Fluid shear stress induces differentiation of Flk-1-positive embryonic stem cells into vascular endothelial cells in vitro

Kimiko Yamamoto,1 Takaaki Sokabe,1 Tetsuro Watabe,2 Kohei Miyazono,2 Jun K. Yamashita,3 Syotaro Obi,1 Norihiko Ohura,1 Akiko Matsushita,1 Akira Kamiya,2 and Joji Ando1

1Department of Biomedical Engineering and 2Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Tokyo; 3Stem Cell Research Center, Institute for Frontier Medical Science, Kyoto University, Kyoto; and 4Interdisciplinary Science Center, Nihon University, Tokyo, Japan

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Yamamoto, Kimiko, Takaaki Sokabe, Tetsuro Watabe, Kohei Miyazono, Jun K. Yamashita, Syotaro Obi, Norihiko Ohura, Akiko Matsushita, Akira Kamiya, and Joji Ando. Fluid shear stress induces differentiation of Flk-1-positive embryonic stem cells into vascular endothelial cells in vitro. Am J Physiol Heart Circ Physiol 288: H1915–H1924, 2005. First published December 2, 2004; doi: 10.1152/ajpheart.00956.2004.—Pluripotent embryonic stem (ES) cells are capable of differentiating into all cell lineages, but the molecular mechanisms that regulate ES cell differentiation have not been sufficiently explored. In this study, we report that shear stress, a mechanical force generated by fluid flow, can induce ES cell differentiation. When Flk-1-positive (Flk-1+) mouse ES cells were subjected to shear stress, their cell density increased markedly, and a larger percentage of the cells were in the S and G2-M phases of the cell cycle than Flk-1− ES cells cultured under static conditions. Shear stress significantly increased the expression of the vascular endothelial cell-specific markers Flk-1, Flt-1, vascular endothelial cadherin, and PECAM-1 at both the protein level and the mRNA level, but it had no effect on expression of the mural cell marker smooth muscle α-actin, blood cell marker CD3, or the epithelial cell marker keratin. These findings indicate that shear stress selectively promotes the differentiation of Flk-1− ES cells into the endothelial cell lineage. The shear stressed Flk-1− ES cells formed tubelike structures in collagen gel and developed an extensive tubular network significantly faster than the static controls. Shear stress induced tyrosine phosphorylation of Flk-1 in Flk-1− ES cells that was blocked by a Flk-1 kinase inhibitor, SU1498, but not by a neutralizing antibody against VEGF. SU1498 also abolished the shear stress-induced proliferation and differentiation of Flk-1− ES cells, indicating that a ligand-independent activation of Flk-1 plays an important role in the shear stress-mediated proliferation and differentiation by Flk-1+ ES cells.

hemodynamic force; blood vessel; vascular endothelial growth factor; mechanical stress; neovascularization

EmBRYOnIC stem (ES) cell lines have been established from the inner cell mass of mouse blastocysts and have the potential to differentiate into all embryonic cell lineages (4, 12). ES cells are now attracting interest as a promising source of cell for regenerative medicine. ES cells have already been shown to be capable of being induced to develop into a variety of cell types, including neural cells, heart muscle cells, blood cells, endothelial cells, and chondrocytes (2, 11, 14, 16). However, a great deal remains to be elucidated concerning the molecular mechanisms of differentiation of ES cells and methods of inducing ES cells to differentiate into various specialized cells in vitro.

Recently, a novel method has been developed to induce selective differentiation of ES cells into both vascular endothelial cells and mural cells [pericytes and vascular smooth muscle (SM) cells] (6, 21). In this method, undifferentiated ES cells are cultured on type IV collagen-coated dishes, and VEGF receptor 2 (VEGF-R2, Flk-1)-positive (Flk-1+) cells are purified by flow cytometry sorting. The addition of VEGF to the cultures promotes endothelial differentiation, whereas mural cells are induced by platelet-derived growth factor (PDGF)-BB. The vascular cells derived from Flk1+ cells have been shown to organize vessels like structures in three-dimensional culture and contribute to the developing vasculature in vivo (22).

Various types of cells have the property of altering their shape and function in response to mechanical forces, such as stretching tension and shear stress. For example, when cultured vascular endothelial cells are exposed to shear stress generated by fluid flow, they become elongated, with their long axes aligned in the direction of flow, and they increase production of vasodilating substances, including nitric oxide, prostacyclin, C-type natriuretic peptide, and adrenomedulin (3). These shear stress-induced changes in endothelial cell function are often accompanied by changes in the expression of related genes (1), and we (20) recently discovered that exposure of human bone marrow-derived endothelial progenitor cells to shear stress affects their differentiation. Shear stress has also been demonstrated to play an important role in embryonic development and organogenesis: intracardiac fluid forces are essential for the formation of a functional heart in zebrafish embryos (8), and the direction of fluid flow on the node of mouse embryos determines the left-right asymmetry in the body plan (13).

During the process of embryonic development, ES cells are exposed to tissue fluid flow or blood flow generated by the beating heart. In the present study, we investigated whether ES cells respond to shear stress in vitro. Mouse Flk-1+ ES cells were subjected to controlled levels of shear stress in a flow-loading apparatus and examined for changes in cell proliferation and differentiation. The effect of shear stress on their ability to form tubelike structures on collagen gel was also examined.

MATERIALS AND METHODS

Cell culture. MGZ5 ES cells [gift from H. Niwa (Riken, CDB, Kobe, Japan)] were maintained, differentiated, and cultured as previously described (21). The cells were initially maintained undiffere-
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>
<th>Amplified Fragment Size, bp</th>
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<tr>
<td>Flk-1</td>
<td>Fwd: TCTGTTGTTCTGGCTGGAGA</td>
<td>248</td>
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<td></td>
<td>Rev: GTATCAATTTCCCAACCCCT</td>
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<td></td>
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<tr>
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<td>369</td>
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<td></td>
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<tr>
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<td></td>
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</tr>
<tr>
<td></td>
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<td>SM α-actin</td>
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<tr>
<td>SM-22a</td>
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<tr>
<td></td>
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VE-cadherin, vascular endothelial cadherin; SM α-actin, smooth muscle α-actin; Fwd, forward; Rev, reverse.

Shear stress experiments. Flk-1–ES cells were exposed to laminar shear stress with a parallel plate-type device, as described previously (10). Briefly, one side of the chamber was formed by a coverslip on which the cells were cultured, and the other side was machined from a polymethylacrylate plate. The two flat surfaces were held ~200 μm apart by a silicone rubber gasket. The chamber was provided with an entrance and an exit for the medium, and the entrance was connected to a reservoir with a silicone tube. The chamber was perfused with a medium (DME (IBL) containing 15% FBS (JRH), 10^3 U/ml leukemia inhibitory factor (ESGRO, Chemicon), 1 mM nonessential amino acid (ICN), and 5 x 10^{-3} mol/l β-mercaptoethanol (Sigma)). To initiate ES cell differentiation, trypticized cells were plated on type IV collagen-coated petri dishes and cultured without leukemia inhibitory factor in differentiation medium by a roller/tube pump (ATTO). The intensity of the wall shear stress (\(\tau\), dyn/cm^2) on the cell layer was calculated by the formula \(\tau = \frac{Q\mu}{A}b\), where \(\mu\) is the viscosity of the perfusate (poise), \(Q\) is the flow volume (ml/s), and \(A\) and \(b\) are the cross-sectional dimensions of the flow path. Because the maximum Reynolds number corresponding to the highest flow rate used in this study was around 40, we assumed that the flow was laminar. The shear stress used in this study ranged from 1.5 to 10.0 dyn/cm^2; all experiments were performed at 37°C in a CO₂ incubator.

Immunohistochemistry and flow cytometry. Culture cells were immunostained as described previously (19). Stained cells were photographed through a microscope (Nikon), and all images were imported into Adobe Photoshop as JPEGs for contrast manipulation and figure assembly.

Expression of various cell lineage marker proteins was measured by flow cytometry. Cells were detached from dishes by incubation in PBS supplemented with 1 mM EDTA (Sigma) at room temperature for 15 min and suspended in PBS with 10% FBS. A total of 200,000

**Fig. 1.** A: cell density, as a function of time, of Flk-1⁺ embryonic stem (ES) cells that were either cultured under static conditions or exposed to VEGF165 or shear stress in the presence or absence of SU1498. Under static conditions, Flk-1⁺ ES cells proliferated, and their proliferation was significantly promoted by the addition of VEGF165 (50 ng/ml, R&D Systems). When exposed to a shear stress of 1.5 dyn/cm², the Flk-1⁺ ES cells proliferated much more than the static cells, reaching almost the same level of proliferation as induced by 50 ng/ml VEGF165. A shear stress of 5 dyn/cm² increased cell density more than the 1.5 dyn/cm² shear stress. SU1498 (30 μmol/l, Calbiochem), a Flk-1 kinase inhibitor, significantly blocked the basal and shear stress-mediated proliferation of Flk-1⁺ cells, indicating that Flk-1 activation plays an important role in both basal and shear stress-mediated proliferation. Data are expressed as means ± SD of data from 5 separate samples. *P < 0.001 versus static control. B: cell cycle analysis. The percentages of cells in the G0/G1 phase, S phase, and G2/M phases were determined by flow cytometry at day 3 of culture under static conditions or after being subjected to shear stress (1.5 or 5 dyn/cm²) for 24 h. The number of cells in the resting state (G0/G1 phase) decreased in response to shear stress in a dose-dependent manner, whereas the number of cells in the mitotic state (S and G2/M phases) increased. Data are expressed as means ± SD of data from 5 separate samples. *P < 0.05 and **P < 0.01 versus static control.
cells was then incubated for 45 min at 4°C with monoclonal antibodies against endothelial cell markers, the VEGFRs Flk-1 (PharMingen) and Flt-1 (Chemicon), the intercellular adhesion molecules vascular endothelial (VE)-cadherin (PharMingen) and PECAM-1 (PharMingen), the mural cell marker SM α-actin (Sigma), the blood cell marker T3 antigen (CD3; PharMingen), and the epithelial cell marker keratin (NeoMarkers). Next, the cells were incubated for 30 min at 4°C with FITC-conjugated anti-mouse IgG (H+L) antibody (Amersham) and analyzed by FACScan™ (Becton Dickinson). Histograms of cell number versus logarithmic fluorescence intensity were recorded for 20,000 cells/sample. Background fluorescence was obtained from the negative control cells stained with the secondary antibody and subtracted from the mean fluorescence of the specific staining patterns. The expression level of each antigen was expressed as the mean channel fluorescence.

Cell cycle analysis. Cells were detached from dishes by incubating in PBS supplemented with 1 mM EDTA. The detached cells were then washed with PBS and fixed with 70% ethanol for 1 h at 4°C. After being washed with PBS, the cells were incubated with PBS containing 0.5% RNase (Sigma) for 30 min at room temperature and then stained with 50 μg/ml propidium iodide (Sigma) for 15 min at room temperature. The fluorescence intensity of this dye was measured in a sample containing 2 × 10⁴ cells, and the cell cycle was analyzed with ModFit LT Cell Cycle Analysis Software (Verity Software House).

ELISA. The amount of VEGF released from Flk-1⁺ ES cells was assayed by ELISA with commercially available kits (Quantikine, R&D Systems). Briefly, 100 μl of perfusate were incubated for 2 h at room temperature in a microplate coated with an anti-mouse VEGF antibody. After the perfusate was washed with the specified detergent, 100 μl of VEGF conjugate were added and incubated for 2 h. After being washed again, the substrate solution was added, and incubation was performed for 30 min. Absorbance at 450 nm was measured with a microplate reader (Bio-Rad), and the concentration of VEGF in each sample was determined from the standard curve.

Immunoprecipitation and Western blot analysis. Immunoprecipitation and Western blot analysis were performed as previously described (19). Briefly, cells were dissolved in lysis buffer containing 0.1% protease inhibitor mixture (Sigma) and centrifuged at 2.6 × 10⁶ g for 30 min. The protein concentration of the lysate was determined with a Protein Assay Kit (Bio-Rad). Protein A/G Sepharose beads (Amersham) were incubated with anti-phosphotyrosine mouse antibody (clone PY69, BD Biosciences) for 4 h, and, after the beads were washed with lysis buffer, equal amounts of protein were incubated with the beads overnight at 4°C while being gently rotated. The beads were subsequently washed, and the immune complexes were eluted in SDS-PAGE sample buffer. Total immune complex samples or total protein samples from total cell lysate were separated by SDS-PAGE, transferred to Immobilon membranes (Millipore), and incubated with anti-Flk-1 rabbit antibody (Santa Cruz). After being washed and incubated with horseradish peroxidase-linked anti-rabbit IgG, immunoreactive proteins were visualized with the ECL Plus detection system (Amersham) and GS363 molecular imager system (Bio-Rad).

Real-time PCR analysis. Total RNA samples were prepared from cells with ISOGEN (Nippon Gene; Tokyo, Japan), and first-strand cDNAs were generated by using Moloney murine leukemia virus reverse transcriptase (GIBCO) and RNA primed with oligo dT primer. After reverse transcription of the RNA into cDNA, real-time PCR was used to monitor gene expression with a Smart Cycler (Cepheid) according to the standard procedure. PCR was performed with a TaKaRa EX Taq R-PCR version (Takara) and SYBR green I (Bio- whittaker), and the primer pairs are shown in Table 1. The temperature profile included initial denaturation for 30 s at 95°C followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, elongation at 72°C, and fluorescence monitoring at 85°C. The specificity of the amplification reaction was determined by performing a melting curve analysis. Relative quantification of the signals was achieved by normalizing the signals of the different genes to β-actin.

Tube formation assay. Collagen gels were formed by adding Biocoat Matrigel (Becton Dickinson) to a 24-well plate and incubation in a CO₂-free incubator at 37°C for 30 min. The gels were then overlaid with 1 × 10⁵ Flk-1⁺ ES cells suspended in differentiation medium and incubated at 37°C under a 5% CO₂ atmosphere. Gels were examined with a phase-contrast microscope equipped with a digital camera Polaroid digital microscope camera, and the images were then imported as TIFF files into NIH Image software. A second
observer measured the total length per image of the tubelike structures (defined as those exceeding 200 μm in length).

Statistical analysis. All results are expressed as means ± SD. Statistical significance was evaluated by ANOVA and a Bonferonni’s adjustment applied to the results of a t-test with SPSS software (SPSS). A value of $P < 0.01$ was regarded as being statistically significant.

RESULTS

Laminar flow promotes Flk-1 ES cell proliferation. Flk-1 ES cells were exposed to laminar flow, and changes in cell density were measured (Fig. 1A). Under static conditions, cell density increased with time and plateaued on day 5. The addition of a maximally effective concentration of VEGF165 (50 ng/ml) markedly increased the cell density of static cells. The application of laminar flow induced a much greater increase in cell density than occurred in the static cells, and the increase in cell density became more prominent as the shear stress increased from 1.5 to 5.0 dyn/cm². The increase in cell density induced by a shear stress of 1.5 dyn/cm² approached the level reached in response to 50 ng/ml VEGF165. The Flk-1 kinase inhibitor SU1498 almost completely suppressed the proliferation of both static and shear stressed cells. These findings suggest that Flk-1 activation plays an important role in both basal and shear stress-induced proliferation by Flk-1 ES cells.

Cell cycle analysis showed that shear stress almost dose dependently decreased the percentage of cells in the G₀ and G₁ phases of the cell cycle and increased the percentage of cells in the S, G₂, and M phases (Fig. 1B). These results indicate that laminar flow stimulates Flk-1 ES cells to proliferate.

Flow-induced Flk-1 ES cell proliferation is shear stress dependent. To determine whether the flow-induced increase in cell density was dependent on shear stress or shear rate, Flk-1 ES cells were subjected to the flow of two perfusates having different viscosities. Cell density increased as the shear rate increased, but it increased to an even greater extent when the viscosity or shear stress was higher at the same shear rate (Fig. 2A). On the other hand, cell densities plotted against shear stress yielded a single line (Fig. 2B). These findings indicate that flow-induced Flk-1 ES cell proliferation is shear stress dependent rather than shear rate dependent.

Shear stress induces differentiation of Flk-1 ES cells into the endothelial cell lineage. Flk-1 ES cells that had either been cultured under static conditions or exposed to shear stress...
(5 dyn/cm²) for 24 h were immunostained for an endothelial cell marker, PECAM-1, and a mural cell marker, SM α-actin (Fig. 3). Under static conditions and culture with 10% FBS, >90% of the cells were positive for SM α-actin (brown). The addition of VEGF₁₆₅, but not PDGF-BB, resulted in the appearance of PECAM-1⁺ sheets of endothelial cells (purple), and the remaining cells surrounding the sheets were positive for SM α-actin. When exposed to shear stress for 24 h, Fig. 4. Effect of shear stress on the expression of various cell lineage marker proteins. Flk-1⁺ ES cells cultured either under static conditions or exposed to shear stress (1.5 or 5 dyn/cm²) were labeled with fluorescent antibodies to Flk-1, Flt-1, vascular endothelial (VE)-cadherin, PECAM-1, smooth muscle (SM) α-actin, CD3, and keratin, and the fluorescence intensity of 2 × 10⁴ cell samples was measured by FACS. Cells were continuously exposed to shear stress for 24–96 h starting on culture day 3, and they were assayed every 24 h. Shear stress accelerated the increases in the expression of Flk-1, Flt-1, VE-cadherin, and PECAM-1. The expression of SM α-actin increased slightly during the first 48 h but gradually decreased at 72 and 96 h. Shear stress had no significant effect on the expression of CD3 or keratin. Data are shown as means ± SD of 4 samples. Similar results were obtained in 3 separate experiments. *P < 0.01 versus static control.
PECAM-1+ sheets appeared in the culture of Flk-1+ ES cells with 10% FBS. Shear stress increased predominantly PECAM-1+ sheets in the presence of VEGF and induced PECAM-1+ sheets even in the presence of PDGF-BB.

The time course of expression of the various cell lineage markers by Flk-1+ ES cells was investigated by flow cytometry (Fig. 4). Under static conditions, the expression of Flk-1, Flt-1, VE-cadherin, and PECAM-1 increased on days 3 and 4 and declined thereafter, whereas the expression of SM α-actin, CD3, and keratin decreased as the days of culture passed. When exposed to shear stress, the expression of Flk-1, Flt-1, VE-cadherin, and PECAM-1 increased markedly, whereas the
expression of SM α-actin increased slightly by days 4 and 5 and then decreased by days 6 and 7 compared with the static controls. Shear stress did not affect the expression of CD3 or keratin.

Gene expression in Flk-1+ ES cells was examined by real-time PCR (Fig. 5). Shear stress markedly increased the mRNA levels of the endothelial cell markers Flk-1, Flt-1, VE-cadherin, PECAM-1, and Tie-2 in a time- and dose-dependent manner but had no effect on the mRNA level of VEGF. The mRNA levels of the SM cell markers SM α-actin and SM-22a showed a slight increase at 24 h in response to shear stress compared with the static controls but no change at 48 and 72 h. These temporal changes in Flt-1, Flk-1, VE-cadherin, PECAM-1, and SM α-actin mRNA levels were consistent with the changes in protein expression shown in Fig. 4. SU1498 almost completely abolished the shear stress-induced increase in the mRNA levels of Flk-1, Flt-1, VE-cadherin, PECAM-1, and Tie-2, and SU1498 also suppressed the shear stress-induced increase in mRNA levels of SM α-actin and SM-22a that occurred at 24 h. Taken together, these results indicate that shear stress promotes differentiation of Flk-1+ ES cells into the vascular endothelial cell lineage and that Flk-1 activation is essential for the differentiation.

Shear stress activates Flk-1 in a ligand-independent manner. Because the experiments in which SU1498 was used revealed that Flk-1 activation is involved in shear stress-mediated ES cell proliferation and differentiation, we investigated whether shear stress induces Flk-1 activation (Fig. 6A).

Tyrosine phosphorylation of Flk-1 occurred when Flk-1+ ES cells were exposed to VEGF, and tyrosine phosphorylation of Flk-1 also occurred in response to shear stress. The shear stress-induced Flk-1 phosphorylation was dose dependent (Fig. 6B), and both the VEGF- and shear-induced tyrosine phosphorylation of Flk-1 were completely blocked by SU1498. Inhibition of tyrosine kinases with genistein or herbimycin A also blocked the shear-induced Flk-1 phosphorylation, whereas prior exposure to neutralizing antibody against VEGF, the extracellular ATP scavenger apyrase, or pertussis toxin was incapable of attenuating the Flk-1 phosphorylation response to shear stress. Shear stress did not affect VEGF production by Flk-1+ ES cells (Fig. 6C). These results indicate that shear stress induces tyrosine phosphorylation of Flk-1 in a ligand-independent manner and that it is not the result of Flk-1 transactivation secondary to G protein-coupled receptor activation or ATP release.

Shear stress promotes tube formation by Flk-1+ ES cells in collagen gels. To investigate whether shear stress affects the ability of Flk-1+ ES cells to form capillary-like tubes, Flk-1+ ES cells cultured under static conditions or exposed to shear stress for 24 h were seeded in collagen gels and microscopically examined for tube formation (Fig. 7A). At 3–6 h, Flk-1+ ES cells cultured under static conditions were present in the form of individual cells or small cellular aggregates, and they exhibited clustering but no tubelike structures at 24 h. By contrast, the shear-stressed Flk-1+ ES cells appeared to be interconnected as early as 3 h after being seeded. The forma-

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**A**

P-Flk-1

Flk-1

Shear stress

VEGF165

SU1498

VEGF Ab

Apyrase

Pertussis toxin

Genistein

Herbimycin A

235 kD

235 kD

**B**

Static

Shear (1.5)

Shear (3.0)

P-Flk-1

Flk-1

**C**

![Graph showing VEGF production under different conditions](image)

The total amount of Flk-1 in whole cell lysates was determined with anti-Flk-1 antibody (below). Both shear stress and VEGF caused tyrosine phosphorylation of Flk-1 that was completely blocked by SU1498 (30 μmol/l). A VEGF neutralizing antibody (1 μg/ml, R&D Systems) did not attenuate the shear stress-induced Flk-1 phosphorylation, indicating that shear stress activates Flk-1 in a ligand-independent manner. Neither apyrase (20 U/ml, Sigma) nor pertussis toxin (100 ng/ml, Sigma) affected the shear-induced Flk-1 phosphorylation, but both genistein (100 μmol/l, Calbiochem) and herbimycin A (1 μmol/l, Calbiochem) completely blocked it. The total amount of Flk-1 protein remained unchanged regardless of the stimulus. Representative immunoblots are shown (n = 3).

**Fig. 6.** Effect of shear stress on Flk-1 phosphorylation and VEGF production. A: shear stress-induced phosphorylation of Flk-1. Flk-1+ ES cells on culture day 3 were treated with vehicle (DMSO) or 30 μmol/l SU1498 and then exposed to VEGF165 (50 ng/ml) or shear stress (1.5 dyn/cm²) for 30 min. Cell lysates were immunoprecipitated with antiphosphotyrosine antibody (PY69, BD Biosciences). Tyrosine phosphorylated proteins associated with Flk-1 (P-Flk-1) were detected by immunoblotting with anti-Flk-1 antibody (Santa Cruz, above). The total amount of Flk-1 in whole cell lysates was also determined with anti-Flk-1 antibody (below). Both shear stress and VEGF caused tyrosine phosphorylation of Flk-1 that was completely blocked by SU1498 (30 μmol/l). A VEGF neutralizing antibody (1 μg/ml, R&D Systems) did not attenuate the shear stress-induced Flk-1 phosphorylation, indicating that shear stress activates Flk-1 in a ligand-independent manner. Neither apyrase (20 U/ml, Sigma) nor pertussis toxin (100 ng/ml, Sigma) affected the shear-induced Flk-1 phosphorylation, but both genistein (100 μmol/l, Calbiochem) and herbimycin A (1 μmol/l, Calbiochem) completely blocked it. The total amount of Flk-1 protein remained unchanged regardless of the stimulus. Representative immunoblots are shown (n = 3).

**B:** shear stress-induced tyrosine phosphorylation of Flk-1 in a dose-dependent manner. C: Flk-1+ ES cells on culture day 3 were exposed to shear stress (1.5 or 5 dyn/cm²) for 24 or 48 h, and the amount of VEGF released was measured by ELISA. Shear stress had no effect on the production of VEGF. Data are means ± SD (n = 5).
tion of tubelike structures had become prominent at 6 h, and an extensive tubular network was observed at 24 h. Quantitative analysis with NIH Image software showed that the total length of the tubes was significantly greater in shear-stressed Flk-1\(^+/\) H11001 ES cell cultures than in the static control Flk-1\(^+/\) H11001 ES cell cultures (Fig. 7 B). Preexposure to shear stress clearly enhanced the formation of tubelike structures by Flk-1\(^+/\) ES cells. Data are means ± SD of 20 images from 3 separate experiments. *P < 0.001 versus static control.

**DISCUSSION**

The results of this study demonstrated that Flk-1\(^+/\) ES cells are flow sensitive and that their proliferation and differentiation into vascular endothelial cells can be promoted by shear stress. Shear stress was also found to enhance the ability of Flk-1\(^+/\) ES cells to form tubelike structures in collagen gel. These findings suggest that the proliferation and differentiation of ES cells are promoted not only by “chemical stimuli” such as VEGF, PDGF, and TGF-\(\beta\) (5, 7, 18, 21) but by fluid mechanical stimuli as well. Blood flow and interstitial fluid flow in the embryo may therefore play a role as key epigenetic factors in determining the cell lineage into which ES cells differentiate.

The proliferation of Flk-1\(^+/\) ES cells was markedly accelerated under flow conditions. Flow has two effects on cells: flow (shear) rate-dependent mass transport and shear stress as a mechanical force. When a bioactive substance such as growth factor is present in the perfusate, the amount of the substance that reaches the cell surface (mass transport) increases with the flow rate, thereby further stimulating cells. Shear stress stimulates cells mechanically and alters cellular functions. To discriminate between these two effects, we performed flow-loading experiments with perfusates having different viscosities. This method allowed us to apply different levels of shear.
stress to cells at the same flow rate. The results showed that the flow-induced proliferation of Flk-1+ ES cells is dependent on shear stress as a mechanical force rather than on mass transport of growth factors, for example, VEGF.

The shear stress-induced proliferation of Flk-1+ ES cells was almost completely blocked by SU1498, which specifically inhibits the enzymatic activity of Flk-1 kinase and downstream events. This indicates that Flk-1 activation and its signal transduction play an important role in the shear stress-induced proliferation of Flk-1+ ES cells. When Flk-1 is phosphorylated with its ligand VEGF, phospholipase C is activated, and it in turn activates the protein kinase C-Raf-MEK-MAPK pathway, which stimulates DNA synthesis in endothelial cells (15). The present study showed that Flk-1+ ES cells do not release VEGF and VEGF receptors in response to shear stress but that they increase their cell surface expression of Flk-1. Thus the increase in Flk-1 expression, but endogenously released VEGF, may at least in part account for the mechanism of the shear stress-enhanced Flk-1+ ES cell proliferation. Interestingly, we observed that shear stress caused tyrosine phosphorylation of Flk-1 that was not blocked by a specific VEGF neutralizing antibody. This means that shear stress activates Flk-1 in a ligand-independent manner and is consistent with the findings observed in bovine aortic endothelial cells (9, 17). Although its molecular mechanism remains to be elucidated, the ligand-independent activation of Flk-1 seems to play an important role in the response of Flk-1+ ES cells to shear stress.

Shear stress increased the expression of the endothelial markers Flk-1, Flt-1, VE-cadherin, and PECAM-1 in Flk-1+ ES cells at both the protein level and the mRNA level. On the other hand, shear stress initially slightly increased and then decreased the expression of SM α-actin, and it did not affect the expression of CD3 or keratin. These findings indicate that shear stress promotes the differentiation of Flk-1+ ES cells into the vascular endothelial lineage rather than into other cell lineages, such as mural cells, blood cells, or epithelial cells. The shear stress-induced upregulation of endothelial cell markers may in part be attributable to the proliferation of a subset of newly differentiated endothelial cells rather than continued recruitment of Flk-1+ ES cells into the endothelial cell lineage. The shear stress-induced differentiation of Flk-1+ ES cells into endothelial cells was significantly suppressed by SU1498, indicating that Flk-1 activation and its downstream signal transduction play an important role in endothelial cell differentiation as well as in the proliferation of Flk-1+ ES cells exposed to shear stress. Further study is needed to identify the signals downstream of the tyrosine phosphorylation of Flk-1 that are involved in the endothelial cell differentiation by Flk-1+ ES cells.

Flk-1+ ES cells have been reported to organize into vessel-like structures consisting of PECAM-1-positive endothelial tubes supported by SM α-actin-positive mural cells when cultured in three-dimensional collagen gel (21). Flk-1+ ES cells injected into chick embryos have been observed to be incorporated into the embryos and contribute to the developing vasculature in vivo (22). In this study, Flk-1+ ES cells exposed to shear stress showed much greater capability to form vessel-like structures in collagen gel than when cultured under static conditions. This seems to be due to the positive effects of shear stress on proliferation and endothelial cell differentiation by Flk-1+ ES cells, and the increase in expression of Flk-1 and the cell-to-cell adhesion molecule VE-cadherin may play important roles in mediating the positive effect of shear stress. These results show that alteration of cell function by a mechanical stimulus such as shear stress can be used as a novel technique to induce differentiation by ES cells. This technique may provide new insight into the development of vascular regenerative cell therapy for ischemic diseases or the development of new types of artificial blood vessels composed of vascular progenitor cells and various biomaterials.

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