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Shear Stress Induces Endothelial Differentiation From a Murine Embryonic Mesenchymal Progenitor Cell Line

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Objective—Recent studies have illustrated that mesenchymal stem cells possess the potential to differentiate along an endothelial lineage, but the effect of shear on mesenchymal differentiation is unknown. Thus, we developed an in vitro shear stress system to examine the relationship between shear stress and the endothelial differentiation of a murine embryonic mesenchymal progenitor cell line, C3H/10T1/2.

Methods and Results—The parallel plate system of fluid shear stress was used. Shear stress significantly induced expression of mature endothelial cell-specific markers in CH3H/10T1/2 cells such as CD31, von Willebrand factor, and vascular endothelial-cadherin at both the mRNA and protein levels with real-time polymerase chain reaction and immunofluorescence analyses, respectively. In addition, shear-induced augmentation of functional markers of the mature endothelial phenotype such as uptake of acetylated low-density lipoproteins and formation of capillary-like structures on Matrigel. Furthermore, shear stress significantly upregulated angiogenic growth factors while downregulating growth factors associated with smooth muscle cell differentiation.

Conclusions—This study demonstrates, for the first time, endothelial differentiation in a mesenchymal progenitor CH3H/10T1/2 cell line resulting from shear exposure. Thus, this analysis may serve as a basis for further understanding the effect of shear on mesenchymal and vascular cell differentiation. (*Arterioscler Thromb Vasc Biol.* 2005;25:1817-1823.)

Key Words: shear stress ■ differentiation ■ endothelial cells ■ C3H/10T1/2 cells ■ CD31 ■ von Willebrand factor

The blood vessel wall is inherently subjected to and affected by the pulsatile hemodynamic stimulus of blood flow within the vascular lumen, and biomechanical forces intrinsically present as a result of this hemodynamic flow are believed to play an important role in vascular development, remodeling, and lesion formation. In particular, cells lining the vascular lumen are constantly subjected to shear stress, a frictional force at the apical endothelial surface exerted by blood flow.^{1,2} Shear stress has been recognized as an important modulator of endothelial phenotype, morphology, gene expression, and, especially, differentiation.^{3,4}

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The ability to influence or control endothelial cell differentiation would have implications for vasculogenesis (neovascularization), understanding the pathogenic physiology behind atherosclerotic lesion formation, use in vascular healing or repair, and use in tissue engineering applications; thus, recent studies have demonstrated the utility of shear stress in provoking endothelial progenitor cell (EPC) differentiation.^{4,5} However, these studies have generally used a fairly heterogeneous starting precursor population. Furthermore, the overall small number of EPCs in circulation and the need for ex vivo expansion before use suggests that the EPC may not be the best progenitor for functional application.⁶

Recent studies have illustrated that mesenchymal stem cells (MSCs) also possess the potential to differentiate along an endothelial lineage.^{7,8} For example, Oswald et al demonstrated endothelial differentiation when MSCs were cultivated in 2% FCS and 50 ng/mL vascular endothelial growth factor (VEGF).⁷ Additionally, other studies have revealed the contribution of MSCs to the formation of new vessels and improvements in cardiac function.^{9,10} Thus, MSCs may hold the potential to differentiate along endothelial lines and to be used for applications traditionally associated with EPC use, such as neovascularization and tissue engineering.

Although there are a handful of studies investigating the effect of growth factors on MSC differentiation, the effect of shear stress on mesenchymal cell differentiation is unknown and difficult to assess using in situ developmental models. Thus, we have developed an in vitro shear stress system to examine the relationship between shear stress and the endothelial differentiation of mesenchymal progenitor cells. The current study uses a murine embryonic mesenchymal progenitor cell line (C3H/10T1/2) that has been shown to have the potential to differentiate into a variety of specialized cells such as osteocytes, chondrocytes, adipocytes, and smooth muscle cells (SMCs).¹¹⁻¹³ Unlike other shear stress-based studies, which may exploit the use of a heterogeneous starting

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From the Molecular Surgeon Research Center, Division of Vascular Surgery and Endovascular Therapy, Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, Tex.

Correspondence to Changyi (Johnny) Chen, MD, PhD, Michael E. DeBakey Dept of Surgery, Baylor College of Medicine, 1 Baylor Plaza, Mail Stop NAB-2010, Houston, TX 77030. E-mail jchen@bcm.tmc.edu

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cellular population, we used this homogenous cell line such that the current study may serve as a basis for the correlation between shear stress and mesenchymal progenitor cell differentiation. Furthermore, unlike other current shear studies that only use a selection of progenitor cells with endothelial markers such as Flk-1,¹⁴ we did not select from progenitor cell subpopulations to obtain a more physiological view of the overall effects of shear on progenitor cell differentiation.

The studies described herein demonstrate that exposure of murine C3H10T1/2 (10T1/2) cells to shear stress promotes differentiation toward an endothelial cell phenotype. Also, this study hypothesizes that the possible mechanism by which 10T1/2 cells undergo endothelial differentiation in conditions of shear stress involves the respective shear-regulated increase of angiogenic factors and decrease of SMC factors.

Methods

Chemicals and Reagents

Trypsin/EDTA and FBS were purchased from Invitrogen (Grand Island, NJ). Eagle's minimal essential medium in Earle's balanced salt solution was obtained from American Type Culture Collection (Rockville, Md). RNAqueous-4PCR kit was acquired from Ambion (Austin, Tex). iScript cDNA synthesis kit and iQ SYBR Green supermix kit were purchased from Bio-Rad (Hercules, Calif). Collagen I and 0.02% EDTA solution were bought from Sigma-Aldrich (St Louis, Mo). Fluorescein isothiocyanate (FITC)-conjugated anti-CD31 (platelet-endothelial cell adhesion molecule [PECAM]-1) and its isotype control were obtained from BD PharMingen (San Diego, Calif). Anti-von Willebrand Factor (vWF) and its isotype control were purchased from DAKO Corp (Carpinteria, Calif). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-acetylated low-density lipoprotein (DiI-acLDL) was purchased from Molecular Probes (Eugene, Ore).

Cell Culture and Shear Stress Experiments

Murine C3H/10T1/2 cells (American Type Culture Collection, Rockville, Md) were incubated on collagen type I-coated tissue culture plates. This particular cell line did not change markers of smooth muscle or endothelial differentiation when cultured on collagen type I matrices under static culture conditions. Plates were cultured with Eagle's minimal essential medium in Earle's balanced salt solution and 10% FBS at 37°C in humidified air with 5% CO₂. After the culture reached 80% of confluent density, the cells were exposed to laminar shear stress.

Shear stress experiments were performed using a custom-made parallel plate flow chamber. This type of flow chamber permits checking the cells during and after the flow experiment. The medium was driven by a constant hydrostatic pressure, exposing the cells to a steady fluid shear stress of 15 dyn/cm² for 6 and 12 hours. During the experiment, the system was maintained at 37°C in humidified air with 5% CO₂. Static controls were also concurrently performed.

Real-Time RT-PCR

After 10T1/2 cells were exposed to shear stress, total cellular RNA was extracted. RNA was reverse-transcribed into cDNA. Real-time quantitative RT-PCR primers targeting murine CD31, vWF, CD14, vascular endothelial (VE)-cadherin, VEGF, VEGF receptor (VEGFR)-1 (Flt1), VEGFR-2, VEGFR-3, transforming growth factor (TGF)- β , platelet-derived growth factor (PDGF)-B, and PDGF receptor (PDGFR) were designed by Beacon Designer 2.1 software, and sequences are listed in the Table I (available online <http://atvb.ahajournals.org>). This relative value of target genes to endogenous reference is described as the fold of GAPDH = 2^{- Δ Ct} (see the online data supplement available at <http://atvb.ahajournals.org> for the detailed method).

Flow Cytometry

After a period of shear stress exposure (15 dyn/cm²) for 6 or 12 hours, cells were detached with 0.02% EDTA and incubated with FITC-conjugated anti-CD31 (PECAM-1) antibody or anti-vWF followed by FITC-conjugated swine anti-rabbit IgG (Dako, F0054). Isotype-identical antibodies served as controls to exclude nonspecific binding. Quantitative analysis was performed using a FACScalibur flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson).¹⁵

Immunofluorescence Staining

Following exposure to shear stress, cells were trypsinized and grown on chamber slides in DMEM/10% FBS for 24 hours. These cultures on slides were rinsed twice in PBS and fixed with 4% of paraformaldehyde for 15 minutes on ice. After fixation, the cells were washed twice with PBS and incubated for 1 hour with anti-vWF polyclonal antibody and anti-CD31 (PECAM-1) antibody. Cells were again washed twice with PBS and then incubated with FITC-conjugated secondary antibody. Finally, the cells were washed 3 times with PBS, mounted, and photographed using an Olympus fluorescence microscope equipped with a digital camera.

Acetylated Low-Density Lipoprotein Uptake

To further verify that exposure to shear stress resulted in cellular differentiation toward an endothelial-like phenotype, uptake of DiI-labeled acetylated low-density lipoprotein (ac-LDL), a function associated with endothelial cells, was determined. Cells were incubated with 10 μ g/mL ac-LDL labeled with the fluorochrome DiI at 37°C for 2 hours. These cells were then washed with PBS and fixed with 2% formaldehyde for 10 minutes. Incorporation of DiI-labeled ac-LDL was photographed under microscopy and determined by fluorescence-activated cell sorting (FACS) analysis as detailed above.

Tube Formation

The cells were treated with 0.25% trypsin-EDTA after exposure to shear stress (15 dyn/cm² for 12 hours) and then collected, counted, and resuspended in culture medium with 2% FBS. These cells were seeded (50 000 cells/well) in a 24-well tissue culture plate which had been evenly coated with growth factor-reduced Matrigel (BD Labware, Bedford, Mass). Seeded cells were incubated at 37°C in a 5% CO₂ incubator for 3 and 5 hours. Gels were examined using phase-contrast microscopy (\times 200 magnification), and ImageJ software (<http://www.nih.gov>) was used to determine the total length of tube-like structures in images captured after incubation. Tube formation was quantified by counting the length of the tubular structures (defined as those exceeding 200 μ m in length) in 5 randomly selected fields.¹⁶

Statistical Analysis

Results are shown as mean \pm SD with at least 3 replicates, unless otherwise noted. The statistical significance of the differences was determined with Student *t* test, with a probability value of *P* < 0.05 considered statistically significant.

Results

Effect of Shear Stress on Cell Morphology of C3H/10T1/2 Cells

Shear stress had a distinct morphological effect, changing the cells from a fibroblast-like appearance (Figure 1A) to a more fusiform shape with increasing shear exposure times of 6 (Figure 1B) and 12 (Figure 1C) hours. Also, under static culture conditions, the 10T1/2 cells were randomly distributed with little respect to alignment (Figure 1A), but after 12 hours of shear stress (15 dyn/cm²), the cells tended to align parallel with the direction of flow (Figure 1C).

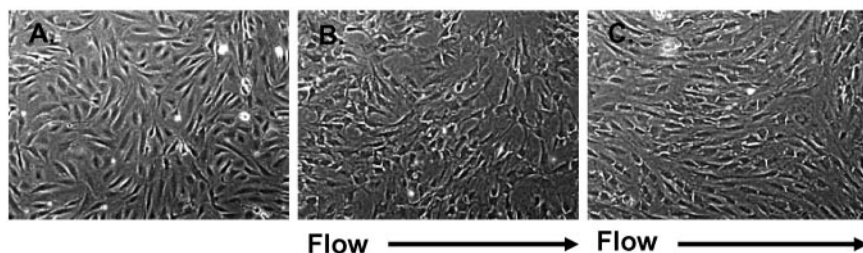


Figure 1. Effect of shear stress on cell morphology and alignment. Phase-contrast photomicrographs of C3H/10T1/2 cells cultured under static conditions (A) or exposed to shear stress (15 dyn/cm²) for 6 hours (B) and 12 hours (C). The 10T1/2 cells aligned parallel to the direction of flow (left to right).

Effect of Shear Stress on CD31 Expression in C3H/10T1/2 Cells

C3H/10T1/2 cells were exposed to shear stress for 6 and 12 hours, and real-time PCR was used to quantify the mRNA level of CD31 (PECAM-1), a glycoprotein localized to the endothelial cell junction and used as a mature endothelial cell marker. As compared with the static control group, CD31 mRNA expression was upregulated by 162-fold and 757-fold after 6 and 12 hours of shear stress (15 dyn/cm²), respectively ($P < 0.01$, Figure 2A). In additional experiments with shear stress for 24 hours, C3H/10T1/2 cells expressed a CD31 mRNA level similar to that of cells with shear stress for 12 hours.

The cellular protein level of CD31 was determined by flow cytometric analysis and immunofluorescence staining. Flow cytometric analysis indicated that the level of protein expression of CD31 increased ≈ 11 -fold after 12 hours shear stress (Figure 2B and 2C). Immunofluorescence studies demonstrated that the CD31 protein expression was markedly increased in shear exposed cells (Figure 2D). These results suggest that shear stress exposure induces the expression of CD31 at both the mRNA and protein levels.

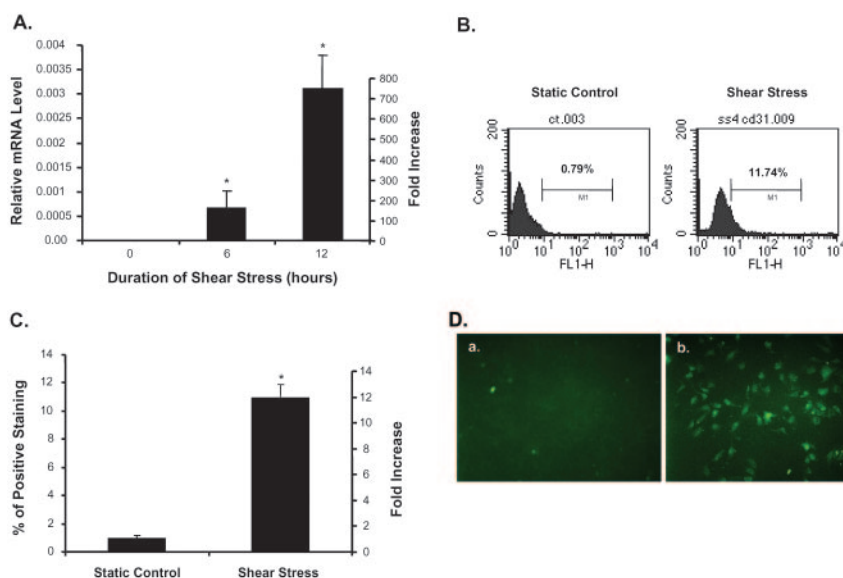


Figure 2. Effect of shear stress on CD31 mRNA and protein levels. A, Murine C3H/10T1/2 cells were kept in static culture or exposed to shear stress (15 dyn/cm²) for 6 and 12 hours. Relative CD31 mRNA expression levels were determined by real-time quantitative PCR. Values of mRNA amounts were normalized to GAPDH expression and expressed relative to its static control. Error bars represent SD ($P < 0.01$). B and C, 10T1/2 cells were cultured either under static conditions or exposed to shear stress (15 dyn/cm²) for 12 hours. The 10T1/2 cells were labeled with fluorescent antibody to CD31, and relative protein levels were determined by flow cytometry. Data in B are representative histograms for staining with anti-CD31 monoclonal antibody. Data in C are mean \pm SD ($P < 0.01$) of CD31-positive cells. Shear stress increased the expression of CD31. D, The 10T1/2 cells were labeled with fluorescent antibody to CD31, and relative protein levels were determined by CD31 immunoreactivity. Cells were cultured either under static conditions (a) or exposed to shear stress (15 dyn/cm²) for 12 hours (b).

Effect of Shear Stress on vWF Expression in C3H/10T1/2 Cells

The mRNA levels of vWF, a glycoprotein derived from endothelial cells that promotes platelet adhesion, are reported in Figure 3A. The mRNA levels of vWF were significantly upregulated by shear stress 97- and 108-fold after 6 and 12 hours of shear stress, respectively, as compared with static control groups ($P < 0.01$). In additional experiments with shear stress for 24 hours, C3H/10T1/2 cells showed a 690-fold increase of vWF mRNA levels as compared with static control cells ($P < 0.01$). Thus, longer shear stress induced much higher vWF mRNA levels in C3H/10T1/2 cells.

Flow cytometric analysis revealed protein expression of vWF was markedly upregulated 214-fold after 12 hours of shear stress as compared with static control (Figure 3B and 3C). The results of immunofluorescence staining are represented in Figure 3D, and in 10T1/2 cells maintained under static control conditions, the immunofluorescence staining was very weak (Figure 3D, a). In contrast, immunofluorescence staining was more apparent in 10T1/2 cells exposed to shear stress (Figure 3D, b). These results suggest that shear

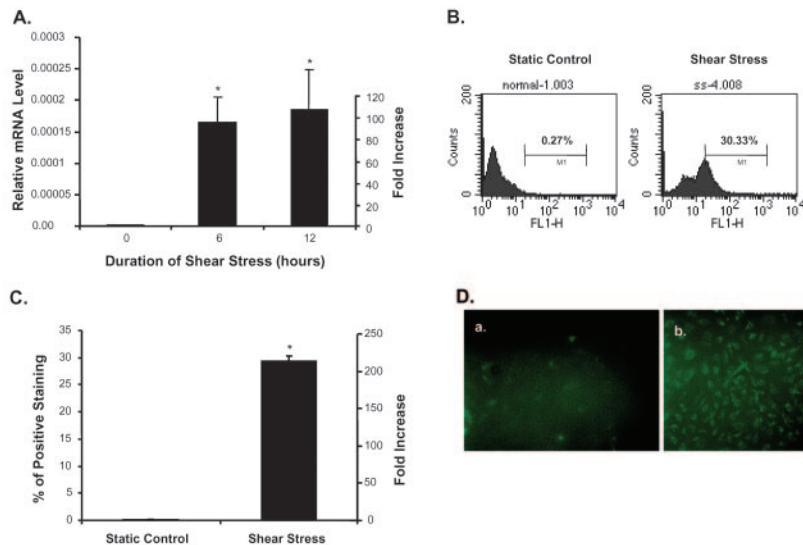


Figure 3. Effect of shear stress on vWF mRNA and protein levels. A, Murine C3H/10T1/2 cells were kept in static culture or exposed to shear stress (15 dyn/cm²) for 6 and 12 hours. Relative vWF mRNA expression level was determined by real-time quantitative PCR. Values of mRNA amounts were normalized to GAPDH expression and expressed relative to its static control for that experimental condition. Error bars represent SD ($P<0.01$). B and C, 10T1/2 cells were cultured either under static conditions or exposed to shear stress (15 dyn/cm²) for 12 hours. The 10T1/2 cells were labeled with fluorescent antibody to vWF, and relative protein levels were determined by flow cytometry. Data in (B) are representative histograms for staining with anti-vWF monoclonal antibody. Data in (C) are mean \pm SD ($P<0.01$) of vWF-positive cells. Shear stress increased the expression of vWF. D, 10T1/2 cells were labeled with fluorescent antibody to vWF, and relative protein levels were determined by vWF immunoreactivity. 10T1/2 cells were cultured either under static conditions (a) or exposed to shear stress (15 dyn/cm²) for 12 hours (b).

stress exposure induces the expression of vWF at both the mRNA and protein levels.

Additionally, shear stress increased VE-cadherin mRNA levels by 23-fold at 12 hours and 55-fold at 24 hours as compared with static controls ($P<0.01$). Monocyte marker CD14 was significantly decreased by 74% after shear stress exposure as compared with static control ($P<0.01$).

Effect of Shear Stress on ac-LDL Uptake and Tube Formation in C3H/10T1/2 Cells

The uptake of DiI-labeled ac-LDL and tube formation are specific functional markers for endothelial cells *in vitro*. To further verify that shear stress could induce an endothelial-like phenotype in 10T1/2 cells, we measured the uptake of DiI-acLDL by 10T1/2 cells in both static and shear stress conditions. Flow cytometric analysis demonstrated that the uptake of DiI-acLDL by shear stressed cells was increased 10-fold as compared with those cultured under static conditions (Figure 4A). Immunofluorescence studies also revealed that shear stressed cells were positive for DiI-acLDL uptake (Figure 4B, b).

Capillary-like tube formation on Matrigel was also enhanced in 10T1/2 cells exposed to shear stress. Cells displayed increased formation of a tube-like network when examined microscopically at 3 (Figure 5A, c) and 5 hours (Figure 5A, d) after being plated on Matrigel following 12 hours of shear exposure. Mean total tube length exhibited by shear stressed cells was increased 11- and 3-fold more than cells cultured under static control conditions (Figure 5B). These findings suggest that shear stress augments the ability of 10T1/2 cells to perform functions suggestive of an endothelial phenotype.

Effect of Shear Stress on Growth Factor Regulation

In an attempt to elucidate a possible mechanism by which differentiation toward a mature endothelial phenotype ensues, the current study analyzed differences in growth factors

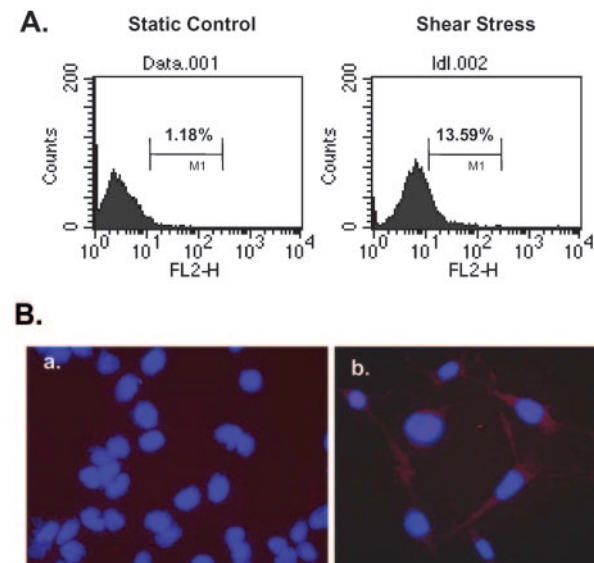


Figure 4. Effect of shear stress on acetylated LDL uptake. A, Murine C3H/10T1/2 cells were cultured either under static conditions or exposed to shear stress (15 dyn/cm²) for 12 hours. Flow cytometric analysis demonstrated uptake of DiI-acLDL increased by 10-fold in the shear exposed (right) group as compared with static conditions (left). B, Fluorescence images of DiI-acLDL uptake by 10T1/2 cells cultured under static conditions (a) or exposed to shear stress (b) for 12 hours revealed that most of the shear stressed cells were positive for ac-LDL uptake (red).

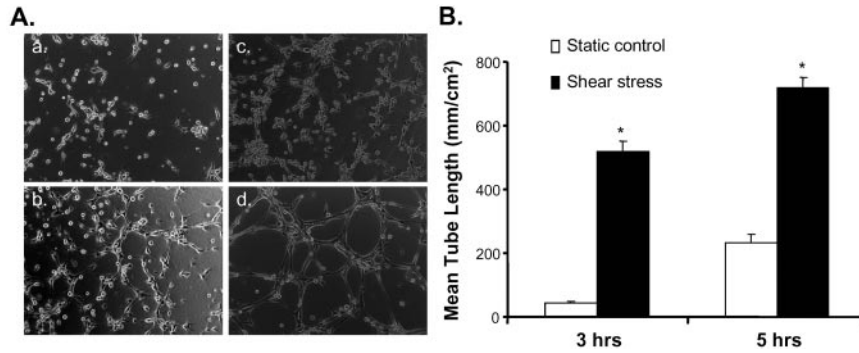


Figure 5. Effect of shear stress on tube formation on Matrigel. A, Murine C3H/10T1/2 cells that had been incubated under static conditions or exposed to shear stress (15 dyn/cm² for 12 hours) were seeded onto Matrigel. Representative photomicrographs are of cells exposed to shear conditions and then cultured on Matrigel for 3 hours (b) and 5 hours (d). Corresponding static control cells were also incubated on Matrigel for 3 hours (a) and 5 hours (c). B, ImageJ was used to determine the total length of tube-like structures in images captured after incubation for 3 and 5 hours. Exposure to shear stress clearly enhanced tube-like structure formation by 10T1/2 cells. Values are mean±SD of 5 images. *P*<0.001 vs static control.

within cultures of 10T1/2 cells exposed to shear as opposed to those cultured strictly under static conditions. First, an angiogenic growth factor, VEGF, and its associated receptor, VEGFR-1, were examined by RT-PCR analysis. Cultures of 10T1/2 exposed to 12 hours of shear exhibited a 7-fold increase in VEGF as compared with static controls (Figure 6A). Furthermore, the mRNA level of VEGFR-1 was upregulated by 41% after 12 hours of shear exposure (Figure 6B). VEGFR-2 and VEGFR-3 were expressed in neither the static control nor the shear-exposed cells (data not shown).

Also, growth factors more closely associated with SMC differentiation and proliferation were considered. The mRNA levels of TGF-β, PDGF-B, and PDGFR were checked by RT-PCR in shear stressed versus static cultures. TGF-β, PDGF-B, and PDGFR displayed decreases of 52%, 85%, and 99%, respectively, in cultures exposed to 12 hours of shear as opposed to controls (Figure 6C, 6D, and 6E). These findings

suggest that shear stress may cause an upregulation of growth factors associated with endothelial differentiation and a downregulation of growth factors associated with SMC differentiation.

Discussion

The current study demonstrates that shear stress significantly induces expression of mature endothelial cell-specific markers such as CD31, vWF, and VE-cadherin at both the mRNA and protein levels in a murine embryonic mesenchymal progenitor cell line. In addition, shear induces augmentation of functional markers of the mature endothelial phenotype such as uptake of ac-LDL and formation of tube-like structures on Matrigel. Then, in an attempt to elucidate a possible mechanism by which differentiation toward an endothelial lineage takes place, we have shown that shear stress significantly upregulates angiogenic growth factors while down-

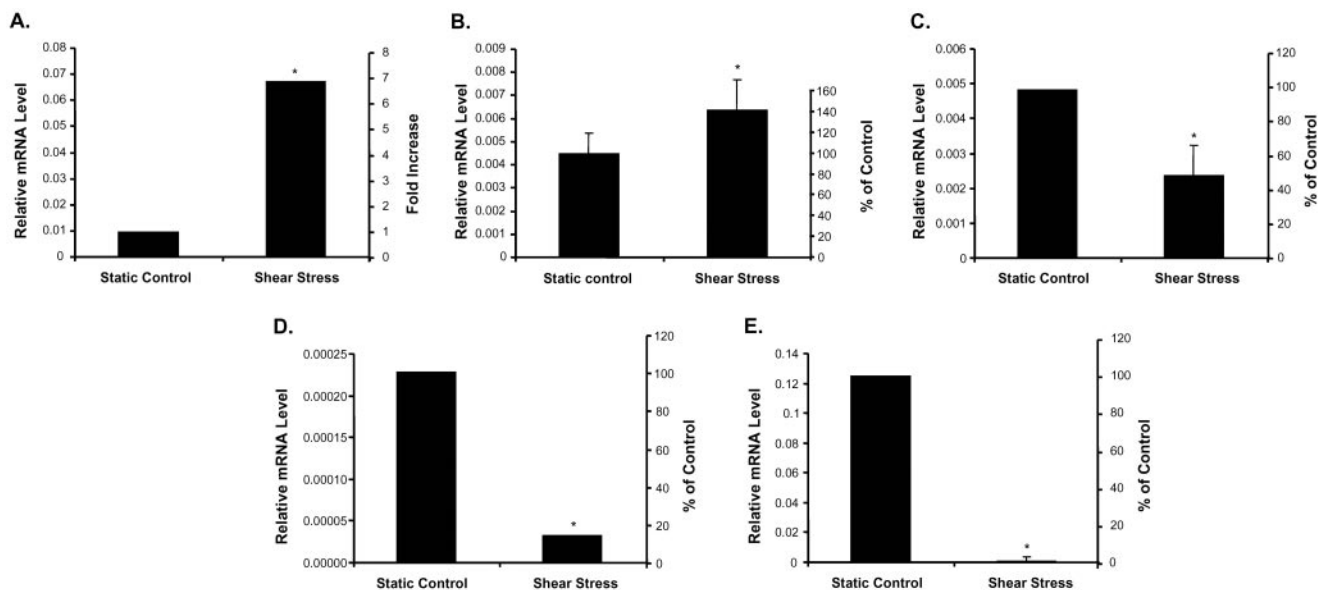


Figure 6. Effects of shear stress on angiogenic and SMC regulating factors. Murine C3H10T1/2 cells were kept in static culture or exposed to shear stress (15 dyn/cm²) for a 12-hour period. Relative mRNA expression levels of VEGF (A), VEGFR-1 (B), TGF-β (C), PDGF-B (D), and PDGFR (E) were determined by real-time quantitative PCR. Values of mRNA amounts were normalized to GAPDH expression and expressed relative to its static control for that experimental condition. Error bars represent SD (*P*<0.01).

regulating growth factors associated with SMC differentiation.

Previous studies have illustrated that growth factors (such as VEGF) can induce MSCs along an endothelial cell lineage,⁷ but the effect of shear stress on mesenchymal to endothelial differentiation has not been studied. Blood vessels are constantly exposed to shear stress, and studies have suggested that laminar shear stress is responsible for phenotype modulation, vascular remodeling, gene expression, prevention of atherogenesis, and endothelial cell differentiation.^{3,4,17} This differentiation was apparent with respect to morphology and alignment in the current study whereby shear caused the usually fibroblastic C3H/10T1/2 cell seen in static culture to adopt a more fusiform morphology with the long axes parallel to the direction of flow. This morphology and alignment are generally comparable to other studies that have used current shear stress models which relatively mimic *in vivo* flow conditions.^{4,18}

The presence of various surface markers indicative of a mature endothelial phenotype is often used as validation for endothelial differentiation. CD31 (PECAM-1) is a glycoprotein expressed by endothelial cells at the endothelial cell junction where it forms Ca²⁺-independent cell-cell adhesions. vWF is a glycoprotein of protomeric subunits derived from endothelial cells and is found circulating as multimers in a wide size range (0.45× to >12×10⁶ Da). The current study illustrated an increase in both CD31 and vWF mRNA and protein levels in 10T1/2 cells exposed to conditions of shear stress. Additionally, mRNA levels of VE-cadherin, another major endothelial adherent junction adhesive protein, and monocyte marker CD14 were significantly increased and decreased, respectively, as compared with static controls. Thus, the present study suggests that shear stress induces differentiation along an endothelial lineage in a murine mesenchymal progenitor cell line. This is the first study that has demonstrated endothelial differentiation at both mRNA and protein levels in a mesenchymal progenitor caused by exposure to shear, but other studies have used similar mechanisms to confirm endothelial differentiation in endothelial cells and EPCs using the above-named expression markers.^{4–7,18} For example, others have shown a direct relationship between CD31 expression and physiological levels of shear stress in endothelial cells,^{19,20} whereas VE-cadherin was upregulated in shear-exposed EPCs.⁴ Unlike these other studies, which have used nonphysiological levels of shear stress,⁴ we have exhibited mesenchymal progenitor to endothelial differentiation using physiological levels of shear stress at 15 dyn/cm².

C3H/10T1/2 cells have the potential to differentiate into a variety of specialized cells such as osteocytes, chondrocytes, adipocytes, and SMCs.^{11–13} However, whether these cells could differentiate into endothelial lineage is unknown. Based on our knowledge, current study is the first report showing that shear stress induces C3H/10T1/2 differentiation into endothelial cell phenotypes including endothelial markers and angiogenic properties. However, this event may be at the stage of partial differentiation with current *in vitro* culture conditions. For example, flow cytometry analysis showed only 11% to 13% shear stressed cells expressing CD31 and vWF proteins. Another possibility is that C3H/10T1/2 may

have subpopulations which have differential potential for differentiation into different types of cells. This interesting hypothesis has not been explored in other cell types.

In addition to increasing surface marker expression suggestive of a mature endothelial phenotype, we have demonstrated that shear stress induces functional markers also indicative of endothelial cells within a mesenchymal progenitor cell line. Compared with static controls, both the uptake of DiI-acLDL and tube formation on Matrigel were enhanced in 10T1/2 cells exposed to shear stress. Whereas some studies have found that uptake of ac-LDL by endothelial cells was drastically decreased by conditions of shear flow,²¹ the majority of studies using endothelial cells and EPCs have illustrated results similar to ours with regard to cellular uptake of ac-LDL in conditions of shear.^{4,5,18} Likewise, the bulk of studies have also displayed similar tube-formation results after a progenitor was placed in conditions of shear to augment differentiation.^{3,5,7} Differences in functional differentiation are most likely correlated with other environmental factors, such as plating medium, presence or absence of growth factors, and length of shear exposure.¹ Thus, future studies should delve into the elucidation of the combined conditions most conducive for endothelial differentiation.

Finally, drawing from other studies that have demonstrated that mesenchymal to endothelial differentiation is enhanced when angiogenic growth factors such as VEGF were present in culture,⁸ we hypothesized that a possible mechanism by 10T1/2 differentiation toward an endothelial lineage that occurs may involve the shear-regulated increase of angiogenic factors as a positive autocrine mechanism. In fact, our studies elucidated those mRNA levels of VEGF and receptor VEGFR-1 were significantly upregulated in cultures of 10T1/2 cells exposed to conditions of shear stress. Because C3H/10T1/2 cells also have the potential to differentiate along SMC lines,^{12,22} we also hypothesized that the differentiation along endothelial lines may involve the shear-induced negative regulation of factors that have been associated with SMC differentiation such as TGF- β and PDGF-B.^{23,24} Indeed, a significant downregulation of growth factor mRNA associated with SMC differentiation was exhibited when cultures were exposed to shear. Thus, the simultaneous shear-regulated increase in angiogenic growth factors and decrease in SMC differentiation factors may induce the differentiation of the murine mesenchymal progenitor cell line along an endothelial lineage. Although studies suggest that VEGF plays a prominent role in endothelial differentiation, Gloe et al have shown contrary data whereby VEGF did not have a significant role in shear-dependent differentiation, instead they implicated bFGF in endothelial cell differentiation.³ Differences between studies may be accounted for in their starting population of mature endothelial cells as opposed to our progenitor cell starting population and in the time period of shear application. Regardless, both studies point to the importance of growth factors in regulating shear-induced differentiation. Further research into the possible mechanisms underlying the shear-induced differentiation of progenitor cells is warranted to confirm the role of growth factors such as VEGF. Future studies should concentrate on identifying the functional characteristics of VEGF in this shear system by

using experiments with conditioned medium from shear-exposed cells and by attempting to disrupt VEGF signaling at various points in the signaling cascade.

In summary, our findings raise the possibility that shear stress induces the expression of several endothelial cell surface markers at both the mRNA and protein levels and increases functional endothelial markers in a murine embryonic mesenchymal progenitor cell line. These data implicate the differentiation toward an endothelial lineage. Thus, this study indicates a novel means by which shear may induce mesenchymal to endothelial differentiation. Furthermore, this study suggests that a possible mechanism by which shear stress induces this differentiation is attributable to the respective shear-regulated increase of angiogenic factors and decrease of SMC differentiation factors. This study may serve as a starting point in understanding the effect of shear on mesenchymal progenitor differentiation. Future studies within this area will undoubtedly provide more insight into mesenchymal to endothelial differentiation, which will have implications for numerous areas of research including neovascularization, vascular healing, and tissue engineering.

Acknowledgments

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Real Time RT-PCR

After 10T1/2 cells were exposed to shear stress, total cellular RNA was extracted using RNAqueous®-4PCR Kit. RNA (0.5 µg total) was reverse-transcribed into cDNA using the iScript cDNA synthesis kit following the manufacturer's instruction. Real-time quantitative RT-PCR primers targeting murine CD31, vWF, CD14, VE-cadherin, VEGF, VEGFR-1 (Flt1), VEGFR-2, VEGFR-3, TGF-β, PDGF-B, and PDGFR were designed by Beacon Designer 2.1 software, and sequences are listed in Table I. The SYBR Green I assay and the iCycler iQ Real-time PCR detection system (Bio-Rad) were used for detecting real-time quantitative PCR products from 50 ng of reverse-transcribed cDNA. PCR cycling conditions were as follows: initial 95°C for 90 sec, then 40 cycles using 95°C for 20 sec and 60°C for 1 minute. Melt curve analysis was performed on the iCycler over the range 55°C-95°C by monitoring iQ SYBR green fluorescence with increasing temperature (0.5°C increment changes at 10 sec intervals). Specific products were determined as clear, single peaks at their melt curves. PCR reactions for each sample were done in triplicate for both the target gene and the GAPDH control.

The level of target gene expression was determined by the comparative Ct method, whereby the target is normalized to the endogenous reference GAPDH.¹⁹ The Ct value is the cycle number at which the fluorescence level reaches a threshold. The ΔCt is determined by subtracting the Ct of the GAPDH control from the Ct of the target [$\Delta Ct = Ct(\text{target}) - Ct(\text{GAPDH})$]. This relative value of target to endogenous reference is described as the fold of GAPDH = $2^{-\Delta Ct}$.

Table I. Primer sequences for real-time PCR analysis

Gene	Accession No.	Forward Primer	Reverse Primer
CD31	NM_008816	TCCCTGGGAGGTCGTCCAT	GAACAAGGCAGCGGGTTTA
vWF	NM_011708	GCTTGAAGTGTGGACGGAGAGG	TGACCCAGCAGCAGGATGAC
VE-cadherin	X83930	TCCTCTGCATCCTCACTATCACA	GTAAGTGACCAACTGCTCGTGAAT
CD14	BC057889	TCTCAGTTACAACAGGCTGGATAG	AACTTCTCCGAGTGGGATTCAGA
VEGF	BC061468	TGTAACGATGAAGCCCTGGAGTG	GTGCTGGCTTTGGTGAGGTTTG
VEGFR-1	NM_010228	GCCAGACTCTCTTTCTCAAGTGC	GGGGAGTGATGCTCAGCCT
VEGFR-2	NM_010612	TAGCTGTGCTCTGTGGTTCT	TGTCAGTATGTCTTTCTGTGTGCT
VEGFR-3	NM_008029	AACTCTCCAGCATCCTGACCATC	AGCCACTCGACACTGATGAAGG
TGF-β	BC013738	ACCAACTATTGCTTCAGCTCCAC	GTGTGTCCAGGCTCCAAATATAGG
PDGF-B	NM_011057	CTGCCTCTTTCCACTCCCCTAC	GCACATGAGGAAGAAGACACAGAG
PDGFR	BC055311	ACGGCTGAGCTGAGTGATTTCG	ACGGCTGAGCTGAGTGATTTCG
GAPDH	M32599	CGTGCCGCCTGGAGAAACC	TGGAAGAGTGGGAGTTGCTGTTG