INTRODUCTION

The onset of hematopoiesis in the mouse is defined by the appearance of discrete blood islands in the extra-embryonic yolk sac at approximately day 7.5 of gestation (Metcalf and Moore, 1971; Russel, 1979). Large nucleated erythroid cells that express the embryonic forms of globin, a population commonly referred to as primitive erythrocytes, represent the predominant mature hematopoietic cells at this early stage of development (Barker, 1968; Metcalf and Moore, 1971; Brotherton et al., 1979). Shortly following the establishment of primitive erythropoiesis, other precursors including those capable of generating definitive or adult type erythroid cells as well as those able to generate the various myeloid and lymphoid lineages can be detected (Moore and Metcalf, 1970; Johnson and Barker, 1985; Wong et al., 1986; Liu and Auerbach, 1991; Cumano et al., 1993; Huang et al., 1994). Although they are present in the embryonic yolk sac, most of these precursors do not generate significant numbers of progeny until hematopoietic activity shifts to intra-embryonic sites, specifically the developing fetal liver. Multipotential stem cells with long-term repopulating capacity are first detectable following the establishment of the intra-embryonic phase of hematopoiesis and are found initially in the aorta-gonad-mesonephros (AGM) region (Muller et al., 1994) and subsequently in the fetal liver (Jordan et al., 1990; Muller et al., 1994) of the developing embryo.

The observation that the primitive erythroid population develops earlier in ontogeny than long-term repopulating cells is difficult to incorporate into existing models of hematopoietic differentiation which typically position the multipotential stem cell as the most immature cell within the system (Keller, 1992; Uchida et al., 1993). This sequence of developmental events is, however, consistent with studies which suggest that embryonic hematopoiesis is initiated at independent sites, an extraembryonic site predominantly for the early wave of hematopoiesis.

SUMMARY

We have investigated the expression pattern of the Flk-1 receptor tyrosine kinase in mouse embryonic and fetal hematopoietic tissues as well as on hematopoietic precursor cells derived from these tissues. RNA analysis indicated that flk-1 was expressed in the yolk sac at day 10 of gestation, in the whole embryo at day 10 and 12 of gestation, in the liver throughout fetal life and in embryoid bodies (EBs) generated from ES cells differentiated in culture. Flk-1 message was also detected in erythroid and macrophage colonies generated from precursors of yolk sac, fetal liver, adult marrow and EB origin. Using an antibody directed against the extracellular portion of the molecule we have found that up to 50% of cells from EBs differentiated for 4 days express Flk-1. Following the development of this early Flk-1+ population the number of receptor-positive cells declines progressively to represent less than 5% of the EBs by day 12 of differentiation. Kinetic analysis revealed that the establishment of the EB Flk-1+ population precedes the development of cells which express CD34, Ly6A (Sca-1) and AA4.1. Cell sorting experiments demonstrated that all day-4 EB-derived hematopoietic precursors are Flk-1+ whereas greater than 95% of those found within the day-12 EBs are Flk-1+, suggesting that the precursor population which expresses this receptor represents an early but transient wave of hematopoietic development. Analysis of yolk sac and whole embryos at day 8.5 of gestation revealed a small but distinct Flk-1+ population that contained hematopoietic precursors. Day-12.5 fetal liver contained few Flk-1+ cells that showed little hematopoietic potential. Together these findings indicate that Flk-1 is expressed on an early population of hematopoietic precursors that may represent the onset of embryonic hematopoiesis.

Key words: embryonic, hematopoiesis, Flk-1 receptor tyrosine kinase, mouse
primitive erythropoiesis and an intraembryonic site for the later developing multipotent stem cells and definitive hematopoiesis (Dieterlen-Lievre, 1975; Lassila et al., 1982; Kau and Turpen, 1983; Muller et al., 1994; Godin et al., 1995; Chen and Turpen, 1995; Cumano et al., 1996; Medvinsky and Dzierzak, 1996). Alternatively, it is possible that the repopulating stem cell is not the most immature cell within the hematopoietic hierarchy, but rather develops from some earlier precursor population that migrates throughout the developing embryo and ultimately generates all lineages including the primitive erythrocytes. To date there is little experimental evidence to support this interpretation, largely due to the fact that the sequence of events leading to the development of the hematopoietic system and the true potential of the earliest hematopoietic cells are not well defined.

In the early embryonic yolk sac, cells with hematopoietic potential are thought to arise from extraembryonic mesoderm-derived precursors. These mesodermal cells destined for extraembryonic sites migrate through the primitive streak eventually contributing to the visceral yolk sac as well as other extraembryonic structures. Visceral yolk sac mesoderm, located adjacent to a layer of visceral yolk sac endoderm, differentiates to give rise to hematopoietic and endothelial cells which can be detected by day 7.5 of gestation. This close developmental association of the hematopoietic and endothelial lineages within the early yolk sac has led to the hypothesis that they share a common ancestor, a cell referred to as the hemangioblast (His, 1900; and reviewed by Risau and Flamme, 1995). Although this hypothesis was put forward almost 100 years ago, a bipotential cell with both endothelial and hematopoietic potential has not yet been identified.

Characterization of the developmental potential of the earliest hematopoietic precursors requires access to these cells as they develop from the mesoderm. Such approaches typically involve physical isolation of the cells of interest, often taking advantage of the differential expression of proteins on early precursor populations. For example, markers such as AA4.1 and Ly6A have been used extensively for the isolation of stem cells and precursors from fetal liver (Jordan et al., 1990) and adult marrow (Spangrude et al., 1988). To date, however, there are no studies documenting that these or any other ‘stem cell’ markers are expressed on the earliest embryonic hematopoietic precursors. One candidate marker for this population is the receptor tyrosine kinase, Flk-1 (Matthews et al., 1991; Millauer et al., 1993; Yamaguchi et al., 1993), as gene targeting studies demonstrated dramatic defects in both the hematopoietic and endothelial lineages in mice lacking this receptor (Shalaby et al., 1995). Additionally, expression studies of the early mouse embryo have shown flk-1 to be present in the extraembryonic mesoderm destined to give rise to the hematopoietic and endothelial components of the yolk sac blood islands (Yamaguchi et al., 1993; Millauer et al., 1993; Palis et al., 1995; Dumont et al., 1995). While these reports have documented that flk-1 expression persists in the endothelium of the blood islands, they did not demonstrate expression in cells of the hematopoietic lineage.

In this study we have used RNA analysis as well as an antibody directed against the extracellular domain of Flk-1 to define its expression patterns in various hematopoietic precursor populations. Hematopoietic cells from ES cells differentiated in culture as well as those from whole embryos, yolk sac and fetal liver were analyzed for the presence of Flk-1. Through this approach we show that Flk-1 is expressed on precursors of multiple hematopoietic lineages early in development and that this expression is abrogated with time. These findings define a wave of early hematopoietic precursors characterized by Flk-1 expression and suggest that this population could represent the earliest stages of hematopoietic commitment.

MATERIALS AND METHODS

Growth and differentiation of ES cells

Embryonic stem cells (CCE; Robertson et al., 1986) were maintained by growth and passage on gelatinized flasks in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum (Gemini Bio-Products Inc.), penicillin, streptomycin, 1% supernatant containing LIF and 1.5x10^-4 M monothioglycerol (Sigma). Cells were transferred to Iscove’s modified Dulbecco’s medium (IMDM) containing the above components 2 days prior to the initiation of differentiation. After 2 days in IMDM, ES cells were trypsinized into a single cell suspension and plated at variable densities (300-4500 cells/ml) into differentiation medium containing IMDM supplemented with 15% fetal calf serum (Hyclone), 2 mM glutamine (Gibco/BRL), 0.5 mM ascorbic acid (Sigma), and 4.5x10^-8 M monothioglycerol. The differentiation cultures were carried out in Petri dish grades and maintained in a humidified chamber at 37°C CO2/air mixture at 37°C. For long term differentiation cultures of ES cells (>6 days), cells were plated in differentiation medium, as described above, supplemented with 1.0% methyl cellulose (Fluka), 1% supernatant containing c-Kit ligand, 1% supernatant containing IL-3 and 25 ng/ml IL-11. Long term cultures were fed after 7–8 days with differentiation medium containing 25% methyl cellulose. LIF and c-Kit ligand were derived from medium conditioned by CHO cells transfected with LIF and KL expression vectors (kindly provided by Genetics Institute). IL-3 was obtained from medium conditioned by X63 Ag8-653 myeloma cells transfected with a vector expressing IL-3 (Karaysu and Melechers, 1988). IL-11 was purchased from R&D systems.

Generation of anti-flk-1 antibodies

Antibodies against murine Flk-1 were raised by immunization of Wistar rats with rat-1 cells stably transfected with the complete coding sequence of murine Flk (Millauer et al., 1994). The rats were immunized subcutaneously in the hind legs with 10^7 cells emulsified in complete Freund’s adjuvant. Immunizations were repeated 4 times at 3-day intervals. 15 days following the first immunization the rats were killed and the inguinal lymph node cells were harvested and fused with murine SP2/0 myeloma cells. Hybridomas resulting from the fusion were grown in RPMI-1640 with 10% FCS, hypoxanthine, aminopterin and thymidine (HAT) as described previously (Bühring et al., 1991). Culture supernatants from the resulting hybridomas were screened by FACS on Flk-1-transfected and non-transfected control rat-1 cells. Hybridomas that secreted antibodies which reacted with the transfected but not with the control cells were selected and cloned twice by limiting dilution. The plating efficiency following the second cloning step was approximately 95% and the proportion of antibody producing cells was 98-100%. The isotypes of the antibodies produced were determined by FACS analysis with FITC-conjugated isotype-specific goat anti-rat antibodies. Five of the hybridomas (74C4, 74A6, 74C3, 74B6 and 88D4) secreted IgG1 antibodies whereas four (73D5, 89B3, 89B1, and 5C3) secreted IgG2a antibodies. The antibody produced by hybridoma 89B3A5, a subclone of 89B3, was selected for these studies as it worked best for FACS analysis and does not bind the closely related receptor FLT-1.

FACS sorting and analysis

EBs grown in methyl cellulose or in liquid culture (identical conditions without methyl cellulose) were harvested and trypsinized for approximately 3 minutes. Alternatively, cells were placed in a collagenase
The PCR primer s used to detect specific GACCGA GCG 3' previously (Choi et al., 1993). The oligonucleotides used for detecting Northern blot and RT-PCR analyses were performed as described previously.

**RNA isolation and expression analysis**

Northern blot and RT-PCR analyses were performed as described previously (Choi et al., 1993). The oligonucleotides used for detecting specific β-actin cDNA were as follows: forward; 5' ATGAAGATCTT-GACCAGAGCG 3' and reverse; 5' TACTTGGCGTAGGAGGAC 3'. The PCR primers used to detect flk-1 were: forward; 5'TGGTGATCT-GAAAAGAGCGC3' and reverse 5'CATTCTTTCTCCGATAGG3'.

**RESULTS**

**RNA analysis of flk-1 expression in hematopoietic tissues and specific lineages**

As a first step in defining the expression pattern of flk-1 in early development, we analyzed embryonic and fetal hematopoietic tissues as well as developing EBs for the presence of specific mRNA. Northern analysis demonstrated that flk-1 was expressed in whole embryos (day 10 and 12 of gestation), in the yolk sac (day 10 of gestation) and in the liver throughout fetal life (Fig. 1A). Expression in fetal liver was downregulated with increasing age of gestation coincident with the decline of hematopoietic activity in the organ. flk-1 was not expressed in undifferentiated ES cells but its expression was upregulated significantly as these cells differentiated and formed EBs. Low levels of flk-1-specific mRNA were detected in the EBs within 72 hours of differentiation (EB3; Fig. 1A). The level of expression increased dramatically over the next 24 hours (EB4), and then persisted for the duration of the study (day 6). The upregulation of flk-1 expression between days 3 and 4 of differentiation is consistent with findings in previous studies (Yamaguchi et al., 1993; Vittet et al., 1996) and coincides with the onset of hematopoiesis within the EBs (Keller et al., 1993).

To demonstrate that flk-1 is expressed in hematopoietic cells, colonies consisting of different erythroid and myeloid lineages generated in methyl cellulose cultures were analyzed by PCR for the presence of flk-1 message. Primitive and definitive erythroid, macrophage, neutrophil and mast cell colonies derived from either EBs, yolk sac, fetal liver, or adult bone marrow were used for this analysis. flk-1 was expressed in EB-derived primitive erythroid colonies as well as in definitive erythroid colonies generated from yolk sac, fetal liver and adult bone marrow precursors (Fig. 1B). In addition, flk-1 expression was readily detectable in macrophage colonies that were generated from fetal liver and adult bone marrow. In contrast to the erythroid and macrophage colonies, only trace levels of flk-1 expression were found in colonies of mast cells and neutrophils. Together these findings suggest that flk-1 is expressed in specific embryonic, fetal and adult hematopoietic lineages and as such could provide a unique marker for their isolation.
Cell surface expression analysis of ES cells and day-3 to -14 EBs

To determine if the flk-1 RNA levels detected in the EBs reflect protein levels, cells from EBs at different developmental stages (day 3 to day 14) were analyzed for the presence of the Flk-1 receptor with an antibody specific for the extracellular domain of the molecule. The pattern of Flk-1 surface expression was compared to that of other proteins known to be present on early hematopoietic precursors and stem cells. These include CD34, which is expressed on various precursor populations as well as on endothelial cells (Civin et al., 1984; Katz et al., 1985; Baum et al., 1992; Krause et al., 1994; Young et al., 1995); c-Kit which is expressed on stem cells (Orlic et al., 1993) and numerous hematopoietic precursors (Ogawa et al., 1991) and is required for normal hematopoietic development (reviewed by Russel, 1979), AA4.1 which is expressed on fetal liver and yolk sac stem cells and precursors (Jordan et al., 1990; Huang and Auerbach, 1993; Carlsson et al., 1995; Godin et al., 1995) and Ly6A (Sca-1) which is expressed on fetal liver (Morrison et al., 1993) and adult bone marrow stem cells and precursors (Spangrude et al., 1988; Trevisan and Iscove, 1995). Flk-1+ cells were first detected in day-3 EBs at which stage approximately 2-3% of the cells expressed this receptor (Fig. 2). Within 24 hours of differentiation the number of Flk-1+ cells increased dramatically to represent approximately 40% of the EB population. This rapid increase in receptor expression between days 3 and 4 of differentiation is consistent with the observed increase in flk-1 RNA (Fig. 1A). Beyond day 4 of differentiation there was a gradual but steady diminution of the Flk-1+ population, decreasing to near 30% in day-6 EBs and continuing to decline to <5% in day-14 EBs.

CD34+ cells were detected (8%-9%) in EBs as early as day 6 of differentiation. This population increased to represent approximately 20% of day-10 EBs and then declined to less than 10% by day 14 of differentiation. c-Kit was found to be expressed on ES cells and appeared to persist on subpopulations of EB-derived cells throughout the 14-day time course. The staining pattern observed with the c-Kit antibody was somewhat unusual in that most of the cells within the population appeared to express some level of the receptor. Consequently the values given in the figure represent that proportion of the population that expresses the highest level of c-Kit. Like c-Kit, Ly6A was also detected on undifferentiated ES cells. However, following the onset of differentiation, the proportion of Ly6A+ cells within the EBs declined to undetectable levels and remained low until day 10 of differentiation. At this time

![Graph showing cell surface marker analysis of developing EBs](image)

Fig. 2. Cell surface marker analysis of developing EBs. EBs differentiated for 0-14 days were stained with antibodies to Flk-1, CD34, c-kit, AA4.1, and Ly6A. Y axis represents relative cell number. X axis represents fluorescence intensity. Lighter lines indicate negative control (2° reagent only) while darker lines represent results obtained with the addition of the specific antibody. The percentage of cells that fall within the indicated gate are noted. Values of less than 2% represent background staining.
point approximately 10%-15% of the cells were found to express Ly6A. Cells expressing AA4.1 were the last to develop within the EBs and were only detectable at day 14 of differentiation, making-up almost 10% of this population. The findings from this kinetic analysis clearly demonstrate the sequential development of populations expressing specific markers, with Flk-1 preceding CD34, Ly6A and AA4.1.

**Fine kinetic analysis of Flk-1 expression in EBs**

The previous analysis demonstrated a rapid expansion in the Flk-1+ population between days 3 and 4 of EB differentiation. To further define the kinetics of this expansion, EBs were harvested every 6 hours over this 24-hour period and the cells were analyzed for the expression of Flk-1 (Fig. 3). As observed in the previous experiment (Fig. 2), approximately 2% of the day-3 EB cells were Flk-1 positive. Changes in the size of the Flk-1+ population were easily detected at 6-hour intervals as it increased from 2% at day 3 to 20% at day 3.25 to 34% at day 3.5 to 50% at day 3.75 and finally to 55% at day 4.0 of differentiation. These rapid changes not only demonstrate a remarkable rate of development and maturation, but also indicate a high degree of synchrony within the EBs with respect to the establishment of the Flk-1+ population. This increase in Flk-1 expression precedes the development of first hematopoietic precursors (primitive erythroid and macrophage) which are routinely detected in EBs at day 4 of differentiation. (Keller et al., 1993; and Fig. 5A, below).

**Cell surface expression of Flk-1 in embryonic and fetal tissues**

The major site of hematopoietic activity shifts from the yolk sac to the fetal liver at approximately day 10 of gestation. To compare and contrast the results obtained from the ES system to in vivo hematopoesis, cells from day-8.5 embryos taken together with the yolk sac (EMB/YS) and cells from day-12.5 fetal liver (FL) were analyzed for the presence of the markers described above. Approximately 7% of cells from day-8.5 embryonic tissues expressed Flk-1 whereas less than 5% of the fetal liver population was positive for this receptor (Fig. 4). Low but detectable levels of CD34+ cells were consistently detected in the day-8.5 tissues, however no significant numbers of Ly6A+ or AA4.1+ cells were found at this stage of development. Day 12.5 fetal liver contained readily detectable levels of Ly6A+ cells as well as small but distinct populations that expressed CD34 and AA4.1. Cells from the day-8.5 tissues as well as from the day-12.5 fetal liver expressed relatively high levels of the c-Kit receptor. A comparison of these findings to those obtained from the EBs reveal similarities between the expression patterns found in day-6 EBs and day-8.5 embryonic tissue as well as between those in the later stage EBs (days 10-14) and the day-12.5 fetal liver. These similarities further support the notion that hematopoietic development within the EBs parallels that found in the normal embryo.

**Hematopoietic potential of Flk-1+ EB-derived cells**

As the expression of Flk-1 correlated well with the onset of hematopoietic activity in EBs, the next series of experiments were aimed at defining the potential of this population. EBs at different stages of development were dissociated into single suspensions, the cells stained with the anti-Flk-1 antibody and sorted into positive and negative populations. The resulting populations were then plated into methyl cellulose in the presence of a broad spectrum of cytokines known to support the growth and differentiation of precursors of most hematopoietic lineages.

Analysis of day-4 EBs indicated that virtually all of the earliest detectable precursors, specifically the primitive erythroid (Ep colony-forming cells; CFC), the macrophage (Mac-CFC) as well as those able to generate bi-lineage erythrocyte/macrophage (E/Mac-CFC) colonies were found in the Flk-1+ population (Fig. 5A). Occasionally a small number of precursors was detected in the Flk-1+ population, however, when considered together it is unclear if they represent contaminants from the positive fraction or true Flk-1+ cells.

By day 6 of differentiation, the EBs contained a broader spectrum of precursors than found at day 4 and included those of the definitive erythroid lineage (Ed-CFC), those able to generate colonies of macrophages and neutrophils (GM-CFC) as well as those that give rise to multilineage colonies (Mix-CFC). In contrast to the findings at day 4 of differentiation, day-6 EB-derived precursors segregated into both the Flk-1+ positive and negative fractions (Fig. 5B). The Flk-1+ population contained precursors of multiple lineages including, Ep-CFC, Ed-CFC, Mac-CFC, E/Mac-CFC, GM-CFC and Mix-
appears to be downregulated with increasing stages of precursor maturation within the primitive erythroid lineage.

With the significant reduction in the proportion of Flk-1+ cells in the late stage EBs (<5% at day 12), one observes a dramatic shift in hematopoiesis from the Flk-1+ to the Flk-1- EB population. As shown in Fig. 5C, most precursors in day-12 EBs were found in the Flk-1- fraction. This included mast cell precursors (Mast-CFC) and bipotential mast/erythroid precursors (E/Mast-CFC) which are not present in day-4 and -6 EBs, as well as precursors with similar potential to those found in the earlier stages, such as E²-CFC, Mac-CFC, GM-CFC and Mix-CFC. Similar to our earlier reported findings (Keller et al., 1993), E²-CFC were not detected in the day-12 EBs, suggesting that hematopoiesis is switching from an embryonic to a fetal program. A small number of Mac-CFC were found in the day-12 EB Flk-1+ fraction, although their number was somewhat variable from one experiment to the next. A comparison of the total number of Flk-1+ hematopoietic precursors (frequency X total cell number) in the three different EB populations demonstrates a remarkable and rapid change from essentially 100% of the total precursor pool at day 4 of differentiation to less than 1% at day 12 (Fig. 6).

These findings clearly demonstrate that the Flk-1 receptor is present on subpopulations of hematopoietic precursors found in EBs differentiated for 4 to 6 days. To determine if these precursors can respond to the Flk-1 ligand, VEGF, cells from day-4 and day-6 EBs were cultured in the presence of VEGF or in the presence of VEGF plus additional cytokines. In three independent experiments, VEGF alone or in combination with other factors failed to stimulate the growth of hematopoietic precursors from either EB population (not shown).

**Hematopoietic potential of Flk-1+ embryonic and fetal-derived cells**

The previous studies using the in vitro ES differentiation model demonstrated a wave of Flk-1-positive hematopoietic precursors present at the onset of hematopoiesis. To compare this pattern of development to that found in the normal embryo, similar sorting experiments were performed on cells derived from day-8.5 embryonic tissues (whole embryo and yolk sac) and day-12.5 fetal liver. Approximately 5% of the day-8.5 embryonic population expressed Flk-1 in the experiments shown in Fig. 7A. At this stage of development the blood islands of the yolk sac are readily detectable indicating that the primitive erythroid lineage is well established. Consequently it is not surprising that most of the E²-CFC isolated at this time appear to be the late stage precursors that generate relatively small colonies and do not express the Flk-1 receptor (Fig. 7B). Colonies generated from these Flk-1+ E²-CFC appear identical to those derived from Flk-1- E²-CFC found in the day-6 EBs. A number of other precursor populations including E²-CFC, Mac-CFC, E/Mac-CFC, Mast-CFC, and E/Mast-CFC were detected in both fractions. The most abundant precursors within the Flk-1+ fraction were those of the macrophage lineage (Mac-CFC), indicating that this population maintains Flk-1 expression longer than precursors from other lineages, a finding similar to that observed with the late stage EBs. When the relative number of cells found in each fraction is taken into consideration, it is clear that the majority of the total precursor pool (>95%) at this stage of development is Flk-1- (Fig. 6). This finding suggests that the shift to the Flk-1- pattern of hematopoiesis observed in the late stage EBs has, to a large

**Fig. 4.** Cell surface expression of embryonic and fetal tissues. Day-8.5 whole embryos and yolk sac, and day-12.5 fetal liver cells were stained with antibodies to Flk-1, CD34, c-kit, AA4.1 and Ly6A. Y axis represents relative cell number and X axis represents fluorescence intensity. The lighter line indicates negative control (2° reagent only) while the darker line represents results obtained with the addition of the specific antibody. The percentage of cells that fall within the indicated gate are noted. Values of less than 2% represent background staining.
Day 8.5 embryonic/extraembryonic tissues and for day-12.5 fetal liver cells respectively.

**DISCUSSION**

The findings presented in this study provide the first conclusive evidence that subpopulations of embryonic hematopoietic precursors express the Flk-1 receptor tyrosine kinase. Previous in situ analyses have demonstrated flk-1 expression in the mesodermal layer of the yolk sac, but did not show significant expression within the hematopoietic components of the blood islands (Millauer et al., 1993; Yamaguchi et al., 1993; Dumont et al., 1995; Palis et al., 1995). Earlier studies showed flk-1 expression in fetal liver populations enriched for stem cells and precursors, but did not demonstrate that the hematopoietic precursors themselves expressed this receptor (Matthews et al., 1991). Our results not only show that Flk-1 is present on hematopoietic precursors but also suggest that this receptor provides one of the earliest markers for these cells as it precedes the onset of expression of other markers such as CD34, AA4.1 and Ly6A within the developing EBs. The low levels of Ly6A expression in day-3 and -4 EBs can be attributed to the persistence of ES cells which do express this marker (Fig 2, day 0). In addition to Flk-1, significant numbers of c-Kit+ cells are also found in day-4 EBs suggesting that these early precursors may co-express these receptors. The fact that we observed a similar progression of surface marker expression in the developing embryo/yolk sac and fetal liver
Fig. 7. Flk-1 expression and hematopoietic potential of day-8.5 embryonic/extraembryonic tissues and of day-12.5 fetal liver cells. Day 8.5 embryonic/extraembryonic tissues (A) and day-12.5 fetal liver cells (C) were stained for Flk-1 expression. Lighter line represents negative control (2° reagent only) while darker line represents results obtained with the addition of the 1° antibody. Y axis represents relative cell number and X axis represents fluorescence intensity. Horizontal bars in A and C represent sorted populations. Day-8.5 tissues (B) or day-12.5 fetal liver cells (D) were sorted into Flk-1 positive and negative populations and plated into methyl cellulose cultures. Bars represent standard deviations of colony counts from three cultures.

indicates that this pattern reflects normal developmental changes during the ontogeny of the hematopoietic system.

One of the most dramatic ontogenic changes found within the hematopoietic system is the transition from an embryonic to a fetal program defined by the switch from the production of primitive erythrocytes and macrophages in the yolk sac to the generation of a broader spectrum of lineages, including the definitive erythroid, in the fetal liver (Moore and Metcalf, 1970; Russel, 1979). Findings from this study indicate that expression of flk-1 appears to define the earliest stages of embryonic hematopoietic commitment, as the highest numbers of Flk-1+ precursors were found at the onset of hematopoietic development within the EBs. The early development and subsequent rapid expansion of the Flk-1+ population is consistent with the interpretation that these precursors establish the hematopoietic system. As such they would represent the most primitive cells within the hematopoietic hierarchy.

A direct comparison of the Flk-1 expression patterns found on day-3 to -4 EB-derived cells to similar populations in the normal embryo is not possible, as the stages of hematopoietic commitment represented by these EBs are difficult to access in vivo. However, in situ hybridization studies (Millauer et al., 1993; Yamaguchi et al., 1993; Dumont et al., 1995; Palis et al., 1995) which demonstrate extensive flk-1 expression prior to and during the establishment of the yolk sac blood islands suggest that a comparable Flk-1+ population does develop in early embryos. A central role for a Flk-1+ precursor population in the establishment of hematopoiesis in vivo has been demonstrated by gene targeting studies which show that mice lacking this receptor fail to develop yolk sac blood islands and have markedly reduced numbers of hematopoietic precursors (Shalaby et al., 1995). A growing body of evidence suggests that hematopoiesis is initiated independently in the extraembryonic yolk sac and within the developing embryo in the area of the paraaortic splanchnopleura which at later stages of development becomes the AGM region (Muller et al., 1994;
Godin et al., 1995; Cumano et al., 1996; Medvinsky and Dzierzak, 1996). While the knock-out studies demonstrate a requirement for Flk-1 in the initiation of extraembryonic hematopoiesis in the yolk sac, they do not define its role in hematopoietic commitment in the intraembryonic AGM region, as the mutant embryos die prior to the establishment of significant activity at this site. Other experimental approaches, such as the generation of chimeric mice with Flk-1<sup>−/−</sup> ES cells, will be required to determine if this receptor is essential for the development of definitive hematopoiesis.

The demonstration that hematopoietic precursors express Flk-1, a receptor initially thought to be expressed specifically in cells of the endothelial lineage (Millauer et al., 1993; Yamaguchi et al., 1993; Dumont et al., 1995) adds further support to the notion that these populations arise from a common ancestor, the hematogonial. If this bipotential precursor does exist, it likely represents a transient population, present in EBs or developing embryos at the earliest stages of detectable Flk-1 expression. The remarkable expansion of the Flk-1<sup>+</sup> population observed between days 3 and 4 of EB differentiation could represent the establishment and expansion of the hematopoietic and endothelial cell lineages. This interpretation is consistent with studies which demonstrate a rapid development of hematopoietic (Keller et al., 1993) and endothelial (Vittet et al., 1996) populations between day 4 and 6 of EB differentiation.

The loss of flk-1 expression on the hematopoietic precursors with increasing maturation of the EBs could signal a change in the growth regulation of these populations. Although we were unable to demonstrate a growth promoting effect of VEGF on EB-derived precursors, the knock-out studies of Shalaby et al. (1995) clearly show that Flk-1 plays an important role in the establishment of the hematopoietic system. It is possible that the interaction of VEGF with Flk-1 is required at a unique early stage of development, perhaps in the acquisition of hematopoietic potential from a mesodermal precursor cell. As the hematopoietic cells develop, they could lose responsiveness to VEGF and subsequently downregulate Flk-1 as they express other receptors and acquire responsiveness to their corresponding ligands. Obvious candidate molecules would be the c-Kit encoded receptor and its ligand, steel factor (SLF) as studies have shown that this receptor is not required for the establishment of the hematopoietic system but is expressed on yolk sac derived precursors and fetal liver stem cells (Ogawa et al., 1993; Ikuta and Weissman, 1992) and is essential for progression through the fetal hematopoietic program (Nocka et al., 1989; and reviewed by Russel, 1979 and Paulson and Bernstein, 1995).

Although the majority of hematopoietic precursors display this downregulation of flk-1 expression, Flk-1<sup>+</sup> cells do persist in the later stage embryos (days 10 and 12) and the fetal liver as demonstrated by RNA analysis. Much of this flk-1 expression likely represents the presence of endothelial cells in these tissues. However, it is also conceivable that small subpopulations of Flk-1<sup>+</sup> hematopoietic precursors do persist through these developmental stages into adult life. Indeed, our analysis of adult marrow derived colonies demonstrated flk-1 expression in macrophage and to a lesser extent in erythroid colonies suggesting that some of these precursors do retain expression of this receptor. Additional evidence supporting this notion has been provided by a recent study which demonstrates that VEGF can enhance the growth of colonies from subsets of granulocye and macrophage progenitors (Broxmeyer et al., 1995).

Based on the findings presented in this report, we propose a model of hematopoietic development as outlined in Fig. 8. In this model the putative hemangioblast expresses the Flk-1 receptor and would likely respond to its ligand VEGF. Flk-1 expression is maintained on the endothelial as well as on the earliest hematopoietic progeny of this precursor. However, unlike cells of the endothelial lineage that maintain flk-1 expression, cells of the hematopoietic lineages selectively lose expression. The earliest loss of expression is associated with maturation of the primitive erythroid lineage, with the late stage precursors expressing little or no Flk-1 receptor. A later loss of Flk-1 is associated with maturation of the hematopoietic system as a whole and may be one of the earliest indicators of the switch from embryonic to fetal hematopoiesis. Although not shown in the model, it is also possible that subsets of precursors maintain flk-1 expression into adult life and as such could represent the persistence of an embryonic hematopoietic program in the adult animal.

In conclusion, the findings in this study demonstrate that flk-1 expression provides a marker for the earliest detectable hematopoietic precursors. Access to the earliest developing Flk-1<sup>+</sup> cells provides a unique opportunity to fully define their developmental potential and ultimately elucidate the relationship between the hematopoietic and endothelial lineages. In addition, methods for the isolation of these precursors provide a means to further define the role of VEGF and Flk-1 in the establishment and maintenance of early hematopoietic cells and possibly define novel commitment steps between these cells and earlier mesodermal precursors.

We wish to thank Carmen Büttner and Marion Kennedy for outstanding technical assistance, Birgit Millauer for providing the flk-1 transfected rat-1 cells, Xixia Zhu for help in generating the anti-Flk-1 antibodies and Shirley Sobus and Bill Towend for expert assistance.

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(Submitted 5 March 1997)