EFFECTS OF FLOW ON THE SYNTHESIS AND RELEASE OF FIBRONECTIN BY ENDOTHELIAL CELLS

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SUMMARY

Human umbilical vein endothelial cells at confluence were subjected to steady shear flow. The effect of flow on the synthesis of fibronectin, its release into the medium, and incorporation into the extracellular matrix were investigated. The total content of fibronectin in endothelial cells exposed to flow was found to be lower than that in static controls after periods of 12 to 48 h. In the presence of cycloheximide there was no difference in the fibronectin content of sheared and unsheared cells. Our results suggest that the synthesis of fibronectin is inhibited by the flow-induced perturbation of endothelial cells.

Key words: endothelial cells; flow; shear stress; fibronectin.

INTRODUCTION

Anchorage-dependent human endothelial cells attach and spread on the substratum due to the extracellular matrix of the cells. The extracellular matrix is made up of collagens, proteoglycans and glycoproteins-like fibronectin, von Willebrand factor, and vitronectin (19). In recent studies much work has been done on the fibronectin molecule including the isolation of its 140 kD transmembrane receptor (15). Fibronectin has thus emerged as a prototype "adhesion protein."

The vascular endothelium is continuously exposed to both steady and oscillatory shear stress due to blood flow. One of the effects of flow on the endothelium is to induce the formation of stress fibers by the actin microfilaments of the cytoskeleton (9,12,23,24). To a certain degree, these stress fibers may play an important role in protecting the endothelium from hydrodynamic injury and detachment. Fibronectin and actin exhibit a transmembrane linkage which is known to involve a chain of proteins (2,4,11). It seems reasonable to hypothesize that fibronectin levels in endothelial cells are affected by flow-induced perturbation of the endothelial cell membrane.

In this study, the effect of flow on the synthesis and release of fibronectin in endothelial cells was quantified. Subjecting endothelial cells to flow for 12 to 48 h resulted in decreased fibronectin content. Our results suggest that the synthesis of fibronectin is inhibited by flow-induced perturbation of the endothelial cell membrane.

MATERIALS AND METHODS

Materials. Tissue culture media and supplements were obtained from GIBCO, Grand Island, NY; fetal bovine serum from Hyclone, Logan, UT; penicillin and streptomycin from GIBCO, Chagrin Falls, OH; trypsin-EDTA, gelatin, cycloheximide, collagenase, bovine serum albumin (BSA), Trizma Base, thimerosal, Tween-20, 3,3',5,5' tetramethyl benzidine, leupeptin, pepstatin, phenyl methyl sulfonyl and fluoride (PMSF), iodoacetamide, and Lowry assay kit from Sigma Chemical Company, St. Louis, MO; fibronectin and rabbit antihuman fibronectin from Calbiochem Chemicals, La Jolla, CA; goat antirabbit IgG unlabeled and goat antirabbit IgG HRP labeled from Fisher Biotech, Pittsburgh, PA; human fibronectin enzyme immuno assay kit and human plasma fibronectin from Biomedical Technologies, Stoughton, MA.

Experimental procedures. Isolation and culture of human umbilical vein endothelial cells was performed as described previously (7). Cell suspensions were seeded onto glass slides (75 mm X 38 mm) and cover slips (24 mm X 30 mm) that had been pretreated with 0.2% gelatin. Two slides (5.0 X 10^4 to 1.0 X 10^5 cells/slide) or four cover slips (2.5 X 10^4 to 5.0 X 10^4 cells/cover slip) were seeded per cord. Cultures reached confluency after 3 or 4 d and experiments were run with primary cells, 1 d after cultures reached confluency.

Apparatus for induction of flow. To simulate the hemodynamic environment of the circulation and to examine the effect of flow on the endothelium, we used the same flow apparatus described earlier (7,8). The flow circuit was primed with 15 ml of culture medium. To measure the effect of flow on intracellular and secreted fibronectin, a slide with a monolayer of cultured cells was positioned to form one of the plates in the parallel plate chamber of area 16 cm^2. For studies on the extracellular matrix fibronectin, a glass cover slip with a monolayer of attached cells was positioned to form one of the plates in
the parallel plate chamber of 3.8 cm² area. A replicate slide or cover slip, with cells from the same umbilical cord, was maintained in the incubator as a static control. Flow experiments were run for periods of 12 or 48 h with or without cycloheximide in the flow circuit medium.

To test for adsorption of medium fibronectin onto surfaces of the flow circuit, flow circuits were run for periods of 12 to 48 h in the absence of cells, with culture medium containing exogenously added human plasma fibronectin. The concentration of the fibronectin introduced into the medium was 300 ng/ml. This was comparable to the levels of fibronectin secreted into the medium by the sheared cells. After running the flow apparatus for 12 h, the concentration of fibronectin in the medium dropped to 270 ng/ml. No further decrease occurred in the medium fibronectin concentration on running the flow circuit for 48 h. This indicated that 10% of the fibronectin in the medium was adsorbed onto the loop parts during the flow experiments.

**Assay Procedures**

*Human fibronectin enzyme immuno assay.* The medium samples from the flow experiments and static controls were centrifuged at 650 × g for 10 min. The resulting supernatants were frozen at −20°C. At the end of an experiment the sheared cells and controls were enzymatically detached from the slide using trypsin at 37°C for 3 min. The cell pellet was isolated by centrifugation, washed, and resuspended in 1 ml of lysis medium containing protease inhibitors (1 mM iodoacetamide, 1 mM PMSF, 1% NP-40, 0.01% NaN₃, 50 µg/ml leupeptin, 10 µg/ml pepstatin). The sample was then sonicated for about 30 s and stored at −20°C. The supernatants and cell lysates were thawed at 37°C before assay.

The amounts of fibronectin in supernatant and cell lysate were quantitated using a human fibronectin enzyme immunoassay (Biomedical Technologies Inc.). It utilizes a double antibody separation, and the tracer is alkaline phosphatase labeled human fibronectin. All the standards and samples were run in duplicate.

Controls and samples containing fibronectin were incubated with primary rabbit antihuman fibronectin antisera followed by incubation with fibronectin tracer. They were then incubated with goat antirabbit gamma-globulin precipitating antisera and precipitating aid. The pellet was isolated by centrifugation at 2000 g at 4°C. The enzymatic reaction was started by addition of the substrate, which consisted of p-nitrophenyl phosphate in diethanol amine buffer. The reaction was stopped using potassium hydrozide, and the absorbance was measured at 405 nm using a plate reader (Biotek, Winooski, VT).

The intracellular and medium fibronectin was normalized with respect to the total protein on the slide using a Lowry protein assay. The procedure is based on Peterson's modification of the micro-Lowry method and utilizes sodium dodecyl sulphate, included in the Lowry reagent, to facilitate the dissolution of relatively insoluble lipoproteins.

Enzyme linked immuno sorbent assay (ELISA). This assay was carried out in situ and was used to quantitate the effect of flow on the amount of fibronectin incorporated in the extracellular matrix. It was a modification of an enzyme-linked immunoassay used by Van Wachem et al. (22). The sheared slides and controls were incubated in 0.1 M NH₄OH for 30 min at room temperature to detach the cell layer. The isolated extracellular matrix was washed 3 times with Dulbecco's phosphate buffered saline solution and fixed with a buffer containing Na₂CO₃ (1.93 g/liter), NaHCO₃ (2.93 g/liter), and 0.001% thimerosal. Portions of the cover slip with the extracellular matrix were cut using a diamond pencil, weighed, and dropped into 24-well tissue culture dishes. The surface areas of the cover slip fragments were correlated to their weight. The assay was standardized by adsorbing fibronectin solutions of various concentrations onto the wells. The amount of protein adsorbed onto the well was determined by assaying the spent solution with the aforementioned enzyme immunoassay.

**RESULTS AND DISCUSSION**

Endothelial cells synthesize fibronectin, secrete it into the culture medium, and incorporate it into the extracellular matrix (18). To quantitate the effect of flow on the synthesis and release of fibronectin by endothelial cells, cells were subjected to flow for periods of 12 to 48 h. Cells were sheared with paired static controls to obviate differences such as cell origin, seeding density, and in vitro cell age. Sheared cells were found to have lower medium and intracellular and extracellular matrix fibronectin levels than static controls (Fig. 1). Shearing for 12 h lowered the fibronectin in the supernatant by 68 ± 16%, the intracellular fibronectin by 44 ± 24%, and the extracellular fibronectin by 29 ± 7%. Subjecting the cells to flow for 48 h decreased the supernatant fibronectin by 39 ± 16%, the extracellular fibronectin by 16 ± 12%, and the

![Graph showing the effect of flow on fibronectin synthesis and release](image-url)

**Fig. 1.** Effect of flow on the synthesis and release of fibronectin in endothelial cells. A and D are the ratios of the amount of fibronectin released into the medium by cells sheared for 12 and 48 h, respectively, to the amount of fibronectin released into the medium by static controls. B and E are the ratios of the amount of intracellular fibronectin in cells sheared for 12 and 48 h, respectively, to the amount of intracellular fibronectin in static controls. C and F are the ratios of the amount of fibronectin in the extracellular matrix of cells sheared for 12 and 48 h, respectively, to the amount of fibronectin in the extracellular matrix of static controls. *n = 9, P < 0.005.