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Platelet-derived growth factor stimulates the secretion of hyaluronic acid by proliferating human vascular smooth muscle cells

(atherosclerosis/carbohydrates/glycosaminoglycans)

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ABSTRACT **Total glycans from the cell layer and the culture medium of human vascular smooth muscle cells (VSMC) that had been cultivated in the presence of platelet-derived growth factor (PDGF) were isolated and purified by gel filtration after Pronase and DNase digestion and alkali–borohydride treatment. Measurements of the content of neutral hexoses and uronic acids revealed that PDGF stimulates total glycan synthesis by proliferating VSMC in a linear fashion from 24 h to 72 h of incubation. In contrast, total glycan synthesis by human fibroblasts, epithelial cells, or endothelial cells was not affected by PDGF, indicating cell-type specificity. Chemical, biochemical, and enzymological characterization of the total glycans synthesized by VSMC showed that PDGF stimulates the secretion of a 340-kDa glycan molecule in a time-dependent manner from 24 h to 72 h. This molecule is highly acidic, shares a common structure with hyaluronic acid, and exhibits a potent antiproliferative activity on VSMC. These results suggest that VSMC in response to PDGF are capable of controlling their own growth and migration by the synthesis of a specific form of hyaluronic acid with antiproliferative potency, which may be involved in the regulation of the local inflammatory responses associated with atherosclerosis.**

The intimal lesions of advanced atherosclerosis are characterized by an increased mass of vascular smooth muscle cells (VSMC), which implies an increment in proliferation and/or migration of VSMC. This involves a transition of VSMC from a quiescent/differentiated to a proliferating/dedifferentiated phenotype. The mechanisms underlying the migration of VSMC into the intima and their modulation into a proliferative phenotype in atherosclerosis have been reviewed by Ross (1).

Among several growth factors, platelet-derived growth factor (PDGF) has been shown to be essentially involved in VSMC proliferation (2, 3) and migration (4, 5). PDGF is generally not expressed in the normal artery, whereas it is upregulated in lesions of atherosclerosis. The lesions at all stages of development from both humans and nonhuman primates contain the PDGF-BB isoform. The capacity to respond to this mitogen is associated with increased numbers of PDGF-receptors on adjacent intimal VSMC in the same lesions (6, 7).

Increasing evidence suggests that the storage and activity of PDGF is influenced by extracellular matrix molecules. For example, heparin, a product of endothelium and smooth muscle, can bind and inactivate PDGF and thus inhibit the PDGF-induced migration and proliferation of VSMC (8). Furthermore, it has been shown that arterial proteoglycans from human atherosclerotic lesions contain a small but significant amount of heparin-like (heparan sulfate) glycosaminoglycans (GAGs) (9), which are also produced *in vitro* by vascular endothelial cells (10, 11) and VSMC (12). The above evidence indicates that GAGs are implicated in the regulation of the inflammatory responses involved in atherosclerosis. However, there is no information related to the precise characterization of the total glycan molecules that are produced by VSMC during the development of atherosclerotic lesions.

METHODS

Cell Cultures. Primary human lung VSMC and fibroblasts were established from sterile lung biopsies and cultivated in RPMI 1640 medium supplemented with 5% (vol/vol) fetal calf serum (FCS) and 4 mM L-glutamine as described (13, 14). Human endothelial and epithelial cells were obtained from Clonetics (San Diego). Subconfluent cultures (75–80%) ($\approx 2 \times 10^7$ cells) were used between passages 2 and 6 and cells were growth-arrested by starvation for 48 h in low-serum medium (RPMI 1640 supplemented with 0.1% FCS); the medium was replaced every 12 hr. Quiescent cells were then challenged with human recombinant PDGF-BB (10 ng/ml; GIBCO/BRL/Life Technology) and incubated further for various times up to 72 hr.

Isolation and Purification of Total Glycans. Total glycans were isolated and purified from VSMC, fibroblasts, epithelial cells, and endothelial cells at 12, 24, 48, and 72 hr after the addition of PDGF-BB. The supernatants (25 ml) were collected separately, and the cells with associated extracellular matrix (cell layer) were washed twice with 10 ml of ice-cold phosphate-buffered saline and harvested by scraping. Unstimulated cells were collected at identical time points and served as controls. Total cell number was determined in a Neubauer chamber. The total glycans were isolated and purified from the cell layers and from their respective supernatants by the same procedure. Lipids were extracted with 4 volumes of 1:2 (vol/vol) chloroform/methanol as described by Svennerholm and Fredman (15), whose procedure also inacivates all hydrolytic enzymes. Centrifugation was carried out at 3200 $\times g$ for 20 min at 3°C and the organic solvents were removed from the resulting pellet by the addition of 10 ml of ethanol. The mixture was again centrifuged at 3200 rpm for 20 min at 3°C, and the pellet was dried at 40°C for 4 hr. The pellet was resuspended in 1 ml of 0.1 M Tris·HCl, pH 8.0/1 mM $CaCl_2$ and subjected to protein digestion with 0.1 kallikrein units (KU) of Pronase (protease from *Streptomyces griseus*, Calbiochem). The Pronase solution was preincubated for 30 min at 60°C to eliminate any glycosidase activity. Digestion was

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Abbreviations: PDGF, platelet-derived growth factor; VSMC, vascular smooth muscle cells; FCS, fetal calf serum; GAGs, glycosaminoglycans.

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carried out as described by Finne and Krusius (16) for 72 hr at 60°C by adding equal amounts of Pronase at 24-hr intervals. The sample was then adjusted to contain 150 mM NaCl and 10 mM $MgCl_2$, and DNA digestion was accomplished by adding 400 KU of DNase I (EC 3.1.21.1, Boehringer Mannheim) and incubating for 16 hr at 37°C. At the end of the incubation period, the $CaCl_2$ concentration of the solution was adjusted to 1 mM, and the reaction was stopped by adding 0.1 KU of Pronase and incubating the mixture at 60°C for 24 hr. The pH was adjusted to 10.0–11.0 by the addition of 10 mM NaOH, and the glycans were subjected to elimination reaction in the presence of 1 M $NaBH_4$ for 16 hr at 45°C (17). At the end of the incubation period, samples were neutralized with 50% (vol/vol) acetic acid. Glycans were then separated from degradation products by gel filtration on a Sephadex G-25 column (0.6 × 25 cm) eluted with 10 mM pyridine acetate (pH 5.0). Fractions of 0.5 ml were collected and analyzed for the neutral hexose (18) and the uronic acid (19) content.

Amino Acid Analysis. The dried glycan samples (20 μg of neutral hexoses) were hydrolyzed in 6 M HCl at 110°C in vacuum-sealed glass tubes for 16 hr. Their amino acid, GlcNAc, and GalNAc composition were determined by using the Pico-Tag method (20, 21) on a Waters HPLC system.

HPLC Analysis. Further fractionation of the glycans was performed by HPLC (Bio-Rad) with a Bio-Gel TSK 40XL gel filtration column (300 × 7.8 mm). Elution was carried out with 7 mM Tris, pH 7.4/200 mM NaCl at a flow rate of 0.3 ml/min. The apparent molecular mass of the glycans was estimated by using the following molecular weight markers: hyaluronic acid, 225 kDa (Sigma); chondroitin sulfates, 19.7–101.6 kDa (Sigma); and heparins 4.44–11 kDa (Roche). The molecular weight of the markers had been determined by ultracentrifugation (22). During the HPLC gel filtration, the absorbance was monitored at 206 or 232 nm with an ultraviolet/visible monitor (Bio-Rad, model 1706) and a refractive index monitor (Bio-Rad, model 1755).

Treatment of the Purified Glycans with GAG-Degrading Enzymes. Lyophilized glycans (50 μg of neutral hexoses) were incubated in a final volume of 35 μl as follows: (*i*) heparinase samples were dissolved in 0.1 M sodium acetate, pH 7.0/3 mM $CaCl_2$ and incubated with 4×10^{-4} units of heparin lyase (EC 4.2.2.7, *Flavobacterium heparinum*, Seikagaku, Tokyo) for 15 hr at 35°C; (*ii*) heparitinase samples were dissolved as above and incubated with 4×10^{-4} units of heparan sulfate lyase (EC 4.2.2.8, *Flavobacterium heparinum*, Seikagaku) for 16 hr at 43°C; (*iii*) chondroitinase ABC samples were dissolved in 0.1 M Tris·HCl, pH 8.0/0.05 M sodium acetate and incubated with 5×10^{-4} unit of chondroitin ABC lyase (EC 4.2.2.4, *Proteus vulgaris*, Sigma) for 16 hr at 37°C; (*iv*) keratanase samples were dissolved in 50 mM Tris·HCl (pH 7.4) were incubated with 0.1 unit of keratan-sulfate endo-1,4-β-galactosidase (EC 3.2.10.3, *Pseudomonas* sp., Sigma) for 16 hr at 37°C; and (*v*) hyaluronidase samples were dissolved in 0.02 M sodium acetate and buffered with acetic acid to pH 5.0 and incubated with 5 units of hyaluronate lyase (EC 4.2.2.1, *Streptomyces hyalurolyticus*, Sigma) for 6 hr at 60°C.

Incubation times and enzyme concentrations used were those required for the complete degradation of their respective standard substrates as estimated by preliminary investigation. In this preliminary study, the standard GAGs (50 μg)—chondroitin sulphate A (bovine trachea), chondroitin sulfate B (porcine skin), chondroitin sulfate C (shark cartilage), hyaluronic acid (bovine trachea), keratan sulfate (bovine cornea), heparan sulfate (bovine intestinal mucosa), and heparin (all from Sigma)—were treated with all of the above-mentioned GAG-degrading enzymes after appropriate incubation procedures. Substrates incubated separately with their respective buffers served as controls. Digestion was initially evaluated by PAGE as described (23) and then by HPLC gel filtration with continuous recording of the absorbance at 206 and 232 nm and of the refractive index.

Proliferation Assay. The effect of the isolated glycans on VSMC proliferation was measured by the amount of [^{3}H]thymidine incorporated into the DNA of VSMC as described (24). Cells were incubated for 12 hr with different amounts of: (*i*) the 340-kDa glycan fraction, (*ii*) hyaluronic acid, and (*iii*) PDGF-BB (10 ng/ml). [*methyl*-^{3}H]Thymidine [1 μCi (37 kBq)/ml] was then added to the culture medium, and cells were further incubated for 36 hr, after which cells were washed with medium and ice-cold methanol. Proteins and DNA were precipitated with 0.1 M trichloroacetic acid, washed with double distilled water, dissolved in 0.3 M NaOH, and measured for their radioactive content.

RESULTS

PDGF Stimulates Total Glycan Synthesis by VSMC. Total glycans were isolated either from the cell layer or the culture medium of subconfluent VSMC, fibroblasts, endothelial cells, and epithelial cells at 0, 12, 24, 48, and 72 hr after challenging with 10 ng of human recombinant PDGF-BB per ml. Unstimulated cell cultures obtained at identical time points were used as controls. Total glycans were purified from the digestion products after treatment with Pronase, DNase, and alkali-borohydride, on a Sephadex G-25 column as a single peak, with 90% recovery (data not shown). Amino acid analysis and measurement of the absorbance at 260 and 280 nm confirmed that the isolated glycans were free of nucleic acids and proteins. The amount of total glycans was calculated by measuring the content of neutral hexoses and uronic acids of the peak fractions. PDGF caused an increase in total glycan

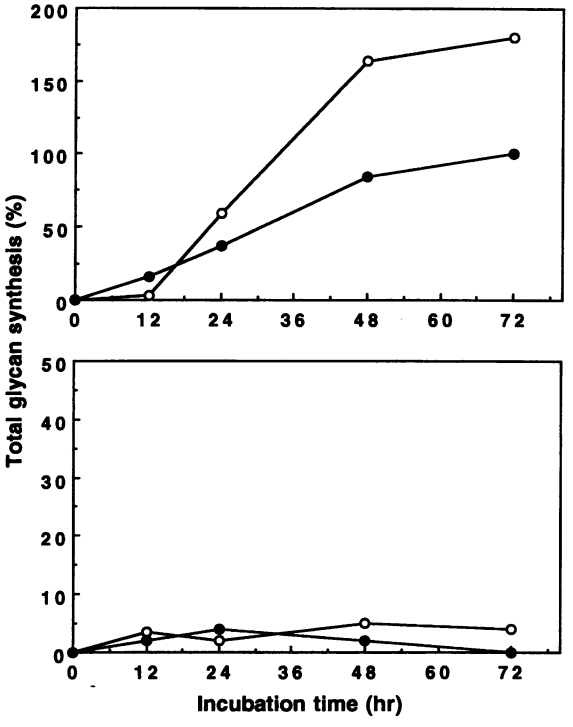

FIG. 1. Total glycan synthesis by proliferating (*Upper*) and confluent (*Lower*) VSMC cultures. Cells were cultivated in the presence of PDGF-BB at 10 ng/ml for various periods of time. Total glycans were isolated and purified from the cell layer (○) and the supernatant (●) as described in text. Total glycan synthesis in the presence of PDGF is expressed as percent of control (incubation without PDGF) and was calculated from the amount of neutral hexoses and uronic acids produced by the same cell number. Values are means of double measurements in three different preparations. Maximal error was always <15%.

synthesis by VSMC in both the cell layer and the culture medium (Fig. 1 *Upper*). In the cell layer, this increase became apparent 24 hr after the addition of PDGF and reached maximal levels (2.5-fold of control) 48 hr after stimulation, thereafter remaining constant up to 72 hr of incubation. The amount of total glycans secreted in the medium was also significantly increased in the presence of PDGF in an almost linear fashion from 24 to 72 hr of incubation. In contrast, when PDGF was added to confluent, nonproliferating VSMC cultures, there was no increase in the amount of total glycans measured in the cell layer or the culture medium (Fig. 1 *Lower*).

To investigate whether the effect of PDGF on total glycan synthesis by VSMC is cell-type specific, we measured the total glycan synthesis by subconfluent cultures of human fibroblasts, endothelial cells and epithelial cells 48 h after stimulation with PDGF. In this case, PDGF failed to significantly increase total glycan synthesis by these cells either in the cell layer (Fig. 2 *Upper*) or in the culture medium (Fig. 2 *Lower*).

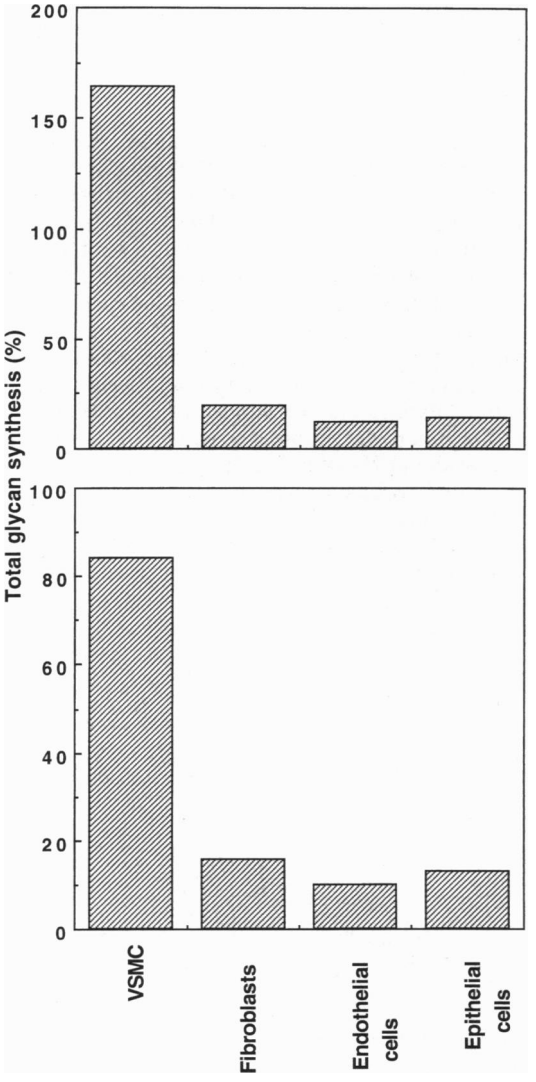

FIG. 2. Total glycan synthesis by VSMC, fibroblasts, endothelial cells, and epithelial cells 48 hr after stimulation with PDGF at 10 ng/ml expressed as percent of control (incubation without PDGF). Values were calculated from the amount of neutral hexoses and uronic acids of the total glycans isolated from the cell layer (*Upper*) and from the culture medium (*Lower*) of the same cell number. They are means of double measurements in two different preparations. Maximal error was always <15%.

PDGF Stimulates the Secretion of Acidic Glycans by VSMC. The nature of the total glycans produced by VSMC in the presence or absence of PDGF was investigated by measuring their content of uronic acids and neutral hexoses. It was found that in the total glycans isolated from the cell layer, the ratio of uronic acids to neutral hexoses was not significantly altered in the presence or absence of PDGF from 12 to 72 hr of incubation (Fig. 3 *Upper*). Similarly, this ratio remained constant up to 72 hr of incubation when measured in the total glycans secreted in the culture medium, in the absence of PDGF (Fig. 3 *Lower*). In contrast, the ratio of uronic acids to neutral hexoses measured in the total glycans isolated from the culture medium of PDGF-stimulated VSMC increased in a linear fashion from 24 hr to 72 hr of incubation (Fig. 3 *Lower*).

HPLC Analysis of the Total Glycans Secreted in the Culture Medium by PDGF-Stimulated VSMC. The total glycan molecules secreted in the culture medium by VSMC 72 hr after stimulation with PDGF were further analyzed by HPLC gel filtration. The molecular mass of the ensuing glycan fractions was estimated by using chondroitin sulfates of 19.7, 28.8, 43.6, 75.8, and 101.6 kDa; heparins of 4.44, 8, and 11 kDa; and hyaluronic acid of 225 kDa as molecular weight markers. The total glycans were eluted as a major peak with K_{av}, the portion coefficient between the liquid phase and the gel phase, = 0.082 (Fig. 4); $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume and V_t and V_o are as defined in Fig. 4. The apparent molecular mass of this glycan fraction was estimated to be 340

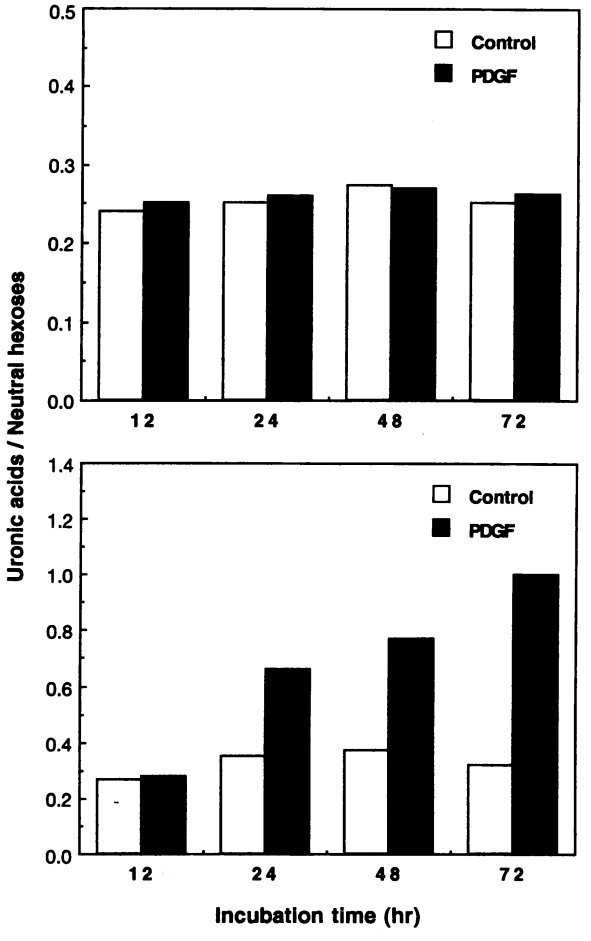

FIG. 3. Ratio of uronic acids to neutral hexoses in total glycans isolated and purified from the cell layer (*Upper*) and the supernatant (*Lower*). Cells were cultivated in the absence (control) or presence of PDGF-BB at 10 ng/ml for various periods of time. Values are means of double measurements in three different preparations. Maximal error was always <10%.

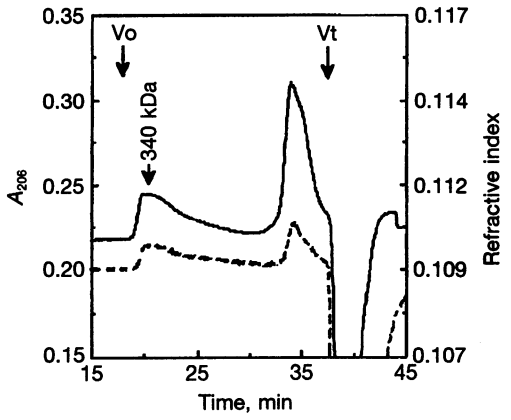

FIG. 4. HPLC gel filtration of total glycans. Total glycans were isolated from the supernatant of VSMC 72 h after incubation with PDGF as described in text. Seven micrograms of neutral hexoses in ddH_2O were applied on a Bio-Gel TSK 40XL gel filtration column (300 × 7.8 mm) equipped with a preceding column (40 × 7.8 mm). Elution was carried out with 7 mM Tris, pH 7.4/200 mM NaCl at a flow rate of 0.3 ml/min. Absorbance at 206 nm (—) and refractive index (---) were continuously recorded by a Bio-Rad HPLC system. V_o and V_t are the void volume and the total volume, respectively, of the gel bed. The refractive index was measured in volts.

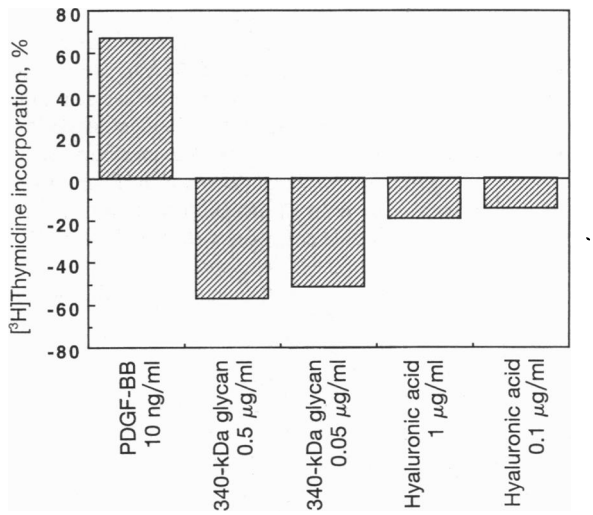

FIG. 5. [^{3}H]thymidine incorporation in VSMC after treatment with PDGF at 10 ng/ml, the 340-kDa glycan fraction, and commercially available hyaluronic acid. Results are expressed as percent of control (incubation in the presence of 0.5% FCS) and are the mean of two different experiments performed in quadruplicate. The maximal error was always <10%.

kDa. The peak which was eluted near V_t (K_{av} = 0.74) corresponds to glycan species with a molecular mass of <3.5 kDa and may be attributed to small degradation products during the isolation and purification procedure.

The total glycans isolated from the culture medium of VSMC 24 hr and 48 hr after stimulation with PDGF were also analyzed by HPLC gel filtration (data not shown). The elution pattern obtained was almost identical with the elution pattern shown in Fig. 4. However, there was an increase in the area under the curve corresponding to the 340-kDa glycan fraction from 24 hr to 72 hr. This increase coincides with the increase in the ratio of uronic acids to neutral hexoses observed from 24 hr to 72 hr, shown in Fig. 3 *Lower*.

Enzymological Characterization of the 340-kDa Glycan with GAG-Degrading Enzymes. To examine whether the 340-kDa glycan fraction shares common structural features to other well-characterized GAGs, enzymatic treatment with the specific GAG-degrading enzymes heparinase, heparitinase, chondroitinase ABC, keratanase, and hyaluronidase was performed. Digestion of the total glycans was initially evaluated by PAGE on 4–20% linear gradient gels stained with Alcian blue (data not shown) and then by HPLC gel filtration under continuous measurements of the absorbance at 206 and at 232 nm and of the refractive index. Digestion (percent) was calculated from the ratios of the area under the curve of buffer-treated substrates over the area under the curve of enzyme-treated substrates of (*i*) the absorbance at 206 nm, (*ii*) the absorbance at 232 nm, and (*iii*) the refractive index (Table 1). All GAG-degrading enzymes used could completely digest their respective specific substrates (chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, hyaluronic acid, keratan sulfate, heparan sulfate, and heparin). However, 3% of hyaluronic acid and 2% of chondroitin sulfate A could not be digested by their respective specific enzymes, which may be attributed to impurities. The 340-kDa glycan fraction could only be digested with hyaluronidase by 82%, indicating that the major part of this molecule shares a structure common to hyaluronic acid.

Functional Studies. The biological role of the 340-kDa glycan fraction was investigated by measuring the incorpora-

Table 1. Enzymatic treatment of 340-kDa glycan with GAG-degrading enzymes

	% digestion				
	Chase ABC	Heparinase	Heparitinase	Hyaluronidase	Keratanase
340-kDa glycan	0	0	0	82	0
CSA	98	0	0	0	0
CSB	100	0	0	0	0
CSC	100	0	0	0	0
H	0	100	0	0	0
HA	0	0	0	97	0
HS	0	30	100	0	0
KS	0	0	0	0	100

The purified 340-kDa glycan or the standard GAGs were digested with specific GAG-degrading enzymes as described in text. The digestion was monitored by HPLC gel filtration analysis. Absorbance at 206 and 232 nm and the refractive index were continuously recorded, and their area under the curve was measured by integration analysis with the Bio-Rad HPLC, version 2.3, software system. Digestion (%) was calculated from the ratios of the area under curve of buffer-treated substrates over the area under curve of enzyme-treated substrates of: (*i*) the absorbance at 206 nm, (*ii*) the absorbance at 232 nm, and (*iii*) the refractive index. In all cases (*i*, *ii*, and *iii*) the % digestion calculated was identical. The results shown are the means of two determinations from three different preparations; the maximal error was <10% of each value. CSA, chondroitin sulfate A; CSB, chondroitin sulfate B; CSC, chondroitin sulfate C; HA, hyaluronic acid; H, heparin; HS, heparan sulfate; KS, keratan sulfate; Chase ABC, chondroitinase ABC.

tion of [^{3}H]thymidine into the DNA of VSMC (Fig. 5). PDGF induced the proliferation of VSMC by 66.5% as compared with control (incubation in the presence of 0.5% FCS). Commercially available hyaluronic acid at 1 μg/ml inhibited the incorporation of [^{3}H]thymidine by 19.5%. The 340-kDa glycan fraction (0.5 μg/ml) inhibited cell proliferation by 57%. The antiproliferative effect of the 340-kDa glycan fraction was 3- to 4-fold greater than commercially available hyaluronic acid.

DISCUSSION

The effect of PDGF on total glycan synthesis by VSMC was studied by isolation; purification; and chemical, biochemical, and enzymological characterization of the total glycan molecules that are produced by human VSMC in culture in response to this mitogen. PDGF quantitatively and qualitatively influences the total glycan synthesis by VSMC. Total glycan synthesis by PDGF-stimulated VSMC is increased from 24 hr to 72 hr in the cell layer and in the culture medium. This effect of PDGF is apparently cell-type specific, since the growth factor did not influence the amount of total glycans synthesized by human fibroblasts, endothelial cells, or epithelial cells. In proliferating VSMC, PDGF stimulates the secretion of a hyaluronic acid species with a molecular mass of 340 kDa, exhibiting 80% homology to commercial hyaluronic acid. Amino acid analysis of the 340-kDa hyaluronic acid showed that this molecule is free of protein. This glycan was shown to have an antiproliferative activity on VSMC that was almost 4-fold greater than that of commercially available hyaluronic acid. Similar to our findings, a species- and cell-type specific acitivity of PDGF isoforms on GAG synthesis has been shown by others (25–27) and has been interpreted as a direct effect of PDGF on GAG chain-elongating enzymes.

The production of this highly acidic molecule by VSMC in response to PDGF coincides in time with the observed decrease in pH of VSMC culture medium and cell growth arrest observed 48 hr after the addition of the mitogenic stimulus (unpublished results). Change of the medium and PDGF supplementation are required for further cell proliferation. These results suggest that VSMC, in response to stimulation by growth factors such as PDGF, control their proliferation by secreting in the culture medium a specific hyaluronic acid, which lowers the pH of the medium and arrests cell growth. This agrees with evidence for a role of natural components, such as heparin and heparan sulfate species, which may exist within the vessel wall and oppose the action of growth factors and hence prevent VSMC proliferation (12, 27, 28).

The antiproliferative function of the 340-kDa hyaluronic acid molecule secreted by VSMC in response to PDGF also agrees with the known biological role of hyaluronic acid that is associated with cell migration. The secretion of this molecule by proliferating VSMC after stimulation by PDGF could reflect an autoregulatory mechanism by which VSMC control the mitogenic effect of PDGF. Since the development of atherosclerotic lesions involves the transition of VSMC from a quiescent to a proliferating phenotype, the 340-kDa glycan fraction may play a functional role in the pathophysiology of atherosclerosis. During the development of the atherosclerotic lesions, damage of the endothelium enhances the appearance of platelets and macrophages at the site of injury and the release of high concentrations of growth factors by these cells. It is possible that VSMC may try to counteract these pathologic alterations and mobilize antiproliferative mechanisms, by augmenting the synthesis and release of specific molecules, such as the above described 340-kDa hyaluronic acid species. Ultimately, VSMC migration to the luminal surface of the blood vessel wall and the ensuing proliferative response would be determined by the net balance between the elevated concentrations of mitogenic factors, such as PDGF, and the increased levels of free hyaluronic acid. Thus, this specific form of hyaluronic acid may be positioned to act as a negative control element during the regulation of cell proliferation and as a positive control element during the regulation of cell migration. We have recently reported that PDGF induces proliferation of nontransformed mesenchymal cells by inducing the expression of the interleukin 6 (IL-6) gene (29). When one considers the negatively regulating potency of hyaluronic acid on PDGF-dependent proliferation, events subsequent to the induction of the IL-6 gene and that of other interleukins involved in inflammation and wound-healing may also be controlled by hyaluronic acid-rich GAGs.

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