A Fucosylated Chondroitin Sulfate From Echinoderm Modulates *in Vitro* Fibroblast Growth Factor 2–Dependent Angiogenesis

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Abstract

Fucosylated chondroitin sulfate (FucCS), a glycosaminoglycan obtained from sea cucumber, has the same structure as mammalian chondroitin sulfate, but some of the glucuronic acid residues display sulfated fucose branches. This new polysaccharide has a more favorable effect than heparin on vascular cell growth. It inhibits smooth muscle cell proliferation as heparin, and it has a potent enhancing effect on endothelial cell proliferation and migration in the presence of heparin-binding growth factors. We now extend our studies to the effect of this glycosaminoglycan on endothelial cells to an *in vitro* angiogenesis model on Matrigel. FucCS, in the presence of fibroblast growth factor-2 (FGF-2), strongly increases the capacity of endothelial cells to form vascular tubes on Matrigel with a well-organized capillary-like network and typical closed structures. Comparison between the activity of native and chemically modified chondroitin sulfate from sea cucumber reveals that the sulfated fucose branches are the structural motif for the proangiogenic activity. Heparin does not induce angiogenesis in this experimental model. We also have evidence for the proposition that endothelial cell proliferation is not the sole event involved in the *in vitro* FGF-2-induced angiogenesis. It implies a variety of other modifications of the endothelial cells and of their interaction with the extracellular matrix, such as integrin expression and actin cytoskeleton reorganization. Finally, the proangiogenic effect of FucCS, concomitant with its capacity to prevent venous and arterial thrombosis, in animal models makes this new glycosaminoglycan a promising molecule with possible beneficial effects in pathological conditions affecting blood vessels such as the neovascularization of ischemic areas.

Received 07/05/02; revised 10/23/02; accepted 11/06/02.
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Grant support: La Fondation pour la Recherche Médicale (FRM); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq: FNDCT, PADCT, and PRONEX) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) (to P.A.S. Mourão).
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Introduction

Angiogenesis is the growth of new capillary blood vessels from preexisting capillaries and postcapillary venules (1). In response to angiogenic stimuli, endothelial cells degrade the extracellular matrix, migrate into the perivascular space, proliferate, and align themselves into patent blood vessels (2, 3). Cell surface integrins, which are the major receptors for extracellular matrix, are implicated in the process (4).

In vitro models using Matrigel (a reconstituted basement membrane) (5) or collagen gel (6) have been widely used to identify the different steps of angiogenesis, including the participation of cell surface and extracellular proteins in tube formation (7, 8) and the inductive effect of different growth factors. Among them, fibroblast growth factor-2 (FGF-2) plays a major role in angiogenesis. It increases production of proteases and their receptors necessary for basement membrane degradation; it stimulates proliferation, migration, and differentiation of endothelial cells to form tubes during vessel elongation; and it induces expression of adhesion molecules at the cell surface (9–12) and regulates actin cytoskeleton organization (13).

Proteoglycans are also major molecules involved in angiogenesis, mostly ascribed to highly sulfated heparan sulfate chains (14). These molecules possess a growth factor-dependent activity. Heparin, in the presence of heparin-binding growth factors such as FGF-1 and FGF-2, modulates endothelial cell proliferation (15–18) and migration (19, 20) with mainly an enhancing effect on FGF-1 and an inhibitory effect on FGF-2 mitogenic activity. Besides heparin and highly sulfated heparan sulfate, few other sulfated polysaccharides can influence vascular endothelial cell growth such as sulfated fucans from marine algae (21). More recently, we demonstrated that fucosylated chondroitin sulfate (FucCS), a glycosaminoglycan extracted from the sea cucumber *Ludwigothurea grisea*, also modulates vascular cell growth (22). We found that this new glycosaminoglycan has a more favorable effect than heparin on vascular cell growth. It inhibits smooth muscle cell proliferation as well as heparin and it has a potent enhancing effect on endothelial cell proliferation and migration induced by FGF-2.

We now extend our studies on the FucCS to an *in vitro* angiogenesis model on Matrigel. The objective is not only to test the effect of this glycosaminoglycan on the formation of vascular tube, but also its role on actin cytoskeleton reorganization and integrin expression. In addition, comparison between the activity of native and chemically modified echinoderm glycosaminoglycan may allow us to identify the specific structural motifs in the molecule responsible for the biological activity, as we already demonstrated for its anti-

coagulant (23) and antithrombotic (24, 25) activities and for its effect on vascular cell proliferation (22). Finally, studies with this new polysaccharide may help us to delineate a clearer picture of the overall mechanisms involved in angiogenesis, including the specific contribution of endothelial cell proliferation, modifications of the extracellular molecules, and involvement of growth factors, besides the obvious practical implications derived from a well-characterized biological activity of a new molecule.

Results

Effect of FucCS on Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) do not form tubes after growing for 18 h on Matrigel (Fig. 1A). However, if the cells were previously incubated for 72 h with 5 ng/ml FGF-2 in 5% fetal bovine serum (FBS), they organize some capillary-like network after 18 h on Matrigel (Fig. 1B). When 10 μg/ml FucCS replaced FGF-2 in the incubation medium, HUVEC do not form tubes (not shown but similar result as in Fig. 1A). Especially noteworthy, after the simultaneous incubation of HUVEC with FucCS and FGF-2, the cells organize an intense capillary-like network on Matrigel, with typical closed structures (Fig. 1C), denoting intense angiogenic activity. Cells incubated with the carboxyl-reduced derivative from FucCS still form the tubes with closed structures (Fig. 1D), but in lower amounts when compared with native FucCS. Finally, incubation of HUVEC with the desulfated (Fig. 1E) or defucosylated (Fig. 1F) derivatives from FucCS does not increase the partially organized capillary-like network already observed with FGF-2 alone (Fig. 1B). Heparin has no effect under the same experimental conditions (data not shown). Indeed, the results obtained in the presence of FGF-2 and heparin (10 μg/ml) are similar to those obtained with FGF-2 alone.

The involvement of β_1 and α_6 integrin subunits on HUVEC organization into tubular structures on Matrigel has been proposed (7). Therefore, we decided to investigate the possible effect of FucCS on the expression of these subunits by using flow cytometry and monoclonal antibodies (mAbs). FucCS (10 μg/ml) and FGF-2 (5 ng/ml) are able separately to increase α_6 integrin subunit expression by HUVEC (Fig. 2, A and B). After incubation of HUVEC with the echinoderm chondroitin sulfate plus FGF-2, a significantly higher expression (3-fold) of α_6 is observed when compared with the control, which does not contain the polysaccharide ($P < 0.0001$) (Fig. 2B). Desulfation and defucosylation, but not reduction of the glucuronic acid residues, reduce markedly the effect of FucCS on expression of α_6 integrin subunit by HUVEC.

Native and chemically modified FucCS have no significant effect on expression of β_1 integrin subunit expression by HUVEC, both in the presence and in the absence of FGF-2. Heparin has no effect on both β_1 and α_6 integrin subunit expression by HUVEC (data not shown).

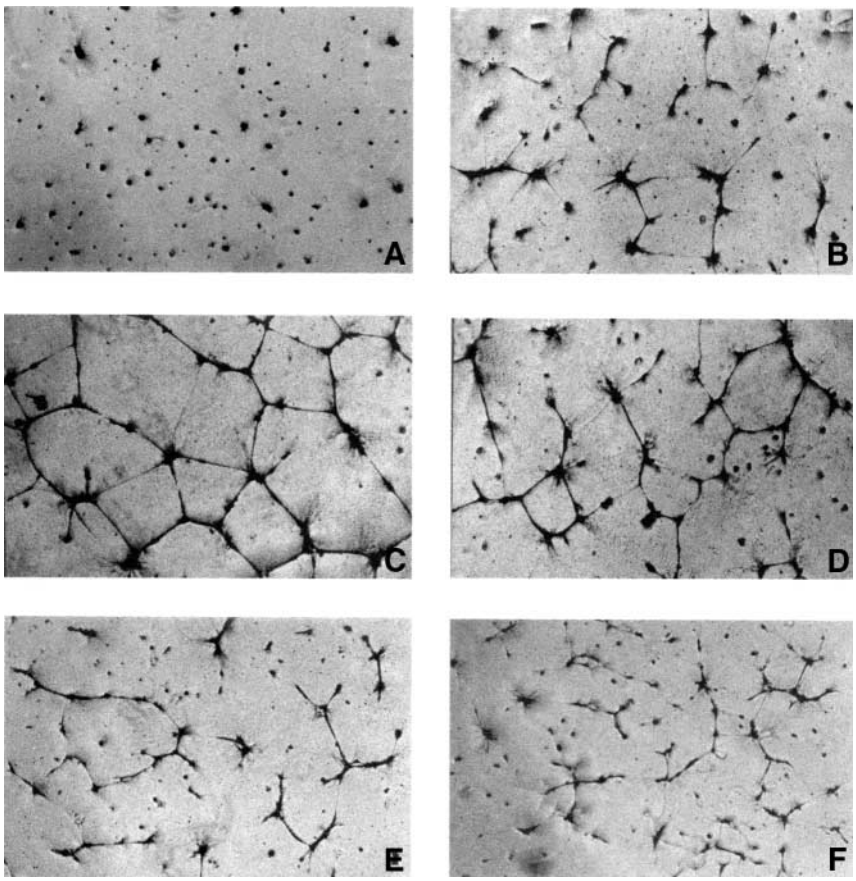

FIGURE 1. Effect of native and chemically modified FucCS on HUVEC tube formation on Matrigel modulated by FGF-2. HUVEC were preincubated for 72 h with 5 ng/ml FGF-2 and 10 μg/ml native or chemically modified FucCS. The cells were then seeded in 48-well plates coated with Matrigel (3×10^5 cells/well). After 18 h of culture at 37°C, they were fixed with glutaraldehyde to stop their reorganization and stained with Giemsa. Untreated cells (**A**); cells preincubated with FGF-2 alone (**B**); or with the growth factor plus native (**C**), decarboxylated (**D**), desulfated (**E**), or defucosylated (**F**) chondroitin sulfate from sea cucumber. Photos (original magnification, ×40) are representative of five independent experiments.

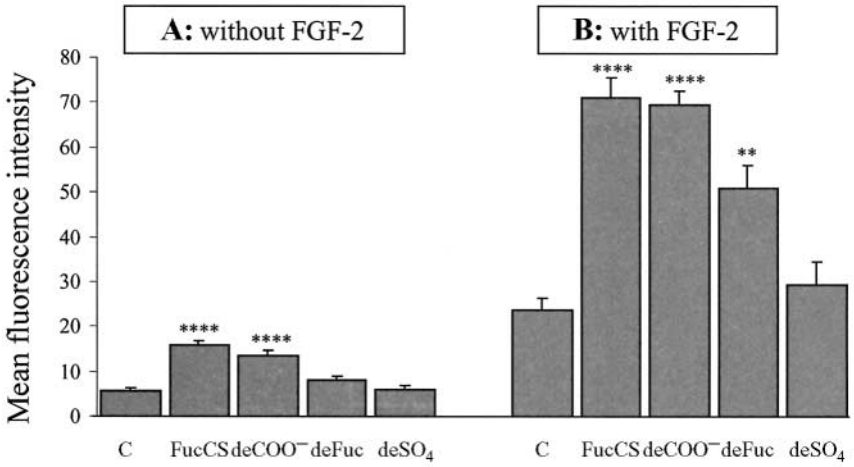

FIGURE 2. Effects of native and chemically modified FucCS on expression of α_6 integrin subunit by HUVEC. HUVEC were incubated with 10 μg/ml native or chemically modified FucCS in the absence (**A**) or in the presence (**B**) of 5 ng/ml FGF-2. After 72 h, HUVEC were analyzed by flow cytometry to quantify the α_6 integrin subunit as described in "Materials and Methods." The results are means±SE of four independent experiments. $deCOO^-$, deFuc, and $deSO_4$ are carboxyl-reduced, defucosylated, and desulfated derivatives from the FucCS, respectively. Columns C correspond to the controls without polysaccharide, in (**A**) without FGF-2 and in (**B**) with FGF-2. Values obtained for each polysaccharide in the flow cytometry assays, which differ significantly from the control, are labeled as ** ($P < 0.01$) and **** ($P < 0.0001$).

FucCS and Actin Cytoskeleton Reorganization

Untreated HUVEC, incubated with 5% FBS for 72 h in the absence of FGF-2, display only a ring of polymerized actin at their membrane periphery when stained with fluorescein phalloidin and examined by fluorescence microscopy (Fig. 3A). No difference is observed when it is compared with the HUVEC incubated with 10 μg/ml FucCS for 72 h (data not shown but similar result as in Fig. 3A). When HUVEC were incubated with FGF-2 alone (5 ng/ml) for 72 h, we observed a slight increase in their stress fibers at the membrane periphery (Fig. 3B), but if HUVEC were incubated simultaneously with native FucCS and FGF-2 for 72 h, we observed high amounts of actin stress fibers at their cytoplasms and a typical fibroblast-like morphology of the cells (Fig. 3C). When we replaced native FucCS by its carboxyl derivative, cells show a more diffuse pattern of actin staining, but they still retain the fibroblast-like shape (Fig. 3D). However, HUVEC incubated with the desulfated (Fig. 3E) or defucosylated (Fig. 3F) derivative from the echinoderm chondroitin sulfate show only a ring of polymerized actin at their membrane periphery as observed for control cells (Fig. 3A).

We further investigated the effect of FucCS and/or FGF-2 on HUVEC cytoskeleton organization with the use of H-7, wortmannin, and PP2, which are protein kinase C (PKC), phosphatidylinositol 3-kinase (PI_3K), and Src kinase inhibitors, respectively (26–29). When the PI_3K inhibitor wortmannin was added to the incubation medium containing FGF-2, the cells display few actin stress fibers on their membrane periphery (Fig. 4A) as for the control cells (Fig. 3A). If, however, FucCS is simultaneously added to the medium, the cells display a fibroblast-like cell shape (Fig. 4B) but with lower amounts of actin stress fibers when compared with the image obtained in the absence of inhibitor (Fig. 3C).

If H-7, a PKC inhibitor, is used instead of wortmannin, cells incubated with FGF-2 display only a diffuse actin staining in the cytoplasm (Fig. 4C) but distinct from the pattern observed in the absence of the inhibitor (Fig. 3B). Several membrane ruffles and diffuse staining patterns of actin, more intense around the nuclei, are observed when FucCS is added simultaneously with FGF-2 (Fig. 4D). Cells under the same conditions but without H-7 show high amounts of stress fibers and a fibroblast-like shape (Fig. 3C).

Finally, if the inhibitor of Src kinase, PP2, is used, HUVEC show only a ring of polymerized actin at their membrane periphery (Fig. 4E) or a fibroblast-like morphology, together with small amounts of actin stress fibers (Fig. 4F), when incubated with FGF-2 alone or with the simultaneous addition of FucCS, respectively.

Discussion

FucCS is a Proangiogenic Compound

Observation of the morphological organization that endothelial cells undertake once growing on Matrigel is a well-established experimental model to study *in vitro* angiogenesis (5). Matrigel is a reconstituted basement membrane containing mainly laminin and type IV collagen. No endothelial cell organization is observed in the absence of FGF-2, whereas a partially organized cellular network is obtained with 5 ng/ml FGF-2 pretreated HUVEC. If the endothelial cells were previously incubated with 10 μg/ml FucCS and FGF-2, we observe a strong increase in the vascular tubes formed on Matrigel, with a well-organized capillary-like network and typical closed structures. These observations demonstrate that FucCS has a potent *in vitro* proangiogenic activity in the presence of FGF-2. The carboxyl-reduced derivative from the echinoderm chondroitin sulfate is less potent but certainly more active than FGF-2 alone. The desulfated and defucosylated molecules do not induce neovascularization on Matrigel. Therefore, we can conclude that the sulfated fucose branches, the distinguished feature of the echinoderm chondroitin sulfate, are the structural motif for the proangiogenic activity of this polysaccharide. We already observed that these sulfated fucose branches are also necessary for the anticoagulant (23) and antithrombotic (24, 25) activities of this glycosaminoglycan. Especially noteworthy is also our observation that heparin does not stimulate angiogenesis on Matrigel under the same experimental conditions used to test FucCS (30). Both are

sulfated polysaccharides but their properties are not a single consequence of sulfate content as they are related to physicochemical characteristics such as the sulfation sites, the saccharide chain. As a matter of fact, the fucose branches are clearly involved in the proangiogenic effect of the FucCS as evidenced by the experiments done with the defucosylated polysaccharide.

In the last few years, several authors, using a variety of experimental models, have reported that sulfated polysaccharides interfere with angiogenesis. These studies have been focused on two major aspects. One is the search for compounds with potent proangiogenic activity. Sulfated polysaccharides with this property may have a beneficial effect on pathological conditions where vascular walls are denuded of endothelial cells. In contrast, a sulfated polysaccharide capable of preventing angiogenesis may have an application as an antitumor compound. FucCS falls in the first category: its concomitant anticoagulant action (23) and its capacity to prevent venous (24) and arterial (unpublished data) thrombosis on animal experimental models increase even more the possible beneficial effect of this echinoderm glycosaminoglycan in pathological conditions affecting blood vessels.

Endothelial Cell Integrin Expression and Cytoskeletal Organization

Initially, we studied the expression of α_6 integrin subunits induced by FucCS on HUVEC in the presence or absence of FGF-2. Interaction between endothelial cells and elements of their environment, such as basement membrane, is mediated by integrins. Again, FucCS added to the culture medium is enough to induce expression of α_6 but not at similar levels as obtained by the simultaneous addition of the glycosaminoglycan and the growth factor.

Finally, we examined actin cytoskeleton reorganization induced by FucCS alone or combined with FGF-2. On differentiated endothelial cells, FGF-2 induces the formation of a complex between Src kinase and focal adhesion kinase (29), which will result in changes in actin cytoskeleton organization and is a prerequisite for cell migration. No significant modification of polymerized actin is induced by addition of the echinoderm glycosaminoglycan to the endothelial cells. It requires the simultaneous addition of FGF-2 to the culture medium to induce high amounts of cytoplasmic actin stress fibers, together with modification of endothelial cells to assume a typical fibroblast-like morphology.

H-7, wortmannin, and PP-2, which are respectively PKC, PI_3K, and Src inhibitors, influence actin dynamics by decreasing stress fiber formation. When PI_3K or Src inhibitors were added to the incubation medium containing FucCS and FGF-2, a few stress fibers were observed on the cell membrane periphery but the cell shape was fibroblast-like. In contrast, if the PKC inhibitor was used, only a diffuse staining pattern around the nucleus was observed in the cytoplasm with no fibroblast-like shape of the cell. So the PKC pathway appears predominant with regard to PI_3K and Src pathways in the cytoskeleton organization induced by FGF-2 and enhanced by FucCS.

Overall, these combined results indicate that addition of FucCS to culture medium containing 5% FBS is not enough for the endothelial cells to undertake all the quantitative and qualitative modifications necessary to achieve *in vitro* angiogenesis on Matrigel. It induces expression of α_6 integrin subunits at a low extension but not the changes in actin cytoskeleton organization. This last event requires the simultaneous addition of FGF-2, as previously demonstrated for endothelial cell proliferation (22). Furthermore, our results emphasize that, besides cell proliferation, angiogenesis involves a complex array of events, such as surface protein expression and actin cytoskeleton reorganization.

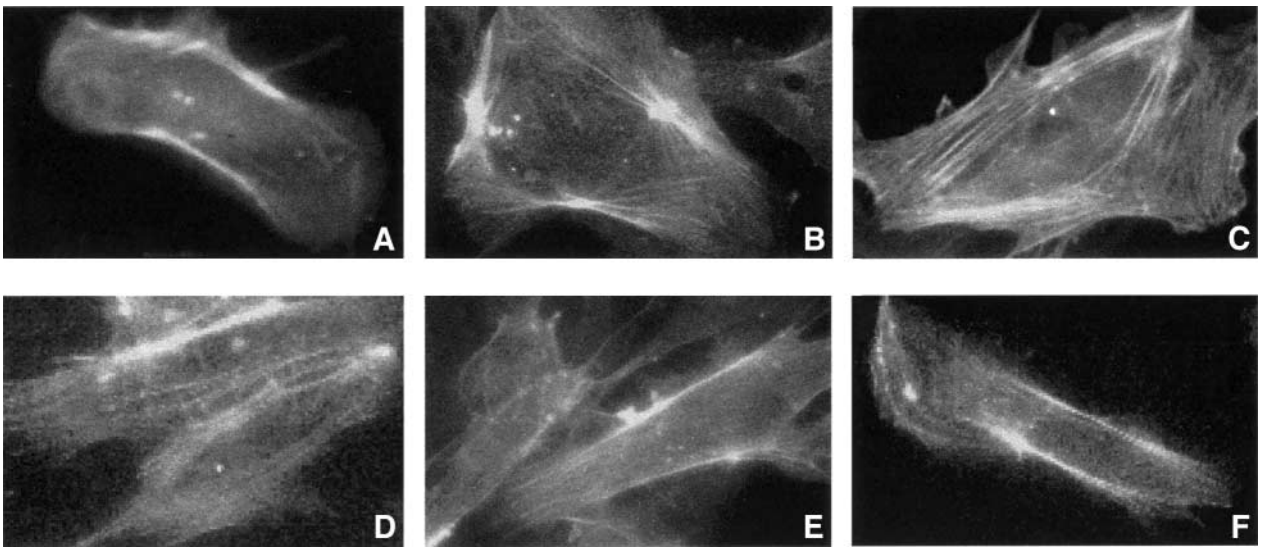

FIGURE 3. Effects of FucCS on FGF-2-induced actin cytoskeleton reorganization. HUVEC were seeded in Lab-Tek chamber slides precoated with 0.5% gelatin at a density of 0.2×10^5 cells/well. After 24 h, the culture medium was replaced by a new 5% FBS medium (**A**) plus 5 ng/ml FGF-2 (**B**–**F**). A total of 10 μg/ml of FucCS was simultaneously added: native in **C**, decarboxylated in **D**, desulfated in **E**, and defucosylated in **F**. After 72 h, the actin cytoskeleton was stained with FITC-phalloidin as described in "Materials and Methods." Photos (original magnification, ×630) are representative of four independent experiments.

In conclusion, we observed an unequivocal effect of a FucCS extracted from sea cucumber in inducing *in vitro* angiogenesis. The effect requires the presence of sulfated fucose branches on the polysaccharide and is dependent on the presence of FGF-2. Heparin does not induce angiogenesis under the same experimental conditions used to test the FucCS. These results not only have obvious practical applications but may also help to delineate and distinguish closely related events on mammalian systems, such as modifications induced on the endothelial cells and their organization on new capillary vessels.

Materials and Methods

Materials

The FucCS was extracted from the body wall of the sea cucumber *L. grisea* by papain digestion and purified by procedures previously described (31–33). This glycosaminoglycan is a homogenous fraction with an average molecular weight of 40,000. Partial removal of sulfated fucose branches from the FucCS was performed by mild acid hydrolysis. Desulfation of the polysaccharide by solvolysis in DMSO/methanol at 80°C for 6 h and reduction of the hexuronic acid carboxyl groups in the polysaccharide by 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide-$NaBH_4$ were performed as described previously (24). The molar ratios of glucuronic acid:galactosamine:fucose:sulfate are 0.30:0.27:0.36:0.70 for native FucCS; 0.30:0.27:0.09:0.60 for defucosylated; 0.06:0.30:0.30:0.70 for carboxyl-reduced; and 0.30:0.30:0.19:<0.01 for desulfated chondroitin sulfate.

Medium M199 (containing Hank's salts, L-glutamine, and 25 mM HEPES), RPMI 1640, antibiotics (penicillin and streptomycin), L-glutamine, HBSS with phenol red, PBS, HEPES, 0.05% trypsin/0.02% EDTA, and versene were from Life Technologies, Inc. (Cergy-Pontoise, France); HBSS without phenol red was from Eurobio (Ulis, France); FBS was from ATGC (Noisy-le-Grand, France); collagenase A was from Boehringer Mannheim (Mannheim, Germany); and gelatin was from Sigma Chemical Co. (St. Louis, MO). Growth factor-reduced Matrigel (without detectable FGF-2) was from Becton Dickinson Labware (Bedford, MA). Human recombinant angiogenic growth factor FGF-2 was from Valbiotech (Paris, France). Fluorescein phalloidin was from Interchim (Montluçon, France). Wortmannin, H-7, and PP2 were from Calbiochem (Meudon, France). Lab-Tek chamber slides were from Poly Labo (Strasbourg, France). Rat anti-human CD49f (α_6 integrin subunit) mAb GoH3 ($IgG2_a$) was from PharMingen (San Diego, CA); mouse anti-human CD29 (β_1 integrin subunit) mAb 2A4 (IgG1) was from Caltag (San Francisco, CA).

Cell Culture

Endothelial cells were isolated from human umbilical cords by enzymatic digestion with 0.1% collagenase according to the method described by Jaffe *et al.* (34) and modified by Giraux *et al.* (21). The cells were grown in equal volumes of M199 and RPMI 1640 supplemented with 20% FBS, 2 mM L-glutamine, 80 units/ml penicillin, and 80 μg/ml streptomycin. They were seeded into T25 flasks (BD Biosciences, Bedford, MA), precoated with 0.5% gelatin, and incubated in humidified 5% CO_2 air at 37°C. They were fed every 2 days and transferred at a split ratio of 1:2 or 1:3 by treatment with

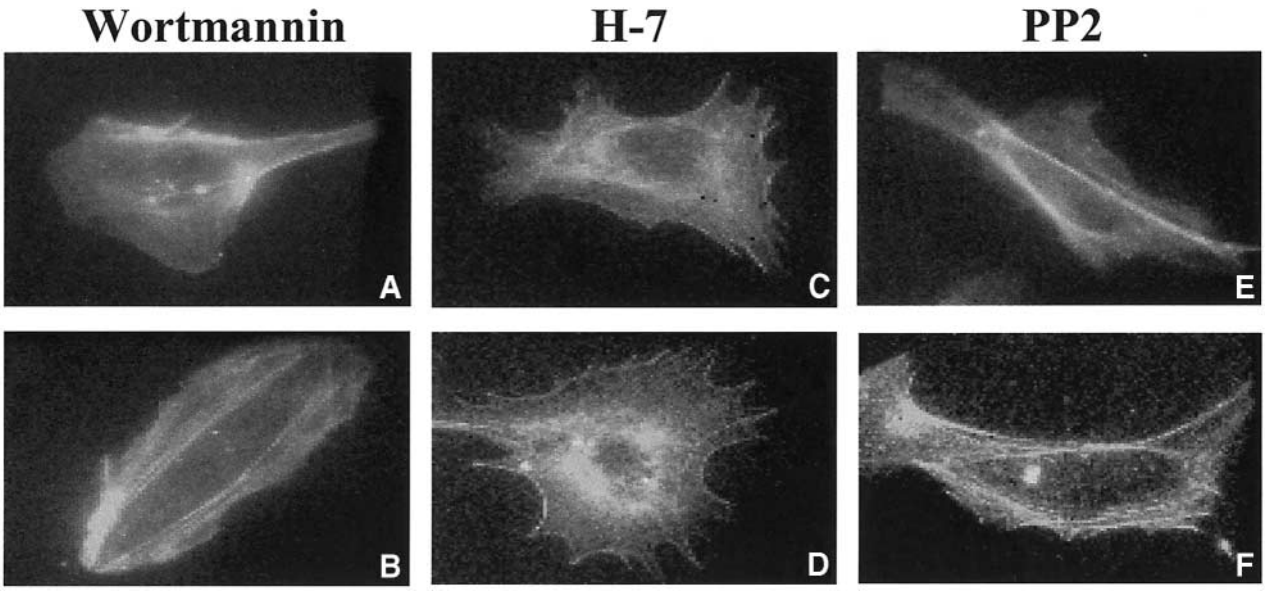

FIGURE 4. Effects of PI_3K, PKC, and Src kinase inhibitors on actin cytoskeleton organization induced by FGF-2 and FucCS. HUVEC were seeded in Lab-Tek chamber slides precoated with 0.5% gelatin at a density of 0.3×10^5 cells/well in the presence of a culture medium containing 5% FBS. After 24 h, the medium was renewed, but now in the presence of the following inhibitors: 300 nM wortmannin (**A** and **B**), 30 μM H-7 (**C** and **D**), or 2 μM PP2 (**E** and **F**). After 30 min, 5 ng/ml of FGF-2 alone (**A**-**C**-**E**) or together with 10 μg/ml of FucCS (**B**-**D**-**F**) were added to the culture media. The media were renewed 48 h later with the same concentrations of inhibitor, growth factor, and FucCS. After 72 h, the actin cytoskeleton was stained with FITC-phalloidin as described in "Materials and Methods." Photos (original magnification, ×630) are representative of four independent experiments. Wortmannin concentrations: 0, 50, and 300 nM; H-7 concentrations: 0, 15, and 30 μM.

PBS without Ca^{2+} and Mg^{2+}, then detached by digestion with 0.05% trypsin/0.02% EDTA. Endothelial cells were identified by their typical cobblestone morphology. Third-passage endothelial cells were used throughout the study.

Preincubation of Endothelial Cells With FucCS and/or FGF-2

HUVEC were seeded in six-well plates precoated with 0.5% gelatin at a density of 3×10^5 cells/well. After 24 h, the culture medium was replaced by a new medium containing 10 μg/ml polysaccharide, supplemented with 5% FBS, with or without 5 ng/ml FGF-2; this latter medium was renewed 48 h later. After 72 h, HUVEC were detached with versene plus 0.01% collagenase, and digestion was stopped with buffered Hank's-2% FBS (4°C) supplemented with sodium bicarbonate. HUVEC were centrifuged at $200 \times g$ for 8 min at 4°C and were then washed with buffered Hank's-2% before centrifugation at $200 \times g$ for 8 min at 4°C. After a second washing, HUVEC were used for angiogenesis assay on Matrigel and for quantification of integrin subunits using flow cytometry analysis (see below). The polysaccharide concentration used was chosen according to previous experiments on HUVEC proliferation and migration assays showing, in a range of concentrations from 0.1 to 100 μg/ml, a maximum enhancing effect of the FucCS on FGF-2 for concentrations of 10–50 μg/ml. Heparin, in the same range of concentrations, had a slight negative effect in the proliferation assay and no effect in the migration assay (22).

Angiogenesis Assay on Matrigel

Plates of growth factor-reduced Matrigel (48 wells) were prepared by adding 150 μl/well of thawed Matrigel (10 mg/ml) to a refrigerated plate. The gel was allowed to solidify for 1 h at 37°C. Previously untreated HUVEC and HUVEC treated for 72 h with FGF-2 with or without polysaccharide (see above) were suspended in medium containing only 5% FBS (without FGF-2 or polysaccharide) and seeded on Matrigel (3×10^4 cells/well). Cell culture was carried out at 37°C in humidified air supplemented with 5% CO_2 for 18 h. These cells were then fixed with 1.1% glutaraldehyde for 15 min. The Matrigel was dehydrated with 75% ethanol at −20°C for 1 h then with 96% ethanol for 3 min at room temperature. The cells were stained with Giemsa for 3 min. Tube formation was examined by phase-contrast microscopy.

Analysis of Integrin Subunits Using Flow Cytometry

Suspension of HUVEC cells (10^5 cells), pretreated with FucCS and/or FGF-2 (see above), was incubated for 30 min with mAb anti-human α_6 or β_1 integrin subunit, conjugated to fluorescein or phycoerythrin. The HUVEC were then analyzed in a FACSCalibur flow cytometer (Becton Dickinson), equipped with an argon laser 5 W and operated at 200 mW power with a wavelength of 488 nm. Fluorescein and phycoerythrin fluorescences were detected by using 530/30 and 585/42 band pass filters, respectively. The values are expressed as means ± SD of three independent experiments performed in duplicates.

Analysis of Actin Cytoskeleton Using Immunofluorescence Microscopy

HUVEC in 5% FBS were seeded on Lab-Tek chamber slides precoated with 0.5% gelatin (ranging from 2 to 3×10^4 cells/well). After 24 h, a new medium containing 5% FBS plus 5 ng/ml FGF-2 and/or 10 μg/ml polysaccharide was added to the cultures and renewed after 48 h, until a total of 72 h incubation period was completed. Then, the cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 3% BSA in PBS for 30 min. All these procedures were carried out at room temperature. Finally, actin cytoskeleton was stained by incubation with fluorescein phalloidin (1 unit/well) for 20 min. After extensive washing on PBS, the lames were mounted, the slides were examined and photographed under a Leica fluorescence microscope (Heidelberg, Germany).

For the experiments done in the presence of inhibitors, culture media containing a final concentration of 30 μM H-7, 300 nM wortmannin, or 2 μM PP2 were preincubated with the cells for 30 min and then 5 ng/ml FGF-2 and/or 10 μg/ml polysaccharide were added to the culture media. These media were renewed after 24 h and the cells were allowed to grow for an additional 48 h. Once a total 72 h incubation period was completed, the cells were analyzed for actin immunofluorescence as described above.

Statistical Analysis

To minimize the variations from one cord cell pool to another, the control in each assay was done on cells from the same pool of HUVEC.

The effects of the polysaccharides on HUVEC α_6 integrin subunit expression were compared with an ANOVA test. For the flow cytometry assays, the comparison of each polysaccharide (native and modified) effect was performed with a Fisher's protected least significant difference test.

Acknowledgments

The authors are indebted to the nursing services of Necker, St. Vincent de Paul, and Cochin Hospitals (Paris) for providing umbilical cords.

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A Fucosylated Chondroitin Sulfate From Echinoderm Modulates *in Vitro* Fibroblast Growth Factor 2–Dependent Angiogenesis [1] 1 La Fondation pour la Recherche Médicale (FRM); Conselho Nacional de Desenvolvimento Cienti?fico e Tecnológico (CNPq: FNDCT, PADCT, and PRONEX) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) (to P.A.S. Mourão).

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Mol Cancer Res 2002;1:96-102.