Fluid Shear Stress Increases the Production of Granulocyte-Macrophage Colony-Stimulating Factor by Endothelial Cells via mRNA Stabilization

Keisuke Kosaki, Joji Ando, Risa Korenaga, Takahide Kurokawa, Akira Kamiya

Abstract—To investigate whether the production of colony-stimulating factors (CSFs) by vascular endothelial cells is regulated by hemodynamic force, we exposed cultured human umbilical vein endothelial cells (HUVECs) to controlled levels of shear stress in a flow-loading apparatus and examined changes in the production of CSFs at both the protein and mRNA level. Exposure of HUVECs to a shear stress of 15 and 25 dyne/cm² markedly increased the release of granulocyte-macrophage CSF (GM-CSF) detected by ELISA to 5.0 and 9.5 times, respectively, the amount released by the static controls at 24 hours, but it had no significant influence on the release of granulocyte CSF or macrophage CSF. The results of reverse transcriptase–polymerase chain reaction demonstrated that GM-CSF mRNA began to increase as early as 2 hours after initiation of 15 dyne/cm² shear stress and continued to increase with time, reaching a peak of about four times the control levels at 24 hours. This increase in GM-CSF mRNA levels in response to shear stress depended on protein synthesis, because it was blocked by cycloheximide. Neither nuclear run-on assay or luciferase assay using a reporter gene containing GM-CSF gene promoter showed any significant change in transcription of the GM-CSF gene even after 24-hour exposure to a shear stress of 15 dyne/cm². Actinomycin D chase experiments using a competitive polymerase chain reaction showed that shear stress extended the half-life of GM-CSF mRNA from ~23 to 42 minutes in HUVECs. These findings suggest that fluid shear stress increases the production of GM-CSF in HUVECs via mRNA stabilization. (Circ Res. 1998;82:794-802.)

Key Words: granulocyte-macrophage colony-stimulating factor ■ shear stress ■ endothelial cell ■ mRNA stability

Granulocyte-macrophage CSF, a member of the hematopoietic growth factor family, is produced and released by monocytes, macrophages, fibroblasts, and ECs and stimulates the proliferation and differentiation of granulocyte/macrophage progenitor cells. Recently, it has been learned that in addition to its effect on hematopoietic cells, GM-CSF can act on nonhematopoietic cells. Specific GM-CSF receptors have been detected on the surface of ECs, fibroblasts, trophoblasts, and keratinocytes, with ECs appearing to be particularly strongly influenced by GM-CSF. This cytokine induces the proliferation and migration of ECs in vitro and causes new capillary formation in vivo. It also upregulates the synthesis of prostacyclin and the expression of class I myosin heavy chain antigens and affects the adhesion of leukocytes to ECs by modulating adhesion molecule expression on the surface of ECs. In addition, GM-CSF activates the Na⁺-H⁺ exchanger and the transcription of the oncogene c-fos in ECs. Thus, GM-CSF produced by ECs may act on ECs themselves in an autocrine or paracrine manner and may play an important role in the regulation of blood vessel functions.

A variety of EC functions were thought to be controlled mainly by chemical stimuli, such as hormones, cytokines, and neurotransmitters, but recently, they have been found to also be regulated by mechanical stimuli generated by blood flow or blood pressure. A number of studies have indicated that the fluid shear stress created by blood flow modulates EC morphology and functions and, at the same time, alters the expression of the related EC genes. For instance, shear stress upregulates or downregulates the mRNA levels of genes coding tissue plasminogen activator, PDGF, endothelin, MCP-1, transforming growth factor-β, superoxide dismutase, angiotensin-converting enzyme, nitric oxide synthase, C-type natriuretic peptide, adrenomedulin, thrombomodulin, heparin-binding epidermal growth factor, tissue factor, intercellular cell adhesion molecule-1, and VCAM-1. More recently, cis-acting elements and transcriptional factors involved in the molecular mechanism of shear stress–mediated regulation of EC genes have been identified; these are SSRE and NF-κB, which have been implicated in the upregulation of the PDGF-B gene by shear stress, and TRE and AP-1, which are involved in the MCP-1 and VCAM-1 gene responses to shear stress. A great deal of attention has been focused on such EC gene responses to shear stress in relation to the mechanisms of blood flow–

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dependent phenomena, such as angiogenesis, vascular remodeling, and atherosclerosis, but a great deal still remains to be elucidated.

ECs constitutively produce small amounts of GM-CSF, but they markedly increase production in response to chemical stimuli, such as TPA, IL-1, TNF, lipopolysaccharide, and acetylated LDL. However, it was uncertain whether production of GM-CSF by ECs is affected by blood flow or shear stress, a critical physiological stimulus. To answer this question, we applied controlled levels of shear stress to cultured HUVECs in a flow-loading apparatus and examined changes in GM-CSF production at both the protein and mRNA levels. We also investigated the effect of shear stress on GM-CSF gene transcription and mRNA stability.

Materials and Methods

Cell Culture

Primary cultures of HUVECs were obtained from human umbilical cord veins by collagenase treatment and grown in a 1% gelatin-coated flask in medium 199 containing 15% FBS (Hyclone Laboratories), 2 mmol/L L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 50 μg/mL heparin, and 30 μg/mL EC growth factor (Becton and Dickinson). Cells were routinely passaged by trypsinization in a 0.05% trypsin/2 mM/L EDTA solution. At every passage, cell number was counted with a Coulter Counter (type ZM, Coulter Electronics Limited), and cumulative population doubling passage, cell number was counted with a Coulter Counter (type ZM, Coulter Electronics Limited), and cumulative population doubling was determined. Cells with 8 to 14 cumulative population doubling were confirmed to be free of endotoxin by the limulus gelatin test.

Flow-Loading Apparatus

ECs were exposed to laminar flow in a parallel-plate type of flow chamber, as previously described. Briefly, one side of the flow chamber consisted of a glass plate on which the cultured ECs rested, and the other side was a polycarbonate plate. These two flat surfaces were held 200 μm apart by a polytetrafluoroethylene gasket. The intensity of shear stress (τ, dyne/cm²) acting on the EC layer was calculated by the following formula: τ=6μQ/abh, where μ is the viscosity of the perfusate (poise), Q is flow volume (ml/s), and a and b are cross-sectional dimensions of the flow path (cm). A closed circuit was arranged with a silicone tube, and a depulser was placed between the pump and flow chamber to eliminate bubbles generated by the pump. Medium was constantly circulated with a roller/tube pump (Atto Co) at 37°C in an atmosphere of 95% room air and 5% CO2.

In preliminary experiments, the characteristics of flow through the chamber containing the coverslip were visually examined at various perfusion rates by circulating medium with suspended polystyrene flakes. Flow patterns, as analyzed with a high-speed video camera (MHS200, NAC), showed no visible turbulence. Since the maximum Reynolds number corresponding to the highest flow rate used in the present study was ~40, we assumed the flow to be laminar.

In some experiments, 5% dextran (molecular weight, 162 000; Sigma Chemical Co) was added to the perfusate to raise the viscosity as much as four times that of the control medium. All conditioned culture media and perfusates obtained after flow-loading experiments were confirmed to be free of endotoxin by the limulus gelatin test.

ELISA

The amount of CSFs released by the ECs was assayed by ELISA using commercially available kits (Ohtsuka Assay Co and R&D Systems). Briefly, 100 μl of perfusate was incubated in a microplate coated with anti-human monoclonal antibodies against each CSF for 2 hours. After a washing with the specified detergent, each CSF conjugate was added and incubated for 2 hours. After the washing, the color reagent was added, and incubation was performed for 20 minutes. Absorbance at a specified wavelength was measured with a microplate reader (model 3550, Bio-Rad), and the concentration of CSF in each sample was determined from the standard curve.

RT-PCR Analysis

RT-PCR was performed to quantify the mRNA levels of each CSF, as previously described. Briefly, total RNA was isolated from the cells by the acid guanidinium thiocyanate–phenol–chloroform extraction method. Reverse transcription of RNA was carried out in 20 μL of a reaction mixture containing 1.0 μg total RNA, 200 U Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL), 0.5 μg oligo(dT)12–18 (Perkin Elmer-Cetus), 40 U ribonuclease inhibitor (Perkin Elmer-Cetus), 2.5 mmol of each dNTP mixture, and 0.001% gelatin was added to each sample. Each temperature cycle consisted of 95°C for 1 hour, heated at 95°C for 5 minutes, and chilled at 4°C for 5 minutes. The cDNA samples were then ampliﬁed by PCR with primer pairs for each CSF and GAPDH (Table). Eighty microliters of a solution containing 0.25 U of Ex Taq DNA polymerase (Takara), 370 kBq of [α-32P]dCTP (≈11 TBq/ mmol) (Amersham), and 0.1 pmol of each primer in an Ex Taq buffer of 10 mmol/L Tris–HCl (pH 8.3), 75 mmol/L KCl, and 3 mmol/L MgCl2. The mixture was incubated at 37°C for 1 hour, heated at 95°C for 5 minutes, and chilled at 4°C for 5 minutes. The cDNA samples were then amplified by PCR with primer pairs for each CSF and GAPDH (Table). Eighty microliters of a solution containing 0.25 U of Ex Taq DNA polymerase (Takara), 370 kBq of [α-32P]dCTP (≈11 TBq/ mmol) (Amersham), and 0.1 pmol of each primer in an Ex Taq buffer of 10 mmol/L Tris–HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, and 0.001% gelatin was added to each sample. Each temperature cycle consisted of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Ten microliters of amplified product was sampled every cycle and electrophoresed on a 5% polyacrylamide gel (Sigma).

For quantification of PCR products, the radioactivity of each band was measured with a GS363 Molecular Imager System (Bio-Rad) and plotted against the number of PCR cycles on a semilogarithmic scale, forming a sigmoid curve. From the curve, the cycle in which the operating range of the PCR was linear was selected, and the ratio of radioactivity between CSF and GAPDH in the cycle was calculated as a parameter of relative CSF mRNA levels.

To confirm that CSF mRNA and GAPDH mRNA were correctly amplified by those primers, the PCR product was cloned into the pCR II vector using the TA cloning system (Invitrogen) and

Selected Abbreviations and Acronyms

AP-1 = activator protein-1
AUBP = AU-rich element–binding protein
CSF = colony-stimulating factor
EC = endothelial cell
G-CSF = granulocyte CSF
GM-CSF = granulocyte-macrophage CSF
HUVEC = human umbilical vein EC
IL-1 = interleukin-1
M-CSF = macrophage CSF
MCP-1 = monocyte chemotactic protein-1
NF = nuclear factor
PCR = polymerase chain reaction
PDGF = platelet-derived growth factor
PMA = phorbol 12-myristate-13-acetate
RT-PCR = reverse transcriptase–PCR
SSRE = shear stress–responsive element
TNF = tumor necrosis factor
TPA = 12-O-tetradecanoylphorbol 13-acetate
TRE = tumor-promoting agent response element
UTR = untranslated region
VCAM-1 = vascular cell adhesion molecule-1
sequenced by the cycle-sequencing methods using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer-Cetus). The sequences showed complete homology to the parts of each gene of interest.

**Nuclear Run-on Assay**

HUVECs, incubated under static conditions or exposed to shear stress for 6 hours, were washed with ice-cold PBS, scraped, and pelleted by centrifugation at 1500 rpm for 5 minutes. The cell pellet was resuspended in 1 mL NP-40 lysis buffer (10 mmol/L Tris-HCl [pH 7.4], 10 mmol/L NaCl, 5 mmol/L MgCl₂, and 0.5% [vol/vol] NP-40), incubated for 5 minutes on ice, and centrifuged at 3000 rpm for 5 minutes. The nuclear pellet was washed once with 1 mL NP-40 lysis buffer and centrifuged again at 3000 rpm for 5 minutes. Nuclei were resuspended in 100 μL of 50 mmol/L Tris-HCl (pH 8.3), 5 mmol/L MgCl₂, 0.1 mmol/L EDTA, and 40% (vol/vol) glycerol and frozen in liquid N₂. The nuclei were thawed and reacted in 100 μL reaction buffer consisting of 10 mmol/L Tris-HCl (pH 8.0), 5 mmol/L MgCl₂, 300 mmol/L KCl, 0.5 mmol/L ATP, 0.5 mmol/L CTP, 0.5 mmol/L GTP, and 3.7 MBq [³²P]UTP (~111 TBq/mmol) for 30 minutes at 30°C. The [³²P]-labeled RNA was precipitated by trichloroacetic acid and purified with phenol/chloroform extraction.

Plasmids containing human GM-CSF or human GAPDH fragment (Clontech Laboratories, Inc) were linearized by restriction enzyme HUVECs, incubated under static conditions or exposed to shear stress for 6 hours, were washed with ice-cold PBS, scraped, and pelleted by centrifugation at 1500 rpm for 5 minutes. The cell pellet was resuspended in 1 mL NP-40 lysis buffer (10 mmol/L Tris-HCl [pH 7.4], 10 mmol/L NaCl, 5 mmol/L MgCl₂, and 0.5% [vol/vol] NP-40), incubated for 5 minutes on ice, and centrifuged at 3000 rpm for 5 minutes. The nuclear pellet was washed once with 1 mL NP-40 lysis buffer and centrifuged again at 3000 rpm for 5 minutes. Nuclei were resuspended in 100 μL of 50 mmol/L Tris-HCl (pH 8.3), 5 mmol/L MgCl₂, 0.1 mmol/L EDTA, and 40% (vol/vol) glycerol and frozen in liquid N₂. The nuclei were thawed and reacted in 100 μL reaction buffer consisting of 10 mmol/L Tris-HCl (pH 8.0), 5 mmol/L MgCl₂, 300 mmol/L KCl, 0.5 mmol/L ATP, 0.5 mmol/L CTP, 0.5 mmol/L GTP, and 3.7 MBq [³²P]UTP (~111 TBq/mmol) for 30 minutes at 30°C. The [³²P]-labeled RNA was precipitated by trichloroacetic acid and purified with phenol/chloroform extraction.

Plasmids containing human GM-CSF or human GAPDH fragment (Clontech Laboratories, Inc) were linearized by restriction enzyme digestion and denatured at 95°C. The DNA was spotted onto nylon membranes and fixed with 0.4 N NaOH. Radiolabeled RNA was digested and denatured at 95°C. Blots were washed twice in 2× SSC at 42°C, once in 1× SSC at 42°C, and twice in 0.1× SSC at 42°C, before incubation at 30°C for 10 minutes with 32P-labeled "782–762" segment was cloned into pBluescript DNA linearized with XhoI was performed in vitro using T3 RNA polymerase in the presence of [α-³²P]UTP. Labeled RNA had a specific activity of ~1×10⁷ cpm/μg.

The competitive PCR was performed to quantify mRNA concentrations. After the total RNA (1 μg) obtained from the sample was reverse-transcribed into cDNA, the products were mixed with serial dilutions of the competitor DNA (pGL-GMCSF, ~2.5 kb) spanning a range of concentrations from 0.01 to 10 pg. These mixtures were amplified in the presence of [α-³²P]dCTP for 36 cycles (94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 60 seconds) and subjected to electrophoresis on 5% polyacrylamide gel. The sense (5'-TGGGTCAAGCGTCTGCTCTTGGGACT-3') and antisense (5'-CTGGAGGTCAACATTCTGGGAGACT-3') primers locate exons 1 and 2 of the GM-CSF gene, respectively, and an intron of 97 bp is present between the two exons. Accordingly, the size of the amplification product from the competitor DNA consists of 290 bp, and that from the cDNA consists of 193 bp. mRNA concentrations in the test samples were estimated by comparing the intensity of bands derived from amplification of the competitor cDNA (290 bp) with those derived from the RNA sample being tested (193 bp). The concentration of competitive cDNA that gives rise to a band with a radioactivity of 1.47 (290/193) times the test sample corresponds to the concentration of mRNA in the test sample.

**Hel Shift Assay**

Cytoplasmic extracts of static and shear-stressed HUVECs (15 dyne/cm², 24 hours) were prepared by freeze-thaw lysis in 25 mmol/L Tris-HCl (pH 7.9), 0.5 mmol/L EDTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride, followed by centrifugation at 15 000 rpm for 15 minutes. The DNA was synthesized using complementary oligonucleotide containing 54 nucleotides from the AT-rich 3' UTR of human GM-CSF (positions 641 to 694) and including seven repeats of the ATTATA sequence (GATCAGTAATATTATATATTATATATATATATATATATTATA). This "X" AT DNA segment was cloned into EcoRI-digested pBluescript II (Stratagene), and sequencing was performed to verify sequence fidelity. Transcription of pBluescript DNA linearized with XhoI was performed in vitro using T3 RNA polymerase in the presence of [α-³²P]UTP. Labeled RNA had a specific activity of ~1×10⁷ cpm/μg.

The cytoplasmic extracts (10 μg of protein, 7×10⁶ cells) were incubated at 30°C for 10 minutes with 10 ⁴ cpm [³²P]-labeled "7× AT" RNA (1×10⁷ cpm) in 10% glycerol, 12 mmol/L HEPES (pH 7.9), 15 mmol/L KCl, 0.25 mmol/L EDTA, 0.25 mmol/L dithiothreitol, and 5 mmol/L MgCl₂. For competition experiments, these extracts were incubated with unlabeled probe before incubation with ¹³C-labeled RNA. Non-specific binding was reduced by adding 2 μg of yeast tRNA. The reaction mixture was cross-linked with 254-nm UV radiation using a UV chamber (GS Gene Linker, Bio-Rad). RNase A was added to a final concentration of 1 μg/μL, and the mixture was incubated at 37°C for 30 minutes. An equal volume of 2× sample buffer containing 2-mercaptoethanol was added to each sample and boiled at 100°C for 3 minutes. The samples were then electropho-

**Oligonucleotide Primers Used for RT-PCR**

<table>
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<tr>
<th>Gene</th>
<th>Oligonucleotide Sequence</th>
<th>Position</th>
</tr>
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<tr>
<td>GM-CSF</td>
<td>Sense primer 5'-GAGCATGTGAATTGCCATCACGAGGAG-3'</td>
<td>91–114</td>
</tr>
<tr>
<td></td>
<td>Antisense primer 5'-CTCCTGAGCTGCTCCAGCAGTCAA-3'</td>
<td>432–406</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Sense primer 5'-CACAGTGCACTTGAGGAGCAGGAGA-3'</td>
<td>61–87</td>
</tr>
<tr>
<td></td>
<td>Antisense primer 5'-CATTCGGCTCTCCTACGTGCGGAGAT-3'</td>
<td>477–448</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Sense primer 5'-CATGACAAAGGCTGCTGCGGA-3'</td>
<td>388–408</td>
</tr>
<tr>
<td></td>
<td>Antisense primer 5'-AAGCTCTGGAGGAGTCTCCTG-3'</td>
<td>782–762</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense primer 5'-ACATCATCTCTTGCTCCTACTG-3'</td>
<td>608–629</td>
</tr>
<tr>
<td></td>
<td>Antisense primer 5'-AGTGGGTCTGCCGCTGAGATC-3'</td>
<td>868–847</td>
</tr>
</tbody>
</table>

Base-pair positions cited are those given in the original published sequences for GM-CSF, G-CSF, M-CSF, and GAPDH.
resed in polyacrylamide gels containing 0.1% SDS, and the \(^{32}\)P-labeled protein was analyzed by a image analyzer.

**Statistical Analysis**

Differences in CSF production between the static control and flow-loaded samples were evaluated by ANOVA followed by Bonferroni’s multiple comparison test by using SPSS (version 6.07J for Windows, SPSS Inc). Significance was assumed at \(P<0.05\). To assess the shear rate or shear stress dependence of GM-CSF mRNA levels, the ratio test of the composite hypothesis of Neyman and Pearson was used, as previously reported by Ando et al.40

**Results**

**Laminar Flow Increases GM-CSF Production in HUVECs**

Confluent monolayers of HUVECs were subjected to laminar flow with a shear stress of 15 dyne/cm\(^2\), and the amounts of GM-CSF, G-CSF, and M-CSF released into the perfusates were measured by ELISA. HUVECs released a small amount of GM-CSF under static conditions but markedly increased the production in response to laminar flow (Figure 1). The cumulative production of GM-CSF was increased to 5-fold the level of production in the static controls at 8, 24, and 48 hours after the initiation of flow. The rate of production tended to diminish after 24 hours. The increase in GM-CSF production became more prominent as shear stress increased from 15 to 25 dyne/cm\(^2\). On the other hand, neither G-CSF nor M-CSF production changed significantly, even under flow conditions.

The stimulatory effect of shear stress on GM-CSF production was compared with that of PMA (Sigma). The amount of GM-CSF released after 24-hour exposure to a shear stress of 25 dyne/cm\(^2\) corresponded roughly to one-third that after 24-hour stimulation with a maximally effective concentration of PMA (100 ng/mL) (161.0±6.0 versus 482.2±6.4 pg/10\(^6\) cells; mean±SD, \(n=6\)).

**Laminar Flow Increases GM-CSF mRNA Levels in HUVECs**

HUVECs were exposed to laminar flow with a shear stress of 15 dyne/cm\(^2\) for 24 hours, and the levels of CSF mRNA were determined by RT-PCR. The GM-CSF band on the gel was much thicker for shear-stressed cells than for static control cells (Figure 2A), indicating that shear stress increased GM-CSF mRNA levels in the HUVECs. The G-CSF band became slightly thinner after exposure to shear stress, whereas the M-CSF band became slightly thicker (Figure 2A). Quantitative analysis of these CSF bands by densitometry revealed that the ratio of the levels of GM-CSF, G-CSF, and M-CSF mRNA in shear-stressed cells to their levels in static control cells was 3.70±0.22 (\(P<0.01\)), 0.71±0.10 (\(P=NS\)), and 1.61±0.13 (\(P=NS\)) (mean±SD, \(n=6\)), respectively. GM-CSF mRNA levels began to increase as early as 2 hours after the initiation of laminar flow and continued to increase over time, reaching a peak of about four times the static control levels at 24 hours, and then decreased but were still much higher than the control level at 48 hours (Figure 2B).

To determine whether upregulation of GM-CSF mRNA by shear stress depends on new protein synthesis, we treated the HUVECs with the protein synthesis inhibitor cycloheximide before exposing them to shear stress. The 1.5-hour pretreatment did not change the basal level of GM-CSF mRNA.

**Figure 1.** Effect of laminar flow on the production of GM-CSF, G-CSF, and M-CSF in HUVECs. HUVECs were exposed to laminar flow, and the concentration of each CSF in the perfusates was measured by ELISA. The open bars indicate static control cells. The solid bars and hatched bars represent cells exposed to a shear stress of 15 or 25 dyne/cm\(^2\), respectively. HUVECs markedly increased production of GM-CSF, but not G-CSF or M-CSF, in response to laminar flow. Data are mean±SD of six samples in two separate experiments. *\(P<0.01\) vs static control. The difference between 15 and 25 dyne/cm\(^2\) was also statistically significant (\(P<.01\)).

**Figure 2.** RT-PCR analysis of GM-CSF, G-CSF, and M-CSF mRNA levels in HUVECs. A, Total RNA was extracted from HUVECs either incubated under static conditions (S) or exposed to laminar flow at a shear stress of 15 dyne/cm\(^2\) (F) for 24 hours and reverse-transcribed into cDNA. The cDNA was amplified by PCR using specific primers and electrophoresed on a polyacrylamide gel. Values in parentheses indicate the molecular size of the amplification products. GM-CSF mRNA levels markedly increased after exposure to shear stress. B, Time course of the changes in GM-CSF mRNA levels after the initiation of shear stress is shown. GM-CSF mRNA levels increased in response to shear stress, peaking at ~3.7 times the control levels at 24 hours. Data are mean±SD of three separate experiments. *\(P<.01\) vs static control.
Cycloheximide blocked the upregulation of GM-CSF mRNA by shear stress (Figure 3), indicating that the upregulation process depends on the synthesis of new protein.

**Flow-Induced Elevations of GM-CSF mRNA Levels Are Shear Stress Dependent**

To determine whether the increase in GM-CSF mRNA levels is shear stress or shear rate dependent, HUVECs were subjected to the flow of two perfusates with different viscosities for 6 hours. The mRNA levels increased as shear rate increased, but the levels increased to an even greater extent when viscosity or shear stress was higher at the same shear rate (Figure 4, top). On the other hand, mRNA levels plotted against shear stress formed a straight line (Figure 4, bottom). These findings indicate that flow-induced elevations in GM-CSF mRNA levels are shear stress rather than shear rate dependent.

**Transcription of the GM-CSF Gene Is Unaffected by Shear Stress**

We performed a nuclear run-on assay to determine whether shear stress directly affects GM-CSF gene transcription. Nuclei were prepared from static control or shear-stressed HUVECs (15 dyne/cm², for 24 hours), and transcription was allowed to proceed in the presence of [³²P]UTP. Purified radiolabeled RNA was hybridized to cDNA immobilized on nylon membranes. Transcription of the GM-CSF gene, which could be detected distinctly in static control cells, was unchanged even after exposure to shear stress (Figure 5A).

We also performed a luciferase assay to evaluate the effect of shear stress on GM-CSF gene transcription in vitro. HUVECs transfected with the reporter gene, pGL-GMCSF (−2.5 kb) or pGL-GMCSF (−4.3 kb), were exposed to a shear stress of 15 dyne/cm² for 24 hours, and luciferase activity was measured. pGL-GMCSF (−2.5 kb) contains the sequence SSRE (GAGACC) at +610 to +615 in an intron between exons 2 and 3, and pGL-GMCSF (−4.3 kb) has the TRE sequence (TGACTCA) at −3.0 kb. Both SSRE and TRE have been found to function as a cis element for shear responsiveness. Luciferase activity, however, did not change significantly even after exposure to shear stress (Figure 5B). These findings indicate that shear stress does not affect transcription of the GM-CSF gene in HUVECs and that neither SSRE nor TRE is involved in the upregulation of GM-CSF gene expression by shear stress.

**Shear Stress Increases GM-CSF mRNA Stability**

We performed actinomycin D chase experiments to determine whether GM-CSF mRNA is regulated by shear stress at posttranscriptional levels. HUVECs were treated with actinomycin D either being incubated under static conditions...
or being exposed to a shear stress of 15 dyne/cm² for 24 hours, and the changes in concentration of GM-CSF mRNA were measured by competitive PCR. GM-CSF mRNA decreased as exposure time to actinomycin D increased, but the rate of decrease was lower in the shear-stressed cells than in the static control cells. The estimated half-life of GM-CSF mRNA in the shear-stressed cells was $42.3 \pm 1.0$ minutes (mean ± SD, n = 3), as opposed to $23.1 \pm 1.3$ minutes in static control cells ($42.3 \pm 1.0$ vs $23.1 \pm 1.3$ minutes; mean ± SD, n = 3, P < .01).

**Figure 6.** Effect of shear stress on GM-CSF mRNA stability. HUVECs were either incubated under static conditions or exposed to a shear stress of 15 dyne/cm² for 24 hours and then treated with actinomycin D for 30, 60, and 90 minutes. Competitive PCR was performed to quantify the changes in GM-CSF mRNA levels after treatment. The half-life of GM-CSF mRNA was significantly longer in shear-stressed cells than in static control cells ($42.3 \pm 1.0$ vs $23.1 \pm 1.3$ minutes; mean ± SD, n = 3, P < .01).

**Effects of Shear Stress on the Binding Activity of AU-Binding Factors in HUVECs**

To examine whether any AU-binding factors are involved in the modulation of GM-CSF mRNA stability, we analyzed HUVECs for the AU-binding factors that specifically bind the AU-rich region in the 3' UTR of GM-CSF mRNA. Gel shift assay showed that $\sim 40$ kD proteins in the cytoplasmic extracts specifically cross-linked to the 7-3 AU motif (Figure 7). The binding activity of the AU-binding protein decreased by exposure of the cells to shear stress. Treatment of the cells with actinomycin D produced a small increase in the binding activity of the protein, but protein binding was unaffected by shear stress.

**Figure 7.** Effect of shear stress on AU-rich binding factors. Cytoplasmic extracts from the HUVECs were incubated with [32P]UTP-labeled 7×AU RNA. Lanes are as follows: 1, no extracts; 2 and 3, extracts from static control cells; 4 and 5, extracts from cells exposed to shear stress at 15 dyne/cm² for 24 hours; 6 and 7, extracts from static cells treated with actinomycin D (5 mg/mL, 1.5 hours); and 8 and 9, extracts from shear-stressed cells treated with actinomycin D. In lanes 3, 5, 7, and 9, relevant unlabeled oligonucleotide was added as a competitor in 300-fold excess. At $\sim 40$ kD, proteins (arrow) in the cytoplasm formed distinct complexes with a 7×AT motif. Binding activity was unaltered by shear stress regardless of actinomycin D treatment. Similar results were obtained in three separate experiments.
Discussion

The present study demonstrated that laminar flow increases the production of GM-CSF in HUVECs at both the protein and mRNA level. It was already known that GM-CSF production by ECs is greatly regulated by chemical stimuli, such as TPA, IL-1, TNF, lipopolysaccharide, and modified LDL, but the present study demonstrated for the first time that GM-CSF production by ECs is regulated by a mechanical stimulus, shear stress.

There are two aspects of the effect of flow. One aspect is shear stress as a mechanical stimulus that deforms ECs, and the other aspect is change in mass transport. If a substance that stimulates ECs is present in the perfusate, the amount of that substance that reaches the cell surface will increase as flow rate or shear rate increases, leading to more potent stimulation. To differentiate between the two aspects, flow-loading experiments were performed with two perfusates having different viscosities, enabling us to apply different levels of shear stress to ECs at the same shear rate. The results showed that GM-CSF mRNA levels increased as shear rate increased but that at any given shear rate, the increases were always larger in the flow with higher viscosity or higher shear stress. This indicates that the stimulatory effect of laminar flow on GM-CSF mRNA levels is shear stress rather than shear rate dependent.

To date, indications that GM-CSF gene expression can be regulated transcriptionally or posttranscriptionally have been found in several cell lines. Many transcription factors (NF-κB, NF-GMa, NF-GMb, NF-GM2, Elf-1, and NF-AT/AP-1) and cis elements implicated in the cytosine-mediated regulation of GM-CSF gene expression have been identified. For instance, the sequence CATTA/T that is present −37 to −48 upstream from the transcription start site has been shown to be a critical positive regulatory element in cell line MLA144 stimulated with PMA, and the AP-1 consensus element (TRE) at −3 kb in the 5′ upstream region has been identified as an enhancer in PMA-treated T cells. In the present study, however, our run-on assay revealed that shear stress did not influence GM-CSF transcription in HUVECs. Furthermore, luciferase assay using the reporter gene containing 1.2 kb or 2.5 to 4.3 upstream from the GM-CSF 5′ UTR, were found in human embryonic lung fibroblasts. To clarify the role of AUBPs in the modulation of GM-CSF mRNA stability by shear stress in the present study, we analyzed the interactions between mRNA containing the 7×AU motif and proteins from cytoplasmic extracts. An AUBP of ~40 kD was identified in HUVECs, but their binding activity was unaffected by shear stress. Thus, the AU-rich element may be inadequate to function as the SSRE. There are other types of mRNA-binding proteins besides AUBPs that are thought to influence mRNA stability: poly(A)-binding protein, a protein that binds to the coding region determinant of mRNA. Thus, regulation of GM-CSF mRNA stability appears to be rather complicated, not simple, and SSREs may be more widely distributed in GM-CSF mRNA. Further study will be required to identify the cis elements involved and the proteins targeting the elements.

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40. Ando J, Ohtsuka A, Korenaga R, Kawamura T, Kamiya A. Shear stress rather than shear rate regulates cytoplasmic Ca2+ responses to flow in...


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