Fluid shear stress induces arterial differentiation of endothelial progenitor cells

Syoataro Obi,1,2 Kimiko Yamamoto,1,3 Nobutaka Shimizu,1 Shinichiro Kumagaya,1 Tomomi Masumura,1 Takaaki Sokabe,4 Takayuki Asahara,3 and Joji Ando1

1Department of Biomedical Engineering, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; 2Department of Regenerative Medicine Science, Tokai University School of Medicine, Isehara, Japan; 3Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency and 4Okazaki Institute for Integrative Bioscience, Okazaki, Japan

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Obi S, Yamamoto K, Shimizu N, Kumagaya S, Masumura T, Sokabe T, Asahara T, Ando J. Fluid shear stress induces arterial differentiation of endothelial progenitor cells. J Appl Physiol 106: 203–211, 2009. First published November 6, 2008; doi:10.1152/japplphysiol.00197.2008.—Endothelial progenitor cells (EPCs) are mobilized from bone marrow to peripheral blood and play central roles in the homeostasis of the endothelium. In the process, EPCs are exposed to the shear stress generated by blood flow and tissue fluid flow. Our previous study showed that shear stress promotes differentiation of EPCs into mature endothelial cells. In this study, we investigated whether EPCs differentiate into arterial or venous endothelial cells in response to shear stress. When cultured EPCs derived from human peripheral blood were exposed to controlled levels of shear stress in a flow-loading device, the mRNA levels of the arterial endothelial cell markers ephrinB2, Notch1/3, Hey1/2, and activin receptor-like kinase 1 increased, but the mRNA levels of the venous endothelial cell markers EphB4 and neuropilin-2 decreased. Both the ephrinB2 increase and the EphB4 decrease were shear stress dependent rather than shear rate dependent. EphrinB2 protein was increased in shear-stressed EPCs, and the increase in ephrinB2 expression was due to activated transcription and not mRNA stabilization. Deletion analysis of the ephrinB2 promoter indicated that the cis-element (shear stress response element) is present within 106 bp 5’ upstream from the transcription initiation site. This region contains the Sp1 consensus sequence, and a mutation in its sequence decreased the basal level of transcription and abolished shear stress-induced ephrinB2 transcription. Electrophoretic mobility shift assays and chromatin immunoprecipitation assays showed that shear stress markedly increased binding of Sp1 to its consensus sequence. These results indicate that shear stress induce differentiation of EPCs into arterial endothelial cells by increasing ephrinB2 expression in EPCs through Sp1 activation.

THE ENDOTHELIAL CELLS that line blood vessels have a variety of functions and play central roles in the homeostasis of the circulatory system. Endothelial cell functions are regulated not only by chemical mediators, such as hormones, cytokines, and neurotransmitters, but also by mechanical forces, including the shear stress generated by blood flow and the stretch tension produced by blood pressure. Endothelial cell responses to these hemodynamic forces are essential to maintaining the vascular system in a physiological state, and they play especially important roles in blood-flow-dependent phenomena, such as angiogenesis, vascular tone control, and vascular remodeling. Impairment of endothelial cell responses to hemodynamic forces leads to the development of various vascular diseases, such as hypertension, thrombosis, and arteriosclerosis (7, 9).

Endothelial progenitor cells (EPCs) contributing to postnatal neovascularization were first isolated from the peripheral blood of adult humans a decade ago (5). EPCs are thought to be mobilized from bone marrow into peripheral blood, where they attach to existing endothelial cells and then transmigrate across the endothelium into tissues, where they proliferate, differentiate, and induce angiogenesis (4, 19, 29). Ever since their seminal description, increasing effort has been devoted to the study of EPCs. Clinically, depletion of circulating EPCs has been found to be a marker of cardiovascular damage, including of atherogenetic processes, and cell therapies using EPCs, which stimulate angiogenesis in ischemic tissues, have proven beneficial in patients with coronary and peripheral artery disease (35, 44). However, there is controversy about the definition of EPCs, and, because of the absence of a single specific marker for EPCs, the methods used to isolate EPCs have never been standardized. For practical reasons, several groups have defined EPCs as peripheral blood-derived mononuclear cells that adhere to fibronectin and display typical functional properties of endothelial cells, such as uptake of acetylated low-density lipoprotein (acLDL) and binding of Ulex lectin (5, 10, 18).

During the process of EPC incorporation into tissues and neovascularization, the cells are exposed to shear stress generated by flowing blood and tissue fluid flow (38, 41). We have previously shown that shear stress increases EPC proliferation, differentiation into mature endothelial cells, and tube formation in collagen gels (46). These findings suggest that EPCs, as well as mature endothelial cells, recognize shear stress and transmit the signal into the cell interior, which results in cell responses. However, there have been few studies on EPC responses to shear stress, including signal transduction and gene responses (32, 47, 50).

Arterial endothelial cells and venous endothelial cells differ both functionally and molecularly (2). On the molecular level, arterial endothelial cells express ephrinB2, neuropilin-1 (NRP1), delta-like-4, Notch1, and activin receptor-like kinase 1 (ALK1) (25, 26, 34, 36, 43), whereas venous endothelial cells express EphB4, NRP2, and chicken ovalbumin upstream promoter transcription factor 2 (13, 16, 48). EphrinB2 and ephrinB2 have a ligand-receptor relationship: EphrinB2 is a...
transmembrane ligand, and EphB4 is its tyrosine kinase receptor. Upon binding of these two molecules, signals are transmitted bidirectionally into both EphrinB2-expressing cells and EphB4-expressing cells. They are thought to be key molecules in the process of arteriovenous differentiation by endothelial cells (23, 39). EPCs express both ephrinB2 and EphB4, but the physiological factors that affect the expression of these two molecules in EPCs remain unclear.

In this study, we investigated whether shear stress affects the arteriovenous differentiation of EPCs. We also investigated the molecular mechanism by which shear stress regulates arterial endothelium-specific ephrinB2 gene expression in EPCs.

MATERIALS AND METHODS

Cell isolation and culture. This study was approved by the institutional review board of the Graduate School of Medicine, the University of Tokyo. Mononuclear cells were isolated from 200 ml of peripheral blood from healthy volunteers by density gradient centrifugation with Histopaque-1077 (Sigma). The mononuclear cells were plated at a density of 5 x 10^5 cells/cm^2 on culture dishes coated with a 100 μg/ml solution of human fibronectin (Sigma) and cultured at 37°C under 5% CO2 atmosphere in endothelial cell basal medium-2 (EBM-2) medium (Clonetics) supplemented with 5% fetal bovine serum, human VEGF, human FGF-2, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid. Five days after plating, the nonattached cells were removed by thorough washing with PBS, and the attached cells were cultured for 5–6 days.

Identification of EPCs. To confirm the EPC phenotype, attached cells were incubated at 37°C for 1 h with 1,1'-diocadetyl-3,3,3',3'-tetramethylinodocarbocyanin-epchlorohydrin-labeled acLDL (Di-acLDL; 10 μg/ml; Biomedical Technologies). The cells were then fixed with 2% paraformaldehyde for 10 min, and, after incubation with FITC-labeled Ulex europeus agglutinin (lectin, 10 μg/ml; Sigma) for 1 h, the cells were viewed through an inverted fluorescence microscope (Nikon). Cells that were both Di-acLDL positive and Ulex-lectin-fluorescence positive were identified as EPCs (46), and nearly all attached cells (>95%) were double-positive. Cell characterization by flow cytometry indicated that the cells were positive for stem cell markers CD34 (39.3 ± 4.0%) and CD133 (37.6 ± 3.2%) and the endothelial markers kinase-insert domain receptor (KDR, in humans, representing type 2 vascular endothelial growth factor receptor, 36.2 ± 2.8%), Flt-1 (type 1 vascular endothelial growth factor receptor, 38.7 ± 3.5%), platelet endothelial cell adhesion molecule-1 (73.9 ± 4.8%), VE-cadherin (33.8 ± 3.5%), and E-selectin (42.9 ± 10.5%) and were positive for the leukocyte marker CD45 (20.6 ± 5.9%) and the monocye marker CD11b (3.6 ± 0.8%).

Shear stress loading experiments. Cells were exposed to laminar shear stress with a rotating-disk-type, flow-loading device, as previously described (73.9 ± 4.8%), VE-cadherin (33.8 ± 3.5%), Flt-1 (type 1 vascular endothelial growth factor receptor, 38.7 ± 3.5%), and E-selectin (42.9 ± 10.5%) and were positive for the leukocyte marker CD45 (20.6 ± 5.9%) and the monocye marker CD11b (3.6 ± 0.8%).

Shear stress loading experiments. Cells were exposed to laminar shear stress with a rotating-disk-type, flow-loading device, as previously described (28). Briefly, a dish containing cultured EPCs was placed on the stage of the device, and a stainless steel disk was placed in the dish. The rotation of the dish caused the medium to flow in a concentric circle, thereby exerting laminar shear stress on the cells. The intensity of the shear stress (τ) was calculated as τ = μρrωd, where μ is fluid viscosity, r is distance from the center of the dish, ω is angular velocity, and d is distance between the disk and the dish. The device generates a gradient of shear stress that depends on the distance from the center of the dish, and the shear stress applied ranged from 0.1 to 2.5 dynes/cm^2 for 24 h. All experiments were performed at 37°C in a CO2 incubator. Since all cells from a single dish, not from regions of the dish at different radii, were used for the assays, the results of the assays represent averaged cell responses to different levels of shear stress within the above range.

Real-time PCR analysis. Total RNA samples were prepared from cells with ISOGEN (Nippon Gene; Tokyo, Japan), and first-strand cDNAs were generated using a Transcriptor First Strand cDNA Synthesis Kit (Roche). After reverse transcription of the RNA into cDNA, real-time PCR was used to monitor gene expression with a Smart Cycler (Cepheid) according to standard procedures. PCR was performed with a Takara EX Taq R-PCR kit (Takara) and primer pairs for ephrinB2, Notch1, Notch3, hairy/enhancer of split-related with YRPW motif 1 (Hey1), Hey2, ALK1, EphB4, NRP2, and GAPDH, respectively (Table 1). The temperature profile consisted of initial denaturation for 30 s at 95°C, followed by 40 cycles of denaturation at 95°C for 5 s, annealing and elongation at 62°C for 30 s, and fluorescence monitoring at 85°C. The specificity of the amplification reaction was determined by performing a melting curve analysis. Relative signal quantification was achieved by normalizing the signal of each gene to that of the GAPDH gene.

Western blot analysis. EPCs were washed with cold PBS and resuspended in Celllytic (Sigma) containing protease inhibitor cocktail (Sigma). Lysates were centrifuged at 26,000 g for 30 min, and the supernatants were mixed with SDS sample buffer (0.2 M Tris-HCl, pH 8.8, 18% glycerol, 4% SDS, 0.01% bromphenol blue, and 10% β-mercaptoethanol) for SDS-PAGE. Gels were transferred to Immobilon polyvinylidene difluoride membranes (Millipore), and the membranes were blocked in Tris-buffered saline with 5% skim milk and 0.1% Tween 20 and incubated for 1 h with the antibodies against ephrinB2 (Santa Cruz Biotechnology) and β-actin (Abcam). The membranes were then washed with PBS and incubated with IgG horseradish peroxidase-conjugated antibody (Amersham), and the blots were developed with an enhanced chemiluminescence kit (Amersham) and analyzed by the GS525 Molecular Imager System (Bio-Rad). Quantitative analysis was achieved by normalizing the signal of ephrinB2 to that of β-actin.

Nuclear run-on assay. A nuclear run-on assay was performed by the method described previously (37). Briefly, nuclear extracts obtained from EPCs were reacted in reaction buffer containing [α-32P]UTP, and 32P-labeled RNA was extracted. Plasmids containing cDNA inserts for ephrinB2 or β-actin (Clontech) were spotted onto nylon membranes, and the DNA was hybridized to the radiolabeled RNA. Autoradiograms were obtained with the GS525 Molecular Imager System (Bio-Rad).

Table 1. Oligonucleotide primers used for gene expression analysis by real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences, 5'-3'</th>
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<tbody>
<tr>
<td>ephrinB2</td>
<td>Fwd  ATGGTGGTGAAGACGCCAGT</td>
</tr>
<tr>
<td>Notch1</td>
<td>Fwd  GCACCGGAGATATTAGGCTTG</td>
</tr>
<tr>
<td>Notch3</td>
<td>Fwd  TCCCATGTCAGGACCCATTCCT</td>
</tr>
<tr>
<td>Hey1</td>
<td>Fwd  CAACATTTGGTGGGCTGAAA</td>
</tr>
<tr>
<td>Hey2</td>
<td>Fwd  AGTACGCGCAGGCTATCAGG</td>
</tr>
<tr>
<td>ALK1</td>
<td>Fwd  GAGCTGACATGCTGCTTGTG</td>
</tr>
<tr>
<td>EphB4</td>
<td>Fwd  ATGAGTCCGCGCAAGTTGACG</td>
</tr>
<tr>
<td>NRP2</td>
<td>Fwd  ATGAGTCCGCGCAAGTTGACG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Fwd  GCACCTGCAAGGCTTACAGG</td>
</tr>
</tbody>
</table>

ALK, activin receptor-like kinase 1; NRP2, neuropilin-2; Fwd, forward; Rev, reverse.
Cloning and sequencing of human ephrinB2 DNA. A 583-mer cDNA encoding exon 1 of the human ephrinB2 gene was radiolabeled with [α-32P]DCTP by using a random primer labeling kit (Takara). A human genomic library (Clontech) was screened by lifting 1.2 × 10^6 phages onto a Hybond-N nylon membrane (Amersham). After a 4-h prehybridization in 5× saline-sodium phosphate-EDTA, 5× Denhardt solution, 0.5% SDS, 10% dextran sulfate, and 0.25 mg/ml salmon testis DNA at 65°C, hybridization was carried out in the same solution with the radiolabeled ephrinB2 probe for 24 h at 65°C. The positive clones were identified by autoradiography with X-ray film. The DNA from the positive clones was isolated, digested with NheI and NsiI (~3,990 bp), and subcloned into pGL3-enhancer vector (Promega) with NheI and Smal. The complete nucleotide sequence was determined with a DNA sequencer (373S-36, Applied Biosystems) and confirmed in Genbank (accession number AL442127). The sequence consisted of 3,940 bp upstream from the transcription start site (~3,940 luc).

Luciferase assay. Reporter plasmids containing the ephrinB2 promoter were used for the transcription assay. A series of deletions was created through the 5'-flanking sequences of the ephrinB2 gene by restriction enzyme digestion, and the deletions were subcloned into the pGL3-enhancer vector. The following constructs were generated: −1,434 luc, −478 luc, −252 luc, and −106 luc. For −1,434 luc, 1,484 bp (~1,434 to +49) of the ephrinB2 upstream region was digested with SpeI and BglII and cloned into the pGL3-enhancer with NheI and BglII. For −478 luc, 528 bp (~478 to +49) of the ephrinB2 upstream region was cloned into the pGL3-enhancer with SacI and HindIII. For −252 luc, 302 bp (~252 to +49) of the human ephrinB2 upstream region was cloned into the pGL3-enhancer with Smal and HindIII. For −106 luc, 156 bp (~106 to +49) of the ephrinB2 upstream region was digested with CpoI and HindIII and cloned into the pGL3-enhancer with Smal and HindIII.

Lipofectamine Plus (GIBCO) was used to transfect the constructs into cultured bovine endothelial cells, and the phRL-TK vector (Promega) was cotransfected to normalize transfection efficiency. After 24 h, cells were either incubated under static conditions or exposed to shear stress, and luciferase activity was determined with a dual-luciferase reporter assay system (Promega) and a luminometer (Berthold).

Synthesis of Sp1 mutation sites. The 112-mer oligonucleotides (~106 to −1) containing specific mutations (underlined) at a consensous binding site for the known transcription factor Sp1 (5'-CCGGT-GACCCGCGCTTCAACACGCAGCGGCGAGCGCGGCC-GCAATTTGCTCTCCGCGCCGGGCGGGGCGGGTCCAGCTTGA- TAGCCGCTTCGACGCGCGCGCGCCGCA-3') were synthesized by PCR and cloned into the pGL3-enhancer with MluI and BglII (Sp1 mut). The mutation was confirmed by DNA sequencing.

EMSA. EMSA was performed by using nuclear extracts obtained from EPCs, as previously described (37). Oligonucleotides containing a Sp1 consensus element (5'-GGCGGGGGCCGGCCGGCCGG-3') and a Sp1-mutated consensus element (5'-GGCGCGGTTCGGGGGC-GCCGG-3') were labeled with T4 polynucleotide kinase and [γ-32P]ATP. Binding reactions between the radiolabeled oligonucleotides and 2.5 μg of nuclear extracts protein were allowed to proceed at 22°C in a total volume of 10 μl binding buffer [10 mM Tris·HCl, 50 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, and 0.05 mg/ml poly[dI·dC]], and the reaction mixtures were separated by 4.7% PAGE in 0.5× Tris·HCl-boric acid-EDTA buffer (45 mM Tris·HCl, 45 mM boric acid, and 1 mM EDTA; pH 8.3) for 1 h at 350 V at 4°C. The protein-DNA complexes were analyzed with a GSS525 molecular imager system (Bio-Rad). In a supershift assay, the antibody against Sp1 (Santa Cruz Biotechnology) was added to the binding reaction, and the mixture was incubated for 60 min at 4°C before the labeled oligonucleotide was added.

Chromatin immunoprecipitation assay. After cross linking 1 × 10^6 cells with 1% formaldehyde at room temperature for 10 min, the cells were washed with ice-cold PBS, centrifuged, and resuspended in lysis buffer (1% SDS, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 50 mM Tris·HCl; pH 8.1), and then sonicated for 10 s four times. Supernatants were then recovered by centrifugation and diluted 1:10 in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 16.7 mM Tris·HCl; pH 8.1). Nonspecific background material was removed by incubating the chromatin resuspension with 75 μl of protein A-agarose/salmon sperm DNA (50% slurry) for 30 min at 4°C. A 20-μl sample of each chromatin supernatant was reserved before the chromatin immunoprecipitation (ChIP) assay as “input” (positive control). Immunoprecipitation was performed overnight with the specific antibody against Sp1 (Santa Cruz Biotechnology) at 4°C with rotation. Then, 60 μl of protein A-agarose/salmon sperm DNA (50% slurry) was added and allowed to react for 1 h at 4°C. The beads were pelleted by centrifugation and washed sequentially with the following buffers: low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris·HCl; pH 8.1), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, and 20 mM Tris·HCl; pH 8.1), and LiCl wash buffer (0.25 mM LiCl, 1% IGEPA-CA630, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris·HCl; pH 8.1). Bead precipitates were washed twice with Tris·HCl-EDTA buffer (1 mM EDTA and 10 mM Tris·HCl; pH 8.0) and eluted with 1% SDS and 0.1 M NaHCO3, and the eluates were pooled and heated for 4 h at 65°C to reverse the formaldehyde cross linking. Supernatants were incubated for 1 h at 45°C with 20 μg/ml proteinase K, and genomic DNA fragments were recovered.

PCR was performed with Ex Taq polymerase (Takara) and the following primers, which amplify the part of the ephrinB2 promoter containing the Sp1 binding site: 5'-ACCAATAGTGCGGA-GGGATG-3' and 5'-AGCGGAGACTGTCGACCGCAG-3'. “Mock” samples contained dilution buffer instead of chromatin (negative control).

Statistical analysis. All results are expressed as means ± SD. Statistical significance was evaluated by ANOVA and a Bonferroni adjustment applied to the results of a t-test performed with SPSS software. P values of <0.05 were regarded as statistically significant.

RESULTS

Shear stress increases the gene expression of arterial endothelial markers in EPCs but decreases the gene expression of venous endothelial markers. Cultured EPCs were exposed to shear stress for 6 or 24 h, and changes in the mRNA levels of the arterial endothelial markers ephrinB2, Notch1/3, Hey1/2, and ALK1 and the venous endothelial markers EphB4 and NRP2, on the other hand, had an increased response to shear stress. The mRNA levels of the venous endothelial markers were dependent on shear stress rather than shear rate.

Flow-induced gene responses of ephrinB2 and EphB4 are dependent on shear stress rather than shear rate. To determine whether the flow-induced changes in ephrinB2 and EphB4 mRNA levels were dependent on shear stress or shear rate, EPCs were subjected to the flow of two media having different viscosities. As the shear rate increased, the ephrinB2 mRNA level increased, whereas the EphB4 mRNA level decreased (Fig. 2, left). However, there was a difference between the degree of response to the high-viscosity medium and the low-viscosity medium; the increase in ephrinB2 mRNA level and the decrease in EphB4 mRNA level were always greater during exposure to the flow of the high-viscosity medium, that

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EphrinB2 protein increases in EPCs exposed to shear stress. EPCs were subjected to shear stress for 12 h or 24 h, and changes in the ephrinB2 protein level were analyzed by Western blot. At 24 h the ephrinB2 protein level had increased to approximately threefold the control level (Fig. 3, A and B).

Shear stress activates ephrinB2 transcription. A nuclear run-on assay was performed to investigate whether shear stress affects the transcription of ephrinB2. Exposure of EPCs to shear stress for 24 h significantly increased ephrinB2 transcription compared with the static control but had no effect on β-actin transcription (Fig. 4, A and B).

Actinomycin D chase experiments were performed to determine whether shear stress affects the stability of ephrinB2 mRNA. The amount of ephrinB2 mRNA decreased as actinomycin D exposure time increased, and there was no difference in the rate of decrease between the static control cells and the cells exposed to shear stress (Fig. 4C). These findings indicate that the increase in the ephrinB2 mRNA levels in response to shear stress is due to activated transcription, not to mRNA stabilization.

A luciferase assay was also performed to confirm the effect of shear stress on ephrinB2 transcription, and shear stress increased the luciferase activity of cells (Fig. 5, 3,940 luc), indicating that shear stress activates ephrinB2 transcription in EPCs.

Sp1 consensus sequence is essential for the shear stress activation of ephrinB2 transcription. A deletion analysis was performed to localize the cis-acting regions within the ephrinB2 promoter that are responsible for the shear stress
activation of ephrinB2 transcription. Transfection with deletion constructs −1,434 luc, −478 luc, −252 luc, and −106 luc resulted in a marked increase in luciferase activity in response to shear stress (Fig. 5). These findings indicate that the cis-acting regions are located within −106 bp 5’ upstream from the transcription initiation site in the human ephrinB2 promoter.

Since this region contains the transcription factor Sp1 consensus sequence (−51 bp to −31 bp), we constructed a chimeric gene with site-specific mutagenesis at the Sp1 consensus sequence and performed a luciferase assay. The mutation decreased the basal ephrinB2 transcription and abolished the shear stress-induced ephrinB2 transcription (Fig. 5, Sp1 mut), indicating that the Sp1 consensus sequence plays a major role in the control of basal ephrinB2 transcription.

Transcription factor Sp1 is involved in the shear stress activation of ephrinB2 transcription. EMSA was performed to identify the nuclear protein that binds the Sp1 consensus sequence. When nuclear extracts from static or shear-stressed EPCs were incubated with a radiolabeled oligonucleotide bearing the Sp1 consensus sequence, distinct protein-DNA complexes formed in nuclear extracts derived from shear-stressed cells (Fig. 6), whereas an oligonucleotide bearing the mutated Sp1 consensus sequence was unable to form protein-DNA complex, and antibody to Sp1 caused a band shift in the protein-DNA complex. These findings indicate that Sp1 is involved in the shear stress activation of ephrinB2 transcription in EPCs.

ChIP assays were performed to study the interactions between Sp1 and the ephrinB2 promoter. Chromatin extracts...
endothelial antigen, KDR, is commonly used as the antigenic prototypical stem cell antigen, CD34 or CD133, and a typical lineage is used for flow cytometry. The combination of immaturity/stemness plus at least one marker of the endothelial blood samples and cell culture. At least one marker of techniques are used to isolate EPCs: flow cytometry of fresh different methodologies have been used to study EPCs. Two (15, 30, 33). Thus far a variety of phenotypic definitions and peripheral blood mononuclear cells that function like EPCs whether true EPCs exist or whether there are only some types of EPCs, called “early EPCs” and “late EPCs” (20).

Early EPCs are identifiable as early as 3 days after plating and organize in clusters, but they have limited proliferative capacity and disappear after 2 wk of culture. Late EPCs survive beyond 2–3 wk, tend to form a confluent cobblestone-like layer, and have a higher proliferative potential. Both early and late EPCs are double-positive for acLDL and Ulex lectin. Some groups use nonattached cells instead of attached cells as the source from which to isolate colony forming unit-endothelial cells as putative EPCs (17). EPCs isolated by flow cytometry probably have little in common with EPCs isolated by culture. Therefore, it must be emphasized that our results apply only to EPCs isolated by the specific protocol used in this study.

The device used generated a gradient of shear stress that depends on the distance from the center of the dish, and the shear stress applied ranged from 0.1 dynes/cm² to 5 dynes/cm². This range corresponds to venous rather than arterial shear stress. Since all of the cells in a single dish, not from individual regions of the dish at different radii, were used for the assays, the results of the assays represent averaged cell responses to

There are ongoing debates about what an EPC is and whether true EPCs exist or whether there are only some peripheral blood mononuclear cells that function like EPCs (15, 30, 33). Thus far a variety of phenotypic definitions and different methodologies have been used to study EPCs. Two techniques are used to isolate EPCs: flow cytometry of fresh blood samples and cell culture (12). At least one marker of immaturity/stemness plus at least one marker of the endothelial lineage is used for flow cytometry. The combination of a prototypical stem cell antigen, CD34 or CD133, and a typical endothelial antigen, KDR, is commonly used as the antigenic phenotype of EPCs. In the cell culture technique, on the other hand, adherent peripheral blood mononuclear cells that attach after plating onto fibronectin are isolated. Cultures of attached cells contain two phenotypically different types of EPCs, called “early EPCs” and “late EPCs” (20).

DISCUSSION

There are ongoing debates about what an EPC is and whether true EPCs exist or whether there are only some peripheral blood mononuclear cells that function like EPCs (15, 30, 33). Thus far a variety of phenotypic definitions and different methodologies have been used to study EPCs. Two techniques are used to isolate EPCs: flow cytometry of fresh blood samples and cell culture (12). At least one marker of immaturity/stemness plus at least one marker of the endothelial lineage is used for flow cytometry. The combination of a prototypical stem cell antigen, CD34 or CD133, and a typical endothelial antigen, KDR, is commonly used as the antigenic phenotype of EPCs. In the cell culture technique, on the other hand, adherent peripheral blood mononuclear cells that attach after plating onto fibronectin are isolated. Cultures of attached cells contain two phenotypically different types of EPCs, called “early EPCs” and “late EPCs” (20).
differently from cells exposed to 5 dynes/cm², the limitations im-
posed by the shear stress device should be borne in mind when evalu-
ating the results.

The results of this study demonstrated that, when cultured
EPCs were exposed to shear stress, the gene expression levels
of the arterial endothelial markers ephrinB2, Notch1/3, Hey1/2, and ALK1 increased, whereas the gene expression
levels of the venous endothelial markers EphB4 and NRP2
decreased. We have previously shown that, as the number
of days of culture increases, EPCs increase their expression of the
mature endothelial cell markers Flk-1, Flt-1, and VE-cadherin
and that shear stress markedly accelerates the increase in
endothelial marker expression. On the basis of our current and
previous observations, EPCs appear to be responsive to shear
stress and to differentiate into mature and arterial endothelial
cells under shear stress conditions. Shear stress has been found
to affect cell differentiation in other cell lines as well, and it has
been shown to induce differentiation of Flk-1-positive embry-
onic stem cells and embryonic mesenchymal progenitor cells
toward the vascular endothelial cell lineage (42, 45). In con-
trast to the study in EPCs, ephrinB2 expression has been shown
to decrease in response to shear stress in human umbilical vein
endothelial cells and human coronary artery endothelial cells
(14). The biomechanical regulation of ephrinB2 expression
may differ between mature endothelial cells and EPCs. Taken
together, the physiological environmental factor shear stress
may play an important role in the regulation of differentiation
programs that lead to blood vessel formation in embryos and
adults.

Flow exerts two effects on cells. One effect is a flow-
velocity-dependent change in mass transport (6). When
some cell-activating substance is present in the perfusate, its
diffusion to the cell surface increases as flow velocity or
shear rate increases, leading to further stimulation of cells.
The other effect is the mechanical cell deformation induced
by shear stress. Flow-loading experiments using two media
having different viscosities make it possible to discriminate
between these two effects (3). Since shear stress is the
product of shear rate and viscosity, cells can be subjected to
different levels of shear stress at the same shear rate by
exposing them to flows of different viscosity. If the flow
effect is shear rate dependent, the response may be similar
regardless of the viscosity, or the response may be greater to
low viscosity than to high viscosity because diffusion de-
creases as viscosity increases. If the flow effect is shear
stress dependent, the response would be greater at high viscosi-
y than at low viscosity. The results of
this study were consistent with the latter; that is, the flow-
induced increases in ephrinB2 mRNA level and decreases in
EphB4 mRNA level were always greater at high viscosity than
at low viscosity at the same shear rate. This means that
the flow-induced changes in ephrinB2 and EphB4 expres-
sion are due to shear stress as a mechanical force rather than
to shear-rate-dependent mass transport.

The results of this study revealed that the increase in
ephrinB2 expression in response to shear stress is attributable
to activation of transcription, not to mRNA stabilization. The Sp1
consensus sequence (GGGGCGGGGC) located at −51 to −31
bp 5′ upstream from the transcription initiation site in the
human ephrinB2 promoter is essential for the response of
ephrinB2 to shear stress. Mutation of the Sp1 consensus
sequence abolished the shear stress activation of ephrinB2
transcription, and EMSA and ChIP assays showed that shear
stress markedly increased Sp1 binding to its consensus se-
quence in the ephrinB2 promoter. Shear stress has been found
to affect the transcription of many genes in mature endothelial
cells, and the cis-elements and transcription factors that are
responsible for shear stress activation of gene transcription
vary from gene to gene (31). Sp1 has also been reported to
be involved in shear-stress-mediated transcriptional upregu-
lation of genes encoding tissue factor, KDR, and plasmin-
gen activator inhibitor-1 (1, 24, 27, 40) and downregu-
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alization of genes encoding purinoceptor P2X₄, membrane
type 1-matrix metalloproteinase, and Toll-like receptor 2 (11, 21,
49). However, the molecular mechanisms by which shear
stress activates Sp1 remain unclear. Many other signals in
addition to shear stress, including viral infection, growth
factors, certain drugs, and cytokines, have been identified as
inducing Sp1 phosphorylation through various signal trans-
duction pathways and kinases, such as p38, AKT, ERK1/2,
DNA-dependent protein kinase (DNA-PK), PKA, PKC-ζ,
casein kinase II, and cyclin-dependent kinase 2 (8). Thus
elucidation of signal transduction pathways and kinases
linking shear stress to activation of Sp1 would provide
deeper insight into the mechanism of shear-stress-mediated
EPC differentiation.

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