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REGULATION OF ENDOTHELIAL CELL GENE EXPRESSION BY HEMODYNAMIC FORCES: Implications for Intimal Hyperplasia and Graft Patency

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Maintenance of proper cellular phenotype (or "genetic state") of the endothelial cell and the smooth muscle cell is critical in preventing loss of vessel patency. Intimal hyperplasia is a common cause of obstructive stenosis following many kinds of vascular reconstruction.[1–5] Both intimal hyperplasia and early atherosclerotic lesions occur most often in areas of flow disturbances such as anastomotic sites[1–3] and in areas with low- or oscillatory-fluid shear stress.[6–11] We have carried out experiments to recreate blood flow forces in vitro and to measure the effects of these forces on endothelial cell gene expression.

In the vessel wall, blood factors, cytokines delivered by macrophages or T cells, and platelet-derived products can all cause excessive proliferation of smooth muscle cells. The endothelium may also regulate the phenotype of the smooth muscle cell by releasing mitogens. Molecular biology techniques are particularly powerful in deciphering the many complex events which occur at the cellular level. Studies of cellular phenotype are often used in tandem with cell culture approaches. In vitro techniques allow the study of cell–cell interactions while largely isolating or decoupling hemodynamic, hematologic, and immunologic contributions to the vascular pathology. However, when cells are taken from the body, whether they are derived from normal or diseased tissue, changes can occur in the cultured cell lines. The three-dimensional structure and confinements of the tissue are lost when cells are grown in plastic dishes. The complex nature of the in vivo environment is only approximated by a growth medium. The delicate balance of growth regulatory factors is only partially recreated by addition of growth factors to the cell culture medium.

An additional complication is that the phenotype of the cell in culture is highly dependent on the mode of isolation, the time in culture, the medium and substrate used to culture the cells, and the initial seeding density of the culture. The challenge of studying cells in vitro is to maintain the basic functions of the cell which are important in understanding the disease process.

To understand the process of intimal hyperplasia, it is important to evaluate how strongly particular genes are being transcribed into messenger RNA (mRNA) by cells in the vessel wall. Measurement of mRNA levels in human cells is complicated by the small numbers of cells available (on the order of 10^6 or fewer cells) from early passage cultures. Northern blotting techniques typically require much greater quantities of total cellular RNA isolated from 10^7 to 10^8 cells grown in culture. The detection limit of autoradiography is about 1 pg of nucleic acid. This limit in detection is especially troublesome for investigations of messages which have only a few copies per cell. 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 and smooth muscle cell mitogens without long-term, large-scale cultivation, since phenotypic drift in culture has been documented in human umbilical vein endothelial cells (HUVEC). For example, the urokinase gene, not normally expressed in primary HUVEC or adult vena cava cultures, becomes quite active[12,13] at higher passage numbers. Also, tissue plasminogen activator (tPA) secretion and tPA mRNA is upregulated many-fold with serial passage in culture,[12–14] while plasminogen activator inhibitor 1 (PAI-1) secretion has been shown to decrease 3 to 10-fold with the use of heparin in combination with endothelial cell growth factor.[15] At higher cell passage number, the quantity of the smaller, polyadenylate-free form of the PAI-1 mRNA increases.[13] Additionally, prostacyclin (PGI_2) production is quickly lost and von Willebrand factor production decreases at high passage number.

With the use of the polymerase chain reaction amplification (PCR) technique utilizing recombinant, heat stable *Thermus aquaticus (Taq)* DNA polymerase, mRNA measurements are possible with small numbers (on the order of 10^6 cells) of cells from primary cultures of HUVEC that have not been passaged in culture or exposed to stimulation by growth factors. The PCR-based mRNA assay utilizes the following approach: total cellular RNA is isolated from cells and target mRNA species are reversed transcribed. The complementary DNA (cDNA) synthesized using the reverse transcriptase enzyme is then prepared for PCR amplification. Primers which are specific for the cDNA are added and the PCR amplification is carried out to produce thousands of copies of the original cDNA. The amplified cDNA is then measured using specific radiolabeled oligonucleotide probes (Fig. 2–1) as an indicator of the quantity of input mRNA species into the assay.

This method of analysis of mRNA levels in cells is more sensitive than standard Northern blotting techniques. Northern blotting requires the use of long cDNA probes which are sometimes difficult to obtain and require a plasmid preparation and nick translation of each probe. With the PCR approach, the target signal is much greater than background and small end-labeled oligo probes allow detection of the product. Measurement of up to six genes is feasible with our approach, since it lends itself to automation. To study the many relationships between growth factors, it is desirable to measure the mRNA levels of several genes simultaneously. Our use of an internal amplification standard, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enhances the

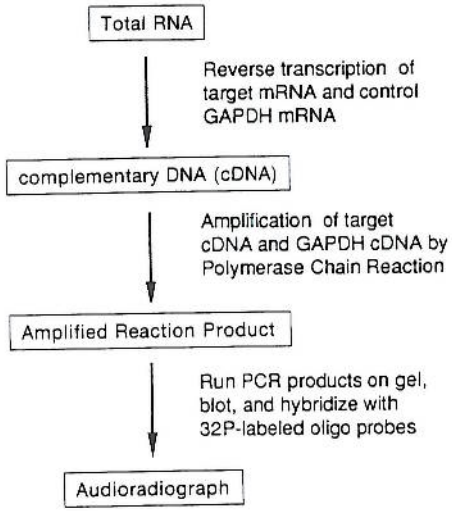

Figure 2–1. Measurements of endothelial mRNA levels in small numbers of human cells is accomplished using a coupled reverse transcription/polymerase chain reaction method. Total cellular RNA is isolated from the cells. The mRNA target and internal control mRNA are then reverse transcribed. The resulting cDNAs are coamplified using PCR and then visualized by standard Southern blotting with radiolabeled DNA probes. The quantity of amplification product serves as an indicator of the amount of the particular mRNA level in the cells.

utility of the technique since the PCR amplification does have some variability from reaction to reaction. As techniques evolve, cRNA constructs added to unknowns[16] may be useful for calibrating our measurements to actual intracellular mRNA copy number. With the internal control gene to normalize for variations in reverse transcription yield and efficiency of amplification, an estimate of the initial mRNA concentration per cell can be made relative to the initial copy number of the GAPDH message.

METHODS

The exposure of monolayers of endothelial cells to defined levels of laminar shear stress used parallel-plate flow channels of known geometry. Replicate primary, confluent monolayers (72 to 86 hours post seeding) of HUVEC were exposed to steady laminar shear stress in individual parallel-plate flow chamber systems with recirculating media driven by a constant hydrostatic pressure head as previously described.[17–19] The cell cultures were mounted on separate parallel-plate flow chambers. The monolayer surface area exposed to shear stress in each chamber was 15 cm^2 with a channel thickness of 200 μm. Flow chambers were connected under sterile conditions to individual flow systems, each filled with 15 ml of medium. The wall shear stress imposed upon the monolayer was evaluated by solution of the Navier-Stokes equation for laminar flow of a Newtonian liquid. The wall shear stress (τ_w = dynes/cm^2) was calculated as follows: $\tau_w = 6\,Q\,\mu/(B^2W)$ where: flowrate, Q = cm^3/sec; viscosity, μ = 0.01 poise; total gap thickness, B = 0.02 cm; and width, W = 2.49 cm. The entrance length needed for steady parabolic flow to be established was less than 1 mm.[20] The Reynolds number for the flow condition was less than 50, ensuring that the flow was truly laminar with no possibility of turbulence.

For each experiment, control cultures were incubated under stationary conditions. Media samples (1 ml) were taken from each system every 4 to 6 hours and stored at −80°C. For PCR analysis of mRNA levels, the flow was stopped and total cellular RNA extracted from the shear stressed monolayers (15 cm^2 of monolayer per slide) within 1 minute after termination of flow. Similarly, RNA was extracted from stationary cultures (15 cm^2 of monolayer per slide). To calculate mean cell density on the slides before RNA extraction, cells were counted in three light micrographs of each monolayer.

Studies of mRNA levels using small numbers of primary human cells were carried out with a reverse transcription/polymerase chain reaction technique (PCR). Isolation of total cellular RNA from small cellular samples was accomplished with a scaled-down adaptation of the guanidine thiocyanate (GTC)/cesium chloride (CsCl) gradient method[21,22] as previously described.[19] Briefly, monolayers were lysed in 5 M guanidine thiocyanate solution with 25 mM sodium citrate and 0.5 per cent (w/v) sodium N-lauroyl sarcosinate and centrifuged at 20,000*g* for 3 hours over a 5.7 M CsCl cushion. The pellet was dissolved in diethyl pyrocarbonate-treated (DEPC) water (0.2 v/v per cent) and extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with cholorform/isoamyl alcohol (24:1). Total RNA was precipitated with 3 M sodium acetate (pH 5.4) and 100 per cent ethanol, vacuum dried, and resuspended in 30 μl of DEPC-H_20. A 2.5 M LiCl precipitation and resuspension was performed prior to the reverse transcription.

To provide an internal standard against experimental variations in the reverse transcription reaction or PCR amplification efficiency, simultaneous reverse transcription and coamplification of the constitutively-expressed mRNA for GAPDH were carried out as previously described.[19] Simultaneous reverse transcription and coamplification of human endothelial cell RNA with primer sets[19,23] for tPA or basic fibroblast growth factor (bFGF) or endothelin-1 (ET-1) and GAPDH produced amplification products of the predicted sizes of 368 bp for tPA, 177 bp for bFGF, 441 bp for ET-1, and 195 bp for GAPDH. Synthesis of tPA (or bFGF or ET-1) cDNA and GAPDH cDNA was carried out simultaneously in 25 μl reactions with 500 U MMLV reverse transcriptase (RT), 0.5 μg of each antisense primer, and 0.5 mM each of all four dNTPs. Each reverse transcription reaction mixture was carried out at 37°C for 20 minutes. The cDNA products were coamplified using the polymerase chain reaction in a total volume of 100 μl. Prior to amplification, a 75 μl volume was added containing 0.5 μg of each sense primer, 0.25 μg of each antisense primer, and 10 mM of each dNTP. Two units of recombinant *Taq* DNA polymerase (AmpliTaq, Beckman) were added to initiate the PCR. Each temperature cycle consisted of 90°C for 1.5 minutes, 50°C for 1 minute, and 72°C for 2 minutes. Amplified products were visualized by Southern hybridization as previously described[19] using T4 kinase ^{32}P end-labeled probes for tPA (or bFGF or ET-1) and for GAPDH.

Immunoreactive endothelin was measured using a radioimmunoassay (Amersham Corporation) employing an ^{125}I-endothelin tracer and antiserum made against synthetic endothelin-1. A calibration curve yielding a LOGIT plot with $r^2 > 0.99$ was generated using synthetic ET-1 added to complete medium (M199 + bovine serum) to control for nonspecific background.

A double antibody enzyme-linked immunosorbent assay (ELISA) technique (American Diagnostica Inc.) using goat antihuman tPA immunoglobin allowed measurement of HUVEC-secreted tPA (free and inhibitor-bound) as previously described.[18] Normal goat IgG was used as a nonspecific blocking agent. Soluble

goat antihuman tPA IgG quenched the tPA-specific signal in the blanking well. HUVEC-conditioned media (undiluted) or antigenic standards (Bowes melanoma single-chain tPA) were added to sample wells in triplicate and to blanking wells. The ELISA was calibrated to the limit of its sensitivity (0 to 1500 pg/ml), yielding a linear calibration curve ($r^2 > 0.99$) with a detection limit of 50 pg/ml.

In HUVEC-conditioned media, PAI-1 exists in latent and active forms with only a very small fraction bound to tPA. An ELISA was used to measure uncomplexed (latent and active) PAI-1 antigen (American Diagnostic Inc.). Briefly, mouse antihuman PAI-1 immunoglobin was bound to a 96-well plate. The plate was washed and loaded with samples of conditioned media (diluted 1:10 and 1:25) or standards. The colorimetric reaction (measured at 490 nm) was performed using biotinylated monoclonal mouse antihuman PAI-1 immunoglobin and horseradish peroxidase conjugated streptavidin with a reaction buffer containing orthophenylenediamine and 0.04 per cent (v/v) 30 per cent-H_2O_2.

RESULTS

Our studies of protein production by endothelial cells exposed to shear stress were carried out in experiments lasting over 24 hours. During the first several hours after the onset of fluid flow, the levels of tPA in the circulating medium at all shear stress levels were the same as those of stationary control cultures (Fig. 2–2). Low shear stress (4 dynes/cm^2) had no effect on tPA secretion over the entire time course of the experiments. After longer exposure to shear stress of 15 or 25 dynes/cm^2, however, the level of tPA produced by shear-exposed cells exceeded that of controls (Fig. 2–2). The increase of tPA in the circulating medium was linear with time for over 20 hours, allowing a least-squares fit to determine the steady-state secretion rate. Steady-state tPA secretion rates of cells exposed to 15 and 25 dynes/cm^2, normalized to matched controls, increased 2.06 ± 0.39 ($n=3$; $p<0.015$)- and 3.01 ± 0.53 ($n=3$; $p<0.015$)-fold over stationary cultures, respectively. The average steady-state secretion rate of tPA by HUVEC in control cultures was 0.168 ± 0.053 ng/10^6 cells-hr ($n=3$).[18]

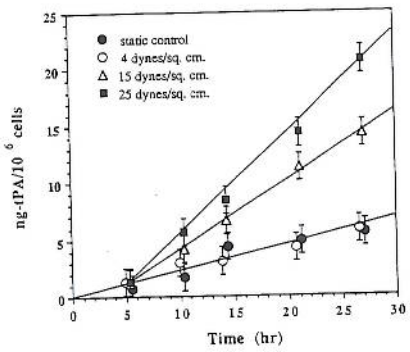

FIGURE 2–2. Cumulative production of tissue plasminogen activator (tPA) by replicate, primary confluent HUVEC monolayers maintained in stationary incubations (●) or exposed to steady laminar shear stress of 4 (○), 15 (△), or 25 dynes/cm^2 (■) using three independent flow systems. Each point is the average of triplicate ELISA determinations. Steady state production rates were calculated for each monolayer by a least-squares fit of cumulative production between 4 and 24 hours.

The secretion of tPA by control cultures was completely inhibited by cycloheximide (5 μg/ml), suggesting that new protein synthesis was continually required in order to maintain constitutive tPA release. This would indicate that the increase of tPA in the circulating medium was not due to release of intracellular stores of tPA. The greatly enhanced convective mass transport at low stresses of 4 dynes/cm^2 (compared the natural convection of stationary cultures) had no effect on the production of tPA. Also, the cyclooxygenase inhibitor indomethacin (50 μM) had no effect on shear-stress–stimulated tPA production indicating that the shear-enhanced tPA secretion was not mediated by a cyclooxygenase product.

Shear stress levels ranging from 4 to 40 dynes/cm^2 caused no significant changes in the PAI-1 secretion rate relative to controls. The average steady-state PAI-1 secretion rate of HUVEC in control cultures was 53 ± 37 ng of PAI-1 per 10^6 cells per hour ($n=7$).[18] Measurements of PAI-1 production over the first 200 minutes after the onset of flow demonstrated that the PAI-1 secretion was not affected by shear stress transiently at early exposure times.

Primary HUVEC secreted about 15,000 pg of ET-1 per 10^6 cells in 24 hours while in stationary culture. Secretion of endothelin was suppressed by laminar shear stress of 25 dynes/cm^2 within 4 hours after the onset of flow (Fig. 2–3). The suppression of endothelin secretion by high shear stress (25 dynes/cm^2) continued for over 20 hours during exposure to flow. In three separate experiments, endothelin production was suppressed only slightly (<15 per cent) by low shear stress of 4 dynes/cm^2 compared to endothelin production of stationary controls.

To investigate the effect of laminar shear stress on mRNA levels in endothelial cells, replicate HUVEC monolayers were either maintained in stationary culture or exposed to shear stress at 25 dynes/cm^2 in individual flow systems. In each experiment, secretion of tPA, PAI-1, and ET-1 (per 10^6 cells) was evaluated for the three individual flow systems and the three matched, stationary cultures. Enhancement of tPA production and suppression of ET-1 secretion were observed after a lag time of several hours of exposure to shear stress (Table 2–1) as was previously observed. In each experiment, the quantity of total cellular RNA isolated from stationary and shear-stressed HUVEC cultures was not significantly different (average variation less than 10 per cent).

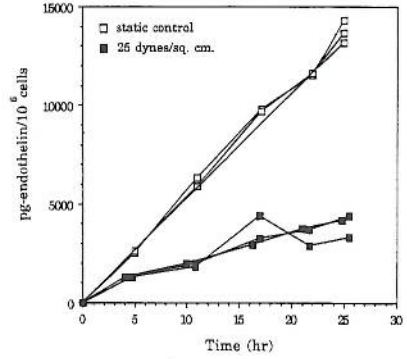

FIGURE 2–3. Cumulative production of endothelin by replicate, primary confluent HUVEC monolayers maintained in three separate stationary incubations (□) or exposed to steady laminar shear stress of 25 dynes/cm^2 (■) using three independent flow systems. Each point is the average of duplicate RIA determinations. Steady state production rates were calculated for each monolayer by a least-squares fit of cumulative production between 4 and 24 hours.

TABLE 2–1. SECRETION RATE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR (tPA), PLASMINOGEN ACTIVATOR INHIBITOR-TYPE 1 (PAI-1), AND ENDOTHELIN-1 (ET) BY HUMAN ENDOTHELIAL CELLS EXPOSED TO LAMINAR SHEAR STRESS OF 25 dynes/cm² [a]

Experiment[b]	Cell Density (10^4 cells/cm²)	tPA Secretion Rate (ng-tPA/10^6 cells/hr)	PAI-1 Secretion Rate (ng-PAI-1/10^6 cells/hr)	ET Secretion Rate (ng-ET/10^6 cells/hr)
1. Control	7.7 ± 0.8	0.170 ± 0.049 (n = 3)	70.2 ± 4.97 (n = 3)	0.545 ± 0.017 (n = 3)
25 dynes/cm²	7.1 ± 0.9	0.399 ± 0.087 (n = 3)	47.8 ± 13.6 (n = 3)	0.132 ± 0.026 (n = 3)
2. Control	6.7 ± 0.8	0.160 ± 0.039 (n = 3)	49.3 ± 3.25 (n = 3)	0.485 ± 0.025 (n = 3)
25 dynes/cm²	7.3 ± 0.8	0.527 ± 0.123 (n = 3)	26.5 ± 3.04 (n = 3)	0.279 ± 0.055 (n = 3)
3. Control	5.9 ± 1.0	0.122 ± 0.013 (n = 3)	53.0 ± 8.71 (n = 3)	0.587 ± 0.057 (n = 3)
25 dynes/cm²	6.2 ± 0.9	0.387 ± 0.073 (n = 2)	58.1 ± 7.60 (n = 2)	0.335 ± 0.061 (n = 2)
Mean rates ± SD				
Control		0.157 ± 0.036 (n = 9)	57.2 ± 10.6 (n = 9)	0.549 ± 0.046 (n = 9)
25 dynes/cm²		0.453 ± 0.108 (n = 8)[c]	42.6 ± 15.5 (n = 8)[d]	0.239 ± 0.099 (n = 8)[c]

[a] The large induction of tPA secretion is in contrast to the suppression of endothelin secretion. Protein secretion rates were determined by a least-squares fit of antigen production data between 4 and 24 hours. Enzyme-linked immunosorbent assays were used to quantify tPA and PAI-1 production, while endothelin production was evaluated using a radioimmunoassay. Analysis of mRNA levels for experiment 1 is shown in Figure 2–4.
[b] Each experiment was conducted with an independent pool of primary HUVEC.
[c] $p < 0.001$.
[d] Not significant.

Using the RT/PCR method, we found that the tPA mRNA level was elevated in endothelial cells exposed to shear stress (Fig. 2–4, upper left). In contrast, when the same Southern blots were stripped and reprobed for the GAPDH amplification product, the transcript level of GAPDH was constant and independent of shear stress (Fig. 2–4, lower left). When this RNA from

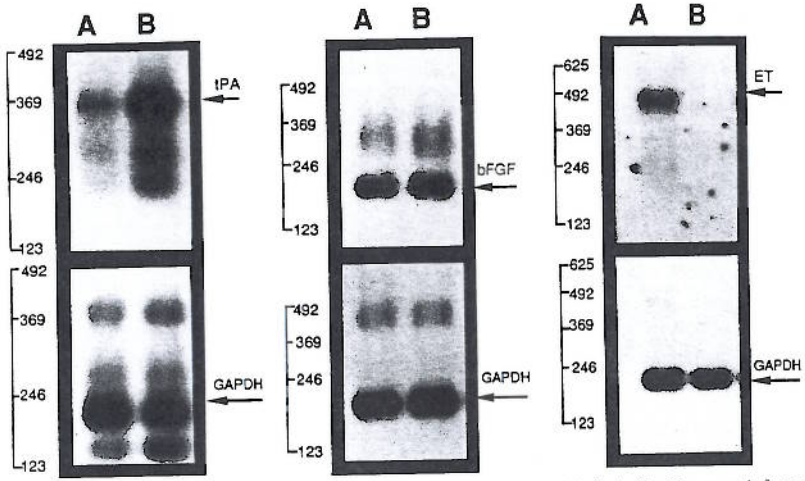

FIGURE 2–4. Analysis of mRNA levels for tissue plasminogen activator (tPA), basic fibroblast growth factor (bFGF), endothelin (ET), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in human endothelial cells maintained in control culture (*lane A*) or exposed to 25 dynes/cm² (*lane B*). The large induction by shear stress of tPA mRNA is in contrast to the suppression of ET-1 mRNA levels by shear stress exposure. This mRNA phenotyping is in agreement with the protein production data (See experiment 1 in Table 2–1).

control and stressed monolayers was used in a similar coamplification experiment with GAPDH to study the effect of shear stress on levels for bFGF mRNA, no large increase in transcript levels such as that seen for tPA was noted for bFGF (Fig. 2–4, middle). Endothelin-1 mRNA levels were sharply reduced in the endothelial cells exposed to 25 dynes/cm² for 24 hours (Fig. 2–4, right). Again, no correspondingly sharp decrease was observed in signal strength for GAPDH product, indicating the uniformity of the coamplification reactions for bFGF and endothelin.

These findings were reproduced in an independent experiment and are unlikely to represent variation in PCR amplification efficiency because of (1) the similar cell numbers in, and RNA amounts extracted from, static and flow-subjected cultures, (2) the comparative constancy of coamplified GAPDH transcript levels observed under different flow conditions, and (3) the observation of increased tPA mRNA levels and increased tPA protein secretion rates detected by the reverse transcriptase/PCR technique in the same RNA and medium samples which showed decreased endothelin secretion and decreased endothelin mRNA levels, indicating opposite flow responses by HUVEC for endothelin and tPA. Our use of the polymerase chain reaction assay technique for quantitative estimates of transcript levels agreed quite well with the results of standard Northern blotting techniques used by Kooistra et al.[24] Those investigators showed that butyrate (1 to 10 mM) can increase HUVEC tPA secretion by a factor of 6- to 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.[19] 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.

DISCUSSION

Several lines of evidence indicate that low shear stress zones in arteries (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.[6–11] Caro et al.[25] showed that low shear stress zones are prone to develop lesions and implicated poor mass transfer properties of low-flow zones in reducing efflux of lipids out of the vessel wall. In addition to lipid efflux, enhanced concentration of platelet-release products in regions of poor mass transport may play as important a role as reduced lipid efflux.[26]

Examining human carotid bifurcations at autopsy, Zarins et al.[10] found that early atherosclerotic lesions were located in regions of flow separations, low shear stress, and nonuniform directional flow. The outer wall of the carotid sinus (with separating flow and low shear stress) had the most plaques. Few lesions were found near the flow divider where shear stress was high and the flow was primarily axially oriented. Ku et al.[11] have correlated intimal thickening (in 12 autopsy specimens) of the outer wall of the proximal internal carotid with low shear stress (mean shear stress = −0.5 dynes/cm²) and a high degree

of flow reversal (instantaneous shear stress = −7 to 4 dynes/cm^2). The inner walls of the internal carotid (mean shear stress = 17 to 26 dynes/cm^2; no flow reversal) had little intimal thickening. Regions of the vessels downstream of the bifurcation had unidirectional flow, high mean shear stress (14 to 45 dynes/cm^2) and no intimal thickening. Similar work by Friedman et al.[27] with pulsatile flow in human aortic bifurcations demonstrated that intimal thickening was inversely related to the magnitude of the shear stress. Sakata and Takebayashi[28] also found that lesions were localized to the outer wall of the carotid artery. In a similar study, Grottum found lesions in the human left coronary artery bifurcation preferentially on the outer wall where shear stress levels were lowest.[29] In human cerebral arteries, the incidence of lesions was also higher on the outer walls of the daughter vessels.[30]

Loss of vessel patency after vascular graft placement or balloon angioplasty involves excessive smooth muscle cell proliferation and often occurs at vessel injury sites where platelets adhere in the early postinjury period. During these early times, Platelet-derived growth factor (PDGF) released by platelets might act as a potent smooth muscle cell mitogen at the site of injury.[31] However, late postoperative intimal hyperplasia involving vascular prostheses occurs most often at the perianastomotic areas, which are most rapidly resurfaced with endothelium from pannus ingrowth and which obtain the earliest protection against platelet deposition.[1–3,32–35] In addition, late postoperative subendothelial vascular smooth muscle cell proliferation occurs in both 60 μm internodal polytetrafluoroethylene (PTFE) arterial grafts in baboons and in Dacron grafts seeded with autologous endothelial cells in dogs despite confuent endothelial linings free of adherent platelets.[36–40] Both endothelial cells and smooth muscle cells express and release PDGF in vitro,[41–43] and increased PDGF mRNA levels are found in carotid plaques and atherosclerotic lesions[44–46] and in the neointima of healing PTFE prostheses in baboons at sites of abnormal smooth muscle cell proliferation.[38,39]

These data suggest that PDGF from endothelial cells or smooth muscle cells or both might cause intimal hyperplasia. However, PDGF is unlikely to be the only mitogen released by EC. Anti-PDGF antibody can neutralize only 30 to 50 per cent of the mitogenic activity of medium conditioned by cultured endothelial cells or by confluent endothelial cells lining PTFE grafts in baboons.[39,43] In pilot studies employing PCR primers and probes specific for PDGF A-chain, we have found no significant change in PDGF A-chain mRNA level in endothelial cells exposed to high shear stress for 24 hours compared to stationary controls (data not shown). This observation is not inconsistent with recent reports[47] of transient induction of PDGF mRNA at short exposure times (under 6 hours) to laminar shear stress which then return to basal levels. The transient nature of flow disturbances found in vivo, which lead to continually disordered flow at anastomotic sites, however, may continually stimulate endothelial cells to release PDGF.

Although HUVEC express mRNA for PDGF A-chain and PDGF B-chain and synthesize a PDGF-like protein, PDGF produced by endothelial cells is unlikely to have autocrine activity, since normal endothelial cells lack the PDGF receptor.[48] Endothelial cells synthesize bFGF which, when added exogenously to endothelial cells, can stimulate tPA and PAI-1 production[49] and cell migration.[50] 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 which

can be induced by bFGF. However, our findings do not exclude the possibility of transient changes of bFGF mRNA at short exposure times.

Non-PDGF factors are likely to account for a significant fraction of EC-secreted mitogenic activity. Endothelin may be an important candidate for the role of a non-PDGF mitogen produced by the endothelium, since the decrease of endothelin gene expression by high shear stress is consistent with the known inverse relationship between fluid shear and intimal hyperplasia. Our results suggest that designing anastomoses to minimize areas of low shear stress or disturbed flow might reduce the severity of intimal hyperplasia.

In the past, observations concerning the geometric localization of hyperplasia at sites of low shear stress have been ascribed to the prolongation of the "residence time" of platelets near the blood/vessel wall interface and possible release of PDGF. In regions of high flow, the concentrations of platelet-released products are reduced by convective mass transport. 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 also play a role in physiological and pathological processes. Blood vessel walls exposed to high flow may have enhanced PGI_2 production[17,51] and enhanced fibrinolytic capacity,[18,19] thus increasing the resistance of high shear zones to fibrin deposition on the vessel wall and platelet-dependent and platelet-independent smooth muscle cell proliferation. Our finding that arterial levels of shear stress suppress endothelin production suggests an additional mechanism whereby reductions in intimal thickening might arise from direct local suppression of endothelial cell expression of the smooth muscle cell mitogen, endothelin. That endothelial cells produce less fibronectin under high flow[52] is also consistent with the in vivo finding of reduced vessel wall thickening in high-flow regions of vessels.

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.[53,54] High serum levels of lipoprotein(a) are correlated with atherosclerosis[55] 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.[56] Fibrin causes disorganization of endothelial monolayers.[57] Fibrin fragment D increases endothelial permeability to albumin, and fibrinopeptide B has been shown to be chemotactic for macrophages in early lesions.[58] In zones of high shear stress, enhanced expression of tPA may protect the vessel wall from shear-induced platelet aggregation[59] with thrombin generation and subsequent fibrin deposition.

SUMMARY

The endothelial cell has been proposed as an ideal vector for human gene therapy, since recombinant proteins can be secreted directly into the blood stream.[60] The seeding of vascular stents and grafts with endothelial cells is currently under study. Several workers using animal models have successfully engineered endothelial cells to express foreign proteins (β-galactosidase, tPA)

in vivo.[61–63] The efficacy of gene-therapy approaches using engineered endothelial cells may be affected by the intrinsic response of endothelial cells to fluid mechanical forces. The analysis of cellular function and metabolism is particularly important when the hydrodynamics of blood flow may play a critical role in the success of the genetically-modified tissue.

REFERENCES

1. Imparato AM, Bracco A, Kim GFE, Zeff R: Intimal and neointimal fibrous proliferation causing failure of arterial reconstruction. Surgery 1972; *72*:1007–1017.
2. LoGerfo FW, et al: Downstream anastomotic hyperplasia. A mechanism of failure in Dacron arterial grafts. Ann Surg 1983; *197*:479–483.
3. Echave V, Voornick AR, Haimov M, Jacobson JH: Intimal hyperplasia as a complication of the use of polytetrafluoroethylene grafts for femoropopliteal bypass. Surgery 1979; *86*:791–798.
4. Clagett GP, et al: Morphogenesis and pathologic characteristics of recurrent carotid disease. J Vasc Surg 1986; *3*:10–23.
5. Liu MW, Roubin GS King SB: Restenosis after coronary angioplasty. Circulation 1989; *79*:1374–1387.
6. Morinaga K, et al: Development and regression of intimal thickening of arterially transplanted autologous vein grafts in dogs. J Vasc Surg 1987; *5*:719–730.
7. Dobrin PA, Littooy FN, Endean ED: Mechanical factors predisposing to intimal hyperplasia and medical thickening in autogenous vein grafts. Surgery 1989; *105*:393–400.
8. Glagov S, Zarins C, Giddens DP, Ku DN: Hemodynamics and atherosclerosis. Arch Path Lab Med 1988; *112*:1018–1031.
9. Karino T: Rheologic and geometric factors in vascular homeostasis. *In* Norman J, ed. Cardiovascular Science and Technology I: Precised Proceedings, Louisville, Oxymoron Press, 1989; 18–20.
10. Zarins CK, Giddens DP, Bharavaj BK, Sottiurai VS, Mabon RF, Glagov S: Carotid bifurcation with flow velocity profiles and wall shear stress. Circ Res 1983; *53*:502–512.
11. Ku DN, Giddens DP, Zarins CK, Glagov S: Pulsatile flow and atherosclerosis in the human carotid bifurcation: Positive correlation between plaque location and low and oscillating shear stress. Arteriosclerosis 1985; *5*:293–302.
12. van Hinsbergh VWM, et al: Production of plasminogen activators and inhibitor by serially propagated endothelial cells from adult human blood vessels. Arteriosclerosis 1987; *7*:389–400.
13. Dichek DA, Quertermous T: Variability in messenger RNA levels in human umbilical vein endothelial cells of different lineage and time in culture. In Vitro Cell Dev Biol 1989; *25*:289–292.
14. McArthur MM, et al: 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 1986; *41*:581–587.
15. Konkle BA, Ginsburg D: 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 1988; *82*:579–585.
16. Wang AM, Doyle MV, Mark DF: Quantitation of mRNA by the Polymerase Chain Reaction. Proc Natl Acad Sci USA 1989; *86*:9717–9722.
17. Frangos JA, Eskin SG, McIntire LV, Ives CL: Flow effects on prostacyclin production by culture human endothelial cells. Science 1985; *227*:1477–1479.
18. Diamond SL, Eskin SG, McIntire LV: Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. Science 1989; *243*:1483–1485.
19. Diamond SL, Sharefkin JB, Dieffenbach CW, Frasier-Scott KF, McIntire LV, Eskin SG: Tissue plasminogen activator messenger RNA levels increase in cultured human endothelial cells exposed to laminar shear stress. J Cell Physiol 1990; *143*:364–371.
20. Frangos JA, McIntire, LV, Eskin SG: Shear stress induced stimulation of mammalian cell metabolism. Biotechnol Bioeng 1988; *32*:1053–1060.
21. Chirgwin JM, Przbyla AE, MacDonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 1979; *18*:5294–5299.
22. Rappolee DA, Wang A, Mark D, Werb Z: Novel method for studying mRNA phenotypes in single or small numbers of cells. J Cell Biochem 1989; *39*:1–11.
23. Sharefkin JB, Diamond SL, Eskin SG, Dieffenbach CW, McIntire LV: Fluid flow decreases preproendothelin mRNA levels and suppresses endothelin-1 peptide release in cultured human endothelium cells. J Vasc Surg 1991; *14*:1–9.
24. Kooistra T, et al: Butyrate stimulates tissue-type plasminogen activator synthesis in cultured human endothelial cells. Biochem J 1987; *247*:605–612.

25. Caro CG, Fitz-Gerald JM, Schroter RC: Atheroma and arterial wall shear: observation, correlation and proposal of a shear dependent mass transfer mechanism for atherogenesis. Proc R Soc Lond [Biol] 1971; *177*:109–159.
26. Folie BJ, McIntire LV: Mathematical analysis of mural thrombogenesis: concentration profiles of platelet-activating agents and effects of viscous shear flow. Biophys J 1989; *56*:1121–1137.
27. Friedman MH, et al: Correlation between intimal thickness and fluid shear in human arteries. Atherosclerosis 1981; *39*:425–436.
28. Sakata N, Takebayashi S: Localization of atherosclerotic lesions in the curving sites of human internal carotid arteries. Biorheology 1988; *25*:567–578.
29. Grottum P, Svindland A, Walloe L: Localization of atherosclerotic lesions in the bifurcation of the main left coronary artery. Atherosclerosis 1983; *47*:55–62.
30. Sakata N, Joshita T, Ooneda G: Topographical study on arteriosclerotic lesions at the bifurcations of human cerebral arteries. Heart Vessels 1985; *1*:70–73.
31. Duel TF: Polypeptide growth factors: Roles in normal and abnormal growth. Ann Rev Cell Biol 1987; *3*:443–492.
32. Chici CC, Klein L, DePalma RG: Effect of regenerated endothelium on collagen content in the injured artery. Surg Gynecol Obstet 1979; *148*:839–843.
33. Edwards WS: Arterial grafts: Past, present, and future. Arch Surg 1978; *113*:1225–1231.
34. Sauvage LR, et al: Interspecies healing of porous arterial prostheses. Arch Surg 1974; *109*:698–705.
35. Hanel KC, et al: Current PTFE grafts: A biomechanical, scanning electron, and light microscopic evaluation. Ann Surg 1982; *195*:456–462.
36. Clowes AW, Kirkman TR, Clowes MM: Mechanisms of arterial graft failure II: Chronic endothelial and smooth muscle cell proliferation in healing polytetrafluoroethylene prostheses. J Vasc Surg 1986; *3*:877–884.
37. Burkel WE, Graham LM, Stanley JC: Endothelial linings in prosthetic vascular grafts. Ann NY Acad Sci 1987; *516*:131–143.
38. Golden MA, Au YP, Kenagy RD, Clowes AW: Growth factor expression by intimal cells in healing polytetrafluoroethylene grafts. J Vasc Surg 1990; *11*:580–585.
39. Golden MA, et al: Platelet derived growth factor activity and mRNA expression in healing vascular grafts in baboons. J Clin Invest 1990; *87*:406–414.
40. Clowes AW, Kirkman TR, Reidy MA: Mechanisms of arterial graft healing. Rapid transmural capillary ingrowth provides a source of intimal endothelium and smooth muscle in porous PTFE prostheses. Am J Pathol 1986; *123*:220–230.
41. Sjolund M, et al: Arterial smooth muscle cells express platelet derived growth factor (PDGF) A chain, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype- and growth state-dependent manner. J Cell Biol 1988; *106*:403–413.
42. Collins T, et al: Cultured human endothelial cells express platelet derived growth factor A Chain. Am J Pathol 1987; *127*:7–12.
43. Limanni A, et al: Expression of genes for platelet derived growth factor in adult human venous endothelium: A possible non-platelet dependent cause of intimal hyperplasia in vein grafts and perianastomotic areas of vascular prostheses. J Vasc Surg 1988; *7*:10–20.
44. Wilcox JN, et al: Platelet derived growth factor mRNA detection in human atherosclerotic plaques by in situ hybridization. J Clin Invest 1988; *82*:1134–1143.
45. Barrett TB, Benditt EP: Platelet derived growth factor gene expression in human atherosclerotic plaques and normal artery wall. Proc Natl Acad Sci USA 1988; *85*:2810–2814.
46. Barrett TB, Benditt EP: (Platelet derived growth factor B Chain) gene transcript levels are elevated in human atherosclerotic lesions compared to normal artery wall. Proc Natl Acad Sci USA 1987; *84*:1099–1103.
47. Frangos JA: Elevation of platelet-derived growth factor in RNA in human endothelial cells by shear stress. Am J Physiol 1991; *260*:H642–646.
48. Kazlauskas A, DiCorleto PE: Cultured endothelial cells do not respond to a platelet-derived growth factor-like protein in an autocrine manner. Biochim Biophys Acta 1985; *846*:405–412.
49. Saksela O, Moscatelli D, Rifkin DB: The opposing effect of basic fibrobiast growth factor and transforming growth factor beta on the regulation of plasminogen activator activity in capillary endothelial cells. J Cell Biol 1987; *105*:957–963.
50. Sato Y, Rifkin DB: Autocrinic activities of basic fibrobiast growth factor: Regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. J Cell Biol 1988; *107*:1199–1205.
51. Grabowski EF, Jaffe EA, Weksler BB: Prostacyclin production by cultured endothelial cell monolayers exposed to step increases in shear stress. J Lab Clin Med 1985; *105*:36–43.
52. Gupte A, Frangos JA: Effects of flow on the synthesis and release of fibronectin by endothelial cells. In Vitro Cell Dev Biol 1990; *26*:57–60.
53. Miles LA, et al: A potential basis for the thrombotic risks associated with lipoprotein(a). Nature 1989; *339*:301–303.
54. Hajjar KA, Gavish D, Breslow JL, Nachman RL: Lipoprotein(a) modulation of endothelial cell surface fibrinolysis and its potential role in atherosclerosis. Nature 1989; *339*:303–305.

55. Dahlen GH, et al: Association of levels of liproprotein Lp(a), plasma lipids, and other lipoproteins with coronary artery disease documented by angiography. Circulation 1986; *74*:758–765.
56. Collen D, Juhan-Vague I: Fibrinolysis and atherosclerosis, Semin Thromb Hemost 1988; *14*:180–184.
57. Schleef RR, Birdwell CR: Biochemical changes in endothelial cell monolayers induced by fibrin deposition in vitro. Arteriosclerosis 1984; *3*:14–20.
58. Singh TM, Kadowaki MH, Glagov S, Zarins CK: Role of fibrinopeptide B in early atherosclerotic lesion formation. Am J Surg 1990; *160*:156–163.
59. Moake JL, et al: Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. Blood 1988; *71*:1366–1372.
60. Zwiebel JA, et al: High-level recombinant gene expression in rabbit endothelial cells transduced by retroviral vectors. Science 1989; *243*:220–222.
61. Nabel EG, et al: Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science 1989; *244*:1342–1346.
62. Nabel EG, Plautz G, Nabel GJ: Site-specific gene expression in vivo by direct gene transfer into the arterial wall. Science 1990; *249*:1285–1288.
63. Dichek DA, et al: Seeding of intravascular stents with genetically engineered endothelial cells. Circulation 1989; *80*:1347–1353.

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IDENTIFICATION OF FAMILIAL AORTIC ANEURYSMS USING DNA TECHNIQUE

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Aortic aneurysms frequently go undetected until rupture, and therefore are a significant cause of morbidity.[1,2] In the United States, aortic aneurysms are the 13th leading cause of death.[3] The mortality from aneurysms that are repaired electively is low (1 to 7 per cent depending on study cited),[4] whereas the mortality from ruptured aneurysms is as high as 90 per cent[5,6] and depends to a large extent on the time it takes for the patient to reach a medical center and for the condition to be diagnosed correctly. The cost to the health care system per survivor of a ruptured aneurysm is approximately tenfold that of elective repair of an aneurysm. The difference in mortality as well as cost indicates that efficient diagnosis of who is at risk, and who is not at risk, will both save lives and reduce cost to the health care system. If aortic aneurysms were genetic and if the gene or genes that harbor the mutations were identified, the powerful techniques of molecular biology could be used to determine who is, and who is not, at risk for developing aneurysms long before the aneurysm or aneurysms make themselves manifest. Early diagnosis of those at risk could also identify a sufficiently large group of patients to evaluate potential drug therapies, such as those that lower blood pressure.

The premise underlying the use of the DNA techniques of molecular biology is that aortic aneurysms are heritable. It was relatively recently that a familial clustering of aortic aneurysms was recognized. The first report of abdominal aortic aneurysms occurring in several members of the same family was by Clifton in 1977.[7] Since the initial report, a number of studies have been published that expanded on the observation and have estimated that from 6 to 36 per cent of aortic aneurysms are clustered in families.[4,8–15] The incidence of aortic aneurysms that are clustered in families, and specifically the incidence of aortic aneurysms in first-degree relatives within the families, strongly suggests a heritable or genetic cause for many, if not most, aneurysms. There are two general approaches using DNA techniques that can be used to identify the gene or genes harboring the defects (mutations) causing aortic aneurysms. The