Fluid Shear Stress Induction of the Transcriptional Activator c-fos in Human and Bovine Endothelial Cells, HeLa, and Chinese Hamster Ovary Cells

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The c-fos protein belongs to a family of transcriptional cofactors that can complex with proteins of the Jun family and activate mRNA transcription from gene promoters containing an activator protein 1 (AP-1) binding element. The shear stress inducibility of the c-fos protein was studied in human and animal cell lines of vastly different origins. Primary human umbilical vein endothelial cells (HUVEC), bovine aortic endothelial cells (BAEC, passage 2-14), HeLa cells, and Chinese hamster ovary (CHO) cells were subjected to steady laminar shear stress using a parallel plate flow apparatus. After 1 h of flow exposure at 25 dyn/cm^2, the c-fos levels in nuclei of shear stress HUVEC, BAEC, HeLa, and CHO were 5.4 ± 2.0 (n = 3), 2.25 ± 1.38 (n = 6), 2.14 ± 0.07 (n = 8), 1.92 ± 0.58 (n = 2) times higher, respectively, than in matched stationary controls. Flow exposure at 4 dyn/cm^2 caused no enhancement of c-fos levels in any of the cell lines tested, but caused significant reduction in c-fos expression in the HeLa cells. The c-fos induction by shear stress could be blocked by pharmacological agents. For example, the flow induction of the c-fos protein levels was blocked by 50% with the preincubation of HUVEC with a protein kinase C inhibitor, H7 (10 μM) and blocked completely in HeLa cells preincubated with the phospholipase C inhibitor, neomycin (5 mM). The minimum time of shear stress exposure required to induce the c-fos protein expression in HeLa cells was found to be as low as 1 min. By Northern analysis, the c-fos mRNA levels were found to be elevated in BAEC, CHO, and HeLa cells exposed to 25 dyn/cm^2 for 30 min. These studies indicate that c-fos induction is a consistent genetic response in a variety of mammalian cells that may alter cellular phenotype in mechanical environments.
Key words: c-fos protein • endothelium • hemodynamics

INTRODUCTION

The mechanical environment in bioreactor systems includes forces generated on cells by bulk liquid flow, solid–liquid interactions, and the effects at gas–liquid interfaces, including the formation and disruption of bubbles.[7,15,22,42] Physical forces also occur in natural and artificial tissue structures and can influence cellular physiology in vivo. For example, fluid shear stress can have a profound influence on the function of cells, ranging from altered metabolism to cell lysis.[5,7,11,24,45] The interactions of various types of cells with fluid flow regimes and gas–liquid interfaces typical of bioreactors have been studied in detail, particularly with respect to cell viability.[13,14] The shear sensitivity of endothelial cells, hybridoma cells, red blood cells, insect cells, and other animal or plant cells has also been studied with respect to cellular viability.[8,12,19] Some of the detrimental effects of hydrodynamic forces in sparged systems can be diminished by the addition of polymers, such as Pluronic F-68, methylcellulose, or serum.[12,28]

In the sublytic regime, mechanical forces involving cellular deformation can also play an important role in intracellular signaling and gene regulation. Direct mechanical deformation of cells has been shown to increase intracellular calcium of several cell types.[38,43] The mechanical stretching and relaxation of rat mesangial cells induces protooncogenes such as c-fos and zif/erg-1 and prostaglandin H_2 (PGH_2) synthase-2 (mitogen inducible form).[1] Hyperosmotic-induced cell shrinking stimulates the expression of the tissue plasminogen activator (tPA) gene in endothelial and HeLa cells.[20] Recently, both fluid shear stress and cyclic strain have been shown to upregulate endothelial nitric oxide synthase gene expression.[3,29,34] In endothelial cells, fluid shear stress is well-recognized to cause changes in morphology, metabolism, gene regulation, protein synthesis, and membrane ion permeabilities.[8,10,30,31] However, less is known about the genetic response of other commonly used animal cell lines.

Mammalian cell lines possess distinct phenotypes that are strongly dependent on the tissue of origin, time in culture, and culture conditions. Given the numerous examples of tissue-specific gene regulation, the response of mammalian cells to physical forces may be quite heterogeneous, depending on the gene, the cell, and the precise nature of the force. For example, shear stress produced a biphasic response of the monocyte chemotactic protein 1 (MCP-1) gene expression in human umbilical vein endothelial cells (HUVEC), HeLa, glioma cell lines, and skin fibroblasts.[40] Different levels of shear stress can modulate gene expression, causing either

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upregulation[17] or downregulation[17,23,37] of the endothelin gene, depending on the magnitude of shear stress. Interestingly, the stretching of endothelial cells causes an induction of the endothelin gene.

The present study has sought to evaluate the changes in expression of the c-fos gene in mortal and immortal human cell lines (HUVEC and HeLa) and mortal and immortal animal cell lines [bovine aortic endothelial cells (BAEC) and Chinese hamster ovary (CHO)] in response to hemodynamic forces. The protooncogenes c-fos and c-jun are the members of the AP-1 (activator protein-1) family of transcription factors, which mediate transcriptional stimulation through their interaction with a specific DNA sequence of TGA(C/G)TCA (20) known as the TPA-responsive element (TRE) or AP-1 binding element.[21] Transcriptional activation of genes through the protein kinase C pathway occurs via this sequence. We report that c-fos protein and mRNA levels are elevated in human and bovine endothelial cells, HeLa, and CHO cells. These results indicate that the responsiveness of cultured animal cells to laminar shear stress is not specific to only those animal cells derived from hemodynamically active origin.

MATERIALS AND METHODS

Cell Culture and Shear Stress Exposures

Primary human umbilical vein endothelial cells were harvested and cultured as previously described[10,33] and grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 20% heat-inactivated newborn calf serum (NCS), 0.30 mg/mL glutamine, 150 U/mL penicillin, and 0.15 mg/mL streptomycin (Gibco Laboratories, Grand Island, NY) (20% complete DMEM). Bovine aortic endothelial cells, HeLa, and CHO cells were grown to confluence in 10% complete DMEM. In some experiments, slides were treated with human plasma fibronectin (Gibco Laboratories, Grand Island, NY) at 2 $\mu g/cm^2$ prior to seeding the cells.

Monolayers were exposed to a steady laminar shear stress of either 4 or 25 dynes/cm^2 in individual parallel plate systems with recirculating medium [20 m*M* *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (HEPES) and 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.4, for HUVEC and 10 m*M* HEPES in DMEM, pH 7.4, for BAEC, HeLa, and CHO cells] as previously described[10,33] or maintained in a CO_2 incubator. No adenosine triphosphate (ATP) was present in the perfusion medium. Prior to shear exposure, primary confluent monolayers of HUVEC were serum-starved for 48 h in 0.5% NCS-supplemented DMEM to establish quiescence in the monolayers. Confluent monolayers of BAEC (passage 2-14) and CHO were serum starved in 1.5% complete DMEM for 48 h. HeLa cells were grown throughout in 10% complete DMEM. After various exposure times, immunocytochemistry or RNA extraction was carried out on the monolayers.

Immunofluorescence Staining and Digital Epifluorescence Microscopy

A solution of 2% paraformaldehyde, 0.5% Triton-X-100 in PBS at 4°C was used to fix cells of the individual glass slides by incubating the slides in this solution for 15 min. The slides were then washed with 3% BSA, 0.5% Triton X-100 in PBS and incubated with 50 μL of sheep polyclonal antibody against c-fos (Cambridge Research, Inc., Wilmington, DE) at a dilution of 1:20 (3% BSA, 0.5% Triton X-100 in PBS) for 2 h. The slides were washed three times with 5 mL of 3% BSA, 0.5% Triton X-100 in PBS solution. Each slide was then incubated with 50 μL of fluorescein donkey anti-sheep IgG (H + L) conjugate (Molecular Probes, Inc., Eugene, OR) (1:20 dilution) for 1 h. The slides were then washed three times with PBS. A Leitz Aristoplan microscope (100× objective) with image intensified-charge coupled diode camera assembly (GenIIsys-CCD72, Dage-MTI, Michigan City, IN) was used for epifluorescence visualization to detect the fluorescence emission. The noise reduction of the video signal was conducted with a Hamamatsu Argus-10 image processor with no gray scale stretching or contrast enhancement. The video signal was recorded on a $\frac{1}{2}$ in. videotape for digital image analysis using a Quickcapture framegrabber (Data Translation, Marlboro, MA) supported on a Macintosh IIci with image analysis software (NIH Image 1.47).

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from control and shear stress stimulated endothelial cells using the Chomczynski method.[6] Briefly, endothelial cell monolayers (15 cm^2) were rinsed and sterile PBS, lysed in 1.0 mL of denaturing buffer (4.0 *M* guanidium isothiocyanate, 0.5% sarkosyl, 25 m*M* sodium citrate, and 0.1 *M* mercaptoethanol), followed by addition of 0.1 mL of 2.0 *M* sodium acetate (pH 4.0). The mixture was extracted twice by phenol and chloroform/isoamyl alcohol (49:1). RNA was precipitated by addition of equal volumes of isopropyl alcohol and ethanol, followed by centrifugation. The RNA pellet was then redissolved in diethylpyrocarbonate-treated water. The PC-fos (human)-1 clone was obtained from the American Type Culture Collection (ATCC). This 9.0-kb insert has homology to the FBJ murine osteosercoma viral (v-fos) oncogene.[25] The insert, containing the entire human c-fos gene, was subcloned into the EcoRI site of the pBR322 vector (13.36 kb). The DNA of this clone was purified and digested with EcoRI and the 9.2 kb fragment was isolated. The cDNA probe was synthesized by the random priming hexanucleotide method using [α-^{32}P]dATP. A

quantity of 5 μg of each total RNA extract was run on each lane of a 1.0% agarose/formaldehyde gel. Ethidium bromide staining indicated complete integrity of the rRNA bands as well as even lane loading. The gels were blotted overnight to Millipore Immobilon N membrane, and then membranes were probed using $\geq 10^5$ cpm/mL of probe in hybridization buffer, followed by high stringency washes. Autoradiography was performed at -75°C for 16 h. Northern blotting of BAEC, HeLa, and CHO total RNA produced a band at 2.2 kb as expected for c-fos mRNA.[39]

RESULTS

Immunofluorescence staining for c-fos in unstimulated cells produced little fluorescence staining in HUVEC (Fig. 1). When matched serum-starved monolayers were exposed to 4 dyn/cm^2 of fluid shear stress for 1 h, a low level of diffuse, whole cell staining was observed in HUVEC (Fig. 1). The level of cell staining for BAECs and CHO cells was not altered significantly by this level of shear stress. However, the level of fluorescence in HeLa cells was significantly reduced (0.41 ± 0.12 times the control value) at low level of shear stress of 4 dyn/cm^2 (Fig. 1). Highly localized nuclear staining of c-fos was observed in the different cell lines exposed to fluid shear stress levels of 25 dyn/cm^2 for 1 h (Fig. 1). The nuclei in the shear stressed HUVEC were 5.4 ± 2.0 times brighter ($p < 0.1$) than matched stationary controls (Fig. 2). BAECs, HeLa, and CHO were 2.25 ± 1.38 ($p < 0.1$), 2.14 ± 0.07 ($p < 0.01$), and 1.92 ± 0.58 ($p < 0.1$) times brighter, respectively, than the matched stationary controls (Fig. 2). The larger stimulation relative to control found with HUVEC (in relation to other cell lines) was due to a lower baseline level under resting conditions.

Given the rapid induction of c-fos protein levels in different cell lines exposed to shear stress, we tested whether c-fos mRNA levels were elevated in shear stressed cells. Northern blot analysis also revealed the induction of c-fos at high levels of shear stress (25 dyn/cm^2) after 0.5 h of flow exposure (Fig. 3). The mRNA level of the 2.2 kb mRNA band for c-fos was elevated in BAEC grown on glass slides or fibronectin-coated slides. A similar induction of c-fos mRNA was observed at 0.5 h in two experiments with CHO cells and also in HeLa cells exposed to a shear stress of 25 dyn/cm^2 (Fig. 3).

For our next set of experiments, we selected HeLa cells for studying the induction of c-fos protein levels under different conditions. HeLa are one of the most commonly used undifferentiated human cell lines, originally obtained from a malignant tumor of the cervix. We selected HeLa as a prototype human cell line because they display little variability in their function or protein production with passage number. It is known that stimulation of cells by chemical agonists like phorbol esters involves large but highly transient elevation of c-fos levels. For example, an induction of c-fos mRNA levels in macrophages occurs after treatment with phospholipase C (PLC) or the activation of the protein kinase C pathway by phorbol 12-myristate 13-acetate (PMA).[2,32] In such studies, the c-fos mRNA levels were elevated as early as 30 min after the addition of the agonists like PMA, phospholipase C, and cholera toxin, reaching a maximum at 1 h and then decreasing to preinduction levels within 3 h in THP-1 monocytic leukemia cells.[2]

We have studied the time course for the induction of c-fos in HeLa cells at three time points of 30, 60, and 180 min of shear stress exposure. It was observed that the c-fos induction due to shear stress followed a course similar to the induction of c-fos by other agonists, with the c-fos levels peaking at about 1 h after exposure to shear and returning to near baseline levels after 3 h of shear stress exposure (Fig. 4). The minimum time required for the c-fos induction in HeLa cells also was studied by exposing the HeLa cells to shear stress for different times and then letting the cells remain in a no-flow environment for up to 60 min. We found that as little as one minute of exposure to shear stress of 25 dyn/cm^2 could induce the c-fos protein levels in HeLa cells, indicating very rapid intracellular signaling with the onset (and perhaps the offset) of fluid flow (Fig. 5).

Because activation of the phospholipase C (PLC) and protein kinase C (PKC) signaling pathways causes an induction of c-fos transcription,[2,16,27] we investigated the effect of inhibitors of these two signaling pathways on the induction of c-fos by shear stress. A 30-min preincubation of the cells with the phospholipase C inhibitor neomycin (5 mM) followed by shear stress exposure for 1 h almost completely blocked the shear induction of c-fos in HeLa cells (Fig. 6). We have also found in a similar experiment that the protein kinase C inhibitor, H7 (10 μM) in HUVEC blocked the induction of c-fos by 50% as compared to untreated HUVEC exposed to high shear stress, which had nuclei that were 3.7 ± 1.6 times brighter than the stationary controls (Fig. 7). The H7-treated cells exposed to high shear stress had c-fos levels that were 1.9 ± 1.2 times higher ($p < 0.01$), indicating that H7 did not completely block the induction of c-fos by high shear stress.

DISCUSSION

This study has sought to measure the level and localization of the transcriptional activator c-fos in anchorage-dependent mammalian cells exposed to well-characterized levels of laminar shear stress using a parallel plate flow system. The protooncogenes c-fos and c-jun help couple the short-term signals elicited at the cell surface to longer term changes in the cellular phenotype by altering the expression of specific target genes. The AP-1 binding proteins have been implicated in a number

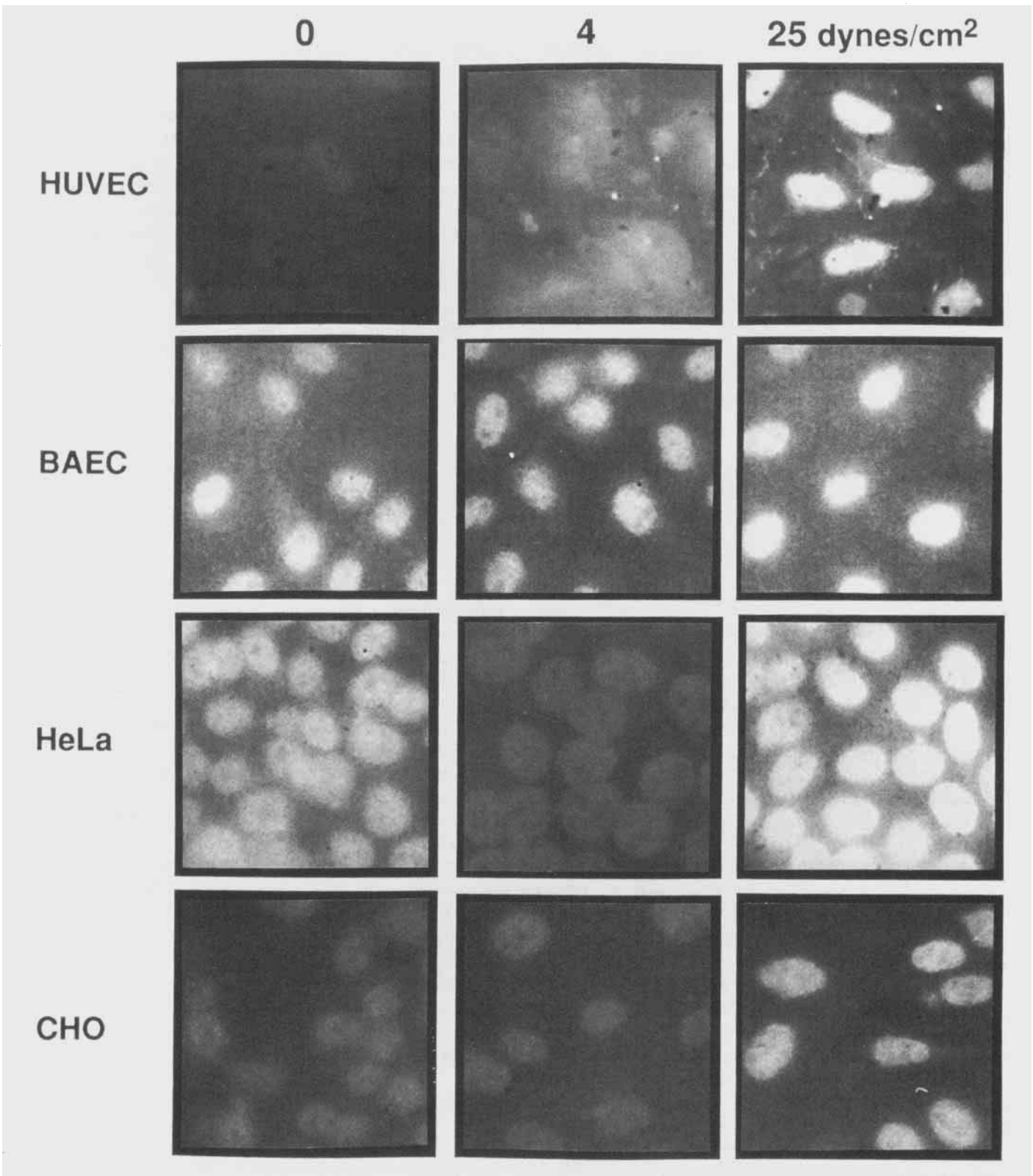

Figure 1. Localization and immunofluorescence intensity of c-fos protein levels in human umbilical vein endothelial cells (HUVEC), bovine aortic endothelial cells (BAEC), HeLa cells, and Chinese hamster ovary (CHO) cells maintained in stationary culture (0 dyn/cm^2) or exposed to fluid shear stress of 4 or 25 dyn/cm^2 for 1 h.

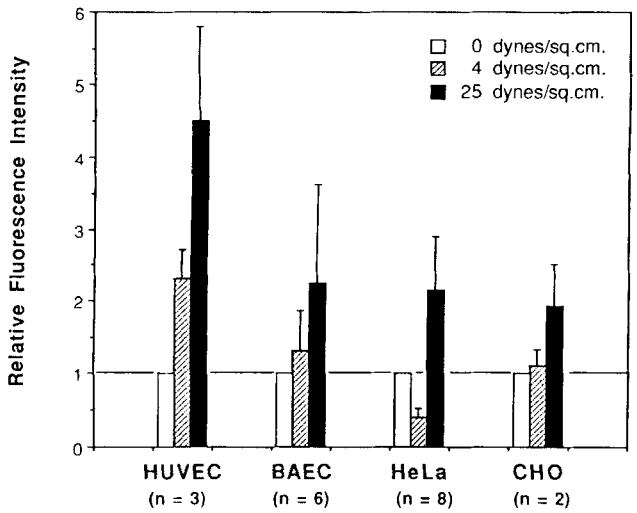

Figure 2. Immunofluorescence intensity of c-fos in HUVEC, BAEC, HeLa, and CHO cells maintained in stationary culture (open bar), or exposed for 1 h to shear stress at 4 (hatched bar) or 25 (solid bar) dyn/cm^2. Average intensity ±SD was determined by digital image analysis for over 30 cells in each monolayer. The average intensity was normalized to the average intensity of the quiescent control cultures maintained in stationary culture to give the relative fluorescence intensity. Each experiment was conducted with an independent harvest of primary HUVEC or independent passage of BAEC, HeLa, or CHO. All monolayers were visualized under identical conditions.

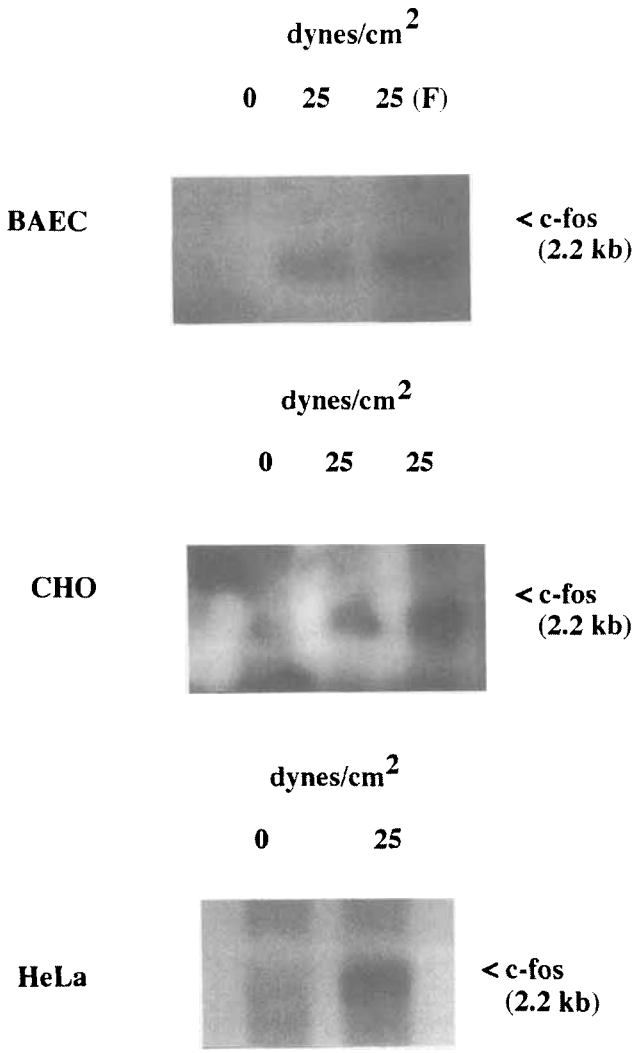

Figure 3. Fluid shear stress causes elevation of c-fos mRNA levels in BAEC, CHO, and HeLa cells exposed to shear stress of 25 dyn/cm^2 for 0.5 h as compared to stationary controls (0 dyn/cm^2). Two monolayers of BAEC at the same passage number, but grown on different substrates, glass, or fibronectin-coated glass (F), were exposed to shear stress. Two replicate monolayers of CHO were subjected to shear stress simultaneously using individual flow loops. A total of 5 μg RNA was subjected to electrophoresis in each lane.

of signal transduction cascades associated with growth, differentiation, and cellular stress.[26,27]

In endothelial cells, the c-fos gene has been shown to be sensitive to various forms of mechanical stimuli. Pulsatile and steady flow induce c-fos expression in endothelial cells.[16] Also, both shear stress[18] and cyclic stress have been shown to stimulate the formation of AP-1 complexes with DNA in BAECs using the gel shift assay. Lan et al.[18] found that the exposure of BAEC to flow led to a biphasic induction of AP-1 binding, reaching a peak at 10–20 min, declining, and then rising again after 2 h exposure. Interestingly, mechanical stress/relaxation induces c-fos expression in cultured rat mesangial cells.[1]

The actual magnitude and temporal nature of a particular mechanical stimulus can have a profound effect on the intracellular signaling pathways and gene regulation of a living cell. Different flow rates may cause different responses, leading to either induction or suppression of a given gene. The inhibition of ET-1 mRNA levels and ET-1 secretion by HUVEC by shear stress of 25 dyn/cm^2 has been well documented.[23,37] However, HUVEC endothelin-1 peptide release is stimulated by about 50% at low levels of shear stress (1.8 dyn/cm^2), whereas 6–25 dyn/cm^2 shear stress for periods of >6 h inhibited ET-1 release.[17] Some genes like monocyte chemoattractant protein 1 (MCP-1) display a biphasic response to flow with the mRNA levels peaking at 1.5 h before gradually decreasing a basal levels after 4 h shear stress.[40] In the context of these studies, the downregulation of c-fos protein level in HeLa at 4 dyn/cm^2 and its upregulation at 25 dyn/cm^2 shear stress is not unprecedented and indicates a role of different intracellular signal transduction mechanisms for gene regulation in response to different flow rates.

The actual mechanisms by which the mechanical stimuli to the cells are transmitted to activate the various intracellular signaling pathways in the cells are not fully resolved at the molecular level. It is possible that shear stress can cause a structural reorganization of the cytoskeleton and selectively adjust gene expression in cells exposed to shear stress. Our studies suggest that perturbation of cells may activate PKC and phospholipase C in some cell types. The extensive and intricate protein framework of the cellular cytoskeleton affects cell shape, cell motility, intracellular transport of macromol-

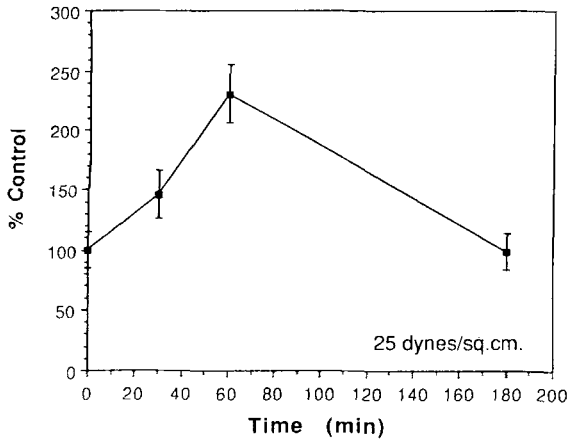

Figure 4. The time course for the induction of c-fos in Hela exposed to continuous laminar shear stress of 25 dyn/cm^2. Replicate cultures were exposed to shear stress using flow systems started in a staggered sequence. All the flow loops were then stopped at the same time for immunofluorescence analysis.

ecules, translation of mRNA into protein, and may influence the regulation of gene expression.[4] The treatment of exponentially growing HeLa cells and quiescent WI-38 cells with cytochalasin D, which disrupts the cytoskeletal actin microfilaments, results in a rapid and marked increase in the transcription of c-fos gene with a concomitant increase of the c-fos mRNA steady state levels.[44]

With respect to the mechanism of gene regulation by flow, a core sequence, GAGACC (or its reverse orientation, GGTCTC) present in the promoter regions of certain genes like PDGF-B, c-fos, c-jun, MCP-1 endothelin-1, ICAM-1, t-PA has been identified as a

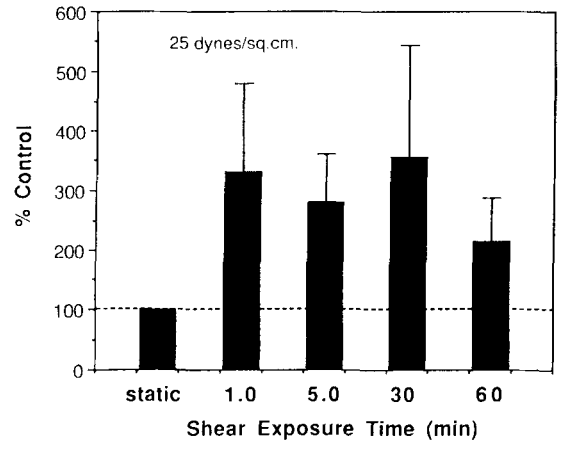

Figure 5. The minimum shear stress exposure time required for c-fos induction (at 1 h) in HeLa cells exposed to a shear stress of 25 dyn/cm^2. After exposure to varying times of shear stress, the cells were allowed to remain under stationary culture conditions up to the 1 h time point, at which time the immunofluorescence analysis was carried out. Average intensity ±SD was determined by digital image analysis for over 30 cells in each monolayer. The average intensity was normalized to the average intensity of the quiescent control monolayers maintained in stationary culture to give relative fluorescence.

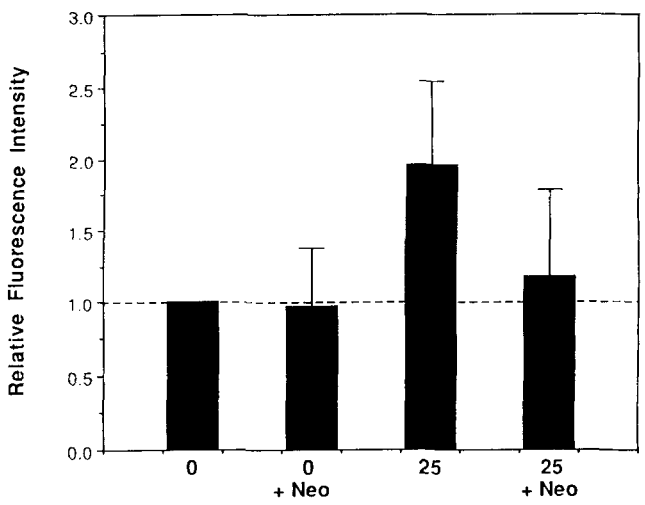

Figure 6. Immunofluorescence of c-fos in HeLa as a function of fluid shear stress in the presence and absence of the phospholipase C inhibitor, neomycin (5 mM). Average intensity values ±SD were determined from 30 cells in each monolayer and normalized as in Figure 2.

shear stress response element (SSRE).[36] However, a recent study has shown that SSRE was not involved in the regulation of the MCP-1 gene, while only one of the two AP-1 elements in the MCP-1 promoter was a functional responsive element to shear stress.[41] The transcriptional factor that binds the SSRE sequence has not yet been identified. We have carried out gel shift assays using DNA containing the SSRE and protein extracts from shear stressed BAEC. The binding of proteins to this sequence was elevated after 1 h of high shear

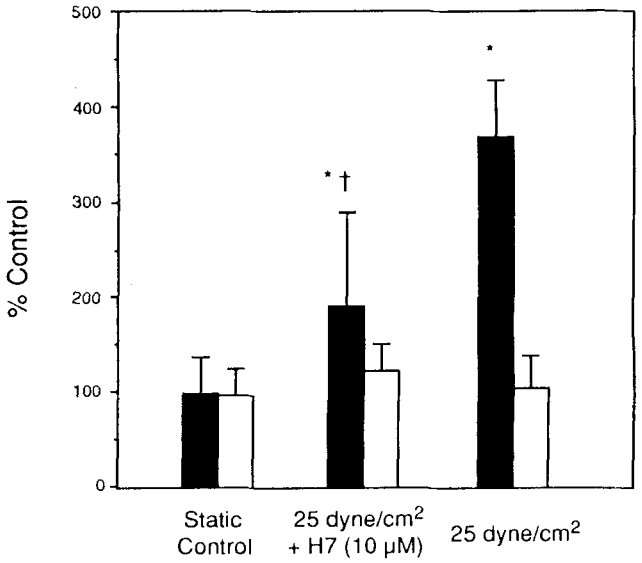

Figure 7. Nuclear (closed bar) and cytoplasmic (open bar) c-fos immunofluorescence as a function of fluid shear stress in the presence or absence of a protein kinase C inhibitor, H7 (10 μM). Average intensity values ±SD were determined from 30 cells in each monolayer and normalized as in Figure 2 (*$p < 0.01$, compared to stationary control; †$p < 0.01$, compared to high shear without H7).

stress exposure of BAECs, as previously described.[36] Interestingly, we have also detected the presence of this transcriptional factor in HeLa cells[35] (data not shown).

Animal cell cultures have been exploited for the production of a variety of useful biological products, and a major biochemical engineering challenge encountered in large scale cell cultivation is the optimization of environmental conditions (biological, chemical, and physical) for cell growth and productivity. From an engineering point of view, the various aspects of the shear sensitivity of cells, including the role of shear stress in the gene regulation and protein production of cells, may have important implications. This study measured the regulation of c-fos protein and mRNA levels in various mammalian cell lines exposed to different levels of well-defined, laminar shear stresses. The shear-stress mediated induction of the c-fos protooncogene was a consistent response in several mammalian cell lines that were tested and may also occur in other cultivation environments.

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