precursors that are capable of in vivo repopulation are conspicuously missing. Several groups, including ours, have tried such experiments and have not been able to reproducibly demonstrate any form of in vivo repopulation by differentiated ESC. To avoid this difficulty, Forrester et al. [43] went so far as to use the ESC to produce chimeric embryos and to use the fetal liver cells to reconstitute irradiated adult animals. Although this procedure is a technique that allows ESC-derived cells to repopulate adult animals, it is laborious and time consuming and it avoids the issue of whether cells with repopulating ability develop in ESC differentiation cultures. This inability to repopulate in vivo is a particularly vexing problem since it is known that precursors of CFC-MIXED are able to form spleen colonies [44], and such precursors are clearly produced in these cultures. There are several hypotheses that can be proposed to explain this failure. For example, the culture system may have such strong signals inducing differentiation that the developing precursors are "*pushed*" rapidly through a stage in which they can produce spleen colonies or repopulate the hematopoietic system; at this stage the cells with the ability to repopulate may be sensitive to the procedure used to prepare single cell suspensions; there may be problems with the homing of the cultured cells; or it may simply be a quantitative problem of not having enough cells in the cultures with this potential. At present there are no data to address this issue.

GENE EXPRESSION DURING ES CELL DIFFERENTIATION

To understand the molecular control of early hematopoietic development, we sought to correlate the expression of genes known to be important in hematopoiesis with the development of hematopoietic precursors during ESC differentiation [38]. The genes for erythropoietin and its receptor are expressed very early after the onset of differentiation before any detectable erythroid precursors appear. As discussed below, this early expression of Epo and its receptor may be critical to the initiation of hematopoietic development. Besides Epo, erythropoiesis requires the expression of the developmentally regulated fetal and adult β-globin genes [34]. During ESC differentiation fetal β-globin is the predominant form expressed during the early stages, whereas in later stages adult globin increases [37,38,45]. The transcription of both globin genes increases dramatically concurrent with the steep rise in hematopoietic precursor development observed during ESC differentiation. In methylcellulose cultures ESC derived mature erythroid colonies, which develop under the influence of IL-3 and Epo, express almost exclusively the adult globin form [38]. These changes in globin expression demonstrate that the regulation of β-globin tran-

scription in this in vitro system is similar to that observed developmentally in vivo.

As indicated above, IL-3 and, to a lesser extent, GM-CSF have been shown to act on the most primitive of the CFCs [46]. IL-3 has even been implicated as being a growth factor for cells that can repopulate in vivo [22,24,47]. Interestingly, our recent data strongly suggest that neither are required for the development of ESC-derived hematopoietic precursors [38]. This is indicated by the observation that IL-3 and GM-CSF genes are transcriptionally silent throughout the differentiation of the ESC, there are no exogenous murine factors in these cultures, and IL-3/GM-CSF are species specific [48,49]. Even though IL-3 is not required for the generation of the precursors, the IL-3 receptor is expressed relatively early during the differentiation of the hematopoietic cells [38]. That this is a functional receptor is verified by the observation that exogenous IL-3 greatly facilitates the expansion and further differentiation of the hematopoietic cells. The combination of IL-3 and Epo promotes a similar, or higher, number of colony forming cells as most batches of human cord serum [unpublished observations]. SLF and its receptor (c-kit) have been reported to have IL-3–like activities on hematopoietic CFU [32,50], and it is possible that they may have a similar function during early hematopoietic development. The expression of c-kit and SLF throughout ESC differentiation and during embryonic development [38,51,52] are all consistent with this hypothesis. That the development of hematopoietic precursors can occur without IL-3 does not imply that IL-3 has no role in the natural development of the fetal hematopoietic system. Small amounts of locally produced maternal IL-3 might enhance the expansion and differentiation of the developing hematopoietic cells.

After analyzing the expression of a number of cytokines and growth factors [38], we can organize them, somewhat arbitrarily, into three groups depending on whether their expression comes before, during, or after the major onset of hematopoietic development. Those genes that are expressed before the onset of hematopoiesis include SLF, c-kit, Epo, Epo receptor, IL-4 receptor, β-globin (fetal), and CSF-1. Those genes that are expressed during the major onset of hematopoiesis include IL-4 and 6, β-globin (adult), and the receptors for IL-3 and CSF-1. Those genes that are expressed after the major onset of hematopoiesis include the IL-1 and G-CSF receptors, CD-45, IL-1α and β, and G-CSF. Although no correlation was made to hematopoietic development, similar experiments suggested that during ESC differentiation the transcription of TGF-β_3, PDGF-B, and IGF-II undergo substantial activation [53].

Although our studies on the role that cytokines and growth factors have during the hematopoietic development of differentiating ESC are only beginning, several points can be made. IL-6, Epo, and the Epo receptor genes show a very early onset of expression before hematopoietic development. Besides the critical roles that Epo and IL-6 have in hematopoietic differentiation, recent data suggest that Epo also stimulates endothelial development [54] and IL-6 may be involved in angiogenesis [55]. This is directly relevant to the system described here because the blood islands which form in the developing cysts are encased in endothelial structures [8,56]. It may be that one of the early functions of Epo and IL-6 is to stimulate the development and activation of endothelial cells that are known to produce many cytokines [28]. IL-6 enhances murine colony formation [57], and IL-6 in combination with IL-4 stimulates colony formation of various hematopoietic progenitor cells [31]. Thus it is conceivable that the combination of IL-4 and IL-6 has important synergistic effects on very primitive hematopoietic cells as well as having crucial roles, independent of hematopoiesis, early in fetal development [58]. More work is necessary to identify the critical factors in this process.

SOME OF THE POTENTIALS OF THIS EXPERIMENTAL SYSTEM

Besides the studies presented above, this system can be productively used to investigate the early stages of vasculogenesis and angiogenesis [56,59], myogenesis [60], neuronal differentiation [8,61], genetic imprinting [62,63], as well as the control of gene regulation [45]. It is also possible that the in vitro differentiation system described herein would be very useful for studying mutations that are lethal in vivo [64,65]. As an adjunct to the widely publicized use of ES cells as a vehicle for introducing null mutations into the germ line [reviewed in 66], this system offers in vitro experimental access to stages of differentiation that were not previously accessible. In terms of hematopoietic development we are able to study in vitro what growth factors

are involved with the differentiation of non-hematopoietic precursors into hematopoietic cells. This system would seem to be ideal for testing novel growth factors or for looking for novel stage specific products either biochemically or by using cDNA subtraction library techniques at various times during the differentiation. This system could become a powerful tool for evaluating the role that various gene products have on this early stage of development by employing transfections with the appropriate expression constructs, or by gene knock out experiments. With this latter approach, those knock outs resulting in lethal phenotypes [e.g., 65] could be productively studied using the in vitro differentiation of ES cells. One of the theoretical potentials, which has yet to be achieved, is to use this system as a source of hematopoietic cells for in vivo repopulation. If this experimental hurdle can be overcome, this would open the door for new experiments and approaches that would have significant clinical interest, especially if self-renewing precursors for muscle cells, fibroblasts, or hematopoietic cells could be demonstrated. If this could be achieved, this would be a system in which easily made genetic modifications could be made in the ESC and transferred in vivo.

In summary, the in vitro differentiation of embryonic stem cells is a system that offers new approaches to the study of early development in a variety of systems. In the coming years we should see a more refined exploitation of this potential in many areas.

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