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Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium

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ABSTRACT **The focal distribution of atherosclerotic lesions in the arterial tree is related to the local shear stress generated by blood flow, but the molecular basis of the atherogenic response of endothelial cells in these lesion-prone areas is still unclear. We report that shear stress mediates a biphasic response of monocyte chemotactic protein 1 (MCP-1) gene expression in vascular endothelial cells (EC). Northern blot analysis indicated that the level of MCP-1 mRNA in human umbilical vein EC (HUVEC) subjected to a shear stress of 16 dynes/cm² (1 dyne = 10 μN) for 1.5 hr increased by 2- to 3-fold when compared with static cells. The MCP-1 gene expression decreased to the basal level at 4 hr and then declined further to become completely quiescent at 5 hr after the onset of shear. Once the gene expression was fully suppressed, it remained quiescent even after static incubation for 1.5 hr and would not respond to reshearing after this static incubation. However, if the postshearing incubation extended from 1.5 to 24 hr, the MCP-1 mRNA returned to the basal level and was then able to increase after the reapplication of shear stress. Nuclear run-on experiments showed that the shear-induced increased MCP-1 mRNA in HUVEC was regulated at the transcriptional level. By using cycloheximide, it was shown that *de novo* protein synthesis was not necessary for the induction of MCP-1 by shear stress. The biphasic response of MCP-1 gene expression was found in experiments in which the applied shear stress was 6, 16, or 32 dynes/cm², and it was observed not only in HUVEC but also in HeLa cells, glioma cell lines, and skin fibroblasts. This *in vitro* study demonstrates that the response of MCP-1 gene to shear stress represents an immediate early gene activation and suggests that this gene is probably suppressed in EC that have been exposed to a constant shear stress.**

Atherosclerotic lesions show a focal pattern of distribution in the arterial tree; they have a predilection in regions such as bends and bifurcations where the blood flow is disturbed with flow separation and where the wall shear stress is low and unsteady (1, 2). Studies of experimental atherosclerosis in animal models also indicate that risk factors such as hyperlipidemia, smoking, and hypertension enhance the occurrence of lesions in these regions by superimposing their effects on the fundamental predilection resulting from hemodynamic forces (3). We have previously demonstrated that the endothelial cells (EC) in these prelesion areas have a higher mitotic rate and a greater permeability to macromolecules such as low density lipoproteins (LDL) than EC in areas experiencing undisturbed laminar flow (4, 5). All of these findings suggest that hemodynamic forces play a key role in atherogenesis. However, the molecular mechanisms underlying these flow-induced atherogenic events in the endothelium at these lesion-prone areas are still unclear.

Because of the difficulties of *in vivo* experiments, the flow chamber was used to study the production of prostacyclin in human umbilical vein EC (HUVEC) (6) and since then has been widely used as an *in vitro* system to monitor the response of cultured EC to hemodynamic forces at cellular and molecular levels. Such *in vitro* studies have demonstrated increasing levels of c-*fos*, c-*jun*, and c-*myc* proto-oncogene transcripts (7) and of transcripts for tissue plasminogen activator (8), platelet-derived growth factor (PDGF) (9, 10), intercellular adhesion molecule 1 (ICAM-1) (11), and transforming growth factor β1 (TGF-β1) (12) in cultured EC subjected to shear stress. Furthermore, in a recent report, Resnick *et al.* (13) showed that a core sequence, GAGGCC, at the 5′ promoter region of *PDGFB* responded primarily to shear stress. Proto oncogenes c-*fos*, c-*jun*, and c-*myc* respond rapidly to growth stimulation in the absence of *de novo* protein synthesis and thus are categorized as immediate early (IE) genes (14). These genes respond not only to shear stress but also to other types of mechanical forces such as pressure overload and stretch in cardiac myocytes (15). The mouse JE gene belongs to IE genes, and its human homologue encodes the monocyte chemotactic protein-1 (MCP-1) (16). MCP-1 is a glycoprotein with a molecular weight of 9–15 kDa, and its biological function is mainly as a chemoattractant specific to monocytes (see ref. 17 for review). MCP-1 is expressed in vascular endothelium, vascular smooth muscle cells, monocytes, and fibroblasts. A number of observations indicate that MCP-1 plays an important role in atherogenesis. Minimally modified LDL can activate the expression of MCP-1 in cultured EC and in mice models *in vivo* (18, 19). MCP-1 has been located in atherosclerotic lesions of human patients, rabbit atherosclerotic models, and hypercholesterolemic primates (20–22). MCP-1 has been shown to increase the adhesion of monocytes to the endothelium (23). Transmigration of monocytes into the subendothelial space induced by LDL was inhibited by an antibody to MCP-1 (24). Due to the critical function of MCP-1 in atherogenesis, it is necessary to address what role, if any, the fluid shear stress plays in the regulation of MCP-1 gene expression. Such a study would contribute to our understanding of how hemodynamic forces in the arterial tree mediate one of the critical cellular events in atherogenesis—i.e., the attachment of blood monocytes to endothelium. Preliminary data of this study have been presented (25).

MATERIALS AND METHODS

Cell Cultures. HUVEC were isolated from the human umbilical cord as described by Jaffe *et al.* (26). The cells were grown in M-199 medium containing 15% (vol/vol) fetal bovine serum supplemented with 2 mM L-glutamine, 100 units of penicillin and 100 μg of streptomycin per ml, and 1 mM sodium pyruvate. To avoid the phenotypic drift of decreasing

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Abbreviations: MCP-1, monocyte chemotactic protein 1; EC, endothelial cell(s); HUVEC, human umbilical vein EC; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule 1; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor β1; TPA, phorbol 12-tetradecanoate 13-acetate; TRE, TPA-responsive element; PKC, protein kinase C.

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expression of various adhesion receptors on the HUVEC, the cells used were mainly prior to passage 2 or 3. Passage 4 cells were used in a few experiments; however, the results obtained were consistent with those from experiments using cells from lower passages. HeLa, glioma, and skin fibroblast cell lines were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All cell cultures were maintained in a humidified 5% CO_2/95% air incubator at 37°C.

Shear-Stress Experiments. A flow system was established according to the design previously described (9) with minor modifications. The fluid used to shear HUVEC was the conditioned medium used to culture the cells. A shear stress of 16 dynes/cm^2 (1 dyne = 10 μN) under laminar flow was generated either by a hydraulic pressure between an upper and a lower fluid reservoir or by a peristaltic pump. There was no difference in the results obtained from experiments using these two different methods to generate the shear stress. The culture medium was circulated through a constant-pressure-head flow loop. HUVEC were cultured on a 38 mm × 76 mm slide to confluence, and the slide was then assembled into the flow chamber. The system was tightly sealed by using a silicon gasket and a vacuum line. The channel in which the medium flew through was 270 mm thick. A surface area of 14 cm^2 on the slide, confined by the gasket, was exposed to the shear stress. This area accommodated $\approx 10^6$ HUVEC, which provided a sufficient quantity of cellular RNA for Northern blot analysis. For the nuclear run-on experiments, however, 5 or 6 slides of HUVEC were needed.

Nuclear Run-on Analysis. Nuclear run-on transcription analysis was performed as described (27) with minor modifications. Briefly, the cells were washed with cold phosphate-buffered saline (PBS) and lysed by using a 0.5% Nonidet P-40 solution. The nuclei were isolated by centrifugation and resuspended in a 40% glycerol buffer. Run-on experiments were performed by incubating the nuclei with [α-^{32}P]UTP at room temperature for 30 min. The run-on RNA was purified by treating the reaction mixture with DNase I and Proteinase K followed by precipitation with ethanol. The labeled RNA was hybridized to 10 μg of linearized, denatured plasmid DNA blotted on membrane in 50% (vol/vol) formamide/0.3 M NaCl/0.03 M sodium citrate/0.01% Ficoll/0.01% polyvinylpyrolidone/0.01% bovine serum albumin/0.1% SDS/100 μg of salmon sperm DNA per ml (hybridization buffer) at 42°C for 24 hr. The same number of counts of RNA were hybridized to each filter.

RNA Isolation and Northern Blot Analysis. Cells from static controls or from shear-stress experiments were washed twice with PBS, and total cellular RNA was isolated by using the guanidinium isothiocyanate method as described (28). In brief, the monolayer on the glass slide was washed and lysed with 4 M guanidinium isothiocyanate. The cell lysates were further extracted with phenol/chloroform/isoamyl alcohol, and RNA was collected. Equal amounts of the isolated RNA from each sample were loaded on a 1.5% formaldehyde agarose gel and subjected to electrophoresis. RNA was transferred to a nylon membrane for hybridization at 42°C in hybridization buffer for 18 hr with a ^{32}P-labeled 0.6-kb cDNA (American Type Culture Collection) encoding human MCP-1 or a 0.96-kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The membrane was then washed and exposed to Kodak X-Omat XAR film at −70°C.

RESULTS

Biphasic Response of MCP-1 Gene Expression to Fluid Shear Stress. We first examined the effect of shear stress on the expression of the gene for MCP-1 in cultured HUVEC. The confluent monolayer was subjected to laminar flow at a shear stress of 16 dynes/cm^2 for various lengths of time. This level of shear stress was selected because it is within the physiological range in the arterial tree (29) and has been shown to regulate the expression of IE genes such as c-*fos* and c-*jun in vitro* (7). RNA was then isolated for Northern blot analysis. Compared to the basal level of expression in static controls, the application of shear stress led to a rapid and transient induction of the MCP-1 gene in HUVEC (Fig. 1*A*). The expression peaked at 1.5 hr and gradually decreased to the basal level at 4 hr. In contrast, the gene encoding GAPDH, a constitutive gene, was expressed at a constant level throughout the time course of the study (Fig. 1*B*). Fig. 1*C* shows the results of quantitative analysis of these blots by laser densitometry. The expression of MCP-1 mRNA in HUVEC exposed to shear stress for 1.5 hr increased 2.5 times compared to that in the static control but decreased to the basal level at 4 hr. In a separate experiment in which HUVEC were exposed to the same level of shear stress for 5 hr (Fig. 2), MCP-1 gene expression was found to decrease further to below the basal level.

To analyze the reprogramming of the MCP-1 gene after the removal of the applied shear stress, HUVEC which had been sheared for 5 hr were subjected to either 1.5 hr of static incubation of 1.5 hr of static incubation followed by 1.5 hr of reapplication of shear stress at 16 dynes/cm^2 (Fig. 2). In both cases, the gene expression remained quiescent. When the postshearing static incubation was extended from 1.5 hr to 24 hr, however, the MCP-1 mRNA returned to the basal level. Furthermore, the cells regained their responsiveness to shear stress with a 2.5-fold increase of MCP-1 mRNA.

MCP-1 Activation Is Transcriptionally Controlled and Does Not Require *de Novo* Protein Synthesis. To determine whether the control of MCP-1 mRNA expression was at the transcriptional or posttranscriptional level, nuclear run-on experiments were performed by using nuclei isolated from static

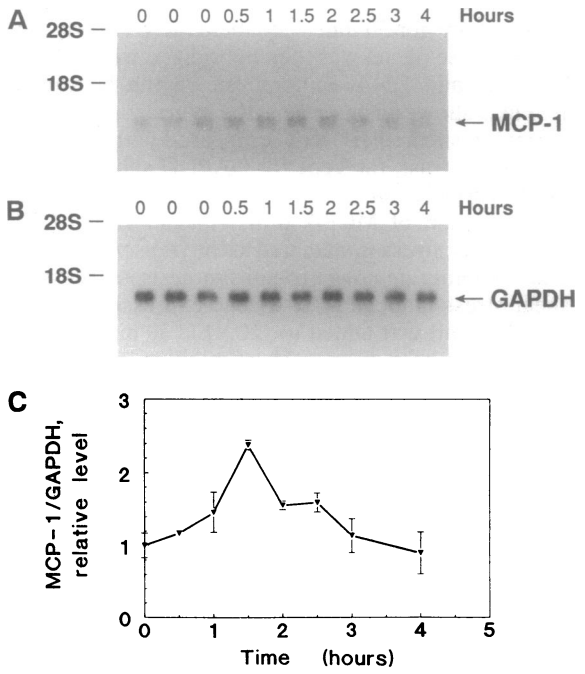

FIG. 1. Shear stress elicits a biphasic response of MCP-1 gene expression in HUVEC. Cells were grown to confluence on the culture slides and were either subjected to shear stress of 16 dynes/cm^2 for a period of time as indicated or kept as static control represented by "time 0." Total cellular RNA was isolated from the cells and subjected to Northern blot analysis. The probe was a ^{32}P-labeled 0.6-kb MCP-1 cDNA (*A*) or a ^{32}P-labeled 0.96-kb GAPDH cDNA (*B*). Shown in *C* are the levels of MCP-1 expression determined by densitometry. Bars indicate SEM.

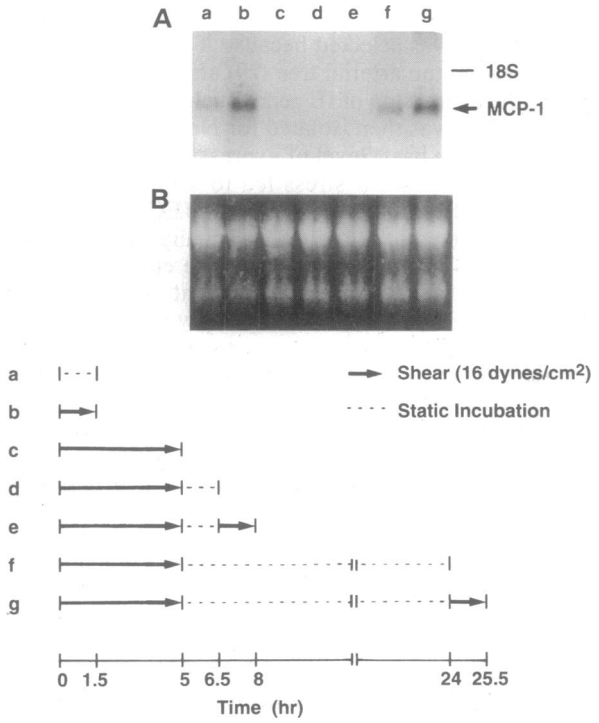

FIG. 2. Northern blot analysis demonstrating the return of MCP-1 mRNA to its basal level after the removal of the applied shear stress. (*A*) HUVEC were grown to confluence and were then subjected to various conditions: static incubation (- - -) and sheared experiments (16 dynes/cm^2) (→). The isolated RNA was then analyzed by Northern blot with the 0.6-kb MCP-1 cDNA as the probe. (*B*) Ethidium bromide staining indicates that comparable amounts of RNA were loaded.

HUVEC or HUVEC that had been exposed to a shear stress of 16 dynes/cm^2 for 50 min. ^{32}P-labeled RNA newly synthesized from these nuclei was then hybridized with MCP-1 and GAPDH plasmid DNA immobilized on the Nytran membrane. Fig. 3 shows an increase in the hybridization of MCP-1 transcripts in sheared HUVEC compared with that of static control. In contrast, the gene for GAPDH showed the same levels of hybridization in static and sheared HUVEC. Thus, the up-regulation of MCP-1 transcripts in HUVEC in response to shear stress is mediated at the transcriptional level.

To test whether *de novo* protein synthesis is necessary for the shear-stress-induced MCP-1 gene expression, cycloheximide (50 μg/ml) was added to HUVEC 30 min prior to the application of shear stress. The inhibition of the cellular protein synthesis by cycloheximide increased the MCP-1 mRNA in the static cells to levels higher than those in cells subjected to shear stress of 16 dynes/cm^2 for 1.5 hr (Fig. 4). When cycloheximide-treated cells were subjected to shear stress, the levels of mRNA in these cells were again higher than those in the treated, static cells, indicating that *de novo*

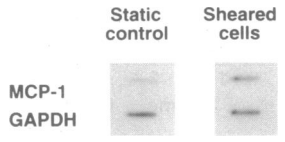

FIG. 3. Shear-stress-induced MCP-1 gene expression is controlled at the transcriptional level. HUVEC were grown to confluence and then either subjected to shear stress of 16 dynes/cm^2 for 50 min or kept as static controls. Nuclear run-on experiments were performed by isolating RNA from these cells and elongating them in the presence of [α-^{32}P]UTP. The labeled RNA were then purified and hybridized to plasmid DNA containing either MCP-1 or GAPDH cDNA.

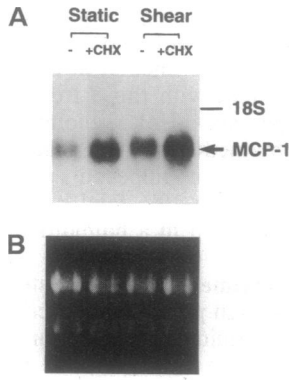

FIG. 4. (*A*) *De novo* protein synthesis is not necessary for the shear-stress-induced MCP-1 gene expression. HUVEC were grown to confluence and either treated with 50 μg of cycloheximide per ml for 30 min (lanes +CHX) or kept as control (lanes −). Cells were then subjected to shear stress of 16 dynes/cm^2 for 1.5 hr (lanes Shear) or kept statically for 1.5 hr (lanes Static). The isolated RNA was then analyzed by Northern blot with the 0.6-kb MCP-1 cDNA as the probe. (*B*) Ethidium bromide staining demonstrates that comparable amounts of RNA were loaded.

protein synthesis is not necessary for the shear-stress-induced MCP-1 gene expression.

The Biphasic Response Is Observed Over a Wide Range of Shear Stress and in Other Types of Cells. To test whether the shear-stress-induced MCP-1 gene expression is dependent upon the magnitude of the applied shear stress, experiments were performed with shear stresses of 6 and 32 dynes/cm^2. These two levels of shear stress, like 16 dynes/cm^2, induced the activation of the MCP-1 mRNA expression in HUVEC, and the levels of induction did not vary markedly with the shear-stress level (Fig. 5).

To examine whether the shear-stress-induced biphasic response of MCP-1 gene expression also occurs in other types of cells, experiments were performed on HeLa cells and glioma cells in which MCP-1 is known to be expressed (17). These cells were grown on gelatin-coated slides to confluence and were subjected to a shear stress of 16 dynes/cm^2 for 1.5 or 5 hr followed by Northern blot analysis. Fig. 6 shows the comparison of the MCP-1 gene expression between these sheared cells and static control cells. The MCP-1 transcripts in both the HeLa and glioma cells increased after the cells had been subjected to shear stress for 1.5 hr and decreased to basal levels when the cells had been subjected to shear stress for 5 hr. Similar results were also obtained in skin fibroblasts (data not shown). Thus, the

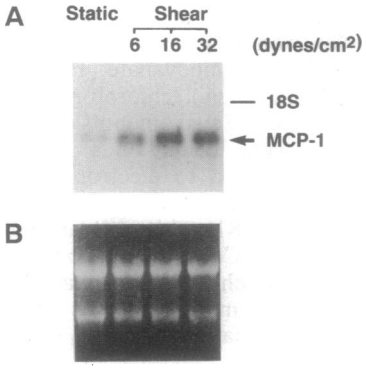

FIG. 5. (*A*) Biphasic response of MCP-1 gene expression is observed over a wide range of shear stress. HUVEC were grown to confluence on the culture slides and were subjected to shear stresses of 6, 16, or 32 dynes/cm^2 for 1.5 hr. Northern blot analysis was performed as in Fig. 1. (*B*) Ethidium bromide staining demonstrates that comparable amounts of RNA were loaded.

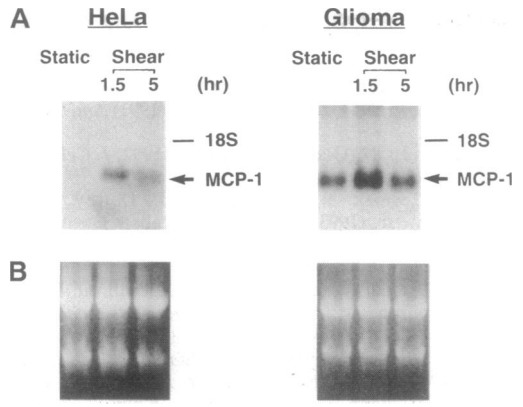

FIG. 6. (*A*) Biphasic response of shear-stress-regulated MCP-1 gene expression in HeLa and glioma cell lines. Cells were grown to confluence on the culture slides and subjected to a shear stress of 16 dynes/cm^2 for either 1.5 or 5 hr. Northern blot analysis was performed as in Fig. 1. (*B*) Ethidium bromide staining demonstrates that comparable amounts of RNA were loaded.

biphasic response of the MCP-1 gene to shear stress is not only tissue-specific for the vascular endothelium but also is found in other types of cells.

DISCUSSION

The responses of the MCP-1 gene to shear stress fulfill the three criteria defined for IE gene activation (30): (*i*) MCP-1 mRNA increases rapidly from the basal level in static cells, within 1.5 hr of stimulation by shear stress and decays rapidly afterwards (Figs. 1 and 2), (*ii*) MCP-1 gene is transcriptionally activated in response to shear stress (Fig. 3), and (*iii*) *de novo* protein synthesis is not necessary for such shear-stress-induced activation (Fig. 4). The activation of c-fos and c-myc transcripts under similar shearing conditions has been previously shown to have peak levels of transcripts at 0.5 hr and 1.5 hr, respectively, and the activation was followed by a rapid decline (7). Thus, the shear-stress-induced activation and down-regulation of MCP-1 and c-myc mRNAs are somewhat later than that of c-fos. However, the transient expression of these mRNAs in HUVEC subjected to laminar flow may not predict a parallel change in translational levels, since numerous examples are known where transient changes at transcription levels do not correspond to changes in protein levels. IE genes can be separated into different groups based on the kinetics of their mRNA accumulation upon serum stimulation. c-*fos* belongs to a group of genes that respond to serum or phorbol ester stimulation within 5 min, and whose mRNAs peak at 30–60 min. The gene for MCP-1 and c-*myc* belong to a second group of genes that reach a peak level at ≈2 hr. The timing of the induction of IE genes in response to shear stress is very similar to that in response to serum growth factor or phorbol ester. Furthermore, shear-stress forces, like serum growth factor and phorbol ester, induce IE genes at the transcriptional level. These similarities raise the questions whether similar signal-transduction mechanisms are involved in these processes and, if they are, whether shear stress mimics serum growth factor and phorbol ester in their proliferating effects. The shear-stress-induced transient regulation seems to be limited not only to IE genes. Genes such as that encoding endothelin 1 are also known to be regulated by shear stress biphasically (31–33). In a recent report, such transient endothelin 1 gene expression was further related to the shear-stress-induced disruption of actin cytoskeleton (34).

Previously, multiple signal-transduction pathways including protein kinase C (PKC), tyrosine phosphorylation, and an independent third signaling mechanism have been shown to be involved in the activation of the gene for MCP-1 (35). The evidence for the involvement of PKC in such induction is that phorbol ester TPA (phorbol 12-tetradecanoate 13-acetate) and other stimulants such as serum, diacylglycerol, lipopolysaccharide, tumor necrosis factor, and interleukin 1 known to activate PKC induce the expression of the MCP-1 gene (35). Starosporine, a PKC inhibitor, blocks all these inductions to some extent (35, 36). Several investigations have suggested that PKC is activated by shear stress based on the following observation. The shear-stress-induced *PDGFB* expression is inhibited by the PKC inhibitor H7 (10). An increased level of inositol 1,4,5-trisphosphate (InsP_3) and an enhanced turnover of diacylglycerol were found in the sheared HUVEC (37, 38); InsP_3 and diacylglycerol are the products of phospholipase C, and elevated levels of diacylglycerol cause the translocation and activation of PKC. At the distal end of PKC activation is the binding of the transcription factor AP-1 to phorbol ester TPA-responsive elements (TRE) (39). If PKC is involved in the shear-stress-induced MCP-1 gene expression, the signal transduction may be mediated through the two copies of TRE with sequences of TCACTCA and TGACTCC located at −129 and −157 bp (upstream from the translation initiation site) of the 5′ promoter region (40). Interestingly, the JE gene is known to be induced by mechanical stretch force in mouse cardiac cells, and PKC has been suggested to be involved in the signal-transduction pathway (15). Furthermore, genes known to be regulated by shear stress, including c-*fos*, c-*jun*, *PDGFA*, *PDGFB*, and genes encoding tissue plasminogen activator, endothelin 1, ICAM-1, and TGF-β1, all contain sequences with homology to TRE (Table 1). It is very possible that the activation of AP-1 and the subsequent binding to the TRE site is one of several mechanisms that mediate the response to shear stress. The consensus sequence for the κB-enhancer element, GGAAGATCCCT located at −148 bp, should not be excluded in this mechanism, since this element is also activated by PKC (41). It should be noted that TRE also responds to serum growth factor and that it has considerable basal activity even in quiescent cells (42). This may explain the basal expression of MCP-1 mRNA in static cells that were cultured in medium supplemented with 15% FBS. However, this serum effect should only modify the basal levels of expression in shear-stress experiments, since the same conditioned medium used in culturing the cells was used to shear HUVEC.

The slow recovery of MCP-1 mRNA from a quiescent level to the basal level after the removal of the shear stress suggests that the gene expression is down-regulated by the continued application of shear stress. Testing with reapplication of

Table 1. Locations of the putative TRE sites [TGA(C or G)TCA] in the 5′ promoter regions of the shear-stress-regulated genes

Gene	Location*	Sequence	Gene	Location*	Sequence
MCP-1	−157	TGACTCC	c-*jun*	−72	TGACATCA
	−129	TCACTCA			
PDGFA	−35	AGACTCC	*EDN1*	−367	TGGCTCA
				−108	TGACTAA
PDGFB	−490	TGAGTCC	*ICAM1*	−1256	TGACTCGCA
	−417	TGACCCA		−1	TGAGCTCC
	−356	TGAGTCC			
	−72	TGACTCC			
c-*fos*	−78	TGAGACA	*TGFB1*	−418	TGACTCT
				−371	TGTCTCA
PLAT	−113	TGACATCA			

PLAT, *EDN1*, *ICAM1*, and *TGFB1* are designations of human genes encoding "plasminogen activator, tissue," endothelin 1, ICAM-1, and TGF-β1, respectively.
*Upstream relative to the transcription initiation site.

shear stress indicates that the return from quiescent to basal activity needs a period of static incubation longer than 1.5 hr—i.e., this does not occur as rapidly as the activation from the basal level. If this biphasic MCP-1 gene expression in HUVEC regulated by shear stress force *in vitro* is also operative in the arterial tree *in vivo*, the results suggest that MCP-1 gene would be quiescent in arterial EC exposed to a constant shear flow. This may be correlated with the pathophysiological observation that the lesion-free areas of the arterial tree are mostly those under steady shear flow.

The response of the MCP-1 gene in HUVEC to shear stress did not vary markedly over the range from 6 to 32 dynes/cm^2 (Fig. 5). This observation and the transient responses found in the epithelial-like HeLa cells, glioma cell line, and skin fibroblasts indicate that the regulation of MCP-1 gene by shear stress is a global event covering many tissue types and a wide range of shear-stress forces. This *in vitro* paradigm of tissue nonspecificity may not be relevant under physiological conditions because the only tissue experiencing wall shear stresses in the range of 6–32 dynes/cm^2 *in vivo* is the vascular EC. It is to be noted again, however, that the MCP-1 gene is probably suppressed in most of these EC that are exposed to a constant shear stress. Otherwise, chemoattraction of monocytes to vascular wall due to MCP-1 expression would have been a physiological rather than an atherogenic event. In areas such as the lateral wall of bifurcations, EC may respond to the disturbed flow by programming differently their IE gene expression.

Resnick *et al.* (13) observed an increase of PDGFB transcripts in bovine aortic endothelial cells subjected to a shear stress of 10 dynes/cm^2 for 4 hr and identified a cis-acting element in the 5′ promoter region of the bovine *PDGFB* gene that responds to this regime of shear stress. Based on mobility-shift assays, the core sequence GAGACC was further identified to bind to transcription factors unique in sheared cells. This shear-stress responsive element (SSRE) is also present in genes encoding ICAM-1, TGF-β1, and tissue plasminogen activator—i.e., genes known to be regulated by shear stress. We also detected SSRE in the 5′ flanking region of the cloned MCP-1 gene. It would be interesting to know whether this sequence regulates collectively with other cis-elements such as TRE in the transcriptional activation of MCP-1 gene by shear stress in human vascular EC. If this is the case, the second phase of the regulation (the suppression of the gene with prolonged shear) may result from either the change of interactions of transcription factors to these elements or a totally independent pathway such as transcript instability due to an A+U-rich sequence in the 3′ untranslated region of mRNA (43, 44). These considerations lead further to the question whether the proposed mechanism obtained from the steady-flow experiments is similar to that under disturbed flow.

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