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SHEAR STRESS INCREASES HEPARIN-BINDING EPIDERMAL GROWTH FACTOR-LIKE GROWTH FACTOR mRNA LEVELS IN HUMAN VASCULAR ENDOTHELIAL CELLS

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Summary: Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a recently identified potent vascular smooth muscle cell (SMC) mitogen. We investigated the effect of shear stress on human HB-EGF mRNA levels in cultured human umbilical vein endothelial cells (HUVEC). In response to shear stress (8 dyne/cm^2), HB-EGF mRNA levels in HUVEC increased rapidly, peaked at 3 h, and returned to near base line at 7 h. The shear stress-induced HB-EGF gene expression in HUVEC is completely blocked by 12-*O*-tetra-decanoylphorbol-13-acetate pre-treatment, suggesting the induction of HB-EGF is mediated by protein kinase C.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a recently identified, 22-kDa protein purified from conditioned medium of macrophage-like U937 cell (1). HB-EGF contains an epidermal growth factor (EGF) domain characterized by three disulfide bonds and is considered a member of the EGF family. HB-EGF binds EGF receptors and is mitogenic for fibroblasts and vascular smooth muscle cells (SMC) but not for vascular endothelial cells (1). Although the mechanism is not known, HB-EGF is a more potent SMC mitogen than EGF and its potency is comparable with that of platelet-derived growth factor (PDGF) (1). HB-EGF gene is transcribed in human umbilical vein endothelial cells (HUVEC) stimulated by cytokines (2), which are known to exist in atheromatous tissue (3,4). Thus, it has been speculated that HB-EGF may have a role in the proliferation of SMC in atheroma (2).

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It has been proposed that in addition to systemic risk factors, local hemodynamic forces may also play an important role in the focal progression of atherosclerosis (5,6). Vascular endothelial cells, which form a continuous monolayer covering the luminal surface of the vascular system, are thought to sense blood flow and mediate various signals to underlying tissues including vascular SMC (7). We and other groups have reported that endothelial cells can produce SMC mitogens in response to shear stress (8,9,10,11,12).

To test whether vascular endothelial cells exposed to shear stress can produce the potent SMC mitogen HB-EGF, we exposed HUVEC to flow and measured HB-EGF mRNA levels. Our results demonstrate that flow induces a rapid and remarkable increase in HB-EGF mRNA levels in HUVEC.

Experimental Procedures

Cell culture and shear stress apparatus.

HUVEC were obtained from Kurabo, Osaka, Japan and grown in EGM-UV medium containing 2% fetal calf serum (2). HUVEC from passage 3-5 were grown to confluency in 28.3 cm^2 culture dishes (60 mm in diameter). A modified cone-plate apparatus was used to generate steady shear stress on HUVEC (8). The entire apparatus was placed in a CO_2 incubator at 37°C in 5 % CO_2/95 % humidified air. The cone was rotated at 100 rpm and the calculated shear stress was approximately 8 dyne/cm^2. This degree of shear stress (8 dyne/cm^2) is known to exist *in vivo*, such as in arterial bifurcations and in stenotic arteries (5,6). To study the mechanism of shear stress-induced HB-EGF gene expression in HUVEC, 12-*O*-tetra-decanoylphorbol-13-acetate (TPA) or *bis*-(*o*-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA-AM) was added to the medium (13).

HB-EGF cDNA probe and Northern blot analysis.

Total cellular RNA was extracted by the LiCl-urea technique (14) from HUVEC and quantitated by measuring absorbance at 260 nm. RNA samples (10 μg) were electrophoresed through 1.2 % formaldehyde/agarose gels and transferred to nylon membranes (Hybond N, Amersham) by standard procedures (15). The membranes were hybridized with a random primed, ^{32}P-labeled, EcoRI-KpnI fragment of HB-EGF cDNA, a gift from Dr. Mu-En Lee (2). The hybridized membranes were then washed and autoradiographed (15). The membranes were subsequently rehybridized with a ^{32}P-labeled β-actin probe to determine an internal standard of total RNA content.

Results

Shear stress increased HB-EGF mRNA levels in HUVEC.

HUVEC were exposed to steady shear stress of 8 dyne/cm^2 using a cone-plate apparatus. The baseline level of HB-EGF mRNA was

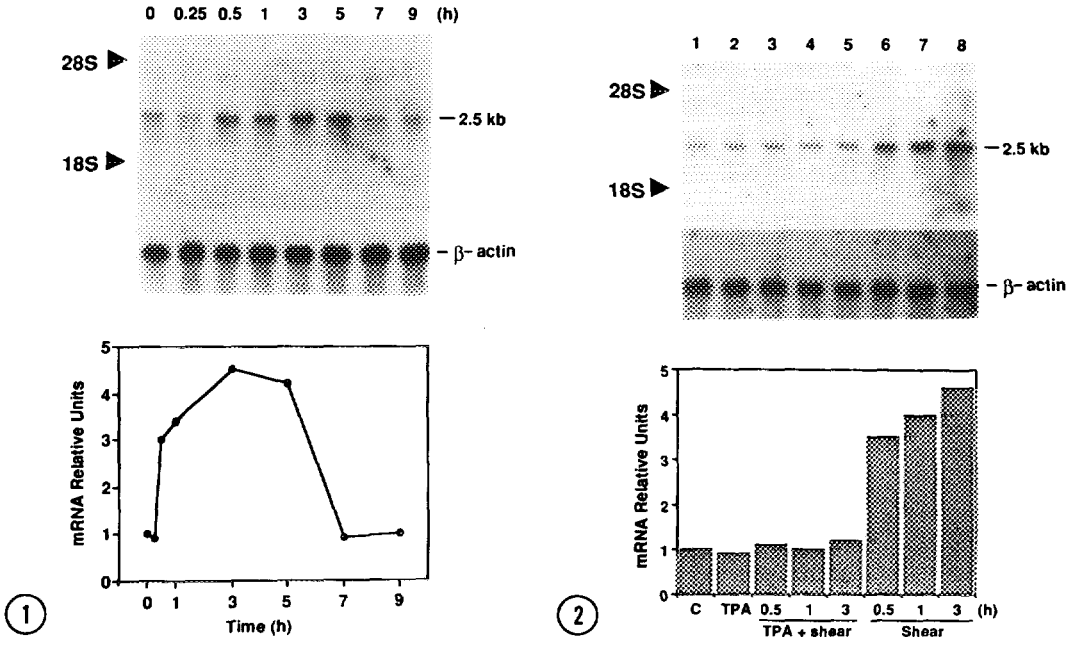

Figure 1. Time course of HB-EGF mRNA induction by shear stress. HUVEC were exposed to shear stress (8 dyne/cm^2), and total RNA was extracted at the indicated times. Northern blot analysis was performed with 10 μg of total RNA probed with human HB-EGF. The membrane was rehybridized with a β-actin probe. To correct differences in loading, the signal density of each RNA sample hybridized to the HB-EGF probe was divided by that hybridized to the β-actin probe. The corrected density for each time point was then divided by that of the control and presented in a relative unit plotted against time.

Figure 2. Shear stress-induced elevation of HB-EGF mRNA was completely inhibited by TPA pre-treatment. HUVEC were pre-treated with TPA (10^{-8} M) for 24 h (lanes 2-5) and subsequently exposed to shear stress of 8 dyne/cm^2 for 0 (lane 2), 30 min (lane 3), 1 h (lane 4), and 3 h (lane 5). HUVEC without TPA pre-treatment were exposed to shear stress of 8 dyne/cm^2 for 0 (lane 1), 30 min (lane 6), 1 h (lane 7), and 3 h (lane 8). Northern blot analysis was performed with 10 μg of total RNA probed with human HB-EGF. The membrane was rehybridized with β-actin probe and relative units were calculated as described in Fig. 1.

low, however within 30 minutes the mRNA level of HB-EGF increased rapidly in response to shear stress and reached a maximum of 4.5 X the baseline level at 3 h (Fig. 1). The increased mRNA levels were sustained for 0.5-5 h followed by a decline to near basal level after 7 h. In contrast, β-actin mRNA levels in HUVEC were not affected by exposure to shear stress of 8 dyne/cm^2.

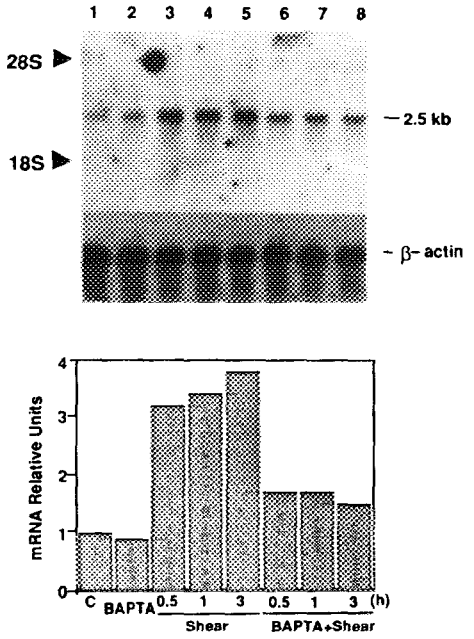

Figure 3. Shear stress-induced elevation of HB-EGF mRNA was partially inhibited by an intracellular calcium chelator. HUVEC were exposed to shear stress of 8 dyne/cm^2 in the absence (lanes 1, 3-5) or presence (lanes 2, 6-8) of BAPTA-AM (10^{-6} M) for 0 (lanes 1,2), 30 min (lanes 3,6), 1 h (lanes 4,7), and 3h (lanes 5,8). Northern blot analysis was performed with 10 μg of total RNA probed with human HB-EGF. The membrane was rehybridized with β-actin probe and relative units were obtained as described in Fig. 1.

Shear stress-induced elevation of HB-EGF mRNA was inhibited by TPA pre-treatment.

To study the role of protein kinase C in shear stress-induced HB-EGF gene expression, HUVEC were pre-treated with TPA (10^{-8} M) for 24 h and subsequently exposed to shear stress of 8 dyne/cm^2 for 0.5-3 h. Shear stress-induced elevation of HB-EGF mRNA was completely inhibited by TPA pre-treatment (Fig. 2), suggesting that shear stress-induced elevation of HB-EGF gene expression is dependent on protein kinase C activity (16).

Shear stress-induced elevation of HB-EGF mRNA was partially inhibited by an intracellular calcium chelator.

To study the role of intracellular calcium in shear stress-induced elevation of HB-EGF, HUVEC were exposed to shear stress of 8 dyne/cm^2 for 0.5-3 h in the absence or presence of BAPTA-AM (10^{-6} M), an intracellular calcium chelator. Shear stress-induced elevation of HB-EGF mRNA levels was partially inhibited

by BAPTA-AM (Fig. 3). This response of HB-EGF mRNA to BAPTA-AM suggests that the existence of intracellular calcium may in part be necessary for shear stress-induced HB-EGF gene expression.

Discussion

We have shown that fluid shear stress increases HB-EGF mRNA levels in HUVEC and this shear-induced HB-EGF gene expression is mediated by protein kinase C activation and may require intracellular calcium. Several studies have shown that shear stress can stimulate phosphoinositide turnover in endothelial cells and may result in the production of second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG) (17,18,19). IP_3 triggers the release of Ca^{2+} from intracellular pools and DAG activates protein kinase C (16). Frangos and colleagues have reported that in HUVEC, protein kinase C mediates shear stress-induced PDGF gene expression (10) and endothelin-1 release (11). It is well known that a complex of transcriptional factors binds to the "AP-1 site" or "TPA-responsive element" and mediates the transcriptional activation of many kinds of genes by TPA (20). Because there is an atypical AP1 consensus sequence (TCAGTCA) in the HB-EGF promoter (21), the AP-1 site may have some roles in the shear stress-induced HB-EGF gene expression.

Recently, fluid shear stress-responsive element (SSRE) in the promoter region of PDGF B chain has been reported (22). This putative sequence appears to bind to nuclear proteins which exist in bovine aortic endothelial cells exposed to shear stress. Interestingly, the core binding sequence (GAGACC) exists in the promoter sequence of human HB-EGF (21). Further studies will be necessary to elucidate the role of SSRE as well as AP-1 site in the shear stress-induced HB-EGF gene expression.

It has been observed that despite being exposed to the same systemic risk factors, atherosclerotic lesions form more frequently at the specific portion of vascular system, such as branch points (5,6). Accordingly, it has been assumed that local mechanical forces may play an important role in determining the rate of atherosclerotic progression. Because vascular endothelial cells are thought to sense blood flow and mediate various signal to underlying tissues (7), SMC mitogens

produced by endothelial cells exposed to shear stress may be responsible for the progression of atherosclerosis at the specific site. Future experiments including *in vivo* studies will be necessary to prove this hypothesis.

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