Hematopoietic Growth Factor Receptor Genes as Markers of Lineage Commitment During In Vitro Development of Hematopoietic Cells

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We have used two in vitro models to identify genes whose expression may serve as markers of lineage commitment during the development of hematopoietic stem cells. One system involves the in vitro development of blastocyst-derived embryonic stem cells into embryoid bodies. The second involves culturing of day 3.5 blastocysts in vitro under conditions that support their development into yolk sac-like cysts. In both cases, hematopoietic cells arise in a manner that closely mimics the normal process occurring in the yolk sac of the early mouse embryo. We have focused our analysis on the expression of mRNAs for 15 hematopoietic growth factor receptor genes and other genes expressed in a hematopoietic lineage-specific manner. Although some growth factor receptor genes are apparently expressed constitutively during in vitro development, there are several classes of genes that undergo a highly consistent pattern of induction in both model systems. Genes induced early include those encoding the shared β subunits of the interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors; those induced at intermediate times include the c-fms, G-CSF receptor, and CD34 genes; and a gene induced late during in vitro development is the IL-7 receptor gene. The defined temporal order for the expression of these genes suggests that they may be useful as markers for multiple stages in the development of different hematopoietic cell lineages during embryogenesis.

The origin of hematopoietic stem cells during embryogenesis and the steps leading to cell fate determination in this lineage are unknown. The initial development of hematopoietic lineage cells during mouse embryogenesis occurs in the yolk sac of the late primitive streak, day 7.5 embryo where blood islands develop in the mesenchyme of the visceral yolk sac. These blood islands contain primitive erythrocytes expressing embryonic globin. The network of blood islands increases with time, and it is postulated that by embryonic day 9 or 10, when circulation begins in the embryo, hematopoietic stem cells that arose in the yolk sac migrate to the embryo and colonize the fetal liver, where a second wave of hematopoietic development ensues. Although the magnitude and complexity of liver hematopoiesis differs from yolk sac hematopoiesis, the latter consisting primarily of erythroid development, it is thought that the stem cells of the yolk sac are fully functional to give rise to all hematopoietic lineages, both myeloid and lymphoid.

As a means to understand the earliest steps of hematopoietic cell lineage commitment, we have used two different in vitro models of early hematopoietic development. One involves the in vitro development of blastocyst-derived embryonic stem cells into embryoid bodies, and the second in vitro culture of day 3.5 blastocysts themselves. Both of these in vitro models offer the potential for analyzing early steps in hematopoietic lineage commitment using cells that are derived from readily available sources. These systems are easier to manipulate and analyze than the technologically challenging stages of early implantation embryos, where the initial events of hematopoiesis take place in vivo.

Embryonic stem cells, derived from the inner cell mass of day 3.5 blastocysts, can undergo a spontaneous program of differentiation in vitro that mimics early stages of embryonic development in vivo. These events include formation of organized cystic embryoid body structures containing primitive endodermal and ectodermal layers, myocardial muscle, and neuronal-like cells, and formation of large fluid-filled cysts that resemble the yolk sac of the egg cylinder stage, postimplantation embryo. These in vitro-derived embryoid bodies can form hematopoietic cells, initially evidenced by the presence of blood islands, and, indeed, are capable of expressing early hematopoietic factor-responsive precursor cells from all myeloid lineages. Additionally, developing embryoid bodies undergo defined patterns of globin and growth factor and receptor gene expression, and may contain lymphoid progenitors. Thus, the earliest molecular and cellular events in lineage commitment can be analyzed in vitro beginning with totipotent embryonic cells.

The in vitro culture of day 3.5 blastocysts under defined culture conditions, described by Hsu, also results in the formation of hematopoietic cells and morphologic patterns that mimic early events of embryogenesis. Under the appropriate conditions, blastocysts cultured in vitro develop normally to early somite stage embryos, each encased in a yolk sac cyst, where early hematopoiesis occurs. Additionally, the cells from blastocysts cultured 3 to 4 days in vitro have been shown to rescue lethally irradiated adult recipients. Thus, this system provides another in vitro model to study the earliest steps of hematopoietic stem cell formation during embryogenesis.

We have analyzed both developing embryoid bodies and in vitro-cultured blastocysts for their patterns of expression of 15 hematopoietic growth factor receptor genes, as well as a set of genes expressed in a hematopoietic lineage-specific manner, to define temporal changes in gene expression during lineage commitment of hematopoietic stem cells. Our results indicate that there is a consistent temporal pattern of expression of certain genes in both in vitro systems that may serve as molecular markers of lineage commitment in developing hematopoietic cells.

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MATERIALS AND METHODS

Embryonic Stem (ES) Cell Culture

ES cell line CCE was routinely maintained on gelatin-coated tissue culture dishes in ES media (80% Dulbecco’s Modified Eagle’s Media [DMEM] + 20% fetal calf serum [FCS] [Gemini Bioproducts, Calabasas, CA], supplemented with 4 mmol/L L-glutamine [JRH Biosciences, Lenexa, KS], 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mmol/L nonessential amino acids [GIBCO, Grand Island, NY], 0.1 mmol/L 2-mercaptoethanol [2-ME] [Sigma, St Louis, MO] plus 0.1% (vol/vol) conditioned medium from cos-7 cells transiently transfected with pSRaLIF human leukemia inhibitory factor (LIF) cDNA. Medium was changed on the second day after passage, and cells were trypsinized to completion as usual for routine passage, centrifuged for 5 minutes at 1,000 rpm, and resuspended in ES medium. Cells dispersed by trypsinization and pipetting were diluted into medium, centrifuged, and resuspended in fresh medium at 2 X 10⁶ cells per 60-mm dish.

Embryoid Body Preparation

The method we used was essentially as described, where ES cells were trypsinized to completion as usual for routine passage, centrifuged for 5 minutes at 1,000 rpm, and resuspended in ES medium (as above) without LIF at 1 to 1.5 X 10⁵ cells/mL in 10 mL in 10-cm bacterial Petri dishes (Baxter, McGaw Park, IL), which generated up to 300 or 400 embryoid bodies per dish. The embryoid bodies were maintained in a humidified 5% CO₂ atmosphere at 37°C for up to 25 days; these cultures were fed every 3 days by allowing embryoid bodies to settle in a tube, replacing medium, and gently pipetting with a wide-bore pipet into fresh Petri dishes.

Blastocyst Harvesting and Culturing Conditions

Blastocysts were flushed from the uterine horns of day 3.5 postcoital ICR mice (Harlan Sprague Dawley) as described, plated individually per well into 24-well cluster plates containing CMRL 1066 medium (GIBCO) supplemented with 10% FCS, 1 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate. After 2 days in culture, when the blastocysts had attached to the plastic, the media was changed daily, with FCS increased to 20%. No antibiotics were added to the medium. Blastocysts that had not attached by day 9 were discarded before the third day of culture. These 24-well cluster plates contained 500,000 blastocysts per well.

RNA Isolation

RNA was isolated from embryoid bodies by either of two methods. In the first method, embryoid bodies were collected by settling, washed once with PBS, then resuspended directly into 4 mol/L guanidine thiocyanate/25 mmol/L sodium citrate pH 7/0.5% sodium lauryl sarcosine/20 mmol/L 2-ME, and sheared by passage through an 18-gauge needle. This sheared lysate was layered onto an equal volume of 5.7 mol/L cesium chloride/10 mmol/L EDTA, and the RNA was pelleted by ultracentrifugation. In the second method, embryoid bodies were collected and washed as above, then lysed in RNAzol (Tel-Test, Inc, Friendswood, TX), and RNA was isolated as described. RNA was resuspended in H₂O and the concentration was determined by absorbance at 260 nm. Cultured and freshly isolated blastocysts were lysed in RNAzol and RNA was isolated as described. Twenty micrograms of glycogen carrier was added to each sample. Because the amount of RNA obtained from cultured blastocysts was too low to quantify by absorbance measurements, an alternative method was used to normalize for the amount of RNA from each time point. We determined the average number of cells per cultured embryo at various times of in vitro culture by harvesting blastocysts in pools of 2 to 10, disrupting them into single cells with 5X trypsin, and counting. The cell counts of various stages of cultured blastocysts were reproducible in four independent trials. The cell number corresponding to one half the number of cells in a day 3 cultured blastocyst was used to normalize other samples for polymerase chain reaction (PCR) analysis; the average number was 560 cells.

Reverse Transcription and PCR

Total RNA (10 µg) was reverse transcribed in 100 µL vol in 50 mmol/L tris-Cl pH 8/10 mmol/L MgCl₂/50 mmol/L KC₁/1 mmol/L dithiotreitol (DTT)/10 µg/mL bovine serum albumin (BSA), 1 mmol/L dNTPs, 1 mmol/L spermidine, 1 U/mL RNasin, 10 pmol/µL random primers (Boehringer Mannheim, Indianapolis, IN), and 20 U AMV reverse transcriptase (Boehringer Mannheim) for 1 hour at 42°C. For ES culture-derived RNA, PCRs were performed using an amount of cDNA reaction equivalent to 500 ng RNA; in some cases, a lower amount was used to achieve a linear range of signal for a particular gene (see figure legends). Primers were chosen to flank intron sequences, to ensure a different size product from contaminating genomic DNA; for growth factor receptor genes, primers were chosen to span the transmembrane domain (see Table 1 for primer sequences). PCR reactions were performed under standard Perkin Elmer-Cetus (Norwalk, CT) PCR conditions (10 mmol/L tris-Cl pH 8.3, 50 mmol/L KC₁, 1.5 mmol/L MgCl₂, 0.001% gelatin, 0.2 mmol/L dNTPs, 0.025 U AmpliTaq DNA polymerase), plus 60 pmol of each primer, for 30 cycles (94°C 30 seconds, 55°C 30 seconds, 72°C 1 minute), followed by 10 minutes at 72°C in a Perkin-Elmer Cetus thermocycler. A portion (25% to 100%) of each PCR product was electrophoresed on 1.5% agarose gels in 1 X TBE, stained with ethidium bromide, photographed, and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) by standard methods. Identity of the PCR products was confirmed by size and hybridization with an internal primer. Routine controls performed in each experiment included a cDNA reaction mix with no reverse transcriptase added (to check for genomic DNA contamination) and a PCR control with no template added (to control for PCR artifacts caused by contamination). Probes were internal oligonucleotides, labeled at the 5' end by 3²P and poly nucleotide kinase. Blots were hybridized in 6× SSC, 5× Denhardt’s solution, 0.1% sodium dodecyl sulfate (SDS), and 100 µg/mL RNA at 42°C for 24 hours, then washed in 6× SSC, 0.1% SDS at 50°C for 1 hour with one change of buffer. Blots were exposed to Kodak O-Mat film (Eastman Kodak, Rochester, NY) at -70°C with intensifying screens.

RESULTS

Establishment and Characterization of Differentiating Embryoid Bodies

In our initial characterization of the development of embryoid bodies from embryonic stem cells, we studied differentiation from two independently derived cell lines, CCE and D3. We found no major differences in the capacity of these two cell lines to form cystic embryoid bodies and blood islands and chose CCE for the majority of our studies. We routinely maintained CCE cells in LIF without feeder cells; we found that the ES cells retained their undifferentiated morphology and expression of the embryonic antigen SSEA under these conditions, just as they do on feeder layers (data not shown). By excluding feeder layers from our culture system, we were able to eliminate the contribution of non-ES cells to the analysis of gene expression patterns.

Although several methods have been described to initiate the formation of embryoid bodies, we used a simple method that involves the seeding of single, fully trypsinized ES cells...
into suspension culture at low density (1 × 10^5 cells/mL).\(^{14}\) Under these conditions, single ES cells expand into small clusters of cells that begin to differentiate within 1 to 2 days, forming a well-defined outer endodermal layer (Fig 1A). Approximately half of these simple embryoid bodies continue to differentiate and form large fluid-filled cysts with an inner ectodermal layer by about 7 to 10 days of suspension culture, as described\(^{26}\) (Fig 1B). Blood islands form in about half of the cystic embryoid bodies. Detection of visibly red cells was most easily achieved by placing cystic embryoid bodies in “organ culture”\(^{17}\) or placing cystic embryoid bodies in individual wells of a 24-well plate (Fig 1D). Embryoid bodies also develop and form hematopoietic cells in methylcellulose.\(^{44,45}\) As reported by others, we observed the outgrowth of nucleated erythroid cells, motile macrophages, and granulocytes in response to hematopoietic growth factors (Fig 2, A through C).

Embryoid bodies derived by the suspension culture method are clonally derived from single ES cells, form synchronously, and are quite uniform in size. We chose this method for derivation of embryoid body cultures for the present RNA analysis, due in part to the ease of generating large cultures of embryoid bodies at specific times of in vitro differentiation, and the reproducibility of the subsequent differentiation.

**Morphologic Characterization of In Vitro Differentiating Blastocysts**

Following a procedure developed by Chen and Hsu,\(^{18}\) we were able to reproducibly differentiate blastocysts into yolk sac cysts in vitro. Approximately 50% of the blastocysts we plated ultimately developed into yolk sac cysts containing blood islands on day 8 to 9 in culture. The developmental stage of the cultured blastocysts was evaluated visually, and RNA was harvested on days 0, 3, 5, 7, and 9 of culture from blastocysts judged to be comparable in maturity. Figure 3, A through E, show the progression of yolk sac cyst development in cultured blastocysts. By day 3 in culture, the blastocysts had attached to the dish, and the inner cell mass was covered by endoderm. After 5 days in culture, blastocysts apparently developed a proamniotic cavity and ectoplacental...
cone. These embryos appeared visually to be identical to those described previously,11 and were reasonably representative of their in vivo counterparts. By day 7 in culture, yolk sac cysts were visible and had a meshlike appearance similar to in vivo yolk sacs in meshlike appearance. We saw no evidence of an embryo proper after 7 days of culture in 20% FCS. After 9 days in culture, the cysts nearly tripled in size and contained visible blood islands (Fig 3E). All blastocysts that were cystic on day 7 developed blood islands by day 9. Anal-
GROWTH FACTOR RECEPTOR GENE EXPRESSION

Fig 4. Expression of lineage-specific genes during in vitro development. (A) Time course of embryoid body development. d3 to d22 correspond to days of in vitro development after initiation of embryoid body culture. (−) RT lane is the PCR product where a mock cDNA was added (no RT added in cDNA synthesis reaction). Input cDNA used was 500 ng (RNA equivalent), except GATA-1, where 100 ng was used. (B) Time course of cultured blastocyst development. blast. lane is RNA from freshly isolated day 3.5 blastocysts. d3 to d9 correspond to days of in vitro culture. Levels of input cDNA were normalized as described in Materials and Methods. The product from a mock PCR reaction (no cDNA added) is shown (H2O, A; (−), B). (+) lane is a positive control for each gene as follows: for c-fms, P388; for G-CSF-R, NF540; for GATA-1, MEL; for epoR, MEL; for IL-7R, clone K. The levels of signal for each gene cannot be compared with the levels of signal for another gene.

ysis of cells derived from yolk sac cysts after cyto-centrifugation and staining with Wright's-Giemsa stain confirmed the presence of macrophages and nucleated erythrocytes (Fig 3F). Cultured blastocysts were harvested for RNA preparation according to the precise visual staging described above. We observed consistent patterns of development from experiment to experiment.

Growth Factor Receptor Gene Expression in Developing Embryoid Bodies and Cultured Blastocysts

To assess the patterns of gene expression during in vitro development of ES-derived embryoid bodies or cultured blastocysts, three independent sets of RNA were generated from time courses of each culture system. We used PCR to analyze levels of gene expression because it allowed us to analyze individual RNA samples in which gene expression levels might be too low to detect by other methods, and because we could look at genes for which we did not possess cloned DNA. However, the PCR technique does not allow for precise quantitative comparisons between RNA samples. To address this shortcoming of the PCR technique, we repeated the analysis of expression of each gene on three independent sets of RNA to look at the overall trend of a given gene's expression patterns; we found that the patterns shown here were remarkably reproducible within each independent time course. In some cases we confirmed these expression patterns by S1 nuclease mapping and the overall temporal pattern of gene expression closely matched that shown by PCR analysis (data not shown). In addition, the amount of input cDNA in the PCR reactions was titrated to achieve a linear range signal. Cultured blastocyst cDNA was normalized to cell number as described in Materials and Methods. Although results using normalized cDNA from pooled cultured blastocysts is reported here, we could detect expression of some of the genes in single whole cultured blastocysts (data not shown).
Our goal was to study molecular events involved in the earliest cell lineage commitment decisions, as totipotent embryonic stem cells or cultured blastocysts produce cells committed to the hematopoietic lineage. Among the genes we chose to analyze, some are known to be expressed in a lineage-restricted manner in adult hematopoietic cells, whereas others have expression profiles on a wider range of hematopoietic lineages and even on nonhematopoietic cells.

**Lineage-specific receptor expression.** The c-fms gene is a myeloid-specific gene that encodes the macrophage colony-stimulating factor (M-CSF) receptor. The expression of this gene was found to be temporally regulated during embryoid body development. c-fms was expressed in ES cells at relatively low levels, then the level of expression increased starting at days 3 to 6 of in vitro development and rose dramatically at day 9 (Fig 4A). This pattern of temporal increase in c-fms mRNA was confirmed by S1 nuclease mapping, where no band was detectable in ES and day 4 embryoid body RNA and a band was present at day 8 of development (data not shown). Induction of the c-fms gene correlated with the appearance of macrophages in the embryoid bodies, as evidenced by hematologic staining (Fig 2C), as well as the presence of the M1/70 macrophage-specific cell surface marker by fluorescence-activated cell sorter (FACS) analysis (data not shown). In cultured blastocysts, c-fms expression was similarly inducible. However, no detectable c-fms message was observed in blastocysts (Fig 4B).

The receptor for the myeloid-specific growth factor granulocyte CSF (G-CSF-R) is found predominantly on precursor and mature neutrophils and myeloid cell lines and is thus fairly lineage specific; however, it is also expressed on human endothelial cells, placenta, and trophoblastic cells. In our study, we found this gene to be expressed at a relatively low level in ES cells and increased temporally in differentiating embryoid bodies, both in vivo and in vitro (Fig 4A). During blastocyst differentiation, the G-CSF-R gene was expressed at very high levels in blastocysts, dropping to low levels at day 3 of culture, and then at elevated levels during the later time points (Fig 4B). The high level of expression in the blastocysts was not surprising given that the G-CSF-R is expressed on human trophoblastic cells.

Another set of lineage-restricted genes that we studied were the c-fms gene, which encodes the macrophage colony-stimulating factor (M-CSF) receptor, and the erythropoietin receptor (epoR). During embryoid body development, the expression pattern of GATA-1 showed a reproducible pattern of temporal increase from day 6 to 9 (Fig 4A). It is noteworthy that the peak of GATA-1 expression that we observed occurred just before the appearance of blood islands in the developing embryoid bodies. The patterns of GATA-1 expression were confirmed by S1 nuclease mapping (data not shown). In cultured blastocysts, GATA-1 was on at a low level in blastocysts, with expression increasing at day 5 in culture and remaining high throughout day 9, when blood islands appear (Fig 4B).

The receptor for erythropoietin was found to be expressed at all time points in embryoid body differentiation, including undifferentiated ES cells (Fig 4A). However, in cultured blastocysts, the epoR was temporally regulated, with no detectable expression until day 5, and the highest level of expression coincided with blood island formation on day 9 (Fig 4B).

Possibly the most striking temporal regulation during embryoid body development was seen with the interleukin-7 (IL-7) receptor. IL-7 acts specifically on primitive precursors of the B- and T-cell lineages. Among the genes that were analyzed, the expression pattern of the IL-7 receptor was unique because there was no detectable expression in ES cells or early time points of embryoid body development, even upon Southern hybridization of 30-cycle PCR products. At days 9 and 10 of development, expression of IL-7R increased dramatically to high levels, which remained high throughout the remainder of the time course (Fig 4A). Such dramatic upregulation of the receptor for a lymphoid-specific growth factor may be a signal for the onset of lymphoid development. Interestingly, the IL-7R gene was expressed at extremely low levels at all time points in cultured blastocysts, increasing significantly at day 9 (Fig 4B). The time course for cultured blastocysts probably did not extend long enough to correspond to the latter stages of embryoid body differentiation.

**IL-3R/GM-CSF-R/IL-5R subunits.** The cytokines IL-3 and GM-CSF are known to have pleiotropic and overlapping activities on early hematopoietic progenitors from the bone marrow, as well as specific activities on more committed cells such as mast cells (IL-3) and granulocytes/macrophages (GM-CSF). IL-5 also has pleiotropic functions, which include promotion of growth and differentiation of eosinophils. It has recently been elucidated that these molecules share common elements in their signal transduction pathways; one component of the high-affinity receptors for both IL-3 and GM-CSF, as well as IL-5, is a common β subunit, AIC12B. There is an α subunit unique for each cytokine, and both subunits are necessary for high-affinity binding. There is also a second β subunit, AIC2A, which can bind to IL-3 alone with low affinity, and can also form a high affinity IL-3R β subunit.

We observed a striking pattern of gene expression of this subunit family during in vitro development. The gene for IL-3Rα and GM-CSF-Rα were expressed constitutively in ES cells, as well as across the entire time course of embryoid body development (Fig 5A). In contrast, the AIC2A (IL-3R-specific β subunit) and AIC2B (common β subunit) genes were dramatically upregulated at days 6 and 7 from a low but detectable level in ES cells and day 3 or 4 embryoid bodies, to a high relative level that remained high for the remainder of the time course (Fig 5A). Among the genes we have analyzed, these β chain genes were the earliest to undergo a dramatic temporal increase in expression level as in vitro development proceeded, and may be considered one of the earliest events of hematopoietic lineage commitment. The IL-5Rα gene was expressed at a very low level in ES cells, increased with time starting at day 7, and displayed a dramatic induction at day 21/22 of embryoid body development (Fig 5A). The expression pattern in cultured blastocysts was somewhat different (Fig 5B). The expression of AIC2A, the IL-3R-specific β subunit, was detectable at a low level at early time points, and increased significantly at day 9. AIC2B, the common β subunit, was dramatically upregulated at day 9 of culture from undetectable levels at the previous time...
Expression of IL-3R/GM-CSF-R/IL-5R subunit genes during in vitro development. (A) Time course of embryoid body development. d3 to d22 correspond to days of in vitro development after initiation of embryoid body culture. (-) RT lane is the PCR product where a mock cDNA was added (no RT added in cDNA synthesis reaction). Input cDNA used was 500 ng (RNA equivalent). (B) Time course of cultured blastocyst development. blast. lane is RNA from freshly isolated day 3.5 blastocysts. d3 to d9 correspond to days of in vitro culture. Levels of input cDNA were normalized as described in Materials and Methods. The product from a mock PCR reaction (no cDNA added) is shown (H2O, A; (-), B). (+) lane is a positive control for each gene as follows: for AIC2A and IL-3Ra, MC9; for AIC2B, CTLL expressing AIC2B cDNA; for GM-CSF-Ra, PT18; for IL-5Ra, CH12. The levels of signal for each gene cannot be compared with the levels of signal for another gene.

Expression of the α subunit gene for IL-3R was detectable in blastocysts and levels remained constant at day 3 and day 5, and subsequently downregulated on days 7 and 9. The GM-CSF-Rα gene was undetectable in blastocysts, then expressed relatively constantly from day 3 to day 9. IL-5Ra expression varied significantly from the embryoid body pattern; there was an extremely low level seen in blastocysts, then higher levels in day 3 and day 5 of culture, and no level of transcript detectable in later time points.

Expression of stem cell-associated genes. We analyzed the expression patterns for a set of genes whose expression has been associated with hematopoietic stem cells or early progenitor cells: c-kit, stem cell factor (SCF), flk-2, and CD34. We examined the expression patterns of these four genes during in vitro development because temporal changes in their expression could be indicative of formation of early hematopoietic precursors. The c-kit gene encodes a protein tyrosine kinase transmembrane receptor whose ligand is a hematopoietic growth factor, termed SCF.

In developing embryoid bodies, SCF mRNA levels increased across the time course, from readily detectable levels in ES cells and day 4 embryoid bodies to high relative levels as in vitro development proceeded (Fig 6A). The levels of this mRNA as well as for c-kit (SCF receptor) were rather high and it was necessary to substantially reduce the amount of input cDNA to achieve a linear signal. In marked contrast to the temporal induction of SCF mRNA levels, the c-kit mRNA level remained constant, with no increase with increasing time of development. We consistently observed constitutive expression of c-kit in three independent experiments. Although this result suggests that these transcripts are constitutively expressed, we cannot rule out the possibility that these transcripts are induced in a subset of cells and downregulated in other cells of the rapidly differentiating embryoid body. Our analysis of steady-state RNA levels on unfractionated cells did not resolve this question. It is clear that overall levels of SCF were induced with in vitro differentiation, while the overall level of c-kit expression remained high but constant. Both c-kit and SCF were upregulated in cultured blastocysts (Fig 6B). Expression of SCF was not detected in blastocysts, peaked at day 5, and remained high throughout the time course. Expression of c-
kit was detected in blastocysts and increased during the time course.

Like c-kit, the flk-2 molecule is a receptor tyrosine kinase that is expressed primarily in populations enriched for hematopoietic stem cells and early progenitors. Expression of the flk-2 gene during embryoid body differentiation was readily detectable, starting in undifferentiated ES cells. The level of flk-2 RNA increased at day 3, decreased at days 6 through 9, then steadily increased from day 12 through day 21 of in vitro differentiation (Fig 6A). This pattern of expression was reproducible in independently derived RNA samples (data not shown). In cultured blastocysts, no flk-2 RNA was detected until day 5 of culture, after which the level remained constant (Fig 6B).

Antibodies recognizing the human CD34 antigen have been used to enrich for early hematopoietic progenitors, including stem cells. The mouse homolog of this cell surface glycoprotein was recently cloned and shown to be expressed by hematopoietic progenitor cell lines, as well as embryonic fibroblasts and brain. The CD34 gene was expressed in ES cells, then was upregulated steadily across the time course of embryoid body development (Fig 6A). The CD34 gene was also upregulated in cultured blastocysts, with no detectable expression in blastocysts, and maximal levels detected on days 7 and 9 (Fig 6B). Expression and upregulation of these stem cell associated genes may indicate the formation and proliferation of cells committed to the hematopoietic lineage.

Expression of other receptors and genes. We also analyzed expression of another set of hematopoiesis-associated genes that do not readily fall into any of the above classes.

The IL-2Rβ gene encodes one subunit of the high-affinity receptor of IL-2, a multifunctional cytokine whose activities include antigen-specific proliferation of T cells, stimulation of B cells, macrophages, natural killer (NK) cells, lymphokine activated killer (LAK) cells, and even action on oligodendrocytes. We found this gene to be expressed at readily detectable levels in ES cells, with levels decreasing at day 4 and then steadily increasing across the entire time course of embryoid body development (Fig 7A). In contrast, no message was detectable in blastocysts, but levels of IL-2R increased across the time course of blastocyst culture (Fig 7B).

The IL-4R has been found on numerous cell types, including nonhematopoietic cells, although the activities of IL-4 are primarily associated with stimulation and proliferation of hematopoietic cells, including B cells, T cells, and, in combination with other cytokines, on myeloid cells. We found
high levels of IL-4R expression in ES cells, embryoid bodies, blastocysts, and cultured blastocysts, with levels increasing slightly with time (Fig 7, A and B).

The receptor for IL-6 is expressed on many cell types including plasmacytoma cells, macrophages, T cells, B-lymphoma cells and pre-B cells. Its ligand, IL-6, is a multifunctional cytokine involved in immune responses, the acute-phase response, and inflammatory responses. IL-6 acts on early hematopoietic progenitors in combination with other cytokines and is thought to induce stem cells to enter cell cycle. In our study, the IL-6R gene was expressed constitutively at readily detectable levels that did not change significantly during in vitro development (Fig 7, A and B).

The c-myb gene was included in our study because it is expressed in early hematopoietic progenitor cells, and it may play an essential role in normal fetal liver hematopoiesis, perhaps by maintaining the proliferative state of early progenitor cells. We found c-myb expressed at readily detectable levels in ES cells and this level remained constant as the embryoid bodies developed. In cultured blastocysts, very low expression levels were observed in blastocysts and day 3 and 5 of in vitro culture, with levels increasing significantly at day 7 and day 9 of culture (Fig 7, A and B).

The results of PCR analysis of growth factor receptor gene expression in developing embryoid bodies and cultured blastocysts are summarized in Table 2.

**DISCUSSION**

**Molecular Markers of Lineage Commitment**

Our goal in this study was to use two in vitro models of early development to examine the expression patterns of a set of genes involved in hematopoiesis. By performing this analysis, we wished to identify genes that could serve as markers of hematopoietic lineage commitment. We focused our study on expression of cell-associated molecules, primarily growth factor receptors, as good candidates for markers of lineage commitment. Our studies confirm an earlier report by Schmitt et al for several receptors in embryoid bodies and provide data on the expression of an additional 10 genes, as well as extend the gene expression analysis to the cultured blastocyst system.

To define markers of hematopoietic lineage commitment, we have chosen two criteria by which to evaluate the genes we analyzed: first, does the gene undergo a consistent pattern of induction during in vitro development and if so, are the patterns of expression consistent between the two in vitro models?
models we have used? Although these criteria may be somewhat arbitrary, by examining our results in this manner it is possible to extract from the large body of data some important observations that lead to a hypothesis about early steps of lineage commitment.

When these criteria are applied to the data we have presented, 7 of the 18 genes that we examine are possible candidates for markers of lineage commitment: AIC2A, AIC2B, SCF, c-fms, G-CSF-R, CD34, and IL-7R. Each of these genes undergoes a significant induction during in vitro development in both in vitro models. Furthermore, there was a highly reproducible order of expression for each of these genes, with some genes being induced at early time points, others at intermediate times, and one gene induced only at later time points. Our prediction is that the order of expression of these receptors will correlate with the functional expression of these molecules, and thus expression of these molecules may serve as markers of lineage commitment.

The earliest genes to undergo a dramatic temporal increase during in vitro development were the AIC2A and AIC2B genes, which encode the two β subunits of the IL-3R, GM-CSF-R, IL-5R family. There was a dramatic induction of both β subunit transcripts at days 6 and 7 of embryoid body development and day 9 of cultured blastocyst development, while the α subunit genes for both IL-3 and GM-CSF were expressed at a relatively constant level. This suggests that induction of the β subunits and consequently acquisition of high-affinity receptors for IL-3 or GM-CSF is an early event during hematopoietic development. Alternatively, there might exist a novel α subunit for an as yet unidentified ligand that uses the common β subunit for signaling, defining a novel growth factor pathway used during development.

The SCF gene was also dramatically induced at early time points in both in vitro systems. In contrast, the SCF receptor gene, c-kit, was constitutively expressed in ES cells and blastocysts and upregulated only in differentiating blastocysts. The upregulation of SCF may reflect induction of a suitable hematopoietic microenvironment, but may not be a good marker for commitment of hematopoietic progenitors because it is probably expressed by cells of the microenvironment.

The myeloid-specific c-fms and G-CSF-R genes were reproducibly induced at intermediate time points of in vitro development in both systems. This expression pattern may reflect the first onset of myeloid development; these expression patterns correlated well with the appearance of macrophages from both embryoid bodies (Fig 2C) and cultured blastocysts (Fig 3F). Cells expressing either of these markers may be the first committed myeloid progenitors.

The CD34 gene was also induced at intermediate time points in both in vitro systems. The expression of the CD34 gene in early hematopoietic progenitor cells and its reproducible induction suggests it could be used as a marker to enrich for early hematopoietic precursors from these sources. Of the genes whose expression changed during in vitro differentiation, the IL-7R gene displayed the most dramatic increase at later stages of development. Because the IL-7R has been shown to be expressed predominantly on pre-B cells, thymocytes, and T cells, this may be evidence of lymphoid lineage commitment occurring in both systems. A recent study demonstrated the presence of lymphoid progenitors in cells developing embryoid bodies, immortalized by retroviral infection and analyzed by adoptive transfer in recipient mice. The fact that the IL-7R gene was completely silent...
until day 9 of embryoid body development is consistent with a late wave of lymphoid lineage commitment.

Comparison of Two In Vitro Systems

Each of the in vitro systems we have described has unique advantages and disadvantages. The in vitro culture of blastocysts involves the tedious isolation of blastocysts from animals for each experiment, while ES cells lines are readily available and can be maintained for many passages by routine techniques. Each blastocyst must be cultured in an individual well for development to proceed, but this allows them to be examined visually to select only for those which formed cystic structures. Embryoid body formation from ES cells, on the other hand, can be accomplished in bulk culture and is less time consuming. The amount of RNA derived from cultured blastocysts is quite low and not readily quantitated, whereas large quantities of ES or embryoid body RNA can be generated.

There are some important differences between these two systems. Cultured blastocysts in vitro may be expected to give somewhat different results than ES cell-derived embryoid bodies because ES cells have been passed many times and may have undergone some differentiation, even in the presence of LIF. Inner cell mass-derived cells from blastocysts have not been subjected to multiple passages and thus may show different patterns of gene expression than ES cells. Additionally, trophoblast cells in the cultured blastocysts may influence subsequent development in vitro that would not be seen with ES cells or embryoid bodies; this was probably the case for the G-CSF-R gene, which is expressed by trophoblast cells.32 However, despite these differences, both systems undergo highly consistent patterns of cellular development in vitro, represented by predictable morphologic changes and reproducible temporal patterns of gene expression. For these reasons, both are useful as models of early hematopoietic lineage commitment.

Although the temporal expression patterns for most genes were similar in both in vitro culture systems, there were some significant differences for certain genes. For example, the eopR and c-myb genes were induced during in vitro development of blastocysts, but both of these genes were already expressed in ES cells and did not change with development. Possibly ES cells, which have been passaged many times in culture, have had certain transcriptional alterations due to long-term culture or selection for stability, whereas the inner cell mass cells of blastocysts were freshly derived in each experiment. Others have recently shown that eopR is expressed in undifferentiated ES cells,54 in agreement with our data. The induction of c-myb in cultured blastocysts is consistent with the formation of early hematopoietic precursors.

The IL-5Rα gene was induced during development in both systems, but the patterns of expression were significantly different. It is interesting to speculate that the dramatic increase at day 21/22 seen in embryoid bodies may correlate with a late wave of lymphoid development, because IL-5R is expressed on early B cells.55 The blastocyst cultures did not extend to this time point. IL-5R would also be expected to be expressed on cells of the eosinophil lineage, but we have no evidence that eosinophils were produced in our culturing systems.

Because we found that many of the genes were analyzed were expressed at detectable levels in the starting ES cells, in contrast to previous studies of some of these genes,12 the differentiation state of the ES cells is an issue. To assure that the ES cells used in our studies represented as closely as possible undifferentiated embryonic cells, cells were maintained in the presence of recombinant LIF and all experiments were initiated with cells at no later than passage 12. The totipotency of the starting cells was also established in separate studies in which passage 14 cells were shown to form chimeric mice capable of transmitting to the germline (R. Murray, unpublished results, 1990). Nevertheless, there may be several explanations for the differences in our results compared with those of Schmitt et al. First, they used different cell culturing conditions that included as a source of LIF, medium from 5637 cells, a cell line that produces additional cytokines including GM-CSF, G-CSF, IL-1, and IL-6 (F.L., unpublished results, 1988). They also physically removed the centers of ES colonies to analyze gene expression in undifferentiated cells. Finally, we cannot rule out that the PCR primers and conditions we used were more sensitive and able to detect lower amounts of RNA.

The constitutive early expression of certain genes involved in hematopoiesis, in ES cells or blastocysts, may indicate that some steps of lineage commitment and differentiation may involve the silencing of certain genes as an alternative mode of regulation to the induction of other genes.54 We also do not know if all of the receptor mRNAs expressed in ES cells lead to the production of functional proteins, or if these molecules are expressed on the surface, although studies are in progress to address this issue.

Conclusion

From our analysis of receptor gene expression during in vitro development, we conclude that a set of hematopoietic growth factor receptor genes may be useful as markers of hematopoietic development, because they undergo a reproducible and ordered pattern of gene induction as in vitro development proceeds. One of the earliest events we have identified is the induction of the β subunit for the IL-3, GM-CSF, and IL-5 receptor group. Our working hypothesis is that the expression of these genes will also be reflected by the expression of functional receptors on the surface of cells, and that these markers may be useful to isolate early cells in the hematopoietic lineage. We are currently using subtractive cDNA cloning to search for genes acting at an even earlier stage in hematopoietic lineage commitment.

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