Shear Stress-induced Release of Basic Fibroblast Growth Factor from Endothelial Cells Is Mediated by Matrix Interaction via Integrin $\alpha_V\beta_3^*$

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Considering that chronic elevation of shear stress results in remodeling of the vasculature, we analyzed whether mechanical load could mediate basic fibroblast growth factor (bFGF) release and whether bFGF would act as mediator of shear stress-induced endothelial proliferation and differentiation. Supernatant media of shear stress-exposed endothelial cells (EC) contained significantly higher amounts of bFGF than medium from static cells. Released bFGF was fully intact with regard to its function as an inductor of proliferation and differentiation. Shear stress-conditioned media induced capillary-like structure formation, whereas static control medium did not. Likewise, only shear stress-conditioned medium induced proliferation of serum-starved EC. Both capillary-like structure formation and proliferation could be inhibited by neutralization of bFGF or its receptor. The release of bFGF was subject to specific, integrin-mediated control, since inhibition of $\alpha_\beta_3^*$ integrin prevented it, whereas inhibition of $\alpha_\beta_2^*$ integrin had no effect. We conclude that shear stress induces the release of bFGF from EC in a tightly controlled manner. The release is dependent on specific cell-matrix interactions via $\alpha_\beta_3^*$ integrins. The effects on cell proliferation and differentiation suggest that release of bFGF is functionally significant and may represent a necessary initial step in adaptive remodeling processes induced by shear stress.

Chronic physical exercise leads to increases in the diameter of existing vessels as well as the formation of new vessels (angiogenesis), thereby enhancing the total number of vessels in the skeletal muscle (1–6). It is believed that shear stress represents an important stimulus for these vascular events. However, little is known about the mechanism of mechano-sensing (the initial step) or about potential mediators involved in this shear stress response. Some growth factors controlling angiogenesis have similar vascular effects as shear stress. Therefore, we hypothesized that shear stress could serve as an adequate stimulus for the release of bFGF from endothelial cells and that bFGF could function as a mediator of shear stress-induced angiogenesis. Because many investigators are claiming that part of the mechano-sensing occurs via specific cell-matrix interactions of integrin and non-integrin receptors (15–19), we further hypothesized that the mechanism of bFGF release could be mediated by such interactions.

To verify these hypotheses, cultured primary porcine aortic endothelial cells (PAEC) were exposed for up to 6 h to physiological levels of shear stress (16 dyn/cm²) with and without the addition of inhibitory peptides GRGDSP (mainly inhibiting $\alpha_\beta_3^*$ integrins) and GRGDNP (mainly inhibiting $\alpha_\beta_2^*$ integrins) (20–22), respectively. We tested whether shear stress mediated the release of bFGF from endothelial cells and whether this release was controlled by matrix receptors. It was additionally studied whether released bFGF increased cellular responses known to be involved in angiogenesis, namely endothelial cell growth and differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Preparation and Culture**

Porcine Endothelial Cells—Shear stress experiments were performed using PAEC, which had been isolated as described before (15). Briefly, fresh aortae were obtained from the slaughterhouse and kept in sterile phosphate-buffered saline until final preparation. The tissue specimens were trimmed and freed of fat and connective tissue, cut longitudinally, and stretched into a frame. A sterile solution of collagenase (0.2 units/ml, Roche Molecular Biochemicals) was applied to the luminal side of the aorta and kept there for 20 min at 37 °C in a moist atmosphere. Finally, the endothelial cells were washed off with culture medium and cultivated under standard conditions (37 °C, 5% CO₂, 20% fetal calf serum in Dulbecco’s modified Eagle’s medium/Ham’s F12).

Human Endothelial Cells—To bioassay bFGF, human umbilical vein endothelial cells (HUVEC) were used because of their higher sensitivity to exogenous growth factors. The central veins from fresh umbilical cords were flushed with sterile, warm, phosphate-buffered saline and play a pivotal role in vascular remodeling. Several investigators have reported a critical participation of this cytokine in new vessel formation (4, 5, 9–13). The expression of bFGF is not affected at the transcription level by shear stress, hypoxia, or hypertension (14), all of which have been shown to stimulate angiogenesis (3, 6), but bFGF is stored at significant concentrations within the endothelium and could well be released in amounts sufficient to initiate angiogenesis after exposure to high shear stress.

Therefore, we hypothesized that shear stress could serve as an adequate stimulus for the release of bFGF from endothelial cells and that bFGF could function as a mediator of shear stress-induced angiogenesis. Because many investigators are claiming that part of the mechano-sensing occurs via specific cell-matrix interactions of integrin and non-integrin receptors (15–19), we further hypothesized that the mechanism of bFGF release could be mediated by such interactions.

PAEC, porcine aortic endothelial cells(s); HUVEC, human umbilical vein endothelial cells (HVEC) were used because of their higher sensitivity to exogenous growth factors. The central veins from fresh umbilical cords were flushed with sterile, warm, phosphate-buffered saline and

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The abbreviations used are: bFGF, basic fibroblast growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; LDH, lactate dehydrogenase; FACS, fluorescence-activated cell sorter.
subsequently filled with collagenase solution (0.2 units/ml). After an incubation of 20 min at 37 °C, the dislodged endothelial cells were flushed out and collected. They were seeded onto plastic culture dishes and cultivated in a moist atmosphere at 37 °C, 5% CO₂, using 20% fetal calf serum in Dulbecco’s modified Eagle’s medium/Ham’s F12 supplemented with 20% endothelial cell growth medium from PromoCell as the culture medium.

Application of Shear Stress

PAEC of passage 1 were seeded onto glass plates that had been pre-coated with laminin I (Sigma/Engelbreth-Holm-Swmar tumor). Confluent plates were transferred into a cone and plate shear apparatus as described previously (15). A laminar shear stress of 16 dyn/cm² was applied for 6 h at 37 °C. In some experiments, peptides inhibiting binding of endothelial cells to the underlying matrix were applied, all at a final concentration of 50 µM. RGD peptides were used to inhibit integrin α₅β₃-mediated binding to vitronectin (GRGDSP, Invitrogen) and α₅β₁-mediated binding to fibronectin (GRGDNP, Invitrogen). In addition to inhibitory RGD peptides, the neutralizing antibodies LM609 (1 µg/ml; Calbiochem) against integrin α₅β₃ and RepoPr (0.5 µg/ml; Lilly) against integrins gp130/μ and α₅β₁ were applied during shear stress. As control, a nonspecific anti-goat immunoglobulin from rabbit (1 µg/ml; G4018, Sigma) was tested. At the end of the experiments conditioned media were collected and stored at −80 °C for further analysis.

Assay for Differentiation of Endothelial Cells after Shear Stress Treatment

At the end of the 6-h application period of shear stress, the endothelial cells were further cultivated under static conditions for up to 24 h in fresh medium. Formation of capillary-like network structures was determined by microscopic inspection and was documented by photography.

Enzyme-linked Immunosorbent Assay Measurements of bFGF

The amount of bFGF released from endothelial cells subjected to shear stress was measured using an enzyme-linked immunosorbent assay, strictly following the protocol provided by the manufacturer (R&D Systems). An aliquot of 100 µl of the respective supernatant was collected for this measurement. For each experiment measurements were done in triplicate.

bFGF Bioassays

Two independent bioassays were used to test the integrity and functional activity of the released bFGF.

Proliferation Assay—HUVECs, passage 2–4 (referred to here as “detector cells”), from static cultures were seeded onto plastic culture dishes (3-cm diameter). The occurrence of functional bFGF in conditioned media from either shear stress-treated (6 h, 16 dyn/cm²) or corresponding static control PAEC (referred to here as “donor cells”) was monitored as the induction of proliferation of serum-starved (16–24 h, 1% fetal calf serum) detector cells. Proliferation of the detector cells was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (24). MTT was added to the growth medium at 0.5 mg/ml (in phosphate-buffered saline) for 2 h. At the end of the experiment, the reduction of MTT to blue formazan was monitored in a photometer at an optical density of 550 nm including a correction for plastic (subtraction of 630 nm values from that at 550 nm).

In some experiments, a peptide (APSHYKKG, Biomol) preventing the dimerization (activation) of the bFGF receptor was added at 1 µg/ml (25). Its specificity in inhibiting only bFGF-induced proliferation was verified in a separate set of experiments. Recombinant bFGF was preincubated with 20% serum alone or in combination with 0.5 mg/ml EGF (positive control stimulus) to HUVEC previously starved of serum. The subsequent proliferation was tested with the MTT assay.

Differentiation Assay—Additionally, conditioned media from the shear stress experiments were tested for their capacity to induce the formation of capillary-like structures in HUVEC, which had never been exposed to shear stress in culture. The detector cells were seeded onto glass beads (density 0.02 g/ml, mean diameter 70 µm; Sigma). After these cells reached confluency, they were embedded in a fibrin gel (5 mg/ml fibrinogen dissolved in conditioned media from either shear stress or static control experiments and solidified with 0.5 units/ml thrombin). After 72 h, capillary-like structures with a minimum length of 70 µm (the diameter of the glass beads serving as size marker) grown into the gel were counted. Similar to the proliferation assay, the specific role of bFGF was confirmed by incubations with inhibitory antibodies (1 µg/ml) against bFGF and VEGF (F3393 and V6627, respectively; Sigma).

Lactate Dehydrogenase Release

To verify that the release of bFGF from endothelial cells was not due to nonspecific cell membrane leakage, measurements of lactate dehydrogenase (LDH) were performed in the supernatant from static cells as well as from shear stress-treated cells in parallel experiments (n = 6). A commercial kit (Promega) was used for LDH determinations; the results are presented as direct readings at OD 485 nm (arbitrary units). For estimation of the sensitivity of the latter assay to detect cell damage, a defined number of HUVEC were serially diluted and lysed with 1% Triton X-100.

Membrane Integrity Measurements

Membrane integrity of the cells exposed to shear stress was tested by incubating PAEC with rhodamine-labeled dextran (molecular mass, 10,000 Da), which is membrane-impermeable, during shear stress experiments or, alternatively, with trypan blue, a dye that is actively excluded by the living cells, directly after finishing the shear stress experiment. After the shear stress experiments were finished, the cells were dislodged non-enzymatically from the glass plates using citrate-buffered saline and measured for uptake of rhodamine-labeled dextran with FACS analysis. The trypan blue staining of the cells was inspected qualitatively using an inverted microscope. All experiments were performed in triplicate.

Western Blotting

Western blotting for identification of bFGF was performed as follows. Cells that were either untreated or exposed to shear stress (16 dyn/cm²) for the indicated time intervals were dislodged non-enzymatically with EDTA (2 mM), KCl (135 mM), and sodium citrate (15 mM). Equal amounts of cell lysates (200 µg in 2% Triton, 50 mM Tris, pH 7.5) were incubated using a specific antibody against bFGF (F3393, Sigma), precipitated with protein G-Sepharose, and collected by centrifugation. The resulting pellets were solubilized with SDS-PAGE buffer and separated on a 12% SDS-polyacrylamide gel using standard procedures. After electrophoresis the gels were transferred to nylon membrane using the semidyed transfer technique. For detection of bFGF, the membranes were probed with an antibody specific for bFGF and visualized on an x-ray film using a second antibody labeled with horseradish peroxidase and the ECL-system (Amersham Biosciences). For documentation, the films were scanned with a video camera based system (Bio-Rad).

Statistical Analysis

Data are presented as means ± S.E. Shear stress experiments and their respective static controls were always performed in paired cultures from the same cell preparation lot. For each experiment a different individual preparation lot was used. Student's t test for paired experiments was used to test for differences, which were considered significant at an error level of p < 0.05. Statistical comparisons within groups were determined using one-way analysis of variance. The number of experiments performed is indicated as n.

RESULTS

After exposure to 6 h of laminar shear stress (16 dyn/cm²), porcine aortic endothelial cells showed a profound alteration of their cellular shape and orientation. In contrast to the cobblestone morphology of the cells in static culture, these appeared to be elongated in the flow direction (as indicated by an arrow in Fig. 1A).

When, after a 6-h initial shear stress exposure, these cells were cultivated for a further 24 h under renewed static culture conditions, they formed capillary-like structures as shown in Fig. 1B. Most of these structures were composed of multiple cells forming a two-dimensional network. As indicated by arrows, oblong unions were detectable, showing cellular migration and differentiation into hollow fibers, so-called capillary-like structures. The formation of these structures was completely inhibited by neutralization of the bFGF receptor (data not shown). Static control cells did not develop capillary-like features (Fig. 1B).
Shear Stress Induces bFGF Release

FIG. 1. Induction of capillary-like structure formation in endothelial cells by shear stress. Although PAEC in static culture showed the characteristic cobblestone pattern, cells that had been subjected to a laminar shear stress of 16 dyn/cm² showed longitudinal cell shapes aligned with the direction of flow (indicated by an arrow in A). During a subsequent 24-h cultivation, again under static culture conditions, capillary-like structures were developed by these cells but not by those never exposed to shear stress (see arrows in B).

In time course experiments, elevated levels of bFGF were detectable in the supernatant after only 30 min of shear stress, increasing continuously during prolonged exposure to shear stress (see Fig. 2A). To determine the origin of the bFGF released by shear stress, Western blots were performed with lysates from cells either exposed to shear stress or kept under static control conditions. A citrate buffer was used instead of trypsin to dislodge the cells, thus preventing enzymatic liberation of bFGF from the matrix pool. Fig. 2B shows that the cellular bFGF content was clearly diminished after shear stress, indicating that the bFGF was liberated from the cytoplasm and not from matrix.

To rule out the possibility that bFGF accumulation in the supernatant from cells under shear stress was due to nonspecific cell damage, the release of LDH was measured. The LDH activities of the respective supernatants showed no significant differences (see Fig. 3A). Using a calibration curve with lysates of known cell numbers, the threshold for the LDH assay was determined to be less than 250 cells/assay (data not shown). Nevertheless, additional dye exclusion experiments were performed using rhodamine-labeled dextran (10,000 Da). Again, no evidence for cell damage was found by comparative FACS analysis between cells exposed to shear stress and static control cells (see Fig. 3B).

Proliferation assays were done to test the biological activity of the released bFGF. The conditioned medium from static PAEC (Fig. 4A, filled circles) did not induce proliferation in growth-arrested HUVEC, whereas exposure to shear stress-conditioned medium (Fig. 4A, filled squares) resulted in a growth response similar to that induced by supplementation of the medium with 20% fetal calf serum (Fig. 4A, filled triangles). To verify that the growth response was due to bFGF, 1 μg/ml of a peptide (APSGHYKG) that inhibits dimerization and activation of the bFGF receptors was added in a subset of experiments (see Fig. 4B). The inhibition of the bFGF receptor completely abolished 48-h cell proliferation induced by conditioned medium from shear stress experiments. The specificity of the bFGF-receptor blockade was tested in separate proliferation assays in which serum-starved HUVEC were exposed to either 1 ng/ml bFGF alone or in combination with 0.1 ng/ml EGF, which served here as a positive control. Administration of the peptide APSGHYKG or the bFGF-neutralizing antibody (F3393) inhibited only the bFGF-induced proliferation but not the remaining cell proliferation induced by EGF (data not shown).

In addition to proliferation, shear stress-conditioned medium induced the formation of capillary-like structures (see Fig. 5, A and B), whereas no differentiation was detectable with supernatants from static control cells. In a subset of experiments, neutralizing antibodies for bFGF (F3393) as well as VEGF (V6627) were added to the conditioned media either from static cultured or from shear stress-treated PAEC. Similar to the effects of the proliferation assays, inhibition of bFGF abolished the induction of capillary-like structure formation by shear stress-conditioned medium, whereas the neutralizing antibody to VEGF had no inhibitory effects (see Fig. 5C).

It is thought that endothelial cells sense mechanical loads via certain cell-matrix interactions. We therefore investigated a possible role in cell-matrix interactions by integrins of the β3 and β1 integrin family. The antagonists used were the peptides GRGDSP, predominantly inhibiting matrix binding via αvβ3, and GRGDNP, mainly inhibiting αvβ1 binding. No significant differences in the release of bFGF were found in the conditioned media of shear stress-treated PAEC during incubation with the peptide GRGDNP (50 μM). In contrast, when cells were incubated with medium containing the GRGDSP peptide (50 μM), the release of bFGF into the medium during shear stress was abolished. This finding was further confirmed by experiments using the antibodies LM609 and ReoPro for inhibition of αvβ3. The antibody blockade of αvβ3 with both LM609 (1 μg/ml) and ReoPro (0.5 μg/ml) reduced bFGF release significantly, whereas the nonspecific control antibody to goat IgG (G4018) had no effect (see Fig. 6). None of the different protocols of peptide or antibody application enhanced cell detachment during shear stress.
**DISCUSSION**

This study demonstrates that shear stress is a potent stimulus for the release of bFGF, which is not due to release from mechanically damaged cells but rather is a well controlled phenomenon that involves specific integrin-matrix interactions. The release of bFGF occurs in sufficient amounts to induce endothelial cell proliferation and differentiation.

In the past, speculations about the release mechanism arose because the bFGF protein sequence does not carry a signal peptide, which would be necessary for penetration of the membrane of the endoplasmic reticulum and thus for secretion in the "classical" way. Although a growing number of proteins lacking a signal sequence are already known, to date no common pathway of secretion has been identified.
Several groups have investigated the enzymatic liberation of bFGF from the extracellular matrix by proteolytic and nonproteolytic activities (8, 26–33). The results presented in Fig. 2, however, clearly demonstrate that the origin of the bFGF released by shear stress is the cytoplasm. Investigating the cellular bFGF content we found that the endothelial cells lost their immunoreactivity for bFGF after shear stress periods as short as 2 h and did not regain appreciable amounts thereafter. This might be explained by the fact that the constitutive bFGF expression is not up-regulated by shear stress. Accordingly, bFGF is not re-synthesized in a manner appropriate to overcome the cellular loss ensuing from enhanced release.

By searching the literature, a list of possible triggers for this liberation of bFGF can be found. However, most of them do not occur physiologically and, therefore, might be only artificial. Such "nonphysiological" stimuli are UV light treatment (34), physico-mechanical treatments (like freeze-thawing, sonication, scrape-loading, or balloon catheter de-endothelialization) (10, 35–39), high density culture (40), and culture on artificial substrates (like polyethylene terephthalate or polytetrafluoroethylene) (41–44). In vivo, one common and accepted hypothesis proposes that so called "sublethal" membrane disruptions may be responsible for the cellular bFGF release, which led to the idea that bFGF represents a kind of "wound hormone" (37).

In accordance with the preceding hypothesis, one might argue that during the shear stress experiments, mechanically induced nonlethal cell damage could have occurred, allowing bFGF to be liberated from the cytosol into the supernatant medium. However, we did not find a significant elevation of LDH in shear stress-conditioned media, which rules out damage of a significant number of cells; our detection limit for this method was calculated to be less than 250 cells. Additionally, rhodamine-labeled dextran (with a molecular mass of 10,000 Da) applied during shear stress was used to further assess the integrity of the cellular membrane. Because intact membranes are impermeable for dextran, it will only enter the cells with nonspecific membrane leaks. Again, we could not find an elevated level of rhodamine fluorescence in endothelial cells exposed to shear stress.

Shear stress as well as other pathophysiological stimuli, e.g., elevated hydrostatic pressure (45), complement pore complex (46), hypoxia (8, 47, 48), inflammatory cytokines (like interferon-α and -γ, interleukin-1, tumor necrosis factor-α) (49–52) nicotine (53), thrombin (8, 12, 54), thrombospondin (55), and lysophosphatidylcholine (56), are all accompanied by enhanced liberation of bFGF from living cells but do not necessarily disrupt the membrane. Our data rather favor the hypothesis of a tightly regulated physiological pathway of bFGF release. This concept is further strengthened by the fact that in our study the release of bFGF could be inhibited by the application of certain RGD peptides. It is quite likely that this integrin dependence reflects a critical involvement of the cytoskeleton and/or focal adhesion points in the generation and propagation of the signal within the cell, leading to enhanced liberation of bFGF. This theory is supported by a recent publication of Albuquerque et al. (59), who showed that estrogen-stimulated endothelial cells release significantly more bFGF when cells are seeded on the extracellular matrix proteins laminin I, collagen IV, and fibronectin compared with collagen I or pure plastic. It has to be emphasized, however, that only inhibition of αβ3 but not of αβ1 integrins reduced the release of bFGF in our experiments. The latter argues against a general role for cell-matrix interactions in mediating the release of bFGF; rather, it indicates a specific signaling pathway. Although the signaling cascade remains unclear at present, it proffers the important possibility of selectively altering the sensitivity of bFGF release toward shear stress by altering the expression of αβ3 integrins or the corresponding matrix binding sites, respectively. Interestingly, Rusnati et al. (57) reported recently that bFGF might serve as a substrate for αβ3 integrin adhesion, and thereby it could be an important mediator in vascular remodeling and angiogenesis. In sum this would mean that shear stress is able to induce bFGF release only under certain conditions, and it might be speculated that such conditions pertain to tissues exhibiting angiogenesis during chronically elevated shear stress. This process goes along with an elevation of endothelial nitric-oxide synthase expression, which is mediated by the non-integrin matrix-receptor for laminin, the 67-kDa laminin-binding protein, as we have reported before (15). The peptide YIGSR, however, which neutralizes the laminin-binding protein (23), had no significant effects on the liberation of bFGF (data not shown), which suggests that different matrix-binding proteins may represent highly precise hinges for shear dependent signaling pathways.

Taking into consideration that bFGF is described as a potent inducer of gene expression of other angiogenic growth factors and their receptors, e.g., VEGF and Flk-1 (58), it can be hypothesized that bFGF might function as a physiological "first and acute response" for endothelial cells to mechanical loads such as shear stress. This first response may later lead to the induction of VEGF-mediated steps. In this early stage of shear-dependent effects, however, we did not find a significant role for VEGF.
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