Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress

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Endothelial progenitor cells (EPCs), circulating in peripheral blood, migrate toward target tissue, differentiate, and contribute to the formation of new vessels. In this study, we report that shear stress generated by blood flow or tissue fluid flow can accelerate the proliferation, differentiation, and capillary-like tube formation of EPCs. When EPCs cultured from human peripheral blood were subjected to laminar shear stress, the cells elongated and oriented their long axes in the direction of flow. The cell density of the EPCs exposed to shear stress was higher, and a larger percentage of these cells were in the G2-M phase of the cell cycle, compared with EPCs cultured under static conditions. Shear stress markedly increased the EPC expression of two vascular endothelial growth factor receptors, kinase insert domain-containing receptor and fms-like tyrosine kinase-1, and an intercellular adhesion molecule, vascular endothelial-cadherin, at both the protein and mRNA levels. Assays for tube formation in the collagen gels showed that the shear-stressed EPCs formed tubelike structures and developed an extensive tubular network significantly faster than the static controls. These findings suggest that EPCs are sensitive to shear stress and that their vasculogenic activities may be modulated by shear stress.

THE FORMATION OF NEW BLOOD vessels in postnatal life has generally been considered to be mediated by the sprouting of endothelial cells (ECs) from preexisting vessels, a process referred to as angiogenesis. However, recent studies have indicated that a vasculogenesis process involving the in situ differentiation of endothelial precursor cells and their subsequent organization into new vessels is also responsible for postnatal neovascularization (10, 22, 27). The existence of bone marrow-derived endothelial progenitor cells (EPCs) circulating in the peripheral blood has been demonstrated in adult humans (6, 26). EPCs have the capacity to circulate, proliferate, and differentiate into mature ECs in response to a variety of growth factors, including VEGF, and other cytokines (7, 16, 18). Transplantation studies have revealed that EPCs can be incorporated into sites of active neovascularization in ischemic hindlimbs and myocardium and contribute to both tumor growth and the formation of new blood vessels (5, 20, 40). However, the role of EPCs in supporting postnatal vasculogenesis is under intensive investigation, and the factors regulating the migration, proliferation, differentiation, and vessel formation of EPCs are not yet known.

During the process of EPC incorporation into tissues and neovascularization, the cells are exposed to fluid shear stress, a mechanical force generated by blood flow or interstitial fluid flow (42). Ample evidence has shown that shear stress modulates mature EC function and gene expression, playing important roles in the homeostasis of vascular functions and the mechanisms of blood flow-dependent phenomena, such as angiogenesis, vascular remodeling, and atherogenesis (12). Accordingly, the present study was designed to examine the effects of shear stress on EPCs. Using a flow-loading apparatus, we applied controlled levels of laminar shear stress to EPCs isolated from human peripheral blood and examined the changes in their morphology, proliferation, and expression of EC-specific marker proteins. We also examined the effect of shear stress on tube formation by EPCs in collagen gels.

METHODS

Mononuclear cell isolation and culture. Mononuclear cells were isolated from 100 ml of human peripheral blood by density gradient centrifugation by using Vacutainer CPT tubes (Becton Dickinson) containing Histopaque-1077 (Sigma), as previously described (6). Cells were then plated on culture dishes coated with 100 μg/ml of human fibronectin (Sigma) at a density of 5 × 10^5 cells/cm² and cultured in EBM-2

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(Clonetics) supplemented with 5% fetal bovine serum, human VEGF, human FGF-2, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid at 37°C in an atmosphere of 5% CO₂. Four days after seeding, ~10% of the cells had attached to the dishes, whereas the remaining 90% were nonadherent. Nonadherent cells were removed by washing with PBS, and the media were reapplied. The culture was maintained through days 2–12.

Identification of EPCs. To confirm the EPC phenotype, attached mononuclear cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated LDL (DiI-acLDL; 10 μg/ml; Biomedical Technologies) at 37°C for 1 h. The cells were then fixed with 2% formaldehyde for 10 min and incubated with FITC-labeled Ulex europeus agglutinin (lectin, 10 μg/ml; Sigma) for 1 h. After staining, the samples were viewed with an inverted fluorescent microscope (Nikon). Cells demonstrating double-positive fluorescence were identified as differentiating EPCs (7, 19, 20). Nearly all adherent cells (>95%) were DiI-acLDL(+) Ulex-lectin(+) (see fig. 1).

Shear stress experiments. Cells were exposed to laminar shear stress by using a rotating disk-type flow loading device, as previously described (4, 32). Briefly, a dish containing cultured EPCs was placed on the stage of the device, and a stainless steel disk was placed in the bottom of the dish. The rotation of the disk caused the media to flow in a concentric circle, thereby exerting a laminar shear stress on the cells. The intensity of the shear stress (τ) was calculated as τ = μrω/d, where μ is the fluid viscosity, r is the distance from the center of the dish, ω is the angular velocity, and d is the distance between the disk and the dish. The device generates gradients of shear stress that are dependent on the distance from the center of the dish. In the majority of experiments, cells from a single dish were used, and the shear stress values were averaged. In some experiments, bands of cells at different radii were separately scraped from the same dish and studied. The shear stress used in this study ranged from 0.1 to 2.5 dyn/cm²; all experiments were performed at 37°C in a CO₂ incubator.

Flow cytometry. The expression of EC-specific marker proteins was measured by using a FACScan flow cytometer (Becton Dickinson). A total of 2 × 10⁵ cells were incubated with monoclonal antibodies against VEGF receptors, such as kinase insert domain-containing receptor (KDR) and fms-like tyrosine kinase-1 (Flk-1) (Sigma), and adhesion molecules, such as vascular endothelial (VE)-cadherin (RDI), for 45 min at 4°C. The cells were then incubated with FITC-conjugated anti-mouse IgG (H+L) antibody (Amersham) for 30 min at 4°C and analyzed by FACS. The accumulated cells were segregated into monocyte-sized and lymphocyte-sized fractions by gating during a light-scatter analysis. Monocyte-sized, but not lymphocyte-sized, cells were positive for lectin and DiI-acLDL, whereas lymphocyte-sized, but not monocyte-sized, cells were positive for leukocyte common antigen CD45 (DAKO). Thus, the gating of the monocyte-sized fraction was programmed to ensure the accumulation of EPCs. Cell number vs. logarithmic fluorescence intensity histograms were recorded for 20,000 cells per sample. Background fluorescence was obtained from the negative control cells stained with FITC-conjugated anti-mouse IgG (H+L) antibody and subtracted from the mean channel fluorescence of the specific staining patterns. The expression of each antigen was expressed as the mean channel fluorescence.

Cell cycle analysis. Cells were detached from dishes by incubating in PBS supplemented with 2 mM EDTA. The detached cells were then washed with PBS and fixed with 70% ethanol for 1 h at 4°C. After washing with PBS, the cells were incubated with PBS containing 0.5% RNase (Sigma) for 30 min at room temperature and then stained with propidium iodide (50 μg/ml) for 15 min. The fluorescence intensity of this dye was measured in a sample containing 20,000 cells, and the cell cycle was analyzed by using ModFit LT Cell Cycle Analysis Software (Verity Software House).

RT-PCR analysis. RT-PCR was performed to determine the EC-specific marker mRNA levels, as previously described (23). Briefly, the reverse transcription of RNA was performed in a reaction mixture containing total RNA, moloney murine leukemia virus reverse transcriptase (GIBCO), oligo(dT)12-18, ribonuclease inhibitor, each dNTP mixture, and dithiothreitol in a first-strand buffer. The mixture was incubated at 37°C for 1 h, heated to 99°C for 5 min, and then chilled at 4°C for 5 min. The cDNA samples were then amplified by PCR by using the following primer pairs: KDR forward: 5′-AGACC-AAAGGGGCACGATTCC-3′, reverse: 5′-CAGCAAAAAC-CAAAAGACCAGAC-3′; Flt-1 forward: 5′-ATTGTTGTATTTGGGCTTTCG-3′, reverse: 5′-CAGGCTCATGAACCTT-GAAAGC-3′; VE-cadherin forward: 5′-GCTGAAAGGAA-AACCAGAAGAC-3′, reverse: 5′-TCGGTATATTCCGT-GAGGTAAG-3′; GAPDH forward: 5′-ACATCATCCCTGCGCTCTACTGCT-3′, reverse: 5′-AGTGGGTGTCGTCTGTT-GAAGTC-3′. A solution containing ExTaq DNA polymerase (Takara), [α-32P]dCTP, and each primer was added to each sample. Each temperature cycle consisted of 30 s, 60°C for 30 s, and 72°C for 1 min. The amplified product was sampled every other cycle and electrophoresed on a 5% polyacrylamide gel. The PCR product sequences confirmed that each mRNA product had been correctly amplified by its primers.

For the quantification of PCR products, the radioactivity of each band was measured by using a GS363 Molecular Imager System (Bio-Rad) and plotted against the number of PCR cycles on a semilogarithmic scale, forming a sigmoid curve. From the curve, the cycle in which the operating range of the PCR was linear was selected, and the ratio of radioactivity between each target gene and GAPDH in the cycle was calculated as a parameter of relative mRNA levels.

Tube formation assay. Collagen gels were formed by adding Biocoat Matrigel (Becton Dickinson) into 24-well plates and incubating in a CO₂-free incubator at 37°C for 30 min. The same batch of Matrigel was used for all of the experiments. The gels were then overlaid with 1 × 10⁵ cells suspended in culture medium and incubated at 37°C in an atmosphere of 5% CO₂. Gels were examined by using a phase-contrast microscope equipped with a digital camera PDLC (Polaroid); the images were then imported as TIFF files into National Institutes of Health (NIH) Image software. A second observer measured the total length of the tubelike structures (defined as those exceeding 200 μm in length) per each image.

Statistical analysis. All results are expressed as means ± SD. The statistical significance was evaluated by using an ANOVA and a Bonferroni adjustment applied to the results of a t-test using SPSS (SPSS). A value of P < 0.01 was regarded as being statistically significant.

RESULTS

EPCs exhibit morphological changes in response to shear stress. EPCs attached, spread, and proliferated on tissue culture plastic coated with fibronectin and showed positive staining for both lectin and DiI-acLDL...
The EPCs showed no definite orientation under static conditions. When they were exposed to laminar shear stress for 24 h, they became elongated and oriented their long axes in the direction of flow (Fig. 1D). The elongated cells also showed positive staining for both lectin and DiI-acLDL (Fig. 1E and F). When shear-stressed cells were returned to the static conditions, the changes reversed in ~24 h (data not shown), indicating the morphological response to shear stress to be reversible.

Shear stress augments EPC proliferation. EPCs were exposed to laminar shear stress for 24 h, and changes in cell density were determined on days 4, 6, 8, 10, and 14 (Fig. 2). Shear stress induced a much larger increase in cell density than occurred in static cells. Statistically significant differences were observed on days 8, 10, and 14 (P < 0.001).

Cell cycle analysis showed that the shear stress decreased the percentage of EPCs in the G0 and G1 phases of the cell cycle and increased the percentage of EPCs in the S, G2, and M phases (Table 1). These results indicate that laminar shear stress stimulates EPC proliferation.

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Shear stress modulates expression of EC-specific markers in EPCs. The cell surface expression patterns of various EC-specific markers were examined in EPCs that had been either cultured under static conditions or subjected to shear stress. Flow cytometric analysis showed a time-dependent increase in the expressions of KDR, Flt-1, and VE-cadherin in EPCs cultured under static conditions (static; Fig. 3). The levels of expression of KDR, Flt-1, and VE-cadherin increased by about six-, five-, and fivefold, respectively, on day 21, compared with the values on day 4.

Shear stress modulated the expression of these EC-specific markers in EPCs. Shear stress markedly increased the levels of expression of KDR, Flt-1, and VE-cadherin (flow; Fig. 3). The levels of KDR, Flt-1, and VE-cadherin expression on days 8, 10, and 14 in the EPCs exposed to shear stress were almost identical to those on day 21 in the EPCs cultured under static conditions.

The mRNA levels of KDR, Flt-1, and VE-cadherin were determined by using RT-PCR. Shear stress markedly increased the KDR, Flt-1, and VE-cadherin mRNA levels in a magnitude-dependent manner at day 6 (Fig. 4). These results indicate that shear stress accelerates the expression of EC-specific markers on EPCs at both the protein and mRNA levels.

Shear stress promotes tube formation by EPCs in collagen gels. To investigate whether shear stress affects the ability of EPCs to form capillary-like tubes, EPCs cultured under static conditions and EPCs exposed to shear stress for 24 h were seeded in collagen gels and examined for tube formation microscopically (Fig. 5A). Control EPCs cultured under static conditions were present in the form of individual cells or small cellular aggregates at 2 h and exhibited clustering on day 1. Tubelike structures were observed on day 4. By contrast, shear-stressed EPCs appeared to be spindle shaped, elongated, and interconnected and had formed tightly adherent cords of cells as early as 2 h after seeding. Formation of tubelike structures became more prominent on day 1, and an extensive tubular network was observed on day 4. Quantitative analysis with NIH Image showed the total length of the tubes at 2 h and on days 4, and 7 to be significantly greater in shear-stressed EPCs than in static control EPCs (Fig. 5B). These findings suggest that shear stress enhances the ability of EPCs to form tubelike structures in collagen gels.

<table>
<thead>
<tr>
<th>Phase</th>
<th>D6</th>
<th>D10</th>
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<tr>
<td>G0–G1</td>
<td>Static 74.70 ± 3.29</td>
<td>Flow 60.79 ± 3.10</td>
<td>Static 82.04 ± 2.80</td>
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<tr>
<td>S</td>
<td>19.39 ± 2.68</td>
<td>22.99 ± 2.11</td>
<td>8.39 ± 0.23</td>
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<tr>
<td>G2-M</td>
<td>5.91 ± 2.03</td>
<td>16.22 ± 0.70*</td>
<td>9.57 ± 2.57</td>
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Values are means ± SD of 4 separate samples. Endothelial progenitor cells either cultured under static conditions (Static) or exposed to shear stress (Flow, 0.1–2.5 dyn/cm²) were stained with propidium iodide on culture days 6, 10, and 14; dye fluorescence was then evaluated by FACS. Cells were exposed to shear stress for 24 h on culture days 5, 9, and 13 and assayed on culture days 6, 10, and 14 (D6, D10, D14). The percentage of cells in each phase, as determined by cell cycle analysis software, is shown. *P < 0.01 vs. static control.
DISCUSSION

The present study demonstrated EPCs to be sensitive to shear stress, i.e., that EPCs change morphologically and functionally in response to shear stress. EPCs elongated and oriented their long axis in the direction of flow when exposed to shear stress (0.1–2.5 dyn/cm²) for 24 h. These morphological changes were observed in mature ECs, such as bovine aortic ECs and human umbilical vein ECs (13, 14). Mature ECs, however, align in the direction of flow in response to shear stress of >100 dyn/cm², but not to such low shear stress as 0.1–2.5 dyn/cm² (24). This indicates that, in terms of morphological responses to shear stress, EPCs are more sensitive than mature ECs.

Shear stress stimulated EPC proliferation. The effect of shear stress on mature EC proliferation, however, remains controversial. Laminar shear stress has been shown to inhibit DNA synthesis in human umbilical vein endothelial cells (HUVECs) in a confluent monolayer (1, 25), but to stimulate DNA synthesis in bovine aortic ECs exposed to shear stress during regeneration after monolayer scraping (3). On the other hand, animal experiments using arteriovenous shunts have shown elevated shear stress to induce an increase in EC proliferation in arteries (28). In the present study, the EPCs did not reach confluence during the 21-day culture period. Thus the absence of cell growth contact inhibition may be related to the behavior difference between EPCs and confluent HUVECs. In this sense, EPCs may not assume a complete endothelial phenotype (at least, not within 21 days). Shear stress may also cause non-EPCs to slough off, causing the EPCs to lose cell contact signals that inhibit proliferation and/or differentiation.

Expression of the VEGF receptors KDR and Flt-1 in EPCs gradually increased with the duration of cell culture. Shear stress markedly augmented the increases in KDR and Flt-1 expression in EPCs. These findings indicate that cells exposed to shear stress acquire enhanced responsiveness to endogenous VEGF. These cells may exhibit greater participation in ongoing angiogenesis. Shear stress has also been shown to cause tyrosine phosphorylation in KDR, without the participation of its ligand VEGF, and to trigger downstream signal transduction, including activation of ras and ERK (11). This means that shear stress activates KDR in the same manner as chemical stimulation with VEGF. Thus activation of KDR, as well as the increase in its expression, may be involved in the mechanism by which shear stress stimulates EPC proliferation.

VE-cadherin is present at endothelial adherens junctions and plays an important role in intercellular adhesion and cell differentiation, growth, and migration. Shear stress increased VE-cadherin expression in EPCs at both the protein and the mRNA level. Similar results were obtained for at least 3 separate experiments. *P < 0.01 vs. static control.

Fig. 4. Effect of shear stress on mRNA levels of EC-specific markers. A: KDR. B: Flt-1. C: VE-cadherin. Total RNA was extracted on D4 and D6 from EPCs that had been either incubated under static conditions (D4 static, D6 static) or exposed to shear stress for 24 h (D6 Flow-L, D6 Flow-H). Bands of cells at the inner or outer half of the same dish (Flow-L, 0.1–1.3 dyn/cm²; Flow-H, 1.4–2.5 dyn/cm²) were separately scraped out and analyzed. After reverse transcription of the RNA into cDNA, the cDNA was amplified by PCR using specific primers and electrophoresed on a polyacrylamide gel. Top: bands show the amplification products for each EC-specific marker and GAPDH mRNA. Bottom: bar graphs (means ± SD) represent the percent changes relative to static controls (D4 static), in band density normalized to GAPDH mRNA levels. Shear stress dose-dependently increased KDR, Flt-1, and VE-cadherin mRNA levels. Similar results were obtained for at least 3 separate experiments. *P < 0.01 vs. static control.
duced to form capillary-like tubes in fibrin and collagen gels, a monoclonal antibody against VE-cadherin inhibited the formation of capillary tubes, and, when the antibody was added to preformed capillary tubes, it disrupted the capillary network (8). The increase in VE-cadherin induced by shear stress may, therefore, contribute to tube formation and thereby lead to neovascularization.

Vascular ECs can organize into tubular capillary-like structures. An in vitro model in which ECs are cultured in three-dimensional collagen gels in the presence of basic FGF and VEGF has been widely used to study the processes involved in the formation of capillary tubes (43). Use of this model revealed that preconditioning EPCs with shear stress enhanced tube formation. EPCs were exposed to shear stress for 24 h on culture day 6 and assayed up to 1 wk later. The positive effect of shear stress on tube formation persisted for an entire week. There may be a “memory” effect of 24-h exposure to shear stress that lasts for, at least, 1 wk. The enhanced tube formation appears to be related to shear-stress-induced augmentation of cell growth, VEGF receptor expression, and VE-cadherin expression, although their exact relationships were not assessed in the present study.

In the present study, peripheral blood-derived mononuclear cells that attach to fibronectin-coated dishes, endocytose DiI-acLDL, and bind Ulex-lectin were used as EPCs. EPCs have been shown to proliferate, differentiate into mature ECs, form tubelike structures in vitro, and contribute to postnatal neovascularization in vivo (2, 7, 19, 20). Identification of EC precursors, however, has been a source of controversy (35, 36). EPCs were initially isolated from enriched CD34 antigen-positive (CD34\(^+/H11001\)) cells from human peripheral blood (6). CD34 has been used as an EPC marker, and other markers such as AC133 and KDR were subsequently proposed as additional tools to further purify EPCs (16, 44). Recent studies, however, have demonstrated that not only CD34\(^+/H11001\) cells but also CD34\(^+/H11002\) cells can function as EPCs; bone marrow- or peripheral blood-derived CD34\(^{-}\) mononuclear cells readily differentiate into EC-like cells in culture and contribute to neovascularization in vivo (17, 38). It has also been shown that EPCs can develop from human peripheral blood CD14-positive monocytes (15, 37). To date, various criteria for EPC selection have been used, including DiI-acLDL\((+)\)-Ulex-lectin\((+)\), CD34\(^+\) (39), CD34\(^+\)A133\(^+\)KDR\(^+\) (34), CD34\(^-\)CD14\(^+\) (17), DiI-acLDL\((+)\)-vWF\((+)\) (29), and CD31\(^+\) (21). In this regard, the present data can be said to have been derived from this population of EPCs; peripheral blood derived DiI-acLDL\((+)\)-Ulex-lectin\((+)\) adherent mononuclear cells.

The results of this study may have clinical relevance. Application of mechanical stress, such as shear stress or cyclic strain, during the production of artificial vas-
cular grafts from vascular cells grown on a polymer matrix has recently been shown to enhance the development and function of the grafts (31). These engineered grafts exhibit superior mechanical strength and good patency when implanted in miniature swine. More recently, injection of muscles with EPCs isolated from human cord blood was shown to enhance neovascularization in the ischemic legs of nude mice (30). Thus manipulating EPCs by means of mechanical stress may be useful in the development of highly efficient tissue-engineered vessels or for the maturation of EPC cultures outside the body for cell therapy in ischemic vascular diseases.

**DISCLOSURES**

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