Heparin Selectively Inhibits the Transcription of Tissue-type Plasminogen Activator in Primate Arterial Smooth Muscle Cells during Mitogenesis*

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How heparin inhibits vascular smooth muscle cell proliferation and migration has not been established. We have investigated the hypothesis that heparin inhibits vascular smooth muscle cell proliferation and migration by interfering with the expression and activity of proteases such as plasminogen activators. In an in vitro mitogenesis model, tissue-type plasminogen activator (tPA) mRNA and protein increase in baboon smooth muscle cells stimulated with fetal bovine serum or phorbol esters. Heparin inhibits smooth muscle cell proliferation and suppresses the induction of tPA mRNA and protein while it has little effect on the mRNA of urokinase-type plasminogen activator, plasminogen activator inhibitor type I, and a number of genes that are also modulated by serum and phorbol esters. The inhibitory effect on tPA mRNA is specific to heparin-like molecules and does not depend on the anticoagulant activity of heparin. The increase in tPA mRNA is due to increased transcription, which is suppressed by heparin. The induction of tPA by serum and phorbol esters is diminished by protein kinase C inhibitors such as H7 or staurosporine and by protein kinase C depletion. Since heparin suppresses the induction of the tPA gene by phorbol esters, these results suggest that heparin may interfere with the protein kinase C pathway.

Heparin is a pharmacological inhibitor of vascular smooth muscle cell (SMC) proliferation and migration, and heparin-like glycosaminoglycans secreted by vascular wall cells possess SMC inhibitory activity and might be endogenous regulators of SMC growth in vivo (1, 2). How these molecules function has yet to be determined, although a number of physiological effects have been described. In vivo, heparin inhibits the initial wave of SMC proliferation induced by arterial injury (3). It also inhibits subsequent SMC migration and intimal thickening (4) and has a striking effect on matrix deposition in the intima (5). It markedly decreases the accumulation of elastin and interstitial collagen while increasing proteoglycans. In vitro, heparin inhibits proliferation (6) and migration of SMC (7). These effects are specific for heparin-like molecules and do not depend on the anticoagulant activity of heparin.

Attempts to define a mechanism of action that accounts for all these effects of heparin have not as yet been successful. Heparin can bind to SMC membranes and be internalized (8). Furthermore, specific species of endogenous heparan sulfate have been recovered in nuclear preparations (9). The significance of these observations is unknown, but they do suggest that heparin or heparan sulfate might act at either external or internal sites in SMC. Recent observations provide evidence that for maximal effect heparin administration in vitro or in vivo must be started before the cells enter S phase and that the heparin block is in G1 (3, 10, 11). Heparin interferes with several G1 functions; in certain kinds of cells, it decreases epidermal growth factor binding and inhibits the expression of several oncogenes including c-myc (12), c-myc and c-fos (13).

One way heparin might affect SMC proliferation, migration, and matrix accumulation is by interfering with either the expression or the activity of extracellular proteases needed for degradation of the surrounding matrix. To test this hypothesis, we have begun to investigate the effect of heparin upon plasminogen activator expression by SMC. The principle function of the two plasminogen activators (PAs), tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), is to convert plasminogen to plasmin (14, 15). Plasmin in turn degrades not only fibrin but also a broad range of matrix molecules; although it does not degrade interstitial collagen, it does activate procollagenase to collagenase (16–18).

In vivo, medial SMC stimulated to proliferate express both uPA and tPA; uPA appears to increase during G1 and tPA later on when the SMC are beginning to migrate from the media to the intima (19). Similar patterns of expression of PAs has been observed in other cell types. For example, uPA expression increases during the pre-replicative period in mouse keratinocytes and 3T3 cells stimulated by epidermal growth factor (20). It also increases in migrating endothelial cells in a wound healing model (21). uPA is mitogenic for some types of cells (22–24). In addition, both uPA and tPA are important for cell migration as shown in studies using appropriate blocking antibodies (25–28).

The possible link between cell proliferation, cell migration, and PAs led us to ask whether heparin has any effect on the expression of tPA and uPA by SMC. In an in vitro mitogenesis model, we studied the effect of heparin on PA expression and DNA synthesis in baboon aortic SMC stimulated with fetal...
bovine serum (FBS). We find that heparin and related molecules inhibit DNA synthesis and the expression of the tPA gene without affecting the expression of several other genes induced by serum.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heparin (isolated from porcine mucosa), dextran sulfate (M, 500000), chondroitin 6-sulfate, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Nonanticoagulant heparin (IC 1772) was obtained from the Institut Choay, Paris. Dermatan sulfate was obtained from ICN (Costa Mesa, CA). Cell culture media were obtained from Sigma unless indicated otherwise. Staurzporine was purchased from Boehringer Mannheim. H7 and H8 were purchased from Seikagaku America Inc. (St. Petersburg, FL). All reagents used for RNA isolation, nuclear run-on transcription assays, and dye-sequencing were of molecular biology grade. Radiosotope were purchased from Du Pont-New England Nuclear.

**Cell Culture**—Baboon aortic smooth muscle cells isolated by the explant method (29) were used between passages 3 and 12. Cells were plated at 20,000/cm² in Dulbecco-Vogt medium containing 5% calf serum-free medium (Dulbecco's modified Eagle's medium/Ham's F-12: 1:1) containing 6 μg of insulin/ml, 5 μg of transferrin/ml, 1 mg of ovalbumin/ml, 200 units of penicillin/ml, 200 μg of streptomycin/ml, and 0.1% sodium pyruvate. The cells had been grown in the serum-free medium 4-7 days at 4°C. The labeling index was determined by counting at least 500 cells well with triplicate wells per treatment condition.

**Northern Analysis**—Total RNA was isolated as described previously (31). Total RNA (15 μg/lane) was electrophoresed in a 1% agarose/formaldehyde gel and then transferred to a nylon membrane (Zeta Probe blotting membrane, Bio-Rad). The membrane was hybridized in a solution (0.15 M NaH2PO4, pH 7.2, 0.28 M NaCl, 7.8% SDS, 1 mM EDTA, 50 mM formamide, 10% polyethylene glycol, and 200 μg/ml of denatured salmon sperm DNA containing 2 × 10^6 cpm/ml of labeled DNA probe. The cDNA probes: tPA (1.6-kb human cDNA) (32), PAI-1 (0.38-kb human cDNA) (gift from Dr. W. E. Holmes, Genentech, Inc., San Francisco), PDGF-A (1.3-kb human cDNA) (34), PDGF α-receptor (2.2-kb human cDNA) (35), PDGF β-receptor (5.57-kb human cDNA) (36), GAPD (1.2-kb human cDNA) (37), c-myc (0.35-kb mouse genomic DNA), GAPD-H (1.3-kb v-fos) (39), c-jun (0.65-kb human cDNA) (40), and actin (1.3-kb bovine cDNA) (41) were labeled by either nick translation or random priming. After hybridization at 42°C for 20-24 h, blots were washed in 2 × SSC, 0.1% SDS at room temperature for 30 min and then in 0.1 × SSC, 0.1% SDS at 65°C for 10-15 min. Signals were detected by autoradiography.

**Nuclear Run-on Transcription Assay**—Cells were washed twice with ice-cold phosphate-buffered saline, scraped, and collected by centrifugation at 4°C. Fresh nuclei isolated from cells that have been lysed in 0.5% Nonidet P-40 were used for the transcription assay (42) with modifications (43). Approximately 5 × 10^6 nuclei were incubated in a 200-μl reaction mixture (25% glycerol, 5 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 150 mM KCl, 1 mM dithiothreitol, 1 unit of RNasain, 300 μM ATP, CTP, and GTP, and 300 μCi of ^32P]UTP (800 Ci/mmol). The reaction mixture was shaken gently at 30°C for 10 min. Nuclei were digested with 100 units of RNase-free DNase I (Phar- macis) in 1 mM CaCl₂ at 30°C for 10 min, and RNA was isolated (31). Equal amounts of radioactivity (10-20 × 10^6 cpm/ml) was added to each filter containing immobilized plasmids (44) and hybridized in solution (10 mM Tris, pH 7.4, 0.2% SDS, 10 mM EDTA, 0.3 M NaCl, 1% Denhardt, 50 μg/ml of tRNA, and 50 μg/ml of denatured salmon sperm DNA) at 65°C for 3-4 days. The filters were washed in 2 × SSC, 0.1% SDS at room temperature for 30 min and then in a 0.1 × SSC, 0.1% SDS at 65°C for 15 min. Signals were detected by autoradiography.

**Isolation and Sequencing a Baboon tPA cDNA Clone**—We isolated a tPA cDNA clone from a baboon aortic SMC λ g11 cDNA library using a human tPA cDNA probe in order to obtain a homologous probe for the nuclear run-on assay. The isolated cDNA clone was subcloned in M13 vector and sequenced by the dye-exchange method (45). The cDNA clone is 1599 base pairs long, it consists of a 21-base 5' noncoding region and a 1578-base pair coding sequence. The deduced baboon tPA amino acid sequence is more than 98% homologous to human tPA. The tPA cDNA clone used for the nuclear run-on assay was a 723-base pair EcoRI restriction fragment containing the 5'-end noncoding region.

**tPA ELISA**—To terminate a nuclear run-on experiment, the medium was removed, centrifuged at 1,600 × g for 5 min and aliquots of the supernatant frozen at -20°C. After washing the cell layer with phosphate-buffered saline, the cells were scraped into extraction buffer (0.1 M Tris, 0.2% Triton X-100, 200 trypan inhibitory units of aprotinin/ml, 10 mM EDTA, 5 mM iodoacetamide, pH 8.1), centrifuged at 14,000 × g for 5 min and aliquots frozen. tPA protein was measured by a double sandwich ELISA (a generous gift from Genentech, Inc.) as described (46). The effect of heparin on the assay was determined by adding heparin during the capture phase of the assay. Heparin (5-50 μg/ml) had no significant effect (>90% recovery) on the assay of single chain tPA (0.05-1.2 ng).

**Statistical Analysis**—Analysis of variance of ELISA results were performed using SPSS/PC+ (SPSS, Inc., Chicago, IL) and BMDP (BMDP Statistical Software, Inc., Los Angeles, CA) and differences tested using Tukey's test.

**RESULTS**

**Effect of Heparin on Mitogenesis**—In an in vitro mitogenesis model, SMC regulation was determined at 28 h after the addition of FBS and heparin. In a total of 16 experiments, the [3H]thymidine labeling index in cells receiving serum-free media was 1.7 ± 0.4% (S.E.). Addition of FBS increased the labeling index to 22.7 ± 3.0% (S.E.) and heparin decreased the labeling index to 12.1 ± 1.8% (S.E.) (p < 0.005, t test). The effect of heparin on the kinetics of SMC entry into S phase was also studied. SMC was given 4-h pulses of [3H]thymidine from 0 to 28 h. SMC entered S phase at 16 h; heparin reduced the number of cells entering S phase without affecting the time of entry (Fig. 1).

**Effect of Heparin on Expression of Genes Induced by FBS**—The addition of FBS to SMC increased the expression of the tPA gene. Significant increases in tPA mRNA were observed at 2 and 4 h (19-fold, mean of 4 experiments) after the addition of FBS, and tPA mRNA remained elevated at 24 h (Fig. 2). FBS had a different effect on the mRNA of uPA (Fig. 2). The results are consistent with the hypothesis that heparin inhibits transcription of the tPA gene.
mRNA level of uPA significantly decreased at 4 h after the addition of FBS and gradually increased back to the basal level by 24 h. Heparin specifically decreased tPA mRNA by 87% ± 6% (mean ± S.E. of 4 experiments) at 4 h and had no significant effect on uPA mRNA in cells stimulated with FBS.

We next examined the effect of FBS and heparin on gene expression of a specific inhibitor to tPA, plasminogen activator inhibitor type 1 (PAI-1). FBS increased PAI-1 mRNA expression of a specific inhibitor to tPA, plasminogen activator inhibitor type 1 (PAI-1). FBS increased PAI-1 mRNA and this increase was not significantly modified by treatment with heparin (less than 50% reduction) (Fig. 2).

Further experiments showed that heparin did not significantly affect the expression of a number of genes that were also modulated by FBS. The mRNAs of the proto-oncogenes, c-fos and c-myc were increased by FBS (Fig. 3a). c-jun expression was also increased by FBS (data not shown). Platelet-derived growth factor (PDGF-A) mRNA was increased by FBS (Fig. 3b), and the PDGF receptor (PDGF-α and PDGF-β subunits) mRNAs were decreased by FBS (Fig. 3c). Heparin alone when added to SMC in serum-free medium had no effect on the expression of genes that we have studied.

**Effect of Heparin on tPA Protein Level**—The changes in tPA mRNA were also reflected in changes in the amount of tPA protein as measured by ELISA. FBS increased cell-associated tPA in the interval 8 to 16 h (Fig. 4), although this was statistically significant only at 16 h. Heparin inhibited this induction. FBS increased tPA in the medium at 16 h 8-fold, and heparin decreased the induction by 60% (1.2 ± 0.5, 10.3 ± 2.4, and 4.2 ± 1.1 ng/mg of cellular protein for control, FBS, and FBS plus heparin, respectively; mean ± S.E., p < 0.05, n = 10). Finally, when heparin was added 1 h before harvest, tPA was decreased only 8% in the cell layer and 0% in the medium compared to a decrease of 99% in the cell layer and 83% in the medium when heparin was added with the FBS at 0 h.

**Effect of Heparin on PMA Induced tPA Gene Expression**—In preliminary experiments we found that inhibitors to protein kinase C blocked the induction of tPA by serum (see, for example, Fig. 8). To explore the possibility that heparin might act on the protein kinase C pathway, we examined the effect of PMA with and without heparin on tPA gene expression in baboon SMC. Others have shown that PMA induces tPA gene expression in human endothelial cells (47), in ovarian carcinoma cell (48), and in HeLa cell (49).

PMA is a weaker mitogen than FBS; it increased the [³H] thymidine labeling index by 2-fold, whereas FBS increased the labeling index 13-fold. Heparin inhibited the PMA-induced labeling index by about 50%. PMA (5 ng/ml), like FBS, induced tPA mRNA but with a slower time course (Fig. 5). tPA mRNA was increased at 8 h and remained elevated at 24 h. The addition of heparin decreased the PMA induction of tPA. However, heparin did not have a significant effect on c-fos or c-jun mRNAs which also were induced by PMA (Fig. 5). Like FBS, PMA increased cell-associated tPA protein and
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Hepran decreased this induction (1.1 ± 0.3, 12.2 ± 3.5, and 5.3 ±2.2 ng/mg for control, PMA and PMA plus heparin, respectively; mean ± S.E., n = 3). Further studies showed that the effect of heparin on tPA mRNA induced by either FBS or PMA was dose dependent. The maximum inhibitory effect was observed at 100 μg/ml of heparin (Fig. 6).

We also determined the effects of FBS and PMA on tPA gene expression in human aortic SMC. The results were similar to those observed in baboon. Both FBS and PMA increased tPA mRNA and heparin decreased this induction (data not shown). These results suggest that the human and baboon tPA genes contain similar regulatory elements.

Induction of tPA mRNA Involves Activation of Protein Kinase C—Many of the effects of PKC are mediated by protein kinase C. Protein kinase C inhibitors such as H7 (50) and staurosporine (51) were used to determine whether the induction of the tPA gene by PMA or FBS might involve the activation of protein kinase C. Results from Northern analysis showed that both H7 and staurosporine decreased tPA mRNA induced by PMA (Fig. 7a). A weak protein kinase C inhibitor, HA1004 (52), was used as a control for H7. HA1004 had little effect on tPA mRNA. However, H7, staurosporine, and HA1004 also inhibit other protein kinases besides protein kinase C. Thus, another approach using pretreatment with PMA to down-regulate the protein kinase C was taken. A second dose of PMA (5 ng/ml) did not increase tPA mRNA in SMC which had been pretreated with either 100 or 200 ng/ml of PMA for 24 h to deplete the protein kinase C activity (Fig. 7b).

Induction of tPA by FBS was also inhibited by H7, but not HA1004 (Fig. 8a). Moreover, 10% FBS did not increase tPA mRNA in SMC which had been pretreated with 100 ng/ml of PMA for 24 h (Fig. 8b). In summary, these results suggest that the induction of tPA mRNA by either PMA or FBS involves the activation of protein kinase C.

Heparin Specificity—Various glycosaminoglycans were used to determine the specificity of the effect of heparin (Fig. 9). Similar results were obtained whether cells were stimulated by either FBS or PMA. Chondroitin sulfate and dermatan sulfate, which did not inhibit mitogenesis, also did not decrease tPA mRNA. However, heparin, IC 1772 (a heparin lacking anticoagulant activity), and dextran sulfate, which significantly decreased mitogenesis, also decreased tPA mRNA.

Effect of Heparin on tPA Transcription—Nuclear run-on
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The results from our studies show that heparin inhibits DNA synthesis in SMC stimulated either by FBS or PMA. We have studied a number of genes that are induced during G1 and among these genes, the induction of tPA mRNA is suppressed by heparin. This inhibitory effect is specific for heparin-like molecules and does not depend on the anticoagulant activity of heparin. Moreover, heparin's inhibitory effect on tPA expression is dose-dependent. Results from nuclear run-on assays demonstrate that the increase in tPA mRNA is due to increased transcription and that heparin blocks this induction. We measured tPA protein by ELISA and found that both FBS and PMA increased cellular tPA protein and that heparin blocks these increases. The fact that tPA transcription and mRNA in the presence of heparin are decreased in parallel with protein suggests that synthesis of tPA is decreased. Whether changes in the rate of degradation of tPA protein occur is not known.

The effects of heparin are unlikely to be attributable to interference with growth factors or other factors in serum, since heparin inhibits FBS-induced tPA expression even if the heparin addition is delayed for 2 h. In addition, PMA induces tPA expression in the absence of serum, and heparin inhibits this induction. However, we cannot rule out the possibilities that heparin binds to an endogenous factor synthesized by the cell or interacts with an extracellular matrix component to activate a cellular response which inhibits tPA gene expression. In fact, extracellular matrix components such as thrombospondin may play a role in the mediation of heparin's inhibitory effect. It has been suggested that heparin inhibits SMC proliferation by inhibiting the incorporation of

thrombospordin into cell-associated matrix (53). In addition, thrombospordin stimulates adhesion and migration of endothelial cells, and these thrombospordin-induced effects are inhibited by heparin (54). Since heparin has been shown to bind to the cell surface and to be internalized (8), heparin might also act internally to inhibit tPA expression. At present we have no evidence to show whether heparin acts extracellularly or intracellularly to inhibit tPA gene expression.

To understand how heparin inhibits tPA expression, we have begun to examine various mechanisms of tPA gene induction. It is possible that the induction of tPA gene expression by either PMA or FBS involves the activation of protein kinase C. The increase in tPA mRNA in response to PMA or FBS is blocked by staurosporine and H7, but not by HA1004. These agents are not specific inhibitors of protein kinase C. However, since the increase in tPA mRNA is also blocked by pretreatment with PMA, these results together provide support for the conclusion that tPA induction by FBS and PMA is mediated by protein kinase C activation. Similar observations have been made in human umbilical endothelial cells (47) and in ovarian carcinoma cells (48).

A number of studies have described how PMA alters gene transcription at the molecular level. In particular, the induction of the collagenase gene by PMA has been studied (55–58). Recent studies from our laboratory show that collagenase is induced in baboon SMC by PMA, and the PMA induction of this gene is blocked by heparin (58). Hence, the collagenase induction pathway may provide a model system for the analysis of PMA induction.

A phorbol ester-responsive element (TRE) has been identified in the promoter region of the human collagenase gene (55). This TRE is the binding site for a trans-acting factor, AP-1 (activating protein 1). AP-1 is a family of proteins and the particular AP-1 involved is a product of c-jun, referred to as JUN/AP-1 (59, 60). JUN/AP-1 forms a heterodimer with FOS (a product of c-fos), binds to the TRE site, and activates collagenase gene transcription (56, 57, 61). AP-1 itself is upregulated by PMA (62, 63). Using a mobility-shift assay we have shown that both PMA and FBS increase AP-1 binding activity in baboon SMC, and that heparin decreases the induced activity.

Since a TRE site has also been identified in the human tPA gene (64), it is reasonable to assume that the tPA and collagenase genes are regulated by a similar mechanism. Although the genomic sequence of baboon tPA is not known, we suspect human and baboon tPA genes contain similar regulatory elements since PMA induces tPA expression and heparin inhibits its induction in both cell types. In summary, our observations that both tPA and collagenase mRNA and AP-1 activity are induced by either PMA or FBS and are all decreased by heparin point to the involvement of AP-1/TRE binding in the mediation of heparin’s effect on tPA and collagenase gene expression. In fact, it is possible that a number of proteases might be regulated by this pathway. It is of interest that a protein immunologically related to major excreted protein, a cathepsin-like molecule inducible by PMA, is expressed by SMC and its secretion is blocked by heparin (35).

Our conclusion that heparin inhibits the protein kinase C-dependent pathway also agree with the experiments of Castellot et al. (11) and Wright et al. (13). In the studies of Wright et al. using 3T3 fibroblasts, PMA induced c-myc and c-fos mRNAs, and heparin blocked this induction. However, heparin did not affect the protein kinase C activity or the phosphorylation of an 80-kDa protein, which is a protein kinase C substrate. Therefore, it was concluded that the heparin block is somewhere distal to the activation of protein kinase C. In our system using baboon SMC, heparin does not affect the expression of c-myc, c-fos, or c-jun induced by either PMA or FBS. The differences in the two studies may be due to using SMC from different species or to using pretreatment of cells with higher doses of heparin.

Is tPA expression necessary for SMC proliferation and migration? Studies by others using antibodies to tPA have shown that tPA expression is important for cell migration through amnion (28). We have tried to block baboon SMC proliferation in an in vitro mitogenesis model, or SMC migration in a wounding model using the antibody to human tPA. However, preliminary results have been negative. We are not sure whether this is because tPA is not accessible to the antibody, or whether tPA expression is not necessary for cell proliferation or migration in a two-dimensional system such as in a tissue culture dish. We are currently investigating the importance of tPA expression in SMC seeded in a three-dimensional matrix gel.

In summary, several intriguing results have emerged from the present study. We have studied a number of genes that are modulated during the G1 phase of the cell cycle and found that tPA is selectively inhibited by heparin. The inhibition of tPA expression may be one of the ways heparin inhibits cell proliferation and migration. By examining how the tPA gene is regulated, we may be able to establish the mechanism of heparin’s action on proliferation and migration.

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REFERENCES
