JOURNAL OF CELLULAR PHYSIOLOGY 143:364–371 (1990)

Tissue Plasminogen Activator Messenger RNA Levels Increase in Cultured Human Endothelial Cells Exposed to Laminar Shear Stress

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Fluid shear stress can stimulate secretion of tissue plasminogen activator (tPA) by cultured human endothelial cells, while plasminogen activator inhibitor type-1 secretion remains unstimulated. To determine whether hemodynamically induced changes in tPA messenger RNA (mRNA) levels also occur, primary cultures from the same harvest of primary human umbilical vein endothelial cells were either maintained in stationary culture or exposed to arterial levels of shear stress (25 dynes/cm^2) for 24 hours. Total cellular RNA was isolated from the shear stressed and stationary cultures and the relative levels of tPA mRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were determined using a coupled reverse transcriptase/polymerase chain reaction method. As indicated by the amount of amplification product, tPA mRNA levels were many fold higher (> 10) in endothelial cells subjected to shear stress for 24 hours than in stationary controls. In contrast, mRNA levels for GAPDH were similar in control and shear stressed cells. The constancy of the measured GAPDH signal indicated that the tPA response was a selective effect of fluid shear stress. When a similar polymerase chain reaction method was used, the mRNA levels of basic fibroblast growth factor (bFGF) were found not to vary in comparison to GAPDH mRNA after 24 hours of shear stress. These results indicate that enhancement of the fibrinolytic potential of endothelial cells in response to hemodynamic forces could involve transcriptional events.

Flowing blood continuously exerts shear stress on the vascular endothelium. Alignment of endothelial cells is observed in vivo in zones of high shear stress (Nerem et al., 1981; White et al., 1983). Over a time period varying from several hours to days, endothelial cells in vitro can align in the direction of fluid flow if the shear stress is greater than about 8 dynes/cm^2 (Dewey et al., 1981; Eskin et al., 1984; Levesque and Nerem, 1985). In cultured porcine aorta endothelial cells, endothelin mRNA levels have been reported to be transiently modulated by fluid shear stress (Yoshizumi, et al., 1989). Short-term responses of the endothelial cell to the onset of shear stress include increase of cytosolic Ca^{2+} (Ando et al., 1988), activation of a membrane potassium current (Olesen et al., 1988), and a rise in prostacyclin production (Grabowski et al., 1985; Frangos et al., 1985).

Several lines of evidence indicate that low shear stress zones in arteries (possibly with reversing flows) are more prone to develop atherosclerotic lesions, intimal hyperplasia, and enhanced thrombogenicity when compared to vascular regions with unidirectional, high shear flow (Caro et al., 1971; Ku et al., 1985; Goldsmith and Turitto, 1986; Dobrin et al., 1989). Since endothelial cells actively participate in thrombotic and fibrinolytic processes and localize these events on or near the cell surface, longer-term alterations of endothelial function by mechanical forces may play a role in physiological and pathological processes.

Production of tissue plasminogen activator (tPA) and plasminogen activator inhibitor type 1 (PAI-1) by endothelial cells is known to be affected by cytokines and other agents. Coordinated induction of both tPA and PAI-1 by thrombin or basic fibroblast growth factor (bFGF) is in contrast with the induction of tPA alone by histamine, activated protein C, and phorbol ester, or

Received October 31, 1989; accepted January 31, 1990.

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the induction of PAI-1 alone by lipopolysaccharide (LPS), transforming growth factor-β (TGF_β), interleukin-1 (IL-1), or tumor necrosis factor-α (TNF_α) (Hanss and Collen, 1987; Saksela et al., 1987; Sakata et al., 1985; Levin and Santell, 1988; Schleef et al., 1988; Emeis and Kooistra, 1986; Sawdey et al., 1989). Cultured human endothelial cells secrete more tPA when exposed to arterial levels of shear stress (> 15 dynes/cm^2), while PAI-1 secretion remains unaffected by shear stress over the physiological range (Diamond et al., 1989). The intent of this investigation was to determine if the stimulation of tPA secretion by increased shear stress is associated with specific increases of tPA mRNA compared to mRNA levels for bFGF and constitutively expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

MATERIALS AND METHODS

Cell culture

Primary human umbilical vein endothelial cells (HUVEC) were cultured as described by Gimbrone (1976). Briefly, four to six umbilical cords (obtained within 6 hours of delivery) were rinsed, cannulated, and filled with collagenase (CLS 1, Worthington Biochemical Corp., Freehold, NJ) in phosphate buffered saline (40–50 U/ml) at 37°C, then incubated for 30 min. The pooled effluent was centrifuged for 10 min at 100*g*, and the pellet was resuspended in complete M199 [Medium 199 (Gibco Laboratories, Grand Island, NY) with 20% heat-inactivated newborn calf serum (HyClone Laboratories, Inc., Logan, UT), 0.30 mg/ml glutamine (Gibco), 0.10 mg/ml streptomycin (Gibco), 200 U/ml penicillin (Gibco), and 0.20 mg/ml of neomycin (Gibco)]. Approximately, 10^4 cells per cm^2 were seeded on glass slides for shear stress experiments as described by Frangos et al. (1985). Studies of butyrate stimulation used primary HUVEC grown to confluence in 150 cm^2 flasks.

Exposure of endothelial cells to shear stress

Replicate primary, confluent monolayers (72–86 hours post-seeding) were exposed to steady laminar shear stress of 25 dynes/cm^2 in individual parallel-plate flow chamber systems with recirculating medium driven by a constant hydrostatic pressure head as previously described (Frangos et al., 1985; Diamond et al., 1989). The cell cultures were mounted on separate parallel-plate flow chambers (monolayer surface area exposed to shear stress in each chamber, 15 cm^2; channel width, 200 μm) and connected under sterile conditions to individual flow systems, each filled with 15 ml of medium. An equal number of monolayers on slides were incubated under stationary conditions. Media samples (1 ml) were taken from each system every 4 to 6 hours and stored at −80°C. After 24-hour exposure to shear stress, the flow was stopped and total cellular RNA from the shear stressed monolayers (15 cm^2 of monolayer per slide) was extracted within 1 min after termination of flow and pooled. Similarly, RNA was extracted from stationary cultures (15 cm^2 of monolayer per slide) and pooled using the same number of slides as used in the RNA extraction from shear stressed cells. To calculate mean cell density on the slides before RNA extraction, cells were counted in three light micrographs of each monolayer.

Enzyme-linked immunosorbent assays

A double antibody ELISA technique was used to measure total human tPA (uncomplexed and inhibitor-bound) in HUVEC conditioned medium (American Diagnostica Imubind™-5 tPA Elisa, New York, NY). HUVEC conditioned media or calibration standards (Bowes melanoma single chain tPA) were assayed in triplicate with background subtraction using blanking wells containing soluble antibody to quench tPA specific response. Horseradish peroxidase-conjugated goat anti-human tPA IgG and the HRP substrate, orthophenylenediamine (OPD), provided a colorimetric reaction product absorbance at 490 nm which was measured using a Bio-tek plate reader. The tPA ELISA was calibrated to the limit of its sensitivity (0 to 1,500 pg/ml), yielding a highly linear calibration curve (typically, $r^2 > 0.99$) with a detection limit of 50 pg/ml. An ELISA using monoclonal mouse anti-human PAI-1 immunoglobin allowed quantification of uncomplexed (latent and active) PAI-1 antigen (American Diagnostica Imubind™ PAI-1 Elisa). Wells were loaded in triplicate with HUVEC conditioned media (diluted 1:10 and 1:25) or with PAI-1 standards. Colorimetric reaction product was produced using biotinylated monoclonal mouse anti-human PAI-1 immunoglobin with horseradish peroxidase-conjugated streptavidin and OPD. No tPA or PAI-1 antigen was detected in complete medium prior to exposure to HUVEC.

Isolation of total RNA from HUVEC monolayers

Isolation of total cellular RNA from HUVEC was accomplished by a scaled down adaptation of the guanidine thiocyanate/CsCl gradient method (Chirgwin et al., 1979; Rappolee et al., 1989). Briefly, monolayers were washed once with PBS and lysed in 1.2 ml of 5 M guanidine thiocyanate (Stratagene, La Jolla, CA) with 25 mM sodium citrate (Fluka Chemical Corp., Ronkonkoma, NY) and 0.5% (w/v) sodium sarcosinate (I.B.I., New Haven, CT). The lysate was layered on a 5.7 M CsCl cushion (0.8 ml) and ultracentrifuged at 200,000*g* for 3 hours (Beckman tube #344625; Beckman rotor TLS-55; Beckman TL-100 ultracentrifuge). The RNA pellet was dissolved in water and then extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform/isoamyl alcohol (24:1). Total RNA was precipitated with 3 M sodium acetate and ethanol, vacuum dried, and redissolved in water. A final precipitation with 2.5 M LiCl followed by a 70% ethanol wash and resuspension of the RNA in water prior to reverse transcription was done to remove residual cesium, phenol, or chloroform. Concentration and purity of the RNA ($OD_{260\ nm/280\ nm} > 1.7$ in all samples) were determined after LiCl precipitation.

Reverse transcription and polymerase chain reaction

A coupled reverse transcription/polymerase chain reaction method used here is a modification of the one described by Jacobsen et al. (1989). Reverse transcription of messages for tPA and GAPDH, or bFGF and GAPDH, were carried out in a 25 μl reaction volume containing 2.0 μg total cellular RNA, 500 units of

TABLE 1. Oligonucleotide primers and probes used for reverse transcription of total cellular RNA, polymerase chain reaction, and Southern blotting of reaction products for tissue plasminogen activator (tPA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and basic fibroblast growth factor (bFGF)[1]

Gene	Oligonucleotide	Sequence	Position (ref.)
tPA	Sense primer	5'-CTGCAGCTGAAATCGGATTCGT-3'	1312–1333†
	Antisense primer	5'-CTGATGATGCCCACCAAAGTC-3'	1679–1659†
	Probe	5'-AACAGAACAGTCACCGACAAC-3'	1531–1551†
GAPDH	Sense primer	5'-CCATGGAGAAGGCTGGGG-3'	386–403*
	Antisense primer	5'-CAAAGTTGTCATGGATGACC-3'	580–561*
	Probe	5'-CTAAGCAGTTGGTGGTGCA-3'	527–547*
bFGF	Sense primer	5'-CAAGCAGAAGAGAGAGGAG-3'	199–217‡
	Antisense primer	5'-CCAACTGGTGTATTTCCTTGA-3'	375–355‡
	Probe	5'-CTGGCTTCTAAATGTGTTACG-3'	280–300‡

[1]Base pair positions cited are those given in the original published sequences for tPA (†Pennica et al., 1983), GAPDH (*Fort et al., 1985), and bFGF (‡Sommer et al., 1987). Target amplification sequence lengths were 368 bp for tPA, 195 bp for GAPDH, 177 bp for bFGF.

Moloney murine leukemia virus reverse transcriptase (MMLV-RT), 0.5 μg antisense tPA (or bFGF) primer, 0.5 μg antisense GAPDH primer, and all four deoxynucleotide triphosphates (0.5 mM each) in a reaction buffer of 50 mM Tris/HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. Each reaction mixture was heated to 70°C for 5 min and placed on ice before adding MMLV-RT as the last step, after which reverse transcription reactions were carried out for 20 min at 37°C and then stopped by denaturing for 10 min at 90°C.

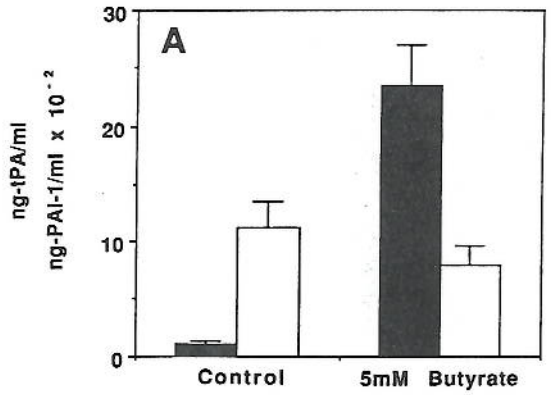
The resulting tPA (or bFGF) cDNA and GAPDH cDNA samples were then coamplified using 20 or 25 cycles of the polymerase chain reaction with sense and antisense primers for tPA (or bFGF) and GAPDH designed from published sequences (Table 1) . For tPA, the target amplification sequence corresponded to the sequence coding for part of the active site of the light chain of tPA. The 100 μl polymerase chain reaction volume contained 2 U recombinant *Thermus aquaticus* (Taq) polymerase (Perkin Elmer-Cetus, Norwalk, CT), 10 mM each of dCTP, dATP, dTTP, and dGTP (Pharmacia Inc., Piscataway, NJ), 0.5 μg of each sense primer, and an additional 0.25 μg of each antisense primer in a reaction buffer of 50 mM KCl, 100 mM Tris/HCl (pH 8.3), 25 mM $MgCl_2$ in 1% (w/v) gelatin. Each temperature cycle consisted of 2 min of denaturing at 92°C, 2 min of primer annealling at 50°C, and 3 min of primer extension at 72°C (Perkin Elmer-Cetus1000 Thermocycler).

From each reaction, 10 μl of polymerase chain reaction product was fractionated by electrophoresis on a 2% SeaKem GTG agarose gel (FMC Corp., Rockland, ME) with bromophenol blue dye marker at 100 V for 3 hours. A "123-laddder" DNA molecular weight standard (Bethesda Research Laboratories, Gaithersburg, MD) was run on each gel. The gels were stained with ethidium bromide, photographed, washed, and then blotted overnight to nylon membranes with 0.45 μm pore size (Nytran, Schleicher & Schuell Inc., Keene, NH). Southern blots of the gels were probed for tPA, bFGF, or GAPDH using oligonucleotides (Table 1) end-labelled with ^{32}P by the T4 polynucleotide kinase reaction. Autoradiographs were exposed at −70°C using Kodak XAR film.

RESULTS

The use of the polymerase chain reaction to measure changes in mRNA levels in primary HUVEC was tested with the chemical agonist butyrate which is known to increase both tPA mRNA levels and tPA secretion in HUVEC (Kooistra et al., 1987). We found that a 24-hour period of exposure to butyrate (5mM) in the culture medium caused over a 20-fold increase of tPA levels in HUVEC conditioned media with little effect (or slight decrease) on PAI-1 levels (Fig. 1A). We found no significant changes in the quantity of total cellular RNA isolated from HUVEC incubated in the presence or absence of butyrate (after LiCl precipitation: 52 ± 2 μg RNA per 150 cm^2 of HUVEC monolayer; $OD_{260\ nm/280\ nm} > 1.8$). For this experiment, the combination of reverse transcription and the polymerase chain reaction amplification of 1 μg of total RNA using 30 amplification cycles was carried out using only the tPA primer set. The amplification reaction for tPA alone (without GAPDH) produced an amplification product which showed a band on electrophoresis at the predicted 368 bp size. This band hybridized to a tPA-specific oligonucleotide probe, producing a unique band on the autoradiograph at precisely the same base pair length (368 bases) of the target amplification sequence predicted by our choice of polymerase chain reaction primers. This identified the amplification product as one generated from tPA cDNA which was derived from tPA mRNA. The amount of tPA amplification product was much greater from RNA of butyrate stimulated cells than from RNA of unstimulated cells (Fig. 1B). This experiment established that changes in tPA mRNA levels in HUVEC found with standard Northern blotting techniques can also be detected in RNA from much smaller numbers of HUVEC using the reverse transcription/polymerase chain reaction technique.

To investigate the effect of arterial levels of laminar shear stress on mRNA levels in primary HUVEC, replicate HUVEC monolayers were either maintained in stationary culture or shear stressed at 25 dynes/cm^2, as described in Materials and Methods. In each experiment, secretion rates of tPA and PAI-1 (per 10^6 cells) were evaluated for each flow system and matched stationary cultures. Changes in HUVEC tPA production were observed after a lag time of several hours of exposure to shear stress (Fig. 2). The average tPA secretion rate from all experiments (Table 2) was 0.476 ± 0.108 ng-tPA/10^6 cells/hr for monolayers exposed to shear stress compared to the secretion rate of 0.165 ± 0.040 ng-tPA/10^6 cells/hr for stationary controls (n = 6, from experiments conducted with two separate pools of HUVEC). The tPA secretion rate by cells exposed to



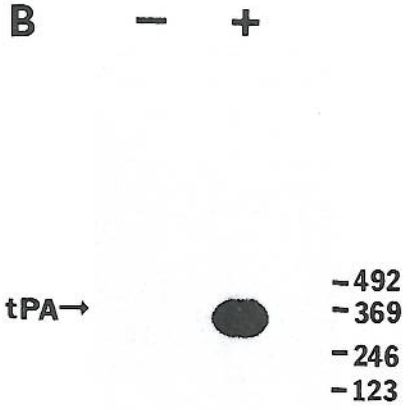


Fig. 1. Effect of 5 mM butyrate on tPA and PAI-1 antigen secretion and tPA mRNA levels of cultured endothelial cells. Primary, human umbilical vein endothelial cells were incubated for 24 hours in complete M199 media (± 5 mM butyrate). Conditioned media was removed at the end of the experiment and frozen at −80°C. Total RNA was isolated from the monolayers using the guanidine thiocyanate/CsCl method. **A:** Conditioned medium was assayed using enzyme-linked immunosorbent assays for total tPA (free and inhibitor-bound) (■) and uncomplexed-PAI-1 ($\times 10^{-2}$; latent and active) (□). **B:** Total RNA from control (−) and butyrate stimulated (+) HUVEC was reverse transcribed (2.0 μg/reaction) and the resulting tPA cDNA was amplified using the polymerase chain reaction as described. The amplification product, run on a 2% agarose gel, was blotted to nylon and probed with a ^{32}P-labelled tPA sense probe. The tPA-specific probe hybridized a unique tPA amplification product at the 368 base pair length of the predicted amplification sequence.

shear stress was 2.88 ± 0.95 times higher (n = 6; $P < 0.005$) compared to stationary controls. The basal PAI-1 secretion rate of 59.9 ± 12.0 ng-PAI-1/10^6 cells/hr (n = 6) was not stimulated by shear stress but was slightly decreased (37.2 ± 14.6 ng-PAI-1/10^6 cells/hr; n = 6) in these experiments.

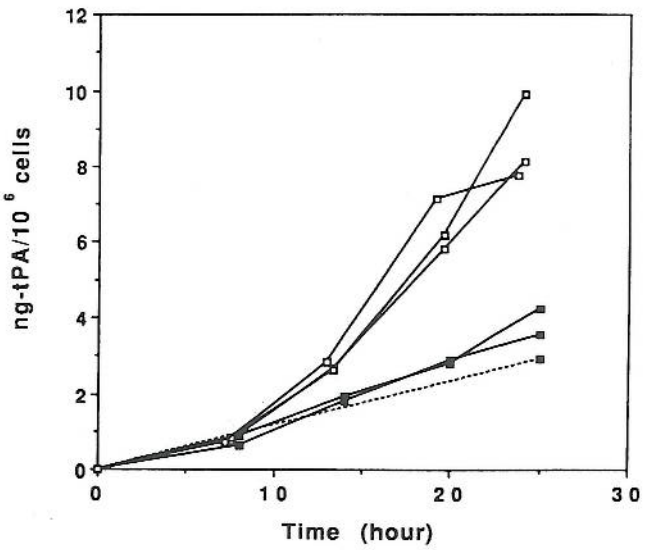


Fig. 2. Effect of laminar shear stress of 25 dynes/cm^2 on tPA secretion by cultured endothelial cells. Six replicate cultures of primary, confluent human umbilical vein endothelial cells were grown on glass slides for each experiment. Three cultures were maintained in independent, stationary incubations (■) (1 ml medium/cm^2-monolayer), while the other three cultures were exposed to laminar shear stress of 25 dynes/cm^2 (□) in independent, parallel-plate flow systems with recirculating medium (1 ml medium/cm^2-monolayer). Samples (1 ml) were taken from each stationary and flow system at intervals of 4–6 hours and frozen for ELISA assays of tPA and PAI-1. Each point is the average of triplicate ELISA determinations. Dotted line: only one data point for culture.

In each experiment, the quantity of total cellular RNA isolated from stationary and shear stressed HUVEC cultures was not significantly different. Coamplification of tPA cDNA and GAPDH cDNA (0.5 μg RNA/reaction tube; 20 and 25 cycles) in the same reaction tube was employed to provide an internal standard to control for variation in RNA loading or the efficiency of the amplification reaction.

Southern blots probed for the amplified tPA product showed a shear stress-induced increase in tPA transcript level estimated at 10-fold or greater (Fig. 3, upper half). In contrast, when the same Southern blots were stripped and reprobed for the GAPDH amplification product, the transcript level of GAPDH was nearly constant and independent of shear stress within each cultured cell line (Fig. 3, lower half). This constancy of the GAPDH transcript level confirmed that the observed marked variation seen in tPA transcript levels was not an artifact of the amplification of small experimental errors or of random sequence-dependent variations in amplification efficiency between tPA and GAPDH primer sets being magnified by the amplification reactions. Quantification of these changes in tPA transcript by densitometry, however, was not performed because the long film exposure time needed to detect the lower tPA levels of static cultures (16 hours) invariably produced autoradiographic images for high shear stress tPA transcripts too dark to be in a linear range of gray level required for accurate densitometry.

When RNA from the same cell groups was used in a similar coamplification experiment with GAPDH to study the effect of shear stress on transcript levels for

TABLE 2. Secretion rate of tPA and PAI-1 by HUVEC and total cellular RNA levels in HUVEC exposed to laminar shear stress or maintained in stationary culture[1]

Experiment[2]	tPA secretion rate (ng-tPA/10^6 cells/hr)	PAI-1 secretion rate (ng-PAI-1/10^6 cells/hr)	Total RNA (μg)
1. Control	0.170 ± 0.049 (n = 3)	70.2 ± 4.97 (n = 3)	21.6
25 dynes/cm^2	0.399 ± 0.087 (n = 3)	47.8 ± 13.6 (n = 3)	24.8
2. Control	0.160 ± 0.039 (n = 3)	49.3 ± 3.25 (n = 3)	29.1
25 dynes/cm^2	0.527 ± 0.123 (n = 3)	26.5 ± 3.04 (n = 3)	29.2

[1]Secretion rates were determined by a least squares fit of antigen production data between 6 and 24 hours. Pooled RNA samples isolated from the control or shear stressed cultures of each experiment were subjected to reverse transcription/polymerase chain reaction analysis as described in Material and Methods. Concentration of RNA was evaluated by $OD_{260\ nm}$ measurement prior to reverse transcription.
[2]Each experiment was conducted with a pool of primary HUVEC.

bFGF, no increase in transcript levels such as that seen for tPA was noted for bFGF (Fig. 4). Instead, variation in the bFGF signal strength in only one lane was matched by a weaker GAPDH signal in that lane, indicating that the bFGF change represented only an experimental variation in overall reaction efficiency.

Although most of the tPA product was found at the predicted base pair size length, smaller-sized tPA (and GAPDH) polymerase chain reaction products were present on autoradiographs of the coamplification reaction products. These may have resulted from incomplete primer extension caused by the competing reactions. With the RNA from control and shear stressed cultures, amplification of only the tPA sequence (1 μg RNA/reaction tube; 20 cycles) produced unique autoradiographic bands at the predicted base pair length and yielded the same results (data not shown) as the coamplification experiment shown in Figure 3.

A short exposure time (2 hours) of the autoradiograph in Figure 1B was sufficient to demonstrate that butyrate stimulated tPA mRNA levels since the enhancement was so large. Due to this short exposure time, the 368 bp amplification product of stationary controls was not visualized, nor were less prevalent, smaller reaction products visible in Figure 1B. Appearance of these smaller reaction products in Figures 3 and 4 was due to the long exposure time needed to visualize the level of tPA mRNA in stationary controls.

DISCUSSION

We have shown that fluid shear stress of 25 dynes/cm^2 sustained for a 24-hour period stimulated tPA secretion by HUVEC with concominant increases in tPA mRNA. In contrast, the mRNA levels for a constitutively expressed mRNA for glyceraldehyde 3-phosphate dehydrogenase and for a potent mitogenic factor, basic fibroblast growth factor, were unaffected by exposure to shear stress. These data indicate that hemodynamic forces can affect endothelial cell function selectively at the level of mRNA expression.

In HUVEC, the majority of tPA synthesized is secreted, indicating that an important regulator of tPA production is the level of mRNA. Stimulation of endothelial tPA secretion by agents such as butyrate or thrombin has previously been shown to correlate with increases in tPA mRNA levels (Kooistra et al., 1987; Dichek and Quertermous, 1989a). In the case of the HUVEC response to shear stress, the large change (> 10 fold) in tPA mRNA levels with only threefold enhancement of tPA secretion suggests that an additional level of post-transcriptional regulation of tPA protein production may be present.

Our use of the polymerase chain reaction assay technique for quantitative estimates of transcript levels using butyrate stimulation of HUVEC agreed quite well with the work of Kooistra et al. (1987). Those investigators showed that butyrate (1–10 mM) can increase HUVEC tPA secretion by a factor of 6–25-fold after a lag time of several hours, with slight attenuation of PAI-1 secretion observed at the higher butyrate concentrations. Using Northern blotting techniques, Kooistra et al. found that tPA mRNA levels increase roughly 30-fold during butyrate stimulation of HUVEC while PAI-1 mRNA levels remain unchanged. Using a butyrate stimulation of HUVEC, we have shown that changes in tPA antigen production correlated with changes in tPA polymerase chain reaction product. Thus, the amount of tPA amplification product can serve as a reliable indication of the tPA mRNA level when compared to the results of more standard methods such as Northern blotting.

Northern blotting techniques, however, typically require much greater quantities of total cellular RNA isolated from 10^7 to 10^8 cells grown in culture. This limitation is especially true for investigations of messages such as tPA which are present on the order of only 10^3 copies per endothelial cell (Zonneveld et al., 1986) compared to actin mRNA likely found at levels 10–100 times higher. To obtain large numbers of cultured human endothelial cells requires the use of endothelial cell growth factor, often in combination with heparin, to grow the cells for several passages. It is more desirable to study endothelial cell production of fibrinolytic mediators, without long-term, large-scale cultivation, since phenotypic drift in culture has been documented in HUVEC. For example, the urokinase gene, not normally expressed in primary HUVEC or adult vena cava cultures, becomes quite active (van Hinsbergh et al., 1987; Dichek and Quertermous, 1989b) at higher passage numbers. Also, tPA secretion and tPA mRNA is up-regulated manyfold with serial passage in culture (McArthur et al., 1986; van Hinsbergh et al., 1987; Dichek and Quertermous, 1989b), while PAI-1 secretion has been shown to decrease 3–10-fold with the use of heparin in combination with endothelial cell growth factor (Konkle and Ginsburg, 1988). At higher passage, the quantity of the smaller, polyadenylate-free form of the PAI-1 mRNA increases (Dichek and Quertermous, 1989b). Additionally, PGI_2 production is quickly lost and von Willebrand factor

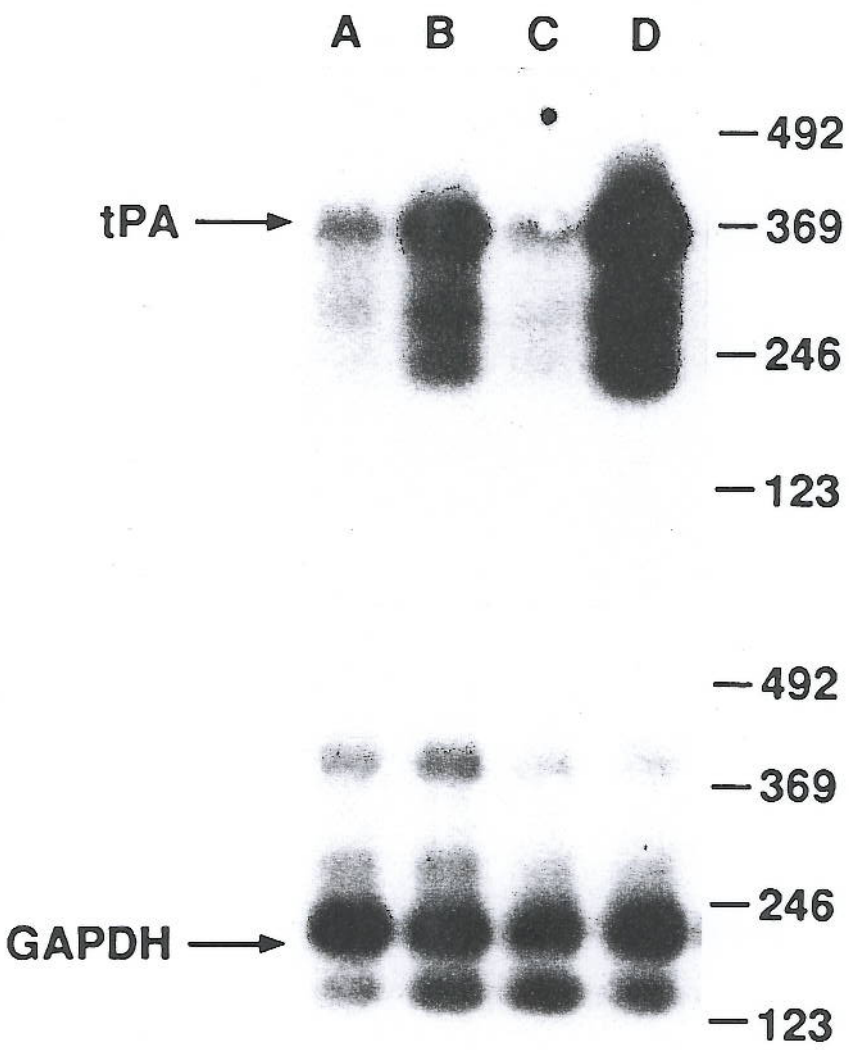


Fig. 3. Effect of laminar shear stress at 25 dynes/cm^2 on tPA and GAPDH mRNA levels in cultured endothelial cells. Total RNA isolated from three stationary cultures was pooled as was RNA from three cultures exposed to fluid shear stress. Matched samples (experiment 1, **lanes A,B:** experiment 2, **lanes C,D**) of RNA from control cells (lanes A,C) and shear stressed cells (lanes B,D) were reverse transcribed (2 μg/reaction) with tPA and GAPDH antisense primers and then coamplified using the polymerase chain reaction with primer sets designed for tPA cDNA and GAPDH cDNA. Amplification products, run on 2% agarose gels, were blotted to nylon and hybridized with ^{32}P-labelled probes for tPA **(upper half)** and GAPDH **(lower half).**

production decreases at high passage number. With the use of the polymerase chain reaction amplification technique utilizing recombinant, heat stable *Thermus aquaticus* (Taq) DNA polymerase, phenotypic analysis is possible with small numbers (on the order of 10^6 cells) of primary cultures of HUVEC which have not been passaged in culture or exposed to stimulation by exogenous growth factors.

The primers we used for the polymerase chain reaction amplification of part of the tPA cDNA were chosen from positions in exons XII and exon XIV (designation of Ny et al., 1984) specifying an amplified product 368 base pairs in length. Because our observed amplification product was a) of the predicted size and b) hybridized correctly to a probe complementary to exon XIII, it is unlikely that the amplified product we observed represented inadvertent amplification of contaminating genomic DNA in our RNA preparations.

Since tPA secretion increased under shear stress and PAI-1 secretion was unstimulated, it is unlikely that autocrine effects mediated by IL-1, TFG-β, and bFGF can account for the changes in tPA secretion because the endothelial response to these cytokines involves increased PAI-1 secretion, sometimes accompanied with decreased tPA secretion (Emeis and Kooistra, 1986; Schleef, et al., 1988; Sawdey et al., 1989; Saksela et al., 1987). Although HUVEC express mRNA for PDGF-A chain and PDGF-B chain and synthesize a PDGF-like protein, PDGF has little autocrine activity since normal endothelial cells lack the PDGF receptor (Kazlauskas and DiCorleto, 1985). Endothelial cells synthesize bFGF which, when added exogenously to

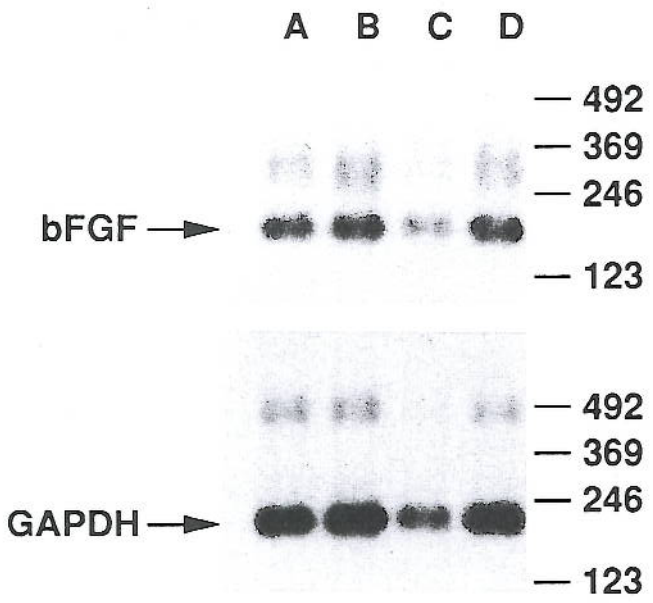


Fig. 4. Effect of laminar shear stress at 25 dynes/cm^2 on bFGF messenger RNA levels of cultured endothelial cells. Total RNA isolated from three stationary cultures was pooled as was RNA from three cultures exposed to fluid shear stress. Matched samples (experiment 1, **lanes A,B**; experiment 2, **lanes C,D**) of RNA (2 μg/reaction) from control cells (lanes A,C) and shear stressed cells (lanes B,D) were reverse transcribed with bFGF and GAPDH antisense primers and then quantitatively amplified using the polymerase chain reaction with primers selected to coamplify bFGF cDNA and GAPDH cDNA. Amplification products, run on 2% agarose gels, were blotted to nylon and hybridized with ^{32}P-labelled probes for bFGF (**upper half**) and GAPDH (**lower half**).

endothelial cells, can stimulate tPA and PAI-1 production (Saksela et al. 1987) and cell migration (Sato and Rifkin, 1988). These effects can be blocked with antibodies against bFGF (Rifkin and Moscatelli, 1989). However, we found the bFGF mRNA levels in shear stressed cells were not elevated, which makes this an unlikely mechanism for tPA induction. This is consistent with the lack of any observed increase in PAI-1 secretion. However, our findings do not exclude the possibility of transient changes of bFGF mRNA at short exposure times.

Atherosclerotic plaque development is known to occur preferentially in low shear regions near arterial branch points (Ku et al., 1985). Recently, a molecular mechanism for the suspected link between impaired fibrinolysis and atherosclerosis has been proposed. Lipoprotein(a), which was shown to compete with plasminogen in binding to the endothelial cell surface receptor, may reduce endothelial cell surface-mediated plasminogen activation (Miles et al., 1989; Hajjar et al., 1989). High serum levels of lipoprotein(a) are correlated with atherosclerosis (Dahlen et al., 1986), thus suggesting the link between impaired fibrinolysis at the cell surface and atherosclerosis possibly initiated by enhanced fibrin deposition on the vessel wall. It may follow that attenuated tPA production in low shear stress regions and subsequently reduced fibrinolysis at the endothelial surface may contribute to atherosclerotic plaque development.

One possible mechanism for hemodynamically enhanced transcription of tPA mRNA is suggested by the large increase in PGI_2 production by HUVEC exposed to shear stress. This enhanced PGI_2 production may result from enhanced availability of arachidonic acid, via the activity of calcium-activated phospholipase A_2 and/or phospholipase C (Ando et al., 1988; Bhagyalakshmi and Frangos, 1989). Given the enhanced incorporation of arachidonic acid into phosphoinositol and diacylglycerol in HUVEC exposed to shear stress (Nollert, et al., 1989), some activation of protein kinase C may occur and activation of protein kinase C is known to enhance tPA gene expression (Levin and Santell, 1988). Although no unified scheme to explain mechanochemical signal transduction, cytoskeletal reorganization during cellular alignment, and modulation of gene expression is yet possible, our data provide some insight into these phenomena at the genetic level. Such information should aid in understanding endothelial mediated modulation of smooth muscle cell proliferation and vasoconstriction in the blood vessel wall.

ACKNOWLEDGMENTS

This work was supported by NIH grants HL 18672 (L.V.M.), HL 23016 (S.G.E.), R01-HL 40680 (J.B.S.), NASA grants NAS 9-17403 (L.V.M.) and NAG 9-207 (L.V.M.), and Grant C-938 (L.V.M.) from the Robert A. Welch Foundation. The opinions stated herein are those of the authors and do not necessarily represent the views of the department of defense.

LITERATURE CITED

Ando, J., Komatsuda, T., and Kamiya, A. (1988) Cytoplasmic calcium response to fluid shear stress in cultured vascular endothelial cells. In Vitro Cell Dev. Biol., *24*:871–877.

Bhagyalakshmi, A., and Frangos, J.A. (1989) Mechanism of shear-induced prostacyclin production in endothelial cells. Biochem. Biophys. Res. Commun., *158*:31–37.

Caro, C.G., Fitz-Gerald, J.M., and Schroter, R.C. (1971) Atheroma and arterial wall shear: Observation, correlation and proposal of a shear dependent mass transfer mechanism for atherogenesis. Proc. R. Soc. Lond. [Biol], *177*:109–159.

Chirgwin, J.M., Przbyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from source enriched in ribonuclease. Biochemistry, *18*:5294–5299.

Dahlen, G.H., Guyton, J.R., Attar, M., Farmer, J.A., Kautz, J.A., and Gotto, A.M. (1986) Association of levels of lipoprotein Lp(a), plasma lipids, and other lipoproteins with coronary artery disease documented by angiography. Circulation. *74*:758–765.

Dewey, Jr., C.F., Bussolari, S.R., Gimbrone, Jr., M.A., and Davies, P.F. (1981) The dynamic response of vascular endothelial cells to fluid shear stress. J. Biomech. Eng., *103*:177–185.

Diamond, S.L., Eskin, S.G., and McIntire, L.V. (1989) Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. Science, *243*:1483–1485.

Dichek, D., and Quertermous, T. (1989a) Thrombin regulation of mRNA levels of tissue plasminogen activator and plasminogen activator inhibitor-1 in cultured human umbilical vein endothelial cells. Blood, *74*:222–228.

Dichek, D., and Quertermous, T. (1989b) Variability in messenger RNA levels in human umbilical vein endothelial cells of different lineage and time in culture. In Vitro Cell. & Dev. Biol., *25*:289–292.

Dobrin, P.B., Littooy, F.N., and Endean, E.D. (1989) Mechanical factors predisposing to intimal hyperplasia and medial thickening in autogenous vain grafts. Surgery, *105*:393–400.

Emeis, J.J., and Kooistra, T. (1986) Interleukin 1 and lipopolysaccharide induce an inhibitor of tissue-type plasminogen activator in vivo and in cultured endothelial cells. J. Exp. Med., *163*:1260–1266.

Eskin, S.G., Ives, C.L., McIntire, L.V., and Navarro, L.T. (1984) Re-

sponse of cultured endothelial cells to steady flow. Microvasc. Res., *28:*87–94.

Fort, P., Marty, L., Piechaczyk, M., Sabrouty, S.E., Dani, C., Jeanteur, P., and Blanchard, J.M. (1985) Various rat tissues express only one major mRNA species from the glyceraldehyde 3-phosphate-dehydrogenase family. Nucleic Acids Res., *13:*1431–1442.

Frangos, J.A., Eskin, S.G., McIntire, L.V., and Ives, C.L. (1985) Flow effects on prostacyclin production by culture human endothelial cells. Science, *227:*1477–1479.

Gimbrone Jr., M.A. (1976) Culture of vascular endothelium. In: Progress in Hemostasis and Thrombosis. T.H. Spaet, ed. Grune and Stratton, New York, Vol. 3, pp. 1–28.

Goldsmith, H.L., and Turitto, V.T. (1986) Rheological aspects of thrombosis and haemostasis: Basic principles and applications. Thromb. Haemost., *55:*415–435.

Grabowski, E.F., Jaffe, E.A., and Weksler, B.B. (1985) Prostacyclin production by cultured endothelial cell monolayers exposed to step increases in shear stress. J. Lab. Clin. Med., *105:*36–43.

Hajjar, K.A., Gavish, D., Breslow, J.L., and Nachman, R.L. (1989) Lipoprotein(a) modulation of endothelial cell surface fibrinolysis and its potential role in atherosclerosis. Nature, *339:*303–305.

Hanss, M., and Collen, D. (1987) Secretion of tissue-type plasminogen activator and plasminogen activator inhibitor by cultured human endothelial cells: Modulation by thrombin, endotoxin, and histamine. J. Lab. Clin. Med., *109:*97–104.

Jacobsen, H., Mestan, J., Mittnacht, S., and Dieffenbach, C.W. (1989) Beta interferon subtype 1 induction by tumor necrosis factor. Mol. Cell. Biol., *9:*3037–3042.

Kazlauskas, A., and DiCorleto, P.E. (1985) Cultured endothelial cells do not respond to platelet-derived growth-factor-like protein in an autocrine manner. Biochim. Biophys. Acta, *846:*405–412.

Konkle, B.A., and Ginsburg, D. (1988) The addition of endothelial cell growth factor and heparin to human umbilical vein endothelial cell cultures decreases plasminogen activator inhibitor-1 expression. J. Clin. Invest., *82:*579–585.

Kooistra, T., van den Berg, J., Tons, A., Platenburg, G., Rijken, D.C., and van den Berg, E. (1987) Butyrate stimulates tissue-type plasminogen-activator synthesis in cultured human endothelial cells. Biochem. J., *247:*605–612.

Ku, D.N., Giddens, D.P., Zarins, C.K., and Glagov, S. (1985) Pulsatile Flow and atherosclerosis in the human carotid bifurcation: Positive correlation between plaqe location and low and oscillating shear stress. Arteriosclerosis, *5:*293–302.

Levesque, M.J., and Nerem, R.M. (1985) The enlongation and orientation of cultured endothelial cells in response to shear stress. J. Biomech. Eng., *107:*341–347.

Levin, E.G., and Santell L. (1988) Stimulation and desensitization of tissue plasminogen activator release from human endothelial cells. J. Biol. Chem., *263:*9360–9365.

McArthur, M.M., MacGregor, I.R., Prowse, C.V., Hunter, N.R., Dawes, J., and Pepper, D.S. (1986) The use of human endothelial cells cultured in flat wells and on microcarrier beads to assess tissue plasminogen activator and factor VIII related antigen release. Thromb. Res., *41:*581–587.

Miles, L.A., Fless, G.M., Levin, E.G., Scanu, A.M., and Plow, E.F. (1989) A potential basis for the thrombotic risks associated with lipoprotein(a). Nature, *339:*301–303.

Nerem, R.M., Levesque, M.J., and Cornhill, J.F. (1981) Vascular endothelial morphology as an indicator of the pattern of blood flow. J. Biomech. Eng., *103:*172–176.

Nollert, M.U., Hall, E.R., Eskin, S.G., and McIntire, L.V. (1989) The effect of shear stress on the uptake and metabolism of arachidonic acid by human endothelial cells. Biochim. Biophys. Acta., *1005:* 72–78.

Ny, T., Elgh, G., and Lund, B. (1984) The structure of the human tissue-type plasminogen activator gene: Correlation of intron and exon structures to functional and structural domains. Proc. Natl. Acad. Sci. USA, *81:*5355–5359.

Olesen, S.P., Clapham, D.E., and Davies, P.F. (1988) Haemodynamic shear stress activates a K^+ current in vascular endothelial cells. Nature, *331:*168–170.

Pennica, D., Holmes, W.E., Kohr, W.J., Harkins, R.N., Vehar, G.A., Ward, C.A., Bennet, W.F., Yelverton, E., Seeburg, P.H., Heyneker, H.L., Goeddel, D.V., and Collen, D. (1983) Cloning and expression of human tissue-type plasminogen activator cDNA in E. Coli. Nature, *201:*214–221.

Rappolee, D.A., Wang, A., Mark, D., Werb, Z. (1989) Novel method for studying mRNA phenotypes in single or small numbers of cells. J. Cell. Biochem., *37:*71–81.

Rifkin, D.B., and Moscatelli, D. (1989) Recent developments in the cell biology of basic fibroblast growth factor. J. Cell Biol., *109:*1–6.

Sakata, Y., Curriden, S., Lawrence, D., Griffin, J.H., and Loskutoff, D.J. (1985) Activated protein C stimulates the fibrinolytic activity of cultured endothelial cells and decreases antiactivator activity. Proc. Natl. Acad. Sci. USA, *82:*1121–1125.

Saksela, O., Moscatelli, D., and Rifkin, D.B. (1987) The opposing effect of basic fibroblast growth factor and transforming growth factor beta on the regulation of plasminogen activator activity in capillary endothelial cells. J. Cell Biol., *105:*957–963.

Sato, Y., and Rifkin, D.B. (1988) Autocrinic activities of basic fibroblast growth factor: Regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. J. Cell Biol., *107:* 1199–1205.

Sawdey, M., Podor, T.J., and Loskutoff, D.J. (1989) Regulation of type 1 plasminogen activator inhibitor gene expression in cultured bovine aortic endothelial cells. J. Biol. Chem., *264:*10396–10401.

Schleef, R.R., Bevilacqua, M.P., Sawdey, M., Gimbrone, Jr., M.A., and Loskutoff, D.J. (1988) Cytokine activation of vascular endothelium: Effects on tissue-type plasminogen activator and type 1 plasminogen activator inhibitor. J. Biol. Chem., *263:*5797–5803.

Sommer, A., Brewer, M.T., Thompson, R.C., Moscatelli, D., Presta, M., and Rifkin, D.B. (1987) A form of human basic fibroblast growth factor with an extended amino terminus. Biochem. Biophys. Res. Commun., *144:*543–550.

van Hinsbergh, V.W.M., Binnema, D., Scheffer, M.A., Sprengers, E.D., Kooistra, T., and Rijken, D.C. (1987) Production of plasminogen activators and inhibitor by serially propagated endothelial cells from adult human blood vessels. Arteriosclerosis, *7:*389–400.

White, G.E., Gimbrone Jr., M.A., and Fujiwara, K. (1983) Factors influencing the expression of stress fibers in vascular endothelial cells in situ. J. Cell Biol., *97:*416–424.

Yoshizumi, M., Kurihara, H., Sugiyama, T., Takaku, F., Yanagisawa, M., Masaki, T., and Yazaki, Y. (1989) Hemodynamic shear stress stimulates endothelin production by cultured endothelial cells. Biochem. Biophys. Res. Commun., *161:*859–964.

Zonneveld, A.J., Change, G.T.G., van den Berg, J., Kooistra, T., Verheijen, J. H., Pannekoek, H., and Kluft, C. (1986) Quantification of tissue-type plasminogen activator (tPA) mRNA in human endothelial-cell cultures by hybridization with a tPA cDNA probe. Biochem. J., *235:*385–390.