

Role of Endothelial Cells in Human Hematopoiesis: Modulation of Mixed Colony Growth In Vitro

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The identification of clonal human multipotent hematopoietic progenitors has permitted an analysis of the growth factor requirements for these cells. Human endothelial cell cultures were used to examine the effects of media conditioned by the endothelial cells on human multipotent (CFU-mix) and committed erythroid (BFU-E, CFU-E) and myeloid (CFU-GM) precursors. These studies demonstrate that endothelial cells produce proteins of approximately 30,000 daltons, with isoelectric focusing points of 4.5 and 7.2,

which stimulate the growth of human BFU-E and CFU-mix. A heat-labile protein(s) of 30,000 and 15,000 daltons stimulated the proliferation and differentiation of granulocyte-macrophage (CFU-GM) colonies. No erythropoietin was detected in endothelial cell supernatants. This suggests that endothelial cells, a normal component of marrow stroma, play an active role in the modulation of human hematopoietic stem cell growth.

ENDOTHELIAL CELLS (EC) are recognized components of human marrow stroma¹ and have been identified as constituents of the adherent layer in long-term cultures of human and murine marrow.^{2,3} The role of these cells in hematopoiesis is not known. There is clinical and experimental evidence, however, that suggests that EC may play a role in the control of blood cell production. The marrow failure of the S1/S1^d mouse strain,⁴ certain cases of congenital hypoplastic anemia,⁵ and in some instances, radiation-induced vasculitis with subsequent hypoplastic pancytopenia⁶ may represent such an association. Furthermore, EC supernatants contain an activity capable of stimulating the development of granulocyte-macrophage progenitors (CFU-GM) in vitro.^{7,8}

In order to assess further the role of EC in hematopoiesis, cultured EC from human umbilical cord vessels were examined for production of hematopoietic factors required for the maintenance and proliferation/differentiation of various hematopoietic precursors. Specifically, the effects of the cells and media conditioned by EC (HuEndo-CM) on the growth of human bone marrow multipotent stem cells (CFU-mix), the erythroid committed progenitors (BFU-E and CFU-E), and CFU-GM in vitro were examined. In addition to erythropoietin (Ep) and colony-stimulating activity (CSA), the optimal growth of CFU-mix in vitro has been demonstrated to depend on the presence of a factor in PHA-LCM (media conditioned by leukocytes in the presence of phytohemagglutinin).⁹ BFU-E are profoundly influenced by a factor designated as burst-promoting activity (BPA), whereas CFU-E primarily require Ep for growth and differentiation.¹⁰

These results demonstrate that EC do not form Ep, but produce factors that stimulate the growth of human CFU-mix, BFU-E, and CFU-GM in vitro. Preliminary experiments to separate these various factors suggest that the CFU-mix hemopoietin is similar to BPA but distinct from CSA.

MATERIALS AND METHODS

Preparation of Mononuclear Cells From Human Bone Marrow (BMNC)

Heparinized (1,000 U/ml) bone marrow was aspirated from paid normal human volunteers after informed consent was obtained. The procedure was approved by the subcommittee on human studies at the Minneapolis VA Medical Center.

The marrow was diluted with Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY) layered over Ficoll-Hypaque cushions (density, 1.077) and centrifuged at 400 g for 30 min. The interface (BMNC) cells were collected, washed twice, counted, and then used as previously described.¹¹ Nonadherent cells were obtained from BMNC: 4×10^6 cells/ml in Iscove's media with 20% fetal calf serum (FCS) (Reheis Labs, Kankakee, IL) was incubated in 25-ml tissue culture flasks for 1 hr at 37°C in 5% CO₂. The nonadherent cells were removed, washed twice, counted, and used.

Hematopoietic Stem Cell Assays

CFU-mix assay. A quantity of $1-1.5 \times 10^5$ BMNC/dish was cultured in the presence or absence of 1-2 IU Ep in triplicate 1-ml aliquots in 35-mm dishes containing 1% methylcellulose, 30% FCS, Iscove's medium, 50 μ M mercaptoethanol with or without 7.5% PHA-LCM, or 2.5%-10% human endothelial cell conditioned medium (HuEndo-CM).⁹ After 15-day incubation at 37°C in 5% CO₂, 100% humidified air, colonies were counted and analyzed for constituent cells using a dissection microscope. CFU-mix colonies were defined as colonies of erythroid and leukocytic elements, with or without megakaryocytes, as opposed to CFU-GEMM-derived

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colonies, which contain all 4 elements.^{12,13} Additional studies involved the culturing of nonadherent BMNC (NA-BMNC) in the presence or absence of Ep, PHA-LCM, and/or HuEndo-CM. In these studies, all nonadherent cells present in 10^5 BMNC were cultured without further adjustment. Therefore, the number of colonies reflects the numbers present in 10^5 BMNC.

BFU-E and CFU-E assays. Erythroid progenitors were assayed in methylcellulose cultures and in plasma clot cultures, as described by Tepperman et al.¹⁴ BMNC ($2-6 \times 10^5$ cells/1.1 ml) were cultured in the presence or absence of 0.5–3 IU Ep. The Ep (25 IU/mg) used in these studies was prepared from human urine and did not contain significant BPA or CSA activities. The cultures were examined at days 7 and 15 for CFU-E- and BFU-E-derived colony formation, respectively.

CFU-GM assay. Nonadherent light-density marrow cells were plated in quadruplicate 1-ml cultures at 10^5 cells/dish in 0.3% agar with or without a source of CSA (placental conditioned medium, 50 μ l/dish) for 7–14 days at 37°C, 7.5% CO₂, humidified air, and colonies of greater than 40 cells were enumerated using a dissection microscope.¹¹ Day 15 colonies were also enumerated in the methylcellulose plates.

Endothelial Cells and Preparation of Conditioned Medium

The endothelial cells were obtained from human umbilical veins after collagenase digestion (Sigma, St. Louis, MO) for 20 min at room temperature as described by Jaffe.^{15,16} After washing, cells were resuspended in "cord media," which contained Dulbecco's modified Eagle's medium, 100 U penicillin, and 100 μ g streptomycin/ml, 2 mM L-glutamine, and 20% calf serum (GIBCO, Grand Island, NY). The cells from each cord resuspended in 8 ml of cord media were plated in a 25-ml tissue culture flask (Falcon, Curtin-Matheson, Minneapolis, MN) or in 1-ml aliquots in 35-mm dishes (Lux, Biolabs, Northbrook, IL) and incubated at 37°C in 5% CO₂ humidified air for 3–6 days until confluent. Immunofluorescence with factor VIII antibody assured the endothelial nature of these cells. Supernatants were then harvested, and serum-free supernatants were subsequently prepared by adding Iscove's medium to the confluent endothelial cells and incubating for another 24–48 hr.

Feeders of EC were prepared by overlaying 1 ml of 0.5% agar in Iscove's medium with 20% FCS in each of the 35-mm dishes at time of confluence. The target BMNC were overlaid in triplicate cultures in 1% methylcellulose containing media with 50 μ M, 2-mercaptoethanol, Iscove's media with 30% FCS, 1.5 U Ep, but without PHA-LCM. The cultures were incubated and colonies counted at day 15.

Erythropoietin Bioassay

Mice rendered hypoxic by exposure to 0.4 atm (19 hr/day, total 219 hr) were injected with test material (1 ml/mouse, 4–5 mice/group) on day 5 posthypoxia. Radioiron (0.5 μ Ci/mouse) was administered on day 7, and the percent RBC ⁵⁹Fe uptake was determined 72 hr later.¹⁷

Column Chromatographic Separation

Two milliliters of HuEndo-CM, dialyzed and concentrated on Amicon YM 10 membranes and containing 14 mg protein was applied to a 1.5 \times 80 cm column of Sephadex G75 (Pharmacia, Piscataway, NJ), equilibrated with 0.02 M phosphate-buffered saline (PBS, pH 7.4), and eluted with 150 ml of PBS; 3-ml fractions were collected at a rate of 6 ml/hr. Fractions were pooled, concentrated with an Amicon B-15 apparatus, sterilized by filtering through a 0.45- μ Millipore filter, and assayed for hematopoietic

stimulating activities. Standard markers included blue dextran (void volume); bovine serum albumin (67,000 daltons); ovalbumin (43,000); cytochrome C (30,000); and carbonic anhydrase (13,000). The protein content of each fraction was measured spectrophotometrically at 280 nm.

Isoelectric Focusing

Flat-bed isoelectric focusing in granulated gel was performed using an LKB 2117 Multiphor apparatus. Three milliliters of dialyzed (against 0.02 M PBS) HuEndo-CM, containing 0.1 M glycine, was applied to a dried gel containing 5 g of Ultradex gel (LKB, Rockville, MD) and 5 ml of pH 3.5–9.5 ampholine (LKB)/100 ml of slurry. After focusing for 18 hr, using 1,050 V (8 W constant power) at 10°C, the fractions were separated with a grid. They were diluted with H₂O and tested for pH. They were then extracted with 3 ml of a 50-mM phosphate-buffered saline, dialyzed overnight against PBS concentrated on Amicon B15 filters, sterilized by filtering through a 0.45- μ Millipore filter, and assayed.

RESULTS

The development of mixed hematopoietic colonies in vitro requires the presence of a factor present in PHA-LCM, or alternatively, a factor produced by EC. Thus, as found by others as well,⁹ Ep alone does not support the growth of CFU-mix colonies (Table 1). In the presence of PHA-LCM, however, approximately 4 mixed colonies/ 10^5 cells are detected (Table 1). Moreover, PHA-LCM is not an absolute requisite factor, as, even in its absence, a feeder layer of EC will suffice, providing more than twice the number of CFU-mix-derived colonies than is seen with PHA-LCM (Table 1).

A soluble factor from cultured EC, termed HuEndo-CM, also acted as a CFU-mix stimulant (Table 1). When assayed in dose-response studies, the stimulant promoted optimal CFU-mix colony growth at a concentration of approximately 5% (v/v) (Fig. 1). In a number of different experiments, this concentration of HuEndo-CM formed a number of colonies similar to that of 7.5% (v/v) PHA-LCM, which is the optimal concentration and, as such, serves as the routine standard in our laboratory (Fig. 1). This activity of HuEndo-CM was also seen using light-density nonadherent bone marrow cell populations.

A somewhat different effect of HuEndo-CM on human bone marrow BFU-E was noted (Fig. 2, data

Table 1. Effect of Human Endothelial Cells (EC) and Supernatants (HuEndo-CM) on Human CFU-mix Growth In Vitro

Additions to Culture	Colonies/ 10^5 Cells*
1.5 IU Ep ($n = 4$)†	0
1.5 IU Ep + 7.5% PHA-LCM ($n = 6$)	3.8 ± 0.5 (2–8)‡
1.5 IU Ep + 5% HuEndo-CM ($n = 6$)	4.9 ± 0.5 (2.6–11)
1.5 IU Ep + EC as feeders ($n = 2$)	8.5 ± 0.8 (3–16)

*Mean \pm SEM.

†Number of experiments.

‡Range of colonies seen in each individual culture assay.

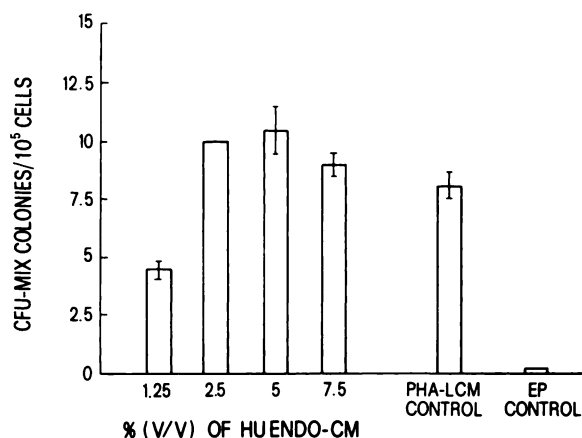


Fig. 1. Effect of HuEndo-CM on human CFU-mix growth in vitro. Bone marrow cells at 10⁵/plate were cultured in 1% methylcellulose containing GEMM media (see Materials and Methods for details) with or without PHA-LCM. Cultures with HuEndo-CM did not contain PHA-LCM. Results are expressed as mean ± SEM of triplicate determinations from a representative experiment.

from one representative experiment). Replicate experiments were performed on three occasions. Low concentrations of HuEndo-CM stimulated the formation of erythroid bursts; however, this stimulatory effect was lost when doses of HuEndo-CM greater than 2.5% (v/v) were employed. The relative decrease in detectable burst-promoting activity of HuEndo-CM at higher concentrations was probably caused by the presence of a heat-labile inhibitor substance, as similar concentrations of a heat-inactivated preparation of HuEndo-CM (boiled for 5 min) subsequently manifested significant BPA activity (see Fig. 2, "H.I."). Alternatively, the heat treatment destroyed some of the BPA, resulting in a lower concentration equivalent. Using the methylcellulose assay, a significant increase in the number of bursts was also seen in cultures containing HuEndo-CM (9 ± 0.9) and PHA-LCM (13.6 ± 1.3) compared to control cultures containing

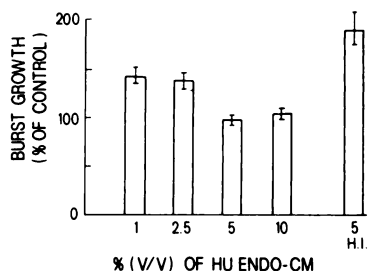


Fig. 2. Effect of human endothelial cell conditioned medium on human erythroid burst (BFU-E) growth in vitro. 5 × 10⁵ marrow cells/plasma clot were cultured for 14 days in the presence of 1.5 IU Ep. Results are from a representative experiment, and the values express mean ± SEM of quadruplicate determinations. "H.I." represents a sample of HuEndo-CM boiled for 5 min. Control: 79 ± 2 bursts/10⁵ cells.

Table 2. Effect of Human Endothelial Cell Conditioned Medium (HuEndo-CM) on Erythropoiesis in Mice

Material Assayed*	Percent RBC ⁵⁹ Fe Incorporation†
Saline	0.34 ± 0.04
0.4 IU Ep	6.54 ± 1.22
HuEndo-CM	0.52 ± 0.21

* 1 ml/mouse.

† Mean ± SEM.

Ep (0.3 U/ml) alone (2 ± 0.1). These results also suggest that BPA is active at low concentrations of Ep.

When the effect of HuEndo-CM on human marrow CFU-E was studied, the results were not consistent. Although, on occasion, some preparations of HuEndo-CM enhanced the growth of CFU-E colonies, mainly it did not (results not shown). We wondered whether HuEndo-CM might have Ep activity, but we were unable to detect it either in vitro (not shown) or in the exhypoxic polycythemic mouse assay system (Table 2). The in vitro assay for Ep utilized the erythroid colony-forming ability of adult sheep marrow cells.¹⁸ This assay is sensitive to concentrations of Ep as low as 0.001 IU/ml, but is quantitative at 0.01 IU/ml level. By contrast, HuEndo-Cm contained significant CSA activity (Fig. 3). As previously demonstrated,¹¹ BMNC depleted of monocytes do not give rise to colonies in the absence of exogenous CSA. Addition of 5% (v/v) placental conditioned medium produces 23 ± 1.2 colonies/10⁵ cells (average of 3 separate experiments), whereas a similar concentration of HuEndo-CM is even more active (35 ± 1.4 colonies/10⁵ cells) and the material stimulates granulocyte/macrophage colony growth in a direct dose-response fashion (Fig. 3). The endothelial-cell-derived CSA was a heat-labile protein destroyed by boiling for 5 min and was present in serum-free supernates from cultured EC (data not shown). The colonies produced with both sources of CSA (placental or endothelial-derived) appeared similar in morphology and size, except that an increase in granulocytic colonies (36%) was noted with HuEndo-CM compared to the standard placental CSA (17%) (Table 3).

Chromatographic separation of the various factors

Table 3. Morphology of Day 10 CFU-GM-Derived Colonies

Additions to Culture	Types of Colonies*		
	Monocytic (%)	Granulocytic (%)	Mixed (%)
HuEndo-CM†	22 ± 0.2	36 ± 0.7	42 ± 0.7
PCM‡	26 ± 1.5	17 ± 1	57 ± 1.5

* Mean ± SEM of triplicate determinations.

† Human endothelial cell conditioned medium.

‡ Placental conditioned medium (control CSA).

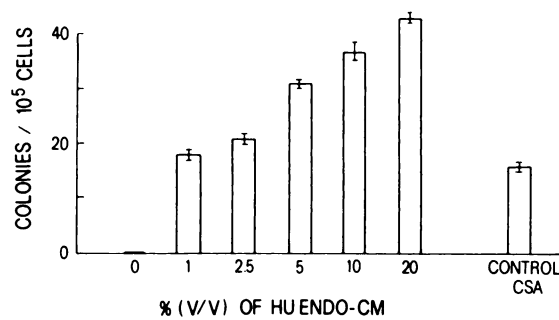


Fig. 3. Effect of HuEndo-CM on human CFU-GM growth in vitro. Results are from one representative experiment and are expressed as mean \pm SEM of quadruplicate cultures. 10^6 nonadherent BM cells/plate were cultured for 10 days in the presence or absence of a source of colony-stimulating activity (CSA). Control CSA represents 5% (v/v) of placental conditioned medium, which is used as the standard in this laboratory.

present in HuEndo-CM (Fig. 4) demonstrated that a peak around 30,000 daltons contained all three growth factors, that is, for CFU-mix, BFU-E, and CFU-GM. A second peak of mainly CSA activity was found in the 13,000–15,000 dalton range, while BPA was poorly separated by this technique. Likewise, isoelectric focusing was not able to clearly separate BPA from the CFU-mix growth factor, as each had similar peaks of activity at pH 4.5 and 7.2 (Fig. 5). No CSA could be detected in any of the fractions obtained with isoelectric focusing, and this was presumably due to precipitation and/or denaturation of this protein during this procedure.

Further experiments examined the possibility that mixed colony growth factor and BPA were similar proteins. We demonstrated that boiled HuEndo-CM (a source of BPA) was equally supportive of the growth

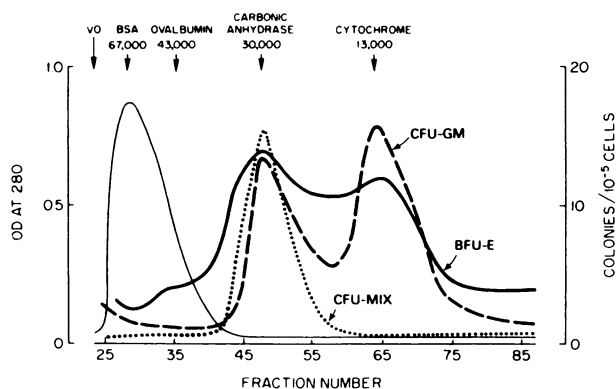


Fig. 4. Column chromatography (Sephadex G75) size separation of the hemopoietins present in HuEndo-CM. Colonies/ 10^6 marrow cells are calculated from the difference between baseline growth and the colony numbers seen with the addition of the various fractions. The standard markers included blue dextran (void volume—VO), bovine serum albumin (BSA) 67,000 daltons, ovalbumin, carbonic anhydrase, and cytochrome with molecular weights of 43,000, 30,000, and 13,000 respectively.

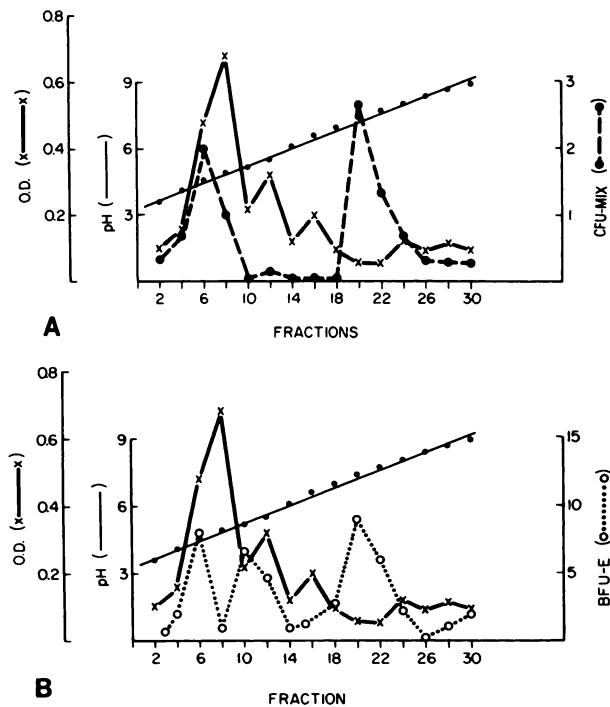


Fig. 5. Separation of hemopoietic growth factors in HuEndo-CM by isoelectric focusing. (A) CFU-mix colonies are expressed as the number of colonies seen after addition of each fraction to Ep containing CFU-mix culture media (without PHA-LCM). (B) BFU-Es were calculated as the number of bursts seen with each fraction added to Ep containing culture media; the number of bursts in the control sample (Ep alone) was subtracted from the above values to give the relative burst numbers expressed in the vertical axis.

of marrow CFU-mix-derived colonies (3.3 ± 2) as compared to PHA-LCM (2.2 ± 0.3) or HuEndo-CM (3.8 ± 0.7) controls.

DISCUSSION

The results presented demonstrate that human endothelial cells are capable of producing factors that sustain and promote development of human multipotential, as well as committed, hematopoietic progenitors in vitro, and as such, suggest that EC may play a regulatory role in the overall production of blood cells.

Endothelial cells represent an important component of the bone marrow microenvironment. In addition to their function as a structural scaffold, possibly controlling the egress of marrow-derived cells into the circulation,^{1,19} EC may serve to regulate, along with other humoral and cellular elements, the numbers of blood cells produced by the bone marrow. It is possible that EC performs this function via the synthesis and/or release of substances that affect the proliferative activity of the hematopoietic precursors. Our initial data also suggest that the effect of HuEndo-CM is not mediated via accessory cell populations, as we demon-

strated the growth of mixed colonies (1.7 ± 0.3 colonies/ 10^5 cells) in cultures of NA-BMNC containing HuEndo-CM. Control cultures with PHA-LCM grew 2.3 ± 0.3 colonies/ 10^5 cells, whereas cultures with Ep alone had no mixed colonies. Quesenberry et al.⁷ have demonstrated production of CSA by endothelial cells. These findings support this view and further indicate that the role of EC may not be limited to only controlling the activity of the granulocyte-macrophage progenitor (CFU-GM), but may also determine the proliferative activities of the multipotential (CFU-mix) as well as the early erythroid committed progenitors (BFU-E). Recently Gordon and coworkers⁸ reported on the effect of medium conditioned by human EC on hematopoietic precursors. They noted production of CSA, and BPA was detected when used at concentrations of 5%. They did not detect mixed colony stimulatory activity; however, the system they used for CFU-mix activity always contained PHA-LCM, a known stimulator of human multipotential colonies *in vitro*. Thus, their study differed from our studies in that we assayed for CFU-mix stimulatory activity in the absence of PHA-LCM.

Whether this function of EC in hematopoiesis is achieved by the production of a single substance that affects all three precursor types or through the synthesis of factors with different affinities for these cellular elements is not known. In this regard, we were unable to clearly separate the CFU-mix growth factor from the BPA and the CSA. All three activities were protease sensitive and not dialyzable, and cofractionated using chromatographic and isoelectric focusing separative methods. However, the CSA activity in the HuEndo-CM was found to be heat-labile, whereas both CFU-mix and the BPA activities persisted following boiling of the mixture for 5 min. This is similar to findings by other investigators demonstrating the heat-stability of BPA and the heat-lability of the CSA.^{11,20,21} Nevertheless, boiled preparations of the mixture were capable of promoting the development of mixed colonies in the presence of an exogenous source of CSA and Ep. This is in agreement with the data of Fauser et al.,²² who demonstrated that boiled preparations of medium conditioned by the T lymphocyte cell line, Mo (Mo-CM), or partially purified BPA preparations of the Mo-CM, were capable of stimulating human CFU-mix growth *in vitro*. The active Mo-CM peak had an

approximate molecular size of 40,000 daltons. Recently, Ruppert et al.²³ demonstrated a peak of approximately 35,000–45,000 daltons in PHA-LCM (as determined by SDS-gel electrophoresis) that stimulated the growth of pluripotent as well as committed precursor cells. Various preparations of CSA and BPA have been shown to have different molecular weights,^{20,21} and it is not surprising to expect similar differences for the CFU-mix growth factor(s).

Iscove and collaborators²⁴ suggest that the early stages of hematopoietic stem cell development may be controlled by molecules that are biochemically very similar and that functionally induce the acquisition of new or additional cell surface receptors for more specific differentiating agents such as erythropoietin. Our inability to separate the three factors produced by EC lends some support to this possibility.

The mechanism underlying the production of the hematopoietic factor(s) by EC is not known. Recently, Bagby et al.²⁵ have shown that a monokine may control the production of CSA by EC. Whether a similar mechanism controls the production of the other activities noted in the present studies is under investigation. Our preparations of EC did not contain monocytes or lymphocytes, as determined by morphological criteria.

It is clear from our studies that EC do not produce Ep. Media conditioned by EC were devoid of Ep activity, as determined by the exhypoxic polycythemic mouse assay and by the fact that they did not support the development of hemoglobinized cells in the absence of Ep in culture. This suggests that EC may play a role in early hematopoietic events, paving the way for the effects of terminal differentiation agents such as Ep. The recent, but still controversial, demonstration of the presence of endothelial cells in the adherent layer of long-term cultures of marrow cells^{2,3} lends additional support to a role of EC in hematopoiesis and may provide a unique tool to investigate the role of these cells in blood cell production in a more physiologic setting.

We speculate that abnormalities in such nurturing functions by endothelial cells may underlie the marrow failure in the S1/S1^d murine model (in which a defective marrow "soil" has been implicated),^{4,26} in rare cases of human congenital hypoplastic anemia,⁵ and in the hypoplastic anemias seen in association with vascular damage.⁶

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