

Effects of Heparin and Related Sulfated Polysaccharides on Tissue Factor Expression Induced by Mitogenic and Non-mitogenic Factors in Human Vascular Smooth Muscle Cells

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Summary

Smooth muscle cells (SMCs) of the intima are generally quiescent and non proliferative. Their proliferation due to different stimulations occurs in myointimal hyperplasia and is regularly present in atherosclerosis or after transluminal angioplasty leading to vascular occlusive stenosis. In the course of these pathologies, the Tissue Factor (TF) synthesis was upregulated and rapidly expressed at the membrane of the SMCs. Heparin is known to inhibit SMCs proliferation induced by FCS. We evaluated the inhibitory effect of heparin on the expression of TF induced by various mitogenic (FCS, PDGF-BB and EGF) and non-mitogenic (bacterial LPS) agents. Inhibition by heparin of SMCs proliferation induced by the same agonists was also determined.

Quiescent human vascular SMCs from normal adult arteries were treated for 1 h by heparin and related sulfated polysaccharides before stimulation by the agonists. All the agonists up-regulated the expression of TF antigen and activity. TF expression induced by the growth factors was inhibited by heparin (IC 50: 10-30 µg/ml), and other sulfated polysaccharides (IC 50: 1-5 µg/ml). SMCs proliferation, late activation of the extracellular signal-regulated kinases (ERK1/2), and PKC activity were inhibited by heparin (IC 50: 30-50 µg/ml) in SMCs stimulated by FCS but not in SMCs treated by PDGF or EGF. In contrast, heparin had no effect on LPS-induced TF expression nor on LPS-induced PKC activation. These results indicate that, besides its well known effect on SMC proliferation, heparin displays an inhibitory effect on cell mediated blood clotting processes through regulation of the TF expression.

Introduction

Tissue Factor (TF) is the initiator of blood coagulation *in vivo* (1). Immunohistochemical analysis and *in situ* hybridization have demonstrated that TF is not expressed, or is found at low levels in the intima or in the media of normal adult blood vessels (2-3). When atherosclerotic plaques are examined by similar methods, TF mRNA and protein are found in mesenchymal-like intimal cells, presumably smooth muscle cells (SMCs) and foam cells, and in monocytes adjacent to cholesterol clefts (3-5). We and others have shown that mitogenic factors for SMCs upregulate the expression of TF by these cells *in vitro* (6-8).

SMCs *in vivo* are found in a quiescent non-proliferative phenotype except for limited periods of time during the evolution of the atherosclerotic lesion or wound healing process after angioplasty (for review, see 9). SMCs migration and proliferation are then induced by a variety of growth factors through different signalling pathways including the activation of tyrosine and serine/threonine kinase such as mitogen activated protein kinases, also called extracellular signal regulated kinases (ERK), and protein kinase C (PKC) (10).

Heparin and glycosaminoglycans of the extracellular matrix in vascular tissue such as heparan sulfate, are known as inhibitors of migration and proliferation of SMCs *in vivo* and *in vitro* (11-14). Other sulfated polysaccharides such as pentosan polysulfate may display antiproliferative activity (15). The intracellular mechanisms involved in the inhibition of cell proliferation by heparin are not well understood. Heparin inhibits the induction of transcription of several genes associated with cell cycle progression, including *c-myb*, *c-myc*, *c-fos* and the expression of the mitochondrial ATP/ADP carrier protein 2F1, and histone H3 (16-19). Heparin also interferes earlier in the signal transduction pathway at different levels, depending on the mitogenic agent. In particular, it was found to inhibit the activation of ERK and/or PKC in rat SMCs (20-23).

Since some of these pathways have been shown to be involved in TF expression (8), we hypothesized that heparin and other sulfated polysaccharides (SPS) could also inhibit TF expression. The aim of this study was to test this hypothesis using various agents which elicit SMCs mitogenic response and/or TF expression by potentially different signalling mechanisms.

Materials and Methods

Materials

Dulbecco's Modified Eagles Medium (DMEM), Foetal Calf Serum (FCS), L-glutamine, penicillin, streptomycin, phosphate buffer saline (PBS), trypsin-EDTA and collagenase, were from Seromed Biochrom KG, France. DMEM and FCS were certified endotoxin-free by the manufacturer. Fungizone was from Bristol Myers Squibb, France. Culture flasks and plates were from Nunclon InterMed, Denmark. Leupeptin, α Pepstatin, Phenyl Methyl Sulfonyl Fluoride (PMSF), Dithiothreitol (DTT), Glycerol, synthetic peptide 4-14 from Myelin Basic Protein (MBP₄₋₁₄), phosphatidyl serine, 1,2 dioleine, ATP and Endotoxin (lipopolysaccharides from E. Coli, Strain 055 : B5) came from Sigma Immuno Chemicals Co, USA. Aprotinin was from Sanofi Choay, France. Bromophenol blue, Triton X 100, EDTA, β Mercapto Ethanol, Tris, NaCl came from Merck, France. Tween 20 was from Fluka Biochemica, France. Sodium dodecyl sulfate (SDS), Protein Assay, Polyvinylidene difluoride membrane (PVDF) 0.2 µM were from Biorad Laboratories, France. Octyl PD glucopyranoside, lumifilm chemiluminescent detection film, came from

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Boehringer Mannheim, France. Monoclonal anti-human Tissue Factor was from Corvas, USA. Elisa Kit Imubind Tissue Factor came from American Diagnostica, USA. Thromborel S was from Behring, France. Human recombinant PDGF-BB and EGF were from Tebu, France. Phospho Plus MAPK antibody kit was purchased from Biolabs, USA. Vitamin K coagulation factors were prepared from human plasma by absorption on barium citrate. Substrate for thrombin (S 2238) was purchased from Chromogenix, Sweden. Standard heparin from pig intestinal mucosa (H, sodium salt average molecular weight MW 15 kDa, 2 SO₃⁻ per disaccharide unit), oversulfated heparin (OSH, MW 15 kDa, 4 SO₃⁻ per disaccharide unit), dermatan sulfate (DS, MW 15 kDa, 1 SO₃⁻) and pentosan polysulfate (PS, MW 6 kDa 4 SO₃⁻) were from Sanofi, France. [γ ³²P] ATP (1000 to 2000 cpm/pmol) was from Amersham, France.

Human Vascular SMCs Culture

Human SMCs were obtained by outgrowth from explants or by enzymatic digestion from the media of normal adult mammary arteries obtained during coronary bypass surgery and cultured as previously described (8). They were used between the second and the seventh passage. Primary SMCs and subcultures were identified by their characteristic "hills and valleys" patterns of growth, by positive immunofluorescence staining with an antihuman SMCs α actin antibody (8).

Treatment of SMCs

Cells were made quiescent by culture in 0.3% FCS medium for 72 h without addition of fresh medium. Control of growth arrest after serum starvation was assessed by flow cytometry (8). Cell viability assessed by the trypan blue exclusion test was >95% after 72 h and the cells were able to re-enter the cycle after addition of serum-rich medium. Quiescent cells were stimulated for various periods of time with heat-inactivated FCS (0.01 to 10% in DMEM buffer), human recombinant PDGF-BB or EGF (0.1 to 100 ng/ml) or LPS (0.1 to 100 μ g/ml), directly added into the wells without changing the culture medium. When indicated, cells were pretreated for 60 min by heparin or related polysaccharides (0.1 to 100 μ g/ml), before the stimulation with FCS 10%, PDGF 100 ng/ml, EGF 100 ng/ml or LPS 10 μ g/ml. Percent inhibition by heparin or related sulfated polysaccharides was determined as: [value obtained in the absence of SPS – value obtained in the presence of SPS/value obtained in the absence of SPS – value obtained in non stimulated cells] \times 100.

Proliferation Assays

Cells were seeded at low density (10⁴ cells/well in 24 well cluster plates) in 1 ml of complete DMEM + 5% FCS. After 24 h, cells were growth-arrested

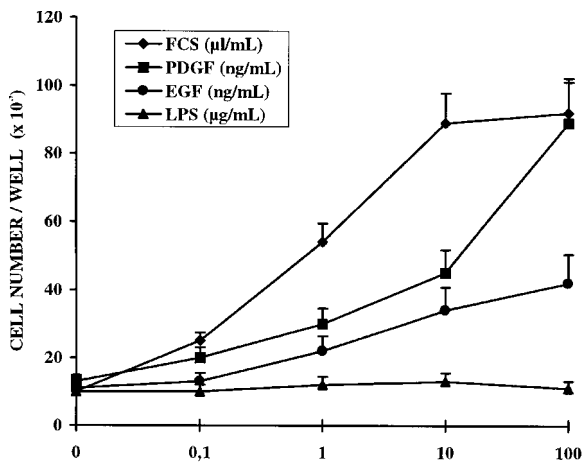


Fig. 1 Effect of FCS, PDGF, EGF and LPS on SMCs proliferation. Quiescent SMCs (10⁴ cells/well) were treated by various agonists at increasing doses for 72 h before counting. Results shown are means \pm SEM (n = 9)

in DMEM + 0.3% FCS. After 72 h, cells of triplicate wells were counted. Cells in the remaining wells were then stimulated with the indicated concentrations of FCS, PDGF, EGF or LPS. SPS were added to triplicate wells at the same time. After 72 h, stimulated cells were trypsinized and counted in a Coulter counter.

Procoagulant Activity and TF Antigen

Quiescent SMCs were stimulated for 5 h with various agonists before TF expression was measured. Procoagulant activity was measured by a chromogenic assay as previously described (8) and expressed in arbitrary units (AU), the reference being a human brain TF preparation (Thromborel), estimated to contain 10⁶ AU/ml (8). The logarithm of the procoagulant activity was linearly related to the absorbance up to 2000 AU/ml. Preincubating the cells with a mouse monoclonal antibody against human Tissue Factor for 30 min at 37^o C inhibited the measured activity by more than 90%.

TF antigen was measured using the Imubind TF kit on cell lysates as previously described (8). Results were expressed as ng of TF antigen per mg of total proteins (ng/mg).

MAPK Activity

MAPK activity was measured after cell homogenization and protein extraction, using an in-gel phosphorylation assay as described (24). Briefly, SMCs were treated with the agonists with or without heparin as indicated and washed with cold PBS. Proteins were extracted in homogenization buffer 20 mM Tris-HCl, pH 7.4, 10 mM 2-mercapto ethanol, 10 mM EGTA, 2 mM EDTA, 1mM PMSF and 0.25 mM sucrose. ERK activity was measured using an in-gel phosphorylation assay (24). Equal volumes of samples were run on a 10% SDS-PAGE gel containing 0.25 mg/ml myelin basic protein (MBP). After electrophoresis, the gels were incubated 1 h at room temperature in 50 mmol/l HEPES-NaOH (pH 7.5) and 20% 2-propanol, followed by 50 mmol/l HEPES-NaOH (pH 7.5) and 5 mmol/l 2-mercaptoethanol (buffer A). Protein was denatured by immersing the gels twice in buffer A containing 6 mol/l urea, for 15 min each time and renatured overnight at 4^o C in buffer A containing 0.05%. Tween-20 with two buffer changes. After a 30-min incubation at room temperature in kinase buffer, phosphorylation was performed by soaking the gels for 45 min in kinase buffer containing 20 μ mol/l [γ ³²P] ATP (1000 to 2000 cpm/pmol). Nonbound radioactivity was removed by six washes with 5% trichloroacetic acid (TCA) and 1% sodium pyrophosphate. Before drying, the gels were stained with Coomassie brilliant blue to check for equal loading of protein. Incorporated radioactivity was analyzed by phosphorimaging.

PKC Activity

PKC activity was assayed on cell homogenate by measuring the incorporation of ³²P from labeled [γ ³²P] ATP into MBP₄₋₁₄ after 10 min of incubation at 30^o C, as described (25). Briefly, growth-arrested SMCs were treated with the agonists with or without heparin as indicated. SMCs were trypsinized and centrifuged (140 g/10 min). The pellet was resuspended at a density of 10⁵ cells/ml and washed twice with ice-cold homogenizing buffer (20 mM Tris-HCl, pH 7.4, 10 mM 2-mercapto ethanol, 10 mM EGTA, 2 mM EDTA, 1mM PMSF and 0.25 M sucrose). After the addition of 0.3% Triton X100, the homogenate was centrifuged at 100,000 g for 1 h at 4^o C. The resulting supernatant was diluted tenfold with elution buffer (20 mM Tris-HCl, pH7.4, 10 mM 2-mercaptoethanol, 0.5 mM EGTA, 0.5 mM EDTA and 0.5 mM PMSF) and assayed immediately or stored at -80^o C for later use. PKC activity was assayed by measuring the incorporation of [³²P] from labeled ATP into MBP₄₋₁₄. Briefly, the incubation mixture (200 μ l) contained 20 mM Tris-HCl buffer (pH 7.4), 0.5 mM MgCl₂, 10 μ M MBP₄₋₁₄, 50 μ g/ml phosphatidyl serine, 5 μ g/ml 1,2 dioleine, 10 μ M ATP [γ ³²P] (10⁶ dpm), and 50 μ l cell fraction. After incubation at 30^o C for 10 min, the reaction was stopped by the addition of 3 ml of 20% TCA. Acid precipitable materials were collected on Watman GF/C filters and extensively washed with ice-cold 20% TCA. The radioactivity on the filters was measured using a liquid scintillation counter.

Results

Effects of FCS, PDGF, EGF and LPS on SMCs Proliferation and Tissue Factor Expression

Figure 1 shows the dose response on SMCs proliferation induced by FCS, PDGF, EGF and LPS. The most potent agonists were FCS followed by PDGF and EGF. LPS was inactive. Consistent with these results, 30 min after cell stimulation, FCS, PDGF and EGF but not LPS induced ERK activation and phosphorylation (Fig. 2). In contrast, PKC was activated not only by FCS, PDGF, EGF but also by LPS.

A preliminary experiment indicated that, after stimulation, the peak of TF activity occurred between 5 and 12 h (not shown), thus a 5-h incubation period was selected for the further experiments. Treatment of quiescent cells by the four agonists increased TF expression in a dose-dependent manner (Fig. 3). FCS was the most active agent and EGF the weakest. For the following experiments, the optimal concentration of each agonist: FCS (10%), PDGF (100 ng/ml), EGF (100 ng/ml) and LPS (10 µg/ml) was used. Procoagulant activity and

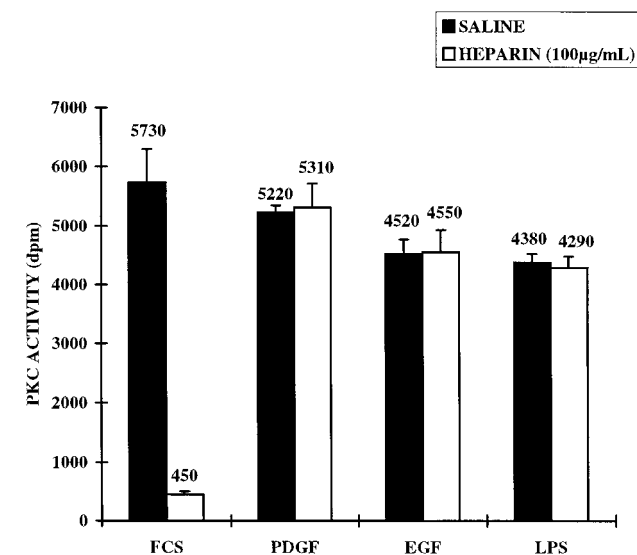
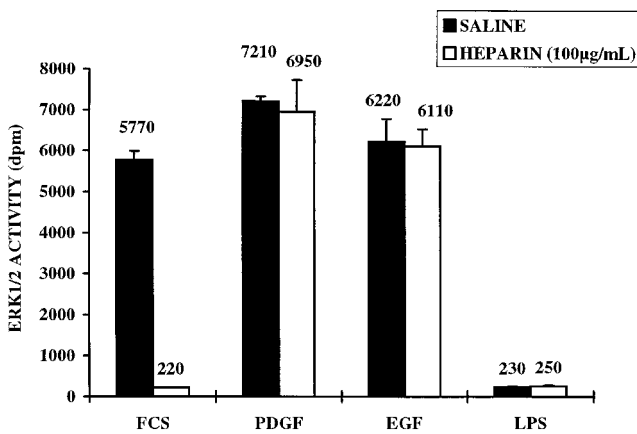


Fig. 2 Effect of FCS, PDGF, EGF and LPS on ERK and PKC activities. SMCs (5.10⁴ cells/well) were stimulated for 10 min by FCS 10%, PDGF 100 ng/ml, EGF 100 ng/ml or LPS 10 µg/ml for PKC assay and for 30 min by the same agonists for ERK determination. Experiments were performed in triplicate in the absence (black columns) or presence (open columns) of 100 µg/ml of heparin. Results shown are means ± SEM (n = 9)

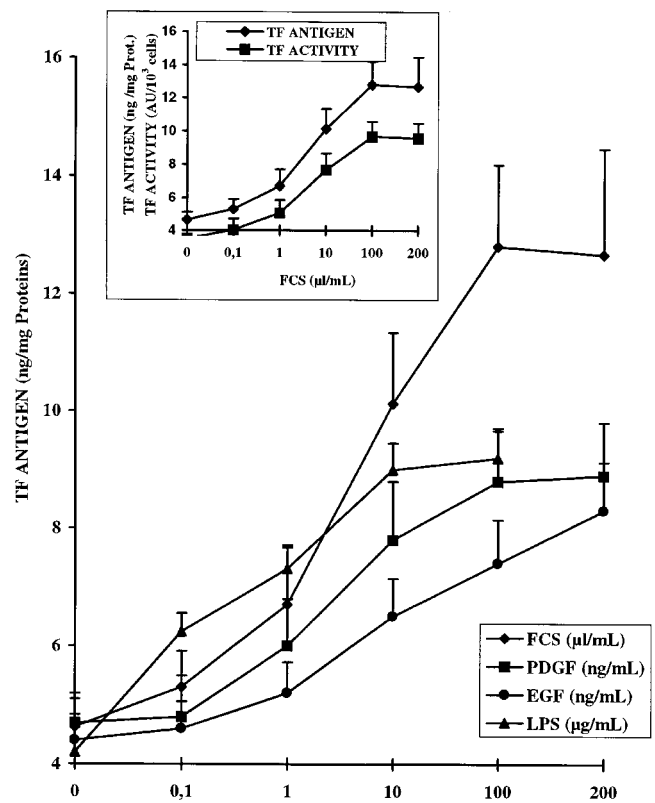


Fig. 3 Effect of FCS, PDGF, EGF and LPS on TF antigen expression in SMCs. Quiescent SMCs (8.10⁴ cells/well) were incubated for 5 h with FCS, PDGF, EGF or LPS. TF expression was determined as described in materials and methods. Inset: correlation between TF activity and TF antigen expression of SMCs incubated with FCS (0.1 to 20%). Similar results were obtained with PDGF, EGF and LPS (not shown). The bars indicate the SEM of 6 different

TF antigen varied in parallel, indicating that TF was synthesized and expressed at the membrane surface (inset Fig. 3).

Effect of Heparin and Sulfated Polysaccharides on SMCs Proliferation and TF Expression

Quiescent SMCs were incubated for 1 h with heparin before stimulation by each agonist. SMCs proliferation induced by FCS was inhibited by heparin in a dose dependent manner (Fig. 4). Almost complete inhibition was obtained with 100 µg/ml and half maximum effect with 30-50 µg/ml. Heparin inhibited both the ERK and the PKC activities induced by FCS (Fig. 2). PDGF- and EGF-induced proliferation was not inhibited by heparin even at the highest concentration tested. Heparin inhibited neither ERK nor PKC activity induced by PDGF and EGF (Fig. 2).

Heparin inhibited the procoagulant activity and TF antigen expression induced by FCS. A maximum of 70% inhibition was obtained with 100 µg/ml and half maximal effect with about 10-30 µg/ml (Fig. 5). Similarly, heparin inhibited TF expression induced by PDGF. The effect of heparin on TF expression induced by EGF was weaker but still quite significant. Heparin had no effect on LPS-induced TF expression nor on LPS-induced PKC activity (Fig. 2).

The effects of SPS on FCS-induced proliferation and TF expression were then examined. As shown in Fig. 6, SMCs proliferation was

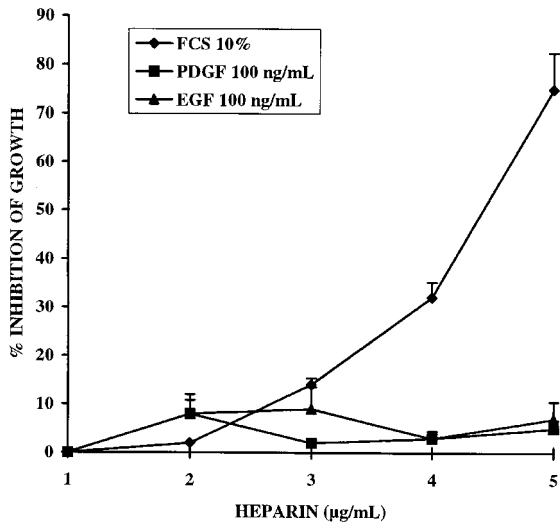


Fig. 4 Effect of heparin on SMCs proliferation induced by FCS, PDGF, EGF, LPS. Cells were treated as in Fig. 1 with increasing concentrations of heparin for 1 h. Cell numbers were determined after 3 days in culture. Results shown are means \pm SEM (n = 9)

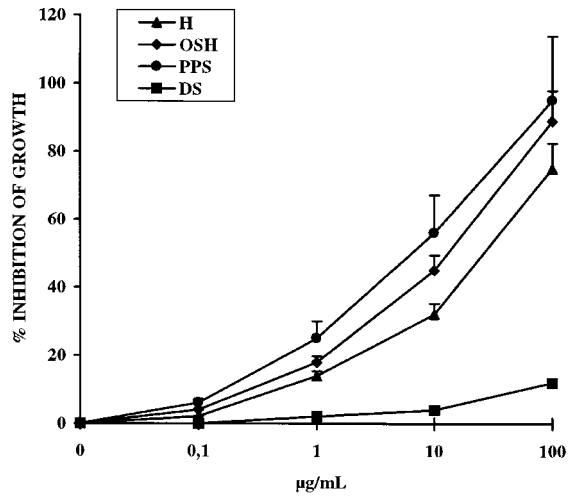


Fig. 6 Effect of heparin and sulfated polysaccharides on SMCs proliferation induced by FCS. Quiescent SMCs (10^4 cells/well) were pretreated by heparin (H) pentosan polysulfate (PPS), oversulfated heparin (OSH) or dermatan sulfate (DS) and then stimulated with FCS (10%) for 72 h before counting. Results shown are means values \pm SEM (n = 9)

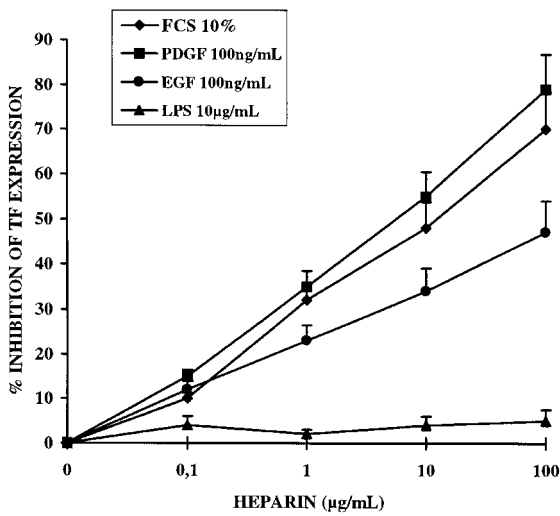


Fig. 5 Effect of heparin on TF antigen expression induced by FCS, PDGF, EGF, and LPS. SMCs were treated as described in Fig. 3 after pretreatment for 1 h by heparin (0.1 to 100 µg/ml). The bars indicate the SEM (n = 6)

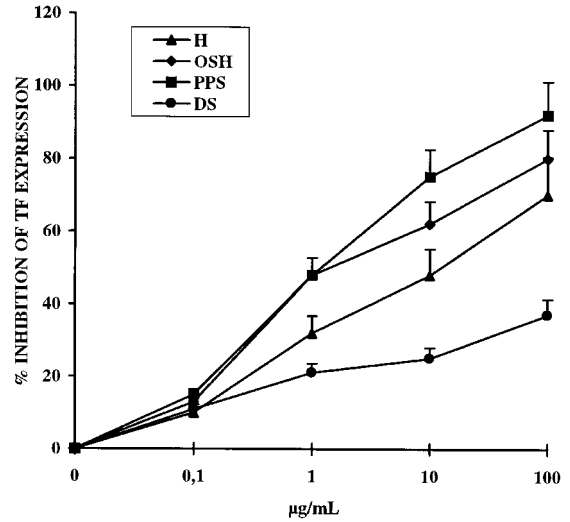


Fig. 7 Effect of heparin and sulfated polysaccharides on TF antigen expression induced by FCS. Quiescent SMCs (8.10^4 cell/well) were pretreated for 1 h by heparin (H), oversulfated heparin (OSH), pentosan polysulfate (PPS), dermatan sulfate (DS) and then stimulated for 5 h with FCS 10% before TF antigen assay. Results shown are means \pm SEM (n = 9)

inhibited in a dose-dependent manner. Pentosan polysulfate was the most potent inhibitor, followed by oversulfated heparin and heparin. Dermatan sulfate had no significant inhibitory effect at the highest dose tested (100 µg/ml). Consistent with these results, ERK phosphorylation was inhibited by sulfated polysaccharides with antiproliferative activity but not by dermatan sulfate (not shown). FCS-induced TF expression was also inhibited by the three polysaccharides, with the same order of potency as that observed on cell growth. Dermatan sulfate only slightly affected FCS-induced TF expression in human SMCs (Fig. 7).

Discussion

Heparin has long been known to be an inhibitor of serum-induced proliferation of cultured vascular SMCs (14, 15). In addition heparin has been reported to have inhibitory effect on TF response in human endothelial cells (26) and monocytes (27-29). In this study, the inhibition by heparin of TF expression induced by various agonists in SMCs is described for the first time. Moreover, we show that the inhibitory potency of the polysaccharides on SMCs proliferation and TF expression is roughly proportional to their sulfate content.

We recently reported that the mitogenic response of human vascular SMCs to PDGF was associated to rapid TF expression (8). We now extend this observation to FCS and to another growth factor, EGF. The question then arises whether proliferation and TF expression are events so closely related that they cannot be dissociated during the response of SMCs to a mitogenic factor. Should this be the case, inhibition of SMCs proliferation by a pharmacological agent would imply a parallel inhibition of TF expression.

Heparin has been shown to inhibit the expression of several proto-oncogenes as well as ERK activation in SMCs stimulated by FCS. In contrast, heparin has no inhibitory effect when EGF is used as an agonist, which elicits cell responses through a PKC-independent pathway (21-22). These results led us to distinguish an "heparin-sensitive PKC-dependent" from an "heparin-insensitive PKC-independent" pathway being distinguished. Our results support this distinction since heparin inhibited SMCs proliferation, late ERK phosphorylation and PKC activation induced by FCS, but was inefficient on any of these cell responses when the cells were stimulated by EGF. Interestingly, heparin inhibited only the sustained ERK phosphorylation measured 30 min after stimulation by FCS, which is required for cell proliferation (30-33), but not early ERK phosphorylation measured at 10 min (not shown).

For PDGF, opposite results have recently been reported in the literature. Pukac et al. (22) have shown that heparin treatment inhibits PDGF-induced rat SMCs growth and late ERK activation by suppression of a PKC-dependent pathway. In contrast, Daum et al. (23) indicate that heparin does not inhibit DNA synthesis or ERK activation induced by PDGF in baboon SMCs. Our results in human cells are consistent with those of Daum since heparin had no effect on proliferation, late ERK phosphorylation or PKC activation. Heparin did not inhibit early ERK phosphorylation induced by PDGF.

In contrast with the diversity of heparin effect on proliferative response and cell signalling, TF expression was inhibited for the three mitogenic agents tested. This was not due to a direct heparin effect on the TF procoagulant assay since (i) heparin has no effect on LPS-induced activity in the same experimental conditions, (ii) the plasma heparin cofactor antithrombin III required for a full anticoagulant activity of heparin was not present in the medium and, (iii) the procoagulant activity and TF protein decreased in parallel. The fact that TF expression induced by EGF and PDGF was inhibited by heparin whereas cell proliferation was not, indicates that the two phenomena can readily be dissociated. This assumption is supported by the lack of effect of heparin on LPS-induced TF expression. LPS, which has no known mitogenic activity, is a potent inducer of TF expression in monocyte/macrophages (34-37) and endothelial cells (38-40). In the present study, we show that LPS is also an agonist for SMCs since it induces PKC activity and TF synthesis. Therefore two pathways can be distinguished for the expression of TF by SMCs: one heparin insensitive elicited by LPS and the other heparin sensitive elicited by mitogenic agents, but uncoupled to the pathway for proliferative response.

The mechanism by which heparin inhibits TF expression remains to be elucidated. A direct effect on the agonists, on their receptors or on early signal transduction events can be ruled out since heparin does not block the whole spectrum of cell responses to the agonists. We have previously shown that ERK activity is required for TF expression induced by PDGF (8). In this study, we show that heparin inhibits TF expression with no major effect on ERK phosphorylation induced by PDGF or EGF. Moreover, PKC activity induced by PDGF or EGF is not inhibited by heparin. This fact is relevant to the study of Taubman et al. (6), who showed that induction of TF by serum growth factors in

rat SMCs was PKC independent. Our results suggest that the effect of heparin lies downstream of the activation of ERK. The regulation of TF gene in various cell types occurs at least at two distinct regions in the promoter: the distal lipopolysaccharide responsive element (LRE), and the proximal serum response region (SRR) (41, 42). The former interacts with nuclear factor kappa (NF- κ B) and activator protein 1 (AP-1) transcription factors upon cell activation by LPS whereas the latter binds Sp1 and Egr-1 upon cell activation by growth factors in human cells. Although the induction of Egr-1 by growth factors has not been demonstrated in human vascular SMCs, it is tempting to speculate that heparin could inhibit TF expression by preventing induction or activation of Sp1/Egr-1 and not of NF- κ B/AP-1.

In conclusion, we have demonstrated that heparin inhibits TF expression induced by growth factors in human vascular SMCs. Whether this effect is relevant to the antithrombotic properties of heparin in cell-mediated blood clotting and in other cell responses such as migration (43) and proliferation, remains to be determined.

Acknowledgments

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