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HEPARIN AND DIBUTYRYL cAMP MODULATE GENE EXPRESSION IN STIMULATED HUMAN SAPHENOUS VEIN SMOOTH MUSCLE CELLS

MASAHIKO YAMAGUCHI, SCOTT DIAMOND[1], HIROYUKI WATANABE, HARALD GALLATI, WENDY BAUR, AND JOHN B. SHAREFKIN

Department of Surgery, New England Medical Center Hospitals, Boston, Massachusetts (M. Y., W. B., J. B. S.); Department of Chemical Engineering, State University of New York, 907 Furnas Hall, Buffalo, New York 14260 (S. D.); Research and Development Division, Takeda Chemical Ind., Ltd., Osaka, Japan (H. W.); and Pharma Division, F. Hoffmann-La Roche Ltd., Basel, Switzerland (H. G.)

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Summary

Increased expression of basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) A chain, and tissue plasminogen activator (tPA) by smooth muscle cells (SMC) has been postulated to mediate the progression of intimal hyperplasia. We tested whether heparin would suppress the expression of these genes in stimulated human saphenous vein SMC. Quiescent cultured human saphenous vein SMC were stimulated for 4 h with heat-inactivated fetal bovine serum (10% by vol) in the presence or absence of heparin (1 to 250 μg/ml). Heparin (50 μg/ml) attenuated the induction by serum of bFGF mRNA, tPA mRNA, and tPA secretion. Nonanticoagulant heparin also attenuated serum induction of bFGF and tPA mRNA levels. To further study the role of second messenger signaling, a more specific mode of SMC stimulation was used with thrombin (3 U/ml) in the presence or absence of dibutyryl cyclic AMP (Bu_2-cAMP; 0.5 m*M*). In contrast to heparin, which had no effect on PDGF expression, Bu_2-cAMP decreased the induction by thrombin of PDGF-A chain mRNA levels. In thrombin-stimulated SMC, Bu_2-cAMP significantly decreased secretion of PDGF-AA protein. Thrombin, however, caused an increase in bFGF mRNA levels which was potentiated by Bu_2-cAMP with associated potentiation by Bu_2-cAMP of intracellular bFGF protein levels. The induction of tPA mRNA and tPA secretion by thrombin was sharply blocked by Bu_2-cAMP. These results suggest that heparin reduces intimal hyperplasia at least partly via partial inhibition of SMC gene expression.

Key words: heparin; cAMP; smooth muscle cell; tPA; PDGF; basic FGF.

Introduction

Surgical reconstructions of diseased vasculature often promote intimal hyperplasia as a result of the wound response of the repaired vessel. Excessive intimal hyperplasia can lead to stenosis and is due to excess smooth muscle cell (SMC) proliferation and migration into the intima of the vessel. Adherent monocytes, aggregated platelets, and activated vascular wall cells can all release growth factors that stimulate SMC proliferation and migration. Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and tissue plasminogen activator (tPA) produced by SMC themselves may also play an important role in intimal hyperplasia.

The effects of PDGF on SMC include mitogenesis, chemotaxis, and induction of collagen secretion (27). PDGF-A chain mRNA is highly expressed in neointimal tissue whereas the expression of PDGF-B chain is not changed (16,22). PDGF may play a major role in the development of intimal hyperplasia because anti-PDGF antibody has been shown to inhibit the development of intimal hyperplasia after angioplasty (14). Wounded vascular cells can also release bFGF which is a potent broad spectrum mitogen that induces cell migration and angiogenesis (18). Infusion of bFGF into the vascular wall provokes SMC proliferation and the formation of new capillaries (10). Additionally, anti-bFGF antibody has been shown to inhibit SMC proliferation after vascular injury (20). In the media of injured rat artery, tPA is found at high levels and probably plays a role in SMC migration from the media to the intima via plasmin-mediated matrix degradation (6). Thus, tPA may also play a role in SMC migration during the initial invasion process of intimal hyperplasia.

Several agents have shown some efficacy in preventing intimal hyperplasia. Heparin inhibits SMC proliferation (4) and migration (21) in vitro and decreases intimal hyperplasia in vivo (7,17) but its mechanism of inhibitory action is not fully understood. Agents that increase intracellular cAMP are known to suppress expression of the gene for PDGF-A chain in endothelial cells and SMC (34). In this study, we tested the effects of heparin on PDGF-A chain, bFGF, and tPA gene expression in human saphenous vein SMC to investigate some of these mechanisms of intimal hyperplasia inhibition by heparin. We also tested the effect of elevated intracellular cAMP levels in human SMC on the expression of these genes.

Materials and Methods

Cell culture. Adult human SMC were obtained by outgrowth from explants taken from the media of the saphenous vein segment discarded after

[1] To whom correspondence should be addressed.

coronary artery bypass operations (34). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mg/ml Fungizone, and 100 mg/ml L-glutamine. SMC were studied in Passage 2 to 8. Before the experiments, confluent SMC were incubated in DMEM with 0.5% FBS for 72 h to obtain a quiescent state as verified by bromodeoxyuridine staining (Amersham, Arlington Heights, IL). The identity of these cultures as SMC was verified by their morphology and by immunohistologic staining with a monoclonal antibody specific for SMC α-actin (30).

Reagents used. Porcine intestinal heparin (Sigma, St. Louis, MO) was added to the culture media 30 min before 10% FBS stimulation. Nonanticoagulant heparin, purified by affinity chromatography of heparin on antithrombin III-sepharose, was a gift from Kabi Pharmacia AB in Sweden. Dibutyryl cAMP (Bu_2-cAMP) (Sigma) was used at a final concentration of 0.5 m*M* in the culture medium. Thrombin was a generous gift from Dr. J. W. Fenton (Wadsworth Center for Laboratories and Research, New York State Dept. of Health, Albany, NY) and was added to culture media at final concentrations of 3 U/ml as a stimulant.

RNA extraction. After stimulation with 10% FBS or 3 U/ml α-thrombin for 4 h, total cellular RNA was extracted from cells in replicate 75-cm^2 flasks using a guanidinium lysis/CsCl gradient ultracentrifugation method (5). Briefly, culture media were removed from the flasks and the SMC layers were each rinsed 3 times with ice-cold phosphate buffer saline (PBS) and lysed in 2.2 ml of guanidinium lysis solution (4 *M* guanidine isothiocyanate, 25 m*M* sodium citrate (pH 7.0), 0.5% *N*-Lauroyl sarcosine, and 0.14 *M* 2-β-mercaptoethanol). The lysates were sheared using 25-gauge needles, layered on a 2.5-ml cushion of 5.7 *M* CsCl containing 0.1 *M* EDTA, and ultracentrifuged at 35 000 rpm at 20° C for 18 h. After centrifugation, RNA pellets were dissolved in 0.1% sodium dodecyl sulfate (SDS)/TE (10 m*M* Tris, 1 m*M* EDTA) solution at 55° C and purified using a sodium acetate/ethanol precipitation method.

Analysis of mRNA by Northern blot. Total RNA (20 μg) prepared from each SMC culture was separated by electrophoresis on a 1% agarose gel containing 1.1% formaldehyde in 1× 3-(N-morpholino)propanesulfonic acid buffer. The RNA was transferred overnight onto 0.45-μm pore nylon (Nytran Schleicher & Schuell) in 20× SSC (3 *M* NaCl, 0.3 *M* Na citrate). The nylon blot was crosslinked by UV irradiation and vacuum baked for 2 h at 80° C. For the Northern hybridizations, the restriction fragments used and the source of human DNA probes were as follows: a) PDGF-A chain, 1.3 kbp EcoRI fragment of pUC-13, gift of Dr. S. Aaronson, National Cancer Institute, Bethesda, MD; b) bFGF, 670 bp cDNA clone from AIDS-KS3 cells, a gift of Dr. B. Ensoli, National Cancer Institute, Bethesda, MD; c) tPA, 1.77 kbp XbaI/BamHI fragment of pdRIPAo; American Type Culture Collection, Rockville, MD (ATCC) no. 40400; d) GAPDH, 0.8 kbp PstI/XbaI fragment of pHcGAP; ATCC no. 57091. The probes were labeled with α-^{32}-P dCTP (3000 Ci/mmol) using a random-primed labeling kit (Oligolabeling kit, Pharmacia). Blotted membranes were hybridized at 42° C overnight with a labeled probe (2×10^6 dpm/ml) in 4× SSPE (720 m*M* NaCl, 40 m*M* Na_2HPO_4, 4 m*M* EDTA), 40% deionized formamide, 4× Denhardts, 0.08% SDS, salmon sperm DNA (100 μg/ml), and 10% dextran sulfate. Hybridized membranes were washed twice in 2× SSC/0.1% SDS at room temperature for 15 min and twice in 0.1× SSC/0.1% SDS at 65° C for 30 min. Exposure of the hybridized membranes to Kodak XAR and/or XRP X-ray film was performed at −70° C with intensifying screens for 1 to 3 days. Blotted membranes were successively rehybridized with other cDNA-labeled probes after stripping the previous hybridized probe by 5 min boiling in 10 m*M* Tris-HCl (pH 7.5)/0.1% SDS. Only the major bands (2.8 kb for PDGF-A chain mRNA and 7 kb for bFGF mRNA) were scanned by densitometry for comparison purposes. Two-dimensional area scanning was used for densitometric analysis. Signal strengths from autoradiographs were measured using an LKB Ultroscan laser densitometer and expressed as a ratio of certain gene signal to constitutive glyceraldehyde 3-phosphate degydrogenase signal calculated by the equation:

$$\text{mRNA Index} = \frac{\text{gene (treatment)/GAPDH (treatment)}}{\text{gene (0.5\% FBS)/GAPDH (0.5\% FBS)}}$$

PDGF-AA ELISA. SMC-conditioned media in replicate wells of 10 cm^2 were obtained 24 h after 10% FBS addition and stored at −70° C until the assay. Ninety-six-well, flat-bottom polystyrene microtiter plates were coated with 200 μl/well of a mouse anti-human PDGF-A monoclonal antibody (clone pfa-5; Dr. H. Gallati, Hoffmann-La Roche) at 5 μg/ml in 100 m*M* $NaHCO_3$ (pH 9.5) at room temperature overnight. Plates were washed 3 times with H_2O and then blocked with 200 m*M* Tris-HCl (pH 7.5), 1% bovine serum albumin, and 0.025% thimerosal for 24 h at room temperature. After removal of the blocking buffer, conditioned media samples and human PDGF-AA standards were titrated in doubling dilutions in a final volume of 200 μl/well in 250 m*M* phosphate buffer (pH 8.0) containing 0.5% bovine serum albumin. To each well, 50 μl of the mouse anti-human PDGF-A antibody (clone pfa-5) conjugated with horseradish peroxidase (HRP) (HRP obtained from Boehringer Mannheim, Indianapolis, IN) were added and incubated overnight at room temperature. The PDGF-AA homodimer has two identical epitopes which allows the use of clone pfa-5 for both antigen capture and detection. After washing the plate, the bound HRP was assayed with substrate buffer (200 μl of 2.5 m*M* H_2O_2 with 1 m*M* 3,3′,5,5′-tetramethylbenzidin in 30 m*M* citrate buffer, pH 4.1) for 10 min before the reaction was stopped with 100 μl of 1 *M* H_2SO_4. The color product was measured at 450 nm.

Measurement of cellular bFGF levels. After incubation with 10% FBS for 24 h, SMC in replicate wells of 10 cm^2 were washed 3 times with ice-cold PBS and disrupted by three freeze/thaw cycles in 1 ml of 10 m*M* Tris-HCl (pH 7.5) and 1.5 *M* NaCl supplemented with 10 mg/ml leupeptin, 20 mg/ml aprotinin, 2 m*M* benzamidin, 1 m*M* EDTA, and 0.2 m*M* phenylmethylsulfonyl fluoride. Cell lysates were collected in Eppendorf tubes and sonicated at 4° C. After spinning at 15 000 rpm at 4° C for 10 min, supernatants were transferred to new tubes and stored at −70° C until the assay. Intracellular bFGF levels were measured by enzyme immunoassay as previously described (33). Briefly, a 96-well microtiter plate was coated with 100 μl/well of anti-human bFGF monoclonal antibody (Takeda Chemical Ind., Ltd. Osaka, Japan) at 10 μg/ml in 100 m*M* $NaHCO_3$ (pH 9.6) at 4° C overnight. The plate was washed with PBS and blocked with 300 ml of Buffer A [PBS containing 25% Block Ace (Snow Brand Milk Products Co., Japan)] at 4° C overnight. After washing with PBS, samples and standard human bFGF (Takeda Chemical Ind., Ltd. Osaka, Japan) diluted in Buffer B (Buffer A containing 100 μg/ml heparin) were added at a final volume of 100 μl/well and incubated at 4° C for 24 h. To each well, 100 μl of anti-human bFGF antibody conjugated with HRP (Takeda Chemical Ind., Ltd.) were added and incubated at 25° C for 2 h. The plate was washed and the bound peroxidase activity was measured using an *o*-phenylenediamine substrate.

tPA enzyme-linked immunoassay (ELISA). SMC conditioned media in replicate wells of 10 cm^2 were obtained at 24 h after 10% FBS addition and stored at −70° C until the assay. A double antibody ELISA technique was used to measure total human tPA in SMC conditioned media (American Diagnostica Imubind-5 tPA ELISA) as previously described (12). The kit was recalibrated (0 to 5000 pg/ml) with a detection limit of 50 pg/ml. SMC-conditioned media (undiluted, 100 μl addition per well) or calibration standards (Bowes melanoma single chain tPA) were assayed in triplicate with background subtraction using blanking wells containing soluble antibody to quench tPA specific response. HRP-conjugated goat anti-human tPA IgG and *o*-phenylenediamine provided a colorimetric reaction product with an absorbance at 490 nm.

Results

Bromodeoxyuridine staining demonstrated that incubation of SMC in 0.5% FBS for 72 h caused near quiescence in the monolayers. After 72 h of serum starvation, only 2.1 ± 0.8% of the monolayer was detected to be in S phase (n = 6). Incubation of these quiescent monolayers with 10% FBS for 24 h caused a sharp increase in S phase cells to 25.7 ± 5.0% of the population (n = 6), indicating that the cells were indeed viable and responsive to FBS.

We tested the effect of porcine intestinal heparin on PDGF-A chain, bFGF, and tPA mRNA levels in serum-stimulated SMC. Using RNA isolated from control and stimulated SMC, we found that the PDGF-A chain cDNA probe hybridized to three mRNA species of 1.4, 2.2, and 2.8 kb as previously reported (9) (Fig. 1). The bFGF cDNA hybridized to four mRNA species of 1.4, 2.2, 3.7, and 7.0 kb as reported (13) and the tPA cDNA hybridized to an mRNA

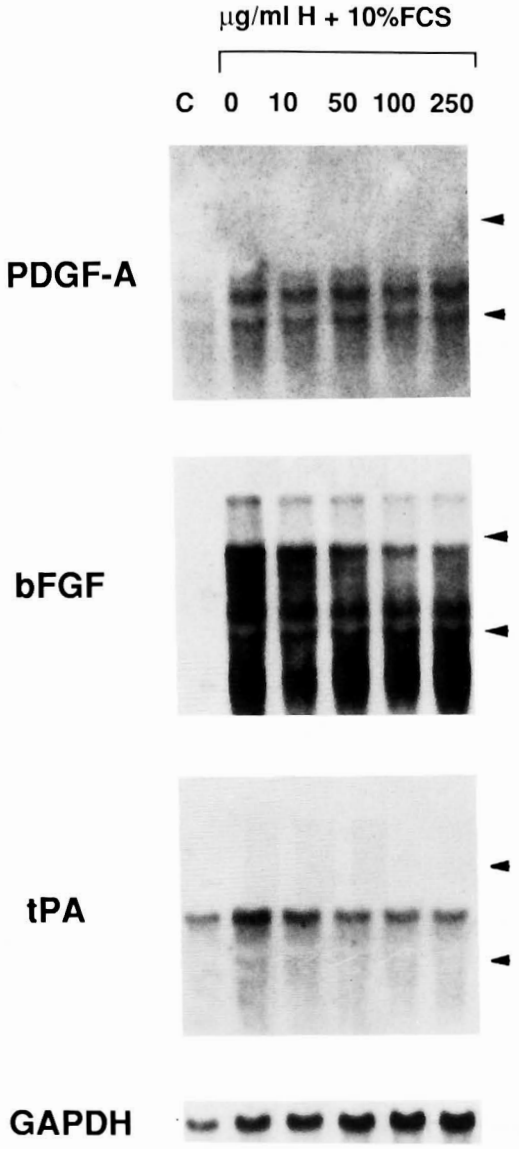

FIG. 1. Autoradiograph of Northern blot hybridization with the cDNA probes (*left*) to evaluate the effect of various doses of porcine intestinal heparin (*H*) on PDGF-A chain, bFGF, tPA, and GAPDH mRNA levels in human saphenous vein SMC stimulated by 10% FBS for 4 h. Control cultures (*C*) were incubated in DMEM with 0.5% FBS before RNA isolation. *Arrows* indicate the positions of 28S and 18S ribosomal RNA.

of 2.7 kb (Fig. 1). Porcine intestinal heparin did not significantly block the large induction of PDGF-A chain mRNA levels by serum at all concentrations tested (Fig. 1). The 2.5-fold increase in the PDGF-A chain mRNA index by 10% FBS addition was not significantly decreased by 50 μg/ml heparin addition (Table 1). However, the large 8.3-fold induction of the bFGF mRNA index by 10% FBS was significantly attenuated by heparin. At a concentration of 50 μg/ml heparin, the stimulation of the bFGF mRNA level was reduced by about 50% (Table 1). Heparin at higher doses caused a dose dependent decrease in the mRNA levels for the higher molecular size species of bFGF. However, lower molecular size bFGF transcripts do not show any decrease in response to heparin treatment. The tPA mRNA index, elevated by stimulation with 10% FBS addition, was also significantly reduced by heparin in a dose-dependent manner (Fig. 1 and Table 1). In similar experiments, we used nonanticoagulant heparin reduced the serum-stimulation of bFGF and tPA mRNA levels.

TABLE 1

EFFECT OF PORCINE INTESTINAL HEPARIN (50 μg/ml) ON PDGF-A mRNA, bGGF mRNA, AND tPA mRNA LEVELS IN HUMAN SAPHENOUS VEIN SMC STIMULATED BY 10% FBS FOR 4 h. THE mRNA CONTENT WAS EXPRESSED AS MEAN ± SD (n = 5) USING THE mRNA INDEX AS DEFINED IN MATERIALS AND METHODS

mRNA Species	0.5% FBS Control	10% FBS Stimulation	10% FBS + 50 μg/ml Heparin
PDGF-A chain	1.0	2.5 ± 1.2	2.1 ± 1.0
bFGF	1.0	8.3 ± 3.1	4.2 ± 1.2[a]
tPA	1.0	1.8 ± 0.4	1.0 ± 0.4[a]

[a] $P < 0.03$ in heparin-treated cultures compared to 10% FBS stimulated cultures.

Inasmuch as heparin and nonanticoagulant heparin blunted the induction of the bFGF mRNA and tPA mRNA levels in serum-stimulated cells, we investigated SMC secretion of PDGF-AA and tPA and intracellular levels of bFGF protein. Secreted PDGF-AA levels in media were increased by 10% FBS stimulation, but were not significantly decreased by heparin (Table 2). We have found that heparin up to concentrations of 100 μg/ml had no significant effect on the secretion of PDGF-AA by serum-stimulated SMC. Levels of intracellular bFGF protein in cell extracts were increased by 10% FBS addition but were not significantly decreased by 50 μg/ml heparin (Table 2). This was unexpected given the significant reductions in bFGF mRNA at 4 caused by heparin. Human donor variability and differing autoradiography exposure times (Fig. 1 and 2) could account for the apparent variability in basal bFGF mRNA levels. SMC secretion of tPA was stimulated by 10% FBS addition and was significantly decreased by heparin (Table 2). Concentration

TABLE 2

EFFECTS OF PORCINE INTESTINAL HEPARIN ON SECRETED PDGF-AA, SECRETED tPA, AND INTRACELLULAR bFGF ($[bGFG]_i$) LEVELS[a]

Protein	0.5% FBS Control	10% FBS Stimulated	10% FBS + Heparin
PDGF-AA	0	0.065 ± 0.017	0.042 ± 0.036[b]
$[bFGF]_i$	4.50 ± 0.57	8.22 ± 2.90	6.98 ± 1.62[c]
tPA	0.03 ± 0.03	0.62 ± 0.03	0.35 ± 0.03[b,d]

[a] Values in ng/ml are expressed as mean ± SD (n = 3). All serum-stimulated cultures had significantly elevated $[bFGF]_i$ levels and secreted significantly ($P < 0.005$) more PDGF-AA and tPA than matched 0.5% FBS controls. Conditiond media (4 ml/10 cm^2 SMC) were collected after 24 h incubation. Cellular lysates (10 cm^2 of SMC lyzed in 1 ml) were obtained after 24-h incubations and then were assayed for intracellular bFGF content. Statistical significance was analysed by Student's t test.

[b] $P < 0.03$ in heparin-treated compared to 10% FBS stimulation. [c] 10 μg/ml heparin pretreatment. [d] 50 μg/ml heparin pretreatment.

of heparin from 1 to 100 μg/ml heparin reduced in a dose-dependent fashion the induction of tPA secretion by serum (n = 3) to levels that were 50% of the serum-induced level of secretion in the absence of heparin.

Because heparin had little effect in blocking the induction of PDGF-A chain mRNA in serum-stimulated SMC, we sought other pharmacologic routes to reduce the expression of this potent mitogen. We evaluated the role of elevated cAMP on gene expression in human SMC using Bu_2-cAMP (0.5 m*M*). As a more specific mode of stimulation in these experiments, thrombin (3 U/ml) was used to stimulate the cells instead 10% FBS. With respect to the genes studied in this work, we found that thrombin (3 U/ml) induced a pattern of gene expression quite similar to that produced by 10% serum. The elevation of cAMP by Bu_2-cAMP strongly attenuated the enhancement of PDGF-A chain mRNA in thrombin-stimulated cells (Fig. 2). In fact, PDGF-AA homodimers could not be detected in the media in thrombin-stimulated SMC pretreated with Bu_2-cAMP (Table 3). Interestingly, the large increase in PDGF-A chain mRNA levels induced by thrombin alone after 4 h was not accompanied by any large change in PDGF-AA protein production over 24 h. However, the combination of thrombin and Bu_2-cAMP caused a large induction of the bFGF mRNA to levels greater than thrombin alone (Fig. 2) with a slight enhancement of intracellular bFGF protein levels. Thrombin-induced tPA mRNA levels and tPA secretion were both significantly decreased by over 50% when thrombin-stimulated cells were pretreated with Bu_2-cAMP (Table 3 and Fig. 2).

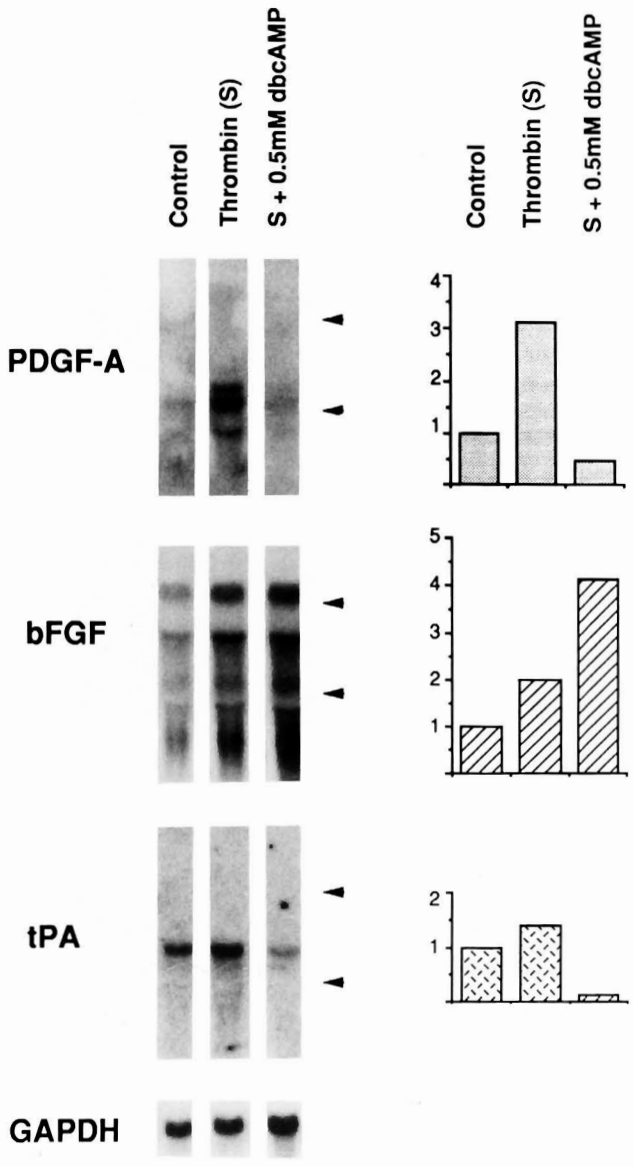

FIG. 2. Autoradiograph of Northern blot hybridization with the cDNA probes (*left*) to evaluate the effect of dibutyryl cAMP (dbcAMP) on PDGF-A chain, bFGF, tPA, and GAPDH mRNA levels in human saphenous vein SMC stimulated by 3 U/ml α-thrombin (*S*) for 4 h. *Arrows* indicate the positions of 28S and 18S ribosomal RNA. Control cultures was incubated in DMEM + 0.5% FBS. Similar results were observed in three other experiments. Average mRNA index for n = 4 is given on the *right* of each blot.

DISCUSSION

We have shown that heparin reduced bFGF mRNA, tPA mRNA, and tPA secretion levels in human saphenous vein SMC stimulated with 10% FBS. Nonanticoagulant heparin had the same inhibitory effect on mRNA levels of bFGF and tPA. These results suggest that heparin may reduce intimal hyperplasia at least partly via inhibition of SMC bFGF and tPA gene expression. In our system, heparin had little regulatory effect on induction of the PDGF gene by either serum or thrombin.

Although thrombin caused a significant enhancement of PDGF-A chain mRNA levels after 4 h of stimulation, there was no corresponding large increase in overall PDGF-AA protein production (Fig. 2 and Table 3) over 24 h. The cells appear to regulate at near constant levels the overall production of PDGF-AA protein over 24 h in spite of the large changes in mRNA levels in great excess of basal levels. The cAMP-mediated block of PDGF-A mRNA induction by thrombin is quite strong. However, the complete absence of PDGF-AA protein secretion by these cells (Table 3) may be due to a posttranscriptional effect mediated by cAMP since PDGF-A mRNA is still present, albeit as slightly lower than basal levels (Fig. 2 *top panel, third lane*). This potential mode of regulation for PDGF-A gene expression—prevention of excess secretion in spite of high mRNA levels and blockade of secretion by elevated cAMP in spite of existing levels of mRNA—was not found for tPA gene expression in SMC. In contrast, changes in tPA mRNA levels closely correlated with changes in tPA protein production. Interestingly, large changes in bFGF mRNA levels (either up or down) at 4 h do not cause correspondingly large changes in intracellular bFGF protein levels after 24 h. Thus, regulatory mechanisms may exist to control the intracellular concentration of bFGF at nearly constant levels. Alternatively, the changes in mRNA levels that we observed may be too short-lived to cause significant changes in protein production. Further studies of the complete time course of mRNA and protein production dynamics are needed to fully elucidate these potential regulatory mechanisms.

Exogenous bFGF is a potent broad spectrum mitogen and induces cell migration or invasion (32) and angiogenesis. However, the function of endogenous bFGF is not fully understood, because bFGF lacks a signal peptide sequence and cannot be secreted. Some evidence would suggest that bFGF can be released to the basement membrane and cell surface through an unknown mechanism (11). Cells transfected with bFGF cDNA display an enhanced

TABLE 3

EFFECTS OF 0.5 m*M* bu_2-cAMP ON SECRETED PDGF-AA, SECRETED tPA, AND INTRACELLULAR bFGF LEVELS IN HUMAN SMCs STIMULATED BY 3U/ml α-THROMBIN FOR 24 h ($n = 3$)[a]

Protein	0.5% FBS Control	0.5% FBS + 3 U/ml Thrombin	0.5% FBS + 3 U/ml Thrombin + 0.5 m*M* bu_2-cAMP[b]
PDGF-AA	0.083 ± 0.038	0.075 ± 0.031	not detected ($P < 0.02$)
$[bFGF]_i$	6.80 ± 2.1	6.6 ± 1.2	8.7 ± 1.2 ($P < 0.05$)
tPA	14.4 ± 0.8	43.8 ± 8.5	14.7 ± 4.2 ($P < 0.01$)

[a] Values in ng/ml are expressed as means ± SD. Conditioned media (4 ml/10 cm^2 SMC) were collected after 24 h incubation. Cellular lysates (10 cm^2 of SMC lyzed in 1 ml) were obtained after 24-h incubations and then were assayed for intracellular bFGF content. Statistical significance was analysed by Student's *t* test.

[a] *P* values for comparison to stimulated cultures.

proliferation rate, higher saturation density, and autonomous cell growth (26,35) indicating that increased levels of endogenous bFGF can affect cell growth. Additionally, anti-bFGF antibody can inhibit cell migration and invasion (24,25,28). These studies suggest that bFGF may act to stimulate the cells in an autocrine fashion. We found that heparin had little effect on intracellular bFGF protein levels after 24 h of serum-stimulation, whereas the same dose of heparin could significantly attenuate the serum-induced increase of bFGF mRNA levels after 4 h of stimulation. It is possible that posttranscriptional regulation might work to keep constant levels of intracellular bFGF over long times in spite of changing bFGF mRNA levels at short times. This level of regulation is consistent with our finding that large increases of bFGF mRNA caused by thrombin and Bu_2-cAMP were accompanied by only slight increases in bFGF protein levels.

Recent studies (1,2) using baboon SMC have shown that heparin specifically decreases 10% FBS-induced tPA and collagenase mRNA levels while having little effect on the induced mRNA levels of plasminogen activator inhibitor type 1 and urokinase. Our results using adult human saphenous vein SMC indicate that the inhibitory effects of heparin on tPA mRNA and tPA secretion are present in cultured human cells. SMC in balloon-injured artery contain increased levels of tPA at a time when the cells are beginning to migrate from the media to the intima (6). Thus, tPA may be required for SMC migration in initial process of intimal hyperplasia. Although heparin reduced tPA levels in stimulated SMC, therapy using heparin against intimal hyperplasia should be combined with other drugs that reduce the expression of PDGF-A by SMC.

The inhibition of intimal hyperplasia by heparin does not depend on its anticoagulant activity because heparin fractions which do not bind antithrombin III are as effective as unfractionated heparin (17). This effectiveness of nonanticoagulant heparin is consistent with our results which demonstrated that nonanticoagulant heparin decreased serum-stimulated bFGF and tPA mRNA levels in a manner similar to porcine intestinal heparin.

Published data (14,22) indicate that PDGF A chain mRNA is more important than PDGF B chain in the development of intimal hyperplasia. Thus, agents that suppress PDGF-A chain expression by SMC might help prevent intimal hyperplasia. Our experiments using Bu_2-cAMP are consistent with reports (19,31,34) that show that increased cAMP levels lead to suppression of PDGF-A chain mRNA levels and PDGF-like protein levels. In our studies with thrombin-stimulated SMC, elevation of cAMP levels caused a reduction in tPA gene expression. Interestingly, elevated cAMP levels in combination with protein kinase C activators in cultured human endothelial cells cause a marked induction of tPA expression (19). Elevated cAMP levels alone tend to reduce tPA expression by endothelial cells (15,19). Inasmuch as thrombin probably activates protein kinase C in SMC (23), our data suggest that the tPA gene is regulated in a cell-specific manner with respect to agonist response in the presence of elevated cAMP.

Recently, studies of combination therapy with heparin and other drugs, such as angiotensin converting enzyme inhibitor or steroids, have been reported to be more effective than either drug alone in the control of intimal hyperplasia (3,8). We have shown that heparin does not decrease PDGF-A chain gene expression in serum-stimulated SMC. Due to lack of heparin effect on PDGF A chain gene expression, optimal anti-intimal hyperplasia therapy may require combining heparinlike drugs with agents to inhibit PDGF-A chain gene expression (possibly phosphodiesterase inhibitors) or PDGF action on SMC.

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