Vascular Endothelium as a Regulator of Granulopoiesis: Production of Colony-Stimulating Activity by Cultured Human Endothelial Cells

By Peter J. Quesenberry and Michael A. Gimbrone, Jr.

Colony-stimulating activity is a regulatory factor(s) that promotes differentiation of hemopoietic stem cells to mature granulocytes and macrophages; in man it has been found that blood monocytes, lymphocytes, and tissue macrophages produce it. In an effort to identify other potentially physiologic tissue sources of colony-stimulating activity, we have studied the capacity of primary cultures of human vascular endothelial cells to produce colony-stimulating activity. Medium conditioned by incubation with endothelial cultures contained activity that promoted granulocyte-macrophage colony formation of nonadherent human and murine marrow cells. Exposure of endothelial cultures to 0.1–5.0 µg/ml S. typhosa endotoxin for 6–72 hr enhanced colony-stimulating activity production. Similarly, incubation of endothelial cells with lysates of human blood granulocytes, or cocultivation with intact granulocytes, resulted in increased colony-stimulating activity levels. In 7–14 day cultures, freshly isolated endothelial cells, incorporated into agar underlayers, consistently stimulated more colony formation by nonadherent human marrow cells than comparable numbers of blood monocytes. These data indicate that: (1) cultured human endothelial cells are a potent source of colony-stimulating activity; (2) they respond to endotoxin and granulocytes and their contents by producing increased amounts of CSA; and (3) they produce more colony-stimulating activity, than human blood monocytes under standardized conditions in vitro. These observations suggest that the vascular endothelium may play a role in the physiologic regulation of granulopoiesis.

COLONY-STIMULATING ACTIVITY (CSA) is necessary for the in vitro growth and differentiation of granulocytes and macrophages,1,2 and is the best candidate to date for a granulopoietin with in vivo physiologic relevance. Monocytes,3,4 macrophages,5 and activated lymphocytes6,7 are sources of CSA with probable in vivo significance, while other reported sources of CSA either have not been adequately characterized as to cell type, or were established cell lines or malignant cells in culture, situations in which physiologic relevance is unlikely.8,9

Knudtzon and Mortenson10 have reported studies suggesting that vascular endothelium might be capable of producing CSA in vitro, although in their experimental model contributions by other cell types were not effectively excluded. Recently developed techniques permit the selective isolation of homogeneous populations of human endothelial cells, which retain their differentiated phenotype in primary culture and are metabolically active.11 These cells are characterized by an epithelioid cytology, monolayer growth pattern, and the presence of endothelial specific organelles (Weibel-Palade bodies); in addition, the presence of angiotensin I converting enzyme and factor VIII antigen serve to distinguish them from smooth muscle cells and fibroblasts, the most probable contaminants in blood vessel-derived cultures.11–15 Thus, these short-term cultures appear to provide an excellent model for the in vitro study of normal endothelial cell physiology.

We have utilized primary cultures of human umbilical vein endothelial cells, free of contaminating cell types, to assess the capacity of vascular endothelium to produce CSA. We have also studied the effect of bacterial and granulocyte products on endothelial CSA production and directly compared the capacity of the endothelial cell and the blood monocyte to release and/or produce CSA under controlled conditions in vitro.

MATERIALS AND METHODS

Isolation and Culture of Endothelial Cells

Human endothelial cells (HEC) were isolated from segments of normal-term umbilical cord veins by limited collagenase digestion of the luminal surface, as previously described in detail.16,17 Cells from 2–6 vessel segments were pooled in Medium 199 (M199; Microbiological Associates, Bethesda, Md., supplemented with 25 mM HEPES buffer (N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) at pH 7.4, 20% heat-inactivated (56°C, 30 min), mycoplasma-free, fetal calf serum (FCS, Microbiological Associates), 60 µg/ml penicillin and 120 µg/ml streptomycin. Aliquots of 0.5–1.0 x 10⁵ cells were replicate-plated in 16 mm diameter plastic culture wells (Cluster-24, Costar, Cambridge, Mass.) and maintained in a humidified atmosphere at 37°C, with medium changes at 24–48 hr intervals. These primary cultures were homogeneous endothelial populations as judged by morphologic, immunologic, and biochemical criteria, including the presence of Weibel-Palade bodies (endothelial-specific organelles), immunofluorescent staining...
of factor VIII-associated antigen, and angiotensin I converting enzyme. Confluent endothelial monolayers showed no evidence of smooth muscle cell overgrowth, and contamination by blood leukocytes was not detected by phase contrast microscopy or in Wright's stained preparations. Mass cultures also were plated in 25 cm² plastic culture flasks (Costar). In most experiments, primary HEC cultures were used after 3-5 days, at which time confluent monolayers of polygonal cells had formed. For comparative studies, virally transformed HEC, originally isolated by transvection of SV 40 DNA, and a diploid line of virally transformed HEC, obtained from the American Type Culture Collection (Rockville, Md.), were cultured in M199 supplemented with 10% fetal calf sera.

*Endothelial Culture Conditioned Media*

Measured volumes of fresh culture medium (M199 + 20% FCS), with or without additives (see below), were incubated with HEC monolayers for periods varying from 6 hr to 5 days to produce "HEC-conditioned medium" (HEC-CM). After removal of the conditioned medium, the number of cells in each culture well was determined by hemocytometer counting of trypsin-versene resuspended cells. Unless otherwise stated, all cultures in a given experiment were replicate-plated at the same initial density; final cell counts of replicate wells usually showed good agreement (±10-15%). In each experiment, samples of culture medium also were incubated in empty culture dishes (no cells) and processed in parallel as "mock conditioned medium" (MOCK-CM). Conditioned media routinely were millipore-filtered (0.45 μm) to remove any cellular debris, and then frozen at −20°C until assayed.

In certain experiments, conditioned media were concentrated by ultrafiltration on Amicon UM-10 membranes (nominal molecular weight cutoff ≤ 10,000), and the effluents and concentrates collected. Samples of HEC-CM also were dialyzed against distilled water or 0.01 M potassium phosphate buffer (pH 8), using three large volume changes over 24-72 hr.

*Colony-Stimulating Activity Assay*

CSA was assayed in vitro in single or double layer soft agar cultures as previously reported. Colony formation and morphology also were assessed in plasma clot culture. To permit the determination of complete dose-response curves of CSA with relatively small samples of HEC-CM, the above systems were adapted to microtiter dishes (Falcon, 3040 Microtest-II TM tissue culture plates). Stimulation of granulocyte-macrophage colony-forming cells (CFU-C) was determined by counting colonies using both 20 and 50 cells as minimum criterion. The use of the 20-cell colony criterion increased the sensitivity of the assay system without qualitatively affecting the results; therefore, unless otherwise stated, the data presented refer to ≥20-cell colonies. When colonies were counted at daily intervals from 5 to 19 days, essentially similar results were obtained; the bulk of the data presented was collected on 7 to 10-day cultures. Routinely 4-10 assay culture wells were counted in each experiment and CSA activity expressed as mean CFU-C (+SEM) per 10⁵ marrow cells. Colony morphology of cells grown in agar was determined by aceto-orcein or Wright-Giemsa staining. The colonies were picked into water in a microhematocrit tube, immediately blown onto glass slides, the slides spun in a cytocentrifuge, and the resulting cell "ringlets" fixed and stained. Morphology of cells grown in plasma clot was determined by benzidine and hematoxylin-eosin staining.

Normal bone marrow cells for the above assays were routinely obtained from normal volunteers, patients undergoing open-hip surgery, or bone marrow transplant donors. Informed consent was obtained in each instance. Rarely, marrow from patients with nonmalignant marrow disorders (e.g., anemia of chronic disease or iron-deficiency anemia) also was utilized. Marrow cells were separated by Hypaque-Ficoll density gradient centrifugation; the light-density fraction was collected and then subjected to adherence separation, by the method of Messner et al. to remove CSA-producing monocytes. In certain experiments, Hypaque-Ficoll separated marrow also was tested prior to adherence separation, and, in others, gravity-sedimented (1-2 hr at 25°C) marrow was tested without being subjected to any other preparative procedure. Murine marrow cells from C57BL/6J (Jackson Labs, Bar Harbour, Maine) or CF1 (Charles River Labs, Wilmington, Mass.) mice were obtained by flushing out the medullary contents of tibiae with tissue culture media and tested with and without Hypaque-Ficoll separation.

*Inhibitor Assay*

Inhibitory activity present in conditioned media was assessed by adding test samples at varying concentrations to human marrow cultures that were stimulated by a feeder-layer of 1×10⁶ human peripheral blood cells, and comparing colony formation to that seen in cultures to which appropriate control media had been added.

*Granulocyte-Lysate Preparations*

Granulocytes were obtained by separating heparinized peripheral blood from normal volunteers by albumin density centrifugation, followed by adherence separation; these preparations consisted of 90%-99% granulocytes. Lysates were prepared from 16 × 10⁶ of these nonadherent, dense (≥ 1.070 g/cm³) human blood cells by rapid freezing (3 times to −70°C) in serum-free M199. Some of these lysates were subjected to centrifugation at 160,000 g for 17 hr, a maneuver that has been reported to enhance the temperature-stability of a "colony-inhibiting activity," present in identically processed granulocyte lysates, which blocks monocyte-CSA production. Granulocyte-lysates (gran-lys) and centrifuged granulocyte-lysates (c-gran-lys) were dialyzed in Spectrapor membrane tubing (molecular weight cut-off ≤ 3500; Spectrum Medical Industries, Inc., Los Angeles, Calif.) against 10 mM sodium phosphate buffer (pH 7.0) and millipore-filtered (0.45 μm) prior to storage at −20°C.

*Effect of Endotoxin or Granulocyte-Lysates on Endothelial CSA Production*

Confluent HEC monolayers were cultured in media containing *S. typhosa* endotoxin (Difco #3940), for from 6 hr to 5 days; controls included HEC cultured without endotoxin, and media incubated with and without endotoxin in the absence of cells (MOCK-CM). Following incubation, the conditioned media were collected, millipore-filtered, and frozen at −20°C for subsequent determination of CSA and inhibitor levels. In other studies, endothelial cells were exposed to 1 μg endotoxin per ml for 6 hr, and the endotoxin media removed, the culture rinsed three times and reincubated with endotoxin-free media. The capacity of these "pulse" exposed HEC to produce CSA over a 72-hr culture period was compared to HEC exposed to the same concentration of endotoxin continuously for 72 hr.

In similar experiments, HEC cultures were exposed to gran-lys or c-gran-lys at a final concentration of 1% (v/v) for varying times. In all experiments, appropriate controls included HEC-CM without any additive, and media with additive but without cells, incubated for the same time intervals.
**Granulocyte-Endothelial Interactions**

Gravity-sedimented heparinized peripheral blood was subjected to Hypaque-Ficoll centrifugation, and the dense pellet (98.8%–99.2% mature granulocytes) was collected and washed 3 times in M199. These granulocyte preparations were then cocultured at varying concentrations for varying time intervals with HEC, and CSA levels in the culture medium compared with those seen with HEC alone or granulocytes alone. The preparations contained approximately 25%–50% erythrocytes (3–1 granulocytes per erythrocyte).

**Comparison of CSA Production by HEC versus Monocytes**

Freshly isolated umbilical vein HEC, prepared as described above but not cultured, and human peripheral blood monocytes separated by Hypaque-Ficoll density centrifugation (83%–85% monocytes), were suspended at equal concentrations in 0.5% agar feeder-layers. These cultures were allowed to condition overlying media for 24–72 hr, or to directly stimulate nonadherent human marrow cells that were plated on top of the feeder-layer, and incubated for 7–16 days. In other experiments, freshly isolated HEC and mononuclear cell suspensions were compared for their ability to condition media in liquid suspension cultures. Statistical significance was determined utilizing Student’s t test. Analysis was carried out on a Hewlett-Packard HP-97 computer with T statistics program ST I-153.

**RESULTS**

**Production of Colony-Stimulating Activity by Endothelial Cultures**

Primary HEC cultures produced CSA that stimulated colony formation, in a dose-dependent fashion, in both human and murine marrow cell assay systems (Table 1). CSA was detected in 23 of 27 samples of unconcentrated HEC-CM in which the final endothelial concentration was greater than 75,000 cells per ml, but was detectable in only 2 of 25 unconcentrated samples from cultures with less than 75,000 cells per ml. In general, CSA levels were higher, on a per cell basis, in HEC-CM collected after 24–72 hr, than after 6 hr. Although activity was variable in conditioned media from low-density cultures, it was routinely observed when HEC-CM samples were concentrated tenfold by ultrafiltration on Amicon UM-10 membranes (Table 1). The CSA in concentrated HEC-CM was active against all types of human marrow cell preparations tested (unseparated; Hypaque-Ficoll separated, light-density fraction; or nonadherent, Hypaque-Ficoll separated), as well as against CF1, or C57BL/6J murine marrow cells (unseparated, or light-density Hypaque-Ficoll separated). Dialysis had little effect on Amicon UM-10 concentrated HEC-CM, and variable effects on unconcentrated HEC-CM, increasing apparent CSA levels in some samples but not in others. CSA was not detected in the Amicon UM-10 ultrafiltrates of active HEC-CM samples. This activity was also stable at 56°C for 30 min.

SV40 transformed HEC* produced CSA active versus both murine and human cells over 24–72 hr of culture, although levels were relatively low compared to those seen with nontransformed HEC. A diploid line of fetal human skin fibroblasts (American Type Culture Collection, CRL-1106) did not produce detectable CSA, over 24–72 hr of culture, as assayed against murine and human marrow.

**Inhibitors of Colony Formation in Endothelial Conditioned Media**

Conditioned media from individual HEC cultures variably showed inhibitory activity when tested against stimulated human marrow cells (Fig. 1). In 9 of 13 separate experiments, inhibitory activity was detectable in HEC-CM samples harvested after 6–72

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**Table 1. Colony-Stimulating Activity in 72-hr Conditioned Media From Human Endothelial Cell Cultures**

<table>
<thead>
<tr>
<th></th>
<th>CFU-c per 10⁶ Marrow Cells*</th>
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<tr>
<td></td>
<td>Human Marrow</td>
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<tr>
<td></td>
<td>(%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HEC-CM</td>
<td></td>
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<tr>
<td></td>
<td>20</td>
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<tr>
<td></td>
<td>9</td>
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<td></td>
<td>4.8</td>
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<td></td>
<td>2.0</td>
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<td>0.9</td>
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*Hypaque-Ficoll separated, nonadherent, normal human marrow or unseparated CF1 mouse marrow cells in soft agar suspension cultures. Colonies of >50 cells were scored as CFU-c, and data expressed as the mean ± 1 SEM.

†Pooled conditioned media (HEC-CM) from 72-hr incubations with primary human endothelial cultures, added at varying concentrations (% v/v) to assay cultures.

‡The above HEC-CM concentrated 10 x by ultrafiltration on Amicon UM-10 membranes before testing.

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**Fig. 1. Inhibitory activity in endothelial culture conditioned media. Pooled HEC-CM from 24-hr incubations were tested at varying concentrations for inhibition of colony formation in Hypaque-Ficoll separated, human marrow (stimulated by cocultivation with 1 x 10⁶ human peripheral blood leukocytes). Data are expressed as CFU-c per 10⁶ marrow cells (mean ± SEM).**
hr incubation and assayed at final concentrations of 4.8% to 29%. In preliminary experiments, dialysis against distilled water did not appear to decrease this inhibition. When tested at 20% concentration against murine marrow that was stimulated by mouse lung-conditioned medium, 72 hr HEC-CM showed relatively insignificant amounts of inhibition (10%–15%).

Morphology of Marrow Colonies

The morphology of human marrow cell colonies stimulated by 72 hr HEC-CM was analyzed after 9 and 15 days of growth, using Wright-Giemsa staining. After 9 days, the percentage of identifiable neutrophils in a given colony ranged from 46% to 100% (71.8% ± 4.7%, mean ± SEM, 13 colonies); small numbers of monocytes and macrophages, but no eosinophils, were seen. After 15 days of growth, occasional predominantly eosinophilic colonies (>85% eosinophils and numerous colonies admixed with eosinophils, were observed. At this time point, the percentage of neutrophils in all colonies examined ranged from 13% to 100% (75.1% ± 5.3%, mean ± SEM, 28 colonies), while the percentage of eosinophils ranged from 0% to 95.7% (mean ± SEM, 15.5% ± 5.5%). Occasional macrophages (range 0%–25.7%, mean ± SEM, 4% ± 1.3%) and monocytes, as well as other unidentifiable cells, also were noted. In experiments with 72 hr HEC-CM from cultures exposed to endotoxin (see below), a total of 20 colonies were analyzed, with qualitatively similar results.

Effect of Endotoxin on Endothelial CSA Production

The survival of primary HEC cultures was not decreased after incubation with S. typhosa endotoxin at concentrations ranging from 0.001 to 5.0 µg per ml, for up to 72 hr (41 paired cultures examined for cell recovery). Endotoxin at levels of 0.001–0.01 µg per ml also had little discernable effect on HEC-CSA production (Fig. 2A). However, endotoxin at concentrations of 0.1–5 µg per ml markedly increased CSA production in 6–72 hr cultures (Fig. 2A, B, and D).

Samples of endotoxin-containing media, incubated in the absence of endothelial cells (MOCK-CM), did not have this effect. Endotoxin stimulated CSA was comparably active against preparations of light-density (Hypaque-Ficoll) human marrow cells before, and after, removal of the adherent (mononuclear) cell fraction (Figs. 2A and B). Endotoxin treatment did not appreciably alter the low level of inhibitory activity present in endothelial conditioned media (Fig. 2C); thus, changes in inhibitor levels did not account for the observed increases in CSA production.

Conditioned media from HEC cultures which were exposed to 1 µg S. typhosa endotoxin per ml contained progressively increasing amounts of CSA at intervals from 6 to 72 hr (Fig. 2D). A pulse-exposure of HEC cultures to 1 µg endotoxin per ml for 6 hr resulted in a lower CSA level after 72 hr of incubation than did
controls.

In 12 of 12 experiments, CSA levels in endothelial culture media were increased during incubations with 1% granulocyte lysate (c-gran-lys), presumably a more stable preparation than granulocyte-lysates, whether ultracentrifuged or not, (a procedure reported to increase the heat-stability of CIA), caused marked increases in endothelial CSA levels than 1 μg endotoxin per ml, as assayed against preparations of both human nonadherent marrow and murine marrow.

Inhibitor levels were not appreciably different in endothelial cultures exposed to granulocyte-lysates than in control cultures (Fig. 3C). The survival of primary HEC cultures was not decreased after incubation with 1% granulocyte lysate (or c-gran-lys) for from 6–72 hr (37 paired cultures examined for cell recovery).

Granulocytes isolated from peripheral blood by hypaque-ficoll density centrifugation (dense pellet, 99% granulocytes) were admixed with confluent HEC monolayers (2 experiments) or admixed with freshly isolated HEC (4 experiments) in suspension culture for 48–72 hr and their effect on CSA levels assessed. When freshly isolated HEC were mixed with granulocytes at a 1:18 or 1:25 ratio, there was an augmentation of CSA production; controls consisted of HEC, media or granulocytes alone. Similarly, if over 2.4 x 10⁶ granulocytes were cocultured with HEC monolayers, increased CSA levels were noted in the media. Visual inspection of these cultures did not indicate any effect of added granulocytes on HEC viability.

The capacity of varying concentrations of freshly isolated HEC suspended in 0.5% agar underlayers to stimulate colony formation by hypaque-ficoll separated nonadherent human marrow overlayed in 0.3% agar was compared to that of similar numbers of monocytes (Table 3); on a cell per cell basis HEC stimulated more colony formation than monocytes over 7–16 days of culture. Similar experiments were carried out in which freshly isolated HEC or monocytes (0.25–2.0 x 10⁶/ml) were suspended in 0.5% agar underlayers or in suspension cultures and allowed to condition overlaying media for 3–4 days. In 3 experiments HEC produced more CSA than equal numbers of monocytes as assayed against hypaque-ficoll separated nonadherent human marrow at concentrations of conditioned media ranging from 0.9% to 29%.

### Table 2. Effect of Pulse Exposure of HEC to Endotoxin

<table>
<thead>
<tr>
<th>Conditioned Media Samples</th>
<th>CSA Activity (CFU-c per 10⁶ cells)</th>
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<tbody>
<tr>
<td>HEC</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>HEC + ENDO-pulse</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>HEC + ENDO-continuous</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>MOCK-CM (no cells)</td>
<td>9 ± 0</td>
</tr>
<tr>
<td>MOCK-CM (ENDO-pulse)</td>
<td>1.3 ± .9</td>
</tr>
<tr>
<td>MOCK-CM (ENDO-continuous)</td>
<td>4 ± 1.4</td>
</tr>
</tbody>
</table>

Replicate-plated HEC cultures were exposed to 1 μg S. typhosa endotoxin per ml for 6 hr, rinsed 3 times and reincubated for 72 hr in endotoxin-free medium (HEC + ENDO-pulse). The CSA levels in conditioned media obtained from these cultures were compared to those from: (1) HEC incubated for 6 hr in endotoxin-free medium, rinsed and then reincubated for 72 hr in endotoxin-free medium (HEC); (2) HEC incubated with media for 6 hr, rinsed, and then reincubated for 72 hr in endotoxin medium (HEC + ENDO-continuous); (3) empty culture wells containing media incubated as described above (MOCK-CM).

CSA levels are expressed as CFU-c per 10⁶ marrow cells stimulated by 9% (v/v) conditioned media. Data represent mean ± SEM values from 3 sets of conditioned media samples assayed separately against the same Hypaque-Ficoll separated, nonadherent human marrow.

CSA production assessed. These lysates were prepared by methods identical to those described by Broxmeyer and others. CSA levels were increased during incubations with 1% granulocyte-lysate for culture intervals varying from 6–72 hr (Figs. 3A and B). Similar results were obtained in other experiments with 5 separate preparations of human marrow cells (3 Hypaque-Ficoll separated, nonadherent; 1 Hypaque-Ficoll separated; 1 nonadherent). The effect of ultracentrifuged granulocyte-lysate (c-gran-lys), presumably a more stable preparation, was also examined; in 12 of 12 experiments, increased CSA levels were observed in 24–72 hr conditioned media samples, compared to appropriate controls.

The capacity of granulocyte-lysates to stimulate CSA production in endothelial cultures was compared to that of endotoxin. In 4 of 7 experiments, HEC cultures incubated with 1% gran-lys produced more CSA, after 24–72 hr, than paired cultures exposed to 1 μg endotoxin per ml (Fig. 3B); in the remaining 3 experiments, comparable levels of CSA were observed in gran-lys and endotoxin treated cultures. In 9 other experiments the effect of c-gran-lys was similarly compared with that of endotoxin in 24–72 hr incubations; in each case, 1% c-gran-lys caused greater increases in endothelial CSA levels than 1 μg endotoxin per ml, as assayed against preparations of both human nonadherent marrow and murine marrow.

### DISCUSSION

Previous studies have demonstrated that human monocytes, macrophages and activated lymphocytes produce colony-stimulating activity (CSA). In man,
from 6 to 72 hr with medium containing no additives (HEC) or medium containing 1% (v/v) granulocyte-lysate (HEC + Gran-lys). Conditioned media samples from 2 separate experiments were assayed, 20% against C57B1/6J murine marrow cells. CSA is expressed as CFU-c per 10⁶ marrow cells (mean ± SEM) using the 50-cell colony criterion. The lower hatched area represents the capacity to block baseline, but not stimulated, monocyte CSA production assayed with C57B1/6J murine marrow. HEC cultures were incubated for 24 hr with the following media: (1) no additives (HEC); (2) 1 µg endotoxin per ml (HEC + ENDO); and (3) 1% (v/v) granulocyte-lysate (HEC + Gran-lys).

Conditioned media samples from 3 separate experiments were assayed at varying concentrations, against Hypaque-Ficoll separated human marrow. CSA levels are expressed as CFU-c per 10⁶ marrow cells (mean ± SEM). MOCK-CM (no endothelial cells), with and without endotoxin or granulocyte-lysate, assayed at concentrations of 4.8%–20%, stimulated 0–3.1 CFU-c per 10⁶ marrow cells. A human peripheral blood leukocyte feeder-layer (1 x 10⁶ cells per ml) stimulated 149 ± 7.5 CFU-c per 10⁶ marrow cells. A human peripheral blood leukocyte feeder-layer (1 x 10⁶ cells per ml) stimulated 149 ± 7.5 CFU-c per 10⁶ marrow cells. A human peripheral blood leukocyte feeder-layer (1 x 10⁶ cells per ml) stimulated 149 ± 7.5 CFU-c per 10⁶ marrow cells. A human peripheral blood leukocyte feeder-layer (1 x 10⁶ cells per ml) stimulated 149 ± 7.5 CFU-c per 10⁶ marrow cells.

The observations presented in this report show that a homogeneous, well characterized population of human endothelial cells, either cultured or freshly isolated, has the capacity to produce CSA that is active against nonadherent human marrow and murine marrow (Tables 1 and 3). Adherence-separated human marrow does not show autostimulation and appears to be devoid of CSA producing cells, thereby suggesting that endothelial conditioned media contains CSA and was not acting through stimulation of a second population of cells to produce CSA. Endothelial-derived CSA appears similar to monocyte CSA in its effect on the morphological pattern of colony formation, i.e., with both, predominantly granulocytic colonies observed early, and, later, increasing number of eosinophil colonies appear. In addition, both endothelial and monocyte CSAs are nondialyzable, relatively heat-stable and can be concentrated on Amicon UM-10 membranes. Inhibitors of colony formation were variably present in endothelial conditioned media (Fig. 1), but their physiologic relevance is uncertain.

The current study also indicates that the production of endothelial derived CSA can be stimulated in vitro by bacterial products, such as endotoxin (Table 2, Fig. 2). This endotoxin effect appears to be time and
of lactoferrin on endothelial CSA production, as induction in response to bacterial infection. Other studies showing not presented), we have been unable to elicit an effect as lactoferrin. In a recent series of experiments (data previously reported) we have been unable to elicit an effect attributable to decreased production of inhibitors of CSA. The stimulatory effect of granulocyte-lysates was not attributable to contamination with bacterial endotoxin, since Limulus lysate assays of these preparations revealed negligible concentrations of endotoxin (less than 0.001 µg/ml). Studies on inhibitor levels from the granulocyte lysate-treated HEC cultures demonstrated no significant change compared with control cultures, thus indicating that alterations in inhibitors of colony formation were not responsible for the observed increase in CSA.

The effect of granulocytes on endothelial CSA production was further illustrated by studies in which coculture of intact granulocytes with freshly isolated HEC or HEC monolayers resulted in augmented CSA levels. The possibility remains that contaminating red blood cells (see Materials and Methods) in these hypaque-ficoll separated granulocyte preparations may have affected endothelial CSA production or that products from their spontaneous lysis in vitro may have influenced in vitro CSA activity. Experiments to clarify these points are underway.

The vascular endothelium forms a vase interface between the body's tissues and the circulating blood, and participates actively in the inflammatory response. Increased vascular permeability is a constant concomitant of acute inflammation, with major permeability changes seen regularly in postcapillary venules. The latter segment of the microcirculation also appears to be the major site of leukocyte transit into tissues. This phenomenon, which can be elicited by various chemotactic stimuli including bacterial products, involves sticking of leukocytes to the vascular lining, followed by migration between endothelial cells. In addition, endothelial proliferation associated with inflammation is influenced by, although not totally dependent upon, neutrophil infiltration. Because of its active role in the inflammatory response and its widespread distribution, the endothelium would appear to be well suited to monitor the presence of bacterial products, such as endotoxin, or the destruction of mature granulocytes. Thus, systemic endothelial CSA production could be envisioned as serving as a humoral regulatory mechanism for the control of granulopoiesis.

Our experiments comparing the production of CSA by human blood monocytes and freshly isolated human endothelial cells, under standardized in vitro conditions, suggest that the broad expanse of vascular endothelium in vivo may constitute a potent source of CSA. The exact roles of endothelial and monocyte–lymphocyte CSA, and their potential interactions in the regulation of granulopoiesis remains to be settled.

### Table 3. Comparison of CSA Production by Freshly Isolated Human Endothelial Cells and Blood Monocytes in a Feeder Layer System

<table>
<thead>
<tr>
<th>Cells per ml in Underlayer</th>
<th>CFU-C/10⁵ Marrow Cells*</th>
<th>Days in Culture</th>
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<tbody>
<tr>
<td>HEC</td>
<td>Monocyte</td>
<td></td>
</tr>
<tr>
<td>1 x 10⁴</td>
<td>25 ± 2</td>
<td>(16 ± 2)</td>
</tr>
<tr>
<td>9</td>
<td>16 ± 0</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>1.25 x 10⁴</td>
<td>30 ± 2</td>
<td>(15 ± 2)</td>
</tr>
<tr>
<td>2 x 10⁴</td>
<td>14 ± 1</td>
<td>(1.7 ± 5)</td>
</tr>
<tr>
<td>59 ± 6</td>
<td>7 ± 2</td>
<td>(2 ± 0.5)</td>
</tr>
<tr>
<td>77 ± 4</td>
<td>45 ± 4.8</td>
<td>(6.8 ± 1.8)</td>
</tr>
<tr>
<td>17 ± 2</td>
<td>(3.5 ± .8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2.5 x 10⁴</td>
<td>27 ± 3</td>
<td>(16 ± 8)</td>
</tr>
<tr>
<td>5 x 10⁴</td>
<td>38 ± 1</td>
<td>(2 ± 2)</td>
</tr>
<tr>
<td>22 ± 3</td>
<td>0 ± 0</td>
<td>(0 ± 0)</td>
</tr>
<tr>
<td>16 ± 3</td>
<td>(2.5 ± 1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>8 ± 1</td>
<td>12 ± 2</td>
<td>(7 ± 1)</td>
</tr>
<tr>
<td>68 ± 5</td>
<td>37 ± 3</td>
<td>(6.5 ± 1)</td>
</tr>
<tr>
<td>26 ± 2</td>
<td>(18 ± 2)</td>
<td>0 ± 0 (0 ± 0)</td>
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</table>

*Normal nonadherent, hypaque-ficoll separated human marrow cells were used for all assays; routinely, a 20 cell colony criterion was applied. Numbers in parentheses represent values using 50 cells as a colony criterion.
†Difficult to ascertain smaller aggregates due to large size of colonies. 
‡Values not significantly different (p > 0.05); all other values significantly different at p < 0.05 to p < 0.001.
Finally, CSA production by the endothelial lining of bone marrow sinusoids also may participate in the local (intramedullary) regulation of granulopoiesis. The intimate relationship of the sinusoidal endothelium to marrow progenitor cells, and its role as a conduit to the peripheral blood, place it in a unique position to monitor and modulate granulocyte production by short-range mechanisms that might include prostaglandin and CSA production.

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REFERENCES

31. Quesenberry PJ, D’Amore P: Unpublished observations
32. Quesenberry PJ, Gimbrone M: Unpublished observations
33. Quesenberry PJ, Levin J: Unpublished observations
Vascular endothelium as a regulator of granulopoiesis: production of colony-stimulating activity by cultured human endothelial cells

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