Fluid Flow Releases Fibroblast Growth Factor-2 From Human Aortic Smooth Muscle Cells

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Abstract—This study tested the hypothesis that fluid shear stress regulates the release of fibroblast growth factor (FGF)-2 from human aortic smooth muscle cells. FGF-2 is a potent mitogen that is involved in the response to vascular injury and is expressed in a wide variety of cell types. FGF-2 is found in the cytoplasm of cells and outside cells, where it associates with extracellular proteoglycans. To test the hypothesis that shear stress regulates FGF-2 release, cells were exposed to flow, and FGF-2 amounts were measured from the conditioned medium, pericellular fraction (extracted by heparin treatment), and cell lysate. Results from the present study show that after 15 minutes of shear stress at 25 dyne/cm² in a parallel-plate flow system, a small but significant fraction (17%) of the total FGF-2 was released from human aortic smooth muscle cells. FGF-2 levels in the circulating medium increased 10-fold over medium from static controls (P<0.01). A 50% increase in FGF-2 content versus control (P<0.01) was found in the pericellular fraction (extracted by heparin treatment). Furthermore, a significant decrease in FGF-2 was detected in the cell lysate, indicating that FGF-2 was released from inside the cell. Cell permeability studies with fluorescent dextran were performed to examine whether transient membrane disruption caused FGF-2 release. Flow cytometry detected a 50% increase in mean fluorescence of cells exposed to 25 dyne/cm² versus control cells. This indicates that the observed FGF-2 release from human aortic smooth muscle cells is likely due to transient membrane disruption on initiation of flow. (Arterioscler Thromb Vasc Biol. 2000;20:416-421.)

Key Words: shear stress ■ fibroblast growth factor-2 ■ vascular smooth muscle

After vascular injury, it is believed that regions of denuded endothelium directly expose underlying smooth muscle cells (SMCs) to blood flow. Consequently, SMCs can experience fluid shear stress that may alter their biological properties. Previous work has shown that flow decreases the proliferation of cultured human aortic SMCs (hASMCs) and increases the production of nitric oxide (NO) by these cells. Furthermore, fluid dynamic models of intact blood vessels suggest that SMCs normally experience shear stress as a result of interstitial flow driven by transmural pressure gradients.

Fibroblast growth factor (FGF)-2 is a potent mitogen for SMCs and endothelial cells (ECs). FGF-2 is expressed ubiquitously in the basement membranes of normal human blood vessels and on the apical surface of cultured ECs. FGF-2 exists in several molecular weight isoforms generated by the initiation of translation at alternate upstream CUG codons. The 18-kDa molecular mass form is found mainly in the cytoplasm, whereas higher molecular mass forms (22 to 24 kDa) are found in the nucleus. The biological activity of FGF-2 is mediated through interaction with specific high-affinity cell surface FGF receptors (FGFRs). There are 4 members in the FGFR family, and among these, FGFR-1 is the predominant form expressed by arterial SMCs. Studies have shown that FGF-2 is a mitogen involved in the early SMC proliferation response to vascular injury. It is also known to induce angiogenesis in vitro by stimulating production of plasminogen activator and EC migration. FGF-2 is also implicated as a survival factor for quiescent cells. Inhibition of endogenous FGF-2 production in vascular SMCs has been shown to induce apoptosis.

The release of FGF-2 is of interest, because it lacks a hydrophobic leader sequence necessary for secretion by the classic exocytotic pathway. It has been shown that FGF-2 can be released passively by mechanical stimuli, such as scraping, mechanical strain, or other mechanisms involving transient pore openings in the cell membrane. Membrane disruption of cells allows for leakage of FGF-2 from the cytosol. However, other studies have shown that cells that are not subjected to injurious stimuli can also release FGF-2. Once released, FGF-2 can bind with high affinity to receptors or with lower affinity to heparan sulfate proteoglycans on the cell surface or in the surrounding matrix. This low-affinity interaction is believed to allow for growth factor storage, creating a reservoir of available FGF-2.

In the present study, we examined whether fluid shear stress can act as a modulator of FGF-2 release from human SMCs. The results that follow indicate that fluid flow can...
mediate the release of FGF-2 from SMCs. It is released from inside the cell, where FGF-2 is known to be sequestered, into the surrounding cell periphery and the circulating medium.

**Methods**

**Cell Culture**

hASMCs were derived from abdominal aortic tissue of a 9-year-old child or purchased (Cascade Biologics). Cells were characterized as SMCs by positive immunostaining for α-actin and cultured in DMEM (GIBCO-BRL) supplemented with 20% FBS (Hyclone Laboratories), 2 mmol/L l-glutamine, 200 μg/mL penicillin, and 100 μg/mL streptomycin (GIBCO-BRL, complete medium). Cells used for experiments were between P4 and P10 and were seeded at 10^4 cells per cm² on glass slides (38×75 mm) previously coated with human fibronectin (1 μg/cm², Collaborative Biomedical Products). Two days after seeding, cells were used in experiments.

**Shear Stress Exposure**

Under sterile conditions, slides with hASMCs were mounted onto polycarbonate flow chambers with a silicone rubber gasket and held together with clamps. This assembly was connected to 2 reservoirs with polytetrafluoroethylene (Teflon) tubing, filled with 15 mL complete medium, and transferred to a room maintained at 37°C. A vacuum at the periphery of the slide replaced the clamps on the slide/gasket/chamber assembly, and flow was begun by adjusting the vertical distance between reservoirs, which established the hydrostatic pressure head across the chamber, resulting in a shear stress of 1 to 25 dyne/cm².20–22 Humidified air with 5% CO₂ was supplied at the upper reservoir of the flow apparatus. Cells were exposed to shear stress for times of 15 minutes to 24 hours. Because cells at the periphery of the flow field in contact with the gasket are damaged by positive immunostaining for α-actin and cultured in DMEM (GIBCO-BRL) supplemented with 20% FBS (Hyclone Laboratories), 2 mmol/L l-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO-BRL, complete medium). Cells used for experiments were between P4 and P10 and were seeded at 10^4 cells per cm² on glass slides (38×75 mm) previously coated with human fibronectin (1 μg/cm², Collaborative Biomedical Products). Two days after seeding, cells were used in experiments.

**Cell-Free Controls**

To study whether FGF-2 is lost as a result of adherence to system components, cell-free experiments with exogenously added FGF-2 were performed. Recirculating flow loops were assembled as previously described, except that cell-free slides were attached to flow chambers, and reconstituted recombinant human FGF-2 (R&D Systems) was added to the complete medium to give initial concentrations of 200, 75, and 30 pg/mL. This medium was collected from the recirculating system (and from static controls) to determine the FGF-2 concentration in the medium versus time.

**Measurement of FGF-2**

FGF-2 was assayed from 3 sources: (1) circulating medium, (2) heparin extracts (cell surface–associated or pericellular), and (3) cell lysates (intracellular). Circulating medium samples were collected to determine a time course for FGF-2 release. To obtain FGF-2 localized in the pericellular region, cells were treated with heparin (10 μg/mL in PBS, Sigma Chemical Co)22 for 30 minutes after flow at room temperature in a laminar flow hood, and the extract was collected for analysis. Because heparin can bind to FGF-2, it can competitively displace extracellular FGF-2 that is bound with low affinity to heparan sulfate proteoglycans. For intracellular FGF-2 measurements, cells were removed from the slide by brief incubation with 0.05% trypsin-EDTA (postheparin treatment), washed with PBS, and resuspended in 0.5% Triton X-100 (Sigma) containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 1 μg/mL aprotinin; Sigma). Lysates were kept on ice for 30 minutes, and all samples were stored at −20°C for later analysis. Before cell lysis, a 100 μL aliquot was removed to obtain a cell count (Coulter Counter). Medium, heparin extract, and lysate samples were all assayed for FGF-2 content by use of a quantitative sandwich ELISA (R&D Systems).

**Assessment of Cellular Injury**

To determine whether flow-induced FGF-2 release is caused by transient cell membrane disruption, cells were exposed to shear stress in complete medium containing 2.5 mg/mL FITC-dextran (Sigma, molecular weight 10 000) for 15 minutes or for 1 hour. After flow exposure, slides were rinsed 3 times with PBS to remove excess FITC-dextran and immediately photographed under a fluorescence microscope (Olympus IMT-2). Then, cells were trypsinized, washed, and resuspended in PBS for flow cytometric analysis. Cells were analyzed for fluorescence intensity by using a FACScan flow cytometer (Becton Dickinson). FITC fluorescence was recorded on the FL1 channel, and mean fluorescence values were obtained.

**Statistical Analysis**

All measurements are reported as the mean±SEM of ≥3 independent experiments. For comparison between groups, a 2-sample Student t test was used. For comparison among groups, 1-way ANOVA was performed. Differences were considered statistically significant at P<0.05.

**Results**

In Figure 1, a time course for FGF-2 release into the medium is shown for a shear stress level of 25 dyne/cm². At 15 minutes, there is a 10-fold increase in the FGF-2 concentration in the medium with respect to static controls (P<0.01), which decays by 24 hours to a 3-fold increase over baseline. To assess whether the decay in FGF-2 seen in Figure 1 is the result of FGF-2 adherence to system components, cell-free controls were run with complete medium containing recombinant FGF-2 (R&D Systems). Figure 2 demonstrates that in the absence of cells, FGF-2 levels drop as much as 50% within 4 hours. This loss is likely caused by protein adsorption to the surface (Teflon tubing and glass) of the flow apparatus. A concentration of 30 pg/mL of FGF-2 in Figure 2 corresponds to the maximum observed that was released by...
the cells under shear stress in Figure 1, because 10⁶ cells are exposed to a volume of 15 mL circulating medium.

Figure 3 shows the relation between shear stress level and FGF-2 release into the medium after 15 minutes of flow exposure. Results indicate that all shear stresses tested (1, 5, and 25 dyne/cm²) result in significant increases in FGF-2 in the medium with respect to the static control. Because there is no direct relation between the shear stress level and the amount of FGF-2 released, the response may be flow dependent rather than shear stress dependent. It is also possible that the response is saturated by a shear stress of 1 dyne/cm².

The amounts of FGF-2 isolated from heparin treatment after 15 minutes of flow exposure to various shear stress levels are shown in Figure 4. There is a significant 1.5-fold increase at 25 dyne/cm² versus control ($P < 0.01$). The response is not directly dependent on the shear stress levels studied, because there are no significant differences between 1, 5, and 25 dyne/cm². In comparing Figures 3 and 4, heparin-extractable FGF-2 released by 25 dyne/cm² shear stress is 1.5-fold greater than control (Figure 4), whereas FGF-2 released by the same shear stress level into circulating medium is 10-fold greater than control (Figure 3). However, the amount of FGF-2 extracted by heparin is 6 times greater than that released into the circulating medium.

In Figure 5, the amount of intracellular FGF-2 is shown (lysate fraction) in the bars on the left. After 15 minutes, there is a significant decrease in the intracellular level of FGF-2 in sheared cells ($P < 0.05$). This decrease corresponds to the increased FGF-2 in the medium and in heparin treatment fractions after shear stress. The bars on the right in Figure 5 display the total amount of FGF-2 by combining all 3 fractions. There is no difference in the total FGF-2 between static and flow treatments, which indicates that FGF-2 that is released by flow is not synthesized de novo. The fraction of FGF-2 released into the pericellular region (Figure 4) and the medium (Figure 3) represents ~17% of the total FGF-2 (Figure 5, right), which is significant. However, most of the FGF-2 remains intracellular (compare y-axes in Figures 3, 4, and 5).

To determine whether membrane permeability is compromised and thus causes FGF-2 release from hASMCs on flow

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**Figure 2.** Time course for the decay of exogenously added FGF-2 in the recirculating flow system. Cell-free experiments were performed with complete medium containing recombinant FGF-2 at initial concentrations of 200, 75, or 30 pg/mL. Recirculating medium was collected from the flow system at indicated times.

**Figure 3.** Effect of shear stress level on FGF-2 release into the medium. Cells were exposed to flow (1, 5, and 25 dyne/cm²) or static control conditions for 15 minutes, and then medium samples were collected for FGF-2 measurement. *$P < 0.04$*, **$P < 0.05$**, and ***$P < 0.01$*** vs control values. Results are expressed as mean ± SEM.

**Figure 4.** Effect of shear stress level on FGF-2 release into the pericellular region (heparin-extractable FGF-2). Cells were exposed to flow (1, 5, and 25 dyne/cm²) or static control conditions for 15 minutes, followed by heparin treatment (10 μg/mL) under static conditions for 30 minutes. *$P < 0.01$*** vs control values. Results are expressed as mean ± SEM.

**Figure 5.** Effect of shear stress on the intracellular amount of FGF-2. Cells were exposed to flow (25 dyne/cm²) for 15 minutes and were subsequently lysed to determine intracellular FGF-2 concentrations. The solid bars show intracellular FGF-2 amounts, and the hatched bars represent the total FGF-2 detected from all 3 fractions (lysate, conditioned medium, and heparin extract). *$P < 0.01$*** vs control values.
exposure, FITC-dextran was added to the medium as an indicator of cellular injury. Cells with membrane disruptions take up the FITC-dextran. After flow or control treatments for 15 minutes, cells exposed to FITC-dextran were washed and then trypsinized for flow cytometry analysis. In Figure 6, the peak to the left represents cells exposed to static control conditions, and the peak to the right represents cells exposed to shear stress at 25 dynes/cm² for 15 minutes. Normalized mean fluorescence of shear-stressed cells increased 1.6-fold after 15 minutes ($P < 0.01$) and was still elevated, but not further increased, after 1 hour (Figure 7). These results suggest that a rapid transient membrane disruption was caused by initiation of shear stress and is likely the cause of FGF-2 release. Because control slides were treated identically and because shear stress at 25 dynes/cm² still caused a significant increase in FGF-2 levels over controls, injured cells along the edge of the gasket cannot account for the observed release. Additionally, cell detachment on flow may cause FGF-2 to be released into the medium. However, cell numbers after control (462,000 ± 33,000 cells) and flow treatment (464,000 ± 32,000 cells) were not statistically different ($n = 11, P = 0.90$). Therefore, the mechanism by which FGF-2 is released on shear stress is potentially due to transient disruptions in the plasma membrane of the cells.

**Discussion**

Fluid flow induced significant increases in the FGF-2 content in the circulating medium and in the pericellular region. At a shear stress level of 25 dynes/cm², there was a 1.5-fold increase in the pericellular FGF-2 content, which represents release of 15% of the total FGF-2 in the cell (Figure 4). The release was rapid in view of the fact that it occurred at ≤ 15 minutes and thus did not depend on de novo synthesis of FGF-2. This release was not sustained, as seen by the decay in medium levels with time (Figure 1), which can be explained by the adsorption of FGF-2 to the surfaces of the experimental system. The half-life of FGF-2 in the flow system (Figure 2) is =4 hours. This means that FGF-2 will adsorb to surfaces after prolonged contact, and data points beyond 4 hours represent significant loss due to this adsorption. Because the release response found in the present study was rapid, adsorption did not represent a significant loss in the first 15 minutes. Furthermore, FGF-2 loss by protein adsorption would lead to an underestimation of the amount actually released into the circulating media at longer times.

Fluid flow released 15% of the total FGF-2 into the pericellular region, as assessed by heparin extract analysis. Heparin treatment displaces FGF-2 that is bound to low-affinity heparan sulfate proteoglycans. There are several potential explanations for this increase. First, flow may cause FGF-2 to be released from the cytosol into the medium, but as the FGF-2 is secreted, it may bind to available membrane low-affinity sites. Previous studies have indicated that medium measurements of FGF-2 underestimate the true level released because of this phenomenon. In addition, FGF-2 that is released in the medium may circulate and rebind to the cell surface. This effect was found to occur in vivo in studies in which heparin treatment released FGF-2 that later rebounded preferentially to sites of vascular injury. Another possible explanation is that there is a shift of FGF-2 in the pericellular region from high-affinity sites to low-affinity sites. Studies indicate that the half-time for FGF-2 removal by heparin treatment is on the order of minutes from heparan sulfate proteoglycans but hours from FGF-2 receptors. Because the present study involved treatment with heparin for 30 minutes, it is likely that mainly low-affinity–bound FGF-2 was extracted. Therefore, if fluid flow caused a transfer of FGF-2 from high-affinity to low-affinity sites, this would be detected by increased extraction on heparin treatment. Affinity modulation of FGFRs has been shown to occur in cultured vascular SMCs.

A previous study has shown that shear stress can influence the release of growth factors, such as FGF-2 and platelet-derived growth factor, which is in agreement with our results. However, that study did not consider the possibility of cell loss or detachment on flow (which apparently occurred in their system) as the source of released FGF-2. Furthermore, a residual antibody-binding activity assay that did not give quantitative results was used to detect FGF-2 in that study. The experimental system in the present study exposed hASMCs to steady laminar flow and did not cause cell detachment from the surface.
The rapid release of FGF-2 on flow exposure was in agreement with a study on mechanical strain–induced FGF-2 release by Cheng et al. They found that strain could release significant amounts of FGF-2 (nearly 15% of the total within 15 minutes) into the conditioned medium, but only above certain threshold amplitudes. The release was dependent on the degree of strain, whereas our results did not show dependence on the magnitude of shear forces tested. It was also shown through the use of a fluorescent dextran marker that mechanical strain–induced FGF-2 release was caused by membrane disruption. Membrane disruption was detected in electrically stimulated cardiac myocytes through a similar technique and was believed to be the cause of FGF-2 release. In the present study, membrane disruption was also detected through the use of a fluorescent dextran marker. However, dextran uptake could not be observed by microscopy (data not shown) but could be detected by flow cytometry (Figure 6). This indicates that the membrane response to shear stress is not as damaging as strain or other forms of mechanical stimulus, yet it is still capable of releasing FGF-2. Yu and McNeil have demonstrated that transient membrane wounds occur in aortic ECs in vivo and can potentially result in the release of growth factors. Reidy has suggested that SMCs at the luminal surface exposed to blood flow after arterial injury may release FGF-2 through nonlethal trauma to the membrane. Our results confirm that hypothesis by showing that transient membrane disruptions can occur in aortic SMCs on the initiation of flow exposure and result in the release of FGF-2.

Aside from mechanical forces, biochemical stimuli can also induce the release of FGF-2. Terminal complement protein C5b-9 has been shown to release FGF-2 and platelet-derived growth factor from ECs. However, this release is the result of membrane damage caused by the complement protein itself. In a recent study, the mitogenic effect of angiotensin II was shown to be mediated by FGF-2 in a rat carotid artery model. However, it was suggested that angiotensin II itself may cause SMC injury and thus FGF-2 release.

The release of FGF-2 can be induced by NO treatment, but at cytotoxic levels, suggesting again that cellular injury is the mechanism. Previous studies have shown that flow can stimulate NO production in SMCs and that this production can be blocked with a NO synthase inhibitor. Therefore, NO generated by flow may act as a messenger to induce FGF-2 release. We tested this hypothesis by blocking NO production under flow conditions by incubating cells with 100 μmol/L N-amino-L-arginine for 60 minutes before and during flow exposure. Preliminary results indicated that inhibition of NO synthesis had no effect on the FGF-2 released by shear stress (data not shown). Furthermore, hASMCs were treated with sodium nitroprusside, a known NO donor in aqueous solution, in the absence of flow. Preliminary results indicated that exposure to sodium nitroprusside (10 to 1000 μmol/L) did not increase FGF-2 levels in the medium. Thus, induced FGF-2 release is not a result of NO production.

In ECs, shear stress has been shown to increase FGF-2 mRNA levels by Northern blot analysis. The FGF-2 mRNA level was maximal after 6 hours of 36-dyne/cm² shear stress exposure. The study did not assess corresponding protein levels. Because of the rapid response seen in FGF-2 released from SMCs under flow conditions and the conservation of total FGF-2 content between flow and control samples (Figure 5), it is not likely that extracellular FGF-2 increases are the result of increased FGF-2 mRNA expression. The amount of FGF-2 found in the cytoplasm did significantly decrease under flow conditions (Figure 5). This suggests that FGF-2 is being depleted from intracellular pools for release and is consistent with the rapid FITC-dextran uptake from the perfusing medium. Recently, it has been shown that ECs exposed to shear stress for a short time (3 minutes) experience a reduction in cell height of ≈1 μm. This finding suggests that cells exposed to shear stress rapidly alter their membrane conformation. A similar deformation in hASMCs may be the cause of transient membrane disruption, leading to FGF-2 release.

In conclusion, our data suggest a method other than injury by which FGF-2 can be released from SMCs in vitro. A significant portion of the total FGF-2 was released either to pericellular regions or into conditioned medium after just 15 minutes of flow exposure. Under conditions such as balloon angioplasty, vascular SMCs may be exposed to blood flow conditions that could alter the release of FGF-2. Better understanding of the controlling mechanisms for FGF-2 export may lead to new strategies aimed at regulating its release under pathophysiological conditions.

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References


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