Platelet-derived Growth Factor and Heparin-like Glycosaminoglycans Regulate Thrombospondin Synthesis and Deposition in the Matrix by Smooth Muscle Cells

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ABSTRACT Platelet-derived growth factor (PDGF), a smooth muscle cell (SMC) mitogen, and heparin-like glycosaminoglycans, known inhibitors of SMC growth and migration, were found to regulate thrombospondin synthesis and matrix deposition by cultured rat aortic SMC. The synthesis and distribution of thrombospondin was examined in growth-arrested SMCs, in PDGF-stimulated SMCs, and in heparin-treated SMCs using metabolic labeling and immunofluorescence techniques. Thrombospondin synthesis in response to purified PDGF occurred within 1 h after addition of growth factor to growth-arrested SMCs, peaked at 2 h, and returned to baseline levels by 5 h. The induction of synthesis of thrombospondin by PDGF was dose dependent, with a maximal effect observed at 2.5 ng/ml. Actinomycin D (2 μg/ml) inhibited thrombospondin induction by PDGF, suggesting a requirement for new RNA synthesis. In the presence of heparin and related polyanions, the incorporation of thrombospondin into the SMC extracellular matrix was markedly reduced. This effect was dose dependent with a maximal effect observed at a heparin concentration of 1 μg/ml. Heparin did not affect the ability of SMCs to synthesize thrombospondin in response to PDGF. We interpret these data to suggest a role for thrombospondin in the SMC proliferative response to PDGF and in the regulation of SMC growth and migration by glycosaminoglycans.

Proliferation of vascular smooth muscle cells (SMCs)1 is an important component of the atherosclerotic process. Accordingly, we have begun studies aimed at achieving an understanding of the molecular basis of SMC growth regulation, with emphasis on the role played by extracellular matrix (ECM) molecules. The vessel wall contains sources of molecules stimulatory to SMCs, such as platelet-derived growth factor (PDGF) (69), as well as sources of inhibitory heparin-like glycosaminoglycans (GAGs) (3, 4, 7, 20). Current concepts of SMC growth regulation in vivo invoke an antagonistic interplay between growth inhibitors and stimulators which may determine the extent of SMC response following arterial injury (4).

Heparan sulfate glycosaminoglycans are biosynthetic products of both vascular endothelial cells (21, 61) and SMCs (21, 48, 80), and belong to a heterogenous family of polyanions which can vary greatly in size, charge density, and sulfation (15). Clowes and Karnovsky (6) demonstrated that heparin was a potent inhibitor of neointimal SMC growth following experimental arterial injury in the rat; the effect was not related to the anticoagulant activity of heparin (24). The ability of heparin to regulate SMC growth was subsequently verified in vitro by several laboratories (3, 4, 26, 28, 58).

Castellot et al. (3, 4) have shown that conditioned medium from cultured endothelium contained a heparinase-degradable activity which was inhibitory to SMC growth in vitro. The serum component believed responsible for release of this inhibitory activity has been identified as a platelet heparitinase (62), an enzyme capable of releasing free heparan sulfate GAGs from proteoglycans (60, 62). Thus free heparin-like GAGs may be present locally in the vascular wall, as recently demonstrated by Marcum and Rosenberg (48). These GAGs, presumably derived from endothelial cell (3, 4) or SMC (20) proteoheparan sulfates, may act as endogenous chalones to regulate SMC growth. In addition to their effect on prolifera-
growth factor-deprived rat vascular SMCs with nanogram (30, 51, 52, 65, 71, 72). We also show that SMCs cultured in described (46). Cells were maintained in a humidified 37°C/5% CO2 environment in Waymouth’s medium supplemented with 10% fetal calf serum (FCS) (HyClone Laboratories, Sterile Systems, Inc., Logan, UT), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.4 µg/ml butyl-p-hydroxybenzoate. Cells were routinely passaged at a 1:5 split ratio and cultures were used in the second to fifth passage. Growth factor–deprived cells were prepared by maintaining confluent cultures of SMCs in 20-mm diameter Costar 6-well plates (Costar, San Diego, CA), used as a primary antibody, was visualized with rhodamine-labeled rabbit anti-goat IgG (Cappell Laboratories, Cochranville, PA) for 30 min. Cover slips were then mounted onto slides and photographed on a Zeiss Photomicroscope II. For immunolocalization of thrombospondin, goat anti-rabbit fibronectin (Calbiochem-Behring Corp., San Diego, CA), used as a primary antibody, was visualized with rhodamine-labeled rabbit anti-goat IgG (Cappell Laboratories).

RESULTS

Serum-inducible SMC Proteins

Confluent cultures of rat SMCs were made growth factor–deficient by treatment with 0.5% FCS for 72 h, with a serum-free defined medium containing transferrin and insulin (41) for 72 h, or with 10% plasma-derived serum (PDS) for 5–7 d. Subsequent treatment of cells with FCS or purified growth factors yielded qualitatively identical results. All experiments described in this paper were performed on cells maintained in 10% PDS. The labeling index of growth-arrested cells, using rat aortic SMC (maximal stimulation of RNA synthesis at 10 ng/ml).

GAGs: Sodium heparin (type I, 167 USP U/mg) was obtained from Sigma Chemical Co. (St. Louis, MO). Other heparin preparations used in these studies included bovine heparin (158 USP U/mg; RD heparin, Mr 5,000, 69 USP U/mg; and a heparan sulfate fraction, Mr 2,700, 26 USP U/mg) were provided by Hepar Industries (Franklin, OH). Chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan sulfate were purchased from Miles Laboratories, Inc. (Elkhart, IN). Hyaluronic acid was from Sigma Chemical Co. Two other heparin sulfate preparations (HSI from bovine liver, and HSI from human aorta) were gifts of Dr. M. Hooîk, University of Alabama (see reference 32 for a detailed characterization of HSI and HSII). Unless otherwise indicated, experiments were performed with Sigma heparin at 100 µg/ml.

Analytical Methods: Metabolic labeling was performed by culturing cells with 40–60 µCi/ml [3H]thymidine (New England Nuclear, Boston, MA; 1166.5 Ci/mmol) in Waymouth’s medium lacking methionine and supplemented with antibiotics, 0.1 mg/ml bovine serum albumin (BSA), and growth factors or GAGs as indicated. Cells were typically overconfluent at the time of the experiment and were labeled for 1 or 2 h at 37°C. Metabolically labeled culture medium was harvested into protease inhibitors at 4°C, to produce a final concentration of 0.9 mM phenylmethylsulfonyl fluoride, 1.0 mM N-ethylmaleimide, 23 mM EDTA, and 45 µg/ml pepstatin. Cell layers were incubated three times with phosphate-buffered saline (PBS), then lysed in 0.5 M NH4OH at 4°C containing protease inhibitors at a final concentration of 0.9 mM phenylmethylsulfonyl fluoride and 45 µg/ml pepstatin. Radiolabeled proteins from medium and cell layers were precipitated in 10% trichloroacetic acid, dissolved in SDS PAGE sample buffer as described by Laemmli et al. (34), neutralized as required with 1 M NaOH, and resolved on 6% or 8% polyacrylamide gels. All gels were run in the presence of 50 mM dithiothreitol. Gels were fixed, dried, and exposed to X-Omat X-ray film (Eastman Kodak Co., Rochester, NY) at −70°C. Quantitation of radioactive protein bands was achieved by densitometric scanning using a Helena Quick-Scan densitometer (Helena Laboratories, Beaumont, TX). Underexposed gels were scanned to insure densitometric readings in a linear range.

Radioimmune precipitation was performed by harvesting radiolabeled cell layer and medium proteins into PBS containing 0.1% SDS and 0.5% Triton X-100. Rabbit antiserum prepared against human platelet thrombospondin (51, 65) were used to precipitate SMC-derived thrombospondin essentially as described by Mumbey et al. (54). The human platelet thrombospondin used as SDS PAGE standards was prepared as described by Raugi et al. (66).

Preparation of Triton X-100-insoluble “extracellular matrices” was achieved by treatment of metabolically labeled SMC layers with 0.5% Triton X-100 in PBS for 30 min and subsequent washing of the insoluble residue with PBS (22). Treatment of cell layers in this fashion removed soluble proteins and membranes; cell nuclei and cytoskeletons remained associated with the insoluble ECM. Matrices were prepared for SDS PAGE by addition of sample buffer to the culture wells or were used as starting material for the immunoprecipitation of thrombospondin as described above.

Immunofluorescence localization of thrombospondin in SMC cultures was performed essentially as described by Raugi et al. (65), using anti-thrombospondin immunoglobulin prepared and characterized in this laboratory and used previously for immunolocalization studies (65, 81). Briefly, SMCs grown on glass coverslips were fixed for 5 min in cold (4°C) methanol, rinsed, and exposed to 10% whole sheep serum for 10 min at room temperature. Cells were then washed three times in PBS, exposed to rabbit anti-human platelet thrombospondin (1:10 in PBS) for 30 min at room temperature, washed again, and exposed to FITC-labeled sheep antibodies directed against rabbit IgG (Cappell Laboratories, Cochranville, PA) for 30 min. Cover slips were then mounted onto slides and photographed on a Zeiss Photomicroscope II. For immunolocalization of fibronectin, goat anti-rat fibronectin (Calbiochem-Behring Corp., San Diego, CA), used as a primary antibody, was visualized with rhodamine-labeled rabbit anti-goat IgG (Cappell Laboratories).

MATERIALS AND METHODS

Cell Culture: Rat aortic SMC were grown from explants as previously described (46). Cells were maintained in a humidified 37°C/5% CO2 environment in Waymouth’s medium supplemented with 10% fetal calf serum (FCS) (HyClone Laboratories. Sterile Systems, Inc., Logan, UT), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.4 µg/ml butyl-p-hydroxybenzoate. Cells were routinely passaged at a 1:5 split ratio and cultures were used in the second to fifth passage. Growth factor–deprived cells were prepared by maintaining confluent cultures of SMCs in 20-mm diameter Costar 6-well plates (Costar, Data Packaging Corp., Cambridge, MA) in 10% plasma-derived fetal bovine serum (HyClone Laboratories) for 5–7 d.

Growth Factors: Insulin, epidermal growth factor (EGF), and fibroblast growth factor (FGF) were obtained from Collaborative Research (Lexington, MA). Each of the growth factors were tested for mitogenic or mitogenic support activity by the manufacturer. PDGF was obtained from E. Raines and R. Ross, University of Washington, and was purified through phenyl sepharose as described (64). The PDGF was tested for mitogenic activity on Swiss 3T3 cells (half-maximal stimulation of [3H]Tdr incorporation at 1.0 ng/ml) and was active in nanogram amounts in a complementation test mitogenesis assay

Results: Rat aortic SMC were made growth factor–deficient by treatment with 0.5% FCS for 72 h, with a serum-free defined medium containing transferrin and insulin (41) for 72 h, or with 10% plasma-derived serum (PDS) for 5–7 d. Subsequent treatment of cells with FCS or purified growth factors yielded qualitatively identical results. All experiments described in this paper were performed on cells maintained in 10% PDS. The labeling index of growth-arrested cells,
following a 30-h exposure to \[^3\text{H}\]thymidine and autoradiography, was typically <6%. Stimulation by 10% FCS or 5 ng/ml PDGF (in a complementation test assay containing EGF and insulin) increased the labeling index to >80% or >50%, respectively. Treatment of growth-arrested SMCs with PDGF alone did not significantly increase the labeling index, consistent with the putative role of PDGF as a "competence" factor (5, 77).

To assess the effects of FCS on specific protein induction, growth factor–deficient SMCs were treated with 10% FCS for 1 h, then metabolically labeled for 1 h with \[^3\text{S}\]methionine. Radiolabeled proteins were resolved on SDS PAGE and visualized by autoradiography. Five cell layer proteins were consistently induced by FCS treatment (Fig. 1, lanes 1 and 2). Four of these proteins migrated on SDS PAGE with apparent $M_r$’s of 50,000, 55,000, 72,000, and 110,000. The fifth protein was identified as thrombospondin (Fig. 2). The $M_r$ 55,000 protein and thrombospondin were also secreted into the culture medium (Fig. 1, lanes 3 and 4). Other cell layer and secreted proteins were increased or decreased in individual experiments, but are not detailed here. It should be emphasized that our experimental strategy and gel systems were designed to favor the resolution of higher molecular weight abundant proteins induced as early (within 2 h) responses to growth factors.

Thrombospondin was identified as a serum-regulated protein by the following criteria: (a) the induced protein chain (reduced $M_r$ 150,000–180,000 on SDS PAGE) co-migrated with human platelet thrombospondin standards under reducing (Fig. 2) and nonreducing conditions; (b) the induced band was quantitatively immunoprecipitated using an antiserum prepared against human platelet thrombospondin (Fig. 2) or bovine aortic endothelial cell thrombospondin (data not shown); and (c) preincubation of the antibody with excess human platelet thrombospondin completely prevented the immunoprecipitation of the induced band (data not shown). Thus the inducible SMC $M_r$ 150,000 chain appears to be electrophoretically and immunologically identical to human platelet thrombospondin.

**Effect of Heparin on Induction of SMC Proteins by Serum**

Since heparin-like molecules are believed to alter SMC gene expression and reduce proliferation (see the introduction), we asked if heparin-treated SMCs would respond less well to serum (in terms of protein induction) than controls. PDS-treated SMCs were exposed to 100 $\mu$g/ml heparin (Sigma Chemical Co.) for up to 96 h in the presence of 10% PDS. Cells were then stimulated with FCS and labeled as described above; cell layer and secreted proteins were visualized by SDS PAGE and autoradiography. Heparin treatment had no inhibitory effect on the induction of SMC layer or medium proteins by FCS. Instead, heparin appeared to enhance the secretion of thrombospondin into the culture medium (Fig. 3, lane 3). Heparin also induced production of two previously described proteins ($M_r$ 37,000 and 39,000) (42) in both PDS-treated and FCS-stimulated SMCs.

**Effects of Purified Growth Factors on SMC Thrombospondin Synthesis**

To define the component(s) in serum responsible for induction of SMC thrombospondin synthesis, we tested the effects of various purified growth factors on thrombospondin production. PDS-treated SMCs were treated for 1 h with growth factor, then metabolically labeled for 1 h in the presence of fresh growth factor. Among those tested, only PDGF was effective in stimulating thrombospondin production, at least within 2 h (Fig. 4). Other growth factors (5–50 ng/ml EGF, 100 ng/ml FGF, 1 $\mu$g/ml insulin) had no effect (data not shown). After PDGF treatment, thrombospondin production into the culture medium was increased 1.8–3.9-fold, as compared with a 1.6–2.3-fold induction by 10% FCS. Thus 5 ng/ml of purified PDGF could mimic entirely the effects of FCS on thrombospondin production. Because the increase in thrombospondin synthesis after PDGF treatment could be observed in both cell layer and medium compartments (Fig. 4), in subsequent experiments we present only data quantifying thrombospondin levels in the culture medium.

**FIGURE 1** Serum-inducible SMC proteins. PDS-arrested SMC cultures were labeled for 1 h with \[^3\text{S}\]methionine with or without prior exposure for 1 h to 10% FCS. Cell layer (lanes 1 and 2) or medium (lanes 3 and 4) proteins were harvested into protease inhibitors and visualized by SDS PAGE (8% gel) and autoradiography. Protein bands indicated at arrows were consistently enhanced in FCS-treated cells; their molecular weights (x 10$^{-3}$) are indicated. One inducible protein was identified as thrombospondin (TS). Both thrombospondin and an $M_r$ 55,000 protein (55) were secreted into the culture medium. Asterisk indicates the position of migration of fibronectin, another SMC matrix component whose synthesis and secretion is unaltered by FCS treatment.
FIGURE 2 Immunoisolation of thrombospondin from SMC culture medium following induction by serum. The protein band labeled T5 in Fig. 1 was identified as thrombospondin by its migration on SDS PAGE and by quantitative removal of the induced protein chain by immunoprecipitation with anti-human platelet thrombospondin immunoglobulin. Lane 1, higher molecular weight proteins secreted by PDS-treated SMCs; lane 2, induction of an M, 150,000 protein by 1 h treatment with FCS; lane 3, isolation of thrombospondin from SMC culture medium by immunoprecipitation after FCS induction; lane 4, supernatant after removal of the induced band with antibodies against human platelet thrombospondin. Arrow indicates the position of migration of a human platelet thrombospondin standard.

Dose Dependence of SMC Thrombospondin Induction by PDGF

The response of SMC to PDGF was dose dependent within a physiological range. PDS-treated cells were exposed to increasing amounts of PDGF (0.1-5 ng/ml) for 1 h, then metabolically labeled for 1 h in the continued presence of PDGF. Secreted radiolabeled proteins were visualized by autoradiography following SDS PAGE and quantified by scanning densitometry. Thrombospondin synthesis reached maximal levels at 2.5 ng/ml PDGF, with an ED50 at less than 1 ng/ml PDGF (Fig. 5). Concentrations of PDGF of at least 10 ng/ml were required to maximally stimulate mitogenesis in rat SMCs in a complementation test assay (data not shown). Thus induction by PDGF occurs at levels of growth factor that are suboptimal for proliferation.

Time Course of Thrombospondin Production after Exposure of SMCs to PDGF

We next examined PDGF-mediated thrombospondin synthesis as a function of time. PDS-treated SMCs were treated with 5 ng/ml PDGF for 0-8 h, then metabolically labeled for 1 h in the presence of freshly added PDGF. Secreted proteins were visualized by SDS PAGE and autoradiography. As presented in Fig. 6, thrombospondin synthesis in response to PDGF occurred rapidly and transiently; labeled thrombospondin was observed as a secreted cell product within 1 h after stimulation. Synthesis of thrombospondin reached a maximal level after 2 h of PDGF treatment (cells labeled at 1 h after stimulation) and declined to baseline (PDS) levels by 5 h. Thrombospondin synthesis remained low (and non-inducible) for at least 9 h after the initial addition of PDGF (Fig. 6).

Actinomycin Sensitivity of SMC Thrombospondin Induction by PDGF

Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, was tested for its ability to influence the induction of thrombospondin synthesis by PDGF. Addition of 2 µg/ml actinomycin to SMC cultures reduced the synthesis of new RNA (measured by [3H]uridine incorporation into total RNA) by > 80% (data not shown). PDS-treated SMC were exposed to actinomycin D for 30 min prior to exposure of the cells to PDGF. After a 1-h stimulation, cells were metabolically labeled for 1 h in the presence of fresh PDGF and actinomycin. Medium proteins were resolved by SDS PAGE and visualized by autoradiography. As shown in Fig. 7, inhibition of RNA synthesis abolished the ability of PDGF to induce thrombospondin synthesis, suggesting regulation at a transcriptional level.

Analysis of Thrombospondin Distribution in Control and Heparin-treated SMC Cultures

Rat SMC cultures maintained in 10% FCS were metabolically labeled for 2 h in the absence or presence of 100 µg/ml heparin. Radiolabeled proteins were resolved on SDS PAGE followed by autoradiography. As shown in Fig. 8, in heparin-treated cell layers the intensity of the thrombospondin band
FIGURE 4 Effect of PDGF on cell layer and medium SMC thrombospondin levels. PDS-arrested SMC cultures were labeled for 1 h with [35S]methionine after a 1-h exposure to 10% FCS (lanes 2 and 5) or 5 ng/ml PDGF (lanes 3 and 6). In PDGF-treated cells, growth factor was present during the labeling period as well. Cell layer proteins (lanes 1–3) and secreted proteins (lanes 4–6) were visualized by SDS PAGE (6% gel) and autoradiography. Note that treatment of SMCs with FCS or PDGF markedly enhanced thrombospondin (TS) production in both cell layer and medium compartments. Other growth factors had no effect (see text for details).

FIGURE 5 Dose dependence of SMC thrombospondin induction by PDGF. PDS-arrested SMCs were labeled for 1 h with [35S]methionine in the presence of increasing amounts of PDGF after a 1-h pretreatment with PDGF. Thrombospondin (TS) levels in the culture medium were determined by quantitative scanning densitometry following visualization of radiolabeled secreted proteins by SDS PAGE and autoradiography. Thrombospondin synthesis was maximal at PDGF concentrations greater than 2.5 ng/ml.

was markedly reduced (lanes 1 and 2, arrow). Thrombospondin levels were concurrently increased in the medium of cells treated with heparin (lanes 3 and 4).

To verify these observations, we repeated the experiment as described above and determined relative thrombospondin levels in SMC layer and medium compartments following immunoprecipitation of SMC proteins with antiserum to human platelet thrombospondin. Immunoisolated proteins were resolved by SDS PAGE, visualized by autoradiography, then scanned with an optical densitometer. Tracings of gel scans of the immunoprecipitated thrombospondin band are presented in Fig. 8 (right). These data clearly define the effect of heparin on SMC thrombospondin: in the presence of heparin, the distribution of thrombospondin in the culture is distinctly shifted into the medium compartment. This observation was further verified by the results of a dose-response analysis (see below). The effects of heparin appear to be confined to newly synthesized thrombospondin as we were unable to release thrombospondin from prelabeled cells with heparin treatment (data not shown).

Dose Dependence of Thrombospondin Release from SMC Layers by Heparin

A dose–response analysis of the release of newly synthesized thrombospondin from SMC layers was performed by metabolic labeling of cultures for 2 h in the presence of increasing amounts of heparin. Cell layer and medium proteins were visualized by SDS PAGE and autoradiography (top). Thrombospondin (TS) levels were quantified by scanning densitometry (bottom). Note that newly synthesized thrombospondin can be detected in the SMC culture medium within a 1-h exposure to PDGF. Thrombospondin production peaks after 2 h of exposure and returns to baseline levels by 5 h.
FIGURE 7 Actinomycin sensitivity of SMC thrombospondin induction by PDGF. PDGF-arrested SMCs were exposed to 2 μg/ml actinomycin D (lane 3) for 30 min prior to the addition of 5 ng/ml PDGF (lanes 2 and 3). Cultures were then metabolically labeled with [35S]methionine for 1 h in the absence (lane 1) or presence (lane 2) of PDGF and with both PDGF and actinomycin D (lane 3). Secreted proteins were visualized by SDS PAGE (6% gel) and autoradiography. Note that actinomycin-treated SMCs do not produce thrombospondin (TS) in response to PDGF (lane 3).

visualized by SDS PAGE and autoradiography; medium proteins were quantified by scanning densitometry. Fig. 9 (left) presents data on thrombospondin levels in the SMC culture medium. Note that a half-maximal response is achieved at a heparin concentration of 0.1 μg/ml, with maximum release observed at heparin concentrations greater than 1.0 μg/ml. Cell layer proteins from the same experiment are shown resolved by SDS PAGE in Fig. 9 (right). Note the strong correlation between the dose-dependent disappearance of thrombospondin from the cell layer and its increase in the culture medium.

Analysis of Thrombospondin in Triton X-100-insoluble Cellular Matrices

To determine if the increased levels of thrombospondin in heparin-treated culture medium resulted from an inhibition of the interaction of newly synthesized thrombospondin with the SMC extracellular matrix, we prepared and analyzed insoluble SMC matrices from control and heparin-treated cultures. Confluent cultures of SMCs were treated with 100 μg/ml heparin for 4 h, then labeled for 2 h with [35S]methionine. Triton X-100-insoluble cellular and matrix components were resolved by SDS PAGE and autoradiography (Fig. 10A), then scanned with an optical densitometer (Fig. 10B). Matrices prepared from heparin-treated cells contained reduced levels of thrombospondin, suggesting that heparin interferes with the initial binding of newly synthesized thrombospondin to the SMC extracellular matrix.

GAG Specificity of Thrombospondin Release from SMC Layers

Various GAGs were tested for their ability to release newly synthesized thrombospondin from SMC layers. The experiments were performed by labeling confluent SMC cultures for 2 h with [35S]methionine in the presence of 100 μg/ml concentrations of different GAGs. Secreted proteins were visualized by autoradiography after SDS PAGE. Only heparin and heparin-like molecules were effective in increasing the amount of thrombospondin released into the medium (Fig. 11). Heparan preparations of differing anticoagulant activities (Sigma Chemical Co., 167 USP U/mg; Hepar Industries, 158 USP U/mg; RD heparin, 69 USP U/mg) were effective, as were low molecular weight heparin fragments (Mr 2,700, 26 USP U/mg) and dermatan sulfate (from porcine skin). Of the two different preparations of heparan sulfate GAGs tested, only HSI from bovine liver was effective. This heparan sulfate binds to, and releases, endogenous heparan sulfates from hepatocytes in a concentration-dependent manner (32). Another heparan sulfate preparation, HSIII, was ineffective in altering the distribution of thrombospondin within SMC cultures. This molecule does not bind to hepatocytes and is characterized by a lower charge density and sulfate content.
FIGURE 9  Dose dependence of thrombospondin release from SMC layers by heparin. SMC cultures were exposed to increasing amounts of heparin during a 2-h metabolic labeling with $^{35}$S-methionine. Proteins were resolved by SDS PAGE (6% gel) and autoradiography. (Left) Quantitation by scanning densitometry of culture medium thrombospondin after the exposure of cells to increasing amounts of heparin. Note that maximal release of newly synthesized thrombospondin occurs at heparin concentrations greater than 1.0 μg/ml. (Right) SDS PAGE autoradiogram of cell layer proteins after the exposure of cells to increasing amounts of heparin. Arrow indicates position of migration of human platelet thrombospondin. Note that thrombospondin is not visible in the cell layer compartment at heparin concentrations greater than 1.0 μg/ml.

FIGURE 10 Analysis of thrombospondin levels in Triton X-100-insoluble cellular matrices. Control or heparin-pretreated (2 h) SMC cultures were labeled for 2 h with $^{35}$S-methionine in the presence or absence of 100 μg/ml heparin. SMC matrices were prepared by exposing SMC layers for 30 min to Triton X-100 as described in the text. (A) Insoluble components of the SMC matrix were visualized on SDS PAGE autoradiograms. The position of migration of immunoprecipitated SMC thrombospondin (TS) is marked with an arrow. Lane 1, components of matrices prepared from control SMC; lane 2, components of matrices prepared from heparin-treated SMC. (B) Tracings of partial gel scans from lanes 1 and 2 (bar) in A. Note that the amount of newly synthesized thrombospondin present in the matrix of heparin-treated SMC is markedly less than that observed in control matrices.

than HSI (32).

These data suggest that, in addition to polysaccharide content, molecular charge may play a role in determining the relative activity of the GAG preparations. Other GAGs tested (hyaluronic acid, chondroitin-4-sulfate, and chondroitin-6-sulfate) were not effective. Thus the range of GAGs capable of altering the distribution of thrombospondin in SMC cultures is similar to that described for other heparin-mediated SMC activities (inhibition of migration, induction of an $M_c$ 60,000 collagen, and induction of the $M_c$ 37,000 and 39,000 proteins) (42–46). Only anticoagulant or non-anticoagulant
heparins, heparin fragments, and dermatan sulfate are active (42–46). The data emphasize that certain, but not all, heparan sulfate GAGs are capable of modifying SMC phenotype. The availability of heparan sulfates with (HSI) and without (HSII) activity on SMCs may prove useful in efforts to define the active moieties responsible for the interactions of heparin-like GAGs with SMC.

Time Course of Activity of Heparin on SMC Thrombospondin Distribution

The time required for heparin to exert an effect on thrombospondin distribution in SMC cultures was determined by treating the cells for increasing amounts of time with 100 µg/ml heparin (0–23 h), labeling the cells for 1 h in the presence of heparin, and determining the relative amounts of thrombospondin secreted into the culture medium. Medium proteins were resolved by SDS PAGE and autoradiography, then quantified by scanning densitometry. Thrombospondin release into the medium compartment occurred rapidly in response to heparin and was maximal by 4 h of heparin treatment (Fig. 12).

The duration of the thrombospondin effect following removal of heparin from SMC cultures was also determined. SMCs were treated with heparin (100 µg/ml) for 4 h to establish the heparin-treated phenotype. Cells were washed three times with heparin-free medium and incubated for 0–7 h in the absence of heparin. Cultures were then metabolically labeled for 1 h with [35S]methionine. Secreted proteins were harvested, visualized by SDS PAGE and autoradiography, then quantified by scanning densitometry. After the removal of heparin from SMC cultures, levels of radiolabeled thrombospondin in the medium remained elevated over controls for at least 2 h, but were only slightly increased at 4 h (Fig. 12). The heparin effect was completely reversible by 8 h.

Immunofluorescence Localization of Fibronectin and Thrombospondin in SMC Cultures

Thrombospondin has been detected previously in matrices of human aortic SMC in vitro (65). We have verified the presence of thrombospondin in the rat SMC matrix and have examined the distribution of thrombospondin under growth-arrested, PDGF-stimulated, and heparin-treated conditions. Experiments to verify the specificity of the immunolocalization included preimmune and non-immune controls and staining with antibody preabsorbed with excess human platelet thrombospondin. SMCs were maintained for 5–7 d in 10% PDS, then trypsinized and replated onto sterile glass coverslips in the presence of 5% “conditioned” PDS (the original 10% PDS was removed, clarified, and diluted 1:1 with fresh Waymouth’s medium). This procedure allowed SMCs to be replated in a growth-arrested state. Thus, the new ECM made in the absence or presence of growth factor or heparin could be examined. After 24 h of attachment and spreading in 5% PDS, cells were left untreated or treated with 5 ng/ml PDGF or PDGF plus 100 µg/ml heparin for 24 h. Cells were then permeabilized, fixed, and processed for immunofluorescence as detailed above. Cells maintained in PDS exhibited a well-developed fibronectin matrix (Fig. 13a), consistent with their ability to synthesize and secrete fibronectin under these conditions (see Fig. 1). In contrast, PDS-treated SMCs did not synthesize thrombospondin or incorporate it into the ECM (Fig. 13b). However, SMC treated with PDGF for 24 h exhibited both intracellular and extracellular fluorescence when probed for thrombospondin (Fig. 13c). Staining was observed in a perinuclear pattern (presumably representing thrombospondin in Golgi vesicles and secretory granules) as well as in a fibrillar extracellular pattern. SMC simultaneously exposed to PDGF and heparin exhibited only the perinuclear pattern of fluorescence (Fig. 13d). These observations agree with the biochemical data presented above: heparin-treated SMCs respond equally well, compared with controls, to PDGF but fail to incorporate thrombospondin into their ECM.

DISCUSSION

Thrombospondin

Thrombospondin is an M, 450,000 glycoprotein composed of three probably identical disulfide-bonded subunits of M, 150,000 (1, 37, 38, 49). Thrombospondin was first described as a component of platelet α-granules (1) and is now known to be synthesized by endothelium, fibroblasts, granular pneumocytes, and smooth muscle cells in culture (30, 51, 52, 65, 71, 72). While platelet thrombospondin has been assigned a role in platelet aggregation (29, 40) and, possibly, in the binding of platelets to the endothelial substratum (35, 36), the function of cell-derived thrombospondin is not known. Thrombospondin has been shown to interact with collagen (36, 53), heparin (17, 38), fibrinogen (16), and fibronectin (35, 36), and to be incorporated into the ECM of fibroblasts (30, 65) and SMCs (65). Thus thrombospondin may play some, as yet undefined, role as a component of the SMC extracellular matrix. Our data suggest a role for matrix-bound thrombospondin in the SMC growth response, presumably as an extracellular mediator of some PDGF-regulated function.
Thrombospondin as a PDGF-inducible Protein

Transient treatment of fibroblasts (77) and porcine smooth muscle cells (5) with PDGF allows the cell to progress to a point in G₁ in the cell cycle at which additional "progression" growth factors are required. The endowment of PDGF-mediated "competence" to fibroblasts is believed to depend on the induction of a family of proteins by PDGF (9, 63). These PDGF-inducible ("competence") genes and proteins have been identified using metabolic labeling techniques (63), in vitro translation of mRNAs extracted from stimulated cells (27), and specific cDNA probes (9, 10, 31). Several of these proteins have been identified as products of cellular proto-oncogenes (10, 31); at least three (c-fos, c-myc, and an M₁, 29,000 protein) are nuclear in location (12, 18, 59), and at least one (an M₁, 38,000 protein) is secreted (74).

Little is known concerning PDGF-inducible gene sequences from nonfibroblastic cells. In our studies of the effects of PDGF on smooth muscle cell protein synthesis, we have identified at least three major secreted products whose synthesis is regulated by PDGF. These proteins, an M₁, 25,000 protein (45), an M₁, 55,000 protein, and thrombospondin, do not appear to be growth factor-regulated proteins in fibroblasts (56, 63). These data suggest that certain PDGF-inducible proteins may be cell type specific. The early induction of thrombospondin by PDGF (within 1 h) and the sensitivity of the induction to inhibition by actinomycin D raise the possibility that activation of the thrombospondin gene is a primary response of these cells to PDGF. The localization of thrombospondin to the SMC extracellular matrix establishes it as unique among known growth factor-inducible proteins and supports the concept (see below) that ECM molecules may play regulatory roles in cellular growth.
Possible Involvement of a Thrombospondin-rich ECM in SMC Growth

A variety of studies have established a role for cell-matrix interactions in the control of cellular growth (22, 73). Bitterman et al. (2) have suggested that fibronectin may facilitate competence in fibroblasts. Similarly, type IV collagen matrices can reduce the requirement of mammary epithelial cells for growth factors (73). It has been suggested (39, 73) that one function of a growth factor may be to direct the cell's production of a growth-supportive ECM. Specific growth factors have, in fact, been shown to influence production of individual matrix components: both FGF and EGF appear to selectively reduce the production of type I collagen (25, 76, 78), while PDGF has been shown to enhance the production of type V collagen by fibroblasts (55). Finally, as shown in this study, vascular SMCs express thrombospondin rapidly and transiently after exposure to PDGF.

The induction of a thrombospondin-rich ECM by PDGF suggests that extracellular thrombospondin may play an important role in SMC growth and/or migration. In support of this concept, we have found that addition of antibodies against thrombospondin to SMC cultures inhibited the migration of these cells following experimental wounding (47). Mumby et al. (54) have shown that sparsely cultured, rapidly growing fibroblasts, endothelial cells, and SMCs synthesized and secreted higher levels (per cell) of thrombospondin than did confluent, more quiescent cultures. Finally, the immunolocalization of thrombospondin in human aorta is suggestive of a growth-related role for matrix thrombospondin. Wight et al. (81) found that thrombospondin was located predominantly in areas surrounding intimal SMC in sections of atherosclerotic tissue; thrombospondin was not readily detectable in or near SMC in the vascular media. It is likely that intimal SMC represent more recently proliferating cells than do their normal medial counterparts.

Heparin Regulation of Thrombospondin Deposition in the Matrix as a Possible Mediator of SMC Growth and/or Migration

In this report we have shown that heparin-like GAGs inhibit the deposition of newly synthesized thrombospondin into the SMC extracellular matrix and consequently inhibit the formation of a PDGF-inducible, thrombospondin-rich matrix. Similarly, McKeown-Longo et al. (50) have recently reported that heparin inhibits the binding of radiodinated platelet thrombospondin to the fibroblast ECM. This heparin effect may result from an inhibition of the interaction of thrombospondin with the cell surface, with type V collagen (53), with fibronectin (35, 36), or with type I collagen (36), and may involve the heparin binding site in the NH2-terminal region of the thrombospondin molecule (11, 17, 66).

In the experiments presented in this study we have demonstrated that heparin treatment of SMCs in culture does not alter the SMC response to serum factors, at least as judged by the induction of specific proteins, suggesting that heparin does not render SMCs unresponsive to competence-inducing growth factors. However, we have identified an antagonism between heparin-like molecules and PDGF in the regulation of production of a thrombospondin-rich ECM and speculate that such a matrix may be facilitative to SMC growth and/or migration (Fig. 14). If this is true, then the inhibition of thrombospondin deposition by heparin may be a step in the mechanism whereby heparin-like molecules regulate SMC function (Fig. 14). It is possible that thrombospondin may act as an extracellular "integrator" of growth stimulatory and inhibitory signals, since the amount of thrombospondin functionally incorporated into the SMC extracellular matrix is regulated by both PDGF and heparin-like GAGs. The relative amounts of these regulatory signals in the SMC milieu may determine, for any vascular injury, the extent of SMC-thrombospondin interactions and consequently the extent of the SMC growth response. We are currently investigating the role of thrombospondin in SMC growth and/or migration and in the heparin-mediated inhibition of movement and proliferation. Taken together, our data on the effects of heparin on SMC protein induction and distribution (42–45, this paper) strongly reinforce the concept that the character of the ECM may be an important determinant of vascular SMC function.

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