Fucoidan Inhibits Smooth Muscle Cell Proliferation and Reduces Mitogen-activated Protein Kinase Activity

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Objectives and design: *fucoidan has previously been shown to inhibit the proliferation of arterial smooth muscle cells both in animal models and* in vitro. *However, the mechanisms behind the anti-proliferative effects of this polysulfated polysaccharide are not known in detail. Here, the inhibitory effect of fucoidan on rat aortic smooth muscle cell proliferation was examined and compared with the effects of heparin after stimulation with fetal calf serum, platelet-derived growth factor BB, basic fibroblast growth factor, heparin-binding epidermal growth factor, and angiotensin II.*
Materials and methods: *the cultures were analysed with respect to cell proliferation and DNA synthesis by cell counting and measurement of* [3]*H-thymidine incorporation. Phosphorylation of mitogen-activated protein kinase and nuclear translocation of phosphorylated mitogen-activated protein kinase were studied by immunoblotting and immunocytochemistry.*
Results: *fucoidan was shown to be a more potent inhibitor of smooth muscle cell proliferation than heparin. Fucoidan also reduced growth factor-induced activation of mitogen-activated protein kinase and prevented nuclear translocation of phosphorylated mitogen-activated protein kinase.*
Conclusion: *fucoidan is a more potent anti-proliferative polysulphated polysaccharide than heparin and may mediate its effects through inhibition of the mitogen-activated protein kinase pathway in a similar manner as heparin.*

Key Words: Smooth muscle cells; Cell proliferation; Fucoidan; Heparin; MAP kinase.

Introduction

Vascular remodelling occurs during atherogenesis, post-angioplasty restenosis, and chronic rejection of transplanted organs. Intimal hyperplasia is one of many components involved in vascular remodelling and smooth muscle cell (SMC) proliferation is a key factor in this process. After activation, SMCs migrate to the intima, proliferate, and secrete extracellular matrix components.[1–4]

In animal models, various growth factors have been shown to influence the development of intimal hyperplasia. Platelet-derived growth factor BB (PDGF-BB) is a potent mitogen and chemoattractant for SMCs and basic fibroblast growth factor (bFGF) promotes SMC proliferation and re-endothelialisation in injured vessels. SMC proliferation is also controlled by angiotensin II (AT II) and a number of other growth factors have been implicated in autocrine growth control.[5–7] Although knowledge of the mechanisms that control SMC function has increased, effective therapy against SMC proliferation in atherogenesis, restenosis and transplant vascular disease is still lacking.

SMC function is modulated by heparin both *in vivo* and *in vitro*.[8,9] Heparin binds to protease-sensitive binding sites on SMCs and has been suggested to enter the cells by receptor-mediated endocytosis.[10] Heparin inhibits the induction of matrix metalloproteinases and, by interaction with extracellular proteins, it modifies cell-matrix interactions and influences cell migration.[11–13] The mechanisms responsible for the anti-proliferative effects of heparin are not known in detail. Heparin prevents cell cycle progression,[14,15] suppresses induction of activator protein-1 (AP-1)/c-fos and c-jun,[16] and inhibits activation of PKC.[17] Mitogen-activated protein kinase (MAPK) is an important transmitter of growth factor-related signals from both tyrosine kinase receptors and G-protein associated receptors.[18] MAPK includes extracellular signal-regulated kinase (ERK), which is dependent on cell activation by growth factors and regulates cell growth

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and differentiation. Other MAPKs are c-Jun N-terminal kinase (JNK) and p38 MAPK, which function mainly in stress responses, apoptosis and inflammation.[19,20] Recently, the growth inhibitory properties of heparin were shown to be associated with inhibition of growth factor-induced ERK activation in SMCs.[21–24]

Fucoidan is also a potent inhibitor of SMC growth *in vivo* and *in vitro*.[25] It is a polyanionic, sulfated polymer of L-fucose obtained from brown marine algae and has an anticoagulant activity lower than that of heparin.[26] The mechanism by which fucoidan exerts its anti-proliferative action is not known. It has been proposed to bind to growth factors[27] and to alter the expression of fibronectin, thrombospondin,[28] and collagen.[29] Its inhibitory activity depends on molecular weight[30] and, like heparin, fucoidan binds to the cell surface and is degraded by SMCs.[30]

The purpose of this study was to investigate how fucoidan modulates SMC proliferation induced by different growth factors and to find out if it interferes with MAPK/ERK signalling pathways in a similar manner to heparin.

Materials and Methods

Mouse monoclonal antibodies (pMAPK[m]) and rabbit polyclonal antibodies (pMAPK[P]) against phosphorylated p42/44 (phosphoMAPK) were purchased from Bio Labs (Beverly, MA, U.S.A.). Rabbit polyclonal MAPK antibodies were a gift from R. Seger.[31] Porcine mucosa heparin, fucoidan from *Fucus vesiculosus*, bovine serum albumin (BSA), human AT II, and human PDGF-BB were purchased from Sigma (St. Louis, MO, U.S.A.). Fluorescein isothiocyanate (FITC)-conjugated porcine anti-rabbit Fab' fragment, alkaline phosphatase conjugated goat anti-rabbit IgG (AP antibodies) were purchased from Dako (Glostrup, Denmark). Prestained SDS-PAGE size markers and alkaline phosphatase substrate were purchased from Bio-Rad (Richmond, CA, U.S.A.). Biotinylated goat anti-mouse IgG and Vectastain Elite ABC Kit were purchased from Vector Laboratories (Burlingame, CA, U.S.A.). Human basic FGF (bFGF) and human heparin-binding epidermal growth factor (hbEGF) were obtained from R&D Systems (Minneapolis, MN, U.S.A.).

Cell culture and proliferation

SMCs were isolated from the aorta of adult Sprague Dawley rats by digestion with 0.1% collagenase.[32] The cell cultures were maintained in F-12 medium (Gibco Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum (FCS), 200 U/ml penicillin, 0.2 mg/ml streptomycin, and 50 µg/ml ascorbic acid (F12/FCS). They were incubated at 37 °C in 5% CO_2 in air and used in passage 3 to 8. Confluent cell cultures were detached with 0.1% trypsin and 0.02% EDTA and seeded at 2×10^4 cells/cm^2 in F12/FCS. After 24 h of culture, the cells were shifted to F-12 medium supplemented with 0.1% BSA and growth-arrested for 72 h. To determine the effects of fucoidan or heparin on SMC proliferation, cells were plated at low density (5×10^3/well) and cultured in F12/FCS for 48–96–144 h in the presence or absence of 100 µg/ml fucoidan or heparin. Cell number was counted using an automatic cell counter and net growth was determined.

MAPK assay

SMCs grown in 60 mm Petri dishes were serum-starved for 72 h. The cells were then stimulated with growth factors in the presence or absence of heparin or fucoidan for different time intervals, lysed on ice for 10 min in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue), scraped off the dishes and centrifuged for 10 min at 10^3 rpm. The samples were heated to 100 °C for 10 min, cooled on ice and centrifuged. A 20 µl aliquot of each sample was loaded onto 10% SDS-PAGE gels (Bio-RAD mini gel system) and, following electrophoresis, the proteins were transferred to Hybond-C nitrocellulose membrane (Amersham, Buckinghamshire, U.K.). The membrane was washed with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween (TTBS) and incubated in blocking buffer (3% BSA in TTBS) for 1 h. The membrane was thereafter incubated with pMAPK[m] or pMAPK[P] antibodies (diluted 1:2000 or 1:1000, respectively) in TTBS with 0.1% BSA with agitation overnight at 4 °C. The membrane was then washed three times with TTBS and incubated with biotinylated goat antibody and developed with Vectastain Elite ABC Kit or with AP secondary antibodies and detected with AP substrate (BioRad). To estimate loaded amount of MAPK protein, the membranes were also incubated with MAPK antibodies (1:1000) and proteins detected as described above with AP secondary antibodies.

Analysis of DNA synthesis

SMCs were plated in 24 well plates in F12/FCS, incubated for 24 h, and thereafter serum-starved for 72 h

in F-12/BSA. The cells were incubated with growth factors with or without addition of heparin or fucoidan in F12/BSA together with ³H-thymidine (1 μCi/ml) for an additional 24 h, washed three times with PBS on ice, and incubated overnight in 10% trichloroacetic acid (TCA). The cells were washed three times with TCA, solubilised in 0.1 M NaOH for 60 min, and radio-activity measured by liquid scintillation counting.

Electron microscopy

To estimate the influence of fucoidan on SMC morphology, the cells were cultured in 60 mm Petri dishes and incubated in F12/FCS with or without heparin or fucoidan. The cells were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.3) with 0.05 M sucrose for 1 h, scraped off the Petri dishes, and transferred to plastic tubes. The specimens were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate-HCl buffer (pH 7.3) with 0.5% potassium ferrocyanate for 1 h at 4 °C, dehydrated in ethanol, stained in 2% uranyl acetate in ethanol, and embedded in low-viscosity epoxy resin. Thin sections were cut on an LKB Ultrotome IV, stained with lead citrate and examined in a Philips CM 120 Twin electron microscope.

Immunofluorescence microscopy

SMCs were seeded on glass coverslips and cultured as indicated before. The cells were stimulated for different time intervals with 10% FCS together with 100 μg/ml fucoidan or heparin and fixed in 4% formaldehyde in PBS for 1 h at room temperature. The specimens were then washed with PBS and permeabilised in 0.1% Triton × 100. After rinsing in PBS, the cells were incubated in 0.15 M glycine for 15 min, followed by incubation for 30 min in 3% BSA in PBS. The specimens were then exposed to pMAPK^P anti-bodies (1:200) overnight at 4 °C, followed by detection with FITC-labelled secondary antibodies (1:50) for 1 h at room temperature. The primary and secondary antibodies were diluted in PBS with 0.1% BSA. The specimens were mounted in Vectashield and studied in a Nikon fluorescence microscope.

Data analysis

Analysis of SMC proliferation was performed with 12 samples in each group and repeated three times. DNA

Table 1. Effects of fucoidan and heparin on proliferation of smooth muscle cells cultured in the presence of 10% FCS.

Treatment	Times (h)	Mean	SD
0.1% BSA (initial cell number)	0	5200	710
10% FCS		23000	1760
10% FCS + 100 μg/ml fucoidan	48	10600	1260
10% FCS + 100 μg/ml heparin		15700	1590
10% FCS		43600	3330
10% FCS + 100 μg/ml fucoidan	98	11900	1130
10% FCS + 100 μg/ml heparin		25500	2770
10% FCS		68100	2440
10% FCS + 100 μg/ml fucoidan	144	11400	1010
10% FCS + 100 μg/ml heparin		29700	2450

Cells were seeded in 12-well dishes in F12/FCS, cultured for 24 h, transferred to F12/BSA, incubated for 72 h. Thereafter, FCS and drugs were added and the number of cells were counted after the indicated times. The results are given as mean values (n=36). The differences in cell numbers between control and drug treated cultures at the different times were statistically significant (p<0.001).

synthesis experiments were performed with quad-ruplicate samples and each experiment repeated three times. Data from three experiments were pooled and evaluated by ANOVA analysis followed by Newman–Keuls post-hoc tests. A significant difference was considered to exist with p values equal to or less than 0.05. Immunoblotting and immunohistochemistry experiments were performed three times and representative results from one experiment are shown.

Results

Effect of fucoidan and heparin on SMC proliferation

To determine the anti-proliferative activity, we counted SMCs grown in F12/FCS with or without 100 μg/ml fucoidan or heparin at various time points. Fucoidan appeared to be a more potent inhibitor of SMC proliferation than heparin and totally blocked the increase in cell number (Table 1). We also measured DNA synthesis in growth-arrested SMCs stimulated with 10% FCS in the presence of increasing concentrations of fucoidan or heparin. Inhibition of DNA replication by both substances was dose-dependent. At 100 and 500 μg/ml the inhibitory effect of fucoidan was significantly higher than that of heparin (p<0.05 and p<0.01, respectively; Table 2a). In order to exclude non-specific toxic effects of fucoidan and heparin, cell

Table 2. Effects of fucoidan and heparin on DNA synthesis in smooth muscle cells cultured in the presence of different mitogens.

Treatment	Mitogen				
	10% FCS (A)	10 ng/ml PDGF-BB (B)	20 ng/ml bFGF (C)	20 ng/ml hbEGF (D)	200 nM AT II (E)
0.1% BSA	5	18	39	45	46
Mitogen (Control)	100	100	100	100	100
Mitogen + 1 µg/ml fucoidan	105	73	66	146	85
Mitogen + 1 µg/ml heparin	98	84	167	122	115
Mitogen + 5 µg/ml fucoidan	94	91	187	123	86
Mitogen + 5 µg/ml heparin	94	73	130	133	89
Mitogen + 10 µg/ml fucoidan	72	101	177	101	79
Mitogen + 10 µg/ml heparin	92	80	257	91	84
Mitogen + 50 µg/ml fucoidan	55	56	145	92**	63
Mitogen + 50 µg/ml heparin	56	79	204	58	62
Mitogen + 100 µg/ml fucoidan	38*	22***	153	96*	60
Mitogen + 100 µg/ml heparin	49	83	209	65	58
Mitogen + 500 µg/ml fucoidan	19**	5***	25***	46	43
Mitogen + 500 µg/ml heparin	44	74	197	48	44

Inhibitory effects of heparin and fucoidan on DNA synthesis in SMCs induced by 10% FCS (A), 10 ng/ml PDGF-BB (B), 20 ng/ml bFGF (C), 20 ng/ml hbEGF (D), and 200 nM AT II (E). SMC cultures were serum starved for 72 h, stimulated with the different mitogens in the presence of increasing concentrations of heparin or fucoidan together with ^{3}H-thymidine for 24 h. DNA synthesis was determined by scintillation counting as described in Materials and Methods.

The effect of heparin and fucoidan is expressed as percentage of control and was calculated from the following relationship: percentage control of DNA synthesis = (stimulation of DNA synthesis in the presence of inhibitor/stimulation of DNA synthesis in controls) × 100%. Statistical comparison between the same dose of fucoidan and heparin (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

Fetal calf serum (FCS), platelet-derived growth factor BB (PDGF BB), basic fibroblast growth factor (bFGF), heparin-binding epidermal growth factor (hbEGF), and angiotensin II (AT II).

cultures were studied by phase contrast- and electron microscopy. We did not detect any major differences in cell morphology between control cells and cells treated with 0.1–1 mg/ml fucoidan or heparin.

Growth-arrested SMCs were also exposed to 10 ng/ml PDGF-BB, 20 ng/ml bFGF, 20 ng/ml hbEGF, and 200 nM AT II with or without fucoidan or heparin and DNA synthesis analysed. PDGF-BB was the most potent mitogen and increased DNA synthesis 6–8-fold; whereas the other factors showed a 1.5–2.5-fold stimulation. In the presence of PDGF-BB, 100 and 500 µg/ml fucoidan inhibited DNA synthesis almost completely, whereas heparin lacked effect at these concentrations ($p<0.001$; Table 2b). Both polysaccharides enhanced bFGF-induced DNA replication. However, at 500 µg/ml, fucoidan had an inhibitory effect whereas heparin still was stimulatory ($p<0.001$; Table 2c). We observed a variation in the action of the polysaccharides in SMCs stimulated by hbEGF. At concentrations of 1 and 5 µg/ml, fucoidan and heparin stimulated induction of DNA synthesis ($p<0.01$), whereas heparin at 50–500 µg/ml and fucoidan at 500 µg/ml were inhibitory (Table 2d). Both polysaccharides reduced the DNA replicative response in cells stimulated by AT II (Table 2e).

Inhibition of MAPK activity by fucoidan or heparin

MAPK activation by different growth factors was analysed by Western blotting with antibodies specific for phosphorylated MAPK (pMAPK). SMCs were stimulated for different time intervals by 10% FCS in the presence or absence of 100 µg/ml fucoidan or heparin. FCS increased MAPK phosphorylation with a peak after 10–15 min and both fucoidan and heparin reduced pMAPK levels, although fucoidan caused a stronger inhibition (Fig. 1A). Similar results were obtained with two different antibodies against pMAPK.

We also studied the effect of 100 µg/ml fucoidan and heparin on MAPK phosphorylation in SMCs stimulated with the previously described growth factors. All of them increased MAPK phosphorylation with a maximum after 15–30 min, but hbEGF, bFGF and AT II were less effective in enhancing pMAPK levels than FCS and PDGF-BB. Fucoidan attenuated MAPK phosphorylation induced by PDGF-BB and AT II. Heparin decreased MAPK phosphorylation following treatment with hbEGF and AT II. However, in SMCs stimulated by PDGF-BB, pMAPK levels were higher after 30–60 min with heparin than with fucoidan. Both substances enhanced pMAPK levels in cells stimulated by bFGF (Fig. 1B–E).
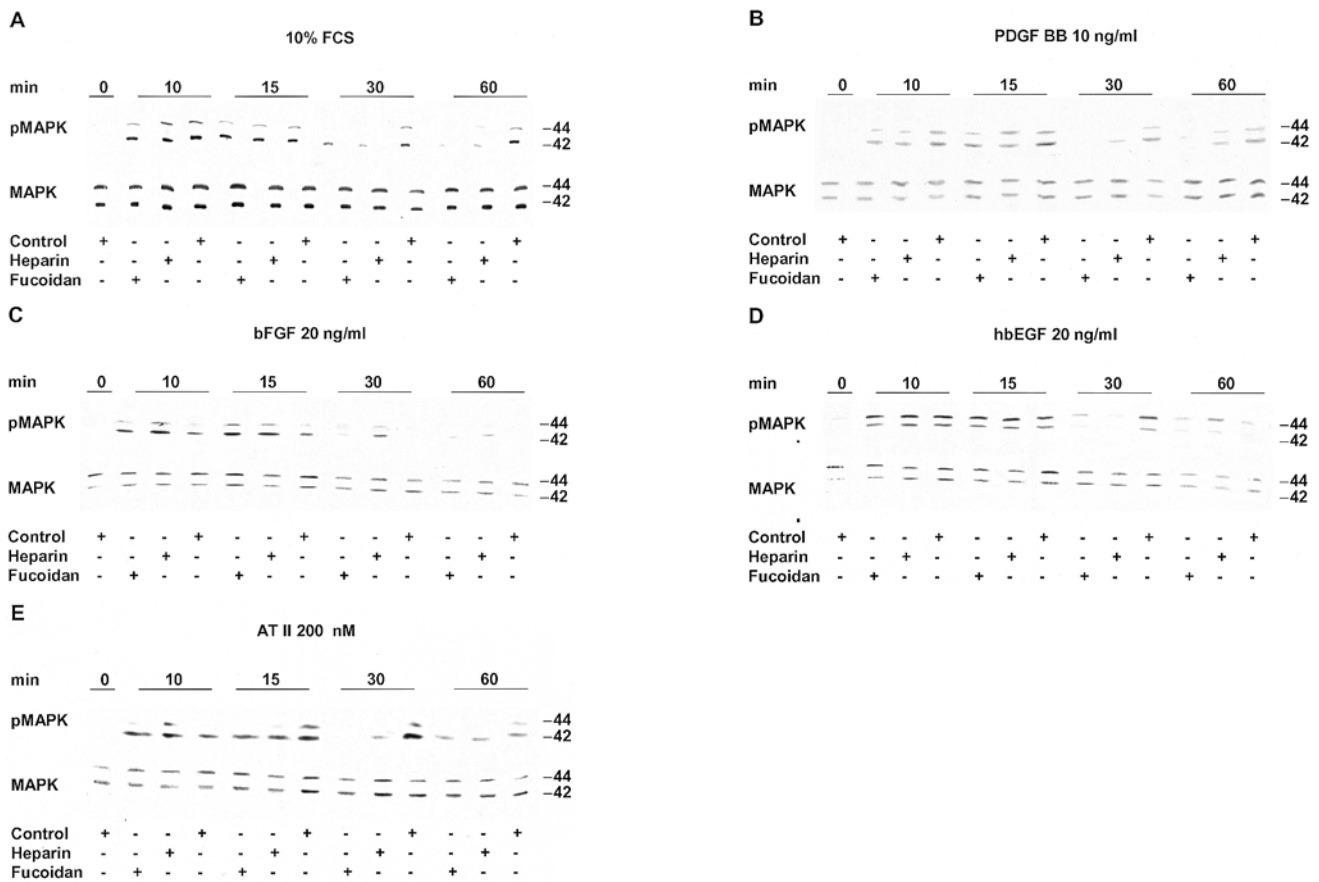
Fig. 1. Immunoblotting analysis of pMAPK expression in SMCs stimulated with 10% FCS (A), 10 ng/ml PDGF-BB (B), 20 ng/ml bFGF (C), 20 ng/ml hbEGF (D), and 200 nM AT II (E) in the presence or absence of 100 μg/ml fucoidan or heparin. SMC cultures were serum-starved for 72 h, stimulated with the different mitogens for 0, 10, 15, 30, and 60 min and proteins isolated for immunoblotting analysis using antibodies against phosphorylated (pMAPK) or total MAPK content (MAPK) as described in Materials and Methods. Representative immunoblots from three experiments are shown.

*Inhibition of nuclear MAPK translocation by fucoidan and heparin*

To investigate if fucoidan prevents nuclear translocation of MAPK, we studied the localisation of pMAPK in SMCs by immunocytochemistry. Cells were stimulated for 15, 30 and 60 min with FCS in the presence or absence of fucoidan or heparin. Immunoreactivity in the nucleus was increased after 15 min and reached a maximum after 30 min. Nuclear pMAPK staining was prevented in cells stimulated with FCS for 30 min in the presence of 100 μg/ml fucoidan or heparin (Fig. 2). After 60 min the inhibitory activity of fucoidan and heparin was decreased.

Discussion

Heparin has been shown to inhibit SMC proliferation both *in vitro* and during the formation of intimal hyperplasia *in vivo*.[14] Similar properties have been attributed to other polysulphated polysaccharides such as pentosan polysulphate and fucoidan.[14,33] In contrast to heparin, the mechanisms responsible for the growth inhibitory effects of these compounds have not previously been examined in detail.

Here, we provide evidence that fucoidan decreases SMC proliferation and DNA synthesis induced by serum and purified growth factors that act either through receptor tyrosine kinases or G protein-coupled receptors. Fucoidan was found to be a more potent inhibitor of DNA synthesis than heparin in SMCs stimulated by FCS, PDGF-BB, hbEGF, and AT II. Especially at higher doses, the effect of fucoidan was more pronounced, and in cells stimulated with PDGF-BB fucoidan inhibited DNA synthesis whereas heparin was without effect. Also with respect to molarity, fucoidan was more effective than heparin, since the approximate molecular weight of fucoidan is higher than that of heparin. This may be due to differences in the degree of sulfation, a factor previously shown
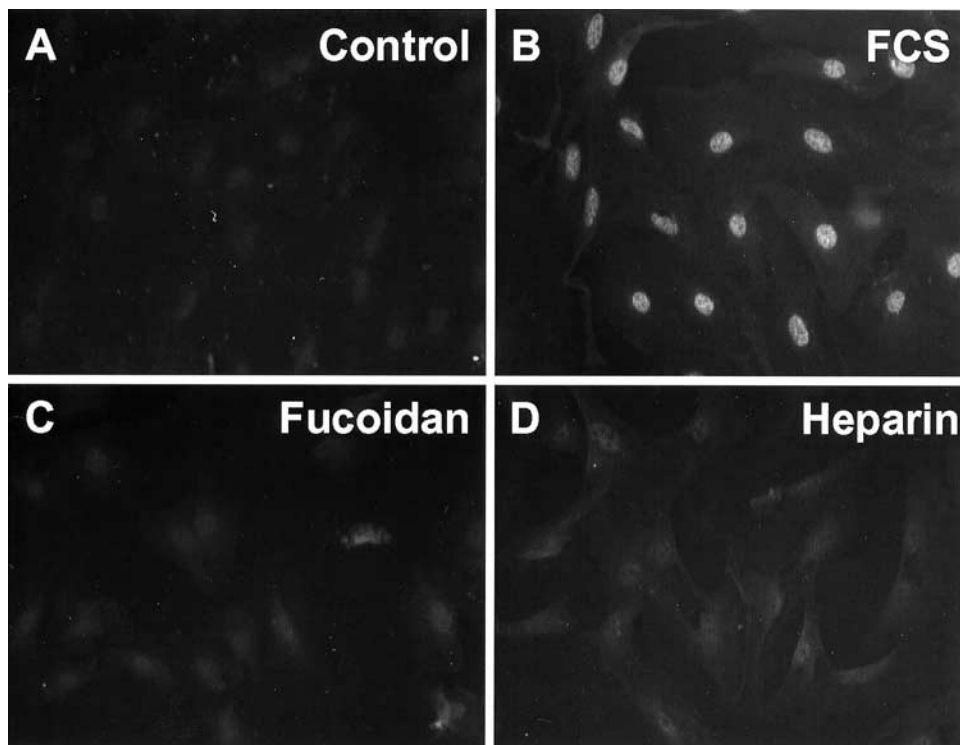
Fig. 2. Immunocytochemical analysis of pMAPK localization in SMCs by immunocytochemistry. Serum-starved SMC cultures (A; Control) were stimulated with FCS (B) in the presence of 100 µg/ml fucoidan (C) or heparin (D) for 30 min and the samples prepared for immunocytochemistry using antibodies against pMAPK as described in **Materials and Methods**. A reduced nuclear pMAPK staining is evident in cells treated with fucoidan and heparin. Fetal calf serum (FCS), platelet-derived growth factor BB (PDGF BB), basic fibroblast growth factor (bFGF), heparin-binding epidermal growth factor (hbEGF), and angiotensin II (AT II).

to determine the anti-proliferative activity of both polysaccharides in addition to the length of the polysaccharide chain.[30,34,35] Commercially available fucoidan from the marine brown algae *Fucus vesiculosus* (the preparation used in this study) is a polymer of α-1-3 L-fucose with only *O*-sulfated groups at C-4.[26] Desulfation of fucoidan has been shown to decrease its anti-proliferative activity[28,34] and 30 sugar units have been demonstrated to be required in order for fucoidan to retain its anti-proliferative activity.[30]

To examine the possible mechanisms responsible for the anti-proliferative effect of fucoidan, we analysed the ability of the polysaccharides to interfere with growth factor-mediated MAPK signalling. The MAPK family of protein kinases is essential for both SMC migration and proliferation. MAPKs transduce signals from different growth factor receptors and the involvement of this pathway in the control of cell cycle progression is well documented.[18] MAPK activity through (ERK) p42/44 provides a link between cell surface receptors and the nucleus since activated p42/44 are translocated to the nucleus where transcription factors are targeted and cell cycle progression mediated.[36–38] Also in SMCs, ERKs have been suggested to be important mediators of growth factor signalling

since inhibitors of the upstream activator of p42/44, MEKK-1, also reduce DNA synthesis induced by various mitogens.[23,39–41] In addition, control of ERK activity has also been suggested to be involved in the growth factor dependent development of intimal hyperplasia[39,42] but not JNK and p38, which seem to be involved in cell activation during inflammation and stress response.[43] Our experiments show that fucoidan and heparin influence phosphorylation and activation of MAPK in SMCs stimulated with growth factors. Fucoidan reduced the expression of pMAPK in cells stimulated with FCS and AT II in a similar manner as heparin. In contrast to heparin, fucoidan also reduced pMAPK levels in SMCs stimulated with PDGF-BB. In agreement with these findings, heparin was previously found to lack inhibitory effect on MAPK activation and DNA-synthesis in SMCs stimulated by PDGF-BB, and it has been suggested that heparin rather inhibits MAPK signalling elicited by G-protein coupled receptors such as the AT II receptor.[21,23,24] Recently, EGF receptor transactivation after stimulation of G-protein coupled receptors was shown to be dependent on proteolytic cleavage of membrane-bound hbEGF, and possibly this is relevant for the antiproliferative effect of heparin.[44]

In agreement with the observed effects of heparin and fucoidan on DNA synthesis induced by hbEGF, heparin inhibited pMAPK levels whereas fucoidan had a weak effect. HbEGF belongs to the epidermal growth factor superfamily of peptide growth and differentiation factors, distinguished by a high affinity for heparin.[45] To our knowledge, the effects of fucoidan on the activity of hbEGF have not been studied previously. EGF stimulation of SMCs was earlier reported to be insensitive to heparin.[21] In contrast, our results indicate that heparin and to some extent fucoidan modulate the action of hbEGF, most likely by growth factor binding. We also found that fucoidan and heparin increased pMAPK expression and SMC DNA synthesis after bFGF stimulation. This effect was previously suggested to be due to stabilisation and protection of bFGF against inactivation by heparin and/or by enhanced FGF receptor binding and oligomerisation, resulting in increased transmembrane signalling and a stronger biological response.[45,46] Release of active transforming growth factor β1 (TGFβ1) from serum is another potential anti-proliferative mechanism for SMCs reported for both polysaccharides.[27,47] However, fucoidan inhibited the action of several growth factors in the absence of serum, suggesting that its effect was not dependent on TGFβ1.

Polysulfated fucans have been shown to arrest SMCs in the cell cycle in a similar manner as heparin.[48] Moreover, it was suggested that heparin and fucans affect cell cycle progression in SMCs after internalisation.[10,48] Regulation of cell cycle progression requires translocation of MAPK to the nucleus, which is preceded by MAPK phosphorylation.[36–38] We observed that fucoidan inhibited both phosporylation and nuclear translocation of ERKs. These results suggest that the inhibitory action of fucoidan on induction of DNA synthesis in mitogen-stimulated SMCs may be mediated through the suppression of ERK activity. However, it cannot be excluded that heparin and fucoidan may also inhibit cell proliferation by affecting additional, parallel signalling pathways.

To summarise, our results confirmed the high anti-proliferative activity of fucoidan and suggest that sulfated fucans may be suitable as model substances for the development of anti-proliferative drugs intended for the prevention of intimal hyperplasia after interventional vascular surgery.

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