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Fluid Shear Stress Regulates the Expression of TGF- β 1 and Its Signaling Molecules in Mouse Embryo Mesenchymal Progenitor Cells

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Abstract

Background—Recently, we have reported that fluid shear stress promotes endothelial cell differentiation from a mouse embryo mesenchymal progenitor cell line C3H10T1/2. However, it is not clear whether the TGF- β 1 system is associated with shear-induced endothelial differentiation. The purpose of this study was to determine the effect of shear stress on the expression of TGF- β 1 and its signaling molecules in C3H10T1/2 cells.

Methods—Murine C3H10T1/2 cells were incubated on collagen type I-coated dishes, and subjected to a steady fluid shear stress of 15 dyn/cm² for 6, 12, and 24 hours. The mRNA levels for TGF- β 1, TGF- β receptors (TGF β R), and Smad molecules were determined with real-time PCR analysis and normalized to GAPDH mRNA levels.

Results—TGF- β 1 mRNA expression was down-regulated by 60% and 66% in shear stress-treated cells at 12 and 24 hours, respectively, compared with static control group ($P < 0.01$). In addition, shear stress significantly decreased TGF β R1 mRNA levels by 30% and 50% in shear stress-treated cells at 12 and 24 hours, respectively ($P < 0.01$). For TGF β R2, shear stress at 6, 12, and 24 hours significantly reduced its expression by 93%, 95% and 97%, respectively, compared with static controls ($P < 0.01$). Furthermore, shear stress significantly decreased mRNA levels of positive signaling molecules Smad2, Smad3 and Smad4 in a time-dependent manner ($P < 0.01$). However, shear stress significantly increased negative signaling molecule Smad7 mRNA levels by 100% at 24-hour treatment compared with static control group ($P < 0.01$).

Conclusions—Fluid shear stress significantly suppresses TGF- β 1 functions through down-regulation of TGF- β 1, TGF β R, positive signaling molecules Smad2, Smad3, and Smad4 and up-regulation of negative signaling molecule Smad7 in a mouse embryo mesenchymal progenitor cell line C3H10T1/2. This study suggests that the negative regulation of the TGF- β 1 system may be involved in shear-induced endothelial cell differentiation in C3H10T1/2.

Keywords

Shear stress; TGF- β 1; TGF- β receptor; Smad; C3H10T1/2; Cell differentiation

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INTRODUCTION

The cell line C3H/10T1/2 is a murine embryonic mesenchymal progenitor cell line which has been extensively used for cell differentiation studies at different conditions. Indeed, C3H/10T1/2 has potential to differentiate into a variety of different specialized cell types including adipocytes, chondrocytes, and osteocytes [1–3]. Recently, our group demonstrated that when C3H/10T1/2 cells are exposed to cyclic strain, this progenitor cell line differentiates into the vascular smooth muscle (SMC) lineage [4]. It is well known that transforming growth factor-beta (TGF β) and Smad signal pathways play critical roles in C3H/10T1/2 differentiation into vascular SMCs. When C3H/10T1/2 cells are supplemented with TGF- β 1 in culture, they possess the ability to differentiate along a SMC lineage [5,6]. In our previous study [7], we showed that fluid shear stress promotes endothelial cell differentiation from C3H10T1/2. The underlying mechanisms are involved in that shear stress significantly up-regulates angiogenic growth factors while down-regulates growth factors associated with SMC differentiation including TGF- β 1. However, it is not clear whether shear stress regulates TGF- β 1 related signaling molecules including TGF- β 1 receptors (TGF β R), positive signaling molecules Smad2, Smad3 and Smad4 as well as negative signaling molecule Smad7. We hypothesized that shear stress may inhibit TGF- β 1 signaling pathways by regulating their gene expression, thereby favoring endothelial cell differentiation in C3H10T1/2 cells. To test this hypothesis in the present study, we analyzed the expression levels of TGF- β 1, TGF β Rs and Smads in response to shear stress in C3H10T1/2.

MATERIALS AND METHODS

Chemicals and Reagents

Trypsin/EDTA and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NJ). Minimum Essential Medium Eagle in Earle's BSS was purchased from American Type Culture Collection (Rockville, MD). RNAqueous-4PCR Kit was obtained from Ambion (Austin, TX). iScript cDNA synthesis kit and iQ SYBR Green supermix kit were purchased from Bio-Rad (Hercules, CA). Collagen I was bought from Sigma-Aldrich (St. Louis, MO).

Cell Culture

Murine C3H10T1/2 cells (American Type Culture Collection, Rockville, MD) were incubated on collagen type I-coated tissue culture plates. Plates were cultured with Minimum Essential Medium Eagle in Earle's BSS and 10% FBS at 37°C in humidified air with 5% CO₂. After the cells reached 80% of confluent density, they were exposed to laminar shear stress performed using a custom-made parallel plate flow chamber as previously described [7]. The medium was driven by a constant hydrostatic pressure roller pump, exposing the cells to a steady fluid shear stress of 15 dyn/cm² for 6, 12, and 24 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 Analysis

After 10T1/2 cells were exposed to shear stress, total cellular RNA was extracted using RNAqueous-4PCR Kit. Total RNA (0.5 μ g) was reverse-transcribed into cDNA using the iScript cDNA synthesis kit following the manufacturer's instruction. The mRNA levels for TGF- β 1, TGF β R1, TGF β R2 and Smads were analyzed by real-time reverse transcriptase polymerase chain reaction (RT-PCR). PCR primers were designed by Beacon Designer 2.1 software (Premier Biosoft International, Palo Alto, CA), and sequences are listed in Table 1. PCR reaction included the following components: 100 nM each primer, diluted cDNA

templates and iQ SYBR Green supermix, running for 40 cycles at 95°C for 20 seconds and 60°C for 1 minute. Each cDNA sample was run in triplicate and the corresponding no-reverse transcriptase (RT) mRNA sample was included as a negative control. The GAPDH primer was included in every plate to avoid sample variations. The mRNA level of each sample for each gene was normalized to that of the GAPDH mRNA. The relative mRNA level was presented as unit values of $2^{[Ct(GAPDH)-Ct(\text{gene of interest})]}$.

Statistical Analysis

Results are shown as mean $\pm$ SD with at least 3 replicates unless otherwise noted. The differences of gene expression between shear stress and control groups were determined with Student's *t*-test. *P* value <0.05 was considered statistically significant.

RESULTS

Shear Stress Decreases mRNA Levels of TGF- β 1 in C3H10T1/2 Cells

TGF- β 1 regulates the differentiation program of a variety of cell types, including mesenchymal cells. We first examined the effects of shear stress on the expression of TGF- β 1 in C3H10T1/2 cells by real time RT-PCR. At basal levels, the expression of TGF- β 1 was relatively low in murine C3H10T1/2 compared to its receptors and its signaling molecules Smads. When the cells were exposed to shear stress for periods of 6, 12 and 24 hours, mRNA levels were measured and compared with the static control group. TGF- β 1 mRNA expression had no change at 6 hours, and however, it was down-regulated by 60% and 66% at 12 and 24 hours, respectively, compared with static controls (P <0.01, Fig. 1).

Shear Stress Reduces the mRNA Levels of TGF β R1 and TGF β R2 in C3H10T1/2 Cells

Two major TGF- β receptors, TGF β R1 and TGF β R2, play a critical role in cellular responses to TGF- β 1 in many cell types. However, it is not clear whether C3H10T1/2 cells could express these receptors and whether shear stress could regulate their expression. In this study, we found C3H10T1/2 expressed relatively high levels of both TGF β R1 and TGF β R2 compared with TGF- β 1, and shear stress significantly decreased their levels. At 12 and 24 hours, shear stress significantly decreased TGF β R1 mRNA levels by 30% and 50%, respectively, compared with static controls (P <0.01, Fig. 2A). However, shear stress had no effect on TGF β R1 mRNA levels at 6 hours. For TGF β R2, shear stress at 6, 12 and 24 hours significantly reduced its expression by 93%, 95% and 97%, respectively, compared with static controls (P <0.01, Fig. 2B).

Shear Stress Regulates the mRNA Levels of Smad2, Smad3, Smad4 and Smad7 in C3H10T1/2 Cells

Smads are major signaling molecules of TGF- β 1 which plays a key role in cell differentiation in several cell types and organ systems. We investigated the possible involvement of Smad molecules in shear stress-treated C3H10T1/2 cells by real time PCR analysis. Indeed, shear stress significantly decreased mRNA levels of Smad2, Smad3 and Smad4 at all time points compared with the static control cells (P <0.01, Fig. 3). However, shear stress significantly increased Smad7 mRNA levels by 100% at 24 hours compared with static control cells (P <0.01, Fig. 4).

DISCUSSION

In the current study, we have investigated the expression of endogenous TGF- β 1, TGF β Rs and Smads in response to shear stress in a mouse embryo mesenchymal progenitor cell line C3H10T1/2. Our data clearly demonstrate that fluid shear stress significantly suppresses TGF- β 1-mediated cell function through down-regulation of TGF- β 1, TGF β R1, TGF β R2,

Smad2, Smad3, and Smad4 and up-regulation of Smad7 in C3H10T1/2. This study suggests that the negative regulation of the TGF- β 1 system may be involved in shear-induced endothelial cell differentiation in C3H10T1/2.

TGF- β 1 is a primary differentiation factor for a variety of cell types. TGF- β 1 is well known to induce differentiation of mesenchymal cells toward a SMC phenotype. The addition of TGF- β 1 generally inhibits myoblast differentiation [8], but stimulates differentiation of embryonic myoblasts [9]. TGF- β 1 was reported to block myogenic differentiation through inhibition of type II TGF- β 1 receptor signaling in C2C12 myoblasts [10]. It is also known that TGF- β 1 inhibits adipose differentiation of preadipocyte cell lines and primary cultures [11,12], and the effects of TGF- β 1 on preadipocyte differentiation are mediated by Smad2 and Smad3, which have distinct functions in the adipogenic differentiation process. Differential expression of Smads occurs at different stages during maturation of chondrocytes [13].

In C3H10T1/2, TGF- β 1 stimulates cell differentiation with the up-regulation of several vascular smooth muscle cell (SMC) differentiation markers such as smooth muscle α -actin (SM α -actin), smooth muscle myosin heavy chain (SM-MHC), smooth muscle protein 22- α (SM22 α), and calponin [14–16]. TGF- β regulates gene expression via serine-threonine kinase receptors at the cell surface and a group of intracellular signaling molecules Smad proteins [17]. In vertebrates, eight members of the Smad family have been identified. TGF- β 1 signaling starts by binding of the ligand with the type II receptor (TGF β R2) and followed by phosphorylation of the type I receptor (TGF β R1). The activated TGF β R1 activates Smad2 and Smad3, which then form a heteromeric complex with Smad4. The Smad4 complex is translocated into the nucleus to regulate the transcription of target genes [18]. However, Smad7 is an inhibitory Smad, which is able to antagonize TGF- β 1 signaling by competing with active Smad complex [19].

Our data showed that shear stress significantly reduced the expression of TGF- β 1, and its receptors TGF β R1 and TGF β R2. These effects could inhibit functional interaction between TGF- β 1 and TGF β R on C3H10T1/2 cells. In addition, the expression levels of Smad2, Smad3 and Smad4 were also reduced in shear stress-treated cells. These data demonstrated shear stress could block TGF- β 1 functions at positive signal transduction pathways. Furthermore, our experiments showed that the mRNA levels of Smad7 were significantly increased in response to shear stress stimulation, thereby enhancing negative signal transduction of TGF- β 1 in C3H10T1/2 cells.

Although we showed that shear stress significantly decreased TGF- β 1 mRNA levels in murine C3H10T1/2 cells in the current study, we did not perform additional experiments to detect TGF- β 1 levels intracellularly as well as its secretion. It could be a limitation of the study. This concern is warranted for further investigations including western blot analysis and immunofluorescence for intracellular TGF- β 1 and ELISA for secreted TGF- β 1. In addition, we did not perform additional experiments to detect TGF β R1 protein levels on the cell surface of these progenitor cells. Further investigations including western blot analysis, immunofluorescence staining or flow cytometry analysis are warranted. Furthermore, current study as well as our previous study [7] showed shear stress significantly induces C3H10T1/2 differentiation into endothelial cells, while inhibiting TGF- β 1-Smad pathways and SMC differentiation potential in C3H10T1/2 cells. Thus, TGF- β 1 and Smad system may have negative effects on endothelial cell differentiation from C3H10T1/2 cells. To further investigate the negative effects of the TGF- β 1-Smad system on shear stress-induced endothelial cell differentiation, it is warranted to design new experiments for C3H10T1/2 cells with shear stress and exogenous TGF- β 1. TGF- β 1 may be expected to inhibit shear

stress-induced C3H10T1/2 differentiation into endothelial cells through activation of Samd signal transduction pathways.

In conclusion, shear stress-induced inhibition in TGF- β 1 signaling pathway is one of important mechanisms by which endothelial differentiation in response to shear stress in a mouse embryo mesenchymal progenitor cell line C3H10T1/2. These findings advance our understanding the molecular mechanisms of shear stress-induced endothelial differentiation in C3H10T1/2. Further studies of shear stress-induced stem cell differentiation and its underlying molecular mechanisms are of clinical significance in areas of neointimal hyperplasia, atherosclerosis, and vascular tissue engineering.

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FIG 1.

Effect of shear stress on the TGF-β1 mRNA levels in C3H10T1/2 cells. Murine C3H10T1/2 cells were kept in static culture or exposed to shear stress (15 dyn/cm²) for 6, 12, and 24 hours. Relative TGF-β1 mRNA expression levels were determined by real time RT-PCR, normalized to GAPDH expression, and compared between shear stress-treated and static control cells. Error bars represent SD. ** $P < 0.001$. n=3.

Figure 2B

FIG 2.

Effect of shear stress on the TGFβR1 and TGFβR2 mRNA levels in C3H10T1/2 cells. Murine C3H10T1/2 cells were kept in static culture or exposed to shear stress (15 dyn/cm²) for 6, 12, and 24 hours. Relative TGFβR1 (**A**) and TGFβR2 (**B**) mRNA levels were determined by real time RT-PCR, normalized to GAPDH expression, and compared between shear stress-treated and static control cells. Error bars represent SD. * $P < 0.05$. ** $P < 0.001$. n=3.

Figure 3B.

Figure 3C.

FIG 3.

Effects of shear stress on the mRNA levels of Smad2, Smad3 and Smad4 in C3H10T1/2 cells. Murine C3H10T1/2 cells were kept in static culture or exposed to shear stress (15 dyn/cm²) for 6, 12, and 24 hours. Relative Smad2 (A), Smad3 (B) and Smad4 (C) mRNA levels were determined by real time RT-PCR, normalized to GAPDH expression, and compared

between shear stress-treated and static control cells. Error bars represent SD. ** $P < 0.001$.
n=3.

FIG 4.

Effect of shear stress on mRNA levels of Smad7 in C3H10T1/2 cells. Murine C3H10T1/2 cells were kept in static culture or exposed to shear stress (15 dyn/cm²) for 6, 12, and 24 hours. Relative Smad7 mRNA levels were determined by real time RT-PCR, normalized to GAPDH expression, and compared between shear stress-treated and static control cells. Error bars represent SD. ** $P < 0.001$. n=3.

Table 1

Primers used for gene expression analysis by real-time PCR

Genes	Accession No	Forward Primer	Reverse Primer
GAPDH	M32599	CGTGCCGCCTGGAGAAACC	TGGAAGAGTGGGAGTTGCTGTTG
TGF- β 1	BC013738	ACCAACTATTGCTTCAGCTCCAC	GTGTGTCCAGGCTCCAAATATAGG
TGFR1	NM_009370	GCCATAACCGCACTGTCATTAC	ACCTGATCCAGACCCTGATGTTG
TGFR2	BC016262	TCTTTTGGATTGCCAGTGCTAACC	AACAAGCCACAGTAACATGACACC
Smad2	NM_010754	GCTCCTCATCCCATTCCTGTTCTG	CTTACTGCCCACACAAACCTTTCC
Smad3	NM_016769	CGTGCGGAGACCCAAACTTTC	GCGGACCGACGGACTGAC
Smad4	NM_008540	CATTCCAGCCTCCCATTTCCTCAATC	CACATAGCCATCCACAGTCACAAC
Smad7	NM_008543	CCCTCCTCCTTACTCCAGATACCC	CCCCAGGCTCCAGAAGAAGTTG