Paracrine mitogenic effect of human endothelial progenitor cells: role of interleukin-8

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Paracrine mitogenic effect of human endothelial progenitor cells: role of interleukin-8. Am J Physiol Heart Circ Physiol 289: H968–H972, 2005. First published April 1, 2005; doi:10.1152/ajpheart.01166.2004.—Endothelial progenitor cells (EPCs) play an important role in repair of vascular injury and neovascularization. Molecular mechanisms underlying vascular effects of EPCs are not fully understood. The present study was designed to test the hypothesis that human EPCs exert a strong paracrine mitogenic effect on mature endothelial cells. Levels of interleukin-8 (IL-8) were significantly higher in conditioned medium (CM) collected from EPCs than in CM derived from mature endothelial cells [umbilical vein endothelial cells (HUVECs) and coronary artery endothelial cells (CAECs)]. CM of EPCs stimulated proliferation of HUVECs and CAECs. This mitogenic effect was partially inhibited by IL-8-neutralizing antibody. In contrast, CM of HUVECs and CAECs had a weak or no mitogenic effect on mature endothelial cells. Our results demonstrate significantly higher levels of IL-8 secretion by human EPCs than by mature endothelial cells. IL-8 appears to be an important mediator of the paracrine mitogenic effect of EPCs.

METHODS

EPC isolation, cell culture, and phenotyping. The protocol for collection and use of human blood samples from healthy subjects was approved by the Institutional Review Board at the Mayo Clinic. EPCs were obtained as previously described (6). Mononuclear cells were isolated from the buffy coat of blood samples from 11 human donors (52 ± 12 yr of age) and plated at a density of ~2 × 10^7 cells/well on six-well plates coated with human fibronectin (R & D Systems) in endothelial growth medium-2 (EGM-2; Cambrex), which was composed of endothelial cell basal medium-2 (EBM-2), 2% FBS, and growth factors. At ~4 wk, subconfluent cell colonies were passaged, and cells were subsequently cultured in EGM-2, yielding outgrowth EPCs. For further experiments, in parallel, human umbilical vein endothelial cells (HUVECs; Clonetics) from three donors and human coronary artery endothelial cells (CAECs; Clonetics) from three donors (29 ± 3 yr of age) were cultured in EGM-2 under identical conditions. The experiments were performed on cells cultured from passages 4–8. Morphological appearance, indirect immunofluorescence, and flow cytometry analysis were utilized to define the endothelial cell phenotype of EPCs as previously described (6).

Collection and analysis of conditioned medium. EPCs, HUVECs, or CAECs (10^6 cells/100-mm-diameter dish) were incubated with EBM-2 (without growth factors and FBS, 6 ml/dish) for 24 h, yielding conditioned medium (CM). The CM was collected for analysis of proteins using Human Cytokine Array V (RayBiotech), which detects 72 cytokines, as previously described (7). The same amounts (5 ml) of CM from EPCs, HUVECs, and CAECs were analyzed in parallel under identical conditions. After the membrane was exposed to Biomax MR film for 5 min, the optical density of the dot was measured by Scion Image. Protein levels in CM were normalized to internal control and expressed as relative densitometric units. An ELISA kit (Quantikine, R & D Systems) was used to quantify IL-8 in the CM. The IL-8 values were expressed as picograms per 1,000 cells.

5-Bromo-2′-deoxyuridine incorporation assay. HUVECs (1,000 cells/well) or CAECs (2,000 cells/well), seeded on 96-well plates (experiments were performed in triplicate), were treated with EBM-2 or CM (200 µl/well) derived from EPCs, HUVECs, or CAECs (10^6 cells in 6 ml of EBM-2 for 24 h). After incubation for 20 h, 10 µM 5-bromo-2′-deoxyuridine (BrDU) labeling solution (from a Cell Proliferation ELISA kit, Roche Applied Science) was added into each well, and the incubation was continued for another 4 h. Cells were fixed and exposed to anti-BrDU and substrate solutions. The reaction product was quantified by measuring the absorbance using a multiwell spectrophotometer (Spectromax340 PC, Molecular Devices). In some experiments, CM was incubated with 50 ng/ml IL-8-neutralizing antibody (R & D System) for 1.5 h at room temperature before it was applied to mature endothelial cells.

Statistical analysis. Values are means ± SD. Differences between mean values of multiple groups were analyzed using ANOVA followed by Bonferroni’s t-test multiple comparison procedure (SigmaStat 2.03).
for Windows). For comparison between two groups, Student’s t-test was used. $P < 0.05$ was considered statistically significant.

**RESULTS**

**Characterization of EPC phenotypes.** EPCs outgrown from mononuclear cells exhibited the “cobblestone” morphology and monolayer growth pattern typical of endothelial cells (Fig. 1A). The endothelial phenotype of the outgrown EPCs was further characterized by expression of endothelial markers (Fig. 1, B–E), such as Flk-1, CD31 (90 ± 8% cells positive, $n = 4$), and VE-cadherin (91 ± 7% cells positive, $n = 3$).

**Paracrine mitogenic effect of EPCs.** Assay by protein array and ELISA showed significantly higher protein levels of IL-8 in CM derived from EPCs than in CM from HUVECs and CAECs (Fig. 2). The protein array analysis also revealed that when cultured in EBM-2, EPCs and mature endothelial cells secreted similar levels of other angiogenic cytokines, such as transforming growth factor-$\beta 2$ (ratio of TGF-$\beta 2$ in CM of EPCs to that in CM of CAECs = 1.0 ± 0.23, $n = 3$) and monocyte chemoattractant protein-1 (ratio of monocyte chemoattractant protein-1 in CM of EPCs to that in CM of CAECs = 1.46 ± 0.65, $n = 3$). CM derived from EPCs increased the number of HUVECs 1.6-fold after 24 h of treatment, whereas CM of HUVECs or EBM-2 control medium did not significantly change cell numbers after 24 h of incubation (Fig. 3). CM of EPCs also significantly increased BrdU incorporation in HUVECs or CAECs compared with CM of HUVECs or CAECs, respectively. This mitogenic effect was partially inhibited by treatment with 50 ng/ml IL-8-neutralizing antibody (Fig. 3). CM of HUVECs also enhanced BrdU incorporation in HUVECs. However, the mitogenic effect was not inhibited by IL-8 antibody (Fig. 3). CM of

![Fig. 1. Phenotyping of human endothelial progenitor cells (EPCs). A: growth of confluent cells into a monolayer with cobblestone appearance at 4 wk. B and C: immunofluorescence confocal microscopy of EPCs (×100 magnification) labeled with Flk-1 and normal mouse IgG (control). Secondary antibody was conjugated to Texas red. Cell nuclei were counterstained with Hoescht 33528. D and E: flow cytometric analysis of EPCs positive for CD31 and VE-cadherin (VE-Cad). Open histograms, test antibodies; filled histograms, isotype-matched control IgG.](http://ajpheart.physiology.org/)

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CAECs did not significantly stimulate the proliferation of CAECs. IL-8 antibody did not alter the effect of CAEC CM on CAEC proliferation.

**DISCUSSION**

The results of the present study demonstrate that human EPCs possess a strong paracrine mitogenic effect. We identified release of IL-8 as an important mechanism underlying the mitogenic effect of EPCs. Our results support the concept that the paracrine effect of EPCs on surrounding endothelium is an essential component of the reported vascular protective and angiogenic function of EPCs.

Late (outgrowth) EPCs are obtained by culturing early EPCs (8, 15). In the absence of a consensus in the literature regarding nomenclature of EPCs, we and others have been using the term EPCs to refer to early and late outgrowth cells (6 – 8). Early and outgrowth EPCs have different morphology and characteristics (8, 15) in vitro; however, both exhibit therapeutic effects in vivo (5, 7). On the other hand, although outgrowth EPCs and mature endothelial cells have similar morphology and endothelial cell marker profile, outgrowth EPCs exhibit biochemical and functional characteristics different from mature endothelial cells, such as high antioxidant capacity and resistance to oxidative stress (6). In the present study, we demonstrated a major difference in paracrine function between mature endothelial cells and outgrowth EPCs.

Recently, release of angiogenic growth factors from early and outgrowth EPCs has been reported (7, 8, 17). However, it is still not clear whether angiogenic paracrine mechanisms contribute to therapeutic neovascularization induced by human EPCs. Mature endothelial cells also produce and release angiogenic factors (3, 18, 21). The autocrine angiogenic effect caused by increased secretion of angiogenic factors from the endothelium has been observed during tumor neovascularization and in angioproliferative diseases (2, 4). However, transplantation of differentiated mature endothelial cells [human microvascular endothelial cells (9), saphenous vein endothelial cells (13), and endothelial cells of gastroepiploic artery (8)], in contrast to EPCs, does not stimulate neovascularization. In the present in vitro study, we provide direct evidence that EPCs exert a stronger mitogenic paracrine effect than HUVECs and CAECs, which at least in part was due to significantly greater release of IL-8 from EPCs.

IL-8 is an ELR (glutamic acid-leucine-arginine) CXC chemokine that has been implicated in chronic inflammatory disorders characterized by persistent angiogenesis (12). IL-8 and its receptors (CXCR1 and CXCR2) are present on endothelial cells (16). Nanomolar to picomolar concentrations of IL-8 have chemotactic activity for neutrophils and lymphocytes and can stimulate chemotaxis as well as proliferation of HUVECs (12). The angiogenic effect of IL-8 (20) is associated with the mitogenic effect on endothelial cells (14, 19), as well as enhancement of endothelial cell survival, matrix metalloproteinase production, and capillary tube formation in vitro (14). In the present study, under serum-deprived conditions, EPCs and mature endothelial cells released similar levels of
suggesting that the contribution of IL-8 to the paracrine mitogenic effect is specific for EPCs. We did not detect significant levels of other angiogenic cytokines, including vascular endothelial growth factor in CM of EPCs and mature endothelial cells. This could be explained by the fact that the CM were obtained after only 24 h of cell culture. In previous reports, higher levels of angiogenic cytokines were detected in CM collected after 72 h of incubation (8, 17).

Our results are consistent with the concept that EPCs may exert a therapeutic effect on regeneration of injured endothelium and neovascularization of ischemic tissues by stimulating proliferation of surrounding cells via a paracrine effect. IL-8 appears to be an important mitogen released from human EPCs. The contribution of IL-8 production and release to an in vivo therapeutic effect of EPCs remains to be determined.

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REFERENCES

10. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, and Epstein SE. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 94: 678–685, 2004.
11. Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, Fuchs S, and Epstein SE. Local delivery of marrow-derived stromal cells


