The Effects of Transforming Growth Factor β_1 Stimulation on Endothelial Cell Physiology are Influenced by Shear Stress

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Abstract

Atherosclerotic plaques preferentially occur at bifurcation regions exhibiting fluid turbulence and low shear stress while straight sections of artery with less complex hemodynamics and relatively high shear stress are protected. In an effort to examine the relationship between shear stress and the effects of cytokine exposure, human umbilical vein endothelial cells (HUVEC) were exposed to continuous pulsatile shear stress for 20 hours prior to a 4-hour Transforming Growth Factor Beta 1 (TGF-β_1) incubation. The expression of several genes was followed at the protein and/or mRNA level to observe the interaction of different levels of shear stress and TGF-β_1. Results showed that expression of TGF-β Receptor II, Thrombospondin-1 and β_1 integrin were each altered under the different levels of shear stress. This alteration appeared to coincide with increased activation of Extracellular Regulated Kinase 1/2 (ERK). These results imply a relationship between shear stress, the level of ERK activation, and the sensitivity of HUVEC to exogenously added TGF-β_1. The finding that a physiologically "low" pulsatile shear stress allowed enhanced TGF-β_1 sensitivity as compared to a physiologically "normal" level without a significant increase in TGF-β RII levels suggests an alternate path by which TGF-β_1 can play a role in endothelial dysfunction and atherogenesis.

Introduction

The development of atherosclerotic plaques has been shown to preferentially occur at sites of reduced fluid shear stress and recirculating flow, such as bifurcation regions (5). Pulsatility may positively reinforce this effect (11,15), possibly through induction of local flow reversal or by means of the waveform created in athero-prone regions of the vasculature (5). Conversely, straight sections of artery with normal levels of shear stress and laminar flow profiles are protected from the development of plaques (4-6,33). While there appears to be a direct correlation between levels of shear stress and plaque development, the underlying interactions and mechanisms responsible for the observed relationship remain to be fully elucidated. Shear stress affects several aspects of endothelial cell physiology including gene expression, signal transduction, cytoskeletal arrangement, and surface expression of adhesion molecules (7,8,26,28,34). This, along with the presence of endothelial cells at the interface of blood flow and the arterial lumen, suggests that shear stress effects and other factors affecting arterial health are likely to be mediated in large part by the endothelial layer.

Transforming Growth Factor β (TGF-β) is a ubiquitous, multifunctional cytokine. Many human cell types, including leukocytes, endothelial cells, and smooth muscle cells, are capable of producing TGF-β and have receptors for it (2,19). Regulatory functions of TGF-β include inhibition of cellular proliferation, promotion of cellular differentiation, and induction of apoptosis (2,17,21). TGF-β also affects the recruitment of leukocytes in response to injury and controls the activation and proliferation of inflammatory cells. Many diseases have been linked to various modulations in TGF-β production, activation, and signaling, including cancer, fibrosis, and atherosclerosis. TGF-β signaling is accomplished by TGF-β Receptor Type II (RII) binding, dimerization with Receptor Type I, and induction of the Smad protein signaling

cascade (16,25). Receptor associated Smads (R-Smads) 2 or 3 are the species activated in response to TGF-β, and subsequently form heterodimers with the co-smad, Smad4 (16,25). Once formed, the heterodimers translocate to the nucleus where they influence gene expression (16,25).

Extracellular Regulated Kinases 1 and 2 (ERK) can exert pressure upon TGF-β induced gene expression (14). Interruption of Smad signaling through ERK pathways is a characteristic of oncogenic Ras, with the consequence being removal of the normal growth arrest induced by TGF-β (14). Integrins can influence the level of ERK activation in human endothelial cells exposed to varying levels of shear stress, with elevated levels of ERK observed as shear stress is increased (31). This effect is presumably achieved through Ras activation, as integrins are associated with this molecule on the cytoplasmic side of the cell membrane.

Due to the apparent relationship between shear stress and atherosclerotic lesion formation in the vasculature, as well as the ability of TGF-β to influence cell physiology, this study examined how shear stress preconditioning influenced cellular responses to exogenously added TGF-β_1. To avoid the measurement of step-change responses, cells were exposed to continuous pulsatile shear stress for 20 hours prior to a 4 hour TGF-β_1 incubation. Expression levels of RII and Thrombospondin-1 (TSP-1) were monitored, as was expression of the cell surface proteoglycan Syndecan-1 (Synd-1). TSP-1 has been linked to the development of atherosclerosis (23) and participates in the conversion of latent TGF-β to the active form (2,21). Due to its ability to be readily cleaved from the cell surface in response to various stimuli (10), Synd-1 may be a valuable indicator of cellular stress. β_1 integrin and the activated forms of ERK (pERK) were followed due to their relationships to shear stress and shear stress effects. Results indicate that differing levels of shear stress preconditioning influences gene expression in response to exogenously applied TGF-β. These interactions and relationships are likely important for further understanding endothelial susceptibility to dysfunction based upon the shear stress environment.

Materials and Methods

Flask and Glass Plate Preparation

T-75 flasks were gelatinized by incubation with M199 (Sigma, St. Louis, MO) containing 0.1% gelatin (Difco, Sparks, MD) at 37C for a minimum of 3 hours. Flasks were washed with 2 volumes of DPBS (Sigma), filled with Endothelial Growth Medium (EGM-Cambrex, Walkersville, MD), and stored in a 37C 5% CO_2 incubator until seeded.

Glass plates (4” x 8”) were ultrasonically cleaned for 15 minutes in a warm water/soap solution, rinsed in ultra-filtered DI water, sprayed with 70% ethanol, placed in sterile 250 x 250 mm Nunc bioassay dishes (2 per dish), and dried in a Forma Biosafety Cabinet while exposed to the UV sterilization lamp. Plates were then gelatinized in the dish by incubating in DPBS containing 1% gelatin for a minimum of 45 minutes, followed by DPBS washes.

Cell Culture

Cryopreserved P3 pooled HUVEC (Cambrex) were plated into gelatinized T-75 flasks (7 x 10^5-1 x10^6 cells/flask) and grown for 24 hours at 37C and 5% CO_2. Cells were collected by trypsinization, pelleted (250 x g, 5 min, 4C), resuspended in 5 ml EGM per flask, and plated onto a gelatinized glass plate. Cells were grown to confluence in a 37C 5% CO_2 incubator.

Plates were then placed into parallel plate flow chambers and subjected to either 4 or 10 dynes/cm^2 shear stress for 20 hours. For treatment experiments, human recombinant TGF-β_1 (Sigma) was added to the media (0.8 ng/ml) for a 4 hour incubation under flow.

Parallel Plate Flow Chambers

Flow chambers were assembled by attaching cell-covered glass plates to a 4"x8" polycarbonate flow manifold (ported on each end) with an aluminum frame and 1.5" metal clamps. A 0.015" silicone rubber gasket was placed between the glass and manifold to create the flow path. Once assembled, the chamber was attached to a flow loop and the complete system placed in an incubator (37C, 5% CO_2). EGM with dextran (400-500 kD, Sigma) was pumped through the system with a digital Masterflex peristaltic pump (Cole-Parmer, Vernon Hills, IL). The viscosity of dextran EGM solutions was determined via capillary viscometry using the equation $\mu=bt + c/t^2$ where μ is viscosity, t is time, and b and c are constants with values of 0.012418 and -1233.47 respectively. Dextran was added to produce a solution with a viscosity of 3 cP. Flow rates were calculated using the equation: $\tau = 6\mu Q / wh^2$, where τ is shear stress, μ is viscosity, Q is flow rate, w is chamber width, and h is chamber height. The flow rate was ramped-up every 15 minutes until the desired level of shear stress was achieved.

Cell Harvest

At the end of the experiment, flow was stopped, the chamber was disassembled, and the glass plate placed in a clean bioassay dish. Cells were washed twice with 25 ml cold DPBS, followed by a wash with 25 ml cold 0.02% EDTA (Fisher, Fair Lawn, NJ) in DPBS. Cells were incubated with 10 ml cold cell dissociation solution for 3 minutes at room temperature. Cells were then scraped into the solution and collected in a pre-chilled 50 ml conical. This was repeated twice more. Cells were pelleted by centrifugation.

Protein Harvest, SDS-PAGE, and Western Blotting

Cell pellets were resuspended in 1 ml cold DPBS and transferred to a 1.5 ml microcentrifuge tube. Cells were pelleted by centrifugation and resuspended in 750 µl Triton X-100 lysis buffer containing Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). Cells were lysed by vortexing for 30 minutes at 4C. Tubes were centrifuged at 21,000 x g for 20 minutes to pellet insoluble material, and the supernatant stored at –20C. Prior to SDS-PAGE, the triton soluble protein fraction was concentrated to approximately 200 µl in Amicon Ultra 5 kD Centrifugal Concentrators (Millipore, Bedford, MA). Total protein was assayed via the microplate BCA method (Pierce, Rockford, IL). Protein was denatured and prepared for SDS-PAGE according to the method of von Jagow and Schägger (32). Aliquots of the denatured protein were stored at –20C. SDS-PAGE was performed using Bio-Rad 4-20% Tris-HCL Ready Gels in a Mini-Protean III apparatus with diluted Bio-Rad 10x Tris/Glycine/SDS stock solution (Bio-Rad, Hercules, CA). 12 µg of total protein were loaded into each lane and gels were run at 200 V (400 mA max) for 50 minutes. Electroblotting was performed at 4C with a Bio-Rad Mini Trans-Blot Cell, 100 V (1 amp max) for 1 hour using diluted Bio-Rad 10x Tris/Glycine stock solution. Proteins were transferred to 0.2 micron nitrocellulose membranes. Membranes were subjected to western blotting for detection of desired targets by ECL Advance (Amersham, Piscataway, NJ). Antibody incubations longer than 1 hour were conducted at 4C.

RNA Isolation and Assay

Cells were resuspended in 1 ml cold RNase free DPBS and transferred to an RNase free 1.5 ml microcentrifuge tube. Cells were pelleted by centrifugation, and subjected to RNA isolation with the Bio-Rad Aqua-Pure kit with RNase free supplies and solutions. Purified RNA samples were stored at –80C until assay by standard methods.

cDNA Synthesis and RT-PCR

cDNA was synthesized from up to 1 µg total RNA per reaction with the Bio-Rad iScript cDNA synthesis kit. cDNA products were diluted 1:10 in PCR grade water (Sigma, St. Louis MO) and stored at –20C. 50 ng/well cDNA was loaded into a 96 well Bio-Rad PCR plate. Forward and reverse primers were designed with standard considerations using Beacon Designer software (Premier Biosoft, Palo Alto, CA) and used at pre-determined concentrations (50-250 nM) with Bio-Rad iQ SYBR Green Supermix according to instructions. Real time PCR was conducted on a Bio-Rad iQ iCycler for 40 amplification cycles. A melt curve was performed for each run. Semi-quantitative data analysis was performed as presented in Figure 1. This semi-quantitative approach was necessary due to the inability to identify a housekeeping gene whose expression was unaffected by all treatment conditions. This lack of a suitable housekeeping gene precluded data analysis via fold induction/repression methods such as $2^{-\Delta\Delta C_T}$. Results are reported as fold changes to de-couple the analysis from sensitivity to error in determination of copy number.

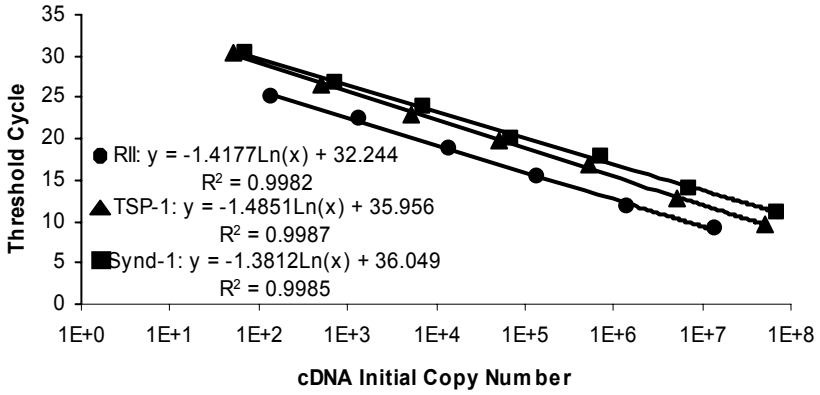

Figure 1. Standard curves relating initial copy number and C_T were prepared via real-time RT-PCR for each gene of interest by purifying PCR products amplified from cDNA with the described primer sets. Purification was performed with a Qiaquick Kit (Qiagen, Valencia, CA), and concentration determined by standard spectrophotometer methods. Copy number was determined using the molecular weight of the expected PCR product. Error bars are smaller than the symbols and represent standard deviations.

Materials, Antibodies, and Primers

Cell dissociation solution was prepared as follows (all reagents from Fisher): (w/w) 0.02% EDTA, 0.44% $Na_3C_6H_5O_7$, 0.04% KCl, 0.06% KH_2PO_4, 0.8% NaCl, 0.005% Na_2HPO_4, 0.1% Dextrose, 0.04% $NaHCO_3$, in ultra filtered H_2O, pH 7.2. The solution was sterilized by 0.2 micron filtration and then 200 µL glycerol was added. Triton X-100 lysis buffer was prepared fresh from a stock of MES buffered saline solution (MBS). MBS was prepared as follows: (w/w) 0.5% MES (Sigma) and 0.3% NaCl in ultra filtered H_2O, pH 6.5. 1% Triton X-100 (Sigma) was added just prior to use. Rabbit polyclonal anti-β_1 integrin (M-106), rabbit polyclonal anti-Syndecan-1 (H-174), and mouse monoclonal anti TGF-β RII (E-6) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at pre-determined

concentrations. Mouse monoclonal anti-pERK (#9106) was purchased from Cell Signaling (Beverly, MA) and diluted 1:2000 for use. Secondary IgG antibodies labeled with HRP (donkey anti-mouse # 711-035-150; donkey anti-rabbit #711-035-152) were purchased from Jackson Immuno (West Grove, PA) and used at pre-determined concentrations. PCR primer sequences were as follows (all obtained from Integrated DNA Technologies, Coalville, IA): RII forward 5'-TCCACCTGTGACAACCAGAAATC-3', reverse 5'-TGGCAAACTGTCTCTAGTGTTATGT-3'. TSP-1 forward 5'-CCAAAGCGTCTTCACCAGAG, reverse 5'- GAGCAGCCTTTGTTCCTGAG. Synd-1 forward 5'-GAGTCGTGGGGTGTGCTG-3', reverse 5'- TCAGTTTGGAGAAACCGAGTC-3'. The functionality of each primer set was verified by determining PCR product size by gel electrophoresis.

Statistical analysis

All results are reported as the mean and standard deviation. Significance of data was determined using a one-way ANOVA with $\alpha = 0.05$.

Results

HUVEC cultivated at either 4 or 10 dynes/cm^2 pulsatile shear stress for 20 hours exhibited differing patterns of gene expression. As shown in Figure 2, RII protein was 8.4 fold and mRNA 74% greater from cultures at 10 dynes/cm^2 than from 4 dynes/cm^2. Similarly, TSP-1 mRNA was increased by 10.9 fold at 10 dynes/cm^2, and β_1 integrin protein levels were 11.7 fold higher.

After incubation with TGF-β_1 for 4 hours, expression of RII, TSP-1, and β_1 integrin were further influenced (Figure 2). Greater changes were observed between 4 dynes/cm^2 plus and minus TGF-β_1 than

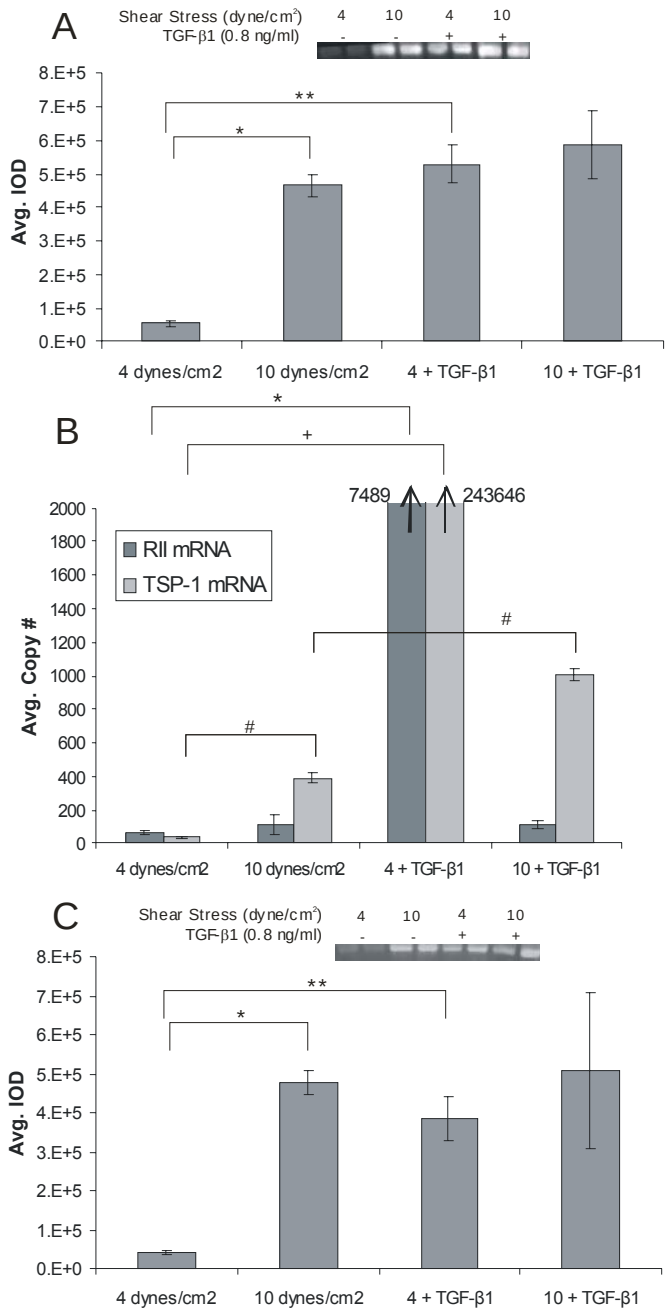

Figure 2. HUVEC were cultivated under pulsatile shear stress for 20 hours and either harvested or treated with 0.8 ng/ml TGF-β_1 for an additional 4 hours under flow. A) RII protein expression analyzed by Western Blot. B) RII and TSP-1 mRNA expression analyzed by RT-PCR. C) β_1 integrin protein expression. Error bars represent standard deviations. *$p \leq 0.005$, **$p \leq 0.01$, $^{+}p \leq 0.001$, $^{\#}p \leq 0.00005$.

in the 10 dynes/cm^2 cases. For 4 dynes/cm^2 cultures, RII protein increased 9.5 and mRNA 112 fold respectively upon the addition of TGF-β_1. TSP-1 mRNA also responded to the presence of TGF-β_1, increasing 6,712 fold over the non-treated 4 dynes/cm^2 case. A 9.4 fold increase was seen for β_1 integrin upon TGF-β_1 addition to 4 dynes/cm^2 HUVEC. At 10 dynes/cm^2, only slightly altered expression was observed. RII protein levels rose by a maximum of 26% in response to TGF-β_1, while mRNA remained essentially constant. TSP-1 mRNA increased by 2.6 fold upon addition of TGF-β_1, and β_1 integrin protein levels were unchanged.

Synd-1 protein and mRNA levels did not show the same dependence on pulsatile shear stress as RII and TSP-1, but did demonstrate an effect of TGF-β_1 for both 4 and 10 dynes/cm^2 cultures. As shown in Figure 3, protein and mRNA levels were essentially the same after 20 hours at either 4 or 10 dynes/cm^2. Despite a lack of shear stress influence, Synd-1 expression was altered in the presence of TGF-β_1. At the protein level, a 2.6 fold increase was observed between 4 dynes/cm^2 cultures upon TGF-β_1 treatment. A more modest increase of 78% was found upon addition of TGF-β_1 to 10 dynes/cm^2 cells. mRNA levels were constant between treated and untreated 4 dynes/cm^2 HUVEC, and exhibited a decrease of 8.2 fold in the 10 dynes/cm^2 cases.

As presented in Figure 4, levels of activated ERK (pERK) were found to be 3.6 fold higher in cultures subjected to 20 hours of 10 dynes/cm^2 as opposed to 4 dynes/cm^2 pulsatile shear stress. This level of pERK represents the amount present upon TGF-β_1 addition to the cultures for the 4 hour incubation prior to harvest.

Discussion

HUVEC cultivated at shear stress

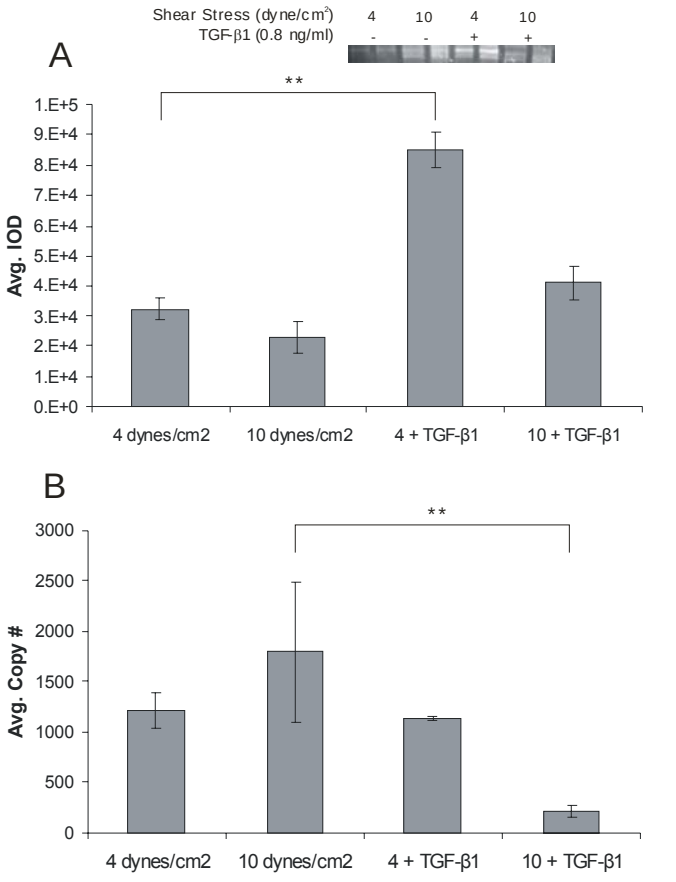

Figure 3. Synd-1 expression in response to 20 hours of pulsatile shear stress +/- 4 hours of TGF- β_1 exposure. A) Protein expression, B) mRNA expression. Error bars represent standard deviations. **p≤0.01.

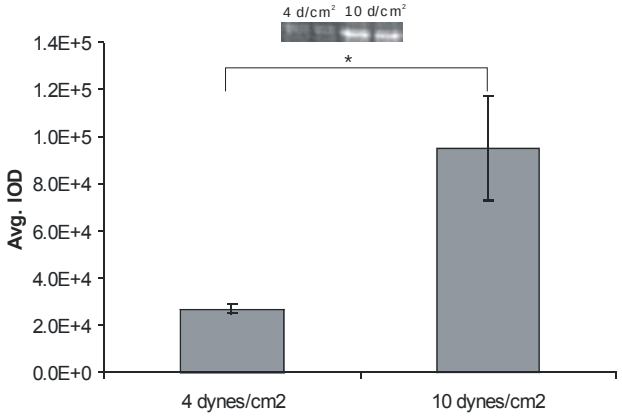

Figure 4. pERK levels from 4 and 10 dynes/cm^2 cells analyzed by Western Blot. Error bars represent standard deviations. *p≤0.005.

levels of 4 and 10 dynes/cm^2 exhibited differing responses to TGF-β_1 treatment as assessed by expression of RII, TSP-1, β_1 integrin and Synd-1 protein and/or mRNA. Based on the data, it appears that shear stress preconditioning directly influenced the fate of TGF-β_1 signaling and the expression of genes that demonstrated TGF-β_1 sensitivity, including TSP-1 and RII. For certain cells, including those from tumors or atherosclerotic plaques, either mutation or loss of RII gene expression has been shown to be involved in disease progression (2,18). Based upon this evidence, it was hypothesized that RII expression would be altered in cells subjected to shear stress conditions similar to those found in atherosclerosis prone arterial segments. For cells from the 10 dynes/cm^2 case, there was little apparent effect on the target markers with TGF-β_1 exposure, while those from the 4 dynes/cm^2 condition demonstrated a large modulation in expression. This is interesting since the level of RII protein present in untreated 10 dynes/cm^2 HUVEC was found to be 9.5 fold higher than from those from 4 dynes/cm^2. If the level of RII protein expression was directly linked to the outcome of TGF-β_1 signaling, then the opposite effects should have been seen. Upon TGF-β_1 treatment, RII mRNA in 4 dynes/cm^2 cells increased more than 100 fold, and the amount of protein rose to a similar level as from both the treated and untreated 10 dynes/cm^2 cases. Similarly, TSP-1 mRNA was markedly increased upon TGF-β_1 treatment of 4 dynes/cm^2 cells, while this effect was attenuated at 10 dynes/cm^2. This finding suggests that TGF-β_1 addition to 4 dynes/cm^2 cells stimulated a strong response through a comparatively small initial number of receptors, and indicates that a factor related to the difference in shear stress preconditioning level influenced the ability of TGF-β_1 to affect HUVEC physiology. This was apparently accomplished in a manner distinct from the level of RII expression, and represents a novel finding.

Shear stress has been shown to influence the physiology of endothelial cells, and notably the level of ERK activation (1,29,30). Most data has been collected for short-term experiments, focusing upon initial activation of signal pathways in response to the onset of shear. Results indicate that an initial spike in ERK activation is seen within 10 to 30 minutes, after which levels return to those in non-sheared cells (4,20). Shear stress preconditioning of Bovine Aortic Endothelial Cells for one day largely eliminated their response to acute shear for time points up to 30 minutes of exposure, but levels of pERK after one hour were similar to those obtained from static cells exposed to acute shear for the same time (3). In both cases, the level of pERK after one hour of acute shear was greater than that obtained from untreated cells. While there is a significant amount of data suggesting that ERK is activated in response to shear and that flow preconditioning influences how cells initially respond to shear challenge, there is less information describing how differing levels of shear applied for long periods of time (24 hours) influences the level of ERK activation, or what the outcomes may be. The results presented here indicate that after 24 hours of pulsatile shear stress exposure, HUVEC from 4 and 10 dyne/cm^2 parallel plate flow chambers exhibited differing levels of ERK activation. Correspondingly, levels of β_1 integrin were also altered. The specific mechanisms of ERK activation in flow stimulated cells have yet to be fully elucidated, however it has been shown that integrins, caveolae, and growth factor receptors play a role in pathway activation (13,22,29). It is interesting that in the current study, the amount of β_1 integrin corresponds to the level of ERK activation. This finding agrees with previous data linking these phenomena to the level of shear stress exposure (31).

Cells have been shown to modulate their TGF-β_1 sensitivity through antagonism of Smad signaling by pERK. It has been demonstrated that for cancer cells, chronic Ras activity causes a phosphorylation of Smad 2/3 proteins in their linker regions (14). This is an inhibitory

effect, blocking translocation of the Smad complex to the nucleus and its activity as a transcription factor. In normal cells, the levels of pERK and activated R-Smad proteins likely achieve a balance and participate in tissue homeostasis. In cells that exhibit chronic Ras activity, this balance is probably disturbed, resulting in cells that demonstrate immunity to the effects of TGF-β (14). For cultures considered in the current study, pERK levels found in 10 dynes/cm^2 HUVEC may represent a normal response to a physiological healthy level of shear stress, and promote normal interaction with activated Smad pathways. A lack of activated ERK in 4 dynes/cm^2 cultures may allow enhanced sensitivity to TGF-β_1, and result in the increased levels of gene expression seen for the markers studied, most notably TSP-1 and RII.

While ERK antagonism of Smad signaling influences receptor mediated TGF-β_1 signaling in cells cultivated under shear stress, other mechanisms also exist. Receptor internalization and trafficking are common mechanisms of signal regulation, and recent evidence suggests that the TGF-β pathway is susceptible to control in these ways (9). Further, expression of the inhibitory Smad (Smad7) has been shown to be inducible by shear stress (27) and to participate in the internalization and degradation of the activated receptor complex by lipid raft-caveolar pathways (9). This mechanism was recently shown to be distinct from clathrin dependent internalization, which was found to promote Smad signal propagation (9). While these methods of Smad signal modulation were not expressly examined in the current study, control of signaling by receptor internalization and degradation via lipid raft-caveolar pathways does not seem to explain the observed results. RII expression was not reduced between the TGF-β_1 treated conditions analyzed, suggesting that it remained intact and able to bind TGF-β_1. Some inhibition of RII signaling by Smad7 may have taken place, which would be consistent with the fact that for 10 dynes/cm^2 cells, the addition of TGF-β_1 produced little effect upon target gene expression. On the other hand, given the association of Smad7 with receptor degradation processes and the lack of reduction in RII protein, this also may be interpreted as playing a minimal role in observed signal modulation.

In summary, the major findings of this study are 1) shear stress directly influenced the expression of RII, TSP-1 and β_1 integrin and 2) shear stress played an important role in determining the response of cells to exogenously added TGF-β_1,. The data implies a relationship between the levels of RII, β_1 integrin, and pERK that affects the fate of Smad proteins activated upon TGF-β_1 addition. Based upon observed levels of RII protein, this appears to be a valid interpretation, although receptor internalization and trafficking were not specifically examined in relationship to the abrogation of TGF-β_1 effects. Regardless of the mechanisms by which shear stress influenced HUVEC responses to TGF-β_1, the finding that a physiologically “low” pulsatile shear stress (4 dynes/cm^2) allowed enhanced TGF-β_1 sensitivity (notably expression of pro-atherogenic TSP-1) as compared to a physiologically “normal” level (10 dynes/cm^2) without a significant increase in RII levels is important. It suggests a path by which TGF-β_1 can play a role in endothelial dysfunction and atherogenesis that is distinct from previously described RII dependent mechanisms.

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