Effect of fucoidan on fibroblast growth factor-2-induced angiogenesis in vitro

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Abstract

Fucoidans are sulfated polysaccharides extracted from brown marine algae. A purified fucoidan fraction exhibits the same venous antithrombotic activity as heparin in rabbits, but with a lower anticoagulant effect. Because of its heparin-like structure, we postulated that fucoidan might modulate heparin-binding angiogenic growth factor activity. We thus studied its effect, at antithrombotic concentrations, on fibroblast growth factor (FGF)-2-induced proliferation and differentiation of human umbilical vein endothelial cells. The fucoidan effect on endothelial cell differentiation was evaluated by studying the expression of surface proteins (i.e. integrin, adhesion molecule) known to be modulated by FGF-2 and involved in angiogenesis, and by quantifying closed areas delimited by vascular tubes formed on reconstituted basement membrane. Fucoidan had no modulatory effect on the mitogenic activity of FGF-2, but significantly increased tubular structure density induced by FGF-2. Fucoidan alone increased α_6 integrin subunit expression with only partially organized tubular structure. In the presence of FGF-2, fucoidan enhanced α_6, β_1 and PECAM-1 and inhibited $\alpha_v\beta_3$ integrin expression. Heparin had no effect in these systems. The most striking effect of fucoidan was observed on α_6 expression and tube formation was abolished by monoclonal anti-α_6 antibodies. Fucoidan plus FGF-2 effect on α_6 expression was markedly decreased by monoclonal anti-FGF-2 antibodies, indicating that fucoidan acts mainly via FGF-2. These results show that, at antithrombotic concentrations, contrary to heparin, fucoidan can enhance vascular tube formation induced by FGF-2 with a modulation of the expression of surface proteins (mainly α_6) involved in angiogenesis.

Keywords: Fucoidan; Heparin; Endothelial cell; FGF-2; Angiogenesis

1. Introduction

Fucoidans are sulfated polysaccharides extracted from marine brown seaweed. We have previously shown that they exhibit the same antithrombotic activity as heparin in rabbits (evaluated in Wessler model), with lower anticoagulant activity and a lesser hemorrhagic risk [1]. These experiments were performed with standard heparin and a fucoidan fraction of the same molecular weight [1]. Given its polyanionic characteristics comparable to those of heparin, fucoidan can interact with a large number of basic amino acid-rich proteins such as blood coagulation inhibitors (antithrombin, heparin cofactor 2, etc.) [2], explaining its anticoagulant activity, and with heparin-binding growth factors (fibroblast growth factor-1, fibroblast growth factor-2, etc.) involved in cell metabolism [3]. Fibroblast growth factor-2 (FGF-2), which is ubiquitous, is thought to play a major angiogenic role in vascular wound repair and collateral vessel formation [4]. In vivo, FGF-2 is stored bound to low-affinity sites provided by heparan sulfate proteoglycans, namely perlecan, located in the extracellular matrix, and syndecan, located on the cell surface [5]. Yayon et al. [6] have shown that heparan sulfate proteoglycans are low-affinity coreceptors for FGF-2 binding to their high-affinity receptors, and constitute a trimolecular complex which is more stable than the complex between FGF-2 and the receptor alone. FGF-2 is released from heparan sulfate chains by heparanase or by competitive binding of

Abbreviations: FBS, fetal bovine serum; FGF-2, fibroblast growth factor-2; HGF, hepatocyte growth factor; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; PECAM-1, platelet endothelial cell adhesion molecule-1; u-PAR, urokinase-plasminogen activator receptor; VEGF, vascular endothelial growth factor.

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soluble heparin-like molecules [5,7]. At the cellular level, the FGF-2 release pathway is unclear, as FGF-2 has no signal peptide. It has been suggested that intracellular FGF-2 may be released passively in response to mild cell damage [8] and some stress conditions associated with tissue injury [9]. In these conditions, released FGF-2 can stimulate endothelial cells. FGF-2 induces many endothelial cell modifications involved in angiogenesis (for a review, see Ref. [10]), namely: (i) increased production of proteases (u-PA, t-PA) and their receptors (u-PAR) involved in basement membrane degradation, (ii) stimulation of proliferation and migration (involving $\alpha_v\beta_3$) during vessel elongation and (iii) cell differentiation to form tubes expressing or over-expressing cell surface proteins (integrins and adhesion molecules). Angiogenesis also depends on interactions between surface proteins and extracellular matrix proteins. In vitro models of angiogenesis using Matrigel (a reconstituted basement membrane rich in laminin) or collagen gel have allowed the identification of surface proteins involved in tube formation, such as α_6 and β_1 integrin subunits [11] and the PECAM-1 adhesion molecule [12]. Many heparan sulfate proteoglycans such as perlecan or syndecan have a potentiating effect on FGF-2 angiogenic activity [13,14]. Heparin has a more controversial effect on angiogenesis, depending on the experimental conditions: it can stimulate collateral circulation formation in ischemic conditions in the presence of FGF-2 [4] and inhibit angiogenesis when administered in combination with steroid compounds [15]. Fucoidan, like heparin, is also a potent antiproliferative agent for rat arterial smooth muscle cells [16], and this could be of interest for the prevention of restenosis. Compounds with antithrombotic activity, weak anticoagulant effects and inhibitory effects on smooth muscle cell proliferation are candidate drugs against restenosis, especially if they are able to promote angiogenesis and to restore the endothelial monolayer. We thus studied the effect of fucoidan on endothelial cell proliferation, surface protein expression and differentiation into vascular tubes in the presence of FGF-2.

2. Materials and methods

2.1. Materials

Medium M199 (containing Hank's salts, L-glutamine and 25 mM HEPES), RPMI 1640 medium, antibiotics (penicillin and streptomycin), L-glutamine, Hank's balanced salt solution with phenol red (HBSS), phosphate buffered saline (PBS), HEPES, 0.05% trypsin/0.02% EDTA and versene were from Gibco BRL (Cergy-Pontoise, France); HBSS without phenol red was from Eurobio (Ulis, France); fetal bovine serum (FBS) was from ATGC (Noisy-le-Grand, France); collagenase A was from Boehringer (Mannheim, Germany); and gelatin was from Sigma (St. Louis, MO, USA). Growth factor-reduced Matrigel™ (without detectable FGF-2) was from Becton Dickinson Labware (Bedford, MA, USA). Human recombinant angiogenic growth factor FGF-2 was from Valbiotech (Paris, France) and murine anti-human FGF-2 monoclonal antibody was from Sigma. Rat anti-human α_6 integrin subunit monoclonal antibodies (NKI-GoH3) were from Chemicon International (Temecula, CA, USA).

Fucoidan, extracted from the brown seaweed *Ascophyllum nodosum* as previously described [17], is a homogenous fraction with an average molecular weight of 16 000 ± 3000 g/mol, as determined by analytical high-performance liquid steric exclusion chromatography. Its chemical composition is 55.7% fucose, 6.3% uronic acid and 29% SO_3Na, and its anticoagulant activity, determined in the activated partial thromboplastin time assay with heparin as standard, is 9.2 IU/mg. Unfractionated heparin (molecular weight: 15 000 ± 3000 g/mol; anticoagulant activity: 173 IU/mg) was from Sanofi Center (Toulouse, France).

Mouse anti-human CD51/CD61 ($\alpha_v\beta_3$ integrin) monoclonal antibody (mAb) 23C6 (IgG_1), anti-human CD87 (u-PAR) mAb A59 (IgG_1) and rat anti-human CD49f (α_6 integrin subunit) mAb GoH3 (IgG_{2a}) were from Pharmingen (San Diego, CA, USA). Mouse anti-human CD29 (β_1 integrin subunit) mAb 2A4 (IgG_1) and anti-human CD31 (PECAM-1, -2) mAb MBC 78.2 (IgG_1) were from Caltag (San Francisco, CA, USA).

2.2. Cell culture

Endothelial cells were isolated from human umbilical cords (HUVEC) by enzymatic digestion with 0.1% collagenase according to the method described by Jaffe et al. [18] and modified by Giraux et al. [3]. The cells were grown in equal volumes of M199 and RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 80 units/ml penicillin and 80 μg/ml streptomycin. They were seeded into T25 flasks precoated with 0.5% gelatin and incubated in humidified 5% CO_2–air at 37 °C. They were fed every 2 days and passaged at a split ratio of 1:2 or 1:3 by treatment with 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. Second-passage endothelial cells were used throughout the study.

2.3. Human endothelial cell proliferation assay

After trypsinization, HUVEC were seeded in 96-well plates at 1.1×10^4 cells/cm^2 (3.6×10^3 cells/well). After 24 h, the culture medium was renewed, with or without sulfated polysaccharides (0.1–50 μg/ml) supplemented with 5% fetal bovine serum (FBS), and with or without FGF-2 (5 ng/ml). Endothelial cells were treated in triplicate, and the medium (with or without polysaccharide and FGF-2) was renewed after 2 days of treatment. After 72 h of incubation, the cells were counted by colorimetric assay. Cell growth

was stopped by adding 1.1% glutaraldehyde for 15 min, and cells were then rinsed with deionized water. The nuclei were stained for 10 min with 0.1% crystal violet [19], and absorbance was measured at 570 nm after 10 min with a microplate reader.

2.4. Endothelial cell treatment and preparation for Matrigel™ and flow cytometric analysis

HUVEC were seeded in 6-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 medium containing the sulfated polysaccharide (0, 0.1, 1 and 10 μg/ml) supplemented with 5% FBS, with or without FGF-2 (5 ng/ml); this latter medium was renewed in the same way 48 h later. After 72 h, HUVEC were detached with versene and versene–0.01% collagenase, and digestion was stopped with buffered Hanks-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% FBS before centrifugation at $200 \times g$ for 8 min at 4 °C. After a second washing step, HUVEC were used

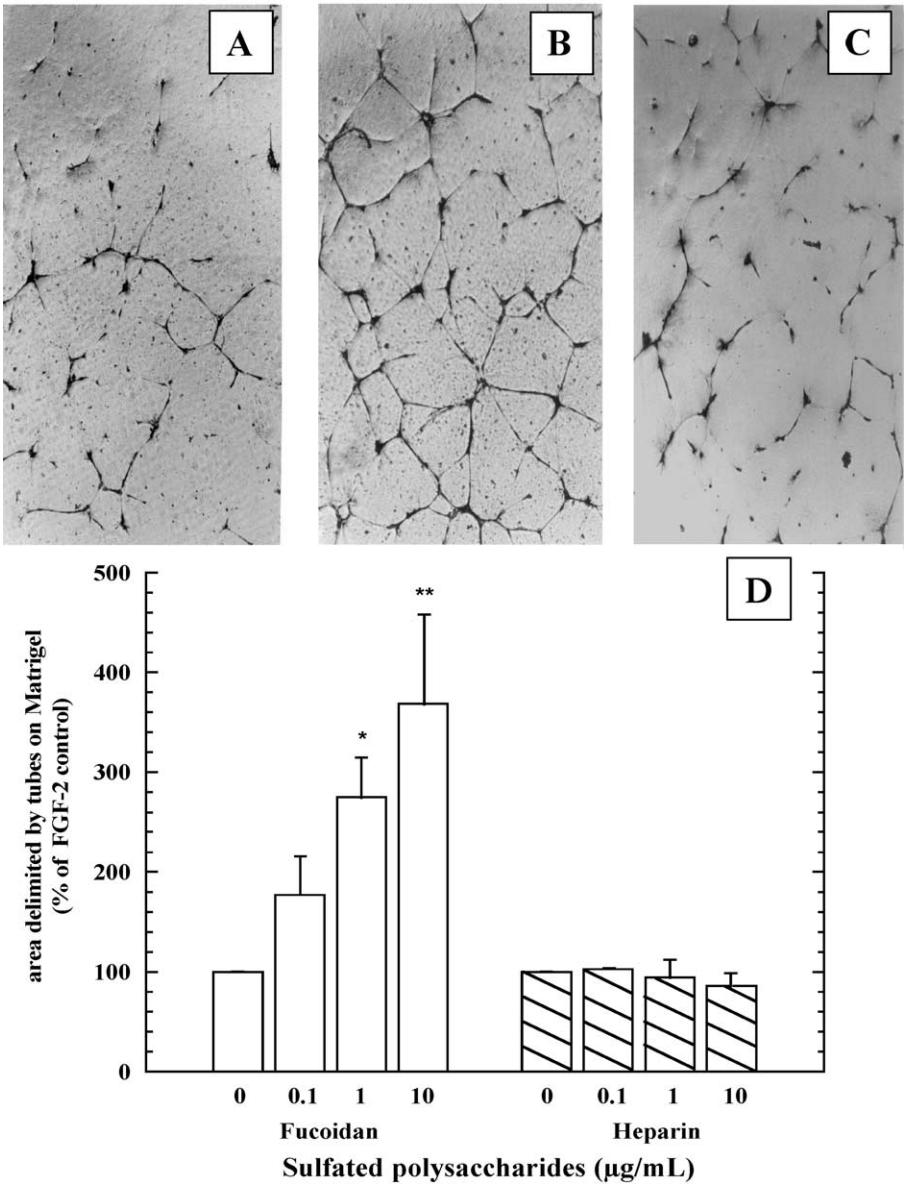

Fig. 1. Effect of fucoidan and heparin on FGF-2-induced tube formation on Matrigel. After 3 days of treatment with FGF-2 (5 ng/ml) combined with fucoidan (0.1–10 μg/ml) or heparin (0.1–10 μg/ml), HUVEC were seeded (3×10^4 cells/well) in 48-well plates coated with Matrigel. After 18 h of culture, HUVEC treated with FGF-2 alone and FGF-2 plus fucoidan or heparin were fixed with glutaraldehyde and stained with Giemsa. Photographs present vascular tube formation from HUVEC previously treated with (A) FGF-2 alone, (B) FGF-2 plus fucoidan at 1 μg/ml or (C) heparin at 1 μg/ml. (D) For different concentrations of fucoidan (white bars) and heparin (hatched bars), in the whole surface of each well, the total enclosed area delimited by the tubes was measured with the Biocom image analysis system. Values are expressed in percentages, 100% corresponding to HUVEC treated with FGF-2 alone. Results are means ± SE of six determinations for fucoidan and four for heparin. (*) and (**) significantly ($p < 0.05$ and $p < 0.01$) different from FGF-2 alone.

for flow cytometric analysis and for tube formation assay (see below).

2.5. Matrigel tube formation assay

Growth factor-reduced Matrigel plates (48-well) 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 ± FGF-2 ± polysaccharide were suspended in medium containing only 5% FBS (without FGF-2 or polysaccharide) and seeded on Matrigel (3×10^4 cells/well). In some experiments, rat anti-human α_6 integrin subunit monoclonal antibodies were added at 10 μg/ml before seeding of untreated or treated HUVEC on Matrigel. Cell culture was carried out at 37 °C in humidified air supplemented with 5% CO_2 for 18 h. The 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. The total closed area delimited by the tubes was examined and quantified in the whole surface of each well by using a microscope (40 × objective) equipped with a monochrome CCD camera connected to a computer equipped with the Biocom image analysis system.

2.6. Flow cytometry

After the second washing step, untreated HUVEC or HUVEC treated for 72 h ± FGF-2 ± polysaccharide were suspended in Hank's-2% FBS at 5×10^6 cells/ml, and 10^5 cells were incubated for 30 min with the different anti-human endothelial cell surface protein mAbs, conjugated to fluorescein or phycoerythrin. Then, HUVEC were analyzed in a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 5-W argon laser and operated at 200-mW power at a wavelength of 488 nm. Fluorescein fluorescence was detected using a 530/30 band pass filter, and phycoerythrin fluorescence was detected with a 585/42 filter.

2.7. Kinetics of α_6 integrin subunit expression

HUVEC (3×10^5 cells/well) were seeded in six-well plates in medium supplemented with 5% FBS and fucoidan (1 μg/ml) in the presence or absence of FGF-2 (5 ng/ml). The control was untreated HUVEC (cultured in the medium supplemented only with 5% FBS). Treatment was stopped after 6-, 12-, 24-, 48- and 72-h incubation, and HUVEC were then prepared as described above for flow cytometry.

2.8. Neutralization of the FGF-2 effect on α_6 expression

To neutralize the FGF-2 effect on α_6 expression, a murine anti-human-FGF-2 monoclonal antibody (20 μg/ml) was added at the endothelial cell treatment step in culture medium for 30 min before adding exogenous FGF-2 (5 ng/ml) and/or fucoidan (1 μg/ml) for 72 h; controls lacked both FGF-2 and fucoidan. Flow cytometry was performed as described above.

2.9. Statistical analysis

The effects of fucoidan and heparin were assessed by ANOVA and Fisher's protected least significant difference test.

3. Results

3.1. Effect of fucoidan and heparin on FGF-2-induced endothelial cell proliferation

After 72 h culture, FGF-2 at 5 ng/ml doubled HUVEC growth compared to FGF-2-untreated controls ($7.49 \times 10^4 \pm 0.6$ cells/cm^2 vs. $3.25 \times 10^4 \pm 0.6$ cells/cm^2). When fucoidan or heparin were added to FGF-2, no significant change was observed, whatever the sulfated polysaccharide concentration used.

3.2. Effect of fucoidan and heparin on FGF-2-induced tube formation on Matrigel

Untreated HUVEC and HUVEC previously treated with heparin in the absence of FGF-2 did not form tubes

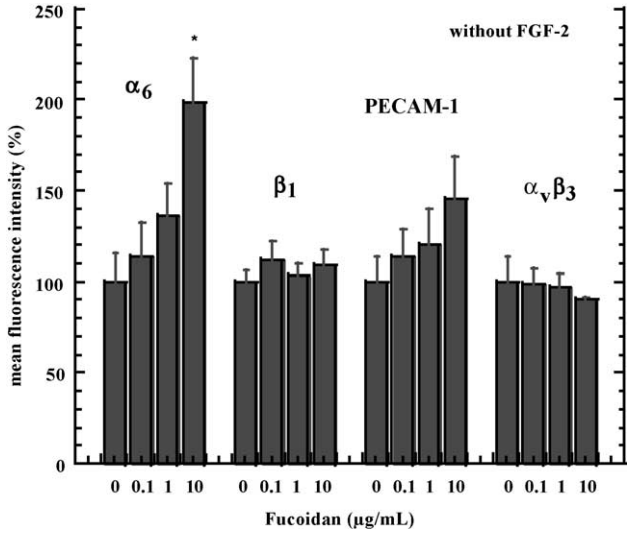

Fig. 2. Effect of fucoidan in the absence of FGF-2 on HUVEC surface protein expression. HUVEC were treated with fucoidan in culture medium. After 72 h of culture, HUVEC were analyzed by flow cytometry to quantify the following surface proteins: α_6, β_1 integrin subunits, PECAM-1 and $\alpha_v\beta_3$ integrin. Mean fluorescence intensities for each protein are expressed in percentages, 100% corresponding to control (c), i.e. untreated HUVEC. Results are means ± SE of four determinations. (*) significantly ($p < 0.05$) different from the control (c) value (in the absence of fucoidan).

after 18 h on Matrigel. When HUVEC were previously treated with fucoidan, some cells migrated, aligned and formed short tubes on Matrigel, but only at the highest fucoidan concentration (10 μg/ml). However, these tubes were not organized in a closed quantifiable area (not shown).

When HUVEC were previously treated with FGF-2 alone (Fig. 1A), vascular tubes were formed in a partially organized capillary-like network. When HUVEC were previously treated with fucoidan plus FGF-2, fucoidan modulated, in a concentration-dependent manner, the density of the capillary-like network induced by FGF-2 (Fig. 1D). At a low concentration (0.1 μg/ml), fucoidan led to a slight but nonsignificant increase in the area delimited by the tubes. In the presence of fucoidan (1 and 10 μg/ml) (Fig. 1B for 1 μg/ml; Fig. 1D), the closed surface delimited by the tubes was increased 2.5- to 3.5-fold ($p < 0.05$ and $p < 0.01$) compared to FGF-2 alone (Fig. 1D). In contrast to fucoidan, heparin did not affect tube formation induced by FGF-2 (Fig. 1D; Fig. 1C for 1 μg/ml).

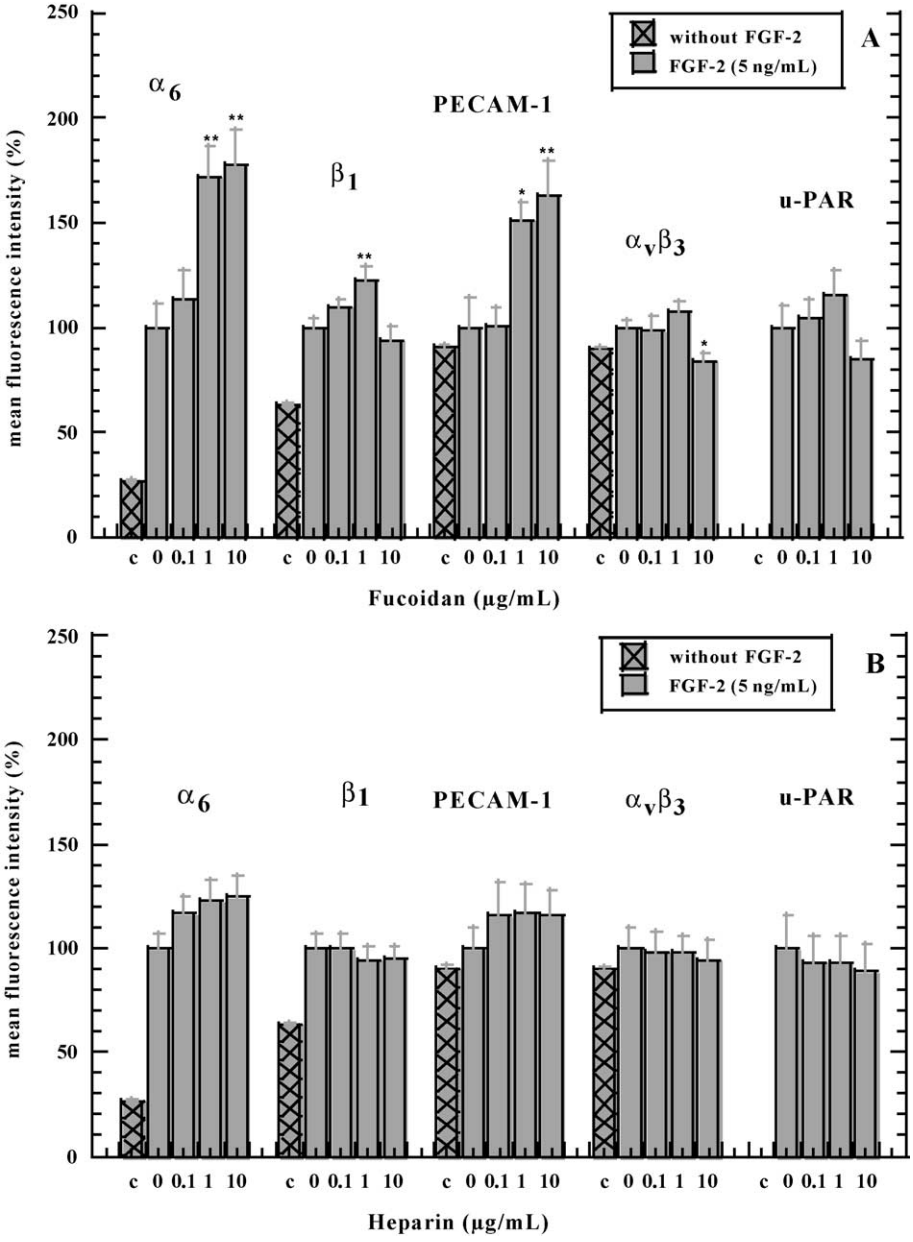

Fig. 3. Effect of fucoidan and heparin on FGF-2-induced HUVEC surface protein expression. HUVEC were treated with (A) fucoidan or (B) heparin combined with FGF-2 (5 ng/ml) in culture medium. After 72 h of culture, HUVEC were analyzed by flow cytometry to quantify α_6, β_1 integrin subunit, PECAM-1, $\alpha_v\beta_3$ integrin and u-PAR expression. Mean fluorescence intensities for each protein are expressed in percentages, 100% corresponding to the value obtained with FGF-2 alone; columns c correspond to the control values obtained without polysaccharide and FGF-2, as in Fig. 2. Results are means ± SE of six determinations. (*) and (**) significantly ($p < 0.05$ and $p < 0.01$) different from FGF-2 alone.

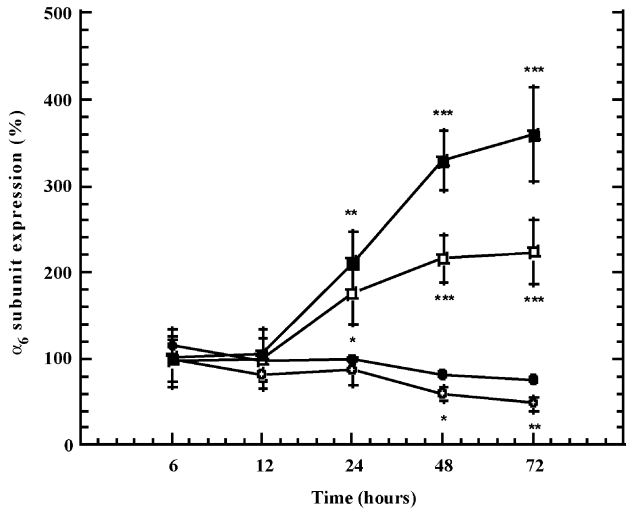

Fig. 4. Kinetics of the fucoidan effect on FGF-2-induced α_6 subunit expression. Cultures without FGF-2 and fucoidan (○), without FGF-2, with 1 μg/ml fucoidan (●), with 5 ng/ml FGF-2 (□), or with both fucoidan and FGF-2 (■). At 6, 12, 24, 48 and 72 h, HUVEC were analyzed by flow cytometry to quantify α_6 subunit expression in terms of the mean fluorescence intensity. Values are expressed in percentages, 100% corresponding to untreated HUVEC (without FGF-2 and fucoidan) after 6 h. Results are means ± SD of three determinations. (*), (**) and (***) significantly ($p<0.05$, $p<0.01$ and $p<0.001$) different from the control.

3.3. Effect of fucoidan and heparin on surface protein expression by HUVEC, in the absence of FGF-2

In the absence of FGF-2, fucoidan increased α_6 subunit expression in a concentration-dependent manner (Fig. 2). At 10 μg/ml fucoidan, this increase reached 198.7 ± 24.1% of the fucoidan-free control value (19.3 arbitrary units ± 2.2 vs. 10.1 ± 1.6; $p<0.01$). At the same concentration, fucoidan induced a slight but nonsignificant increase in PECAM-1 expression. Fucoidan had no effect on β_1 subunit or $\alpha_v\beta_3$ integrin expression. In the same conditions, heparin had no effect, even on α_6 expression. Neither polysaccharide induced u-PAR expression (data not shown).

3.4. Effect of FGF-2 on surface protein expression by HUVEC, in the absence of polysaccharide

Compared to untreated HUVEC, HUVEC treated with FGF-2 (5 ng/ml) exhibited a near 400% increase in mean fluorescence intensity for α_6 (41.0 ± 3.3 arbitrary units vs. 11.0 ± 1.1; $p<0.0001$) and a 64% increase for β_1 (37.9 ± 2.5 arbitrary units vs. 24.1 ± 1.2; $p<0.001$), whereas no increase was observed for $\alpha_v\beta_3$ or PECAM-1. FGF-2 induced u-PAR expression at the cell surface (Fig. 3).

3.5. Effect of fucoidan and heparin on surface protein expression by HUVEC, in the presence of FGF-2

When HUVEC were treated with both FGF-2 and fucoidan (Fig. 3A), α_6 subunit expression was increased by about 70% ($p<0.01$) at fucoidan concentrations of 1 and 10 μg/ml compared to FGF-2 alone. β_1, PECAM-1 and $\alpha_v\beta_3$ expression was modified by FGF-2 plus fucoidan. β_1 expression increased by 22.5 ± 6.7% ($p<0.01$) at 1 μg/ml fucoidan relative to FGF-2 alone ($p<0.01$). PECAM-1 expression also increased by about 60% at 1 and 10 μg/ml fucoidan relative to FGF-2 alone. At 10 μg/ml, fucoidan induced a

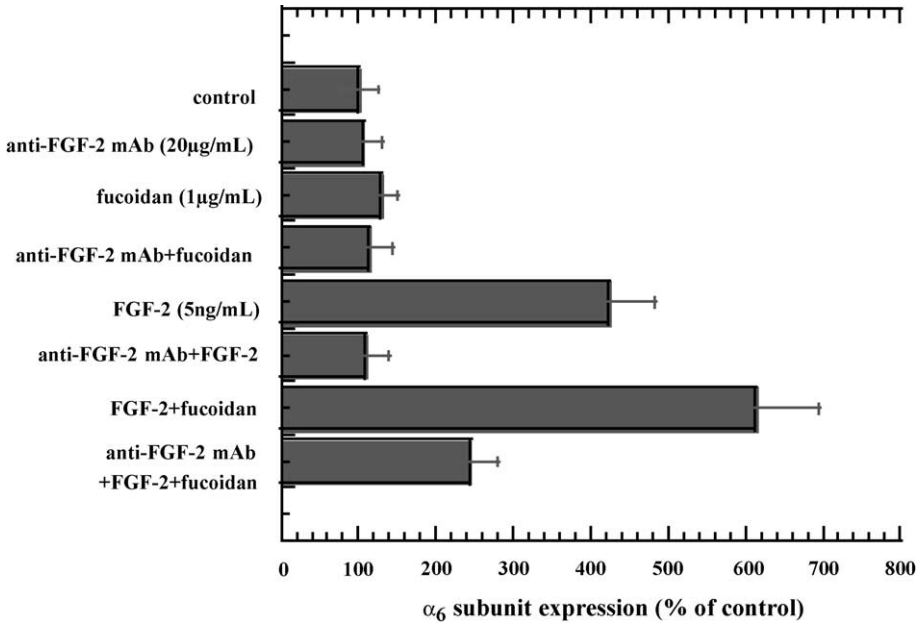

Fig. 5. Neutralization of FGF-2 effect on α_6 subunit expression in the presence of fucoidan. HUVEC were treated with or without fucoidan (1 μg/ml), anti-FGF-2 monoclonal antibody (20 μg/ml) and FGF-2 (5 ng/ml). When used, FGF-2 and FGF-2 plus fucoidan were added 30 min after anti-FGF-2. Results are means ± SE of six determinations and are expressed as percentages, 100% corresponding to control (untreated HUVEC: without FGF-2, fucoidan and antibody).

16% decrease in $\alpha_v\beta_3$ expression ($p < 0.05$) relative to FGF-2 alone. Heparin had no significant effect on the surface protein expression induced by FGF-2 (Fig. 3B).

3.6. Effect of α_6 integrin inhibition on tube formation

Since α_6 is the surface protein on which expression fucoidan had the most striking effect, we tested the effect of a monoclonal anti-α_6 antibody. In these conditions, no tube was observed from HUVEC even previously treated with FGF-2 plus fucoidan (data not shown).

3.7. Kinetics of the fucoidan effect on FGF-2-induced α_6 subunit expression

In the absence of FGF-2 and fucoidan, a slight fall in α_6 expression was observed after 48 h of culture (Fig. 4). In the presence of fucoidan (1 μg/ml), no change was observed in the time course of α_6 expression. FGF-2 significantly increased α_6 expression after 24 h, a plateau being reached after 48 h. The potentiating effect of fucoidan on FGF-2-induced α_6 expression was observed after 24 h of incubation; however, the difference between cells treated with FGF-2 alone and those treated with FGF-2 plus fucoidan became significant after 48 h ($p = 0.018$) and further increased after 72 h ($p = 0.005$), at which time the effect was almost twice that observed with FGF-2 alone (Fig. 4).

3.8. Neutralization of the FGF-2 effect on α_6 expression in the presence of fucoidan

To confirm that fucoidan acts, at least principally, on α_6 expression via FGF-2, we performed flow cytometric assays after 72 h of incubation in the presence of a murine anti-human-FGF-2 monoclonal antibody (20 μg/ml) (Fig. 5). In the absence of FGF-2, no effect was observed when the antibody was used alone or in combination with fucoidan. In the absence of fucoidan, FGF-2-induced expression of α_6 was abolished by the antibody ($p < 0.0001$). When the antibody was used in the presence of fucoidan plus FGF-2, the α_6 expression induced by FGF-2 was markedly reduced ($p < 0.01$) but remained above the levels obtained with untreated and fucoidan-treated cells ($p < 0.05$ and $p < 0.08$, respectively).

4. Discussion

We compared the ability of fucoidan and heparin to modulate angiogenesis in the presence of FGF-2. For this purpose, we studied the effect of the two polysaccharides on several endothelial cell properties related to in vitro angiogenesis, namely proliferation, the ability to form capillary-like tubes and surface protein expression. The fucoidan fraction used throughout this study has been previously shown to have efficient venous antithrombotic activity in rabbits, with lower anticoagulant activity than heparin [1]. The present study was performed with fucoidan concentrations of 1 and 10 μg/ml corresponding to ex vivo fucoidan antithrombotic concentrations. Heparin, the most widely used antithrombotic agent, was tested at the same concentrations.

In the presence of FGF-2 (5 ng/ml), fucoidan, like heparin, had no effect on HUVEC proliferation. This is not surprising, as previous studies [3] have shown that HUVEC proliferation reaches a plateau at 5 ng/ml FGF-2. Thus, at the concentration currently used for in vitro angiogenesis studies [20], no effect on proliferation is observed. However, if a plateau is reached for FGF-2-induced cell proliferation, this does not imply that, at the same concentration, a plateau is reached for FGF-2-induced cell differentiation. Indeed, some authors have shown, using FGF-2 mutants, an uncoupling of the effects of FGF-2 on cell proliferation and differentiation [21]. In order to investigate a fucoidan effect on endothelial cell differentiation, we studied, on the same batch of HUVEC, surface protein expression by flow cytometry and tube formation on Matrigel. In our conditions, Matrigel seems to be an interesting experimental system for studying FGF-2-dependent angiogenesis in vitro. Indeed, no endothelial cell reorganization was observed in the absence of FGF-2, whereas a partially formed network was obtained with FGF-2-treated HUVEC. In the presence of both fucoidan and FGF-2, we observed a noteworthy concentration-dependent increase in tube formation. Heparin had no effect in the same conditions. Jackson et al. [22] have shown that sulfated polysaccharides such as heparin, heparan sulfate, pentosan polysulfate, dextran sulfate and chondroitin sulfate stimulate vascular tube formation, whereas the nonsulfated polysaccharides xylan and dextran have no effect. However, these authors used a model of collagen-induced tube formation which does not require the presence of direct angiogenic agents such as FGF-2. In contrast to our results, Soeda et al. [23,24], using both models of angiogenesis (Matrigel and collagen gel), found that an oversulfated fucoidan inhibited tube formation. Surprisingly, this effect was observed in the absence of FGF-2 in the Matrigel experiment and in the presence of FGF-2 in the collagen gel experiment. These authors reported that native fucoidan had no effect. However, the fucoidans used in these experiments had very high molecular weights (100–130 kDa) [23] relative to our compound. In vitro studies have demonstrated that high molecular weight hyaluronan (a nonsulfated polysaccharide) is antiangiogenic, whereas hyaluronan oligosaccharides stimulate endothelial cell proliferation and tube formation [25]. Desgranges et al. [26] have shown that chemically derived dextrans also enhance the angiogenic effect of FGF-2, whereas synthetic nonsulfated polyanionic compounds [27] and chemically modified sulfated oligosaccharides inhibit FGF-2-induced endothelial cell proliferation and angiogenesis. Thus, the effects of polysaccharides on tube formation seem to be related to their chemical structure and

molecular weight. Moreover, the results may also depend on the model used for the assays.

The cell surface proteins explored—u-PAR, $\alpha_v\beta_3$ integrin, α_6 and β_1 integrin subunits and PECAM-1—are known to be modulated by FGF-2 and involved in tube formation [11,12,28,29]. In the absence of FGF-2, heparin had no effect on surface protein expression, whereas fucoidan significantly increased α_6 integrin expression only at 10 μg/ml. However, this latter increase was smaller than that observed with FGF-2 alone. Indeed, a significant increase in α_6 and β_1 expression is observed in the presence of FGF-2 alone, and u-PAR expression is then detectable, as reported by other authors [28,29]. We did not observe an increase of PECAM-1 expression or a decrease of $\alpha_v\beta_3$ induced by FGF-2, as observed by Klein et al. [28], however, with a higher FGF-2 concentration; but in the presence of FGF-2 plus fucoidan, we observed these effects on PECAM-1 and $\alpha_v\beta_3$. The effects of FGF-2 on $\alpha_v\beta_3$ expression are controversial [30,31]. Nevertheless, interestingly, fucoidan seems to act mainly on α_6 expression to promote vascular tube formation. Indeed, in the presence of FGF-2 and fucoidan, at concentrations at which tube formation was observed, the polysaccharide had a potentiating effect on the expression of proteins modulated by FGF-2 (α_6, β_1 and PECAM-1), with the most striking effect on α_6 integrin subunit. Moreover, anti-α_6 monoclonal antibodies inhibited tube formation from HUVEC previously treated with fucoidan alone or fucoidan plus FGF-2. These data suggest that fucoidan, with or without FGF-2, promotes tube formation by α_6 overexpression. The kinetics of α_6 integrin subunit expression in the presence of FGF-2 alone and of FGF-2 plus fucoidan ran parallel, fucoidan always enhancing the effect of FGF-2. These data suggest that fucoidan acts on α_6 expression mainly via FGF-2. Furthermore, monoclonal antibodies against FGF-2 strongly inhibited the effect of fucoidan plus FGF-2 on α_6 expression. The residual effect observed could be due to a change in the affinity of the FGF-2–fucoidan complex for its receptor in the presence of the anti-FGF-2 antibody. Indeed, the observation that a higher antibody concentration did not modify the results rules out competitive binding for FGF-2 between fucoidan and the antibody (not shown).

Heparin, which did not promote tube formation, had no effect on protein expression in the presence of FGF-2. Okada et al. [32] have reported that serum collected after heparin administration to patients had more pronounced vascular tube-inducing effects on endothelial cells than serum collected before treatment. This increased activity was completely inhibited by neutralization of HGF, whereas neutralization of FGF-2 and VEGF had no effect. HGF may thus play a significant role in heparin-induced angiogenesis, in the same way as FGF-2 in fucoidan-induced angiogenesis.

Thus, fucoidan, a drug with a potent antithrombotic effect in rabbits, combined with minimal anticoagulant and hemorrhagic effects, can promote, mainly via FGF-2, vascular tube formation on a reconstituted basement membrane with a major role of α_6 integrin subunit. Although these results need to be confirmed with in vivo angiogenesis assays, our data, together with those showing inhibition of smooth muscle cell proliferation [16], suggest a potential preventive effect of this polysaccharide on restenosis.

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