Development 125, 725-732 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV4967

A common precursor for hematopoietic and endothelial cells

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Accepted 27 November 1997: published on WWW 22 January 1998

SUMMARY

Embryonic stem cell-derived embryoid bodies contain a unique precursor population which, in response to vascular endothelial growth factor, gives rise to blast colonies in semi-solid medium. Upon transfer to liquid culture with appropriate cytokines, these blast colonies generate both hematopoietic and adherent, stromal-type cells. Cells within the adherent population display characteristics of endothelial lineage including the expression of CD31, *flk-1*, *flt-1*, *tie-2*, the capacity to take up acetylated LDL and the presence of cytoplasmic Weibel-Palade bodies. Mixing studies demonstrated that the hematopoietic and endothelial precursors within the blast colonies develop from the same cell, the blast colony-forming cell. Kinetic analysis showed that the blast colony-forming cell represents a transient cell population that develops early and is lost quickly during embryoid body development. These findings provide strong evidence that the blast colony-forming cell represents the long-hypothesized hemangioblast, the common precursor of the hematopoietic and endothelial lineages.

Key words: Embryonic stem cell, Embryoid body, VEGF, Blast cell, Hemangioblast, Hematopoietic precursor, Endothelial precursor

INTRODUCTION

The establishment of blood islands in the extraembyronic yolk sac marks the onset of hematopoiesis and vasculogenesis in the developing mouse embryo. These blood islands derive from aggregates of mesodermal cells that colonize the presumptive yolk sac at approximately 7 days postcoitum (dpc). Over the next 12 hours, the central cells within these aggregates give rise to the embryonic hematopoietic cells while the peripheral population differentiates to endothelial cells which form the first vascular structures that surround the inner blood cells (reviewed by Wagner, 1980). This close developmental association of the hematopoietic and endothelial lineages within the blood islands has led to the hypothesis that they arise from a common precursor, the hemangioblast (Sabin, 1920; Murray, 1932; Wagner, 1980). The concept of the hemangioblast has gained most support from the observation that the hematopoietic and endothelial lineages share expression of a number of different genes (Fina et al., 1990; Millauer et al., 1993; Yamaguchi et al., 1993; Anagnostou et al., 1994; Kallianpur et al., 1994; Young et al., 1995; Asahara et al., 1997; Kabrun et al., 1997). Recent gene targeting experiments demonstrating that a functional Flk-1 receptor tyrosine kinase is required for the development of the blood islands provide further evidence that these lineages derive from a common precursor (Shalaby et al., 1995, 1997). While both observations are consistent with the concept of the hemangioblast, they do not prove its existence.

Past studies aimed at identifying and characterizing the putative hemangioblast have been hampered by difficulties in accessing the embryo prior to the establishment of the blood islands and by the limited number of cells present at this stage of development. Differentiation of embryonic stem (ES) cells to hematopoietic and endothelial cells in culture offers an alternate approach for studying these early commitment steps as both cellular and molecular analyses have documented that the sequence of events leading to the onset of these lineages in this model system is similar to that found in the normal mouse embryo (Risau et al., 1988; Wiles et al., 1991; Keller et al., 1993; Nakano et al., 1994; Keller, 1995; Vittet et al., 1996). Using this model, we have recently shown that embryoid bodies (EBs) generated from ES cells allowed to differentiate for 3-3.5 days contain a unique precursor population with both primitive and definitive hematopoietic potential (Kennedy et al., 1997). When cultured in the presence of vascular endothelial growth factor (VEGF), *c-kit* ligand (KL) and conditioned medium from an endothelial cell line, D4T, these precursors form colonies consisting of immature or blast-like cells that express a number of genes common to both the hematopoietic and endothelial lineages, including *tal-1*/SCL, CD34 and the VEGF receptor, *flk-1* (Kennedy et al., 1997). The VEGF responsiveness of these embryonic precursors together with the gene expression pattern of their blast cell progeny suggests that this population could have the potential to generate cells of the endothelial lineage in addition to hematopoietic precursors.

In this study we have analyzed the endothelial potential of the blast colonies by transferring them to liquid cultures containing growth factors known to support the growth of both hematopoietic and endothelial cells. Under these conditions, a significant proportion of the colonies generated hematopoietic precursors as well as adherent cells with endothelial characteristics. These observations demonstrate that the blast colony-forming cell (BL-CFC) has both hematopoietic and endothelial potential, and as such could represent the in vitro equivalent of the hemangioblast.

MATERIALS AND METHODS

Cell culture

CCE ES cells were maintained on STO feeder cells or on gelatinized flasks in the presence of recombinant LIF. EBs and blast cell colonies were generated as described previously (Kennedy et al., 1997). For the experiments in this study blast colonies were grown in the presence of either VEGF (5 ng/ml), KL (1% conditioned medium or 100 ng/ml purified) and D4T CM (25%) or VEGF (5 ng/ml) and D4T CM (25%). For the generation of endothelial cells, individual blast colonies were transferred to matrigel-coated (Collaborative Research) microtiter wells containing IMDM with 10% fetal calf serum (Hyclone), 10% horse serum (Biocell), VEGF (5 ng/ml), IGF-1 (10 ng/ml), Epo (2 U/ml), bFGF (10 ng/ml), IL-11 (50 ng/ml), KL (1% conditioned medium), endothelial cell growth supplement (ECGS, 100 μg/ml; Collaborative Research), L-glutamine (2 mM), and 4.5×10^{-4} M MTG (Sigma). Following 3-4 days in culture, non adherent hematopoietic cells were removed by gentle pipetting and used directly for gene expression studies or assayed for precursors in methyl cellulose containing 10% plasma-derived serum (Antech, Texas), 5% protein free hybridoma medium (PFHM2; GIBCO/BRL) together with the following cytokines; KL (100 ng/ml), IL-3 (1% conditioned medium), IL-11 (25 ng/ml), GM-CSF (25 U/ml), Epo (2 U/ml), M-CSF (100 U/ml), G-CSF (1000 U/ml), IL-6 (5 ng/ml), LIF (1 ng/ml) and VEGF (5 ng/ml). IL-11, GM-CSF, M-CSF, IL-6, LIF and VEGF were purchased from R&D Systems. *c-kit* ligand was obtained from medium conditioned by CHO cells transfected with a KL expression vector (kindly provided by Genetics Institute) or purchased from R&D Systems. IL-3 was obtained from medium conditioned by X63 Ag8-653 myeloma cells transfected with a vector expressing IL-3 (Karasuyama and Melchers, 1988). Following removal of the non-adherent population, the adherent cells were cultured for an additional 1-2 weeks in IMDM with 10% fetal calf serum (Hyclone), 10% horse serum, VEGF (5 ng/ml), IGF-1 (10 ng/ml) bFGF (10 ng/ml), endothelial cell growth supplement (ECGS, 100 μg/ml; Collaborative Research), L-glutamine (2 mM), and 4.5×10^{-4} M MTG (Sigma) and then harvested by trypsinization for gene expression.

Cell staining

Blast colonies were picked onto prewashed glass cover slips that were coated with a thin layer of matrigel and cultured in 35 mm Petri dishes in medium containing both hematopoietic and endothelial cytokines described above. Four to seven days following the initiation of the cultures, the non adherent cells were harvested and the adherent cells were cultured for an additional one to two weeks in medium without hematopoietic cytokines (see above). For fluorescence analysis, adherent cells were initially cultured (37ºC) in the presence of 10 μg/ml of DiI-Ac-LDL (Biomedical Technologies, Inc.) for 2 hours. Following this incubation, the cells were washed 3 times and fixed for 10 minutes in PBS containing 3% paraformaldehyde and 3% sucrose. The fixed cells were washed 2-3 times, incubated initially with Biotin-α-mouse CD31 (Pharmingen; #01952D) for 1 hour, washed 5 times (5 minutes each) and then incubated with Streptavidin-FITC for an additional hour. Following this second staining step the cells were washed again (5 times) and the coverslip with the cells was mounted onto a slide for analysis. The images were acquired with a digital confocal microscope using the Slidebook software program (Intelligent Imaging Innovations Inc. Denver, Co.). Images were deconvolved using a nearest neighbor algorithm and displayed with orthographic maximum intensity projection as described by Monks et al. (1997).

PCR analysis

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) were performed as described (Chomczynski and Sacchi, 1987; Chelly et al., 1988). All RNA samples were treated with DNaseI (amplification grade from Gibco/BRL) before cDNA synthesis to eliminate any contaminating genomic DNA. The PCR analysis in the mixing experiment was carried out as described in our previous report (Kennedy et al., 1997). Specific primers used were as follows (Rothwell et al., 1987; Keller et al., 1993; Choi et al., 1994; Vittet et al., 1996).

hprt, sense, 5′CACAGGACTAGAACAACCTGC3′; antisense, 5′GCTGGTGAAAAGGACCTCT3′.

flk-1, sense, 5′CACCTGGCACTCTCCACCTTC3′; antisense, 5′GATTTCATCCCACTACCGAAAG3′.

tie-2, sense, 5′TTGAAGTGACGAATGAGAT3′; antisense, 5′ATTTAGAGCTGTCTGGCTT3′.

flt-1, sense, 5′CTCTGATGGTGATCGTGG3′; antisense, 5′CATGCGTCTGGCCACTTG3′.

c-*fms*, sense, 5′CACAGATAAAATTGGAGCCTA3′; antisense, 5′TGGAAGTTCATGGTGGCCG3′.

PECAM-1, sense, 5′GTCATGGCCATGGTCGAGTA3′; antisense, 5′CTCCTCGGCATCTTGCTGAA3′.

β-major, sense, 5′CTGACAGATGCTCTCTTGGG3′; antisense, 5′CACAAACCCCAGAAACAGACA3′.

β-H1, sense, 5′AGTCCCCATGGAGTCAAAGA3′; antisense, 5′CTCAAGGAGACCTTTGCTCA3′.

neo, sense, 5′CATGATTGAACAAGATGG3′; antisense, 5′TCAGAAGAACTCGTCAAG3′.

hygro, sense, 5′TGCAAGACCTGCCTGAAACC3′; antisense, 5′CTGCTCCATACAAGCCAACCAA3′.

TEM analysis

Permanox (Nunc) tissue culture dishes were treated with 1% gelatin in PBS for 20 minutes and then coated with matrigel for 15 minutes. Blast colonies were picked onto the matrigel-treated area and cultured with 50 μl of medium. After 6-8 day in culture, adherent cells were processed as described by McDowell and Trump (1976). Briefly, the cells were fixed overnight in a phosphate buffer containing 4% formaldehyde/1% glutaraldehyde. Following the fixation step, the cells were postfixed in s-collidine buffer containing 1% OsO_4 for 1 hour, stained en block with uranyl acetate, dehydrated, and embedded in polybed 812 (Polyscience). Thin sections were cut, stained with uranyl acetate and lead citrate and then examined using a JEOL EX 1200 transmission electron microscope.

RESULTS

Developmental potential of blast colonies in liquid culture

Blast cell colonies were generated from 3.0- to 3.25-day-old EBs in methyl cellulose cultures in the presence of VEGF or VEGF and KL as described previously (Kennedy et al., 1997). Following 4 days of growth, individual colonies were transferred to liquid culture in microtiter wells containing cytokines known

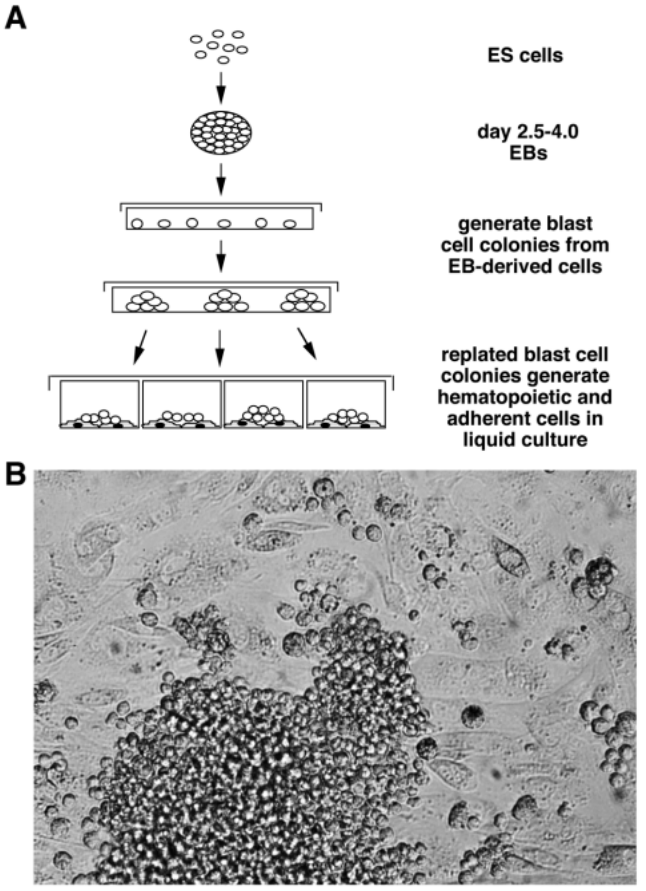

Fig. 1. (A) Scheme of derivation of adherent and hematopoietic cells from blast colonies. (B) Microtiter well containing adherent and round hematopoietic cells 4 days following transfer of the blast colony (magnification ×64).

to support the growth of both hematopoietic and endothelial cells (Fig. 1A). Within 2-4 days of culture in these conditions, both adherent and round non-adherent cells developed from approximately 1/3 of the transferred blast cell colonies (Fig. 1B). Most of the remaining blast colonies generated only non-adherent cells. The non-adherent population in most wells showed signs of hemoglobinization, demonstrating active erythropoiesis. To determine the full hematopoietic potential of the non-adherent population, cells were harvested following 3-4 days of culture and assayed in methyl cellulose in the presence of a broad spectrum of hematopoietic cytokines. From the analysis of 300 cultures containing both non-adherent and adherent cells, approximately 80% were found to contain hematopoietic precursors. The majority of these cultures (97%) had precursors for more than 2 lineages and of these 27% showed both primitive and definitive hematopoietic potential. Colonies containing primitive erythroid cells were identified by cell morphology and by the presence of βH1 globin gene expression. In addition to the predominant multilineage pattern of hematopoietic development, a small number of the cultures (3%) generated only primitive erythroid precursors. Most of cultures that contained no precursor activity when assayed in methyl cellulose (approx. 20% of total) did show some visible erythropoiesis on the adherent cells suggesting the blast colonies seeded in these wells had limited hematopoietic potential.

The morphology of the cells in the adherent layer was somewhat heterogeneous, varying from densely packed medium-sized cells to relatively large, elongated flat cells with a fibroblastic morphology (Fig. 2C). Clusters of macrophages were present in some cultures growing on the fibroblastic type cells. They were easily distinguished from this latter population by their smaller size and less flat morphology. To determine if the elongated flat cells were of the endothelial lineage, adherent populations were analyzed by immunofluorescence, RT-PCR and transmission electron microscopy for cell surface markers, gene expression patterns and subcellular cytoplasmic structures indicative of endothelial cells.

Blast colony-derived adherent cells display endothelial characteristics

Endothelial cells express the platelet endothelial cell adhesion molecule, PECAM-1 or CD31 (Newman, 1994) and display the capacity to take up acetylated low-density-lipoprotein (Ac-LDL, Voyta et al., 1984). Although platelets and megakaryocytes also express CD31 and macrophages can take up Ac-LDL, other types of adherent cells such as fibroblasts are negative for these markers. In the initial analysis, adherent cells derived from blast colonies were analyzed by immunofluorescence for the presence of CD31 and for their ability to take up fluorescenated Ac-LDL, DiI-Ac-LDL. D4T endothelial cells and NIH3T3 fibroblasts were included as positive and negative controls, respectively. Fig. 2 shows the morphology of the cells in the three different populations (A,C,E) and their corresponding staining patterns (B,D,F). D4T cells express high levels of CD31 (green) and take up DiI-Ac-LDL showing a punctate pattern of fluorescence (orange) characteristic of endothelial cells (Fig. 2B). Similarly, blast colony-derived adherent cells express abundant levels of CD31 and also show DiI-Ac-LDL uptake, although they appear to be less active in this capacity than the control endothelial cells (Fig. 2D). This different DiI-Ac-LDL uptake pattern between endothelial D4T and blast-derived adherent cells could reflect differences in the state of maturation of these cell populations. NIH3T3 cells did not express any significant CD31 and were unable to take up DiI-Ac-LDL (Fig. 2F). Macrophages, when present, were easily identified by the fact that they did not express CD31 and that their DiI-Ac-LDL staining was much more intense and homogenous than observed in the larger adherent cells (not shown). Similar staining patterns were observed in 5 independent experiments. The finding that adherent cells from blast colonies express CD31 and take up DiI-Ac-LDL strongly suggests that they are of the endothelial lineage.

In the next study, blast-derived adherent cells were analyzed by RT-PCR for the expression of *flk-1*, *flt-1*, and *tie-2* (also known as *tek*), three genes which encode receptor tyrosine kinases involved in the growth and development of endothelial cells (Matsushime et al., 1987; Shibuya et al., 1990; Dumont et al., 1992 and 1993; Sato et al., 1993; Millauer et al., 1993; Shalaby et al., 1995; Fong et al., 1995). The analysis also included PECAM-1 (CD31) as an additional marker for endothelial cells, c-*fms* as a marker for the presence of macrophages and βH1 and βmajor as markers for contaminating erythroid cells in the adherent population. As a comparison, the expression pattern of these genes was analyzed

in the non-adherent hematopoietic populations. The analyses of 15 adherent and 15 hematopoietic populations from two independent experiments are shown in Fig. 3. Fourteen of the adherent cell samples contained elongated flat cells, some with varying numbers of contaminating macrophages (lane 1 and lanes 3-15). One population consisted predominantly of macrophages (lane 2). Thirteen of the fourteen populations with flat cells expressed readily detectable levels of the three receptor genes, *flk-1*, *tie-2* and *flt-1* as well as high levels of PECAM-1. Four of these populations (lanes 1, 3, 4, and 13) showed no c-*fms* expression, indicating a lack of contaminating macrophages while the remaining ten showed some macrophage component. In contrast to these patterns, the macrophage population (lane 2) expressed abundant levels of c-*fms* but showed little if any expression of the other genes. None of the adherent populations expressed either βH1 or βmajor. All fifteen hematopoietic populations analyzed expressed low levels of *flk-1* while only six showed any detectable levels of *tie-2*. Significant levels of *flt-1* were present in most of the hematopoietic populations and all expressed PECAM-1. Expression of these receptor genes in the hematopoietic populations could reflect the presence of low levels of contaminating adherent cells or the fact that some of these receptors are expressed by subpopulations of early hematopoietic cells (Fong et al., 1996; Kabrun et al., 1997). The expression of c-*fms*, βmajor and βH1 in most or all of the hematopoietic populations is consistent with the presence of cells of the macrophage and erythroid lineages. Together the findings from the gene expression analysis provide further evidence that the blast colony-derived adherent population contains endothelial cells.

As a final study, adherent cells were analyzed by transmission electron microscopy (TEM) for sub-cellular structures characteristic of endothelial cells. One such structure is the Weibel-Palade (WP) body (Weibel and Palade, 1964) which is found exclusively in vascular endothelium. Adherent cells from eight different blast colonies analyzed showed structures morphologically similar to WP bodies (Fig. 4). These structures consisted of single membrane bound, cylindrical, rod-like bodies which contained a number of microtubules in an electron dense matrix. They were very often seen in close association to the Golgi complex, as previously reported (Ghadially, 1988). The average length of these organelles was 1.3 μm and the average diameter was 0.1 μm, well within the range of reported size of WP bodies (Ghadially, 1988). Other ultrastructural features that suggested the adherent cells were endothelial were the presence of broad intercellular junctions and relatively abundant pinocytotic vesicles (not shown). The findings from the TEM studies together with the gene expression and immunofluorescence analysis demonstrate that endothelial cells are present within the blast colony-derived adherent cell population.

Hematopoietic and endothelial cells develop from a common precursor

The generation of hematopoietic and endothelial cells from the same blast colony suggested that both lineages are derived from

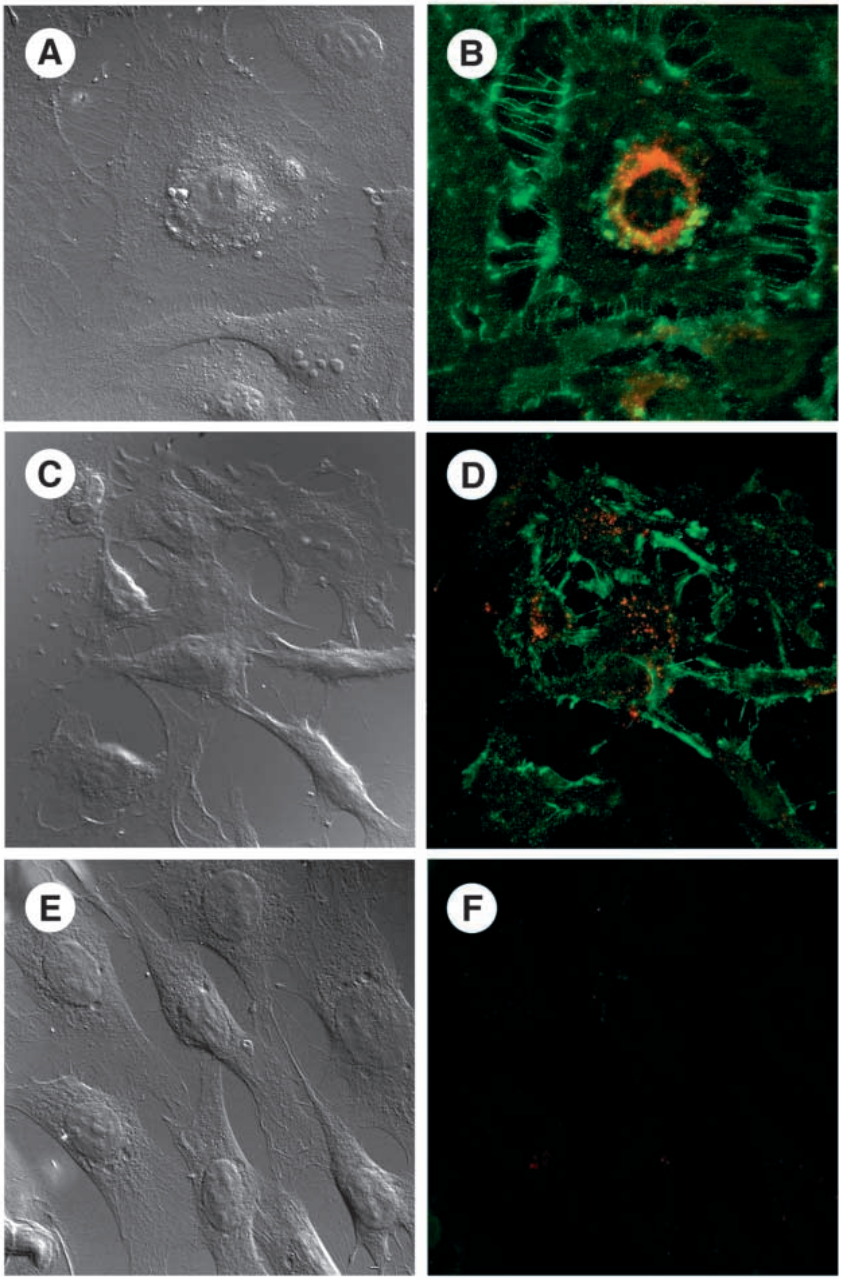

Fig. 2. CD 31 staining and DiI-Ac-LDL uptake of adherent cell populations. A,C and E show the morphology of (A) D4T endothelial cells, (C) blast colony-derived adherent cells (E) and NIH-3T3 cells under Nomarski optics. B,D and F show the same (B) D4T, (D) blast colony-derived adherent cells and (F) NIH-3T3 cells stained for the presence of CD31 (green) and analyzed for the ability to take up DiI-Ac-LDL (orange).

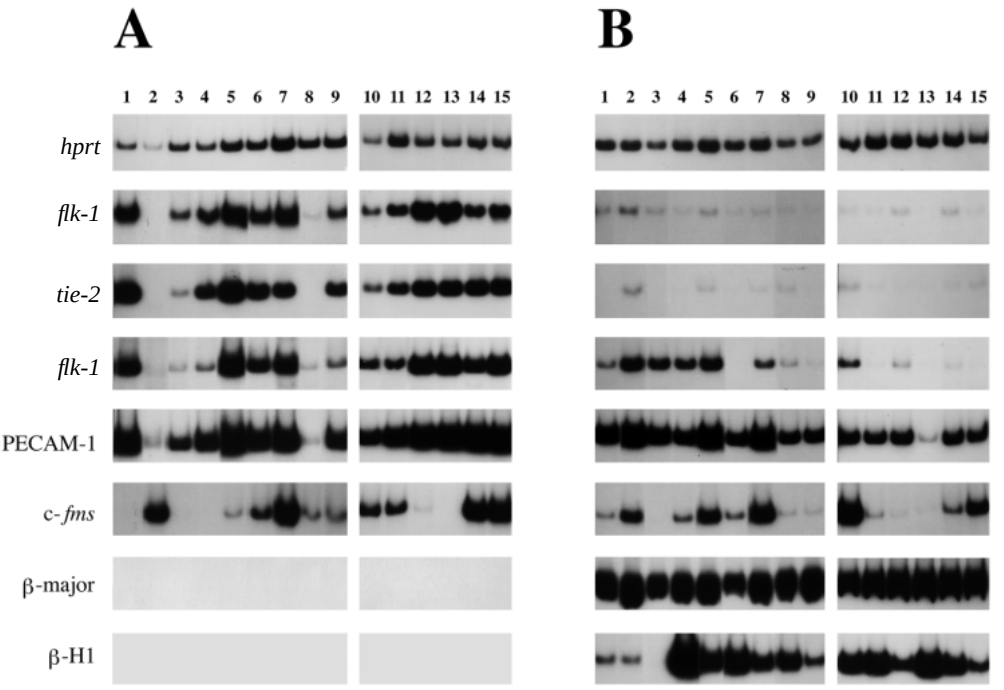

Fig. 3. RT-PCR analysis. Two independent experiments (lanes 1-9 and 10-15) are shown. A, adherent cells; B, hematopoietic cells. Hematopoietic cells represent the non-adherent cell fraction harvested 3-4 days following transfer of the blast colonies. Adherent cells were harvested following a further 2 weeks in culture. Size of each RT-PCR product is as follows; *hprt*, 249 bp; *flk-1*, 239 bp; *tie-2*, 197 bp; *flt-1*, 317 bp; c-*fms*, 274 bp; PECAM-1, 260 bp; β-major, 578 bp; β-H1, 265 bp. Primers for *hprt*, βmajor, and βH1 span the intron, such that approx. 1,100 bp (*hprt*), 1296 bp (βmajor), and 1,053 bp (βH1) genomic DNA fragment will be amplified. None of the samples showed genomic DNA amplification (not shown).

a common precursor, provided that these colonies represent clones. Our previous study demonstrated the clonal origin of primitive and definitive hematopoietic precursors within the blast colonies (Kennedy et al., 1997). To determine if the endothelial and hematopoietic cells derived from the blast colonies belong to the same clone, we used a cell mixing strategy similar to the one used in our previous study. Cells from EBs differentiated from 2 ES cell lines, one carrying the *neomycin* (*neo*) resistance gene and the second carrying the *hygromycin* (*hygro*) resistance gene were mixed for the generation of blast colonies. Adherent endothelial cells and hematopoietic cells generated from individual blast colonies from these mixed cultures were analyzed for the presence of the *neo* and *hygro* genes (Fig. 5). The progeny of 46 blast colonies were analyzed by this approach. Endothelial and hematopoietic populations from 44 of these blast colonies were found to contain the same marker (24 pairs with *neo*, 20 with *hygro*). In one instance, a small *neo* signal was detected in the hematopoietic component of a colony that showed strong *hygro* signals in both populations while a second colony showed almost equal mixing in both the adherent and hematopoietic populations. The fact that the vast majority of endothelial and hematopoietic cells from individual blast colonies contain the same marker, strongly suggests that they arise from a common precursor.

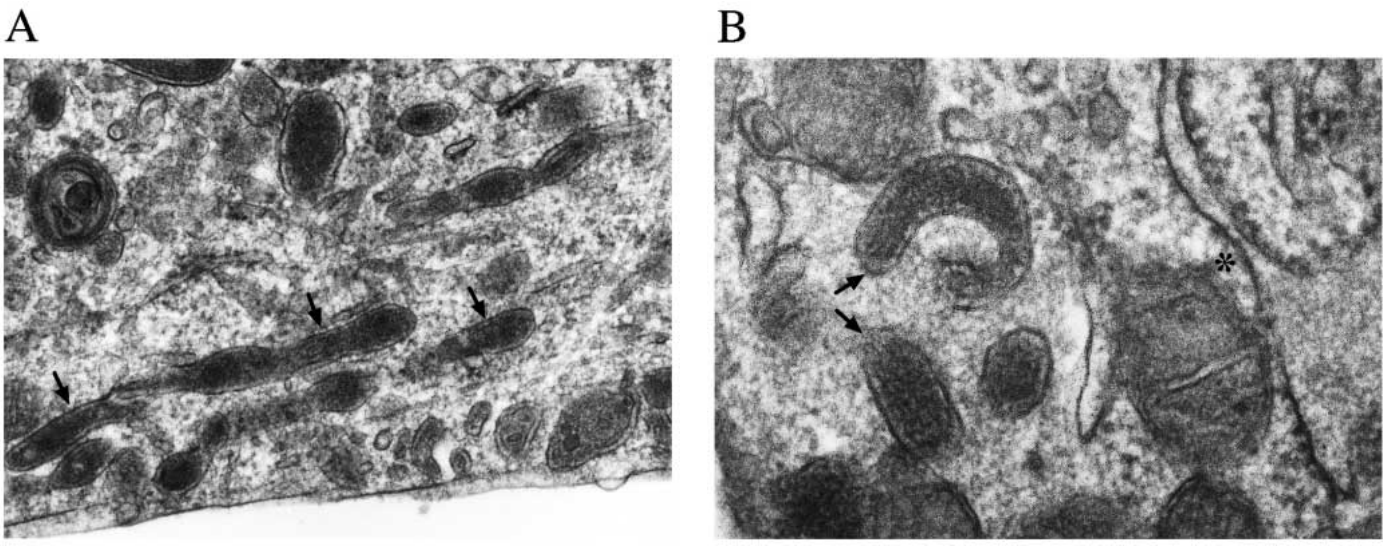

Fig. 4. TEM photomicrographs of adherent cells generated from blast colonies. Rod-shaped, single membrane bound structures with striated microtubules are indicated by arrows. Double membrane bound mitochondria, indicated by asterisks, are shown for comparison. Magnification (A) ×62,500; (B) ×150,000.

The BL-CFC represents a transient cell population

As indicated earlier, only a portion of the blast colonies from day 3 EBs contain both endothelial and hematopoietic precursors, while others appear to be restricted to the hematopoietic lineages. These different potentials could reflect differences in the stage of maturation of the blast colony-forming cells within the day-3 EBs, with those showing both hematopoietic and endothelial potential representing an earlier stage of development than those restricted to hematopoiesis. If this is true, younger EBs might be expected to contain a higher proportion of bi-potential precursors. To test this possibility, blast cell colonies generated from EBs differentiated for various times were analyzed for the presence of both endothelial and hematopoietic precursors. As shown in Fig. 6, approximately 75% of the blast colonies from day 2.5 EBs were able to generate both lineages. The number of blast colonies with both hematopoietic and endothelial potential dropped significantly over the next 12-24 hours of EB development. In 2 of the 3 experiments, a higher proportion of blast colonies from the later stage EBs were restricted to hematopoiesis compared to those generated from earlier EBs (not shown). The findings from this kinetic analysis indicate that BL-CFCs with both endothelial and hematopoietic potential represent a transient population that develops early and persists for short periods of time in differentiating EBs. Furthermore, they indicate that the development of the bi-potential BL-CFC precedes the onset of hematopoiesis (Kennedy et al., 1997) and vasculogenesis (Vittet et al., 1996) within the EBs.

DISCUSSION

We demonstrated in this study that blast cell colonies generated from ES cell-derived embryoid bodies contain both hematopoietic and endothelial cell precursors. Our previous studies have shown that these blast cell colonies contain both primitive and definitive hematopoietic precursors and as such provided the first link for these lineages through the BL-CFC. The demonstration of endothelial precursors in the blast colonies adds a new and important dimension to their developmental potential and strongly suggests that the BL-CFC represents the long-hypothesized hemangioblast.

BL-CFCs likely represent a population of bipotential precursors restricted to the hematopoietic and endothelial lineages. However, it is possible that these cells have other potentials that do not develop in our assay or that are lost as the blast cell colonies develop. In preliminary experiments aimed at addressing this question we found that blast colonies are unable to generate cardiac muscle whereas age-matched EBs from the same cultures efficiently generate

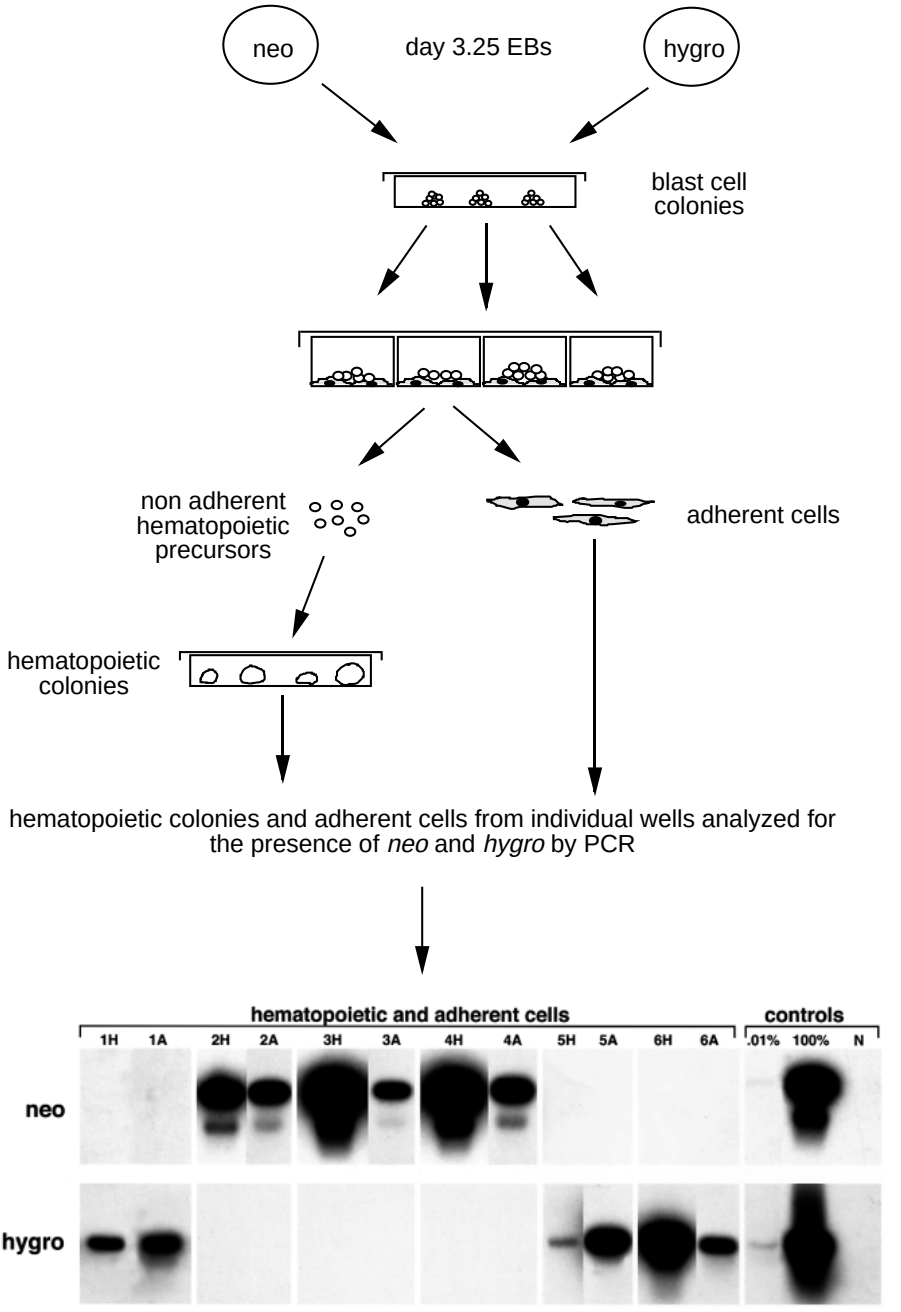

Fig. 5. PCR analysis of hematopoietic and adherent cells from blast colonies generated from a mixture of *neo* and *hygro* containing EBs. Hematopoietic colonies were generated from the non-adherent population that developed 3 days following blast colony transfer. Adherent cells were harvested for analysis following an additional 2 weeks in culture. Analysis of hematopoietic (H) and adherent (A) cells from 6 individual colonies is shown. Controls represent either the *neo* or *hygro* containing ES cells alone (100%) or 0.01% of each population mixed with the opposite population. N represents a sample with all the reagents but no DNA.

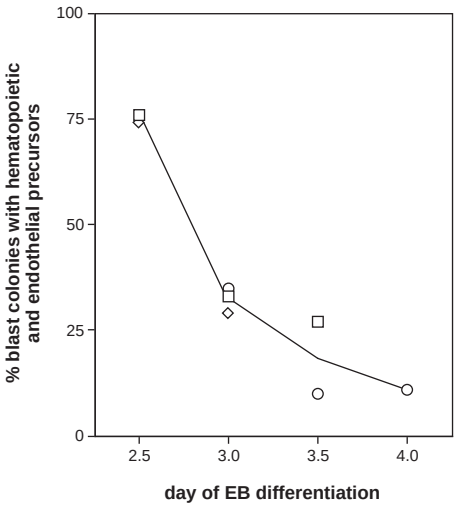

Fig. 6. Kinetic analysis of developmental potential of the BL-CFC. Blast colonies from EBs at different stages of development were transferred to liquid culture and the wells containing both hematopoietic and endothelial cells were scored. Different symbols represent results from three different experiments.

such cells under appropriate conditions. These findings together with previous gene expression analysis demonstrating significant differences between blast cell colonies and EBs (Kennedy et al., 1997) are consistent with the interpretation that the BL-CFCs do indeed have restricted developmental potential. Further characterization of the developmental potential of this putative hemangioblast will require isolation of these precursors directly from the differentiating EBs. The expression of the Flk-1 receptor could provide an ideal marker for their isolation (Kabrun et al., 1997).

The early development of the BL-CFCs within the EBs along with their capacity to generate both hematopoietic and endothelial cells suggests that this population represents the in vitro equivalent of the precursors that establish the yolk sac blood islands in the embryo. The findings that blast colonies develop in response to VEGF strongly suggest the VEGF/Flk-1 interaction is important for the proliferation and differentiation of these precursors in vivo. This interpretation is consistent with gene targeting studies which demonstrate a severe impairment in the development of both the hematopoietic and endothelial lineages in mice lacking Flk-1 or VEGF (Shalaby et al., 1995, 1997; Carmeliet et al., 1996; Ferrara et al., 1996). In addition to a role in proliferation of the putative hemangioblast, the Flk-1 receptor appears to be involved in cell migration as the *flk-1*$^{-/-}$ cells are unable to colonize the developing yolk sac region of these embryos (Shalaby et al., 1997). While it is clear that the interaction of VEGF with Flk-1 is essential for the development of functional blood islands, the residual hematopoietic activity observed in *flk-1*$^{-/-}$ embryos and the presence of both hematopoietic and endothelial cells in VEGF$^{+/-}$ embryos suggests that these molecules are not essential for the commitment of mesoderm to these lineages and that other factors are involved in these early developmental steps. Access to the BL-CFC in vitro provides a novel assay system for such molecules.

The observation that both hematopoietic and endothelial cells develop in individual wells seeded with single blast colonies differs from recent studies of Eichmann et al. (1997) which showed that VEGFR2$^+$ (Flk-1$^+$) chick embryo cells are able to generate either endothelial cells when cultured in the presence of VEGF or hematopoietic cells when cultured without VEGF. Mixed hematopoietic and endothelial cultures were not found in their analysis. The differences from our findings could be related to the fact that the VEGFR2$^+$ cells were already committed to the endothelial or hematopoietic lineages or that conditions able to support the growth of both populations simultaneously have not yet been defined for chicken cells. Given the transient nature of the BL-CFC within the EBs in culture, it is likely that a similar narrow window of development would exist for these precursors in either the chick or mouse embryo. Consequently their isolation from early embryos by cell sorting prior to commitment to the hematopoietic and endothelial lineages might be difficult, if not impossible. Growth of blast cell colonies from developing embryos provides an alternate approach for their identification. Preliminary studies indicate that BL-CFCs are present in the developing mouse embryo for short periods of time, prior to the establishment of the blood islands (J. Palis, G. Keller et al., unpublished observation). The full developmental potential of these embryo-derived BL-CFC is currently under investigation.

In summary, we have identified a novel ES cell-derived embryonic precursor population with both hematopoietic and endothelial potential. These precursors develop early during EB development and persist for a short period of time, preceding the onset of hematopoiesis and vasculogenesis. The identification and characterization of the BL-CFC in conjunction with the availability of many different knock out ES cell lines provides a unique opportunity to delineate the role of specific genes in the establishment of hematopoiesis and vasculogenesis.

We thank P. Faloon, D.H. Fremont, D. M. Pardoll, S. Robertson, A. Smith, and C. Wall for critical comments on the manuscript; J. Yardley, S. Chang, with TEM studies and T. Kolesnikova and L. Landskroner for assistance with graphics and photography. The authors would also like to thank N. Johnson and C. Monks for assistance with the analysis of the immunoflourescent images. This work was supported by NIH grants R01HL488344 (G. K.) and R29HL55337 (K. C.).

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