

Mechanical, cellular, and molecular factors interact to modulate circulating endothelial cell progenitors

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Wang, Chunlin, Chunhua Jiao, Heather D. Hanlon, Wei Zheng, Robert J. Tomanek, and Gina C. Schatteman. Mechanical, cellular, and molecular factors interact to modulate circulating endothelial cell progenitors. *Am J Physiol Heart Circ Physiol* 286: H1985–H1993, 2004. First published January 8, 2004; 10.1152/ajpheart.00431.2003.—It appears that there are two classes of human circulating endothelial cell (EC) progenitors, CD34⁺ and CD34⁻CD14⁺ cells. Attention has focused on CD34⁺ cells, yet CD34⁻CD14⁺ monocytic cells are far more abundant and may represent the most common class of circulating EC progenitor. Little is known about molecular or physiological factors that regulate putative CD34⁻CD14⁺ EC progenitor function, although factors secreted by other blood and cardiovascular cells to which they are exposed probably affect their behavior. Hypoxia and stretch are two important physiological stimuli known to trigger growth factors in cardiovascular cells and accordingly may modulate EC progenitors. To investigate the impact of these environmental parameters on EC progenitors, EC production in CD34⁻CD14⁺ cultures was evaluated. Our data indicate that neither stretch nor hypoxia alters EC production by EC progenitors directly but do so indirectly through their effects on cardiovascular cells. Conditioned media (CM) from coronary artery smooth muscle cells inhibit EC production in culture, and this inhibition is stronger if the coronary smooth muscle cells have been subjected to cyclic stretch. In contrast, cardiomyocyte CM increases EC cell number, an effect that is potentiated if the myocytes have been subjected to hypoxia. Significantly, EC progenitor responses to CM are altered by the presence of CD34⁻CD14⁻ peripheral blood mononuclear cells (PBMCs). Moreover, CD34⁻CD14⁻ PBMCs attenuate EC progenitor responsiveness to the angiogenic factors basic fibroblast growth factor (FGF-2), vascular endothelial cell growth factor-A₁₆₅, and erythropoietin while inducing EC progenitor death in the presence of transforming growth factor- β_1 in vitro cyclic stretch; hypoxia; erythropoietin; monocyte; conditioned medium

IT IS NOW WELL ESTABLISHED that endothelial cell (EC) stem or progenitor cells are present in the adult bone marrow. Experiments with mice whose bone marrow was replaced by genetically marked bone marrow cells indicate that engrafted cells move from the bone marrow through the blood into the endothelium (2, 3, 11, 21). Within the blood, mononuclear cells enriched for CD34 expressing (CD34⁺) and CD34-depleted CD14-expressing (CD14⁺) cells may act as EC progenitors (3, 12, 13, 18, 30, 36, 41). A number of studies suggest that exogenous CD34⁺ cells can integrate into the vasculature of ischemic muscle and myocardium in nondiabetic and dia-

betic rodents (3, 25, 32, 35). Similarly, CD14⁺ cells appear to integrate into the vasculature of ischemic tissue in nondiabetic mice, but an unidentified additional stimulus may be required for integration to occur in diabetic mice (18, 43). In the presence of high levels of monocyte chemoattractant protein-1, but not in its absence, monocytes have been found to form vascular-like channels in ischemic hearts and to reendothelialize denuded carotid arteries (16, 30). Thus, although it has not been established definitively, CD34⁺ and CD14⁺ cells appear to act as two functionally distinct progenitor populations. If true, and the EC stem/progenitor cell hierarchy is analogous to that of the hematopoietic system, CD34⁺-enriched cells could be a primitive progenitor/stem cell-containing population, and CD14⁺ cells a population with more differentiated EC precursor.

Distribution of marrow-derived cells in the vasculature is not uniform in that the fraction of marrow-derived EC is greater in newly formed vasculature than in preexisting vessels (11). This suggests that local factors influence circulating bone marrow-derived cells' differentiation into EC or ability to incorporate into the endothelium. Among local factors, cellular-extracellular matrix interactions play a critical role in blood vessel formation (20). These interactions are mediated in large part by integrins including the fibronectin receptors $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$. The integrin $\alpha_5\beta_1$, the primary fibronectin receptor, is involved in vascular development, and the fibronectin binding integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are important regulators of basic fibroblast growth factor (FGF-2) and vascular endothelial cell growth factor-A (VEGF)/transforming growth factor (TGF)- β_1 -mediated angiogenesis, respectively (15, 20). $\alpha_v\beta_3$ also mediates attachment to laminin and the angiogenic factors VEGF, FGF-2, and TGF- β_1 can increase its expression (7, 39, 40). $\alpha_2\beta_1$ integrin, the major collagen receptor on EC and a receptor for laminin, also promotes angiogenesis (1, 17, 26). The role cellular-extracellular matrix interactions may play in regulating angiogenesis is unknown.

Circulating EC progenitors also interact with other peripheral blood mononuclear cells (PBMCs) and cells of the cardiovascular system. PBMCs can affect EC progenitor function (18), but whether cardiovascular cells perform a similar function is not known. Cardiovascular cells secrete both pro- and antiangiogenic factors, and this secretory milieu changes in response to physiological stimuli, including hypoxia and cyclic stretch (37, 44, 45). For example, hypoxia induces VEGF upregulation by vascular smooth muscle cells (SMC), EC, and cardiomyocytes (9, 27, 29, 45), and stretch upregulates VEGF

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in the intact ventricular wall and in isolated myocytes (28, 38, 47). Several studies demonstrate a modulatory effect of VEGF on EC progenitor function (4, 13); hence, EC progenitor behavior may be influenced indirectly by physiological stimuli acting on cardiovascular cells.

This study examined the interaction of circulating EC progenitors with molecular and physiological stimuli within their local environment. Because CD14⁺ cells may be the cells that actually differentiate and integrate into the vasculature in vivo, this study focused on these cells. To assay responsiveness of cells in CD14⁺ cultures, the number of EC produced in culture under various conditions was monitored. First, to establish an appropriate substrate for conducting in vitro experiments and to identify possible mediators of EC progenitor interaction with their physical environment, the ability of various extracellular matrix molecules to support EC production was investigated. Second, we examined known local regulators of angiogenesis for their effects on circulating EC progenitor behavior. These included the molecular regulators VEGF₁₆₅, FGF-2, TGF- β ₁, and erythropoietin; cells of the cardiovascular system (coronary and aortic SMC, umbilical vein EC, and cardiomyocytes); and physiological stimuli (hypoxia and stretch). Finally, PBMC modulation of EC production by EC progenitors was studied.

MATERIALS AND METHODS

Isolation of PBMC subsets. Highly purified (as assessed by fluorescence-activated cell sorting) CD34⁻CD14⁺ (>99%) PBMCs from pheresis donors were purchased from Poietics (Gaithersburg, MD). These cells are referred to as CD14⁺ cells in the text (Table 1). To obtain CD34⁻ PBMCs, human blood, collected from healthy adult volunteer donors (after giving informed consent) per University of Iowa Institutional Review Board approved protocols, was fractionated by centrifugation on Histopaque 1077 (Sigma, St. Louis, MO) gradients per manufacturer's instructions with modifications as described to obtain PBMCs (18, 35). PBMCs were washed twice in 1.1 mmol/l KH₂PO₄, 3 mmol/l Na₂HPO₄, 0.9% NaCl, 0.13 mol/l sodium citrate with 20 mg/ml BSA and resuspended in the same buffer. CD34⁺ cell depletion was performed using CD34⁺ antibody-coated magnetic beads (Dyna, Lake Placid, NY) as described and per manufacturer's instructions (18, 35). Residual depleted PBMCs, referred to as CD34⁻ PBMCs in the text, typically contain <0.03% CD34⁺ and 8–13% CD14⁺ cells (Table 1).

Culture of EC progenitors. Freshly isolated or thawed cells were plated at 1.5×10^5 cells/cm² for growth factor experiments and $2\text{--}2.5 \times 10^5$ cells/cm² for all other experiments. At these densities, cells are approximately 20% and 25–33% confluent, respectively. Experiments were done in duplicate or triplicate, performed 4–10 times, and media (fresh or conditioned) were replaced after 3 days and every 4 days thereafter unless otherwise indicated. Care was taken to remove all unattached cells when changing media so that subsequent cell attachment did not compromise data interpretation. However, because after 24 h in culture replated unattached cells fail to attach to fibronectin-coated plates, and after 2 days in culture unattached cells

Table 1. Cell populations

	CD14 ⁺ Content	CD34 ⁺ Content	Other PBMC
CD14 ⁺ cells	>99%	<0.05%	<1%
CD34 ⁻ PBMCs	8–13%	<0.05%	87–92%

PBMC, peripheral blood mononuclear cells.

Table 2. High-serum media formulations

Medium Type	High Serum	Low Serum
Medium D (also HUVEC)	M199	M199
	20% HIFBS 0.4% BBE	5% HIFBS 0.1% BBE
Coronary SMC	Medium 231	Medium 231
	10% SMGS	2% SMGS
Aortic SMC	F12K medium	F12K medium
	10 mM HEPES	10 mM HEPES
	10 mM TIS	10 mM TIS
	10% HIFBS	2% HIFBS
	1 × TIS	1 × TIS
	1% BBE	50 μg/ml Ascorbic acid
Cardiomyocyte	50 μg/ml Ascorbic acid	
	DMEM	DMEM
	10% HIFBS	2% HIFBS

HUVEC, human umbilical vein endothelial cells; SMC, smooth muscle cells; M199 (no. 12340, GIBCO-BRL, Gaithersburg, MD); HIFBS, heat-inactivated fetal bovine serum (Hyclone, Logan, UT); BBE, bovine brain extract (Clonetics, San Diego, CA); medium 231 (Cascade Biologicals, Portland, OR); smooth muscle growth supplement (SMGS) (Cascade Biologicals); F12K (ATCC no. 30-2004); DMEM (no. 11965, GIBCO-BRL); TIS, transferrin-insulin-selenium (Fisher).

do not appear to be viable, this is unlikely to be a significant concern (Hanlon and Schatteman, unpublished).

Analysis of substrate effects. For substrate experiments, wells were coated with 5 μg/cm² mouse laminin, rat collagen I, mouse collagen IV, human fibronectin, or undiluted Matrigel (all from Collaborative Biomedical Products, Bedford, MA) per manufacturer's instructions or 1% type B gelatin Bloom 225 (Sigma) or combinations of the above. Substrate comparisons were made in medium D (Table 2) containing 2 × antibiotic-antimycotic (no. 15240–0620, GIBCO BRL, Gaithersburg, MD). To determine the number of EC in CD34⁻ PBMC cultures, spindle-shaped cells in 11 (24-well tray) or 5 (96 well tray) representative fields per well (approximately 5 and 10%, respectively, of each well) were counted at 4–5 days and 12–14 days after plating using phase-contrast microscopy. Spindle shape was used as the criterion for EC differentiation at the 4- to 5-day time point because by 6 days in medium D essentially all spindle-shaped cells express tie-2 and take up acetylated low-density lipoprotein (18, 35). We verified expression of tie-2 (Santa Cruz Biotechnology, Santa Cruz, CA) expression in spindle-shaped cells immunocytochemically after the final count as described (18, 35).

Analysis of conditioned media effects. CD14⁺ cells or CD34⁻ PBMCs were plated on 5 μg/cm² fibronectin-coated wells in conditioned or the corresponding fresh medium (Table 2). At 11–12 days after plating, cells were fixed in methanol, and total and spindle-shaped cells were counted, followed by anti-tie-2 immunolabeling. In additional experiments, cells fixed at days 11–12 were immunostained with anti-endothelial nitric oxide synthase (anti-eNOS; Transduction Laboratories, Lexington, KY) to identify EC and then labeled with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) to visualize all cells. DAPI-labeled nuclei and eNOS⁺/DAPI-labeled cells were counted. In one series of experiments, cells were plated in medium D. On the 4th day, media were replaced with fresh or conditioned myocyte medium and then assayed as above at days 11–12.

Conditioned media (CM) were prepared from passage 4–5 human aortic SMC (ATCC, Bethesda, MD), passage 3–5 human coronary artery SMC (Clontech, Palo Alto, CA), H9C2(2–1) rat cardiomyocytes (ATCC), or passage 2–3 human umbilical vein EC (HUVEC) (Univ. of Iowa Cardiovascular Center Cell Culture Facility). Initially, cells were plated in the high-serum media listed in Table 2. CM from normoxic and hypoxic cells was prepared by plating cells on uncoated or gelatin (for HUVEC)-coated culture dishes. When ~90% confluent, medium was replaced with low-serum medium (Table 2), and CM

were collected 3 days later. For hypoxia CM, low-serum medium was preequilibrated in 5% O₂, and cultures were placed in a humidified hypoxia chamber at 5% O₂-5% CO₂-90% N₂ at 37°C and regassed daily until collection at 3 days (45). Fresh medium was incubated at 37°C for 3 days before use. For stretched and unstretched control CM, cells were plated on collagen I-coated BioFlex plates in high-serum medium (Table 2). At 95–100% confluence, medium was replaced with low-serum medium (Table 2), and 16–20 h later cells were subjected to a 10% average surface elongation at 30 cycles/min for 1 h (47). CM were collected immediately thereafter. For unstretched controls, cells were placed in the flexor unit but were not stretched. All CM were clarified twice by centrifugation at 400 g for 10 min and then supplemented with 25% medium D because few CD14⁺ cells or CD34⁺ PBMCs survive in the low-serum CM alone.

Analysis of growth factor effects. CD14⁺ cells or CD34⁺ PBMCs were plated on 5 μg/cm² fibronectin-coated wells in low-serum medium D containing 20 ng/ml VEGF₁₆₅; 2.5 ng/ml FGF-2: 1.1, 2.2, or 3.3 ng/ml TGF-β₁ (R&D Systems, Minneapolis, MN); or 8 U/ml erythropoietin. Factors were added 16–20 h after plating and every other day thereafter. Total cell numbers were assayed at days 0, 2, 6, and 9 by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (ATCC, Manassas, VA) after 2 h incubation in MTT reagent according to manufacturer's instructions. Standard curves were generated to convert MTT absorbance readings to live cell numbers. Additional wells were fixed at day 9 and immunostained with anti-eNOS (Transduction Laboratories, Lexington, KY) and then labeled with DAPI to visualize all nuclei. Total EC counts were calculated as the product of total cell number (from MTT assay) and fraction of eNOS⁺-expressing cells.

TGF-β₁ determinations. To measure TGF-β₁ concentrations in CM, the TGF-β₁ was first activated with 1 N HCl, then neutralized with 0.2 ml 1.2 N NaOH/0.5 M HEPES. CM were immediately frozen and sent to the Cytokine Core Laboratory (Univ. of Maryland, Baltimore, MD) for ELISA analysis.

Statistics. The mean cell number from replicate wells for each experiment was used for purposes of statistical analysis. Data analysis from studies using impure cells was performed using a paired *t*-test or converted to percentage of control before ANOVA because absolute numbers of cells varied widely among experiments. For all other studies, ANOVA was performed on the raw data. Tukey's honestly significant difference test or Bonferroni post hoc test was performed with *P* < 0.05 considered significant. In some figures and tables, data are represented as mean percentage relative to control conditions. Error bars indicate SE of the mean.

RESULTS

Cell populations. Because our purpose was to study non-CD34⁺ circulating EC progenitors, all experiments were performed on CD34⁺-depleted PBMCs, i.e., CD34⁺ PBMCs. CD34⁺ PBMCs include essentially the entire PBMC population because CD34⁺ cells typically represent <0.1% of human PBMCs. CD34⁺ PBMCs are, thus, a mixed cell population that includes various non-EC progenitor cell types as well as CD34⁺ EC progenitors. Data from this laboratory and others strongly suggest that the CD14⁺ subset of CD34⁺ PBMCs represent the principal source of circulating EC progenitors (12, 13, 18, 30, 33, 36). Hence, CD14⁺ cells were selected from total CD34⁺ PBMCs to obtain an essentially pure population of CD14⁺ cells. Table 1 shows the relevant characteristics of CD34⁺ PBMCs and CD14⁺ cells.

Fibronectin promotes but laminin inhibits differentiation. In vivo, circulating cells interact with a variety of extracellular matrix molecules so the choice of substrates on which to conduct in vitro studies of EC progenitors is not clear. To

address this question, CD34⁺ PBMCs were plated on collagen I, collagen IV, fibronectin, Matrigel, laminin, gelatin, and combinations thereof in medium D as medium D facilitates differentiation of EC progenitors into EC. CD34⁺ PBMC cultures were subsequently examined for the presence of spindle-shaped cells followed by immunostaining for tie-2 or eNOS at 12–14 days.

Many EC (tie-2⁺ spindle-shaped cells) were present on fibronectin, collagen I, and collagen IV, but not laminin (Fig. 1). Cells differentiated more rapidly on fibronectin than on either type of collagen, and no combination of any two substrates yielded more EC than did fibronectin alone (Fig. 1). Cells cultured on combinations of laminin and collagen IV or laminin and fibronectin yielded EC numbers similar to those when plated on laminin alone (data not shown). Surprisingly, although EC normally grow on both Matrigel and gelatin, we twice attempted culture on these substrates, but few cells survived. Additionally, we three times attempted culture of CD34⁺ PBMCs on commercially coated ProNectin F (a recombinant molecule with multiple copies of the human fibronectin RGD sequence) (ICN Labware, Aurora, OH) plates. Cells attached to this substrate, but no EC were observed and cells died within a few days. Because among substrates tested, EC production by CD34⁺ PBMCs was maximal and most rapid on fibronectin, subsequent experiments were performed on this substrate.

Direct effects of physiological stimuli. Because hypoxia and ischemia stimulate vascular growth (14, 45), we tested the hypothesis that EC progenitors contribute to this hypoxia-induced vascular growth by producing more EC. CD34⁺ PBMCs were plated in a normoxic environment for 1 day and then cultured in hypoxia thereafter. In two experiments, hypoxia failed to stimulate EC formation at 5, 7–8, or 11–12 days in the cultures (data not shown). Because cyclic stretch also induces vascularization (46), we attempted to test its effects on EC progenitor differentiation. However, when CD34⁺ PBMCs were plated on a stretchable membrane, they did not adhere well and died shortly thereafter even when the membrane was coated with fibronectin or collagen I.

Cardiovascular cell CM do not affect differentiation. Cardiovascular cells, SMC, and EC secrete angiogenic factors including

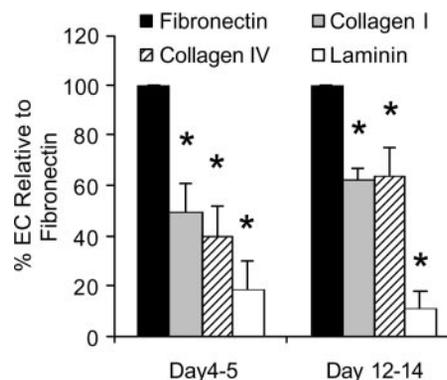


Fig. 1. Endothelial cell (EC) formation on angiogenic substrates. EC formation by CD34⁺ PBMC cultures 4–5 or 12–14 days after plating. Tie-2⁺ spindle-shaped cells were considered EC. Cells were plated on fibronectin, collagen I, collagen IV, or laminin in medium D. Data are expressed as percentage of fibronectin controls. Error bars indicate SE. **P* < 0.05 relative to control. Minimum of 4 experiments performed per group.

TGF- β 1, FGF-2, and VEGF (42). The secretion of pro- and anti-angiogenic factors by cardiovascular cells is modulated by physiological stimuli such as hypoxia and stretch. Therefore, we tested the hypotheses that cardiovascular cells secrete factors that affect EC progenitor cell function and that physiological modulation of the balance of these secreted factors leads to changes in EC progenitor behavior. Experiments were conducted using the total CD34⁻ PBMC cell population so that circulating progenitors remained in a cellular environment resembling their in vivo situation. However, when preliminary data suggested that pure CD14⁺ cells and the mixed CD34⁻ PBMCs (which are 8–13% CD14⁺) behave differently in culture, subsequent experiments were modified to include both cell populations. This allowed the study of CD14⁺ cells specifically, while also providing a glimpse of how cells with which circulating EC progenitors normally interact modulate their behavior. As above, CD34⁺ cells were removed to avoid complications in interpretation that might arise from the presence of a second class of progenitors in the cultures.

Cells were cultured for 11–12 days in CM from aortic or coronary artery SMC, cardiomyocytes, or HUVEC that had been cultured in normoxia, hypoxia, or fresh medium. Initially, pure CD14⁺ cells or CD34⁻ PBMCs were plated on fibronectin in various fresh or CM containing 1–2% serum, but the cells died. In pilot experiments it was found that EC progenitors require a minimum of 5% serum and 0.1% bovine brain extract in medium D, so the fresh and CM were supplemented with 25% medium D in subsequent experiments to achieve these minimum concentrations.

We found previously that in high-serum medium D, essentially all viable cells in 12-day cultures have differentiated into EC (18), but the extent of EC differentiation could differ significantly in the various low-serum media used to culture cardiomyocytes and vascular cells. Thus CD14⁺ cells cultured in the various fresh and CM were DAPI stained and immunolabeled for expression of eNOS to determine both the total number of cells and the percentage of differentiated cells, i.e., eNOS⁺ EC in the cultures. As shown in Table 3, coronary artery SMC, aortic SMC, and HUVEC CM did not significantly affect differentiation of CD14⁺ cells relative to matched fresh media, although the fraction of cells that differentiated was lower than that which we previously observed for cells in (high-serum) medium D (18). Cells were not viable in fresh cardiomyocyte medium, but in cardiomyocyte CM, some cells survived (Figs. 2A and 3A), a small fraction of which differentiated and expressed eNOS (Table 3).

Heart and vascular cell CM modulate survival or growth. The total EC number in the same cultures was compared to assess the effect of CM on overall EC production (Fig. 2A).

Table 3. Percentage of eNOS immunolabeled cells

Stimulus	Aortic SMC	Coronary Artery SMC	HUVEC	Cardiomyocyte
Fresh	92 \pm 5	82 \pm 5	90 \pm 6	0
Normoxia	86 \pm 5	85 \pm 7	87 \pm 9	12 \pm 15
Hypoxia	89 \pm 3	81 \pm 6	91 \pm 2	11 \pm 9
Unstretched	90 \pm 5	84 \pm 6	95 \pm 0.1	16 \pm 10
Stretched	81 \pm 4	83 \pm 5	95 \pm 0.1	19 \pm 8

Values are means \pm SE. CD14⁺ cells after 12 days of culture in conditioned media (CM) from the indicated cells. eNOS, endothelial nitric oxide synthase.

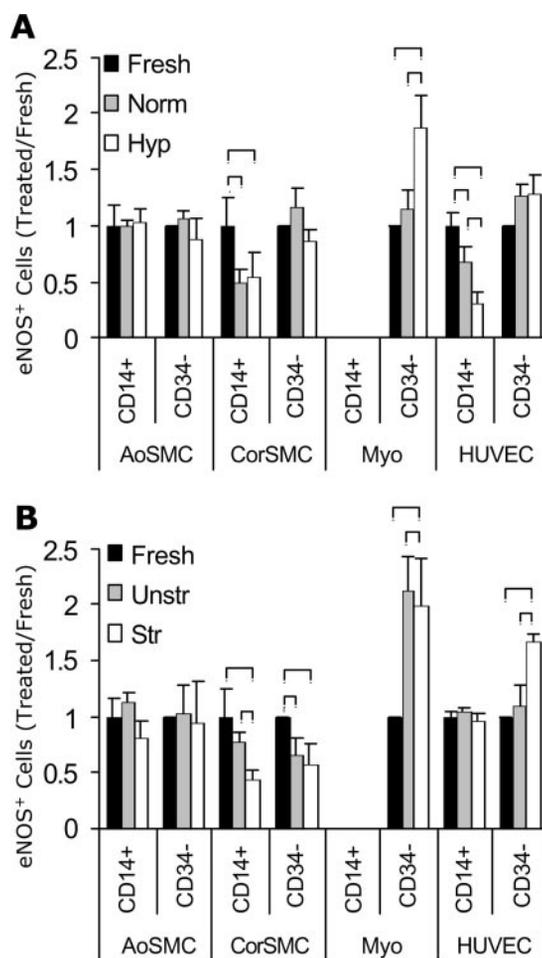


Fig. 2. Cardiovascular cells secrete factors that modulate EC progenitor derived EC formation. Pure CD14⁺ cells or CD34⁻ PBMCs plated in fresh or conditioned media (CM). A: CM collected from the indicated cells after 3-day culture in normoxia (Norm) or hypoxia (Hyp) (5% O₂). B: Fresh medium (Fresh) or CM collected from indicated cells plated on FlexorCell membranes after no (Unstr) or 1 h of cyclic stretch (Str). Minimum of 4 experiments performed per group. Data represent number of endothelial nitric oxide synthase (eNOS)⁺ cells 10–12 days after plating and are expressed as percentage of fresh medium controls. Error bars indicate SE. Comparison bars indicate **P* < 0.05 for the pair. CorSMC, coronary artery smooth muscle cells (SMC); AoSMC, aortic SMC; Myo, cardiomyocytes; HUVEC, human umbilical vein EC.

Culture of CD14⁺ cells in CM from normoxic or hypoxic aortic SMC had no effect on the cells relative to fresh medium. In contrast, culture in CM from coronary artery SMC or HUVEC led to a large decrease in EC cell number. This decrease was observed for CM from both hypoxic and normoxic cells. Surprisingly, CM from hypoxic HUVEC was more inhibitory than that from normoxic HUVEC, a result that was observed in nine of nine assays. As noted above, CD14⁺ cells did not survive in fresh cardiomyocyte medium, but both normoxic and hypoxic cardiomyocyte CM promoted cell survival, although few cells differentiated (Fig. 3A and Table 3).

An identical series of experiments was performed with CD34⁻ PBMCs (Fig. 2A). Again, neither CM from normoxic nor hypoxic aortic SMC had an effect on eNOS⁺ cell number relative to fresh medium. However, whereas coronary artery SMC and HUVEC CM decreased EC numbers in CD14⁺ cultures, in the physiological CD34⁻ milieu, these CM had no

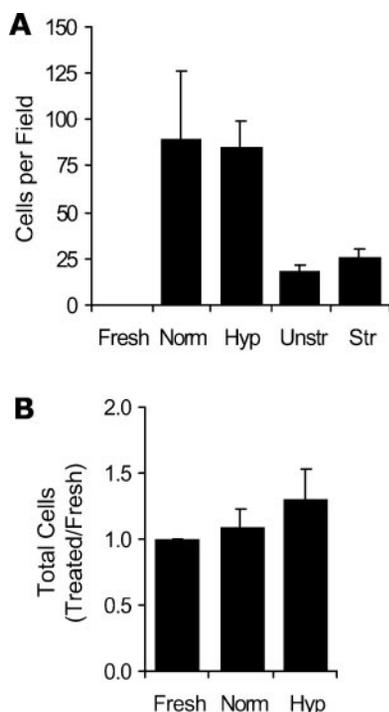


Fig. 3. Effect of myocyte CM on CD14⁺ cell viability. *A*: CD14⁺ cells plated in fresh or the indicated cardiomyocyte CM on *day 0*. Data represent number of cells per field 10–12 days after plating. *B*: CD14⁺ cells plated in medium D on *day 0* and switched to fresh or the indicated cardiomyocyte CM on *day 4*. Data are expressed as percentage of fresh medium controls. Error bars indicate SE. Minimum of 4 experiments performed per group. Norm, normoxia; Hyp, hypoxia; Unstr, unstretched; Str, stretched.

significant effect relative to fresh medium. The most dramatic difference in cultures of CD14⁺ cells compared with CD34[−] PBMCs was observed in cardiomyocyte CM cultures. CD34[−] PBMCs survived and differentiated in fresh cardiomyocyte medium at a rate similar to that of cells in normoxic myocyte CM, and CM from hypoxic cardiomyocytes further increased EC production.

As with the physiological stimulus of hypoxia, CM from aortic SMC subjected to cyclic stretch had no significant effect on EC number in CD14⁺ cell or CD34[−] PBMC cultures relative to CM from unstretched cells (Fig. 2*B*). On the other hand, CM from stretched coronary SMC inhibited EC production in CD14⁺ cultures compared with unstretched controls, but not in CD34[−] PBMC cultures (Fig. 2*B*). A small number of CD14⁺ cells survived in both unstretched and stretched cardiomyocyte CM, but few cells in these cultures expressed eNOS (Fig. 3*A*, Table 3), and stretch had no significant effect on total EC cell number (Fig. 2*B*). In CD34[−] PBMC cultures, wherein viability was not an issue, EC numbers were similar in unstretched and stretched myocyte CM. Finally, CM from stretched HUVEC had no effect on CD14⁺ cells but increased eNOS⁺ cell numbers in CD34[−] PBMC cultures relative to CM from unstretched controls (Fig. 2*B*).

Freshly isolated undifferentiated CD14⁺ cells did not survive in myocyte medium, but CM had a profound effect on survival of these cells (Fig. 3*A*). To determine whether factors in the CM were required for survival after CD14⁺ cell differentiation, CD14⁺ cells were plated in medium D. Four days later, the medium was replaced with either fresh, normoxic, or

hypoxic myocyte CM. After an additional 8 days in culture (i.e., at 12 days), there was no significant difference in the number of viable cells among the three media (Fig. 3*B*), and cell viability was similar to that observed in other media (data not shown).

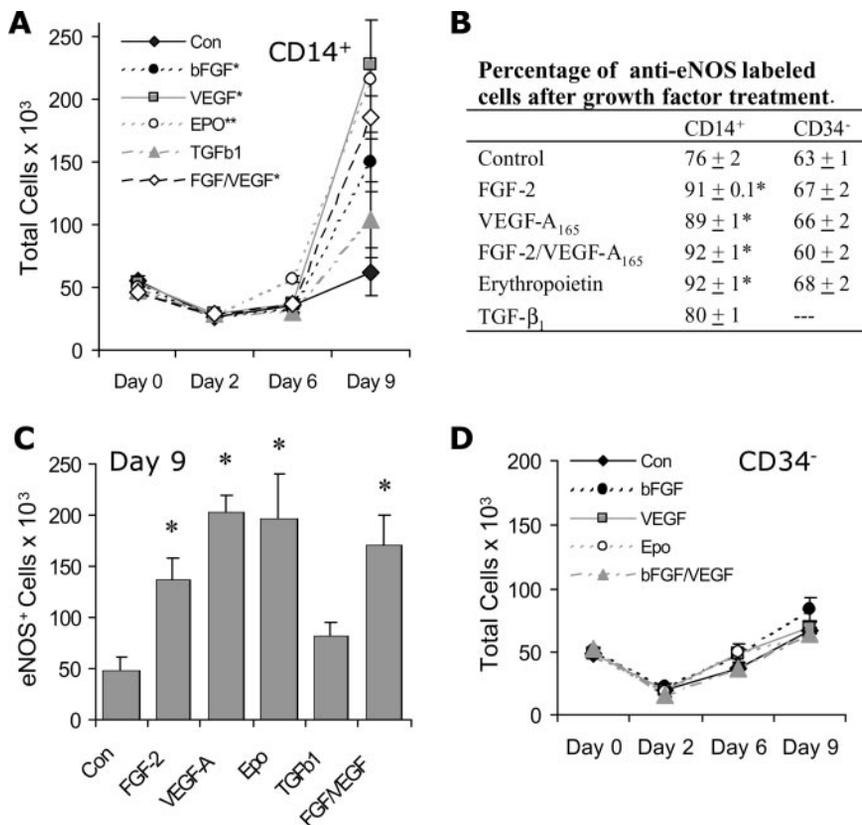
FGF-2, VEGF, erythropoietin, and TGF- β_1 . Heart and vascular cells secrete FGF-2, VEGF, and TGF- β_1 , and growth factor expression is regulated by hypoxia and cyclic stretch. EC progenitor behavior is modulated by interactions with these factors, and this modulation is affected by physiological changes to the cardiovascular cells. Additionally, various fibronectin receptors mediate FGF-2- and VEGF-induced vascularization. Thus we postulated that FGF-2, VEGF, and TGF- β_1 might be involved in EC progenitor interactions with their environment.

We cultured CD14⁺ cells on fibronectin in low-serum medium with or without added growth factors. Growth factors were added 1 day after plating and every 2 days thereafter, and cell numbers were assessed at 0, 2, 6, and 9 days by MTT assay (Fig. 4*A*). Two days after plating, total cell numbers decreased dramatically but similarly in all cultures, after which time cell numbers increased. Neither VEGF₁₆₅, FGF-2, nor the combination of the two significantly affected cell number in 6-day cultures, but by 9 days total cell number increased significantly under their influence, although the effects of VEGF₁₆₅ and FGF-2 were not additive. Stimulation with these growth factors also led to an increase in the percentage of eNOS⁺ cells in the cultures compared with controls at *day 9* (Fig. 4*B*), resulting in an increase in the total number of eNOS⁺ cells as well (Fig. 4*C*). In contrast, TGF- β_1 had no significant effect on either total or eNOS⁺ cell number or on the percentage of eNOS⁺ cells in the cultures (Fig. 4, *A–C*).

Because erythropoietin is a potent stimulator of angiogenesis, has pleiotropic effects on blood cells, and mobilizes EC progenitors *in vivo*, we examined its potential to modulate EC progenitors (5, 6, 10, 19, 22). As with FGF-2 and VEGF, total cell numbers decreased after 2 days, but by 9 days total cell number, percentage of eNOS⁺ cells, as well as total eNOS⁺ cells had increased relative to controls (Fig. 4, *A–C*). However, unlike FGF-2 and VEGF, the stimulatory effects of erythropoietin were already apparent by 6 days in culture, at which time there was a twofold increase in total cell number (Fig. 4*A*).

Because pure CD14⁺ cells respond to CM differently than when in the presence of other leukocytes, we also examined CD34[−] PBMC responses to the same growth factors. When cultured identically to CD14⁺ cells, growth factor stimulation of CD34[−] cells did not lead to an increase in total cell number in the cultures at *days 2, 6, or 9* relative to unstimulated controls (Fig. 4*D*). Also, the percentage of eNOS⁺ cells was significantly decreased relative to corresponding CD14⁺ cell cultures (Fig. 4*B*). Interestingly, however, at 4 days after plating, EC number was 1.8 \pm 0.3-fold higher ($P < 0.05$) in erythropoietin-stimulated cultures relative to controls. Erythropoietin was the only factor among eight tested that significantly stimulated EC production in CD34[−] PBMC cultures at any time through 12 days in culture. In cultures treated with 1.1 ng/ml TGF- β_1 , total cell number decreased slightly, and at 2.2 ng/ml few elongated cells were observed in the cultures (data not shown). Remarkably, at 3.3 ng/ml, only small round cells with scattered elongated cells were present in the cultures (Fig. 5), and all died by *day 6* in culture.

Fig. 4. Effects of growth factors on cultured EC progenitors. CD14⁺ cells or CD34⁻ PBMCs plated in low-serum medium D with or without supplemental growth factor. A and D: total cell number as assessed by MTT assay 0, 2, 6, and 9 days after plating pure CD14⁺ cells (A) or CD34⁻ PBMCs (D) in low-serum (5%) medium D supplemented with the indicated growth factor(s). **P* < 0.05 at day 9, ***P* < 0.05 at days 6 and 9 relative to same day control. B: percentage of eNOS⁺ cells 9 days after plating as assessed by anti-eNOS immunolabeling. Experiments performed 3 times in duplicate or triplicate wells and at least 3 fields per well were counted. **P* < 0.05, relative to CD14⁺ control. C: mean total eNOS⁺ cell number 9 days after plating obtained by multiplying total cell number by the fraction of eNOS⁺ cells. Error bars indicate SEs. VEGF, vascular endothelial cell growth factor-A; bFGF (FGF-2), basic fibroblast growth factor; TGF-β₁, transforming growth factor-β₁; Epo, erythropoietin. **P* < 0.05, relative to control.



The differential response to TGF-β₁ in CD14⁺ cell and CD34⁻ PBMC cultures was striking, so TGF-β₁ levels in CM from coronary artery SMC and HUVEC were examined, both of which depress eNOS⁺ cell production by CD14⁺ cells, and from aortic SMC, which do not affect eNOS⁺ cell numbers. CM was collected from cells after 3 days of culture in low-serum medium. TGF-β₁ protein levels were assessed by ELISA and are listed in Table 4. There was no apparent correlation between the amount of TGF-β₁ protein and EC number in the cultures. Furthermore, TGF-β₁ protein levels were similar in CM from CD14⁺, CD34⁻, and CD34⁻CD14⁻ PBMCs (Table 4).

The effects of a number of other growth factors and growth factor combinations on CD14⁺ cells were investigated in an effort to identify other potential EC progenitor inhibitors. None was identified.

DISCUSSION

Much attention has been paid to identification of adult stem and progenitor cells and the assessment of their therapeutic potential, but physiological modulation of their function has been less well studied. This work focused on interactions of EC

Table 4. TGF-β₁ concentration in fresh and conditioned medium

Cell Type	Medium				
	Fresh (Control)	Normoxia	Hypoxia	Unstretched	Stretched
Aortic SMC	90	279	209		
	81	278	287		
Coronary artery SMC	138	566	522	150	183
	168	456	580	187	189
HUVEC (1% serum)	81	414			
	91	424			
HUVEC (20% serum)	1392	1492			
	1446	1542			
Pure CD14 ⁺	636	505			
	844	1028			
All CD34 ⁻ (includes CD14 ⁺)	636	629			
	844	1170			
CD34 ⁻ CD14 ⁻	636	774			
	844	1217			

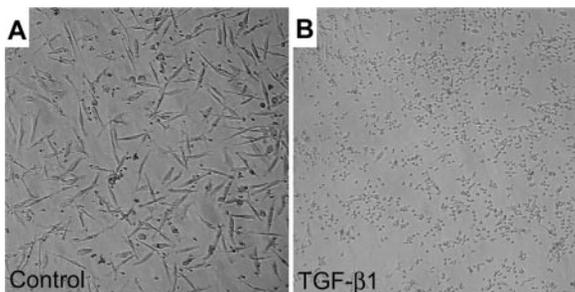


Fig. 5. Inhibition of CD34⁻ cell differentiation by TGF-β₁. Phase contrast micrographs of CD34⁻ PBMC cultures 6 days after plating on fibronectin in medium D with no supplements (A) or 3.3 ng/ml TGF-β₁ (B).

Each concentration (pg/ml) represents the average of triplicate wells from one experiment. TGF-β₁, transforming growth factor-β₁.

progenitors in CD14⁺ cell cultures with environmental factors and culminated in several novel findings. We demonstrated that 1) cardiovascular cells secrete factors that both promote and inhibit EC formation by progenitors; 2) neither cyclic stretch nor hypoxia directly alter progenitor-derived EC formation *in vitro*; 3) both hypoxia and cyclic stretch can modulate EC progenitor function indirectly via altering secretion of soluble factors by other cells; 4) non-EC progenitor PBMCs are strong modulators of EC progenitor behavior, and FGF-2, VEGF, and TGF- β 1 may have roles in this modulation; and 5) erythropoietin is a potent stimulator of progenitor-derived EC formation.

To define suitable conditions for assaying EC progenitors, their ability to differentiate on a variety of substrates was tested. In our standard culture conditions, EC progenitors differentiated preferentially on fibronectin although their failure to differentiate on ProNectin F indicates that the RGD must be presented appropriately. Fewer cells differentiated on collagen IV and collagen I than on fibronectin, and surprisingly, they failed to or differentiated poorly on laminin, gelatin, and Matrigel in our culture conditions.

The principal integrin mediating collagen binding is $\alpha_2\beta_1$, and both collagen I and IV bind to it via the same high-affinity sequence; $\alpha_1\beta_1$ integrin also binds collagen, but it binds to different sites on collagen I than on collagen IV (24). VEGF induces both $\alpha_1\beta_1$ and $\alpha_2\beta_1$, FGF-2 decreases $\alpha_1\beta_1$, and TGF- β 1 induces $\alpha_2\beta_1$ expression (8). Therefore, the effects of these growth factors on cells plated on collagen may differ markedly from those plated on fibronectin.

The fibronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin are expressed on EC and are involved in FGF-2 and VEGF-mediated angiogenesis, respectively (7, 15, 39, 40). Because FGF-2 and VEGF increase EC number in progenitor cell cultures, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ may play a role in EC progenitor differentiation. It is noteworthy that $\alpha_v\beta_3$ also interacts with laminin (23), yet few EC are present in EC progenitor cultures on laminin. The fact that co-coating plates with fibronectin and laminin failed to increase EC number relative to laminin alone suggests that laminin binding inhibits EC production. FGF-2 downregulates expression of $\alpha_v\beta_3$ on EC (23), so a major function of FGF-2 in promoting EC progenitor differentiation could be downregulation of $\alpha_v\beta_3$ expression. Clearly, however, additional extensive study is required to investigate these hypotheses and to understand progenitor-substrate interactions.

That FGF-2 and VEGF might promote EC progenitor survival, growth, or differentiation is not surprising. Growth media that support EC progenitor differentiation include FGF-2 or FGF-2 containing bovine brain extract (4, 12, 41). VEGF is thought to mobilize circulating EC progenitors (4, 31), stimulate differentiation of dendritic cells into an EC phenotype (13), and play a role in the differentiation of the bone marrow-derived EC progenitors described by Verfaillie and colleagues (34). Our data demonstrate directly that both FGF-2 and VEGF stimulate EC production by EC progenitors in CD14⁺ cultures and that their effects are mediated by increased progenitor differentiation (Fig. 4B) and, probably, proliferation of immature progenitor-derived EC (Fig. 4A). They also demonstrate that the effects of FGF-2 and VEGF on cell number are not additive, suggesting that the two factors converge on the same pathway.

An intriguing aspect of the data is the absence of an obvious FGF-2- or VEGF-mediated effect on EC progenitor growth or differentiation in CD34⁻ PBMC cultures. One explanation is that CD34⁻ PBMCs secrete the growth factors at relatively high concentrations, such that additional exogenous growth factors have no effect. Arguing against this are the facts that 1) high-serum CM from CD34⁻ cells promotes EC progenitor differentiation (18), and 2) in the absence of bovine brain extract, CD34⁻ PBMC survival requires supplementation with VEGF and FGF-2. These data suggest that PBMCs actively inhibit EC formation even in the presence of proangiogenic factors and that either 1) cell-cell interactions are required for the inhibitory effect, or 2) factors produced by CD34⁻ promote EC formation but in stressful conditions (i.e., low serum) they inhibit it. It seems more reasonable that cell-cell interactions inhibit EC formation because it is unlikely that it would be advantageous for blood cells to promote progenitor cell differentiation under normal conditions.

These findings also suggest that erythropoietin potentiates proliferation of immature EC progenitor-derived EC but that it may have little role in promoting growth of more mature EC. Moreover, it may be able to transiently disinhibit leukocyte-mediated inhibition or provide a sufficiently potent growth stimulus to overcome the inhibition.

TGF- β 1 produced the most dramatic effect on EC progenitors exhibited by any factor or CM studied. While its addition to CD14⁺ cultures had little apparent impact on the cells, EC progenitors failed to differentiate and eventually died in CD34⁻ PBMC cultures cells containing TGF- β 1. It is possible that CD34⁻ PBMCs secrete high levels of TGF- β 1, so that the addition of more of the growth factor might raise TGF- β 1 to toxic concentrations. However, CM from CD14⁺, CD34⁻CD14⁺, and CD34⁻CD14⁻ PBMCs contain similar TGF- β 1 levels, ruling this out. Moreover, no correlation between the amount of TGF- β 1 present in cardiovascular cell CM and their ability to increase or decrease EC cell number was observed. Thus levels of TGF- β 1 that are tolerated in one setting are not in another. Studies are under way to better understand possible roles for TGF- β 1 in EC progenitor differentiation and survival.

Our data show that the cellular milieu in which EC progenitors find themselves markedly influences their behavior. Circulating PBMCs and three of the four different cardiovascular cell types examined each, in some way, regulated the ability of EC progenitors to produce EC, and PBMCs or the factors they produce interacted with molecules secreted by the cardiovascular cells to coordinately regulate EC production. Thus it is difficult to say exactly how FGF-2, VEGF, TGF- β 1, or any other factors released by cardiovascular cells will influence EC progenitors *in vivo*. If the CD14⁺ cells are the EC progenitors, the fact that the precise nature of microenvironment might be critical to EC formation should be expected because CD14⁺ cells can assume at least two other phenotypes, macrophages or dendritic cells, depending on the environment in which they find themselves. It is noteworthy that no effect of CM on differentiation was detected (Table 3), suggesting that observed changes were mediated through cell survival and/or growth. In the case of cardiomyocytes, the data indicate that factors secreted by cardiomyocytes promote EC progenitor survival but probably do little to increase growth or differentiation.

It makes physiological sense that myocytes promote and coronary SMC inhibit formation of EC. After injury, inflammation presumably causes EC progenitors to leave the blood and enter the myocardium. If the myocardium is hypoxic, proangiogenic signals are released and one or more of these may promote survival of invading EC progenitors. Once the progenitors and resident EC coalesce to form capillary tubes, there is no need for additional EC progenitor differentiation (at least locally), so as SMC are recruited to the vessel, further production of EC may be inhibited. Data showing that hypoxic HUVEC secrete factors that inhibit EC formation are puzzling, yet as noted, this finding was consistent in nine of nine experiments.

It is difficult to definitively prove that CD14⁺ cells are EC progenitors, and it could be that contaminating CD34⁺ PBMCs are the source of EC in our cultures. However, in a previous study we showed that at a constant total cell number of CD34⁺ and CD14⁺ cells if the fraction of CD34⁺ cells in the culture was varied from as little as <0.1% to 18%, the total number of viable EC present in the culture did not change, suggesting that both cells were equally competent to produce endothelial cells (18). The data here also support the hypothesis that CD14⁺ PBMCs are EC progenitors. To obtain CD34⁻CD14⁺ PBMCs, we first deplete PBMCs of CD34⁺ cells. The depleted cells are then subjected to two rounds of CD14 selection. In our hands, <0.1% of total blood mononuclear cells are CD34⁺ initially, and we typically recover ~50% of these. That is, even if the two subsequent rounds of CD14⁺ selection removed no CD34⁺ cells (and this is unlikely), there would be on average no more than 25 CD34⁺ cells in the 5 × 10⁴ cell culture, assuming all CD34⁺ cells survived. If constant through this period, a doubling time of 15 h would be required to produce 5 × 10⁴ CD34⁺-derived eNOS⁺ cells by day 9. In fact, however, the doubling time between 6 and 9 days is at least 27 h (depending on the cell treatment), implying that the doubling time was 10 h before day 6. Not only would this represent very rapid cycling, but it assumes that the cells cycled rapidly and then abruptly nearly tripled their cell cycle time at 6 days.

Thus, even if we ignore our previously published coculture data, we are left with two possibilities: 1) CD14⁺ cells are EC progenitors; or 2) CD14⁺ selection removes no CD34⁺ cells, every CD34⁺ cell survives plating, and doubling time is ≤10 h for days 0–6 and then ≥27 h for days 6–9. Invoking Occam's razor, we think the first interpretation is more reasonable, although the alternative is formally possible.

In summary, the findings presented further support the idea that CD14⁺ PBMCs are a source of EC progenitors. They also demonstrate that EC progenitor regulation is complex and may be mediated via direct cell-cell interactions, extracellular matrix molecules, as well as by soluble factors, including VEGF, FGF-2, erythropoietin, and TGF-β₁. These regulatory components interact and modulate the effects of one another and are themselves further regulated by physiological stimuli. Cellular components of the blood appear to dampen responses to these stimuli, perhaps in the interest of maintaining EC progenitor homeostasis. This multilayered control system makes it difficult to predict the effect that any single factor will have on EC progenitors in vivo.

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