Gene expression profiling of human aortic endothelial cells exposed to disturbed flow and steady laminar flow

ALAN R. BROOKS,1 PETER I. LELKES,2 AND GABOR M. RUBANYI1
1Gene Therapy Research Department, Berlex Biosciences, Richmond, California 94804; and 2Drexel University, Philadelphia, Pennsylvania 19104

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IN BOTH HUMANS AND ANIMAL models atherosclerosis tends to initially develop focally at branch points and bifurcations in the arterial circulation (3, 13, 38). The existence of these predilection sites points to the role of localized, nonsystemic factors in the susceptibility to atherosclerosis. Predilected regions of the arterial circulation are characterized by “disturbed,” oscillatory flow with low levels of shear stress (mean time-averaged shear stress \(\leq 1 \text{ dyn/cm}^2\)) (3, 22). In contrast, regions of the arterial circulation that are more resistant to atherosclerosis are exposed to unidirectional laminar flow (LF) with high levels of shear stress (mean shear stress >10 dyn/cm\(^2\)). These observations have given rise to the hypothesis that local mechanical forces created by the flowing blood play an important role in the initiation and progression of atherosclerosis (for recent reviews see Refs. 4, 15, 60). The single layer of endothelial cells (EC) that forms a barrier between the blood and the underlying smooth muscle cells of the blood vessel is directly exposed to hemodynamic forces. Changes in mRNA levels in cultured EC exposed to high-shear steady LF have been well documented (8, 14, 45). Several of the genes induced by LF have antiatherogenic potential, for example, manganese superoxide dismutase (MnSOD) and EC nitric oxide synthase (58). This has led to the concept that high-shear steady unidirectional LF induces the expression of atheroprotective genes in the endothelium (58) while downregulating proatherogenic genes, for example, vascular cell adhesion molecule 1 (VCAM-1) (2), endothelin converting enzyme (32), and angiotensin converting enzyme (48). Most studies on gene expression changes induced by high-shear LF have looked at single or at most a few genes that were selected because of their involvement in vascular disease. However, two recently published reports describe a more extensive analysis of the changes in mRNA levels induced in human umbilical vein EC (HUVEC) by high-shear steady unidirectional LF (12, 34), while Chen et al. (7) have used the Atlas cancer gene arrays to measure changes in mRNA levels in cultured human aortic ECs (HAEC) exposed to high-shear steady LF. In contrast to LF, little is known about how hemodynamic conditions similar to those found at branch points and bifurcations might affect gene expression in cultured EC. Various flow systems have been developed in an attempt to mimic some of the characteristics of the flow conditions that exist at branch points and bifurcations (for a recent review, see Ref. 53). An in vitro flow system consisting of a cone and plate apparatus has been used to generate either steady LF or turbulent
fl flow (TSS) both at a high shear stress of 10 dyn/cm² (58). Using this model, it was demonstrated that while LF induced sustained upregulation of the mRNA for MnSOD and cyclooxygenase-2, TSS did not (58). A more comprehensive analysis of gene expression changes induced in HUVEC by TSS and LF using cDNA arrays demonstrated that ~100 genes were differentially regulated by these two stimuli (12). Sprague and co-workers (35, 36) have exposed HAEC to steady LF at low shear stress (2 dyn/cm²) and demonstrated an upregulation of VCAM-1 mRNA after 6 h. Exposure of HUVEC to oscillatory LF (>5 dyn/cm², frequency 1 Hz, mean time-averaged shear stress of 0.2 dyn/cm²) in a parallel plate flow system resulted in a transient upregulation of VCAM-1 mRNA that peaked at 6 h and returned to baseline by 24 h. (6). The three flow systems described above impose either turbulent flow, low-shear unidirectional steady LF, or oscillatory LF, but none have combined these mechanical stimuli simultaneously. We have developed a flow system (9, 25, 51) that exposes EC to low-shear, pulsatile, nonsteady, non-unidirectional flow (disturbed flow, DF). HAECs exposed to this flow regimen exhibit rapid upregulation of VCAM-1 mRNA and protein expression that is sustained for up to 24 h (21, 26). Increased VCAM-1 expression has been detected at branch points in the arterial circulation of hypercholesterolemic rabbits prior to atheroma formation (17, 28) and also in human atherosclerotic plaques (44). ApoE-deficient mice exhibit a marked focal increase in VCAM-1 protein expression on the luminal surface of EC only in lesion-prone sites (39). Furthermore, VCAM-1 expression is upregulated in lesion-prone sites in the arteries of normal mice in the absence of diet-induced atherosclerosis (17, 39), indicating that upregulation of VCAM-1 is a good marker for the EC phenotype at atherosclerosis-prone areas. The fact that we observe sustained VCAM-1 mRNA and protein upregulation in response to exposure to DF in this in vitro model suggests that the mechanical forces created in this system may mimic those that exist at atherosclerosis-prone sites in vivo.

In this study we used cDNA microarrays and subtractive cloning methods to analyze changes in steady-state mRNA levels in cultured HAEC exposed to either DF or high-shear steady LF. This comprehensive and unbiased analysis demonstrated significant differences in the gene expression pattern exhibited by HAEC exposed to the two distinct flow patterns and allowed the identification for the first time of a collection of “proatherogenic” genes not reported so far to be regulated by flow/shear stress.

MATERIALS AND METHODS

Cell culture. HAEC were isolated from freshly harvested aortas according to Institutional Review Board-approved protocols and cultured in MCD131 medium supplemented with 25 μg/ml bovine brain extract (Clonetics), 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml Fungizone as previously described (19, 31, 56). The cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO₂-95% air and passaged at a 1:3 ratio with trypsin every 4–6 days. The endothelial nature of all cultures was routinely confirmed by their contact-inhibited, cobblestone morphology, by the ability to endocytose diiodocarbocyanine-labeled acetylated low-density lipoprotein, and by expression of the EC-specific markers von Willebrand factor and platelet EC adhesion molecule (PECAM-1/CD31). First passage cells of three individuals (aged between 15 and 45) who had no evidence of cardiovascular disease were pooled and used at passages 3–8. Additional confirmatory experiments were performed with primary HAEC purchased from Clonetics (donor no. 5043) or from Cascade Biologicals (pool of two donors), which were grown in the media recommended by the supplier and used at passage 4–7.

Flow apparatus. The design of the DF chamber has been described in detail previously (9, 25, 27, 50–53). It consists of a circular chamber (6.5 cm diameter, height of 1.5 cm) into which media enters from a round entrance port and then exits through a round orifice opposite to the entrance port. Flow of media through the chamber (100 ml/min) is driven by a peristaltic pump together with upstream and downstream reservoirs, which serve to generate a pulsatile flow with a sinusoidal pattern. The flow field in the chamber has been extensively characterized with high spatial (<100 μm) and temporal (10 ms) resolution (9, 25, 50–53). Nunc-Thermanox coverslips (13 mm diameter) were placed into the wells of 24-well plates, coated with human fibronectin, and then seeded with 50,000 cells/well of HAEC. Approximately 48 h after reaching confluence, the coverslips were transferred either into another 24-well plate with fresh media (static controls) or glued into depressions in the surface of the flow chamber using 20 μl of biological glue solution (42), which consisted of a mixture of thrombin and fibrinogen, such that the coverslips are flush with the surface of the chamber. The small quantity of thrombin in the glue (0.5 U/coverslip) was not sufficient to upregulate VCAM-1 mRNA expression as determined by TaqMan quantitative RT-PCR (Q-RT-PCR) (data not shown). Three of the coverslips (positions 1, 3, and 5) are located along the “jet stream” between the entrance and the exit of the flow, while coverslips in locations 2 and 4 are located in the areas of “recirculating eddies” which mimic some of the flow conditions in the areas of atherosclerotic predilection (27, 53). All the experiments reported here were performed with cells pooled from locations 2 and 4 in the chamber which experienced shear forces of <0.01 dyn/cm² and are located in the region of the chamber exposed to recirculating eddies. A detailed description of the hemodynamic conditions in this chamber, including numerical analyses of the flow fields (including the spatial distribution of peak mean flow velocities and calculation of shear stresses) in the areas of the recirculating eddies, are described by Samet and Lelkes (51). Photographs of the flow chamber with the location of the coverslips can be found in Lelkes et al. (27). A parallel plate chamber was used to generate steady LF as described (53). Briefly, HAEC were plated on fibronectin-coated single-well tissue culture chamber slides (Nunc). Forty-eight hours after reaching confluence, the cell-lined tissue culture slides were placed into a depression in the bottom half of the chamber. A Teflon gasket was used to create a 0.5-mm space above the cells when the top half of the chamber was attached. Media was circulated through the chamber at 100 ml/min by a peristaltic pump via upstream and downstream reservoirs, resulting in a wall shear stress calculated as 13 dyn/cm². The upstream reservoir was left open to the atmosphere to dampen the pulsatility of the pump, thereby generating steady LF. The height of the chambers was adjusted to create the necessary hydrostatic pres-
sure, and the flow rate through the chamber was calibrated using an in-line flow meter. The media used for all flow experiments was the same as that used to culture the cells, except that the amount of FBS was reduced to 5% (cells were adapted to the lower serum concentration for 24 h prior to initiating the flow experiments). The flow apparatus was placed inside a tissue culture incubator (5% CO₂, 37°C) for the entire course of the experiment (up to 24 h). Media was changed on the static control wells when the flow experiment was started.

**RNA isolation.** At the end of a flow experiment, the HAEC-lined coverslips were removed from the chamber and placed in 12-well plates containing media from the flow system. Microscopic examination showed that the monolayers remained intact in all of the flow experiments. The HAEC-lined coverslips were then washed once with PBS and lysed with Trizol reagent (Life Technologies). RNA was extracted from the lysate according to the manufacturer's instructions and then treated with 1 unit of RNase-free DNase I (Promega) per 20 μg of RNA in 1× transcription buffer (Ambion) at 37°C for 30 min to remove traces of contaminating genomic DNA. The RNA was recovered by extraction with phenol/chloroform/isoamyl alcohol and ethanol precipitation, dissolved in water and quantitated by measuring the absorbance at 260 nm. Alternatively, RNA was extracted using the RNeasy kit (Qiagen) and treated with DNase I on the column prior to elution with water. The quality of every RNA sample was determined by denaturing 250 ng with formamide followed by electrophoresis on a nondenaturing agarose gel. RNA with distinct 18s and 28s rRNA bands with no smearing was considered to be undegraded and suitable for further analysis. The lysate from coverslips in locations 2 and 4 were pooled from 3–5 independent flow experiments to generate the RNA used in array hybridizations and Q-RT-PCR.

**Tumor necrosis factor-a treatment of HAEC.** HAEC were plated on 10-cm tissue culture plates, allowed to reach confluence, and the RNA isolation. At the end of a flow experiment, the HAEC-lined coverslips were removed from the chamber and placed in 12-well plates containing media from the flow system. Microscopic examination showed that the monolayers remained intact in all of the flow experiments. The HAEC-lined coverslips were then washed once with PBS and lysed with Trizol reagent (Life Technologies). RNA was extracted from the lysate according to the manufacturer's instructions and then treated with 1 unit of RNase-free DNase I (Promega) per 20 μg of RNA in 1× transcription buffer (Ambion) at 37°C for 30 min to remove traces of contaminating genomic DNA. The RNA was recovered by extraction with phenol/chloroform/isoamyl alcohol and ethanol precipitation, dissolved in water and quantitated by measuring the absorbance at 260 nm. Alternatively, RNA was extracted using the RNeasy kit (Qiagen) and treated with DNase I on the column prior to elution with water. The quality of every RNA sample was determined by denaturing 250 ng with formamide followed by electrophoresis on a nondenaturing agarose gel. RNA with distinct 18s and 28s rRNA bands with no smearing was considered to be undegraded and suitable for further analysis. The lysate from coverslips in locations 2 and 4 were pooled from 3–5 independent flow experiments to generate the RNA used in array hybridizations and Q-RT-PCR.

**Atlas arrays.** Three micrograms of total RNA was converted to 32P-labeled cDNA using a mixture of primers specific to the set of genes on each array according the manufacturer's instructions (Clontech). This has the advantage of generating a relatively uncomplex hybridization probe containing cDNA from only the limited set of 588 genes on the array, thereby reducing nonspecific cross-hybridization, which can lead to false positives. Labeled cDNA was denatured, prehybridized in solution to human Cot1,000 DNA and then hybridized to the array in ExpressHyb hybridization solution (Clontech) containing 0.1 mg/ml of denatured sperm DNA at 68°C overnight. The arrays used were the human I array (catalog number 7740-1) and the human cardiovascular array (catalog number 7734-1), both of which contain cDNA fragments representing 588 unique human genes and 9 housekeeping genes, each spotted in duplicate. After extensive washing under stringent conditions (68°C in 0.1× SSC) the arrays were exposed to a storage phosphor autoradiography screen (FujiFilm Medical Systems) which were subsequently scanned on a Storm PhosphorImager (Molecular Dynamics). The resulting image files were imported into Array Vision (Imaging Research) image analysis software to locate and quantify spot intensities and perform background subtraction. The signal intensity was calculated as the mean pixel value minus a local regional background and reported for each spot on the array. The resulting intensity values were exported to GepView a proprietary array analysis program developed at Berlex for identification of differentially expressed genes. The signal intensities were normalized to the mean signal intensity of all the spots on the array. Duplicate spots of each cDNA were averaged. To compare two arrays (2 conditions), the fold changes and differences of the corresponding normalized signal intensities were first calculated. The differences were standardized by calculation of a z score = (I₁/I₂) – I₂, where I₁ is the mean, normalized intensity value for a given gene, and I₂ is the standard deviation of the signal intensity differences of all of the genes on the array. The z score is a measure of the statistical significance of the difference in signal intensity of a given gene on the two arrays compared with the average difference for all of the genes on the array. Differentially expressed genes with significant fold changes were identified by setting two thresholds, one for fold changes (2-fold) and one for z score (0.3), and taking the set of genes above both thresholds. It was determined empirically that using a z score of 0.3 eliminates most of the false positives with both intensity values close to background and ratios greater than 2 due to division by low intensity values. The probes for all Atlas array hybridizations were synthesized from RNA that was pooled from at least three independent flow experiments. For the 24-h DF experiments, two hybridizations were performed each with probe derived from different pooled RNA samples, and the mean fold change for each gene that showed significant fold change over static was calculated. A comparison of the fold changes from these two hybridizations showed that more than 70% of the changes were consistent. For the other experimental conditions, single-array hybridizations were performed. For a given RNA sample, the data generated from the human I and cardiovascular Atlas arrays were combined. For those genes that were differentially expressed and present on both types of arrays, the average of the fold change on the two arrays was calculated. The differences in expression between DF and LF at the two time points were calculated from the respective fold change to static as follows. If the fold change to static was positive in both LF and DF or negative in both LF and DF, then the fold change in DF was divided by the fold change in LF, and the reciprocal was taken as required. When the fold change to static was positive for DF and negative for LF or vice versa, then the fold changes were multiplied, taking the sign of the fold change in DF.

**Subtraction cloning.** One microgram total RNA from HAEC exposed for 24 h to either DF or to LF was converted into cDNA using the SMART cDNA synthesis kit (Clontech). The resulting cDNA was amplified by 17 cycles of long-distance PCR according the PCR-Select subtraction cloning protocol (Clontech). This limited number of cycles was carefully optimized to provide sufficient amplified cDNA without over-amplification. After purification the cDNA was digested with RsaI, ligated to special adapters according to the PCR Select protocol, and the PCR-based subtraction procedure was then performed (DF minus LF). Semi-quantitative PCR analysis of the subtracted cDNA demonstrated that the abundance of cDNA for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-tubulin, and ribosomal protein S9 was substantially reduced compared with un subtracted cDNA, whereas that of VCAM-1 and monocyte chemotactic protein 1 (MCP-1) was substantially increased. The subtracted cDNA was cloned into the TA cloning vector pCR2.1TOPo (Invitrogen), and white colonies (containing inserts) were picked at random and grown up in
were generated by RT-PCR using human VCAM-1-specific primers (5’ GAGAGATGCCGAGTATAGA 3’, 5’ GACATAGTGCGACTTTT 3’), and negative control cDNA was generated by PCR amplification of the inserts of plasmids containing the human testis-specific protein (GenBank accession no. X52128) and human semenogelin (accession no. XM_009525). Amplified cDNA was denatured with 0.3 M NaOH, spotted on nylon membranes (Schleicher and Schuell), neutralized with 0.5 M Tris, pH 7.5, then cross-linked with UV radiation. Hybridization of these cDNA arrays with 32P-labeled cDNA probes was performed according to the instructions in the PCR-Select Differential Screening kit (Clontech). This method uses oligonucleotides complementary to the common primer sequences found in all of the arrayed cDNA clones to prevent nonspecific hybridization to the identical sequences present in the labeled cDNA probe. To prepare the hybridization probe, cDNA was synthesized from 0.6 µg of total RNA extracted from HAEC exposed for 24 h to DF, LF, or no flow (static), or from HAEC under static conditions exposed to TNFα or vehicle control for 2 and 21 h using the SMART cDNA synthesis protocol (Clontech). This cDNA was subjected to the minimum number of PCR cycles needed to make sufficient cDNA for labeling (9 cycles of long-distance PCR were used). After purification, half of this minimally amplified cDNA was labeled with 32P using random primers and Klenow enzyme. 32P was incorporated equally into the probes which were subsequently hybridized to identical filter arrays of cDNA inserts that had been prepared as described above. In addition to clones from the subtraction library, these filters contained cDNA fragments from VCAM-1 as a positive control, the two testis-specific genes as negative controls, and seven housekeeping genes (GAPDH, GenBank accession no. X01677; 23 kDa highly basic protein, X56932; ribosomal protein S9, U14971; phospholipase A2, M86400; α-tubulin, K00558; HLA class I histocompatibility antigen C-α chain, M11886; and ribosomal protein S19, M81757). The filters were washed at high stringency (0.2× SSC, 68°C) and imaged on a Storm PhosphorImager (Molecular Dynamics). Signal intensities for each spot were determined as described above for Atlas arrays, except that the signal on the testes-specific cDNA fragments (which was essentially identical to the signal on blank areas of the membrane) was used for background subtraction. For comparing one array to another, the signal intensities were normalized to the mean signal of the seven housekeeping genes instead of normalizing to all genes because of the large number of genes showing increases in DF compared with static or LF. Normalized intensity values were used to calculate fold changes between the three flow conditions (DF vs. LF, DF vs. static, and LF vs. static). When the signal intensity of a given spot was less than twice the average background, that gene was considered to be not detectable under that condition. When expression was not detectable under any of the three flow conditions, that clone was eliminated from further analysis. For those clones whose expression was not detectable under one or two conditions, the intensity value in those conditions was artificially set to twofold over background before calculating the fold change. Hybridizations were performed once using probes derived from RNA that was pooled from at least three independent flow experiments. To confirm this data, arrays containing a subset of the genes were probed in the same way using probes derived from RNA pooled from two independent DF experiments on a different strain of HAEC purchased from Clonetics. In total, some 700 cDNA clones from the forward subtraction library (DF minus LF) were screened for differential expression under flow.

**Assays for DNA synthesis.** Following exposure to DF or LF, HAEC were incubated for an additional 4 h with 1 µCi [3H]thymidine. After three washes with ice-cold PBS, the cells were lysed on ice for 10 min with 10% trichloroacetic acid (TCA). The lysate was transferred into microcentrifuge tubes, centrifuged for 5 min at 10,000 g, and the pellet was resuspended in 10% SDS. Radioactivity in the TCA-insoluble pellet was determined in a liquid scintillation counter. For determining bromodeoxyuridine (BrdU) incorporation, we used a commercially available kit (Zymed). Briefly, following a 24-h exposure to flow conditions, HAEC were transferred to 24-well plates, incubated for an additional 24 h with the BrdU labeling reagent, and subsequently processed according to the manufacturer’s instructions. The coverslips were then mounted on microscope slides, and bright-field images were acquired from three random fields at a magnification of ×125. The number of BrdU-stained nuclei was evaluated by computer-aided image analysis (Image-Pro). A total of three independent experiments were performed, each in duplicate. The significance of differences between the groups was determined using unpaired Student’s t-test.

**Monocyte adhesion assay.** HAEC-lined coverslips (static controls, treated for 5 h with 10 ng/ml TNFα, or exposed to DF for 5 h) were placed into 24-well plates. To each well, 1 ml of MCD8131 medium containing 10% FBS and 5 × 105 monocytic U937 cells (ATCC CRL-1593.2) was added, and the monocytes were allowed to adhere for 30 min at 37°C, 5% CO₂. To investigate the role of VCAM-1 in monocyte adhesion, some of the monolayers were preincubated for 2 h with saturating concentrations of a neutralizing anti-VCAM-1 antibody (11.5 µg/ml) followed by two washes with PBS, prior to addition of the monocytes. At the end of the experiments, nonadhered monocytes were removed by three washes with PBS, and the cells were fixed for 15 min with 3.7% paraformaldehyde. Subsequently, monocytes adhesion was evaluated microscopically. For each experimental condition, three random phase-contrast microscopic images per well, captured with a high-resolution camera (series 68; Dage-MTI, Michigan City, IN) connected to an inverted microscope (Diatoph, Nikon, Tokyo, Japan) through a ×10 objective were projected onto a video monitor. Adhered monocytes per field (0.2 mm²) were counted manually on the screen. A total of three independent experiments were performed, each in duplicate. The significance of differences between the groups was determined by ANOVA followed by the Tukey comparison.

**Quantitative real-time RT-PCR.** To quantitate the mRNA levels of selected genes, the following sets of primers and probes were used. All primer probe sets were designed using the ABI Primer Express software and the sequence of the relevant human mRNA from GenBank. All probes were labeled at the 5’ end with 6-carboxyfluorescein (6-FAM) and contained the quencher dye 6-carboxy-X,N,N’,N’-tetramethylrhodamine (TAMRA) at the 3’ end. VCAM-1: F primer CATGGAATTCGACACCCCAACA, R primer GACCAAGCGGTTGATCTCTCTGG; probe AGGCAGAGTACGGAAACT-
RESULTS

ECs exposed to low-shear, pulsatile, nonunidirectional flow (DF) or steady high-shear LF exhibit distinct gene expression profiles. Confluent monolayers of HAEC plated on fibronectin-coated coverslips were exposed to DF inside a circular flow chamber (21, 27, 53). After exposure to this flow regimen for up to 24 h, the integrity of the monolayer was unaffected (Fig. 1). In addition, confluent monolayers of HAEC plated on fibronectin-coated chamber slides were exposed to steady high-shear LF in a parallel plate chamber (53). To characterize the changes in mRNA levels occurring in HAEC exposed to DF and LF, we used Atlas cDNA microarrays that survey a total of 1,086 distinct human genes, most of which have well-characterized biological functions. These arrays were chosen to encompass a wide range of functional classes as well as genes relevant to the cardiovascular system. An early (2 h) and a late (24 h) time point were examined so as to cover both early changes in mRNA levels which may be transient and the longer term consequences of exposure to mechanical forces.

In all array experiments, HAEC exposed to DF or LF were initially compared with HAEC that had been maintained under static conditions in parallel with and for the same duration as the cells exposed to flow. To correct for differences in probe specific activities and hybridization efficiencies, the array data were normalized to the mean signal of all of the genes on the array. The results were compared to static controls of the same duration as the cells exposed to LF or DF at the 2 h time point, which resulted in significant fold changes in the mRNA levels of 3–8% of the expressed genes (Table 1). It is noteworthy that exposure to LF for 24 h resulted in the downregulation of 50 genes (14.2% of those expressed), which is significantly greater than the number of changes observed under the other flow conditions tested. A list of all of the genes exhibiting significant changes after exposure to DF or LF compared with static conditions is shown in Fig. 2, grouped according to the following functional categories and with the fold changes observed normalized to the mean signal of all of the genes on the array.
to biological function and with a color scale to represent the magnitude of the fold changes. A comparison between LF and DF may give a more accurate picture of gene expression differences between EC in “athero-protected” regions of the arterial circulation and EC in atherosclerosis-prone vascular areas (bifurcations and branch points) and above atherosclerotic lesions. Therefore, a comparison of the relative gene expression in HAEC exposed to DF and LF was made at the two time points and is presented in the last two columns of Fig. 2. Within each functional group in Fig. 2 the genes have been sorted by the magnitude of the fold change in DF relative to LF.

In total, eight cell adhesion molecules (nos. 1–8, in Fig. 2) were regulated either by DF or LF at one or more time points. Consistent with our previous observations on enhanced VCAM-1 protein expression in the same in vitro model (21, 26), the array analysis was able to detect the early upregulation of VCAM-1 mRNA (Fig. 2, no. 6) by DF (7-fold at 2 h) and confirmed that this increase was sustained for at least 24 h. Using quantitative real-time RT-PCR (Q-RT-PCR), we found the mean upregulation of VCAM-1 mRNA by DF at 24 h was 3.5-fold in the same RNA samples used to generate the probes for the microarrays (Table 2). Using a different isolate of HAEC, we measured a 5.3–2.4-fold upregulation of VCAM-1 mRNA by 21 h of DF compared with static controls using Q-RT-PCR (data not shown). This confirms the array data and demonstrates that the sustained VCAM-1 response is consistent between HAEC isolated from different individuals. We were unable to measure a significant downregulation of VCAM-1 expression by LF using the Atlas arrays, because of the already low VCAM-1 hybridization signal under static conditions. However, by Q-RT-PCR, VCAM-1 mRNA levels were decreased by 65-fold upon exposure of the cells to LF (Table 2). E-selectin was also upregulated by DF in a sustained fashion, and this was confirmed by Q-RT-PCR (Table 2). Intercellular adhesion molecule 1 (ICAM-1) was only transiently upregulated by DF but significantly downregulated by LF at the 24 h time point. The other adhesion molecules exhibited downregulation by LF either early or in the case of PECAM, ICAM-2, and MUC18 only at the late time point. It is noteworthy that all eight adhesion molecules exhibit increased expression in DF compared with LF (see last two columns in Fig. 2).

A group of 10 genes (nos. 9–18 in Fig. 2) composed of chemokines/cytokines and their receptors were also found to be expressed at higher levels in HAEC exposed to DF compared with HAEC exposed to LF, mostly due to downregulation by LF. These include monocyte chemotactic proteins (MCPs) (MCP-1, mip-2α) and the neutrophil activating protein (NAP3). The downregulation of MCP-1 mRNA levels by 24 h of LF was also confirmed by semi-quantitative RT-PCR (Table 2). An increase in expression of the receptors for interleukin-2 (IL-2), IL-3, and IL-9 in DF compared with LF was also found. IL-17, Rantes, and interferon-γ, all of which are generally considered as proinflammatory molecules, were reduced in DF compared with LF.

Several genes with known roles in cell cycle control were modulated by DF or LF (Fig. 2, nos. 22–26). We observed a significant upregulation in LF compared with static of the mRNA for the cyclin-dependent kinase (CDK) inhibitor p57kip2, which was confirmed by Q-RT-PCR (Table 2). The tyrosine kinase wee1Hu was also upregulated in LF exposed cells compared with cells under static conditions, but unchanged by DF compared with cells with static conditions. Both of these proteins can function to block cell cycle progression. The upregulation of the growth arrest and DNA damage inducible protein 153 (GADD 153) by LF is consistent with an inhibition of cell cycle progression under high-shear conditions. Cyclin B1 and p55CDC were downregulated by DF but were not affected by LF, suggesting that DF also has an effect upon cell cycle. The downregulation of p55CDC by DF was confirmed by Q-RT-PCR (Table 2).

Several apoptosis-related genes were altered by DF or LF (Fig. 2, nos. 27–34), including a relative decrease of TSP-1 expression under LF conditions, which was confirmed by Q-RT-PCR (Table 2). At the 24 h time point, we also observed a decrease in p53 mRNA levels in HAEC exposed to LF compared with static conditions. A significant increase in the expression of the mRNAs for the heat shock proteins HSP70, -40, and -47 (Fig. 2, nos. 35–37) was observed under LF at the late time point, with little or no change under DF. The increase of HSP70 mRNA levels by LF was confirmed by Q-RT-PCR (Table 2).

Table 1. Quantitative summary of gene expression changes detected on Atlas arrays

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Early Time Point</th>
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<th></th>
<th>Late Time Point</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Expressed genes</td>
<td>Upregulated</td>
<td>Downregulated</td>
<td>Expressed genes</td>
<td>Upregulated</td>
<td>Downregulated</td>
</tr>
<tr>
<td>Static</td>
<td>466(43%)</td>
<td>13(2.0%)</td>
<td>16(1.5%)</td>
<td>624(57%)</td>
<td>19(5.4%)</td>
<td>50(14.2%)</td>
</tr>
<tr>
<td>LF</td>
<td>656(60%)</td>
<td>14(3.8%)</td>
<td>9(0.8%)</td>
<td>352(32%)</td>
<td>11(2%)</td>
<td>19(3.4%)</td>
</tr>
<tr>
<td>DF</td>
<td>371(34%)</td>
<td>13(2.0%)</td>
<td></td>
<td>558(51%)</td>
<td>11(2%)</td>
<td>50(14.2%)</td>
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Expressed genes were defined as those with un-normalized signal intensities (average of the two spots for each gene) that were greater than twofold above background. Data in each cell came from hybridization of probe made from a single RNA sample to the human I and cardiovascular (CV) arrays. Ninety genes are present on both the CV array and the human I array; thus the total number of distinct genes present on both arrays is 1,086 (588 × 2 – 90). Percentages are expressed as percentage of expressed distinct genes. ND, not determined; LF, laminar flow; DF, disturbed flow. Early time point was 2 h; late time point was 24 h.
There appears to be a coordinated reduction of integrin expression under LF (Fig. 2, nos. 38–44) and, therefore, a relative increase under DF. These changes include the two subunits of the fibronectin receptor (α5, β1), as well as integrins-α3, -α6, -αm, and -αv and VE-cadherin. A number of genes involved in thrombosis or fibrinolysis were regulated by either DF or LF (nos. 54–59 in Fig. 2). Among these are gpIIia and the thrombin receptor, both of which were downregulated by LF. The downregulation of gpIIia by LF was confirmed by Q-RT-PCR (Table 2). We also found that the mRNAs for endothelin 1 and endothelin converting enzyme 1 were downregulated under LF. The magnitude of the fold change is represented by the color gradations as listed on the scale. The first four columns show the fold changes to the matched static control from that particular experiment. Array data was processed as described in MATERIALS AND METHODS. The last two columns show the relative expression in DF compared with LF. For more details see MATERIALS AND METHODS.
Table 2. Quantitative real time RT-PCR analysis of changes in the mRNA levels of selected genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
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<tbody>
<tr>
<td>VCAM-1</td>
<td>4.36</td>
</tr>
<tr>
<td>E-selectin</td>
<td>-65.85</td>
</tr>
<tr>
<td>p57kip2</td>
<td>95.25</td>
</tr>
<tr>
<td>p55CDC</td>
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</tr>
<tr>
<td>HSP70</td>
<td>1.20</td>
</tr>
<tr>
<td>gpIIa</td>
<td>9.98</td>
</tr>
<tr>
<td>Jagged-2</td>
<td>-21.41</td>
</tr>
<tr>
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<tr>
<td>MCP-1*</td>
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<tr>
<td>MMP-14</td>
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<tr>
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<td>-25.90</td>
</tr>
<tr>
<td>MCP-1*</td>
<td>NT</td>
</tr>
<tr>
<td>MCP-1*</td>
<td>NT</td>
</tr>
</tbody>
</table>

Quantitative real-time RT-PCR (Q-RT-PCR) analysis of changes in the mRNA levels of selected genes. RNA samples from human aortic endothelial cells (HAEC) exposed to DF, LF, or tumor necrosis factor-α (TNFα) for 24 h, and the corresponding static or untreated controls were assayed for the genes shown by Q-RT-PCR. Quantities of the mRNA for each gene were normalized to the quantity of GAPDH in the same sample that were also measured by Q-RT-PCR. For each treatment, the normalized quantity in the treated group was divided by the corresponding control (static for flow experiments and vehicle treated cells for TNFα) to determine the fold change shown. The fold change for DF is the mean of two independent RNA samples, which each was pooled from three independent flow experiments. The fold change for LF was determined from a single RNA sample that was pooled from three independent flow experiments, whereas the TNFα data is from a single treatment. *Data for MCP-1 was generated by semi-quantitative RT-PCR. NT, not tested.

enzyme 1 (nos. 61 and 62, respectively, in Fig. 2) were downregulated by LF. The levels of mRNA encoding a number of proteins involved in regulating oxidative status were modulated by flow (Fig. 2, nos. 64–67). Both SOD-1 and SOD-2 were downregulated in DF compared with LF at 24 h, whereas glutathione S-transferase exhibited a relative increase in DF compared with LF. The matrix metalloproteinase (MMP)-14 and MMP-7 were downregulated by DF at 24 h, whereas MMP-1 and MMP-8 were increased by LF at the same time point (Fig. 2, nos. 68–71).

The mRNA level of several signaling molecules were affected by the different flow conditions (Fig. 2, nos. 72–81). For example, bone morphogenetic protein 4 (BMP4) and Smad1, both of which are components of the transforming growth factor-β (TGFβ) signaling pathway, were downregulated by DF at the late time point. In addition, various protein kinases were modulated by the different flow conditions at one or both time points. For example, the tyrosine kinase receptor RET and the tyrosine kinase RYK were downregulated by LF. We also observed significant changes in the mRNA levels of several transcription factors (Fig. 2, nos. 82–89), including relative increases of Hox-11 and Hox-7 and relative decreases in b-myb, Fra-1, and egr1 in DF compared with LF. Among the growth factors surveyed on these arrays, only three showed significant changes under flow (Fig. 2, nos. 90–92). The mRNA levels of several receptors were altered by flow; for example, the platelet-derived growth factor (PDGF) receptor α-chain was downregulated by DF, and slightly increased by LF but only at the early time point. The mRNA for two cytochrome P-450 enzymes
Table 2). The class II genes (upregulated by DF but little changed by LF) include Jagged-2, a G protein-coupled receptor with no known function, Wnt13, and translation initiation factor 4E, as well as two genes with unknown functions. Q-RT-PCR confirmed the upregulation of Jagged-2 mRNA by DF and showed that this gene was also downregulated by LF (Table 2).

Of the class III genes (downregulated by LF but little changed by DF), seven are known genes with known biological functions, whereas the other four are present in DNA databases but have no known function. A cDNA probe fragment representing a portion of the 3'-untranslated region of the connexin 40 (Cx40) mRNA detected a significant 70-fold downregulation of the corresponding transcript by LF (Fig. 3, no. 24). Interestingly, a second cDNA fragment from a different region of the Cx40 locus that was present on the same hybridization array did not detect this change (data not shown). Both cDNA probe fragments align perfectly with the genomic sequence for Cx40, although both lie outside of the GenBank Cx40 cDNA (accession no. U03486). The difference in expression pattern detected by the two probes may be explained by alternative splicing. Additional genes in class III include Dickkopf-2, the fibulin-like ECM protein 1, the chemokine HuMig, BMP6 (a member of the TGFβ family), and ribosomal protein S7. Downregulation of thrombin receptor I by LF as measured in this array analysis is consistent with the data from the Atlas arrays (see Fig. 2).

Gene expression profile induced by TNFα is distinct from that induced by DF. To investigate the possible role of nuclear factor κB (NF-κB) activation in gene regulation under DF conditions, we also surveyed gene expression changes induced in HAEC exposed to 10 ng/ml TNFα, a well-characterized activator of the NF-κB pathway (30). Microarray analysis indicated distinct differences in the response of HAEC to TNFα and DF (data not shown). We therefore used Q-RT-PCR to measure changes in the mRNA levels of selected genes induced by 24-h exposure to TNFα and compared this to the response to 24-h exposure to DF (Table 2). As expected, E-selectin and VCAM-1 were upregulated by TNFα, and these genes were also up-
regulated by DF. However, while VCAM-1 mRNA was upregulated more than 70-fold by TNFα, DF induced a much smaller 3.5-fold upregulation of this mRNA. Similarly, we observed large differences in the magnitude of upregulation of E-selectin mRNA by TNFα and DF (10-fold vs. 90-fold, respectively). Although the mRNA level of p55CDC was slightly upregulated by TNFα (2.5-fold), it was strongly downregulated by DF (21-fold). Similarly, HSP70 and Jagged-2 were regulated in opposite directions by TNFα.

Effect of DF and LF on DNA synthesis in HAEC. The gene expression profile of HAEC exposed to LF predicts a reduction in cell proliferation under this flow condition compared with HAEC kept under static conditions. To analyze the functional consequences predicted by the differential gene expression profile, we assessed DNA synthesis in HAEC exposed for 24 h to DF, LF, or static conditions by measuring [3H]thymidine and BrdU incorporation. The results (Fig. 4) demonstrate a statistically significant 25% decrease in [3H]thymidine incorporation and a 44% reduction in the number of BrdU-positive nuclei in cells exposed to LF compared with static controls. In contrast, exposure of HAEC to DF resulted in a significant 60% increase in [3H]thymidine incorporation and a 225% increase in the number of BrdU-positive nuclei compared with HAEC under static conditions (Fig. 4).

Effect of DF and LF on monocyte adhesion to HAEC. The gene expression profile of HAEC exposed to DF predicts an increase in the expression of the proinflammatory molecules VCAM-1, E-selectin, MCP-1, and RP1 under this flow condition. To analyze the functional consequence predicted by the differential gene expression profile, we measured monocyte adhesion to monolayers of HAEC that had been exposed for 5 h to DF, static conditions, or static conditions in the presence of the inflammatory cytokine TNFα. The results (Fig. 5) demonstrate that, as expected, TNFα induced a statistically significant (P < 0.05) 32-fold increase in monocyte adherence. Exposure to DF also resulted in a statistically significant (P < 0.05) 16-fold increase in monocyte adherence. The TNFα- and DF-induced increases in monocyte adherence could be partially blocked by preincubation with a neutralizing antibody to VCAM-1. For both stimuli, the reduction by the antibody was statistically significant (P < 0.05).

DISCUSSION

Vascular EC in straight sections of the arterial system are continuously exposed to high-shear unidirectional LF and are adapted to function normally under these conditions, i.e., form a quiescent, anti-thrombotic monolayer. Numerous studies in vitro have demonstrated that high-shear LF may be “atheroprotective,” mostly by downregulation of proatherogenic genes (8, 14, 45). In contrast, flow conditions at branch points and bifurcations are disturbed with low or oscillatory shear stress (3, 22, 57). Very limited data have been reported on the effects of these hemodynamic flow conditions upon EC function and gene expression, and none of these studies (6, 12, 35, 36, 58) has examined EC exposed simultaneously to low-shear, non-unidirectional, pulsatile flow. We developed a novel in vitro flow chamber that generates low-shear, nonsteady, pulsatile, non-unidirectional flow (disturbed flow, DF). At the flow rate used, the Reynolds number in this chamber was calculated to be about 500, and the Womersley number was calculated to be 10 (51). This defines the average flow in the chamber as essentially laminar, although we cannot exclude the possibility that at peak flow the cells might experience transitional flow. Cells from positions 2 and 4 of the chamber, which are located in the region of recirculating eddies, experience nonsteady, non-unidirectional flow with low computed time-averaged shear stress levels of <0.01 dyn/cm². The cells on both these coverslips are exposed

Fig. 4. Effects of DF and LF upon DNA synthesis in HAEC. Confluent monolayers of HAEC were exposed for 24 h to DF, high-shear LF, or static conditions. At the end of the experiments the proliferative status of the cells was assessed by nuclear incorporation of [3H]thymidine (A) and bromodeoxyuridine (BrdU, B) (for details, see MATERIALS AND METHODS). Results are expressed as means ± SD for at least three independent experiments, each performed in duplicate. *P < 0.001 compared with static. **P < 0.001 compared with LF.
markers of EC phenotype at branch points or bifurcations of the arterial system and early atherosclerosis at these sites.

Cultured EC are no longer exposed to hemodynamic forces, specifically high-shear LF, a key stimulus that is required to maintain their physiological state. Most published data on the effects of flow on EC function and gene expression comes from a comparison of high-shear LF vs. static conditions. We have attempted to obtain a more relevant picture of the gene expression changes induced in EC by “pathological” flow conditions by comparing the changes in mRNA levels induced by DF and steady high-shear LF using microarrays and subtraction cloning. To date, no microarray-based analysis comparing the gene expression changes induced in vascular EC by high-shear LF and low-shear non-unidirectional or disturbed flow (or other conditions similar to DF) has been reported. Subtraction cloning proved to be an effective way to identify flow-regulated genes, as only 5 of the 32 genes discovered with this approach were also identified using arrays of preselected genes. To confirm the microarray data, we used Q-RT-PCR to measure the relative expression of nine selected genes in HAEC exposed for 24 h to DF, LF, and static conditions (Table 2). The results from this analysis agreed well with the array data in terms of both the directions of the change and, for most of the genes, the magnitude of the change. The genes we selected for Q-RT-PCR cover the most relevant functional classes with respect to the effect of DF and LF upon EC, for example, the class of genes involved in cell adhesion (VCAM-1, E-selectin), inflammation (MCP-1), proliferation (p57kip2), apoptosis (TSP-1, Jagged-2, HSP70), and thrombosis (gpIIbIIIa).

Our results demonstrate that DF and LF induce distinct patterns of gene expression. The genes that were regulated by hemodynamic forces could be grouped into nine classes according to their response to exposure to DF and LF for 24 h (Table 3). Although genes downregulated by LF but unaffected by DF (class III) constituted the largest group (50 genes), the finding that 49 genes were regulated by DF (16 in class I, 10 in class II, and 23 in classes V–IX) demonstrates that DF is more than simply the absence of LF. The fact that the DF-induced gene expression pattern is significantly different from that seen under static conditions indicates that although DF represents very low shear stress values, the coexistence of other variables (e.g., pulsatile non-unidirectional, nonsteady flow), is sufficient to induce a biological response in EC. In total, more than 130 genes were regulated by DF or LF at either the early or late time points. To the best of our knowledge, flow regulation of the mRNA levels of not more than 22 of these genes has been reported previously, including the recent microarray-based analyses of HUVEC (12, 34) and HAEC (7). Of particular interest are the class I genes (Table 3; Fig. 3) that were upregulated by DF and downregulated by LF. These genes exhibit the largest difference in expression between DF (that is, similar to conditions that exist at areas of the arterial circulation predilected for athero-
### Table 3. Gene expression changes induced by DF and LF at 24 h grouped by expression profile

<table>
<thead>
<tr>
<th>Expression Class</th>
<th>Regulation Pattern</th>
<th>Number of Genes Found by Atlas Array</th>
<th>Number of Genes Found by Subtraction Cloning</th>
<th>Total</th>
<th>Genes Common to Both Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>DF ↑ LF ↓</td>
<td>1</td>
<td>15</td>
<td>16</td>
<td>4*</td>
</tr>
<tr>
<td>II</td>
<td>DF ↑ LF →</td>
<td>4</td>
<td>6</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>DF → LF ↓</td>
<td>41</td>
<td>11</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>IV</td>
<td>DF → LF ↑</td>
<td>13</td>
<td>0</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>DF ↓ LF →</td>
<td>10</td>
<td>0</td>
<td>10</td>
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<td>DF ↓ LF ↓ ↓</td>
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<tr>
<td>VIII</td>
<td>DF ↑ LF ↑</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>IX</td>
<td>DF ↑ ↑ LF ↑</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Regulated genes (at least twofold change) from Atlas arrays and subtraction cloning were grouped according to their expression profile in LF and DF. −−, no significant change compared to static; ↑, increased compared to static; ↓, decreased compared to static; LF ↓ ↓, magnitude of downregulation in LF at least twofold greater than in DF; DF ↑ ↑, magnitude of increase in DF at least twofold greater than the increase in LF. *VCAM-1, MCP-1, E-selectin, and TSP-1 fell into class I on arrays generated from subtraction clones, but into class II on Atlas arrays. This is a function of the array technology (Atlas was unable to detect the reduction by LF due to the already low signal intensities for these genes with the static probe).

Reports of the downregulation of the thrombin receptor (41), endothelin-1 (54), endothelin converting enzyme-1 (32), and MCP-1 (55) by high-shear LF are consistent with the microarray data presented here. The upregulation of VCAM-1, ICAM-1, and E-selectin by DF is consistent with a previous report showing upregulation of these genes by oscillatory flow, another model of low shear stress (6). However, in contrast to the sustained upregulation of VCAM-1 mRNA by DF, oscillatory flow induced a transient upregulation of VCAM-1 mRNA that peaked at 4 h and declined to baseline by 24 h (6). For several genes the pattern of flow regulation we observed differs from earlier reports. For example, upregulation of ICAM-1 by high-shear LF has been described using HUVEC (37), whereas we observed a downregulation of this gene after 24 h of LF. Similarly, SOD-2 was shown to be upregulated in HUVEC exposed to laminar shear stress (LSS) for up to 6 h (58), whereas we found SOD-2 unchanged by LF but downregulated by DF at 24 h. Urbich et al. (61) found that the mRNA for integrins-α5 and -β1 were increased in HUVEC by exposure to LF, whereas we observed a decrease in the mRNA levels for these genes in HAEC under LF. We surmise that some of these discrepancies may be due to differences in the cell type, flow model, or time point analyzed.

The mRNA levels of several cell adhesion molecules (E-selectin, VCAM-1, ICAM-1, sialophorin, CD44, ICAM-2, PECAM, and MUC18) exhibited a relative increase in expression under DF compared with LF conditions (Fig. 2, nos. 1–8). The importance of VCAM-1 in early atherosclerosis is well established (10), presumably via the ability of this molecule to promote extravasation of monocytes. Our finding that additional adhesion molecules are upregulated under DF compared with LF suggests that these may also play a role in the interaction of the endothelium with inflammatory cells at predilected areas. For example, sialophorin or CD43 (Fig. 2, no. 1) is a member of the sialomucin family, which act as scaffold molecules to present selectin carbohydrate ligands, thereby increasing the strength of the interactions between leukocytes and atheroprotected areas. This class contains genes with clearly defined roles in atherosclerosis, such as VCAM-1, E-selectin, and MCP-1, as well as genes of known function whose involvement in atherosclerosis has yet to be proven, such as TSP-1. There are also genes in class I, such as KIAA0487 and 24775 mRNA, for which a function remains to be identified. The finding that one set of genes can respond to both DF and LF, albeit in different directions, while others respond only to DF or only to LF suggests that distinct signaling pathways are responsible for the response to these two stimuli.

An association between the upregulation of VCAM-1 expression and activation of the transcription factor NF-κB under conditions of steady LF at low shear stress (2 dyn/cm²) has been reported (35, 36). Transcription of the VCAM-1 gene is inducible by cytokines, and this is mediated by binding of NF-κB to the proximal promoter (16, 40). The activation of NF-κB is arguably the most important response of EC to TNFα in terms of induction of gene expression (30, 47), although other signaling pathways are also affected, for example, sphingosine (30, 62). Therefore, if the observed changes in mRNA levels in HAEC in response to DF were due entirely to the activation of NF-κB, then the genes upregulated by DF should also be upregulated by TNFα. By microarray analysis we found that although 8 genes were upregulated by both DF and TNFα, 37 genes were differentially regulated by these two stimuli (data not shown). The differential regulation by DF and TNFα was confirmed for five genes (VCAM-1, E-selectin, p55CDC, HSP70, and TSP-1) by Q-RT-PCR (Table 2). Importantly, these results demonstrate that the gene expression pattern elicited by DF is qualitatively different from that produced by an activator of the NF-κB pathway, suggesting that NF-κB is not the only signaling pathway involved in the response to DF.

In most cases the expression data presented here for genes known to be regulated by flow/shear stress are in agreement with published data. For example, earlier...
and EC (23). The relevance of our model was strengthened by the finding that exposure of HAEC to DF resulted in increased monocyte adherence (Fig. 5). The ability of a neutralizing antibody to VCAM-1 to partially block this increased monocyte adherence supports the concept that the observed changes in cell adhesion molecule gene expression have functional consequences.

The largest functional group of flow-regulated genes comprises mediators of inflammation (Fig. 2, nos. 9–21; Fig. 3, nos. 15 and 26). According to the literature published to date, only one of these genes, MCP-1, has previously been shown to be regulated by flow (55). The finding that the mRNAs for additional proinflammatory molecules (CDC27L receptor, NAP3, mip2α, receptors for IL-2, -3, -9, CD72, IL-6, IL-8, RP1, and HuMig) are present at higher levels in DF relative to LF (mostly due to the downregulation by LF) suggests that exposing EC to DF could contribute to the persistent state of low level inflammation characteristic of atherosclerosis-prone regions (49).

Both the Atlas arrays and Q-RT-PCR revealed that the mRNA for p57kip2 was significantly increased in HAEC exposed to LF for 24 h. High levels of p57kip2 would be expected to block cell cycle progression at the G1→S transition by binding to and inhibiting the activity of CDKs such as CDK2 and CDK4 (24, 33). The LF-induced upregulation of the mRNA for wee1Hu (Fig. 2, no. 25), which is a negative regulator of p34CDC2 (59), would also be expected to inhibit cell cycle progression, but at the G2 checkpoint. These results show that in our experiments LF induced the expression of two cell cycle regulators capable of blocking cell cycle progression at both the G1→S and G2→M transitions. The anti-proliferative effect of high-shear steady LF on EC is well established, and upregulation of the CDK inhibitor p21cip1 by LF has been demonstrated in both HUVEC and bovine aortic EC (BAEC) (1, 29). A careful examination of our Atlas array data showed that the signal for p21cip1 mRNA was increased in LF compared with static at 24 h, but because of the low signal intensity this difference did not meet the criteria for significance. Consistent with previous reports and the gene expression data presented here, we showed a statistically significant decrease in DNA synthesis in HAEC exposed to LF (Fig. 4). The mRNA levels of p57kip2 and wee1Hu were unchanged in DF conditions compared with static and therefore were relatively lower in DF than under LF conditions. This suggests that like static cells, DF exposed cells may have lost the cell cycle block conferred by the increased expression of these genes under LF conditions and therefore may exhibit a proliferative phenotype. In our model the rate of DNA synthesis in HAEC exposed to DF was increased compared with cells under static conditions. Previously, Davies et al. (11), using a model of turbulent flow that generates different hemodynamic conditions to those used in our studies, reported an increase in the number of [3H]thymidine-labeled nuclei in BAEC exposed to low- or high-shear turbulent flow.

Several genes that have been shown to modulate apoptosis and/or angiogenesis were regulated by flow (Fig. 2, nos. 27–37; Fig. 3, nos. 3 and 18). The levels of TSP-1 mRNA were upregulated by DF and downregulated by LF (Fig. 3, no. 3; Table 2). TSP-1 is a pleiotropic molecule, but one of its functions in EC is to block angiogenesis by activating apoptosis (18, 43). The mRNA for p53 was downregulated by LF compared with static but unchanged by DF and therefore relatively higher in DF compared with LF. p53 responds to a variety of stimuli, including DNA damage, and this can result in the induction of apoptosis. A coordinated upregulation of HSPs (HSP70, -40, and -47) was detected after 24-h exposure to LF (Fig. 2, nos. 35–37; Table 2). A number of the HSPs including HSP70 are anti-apoptotic due at least in part to their ability to inhibit formation of the apoptosome complex (5). Jagged-2, a ligand for the receptor notch, and potential anti-angiogenic molecule (63) was upregulated by DF and also downregulated by LF (Fig. 3, no. 18 and Table 2). Thus our results demonstrate flow-induced changes in the mRNA levels of several genes (TSP-1, p53, HSPs, and Jagged-2) all of which are consistent with increased apoptosis under DF conditions. The combination of increased apoptotic rate and increased proliferation rate under DF conditions might lead to increased EC turnover that could result in focal denudation of the blood vessel thereby exposing the procoagulant surface beneath.

The relative increase in mRNA levels of endothelin 1 and endothelin converting enzyme 1 in DF compared with LF conditions (Fig. 2, nos. 61 and 62) would be predicted to result in endothelium-mediated vasosconstriction under conditions of DF. The relative increase in expression of the thrombin receptor (Fig. 2, no. 54; Fig. 3, no. 32) and PAI-1 (Fig. 3, no. 12) in DF compared with LF is predicted to cause a shift to a procoagulant state under DF. Although a decrease in PAI-1 mRNA in HUVEC exposed to shear stress has been reported (20), we extend this observation by demonstrating not only the downregulation under LF but also upregulation by DF such that the net change is more than 10-fold. Increased PAI-1 at sites exposed to DF would be expected to reduce the potential for clot lysis. The level of the mRNA for gpIIIa, one of the subunits of the gpIIbIIIa complex, an important receptor for activated platelets, was reduced under LF compared with static cells (Fig. 2, no. 55; Table 2), which might reduce the capacity to bind activated platelets, leading to reduced platelet deposition in arterial areas exposed to high-shear LF.

Several genes involved in signaling, including two members of the TGFβ family of signaling molecules (BMP4 and Smad1), protein kinases (e.g., RYK, RET, FAST, and MAPK6), and the ligand Dickkopf-2 (Dkk2) were regulated by either DF or LF. This suggests that exposure to flow may alter the capacity of EC to respond to various stimuli.

In summary, these data demonstrate that DF is not simply the absence of LF but in fact represents a distinct biomechanical stimulus that has a profound
impact upon the gene expression profile of human aortic EC in culture. Microarray and subtraction cloning analysis revealed regulation of genes hitherto unknown to be flow regulated. Q-RT-PCR analysis confirmed the data obtained from the microarrays. For some of the genes whose expression was altered by DF or LF, corresponding changes in EC function (monocyte adhesion and DNA synthesis) could be demonstrated. The upregulation of VCAM-1 and increased monocyte adhesion to EC exposed to DF was similar to EC in vivo at atherosclerosis-prone regions, confirming the relevance of our model system for in vivo conditions. Many of the genes upregulated by DF are potentially proatherogenic. In addition, we identified genes whose regulation by LF would be predicted to be antiatherogenic. Thus it is likely that the presence of low-shear non-unidirectional, nonsteady, pulsatile flow (DF) together with the absence of LF at predilected areas may predispose the endothelium to the development of atherosclerosis. Although the described studies lead to important new observations, they represent findings in a model system. The relevance of the gene expression changes detected in these in vitro models to gene expression differences at atherosclerosis-prone sites in vivo must be studied in the future.

We are grateful to Dr. Mark Samet and Mishel Davis for meticulously carrying out the flow experiments and preparing the RNA samples used for some of these analyses. We thank Sarah Himmerich for development of the VCAM-1 TaqMan assay, Marty Bartholdi for microarray image analysis and assistance with the preparation of figures, Mary Rossier and Eileen Paulo for assistance with cell culture, and John McClary for DNA sequencing. This study was supported in part by an American Heart Association Grant-in-Aid (to P. I. Leikes).

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