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FLUID SHEAR STRESS INDUCES SYNTHESIS AND NUCLEAR LOCALIZATION OF C-FOS IN CULTURED HUMAN ENDOTHELIAL CELLS

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Quiescent cultured primary human umbilical vein endothelial cells were exposed to low levels (4 dynes/cm^2) or arterial levels (25 dynes/cm^2) of steady laminar shear stress for one hour. Intracellular *c-fos* protein was measured by immunocytochemistry and quantitative fluorescence video microscopy. The nuclear-localized *c-fos* protein level was 5.4 ± 2.0 fold higher ($p<0.01$) in the cells exposed to arterial levels of shear stress as compared to the cells maintained in stationary culture. In contrast, the cells exposed to low levels of shear stress showed diffuse but slightly elevated levels of *c-fos* (2.4 ± 0.73 times higher than control; $p<0.01$) without preferential nuclear localization. The protein kinase C inhibitor, H7 (10 μM) significantly attenuated the induction of *c-fos* by 50 % in cells exposed to arterial shear stress for 1 hour.

The vascular endothelium is continuously exposed to blood flow and consequently experiences hemodynamic shear stress. Alignment of endothelial cells is observed *in vivo* and *in vitro* in zones of unidirectional high shear stress (1,2). Short term responses of the endothelial cells to the onset of shear stress include activation of a membrane potassium current (3), a burst of prostacyclin production (4,5) and elevation of inositol trisphosphate levels (6). *In vitro* studies have shown that the chronic exposure of endothelial cells to shear stress can cause a sustained increase in tissue plasminogen activator (tPA) secretion (7) and tPA mRNA levels (8), sustained decrease of endothelin mRNA levels and endothelin secretion (9), and transient enhancement of platelet-derived growth factor mRNA levels (10). These genetic responses in endothelial cells exposed to hemodynamic forces are similar to changes seen with stimulation of endothelial cells by exogenous addition of protein kinase C (PKC) activators.

It is known that activation of PKC in mammalian cells can lead to rapid induction of the proto-oncogenes, *c-fos* and *c-jun* (11). These proteins bind each other and form a transcriptional activator which can bind AP-1 promoter sites of

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various genes (12,13). In a recent study, pulsatile and steady flow were found to elevate *c-fos* m-RNA levels in human endothelial cells (14). The activation of PKC and subsequent induction of *c-fos* may be a link between rapid signal transduction events in cells exposed to fluid flow (3-6) and slower changes in genetic expression (7-10) which occur over several hours. In this study, we investigated the effects of shear stress on the intracellular *c-fos* protein levels and *c-fos* cellular localization.

MATERIALS AND METHODS

Cell culture: Primary human umbilical vein endothelial cells (HUVEC) were cultured as previously described (7,15). Briefly, two to four umbilical cords (obtained within 6 hours of delivery) were rinsed, cannulated, and filled with collagenase. (Worthington Biochemical Corp., Freehold, NJ) in phosphate buffered saline (PBS, 40-50 U/ml) at 37°C, then incubated for 30 minutes. The pooled effluent was centrifuged for 10 minutes at 100g, and the pellet was resuspended in complete DMEM with 20% heat-inactivated newborn calf serum, 0.30 mg/ml glutamine, 150 U/ml penicillin, and 0.15 mg/ml streptomycin (Gibco Laboratories, Grand Island, NY) .

Exposure of endothelial cells to shear stress: Primary, confluent monolayers (72-86 hours post-seeding) were serum starved for 48 hours in DMEM with 0.5 % serum. The cells were exposed to steady laminar shear stress in individual parallel plate flow chamber systems with recirculating medium (20 mM HEPES and 1% BSA in PBS, pH 7.4) as previously described (7) or maintained in the incubator. No ATP was present in the circulatory medium. After 1 hour, immunocytochemistry was carried out on the monolayers.

Immunocytochemistry: Cells on each individual slide were fixed by incubating the slides in a solution containing 2% paraformaldehyde, 0.5% Triton X-100 at 4°C for 15 minutes. 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 in PBS) for 2 hours. The slides were then washed three times with 5 ml of 3% BSA, 0.5% Triton in PBS solution. Each slide was incubated with 50 µl of fluorescein donkey anti-sheep IgG (H+L) conjugate (Molecular Probes Inc., Eugene, OR) (1:20 dilution) for 1 hour. The slides were washed 4 times with PBS and view by video microscopy.

Fluorescence video microscopy: A Leitz Aristoplan microscope (100 X objective) was used for epifluorescence visualization. The fluorescence emission was directed toward an image intensified (GenIIsys)-charge coupled diode (CCD-72) camera assembly (Dage-MTI). A Hamamatsu Argus-10 Image Processor was used for noise reduction of the video signal but no grey scale stretching or contrast enhancement was carried out. The data was recorded on 1/2" videotape for digital image analysis (NIH Image 1.47) using a Quickcapture framegrabber (Data Translation) and a Macintosh IIci.

RESULTS

To establish a state of quiescence within monolayers of endothelium, the cells were serum starved for 48 hours. After serum starvation, immunostaining for *c-fos*

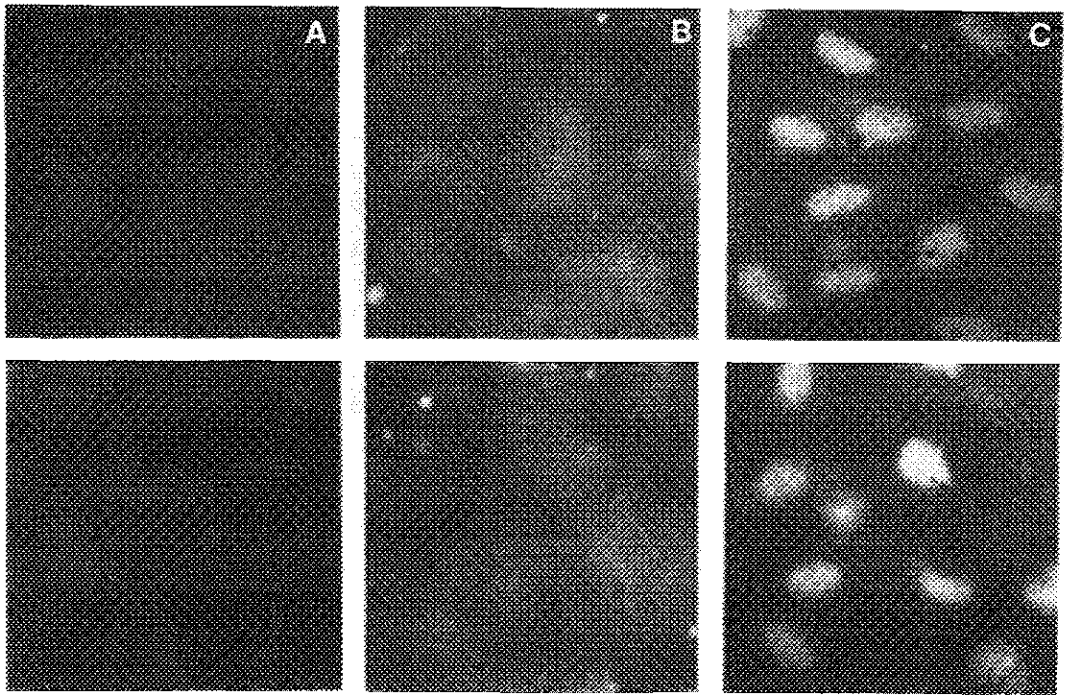

Fig. 1. Localization and immunofluorescence intensity of *c-fos* protein in human umbilical vein endothelial cells maintained in stationary culture (A) or exposed to fluid shear stress of 4 (B) or 25 dynes/cm^2 (C) for 1 hour. The perfusion media was HEPES-buffered PBS with 1 % BSA containing no extracellular ATP. Two representative views of each monolayer are shown. The cells maintained in stationary culture (A) had a detectable but very low level of fluorescence.

in unstimulated cells produced almost no fluorescence (Fig. 1A). When matched serum starved monolayers were exposed to 4 dynes/cm^2 of fluid shear stress for 1 hour, a low level of diffuse, whole cell staining was detected (Fig. 1B). There was no significant preferential localization of *c-fos* protein in stationary controls or in cells exposed to low shear stress of 4 dynes/cm^2. We observed an intense and highly localized nuclear staining of *c-fos* in endothelial cells exposed to arterial levels of fluid shear stress of 25 dynes/cm^2 for 1 hour (Fig. 1C). The nuclei in shear stressed HUVEC were 5.4 ± 2.0 times brighter ($p<0.01$) than matched stationary controls (Fig. 2). In HUVEC exposed to high shear stress for 1 hour, the concentration of *c-fos* in the nucleus was 2.9 ± 1.1 times greater than that in the cytoplasm.

In a separate experiment, HUVEC were preincubated with the protein kinase C inhibitor, H7 (10 μM) for 30 min and then exposed to arterial levels of shear for 1 hour with H7 (10 μM) in the perfusion media. Elevation of *c-fos* levels was significantly reduced by 50 % ($p<0.01$) in H7-treated HUVEC exposed to high shear stress as compared to the *c-fos* levels in untreated endothelial cells exposed to high shear (Fig. 3) which had nuclei that were 3.7 ± 1.6 times brighter than stationary controls. The H7-treated cells exposed to high shear stress had *c-fos* levels which were 1.9 ± 1.2 times higher ($p<0.01$) than stationary controls, indicating that H7 did not fully block the *c-fos* induction by high shear stress.

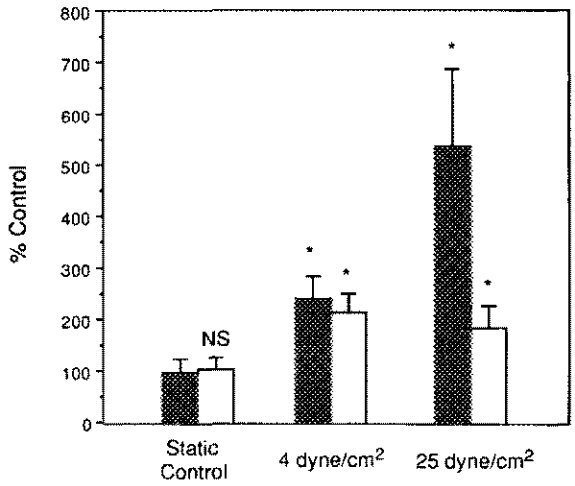

Fig. 2. Nuclear (closed bar) and cytoplasmic (open bar) intensity of *c-fos* immunofluorescence as a function of fluid shear stress. Average intensity ± SD was determined by digital image analysis for 30 cells in each monolayer. Intensity values were normalized to the nuclear intensity of quiescent control endothelium maintained in static conditions. Data were typical of three separate experiments conducted with three independent harvests of HUVEC. A student t-test was used with a bonferoni correction for multiple comparisons (*p<0.01; NS, not significant).

The basal level of *c-fos* protein in the endothelial cells was completely suppressed by serum starvation of the cells for 48 hours. It was found that when the unstimulated, confluent endothelial cells were serum starved for only 24 hours, numerous nuclei contained *c-fos*-immunolocalized fluorescence. Cells were viable after serum starvation for 48 hours as indicated by their ability to synthesize and

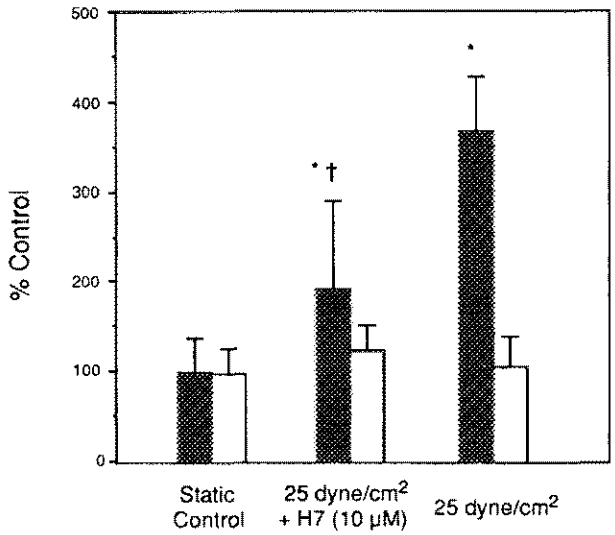

Fig. 3. Nuclear (closed bar) and cytoplasmic (open bar) intensity of *c-fos* immunofluorescence as a function of fluid shear stress in the presence or absence of the protein kinase C inhibitor, H7 (10 μM). Average intensity values ± SD were determined from 15 cells of each monolayer and were normalized as in Fig. 2 (*p<0.01, compared to stationary control; †p<0.01, compared to high shear without H7).

translocate *c-fos* and to maintain adherence to a glass substrate under arterial levels of shear stress. Cellular morphology was normal as accessed by light microscopy.

DISCUSSION

Several studies have shown that fluid shear stress can regulate the expression of endothelial genes (7-10). Changes in secretion of tPA and endothelin appear to be due to changes in the mRNA levels of these particular genes. We report that arterial levels of fluid shear stress can stimulate the synthesis of c-fos protein which was then translocated into the nucleus.

In contrast to arterial levels of shear stress, low shear stress of 4 dynes/cm^2 did not cause a striking induction of c-fos expression or nuclear translocation of c-fos protein. This would indicate that local mixing or boundary layer phenomena is not the cause of the arterial shear stress-induced expression of c-fos since the wall shear rate (400 sec^{-1}) at the low shear stress is still at least three orders of magnitude greater than that found in stationary cultures due to natural convection.

The *c-fos* proto-oncogene product, together with the transcription binding factor *c-jun*, has been shown to form DNA-binding nucleoprotein complexes, related to the regulation of gene expression in a variety of cell types (12, 16-18). The *c-fos* proto-oncogene is an early gene that is induced when cells are stimulated by agents like growth factors, thrombin, ATP, or phorbol esters (19, 20). Our results are consistent with a recent study (14) which showed that flow can cause *c-fos* m-RNA levels to be elevated in HUVEC. The partial inhibition of this response by H7 indicates that PKC (as well as other possible pathways) are involved in shear stress induction of c-fos expression. Some nuclear translocation—a cAMP-dependent process (21)—did occur in H7-treated cells, suggesting that H7 did not completely and/or nonspecifically inhibit cAMP-dependent protein kinase (PKA). In cells stimulated by arterial levels of shear stress, the elevation of *c-fos* synthesis was accompanied by a marked translocation of this newly synthesized protein into the nucleus, suggesting that the activity of cytoplasm retention factors (21) and the DNA-binding affinity of the AP-1 complex changed in HUVEC exposed to flow.

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