# Inhibition of Proteoglycan Synthesis Alters Extracellular Matrix Deposition, Proliferation, and Cytoskeletal Organization of Rat Aortic Smooth Muscle Cells in Culture

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*Abstract.* Arterial proteoglycans have been implicated in several important physiological processes ranging from lipid metabolism to regulation of smooth muscle cell growth. Vascular smooth muscle (VSM) cells are the major producers of proteoglycans in the medial layer of blood vessels. To study functional consequences of alterations in VSM proteoglycan metabolism we used 4-methylumbelliferyl-$\beta$-D-xyloside to inhibit proteoglycan synthesis in primary and early passage cultures of rat aortic smooth muscle cells. Biochemical analysis of cultures labeled with $^{35}SO_4$ showed the drug inhibited synthesis of different classes of proteoglycans by 50 to 62%. Inhibition of proteoglycan synthesis resulted in reduced accumulation of extracellular matrix, as shown by immunofluorescent staining with antibodies to chondroitin sulfate, fibronectin, thrombospondin, and laminin. There was also an inhibition of postconfluent (multilayered) growth of the smooth muscle cells, and a change in the morphology of the cells, with no apparent effect on subconfluent growth. In addition, in drug-treated cells there was a reduction in the number of cytoskeletal filaments that contained $\alpha$-actin, the actin subtype synthesized by differentiated VSM cells. This occurred even though the total content of $\alpha$-actin in the cells was not reduced. The effects of the inhibitor on growth and morphology could be reversed by switching the cultures to normal medium and could be prevented by growing the cells on preformed VSM extracellular matrix. These observations suggest the vascular extracellular matrix may play a role in regulating the growth and differentiation of smooth muscle cells.

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<span>T</span>HE extracellular matrix (ECM)[1] was once regarded as an inert substance whose only function was to provide mechanical stability to tissues. Recent studies have indicated, however, that the ECM is involved in a number of cell functions such as adhesion, migration, proliferation, and regulation of gene expression (2, 15, 23, 46). Several ECM components and their receptors have been identified and purified which has allowed for an examination of ECM function at the molecular level (10, 21, 41).

Proteoglycans are major components of ECM, but detailed knowledge of the specific functions of these molecules is still lacking. Evidence suggests proteoglycans play important roles in cell proliferation (11, 14, 34), cell matrix adhesion (8, 9, 26, 28, 29, 37), and overall ECM architecture (19, 44). One interesting and potentially important property of proteoglycans is their ability to bind to other matrix components such as fibronectin, laminin, and thrombospondin (13, 19, 30, 36, 37, 46).

Vascular smooth muscle (VSM) cells are the major producers of proteoglycans in the arterial wall (44). VSM cells grown in tissue culture have been a useful model to study proteoglycan synthesis. These studies have shown that the bulk of the proteoglycans synthesized by VSM cells are chondroitin sulfate and dermatan sulfate proteoglycans that are secreted into the culture medium (6, 45) to be incorporated into the extracellular matrix produced by these cells. VSM cells also synthesize lesser amounts of cell-associated heparan sulfate (6, 14). Recent studies have revealed VSM cells synthesize at least two types of cell associated heparan sulfate proteoglycan: one that appears to be a basement membrane proteoglycan, and a second type that is a hydrophobic cell surface proteoglycan (Hamati, H., and D. Carey, manuscript submitted for publication).

Previous studies have implicated VSM proteoglycans in the pathogenesis of cardiovascular diseases such as artherosclerosis (1, 42, 43). There is increased accumulation of proteoglycans in the walls of artherosclerotic vessels (43). Experiments in vitro have shown that proteoglycans can bind low density lipoproteins and may allow them to be taken up by smooth muscle cells and macrophages (1, 22). Also, there is evidence that glycosaminoglycans may regulate the proliferation of VSM cells. Commercial heparin (4), synthetic heparin oligosaccharides (5), and heparan sulfate isolated from VSM cell cultures (14) are potent inhibitors of

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1. *Abbreviations used in this paper:* $\beta$-D-xyloside, 4-methylumbelliferyl-$\beta$-D-xyloside; CHAPS, 3-([3-cholamidopropyl)dimethyl-ammonio]1-propane sulfonate); ECM, extracellular matrix; VSM, vascular smooth muscle.

VSM cell proliferation. This suggests heparan sulfate proteoglycans, possibly those made by VSM cells, are involved in keeping VSM cell proliferation low in normal healthy vessels.

In light of their potential importance we have initiated an investigation of the properties and functions of VSM cell proteoglycans. As one approach toward studying proteoglycan function we have used the specific inhibitor of proteoglycan synthesis, 4-methylumbelliferyl-$\beta$-D-xyloside ($\beta$-D-xyloside) to determine what functional consequences could be observed in VSM cell cultures in which proteoglycan synthesis was inhibited. In this paper we present evidence that inhibition of proteoglycan synthesis has no effect on the subconfluent growth of VSM cells, but it substantially inhibits the deposition of extracellular matrix and the postconfluent (multilayered) growth and differentiation of the cells. Effects of proteoglycan synthesis inhibition can be prevented by growing the cells on preformed VSM cell extracellular matrix.

## Materials and Methods

### Cell Cultures

VSM cells were obtained from medial strips dissected from aortas of male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA). The medial strips were dispersed to single cell suspensions by digestion with enzymes. The strips were placed in a solution of 0.3% collagenase (type CLS III; Worthington Biochemical Corp., Freehold, NJ) in DME (5 ml/4 aortas) and incubated in a humidified atmosphere containing 5% $CO_2$ with gentle rotation at 37°C for 1 h. The collagenase solution was removed and 0.1% elastase (Sigma Chemical Co., St. Louis, MO) in DME (3 ml/4 aortas) was added to the remaining tissue and incubation was continued for another hour. Finally, 0.3% collagenase (4 ml/4 aortas) was added to the elastase suspension and incubation was continued for one more hour. The dispersed cells were pelleted by centrifugation and resuspended in DME supplemented with 10% FCS (DME-FCS). The cells were seeded at 50,000 cells/cm² in plastic tissue culture dishes or glass coverslips. Cells were subcultured by trypsinization with 0.125% trypsin in Hank's balanced salt solution (HBSS) without $Ca^{++}$ and $Mg^{++}$. Cell culture supplies and media were purchased from Gibco Laboratories (Grand Island, NY). For the experiments reported here, cells were used as primary cultures or in the first or second passage.

### Proteoglycan Analysis

To assess the effects of $\beta$-D-xyloside on proteoglycan synthesis VSM cells were grown for 4 d in DME-FCS containing either no inhibitor or 1 mM $\beta$-D-xyloside and then incubated in Ham's F12 medium with 10% FCS and 100 $\mu$Ci/ml $^{35}SO_4$ (carrier-free sulfuric acid; ICN Radiochemicals, Irvine, CA) for 24 h in the presence or absence of the inhibitor. At the end of the labeling period the media were removed and dialyzed to remove unincorporated isotope. The cells were then extracted sequentially with 2% 3-([3-cholamidopropyl] dimethyl-ammonio)1-propanesulfonate (CHAPS), 0.1 M Tris-HCl, pH 7.5, and 2% SDS, 0.1 M Tris-HCl, pH 7.5, to solubilize membrane-associated and ECM proteoglycans, respectively. Aliquots of media and cell extracts were subjected to gel permeation HPLC on a TSK-4000SW column (Beckman Instruments, Inc., Palo Alto, CA) eluted with 0.1% SDS, 0.1 M Tris-HCl, pH 7.5, at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and radioactivity was counted by liquid scintillation counting to quantitate $^{35}SO_4$ incorporation into proteoglycans and glycosaminoglycans.

The proteoglycans were identified and characterized by their susceptibility to various chemical and enzymatic digestions. Glycosaminoglycan chains were released from proteoglycans by mild alkaline hydrolysis in 0.2 M NaOH at room temperature for 16–18 h. Nitrous acid hydrolysis was done in 0.25 M $NaNO_2$, 0.18 M acetic acid at room temperature for 2 h. Digestions with chondroitinases ABC and AC were carried out in 0.2 M Tris-0.2 M sodium acetate, pH 7.3, at 37°C for 4 h. Digestion products were analyzed by gel permeation HPLC on a TSK-4000SW column.

### Immunofluorescent Staining

Cells to be immunostained were grown on glass coverslips. The medium was removed and the coverslips were rinsed with PBS (0.15 M NaCl, 0.05 M sodium phosphate, pH 7.5). For staining of ECM proteins the cells were incubated with primary antibodies for 1 h at 4°C before fixation. After rinsing the cells were fixed with 3% paraformaldehyde for 30 min at room temperature. For staining of intracellular proteins the cells were fixed with paraformaldehyde and then permeabilized with ice-cold acetone or 0.05% Triton X-100 for 30 s. The cells were rinsed with Blotto (20 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 5% Carnation nonfat dry milk) (24) and then incubated for 1 h at room temperature with primary antibodies diluted in Blotto. After rinsing with Blotto, the cells were incubated with the appropriate affinity-purified fluorescein-conjugated secondary antibodies (Sigma Chemical Co.) diluted in Blotto for 1 h. The coverslips were rinsed with Blotto and mounted on glass slides. Fluorescein was visualized using a Nikon Diaphot inverted microscope equipped for epifluorescence.

Mouse monoclonal antibodies to vimentin and chondroitin sulfate were purchased from ICN Immunologicals (Irvine, CA). The latter antibody reacts with chondroitin-4 and chondroitin-6-sulfate glycosaminoglycans but not with dermatan or heparan sulfate. Mouse monoclonal anti–smooth muscle $\alpha$-actin, anti-thrombospondin, and anti-fibronectin as well as anti-fibronectin goat antiserum were purchased from Sigma Chemical Co. Anti-laminin rabbit antiserum was produced in this laboratory (3).

### Growth Assays

To examine effects of $\beta$-D-xyloside on VSM cell growth, cells were trypsinized and seeded at a density of 50,000 cells/cm² in four-well Nunc multidishes (Thomas Scientific, Philadelphia, PA) with 2 cm² growth area per well. The cells were grown in DME-FCS in the presence or absence of 1 mM $\beta$-D-xyloside. The cells in quadruplicate wells were counted 24–36 h after seeding and every few days for up to 10 d. For counting, the cells were released from the plates by trypsinization and counted electronically using a Coulter Counter Model ZM (Coulter Electronics Inc., Hialeah, FL).

To examine effects of secreted glycosaminoglycans on VSM cell growth the glycosaminoglycans were purified from conditioned media of $\beta$-D-xyloside–treated cells. Conditioned medium was collected over three consecutive 24-h periods from VSM cells growing in two 100-mm dishes, each containing ∼1.5 × 10⁷ cells. Radiolabeled medium glycosaminoglycans from $\beta$-D-xyloside–treated cells were added as tracer. The conditioned medium was dialyzed vs 0.1 M Tris-HCl, pH 7.5, and applied to 0.75 × 7.5 cm DEAE-5PW column (Beckman Instruments Inc.) and eluted with a linear gradient of 0–1 M NaCl containing 0.1 M Tris-HCl, pH 7.5. The fractions containing the radiolabeled glycosaminoglycans (which eluted at a salt concentration of ∼0.5 M) were pooled, dialyzed extensively vs. $H_2O$, and lyophilized. The dried glycosaminoglycans were dissolved in DME-FCS at approximately four times their concentration in the original conditioned medium and used to feed cells growing in four-well plates as described above.

To assess effects of exogenous ECM on cell growth, VSM cells were seeded at a density of 50,000 cells/cm² on either plastic or ECM-coated dishes (see below) and grown in DME-FCS containing 1 mM $\beta$-D-xyloside.

To prepare VSM cell ECM, postconfluent cultures were extracted with 2% deoxycholate, 0.1 M Tris-HCl, pH 7.5, for 20 min at 4°C. The dishes were washed extensively with ice-cold PBS and then used immediately for immunostaining or as a substrate for cell growth.

### Gel Electrophoresis and Immunoblotting

Gel electrophoresis and immunoblotting were used to estimate the amount of $\alpha$-actin in control and $\beta$-D-xyloside–treated cells. Groups of cultures were trypsinized and counted with a Coulter counter (to determine total cell numbers) or solubilized with 2% SDS, 0.0625 M Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol (for electrophoresis). Extracts from equal numbers of control and drug-treated cells were loaded onto 10% polyacrylamide slab gels prepared according to Laemmli (27). After electrophoresis the resolved proteins were electrophoretically transferred to nitrocellulose (70 V, 40 min), incubated in Blotto at room temperature for 1 h to block nonspecific binding, and stained with anti–$\alpha$-actin antibody (1:400 dilution in Blotto). The bound antibody was visualized with affinity-purified peroxidase-conjugated goat anti–mouse IgG (Bio-Rad Laboratories, Richmond, CA) using 4-chloro-1-naphthol as substrate.
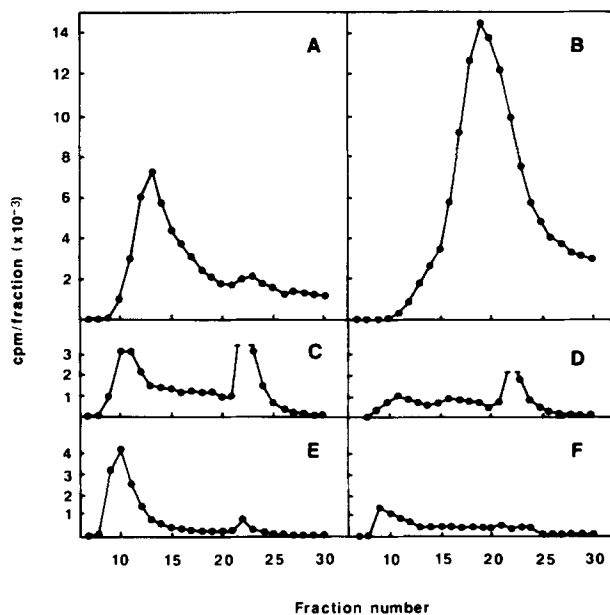
*Figure 1.* Gel permeation chromatography of proteoglycans and glycosaminoglycans of control and β-D-xyloside-treated cultures. Cells were grown in DME-FCS for 5 d without (*A*, *C*, and *E*) or with (*B*, *D*, and *F*) 1 mM β-D-xyloside and labeled for the last 24 h with ³⁵SO₄. After labeling the medium was removed and the cell layers were extracted sequentially with buffers containing 2% CHAPS and 2% SDS. Aliquots of the medium (*A* and *B*), CHAPS extracts (*C* and *D*), and SDS extracts (*E* and *F*) were subjected to gel permeation chromatography on a TSK-4000SW column.

## Results

### Effects of β-D-Xyloside on VSM Proteoglycan Synthesis

To study the effects of β-D-xyloside on proteoglycan synthesis aortic VSM cells were grown in plastic dishes in DME-FCS with or without 1 mM β-D-xyloside for 5 d. During the last 24 h ³⁵SO₄ was added to the medium to label the proteoglycans. At the end of the labeling the medium was removed and the cells were extracted with 2% CHAPS (to solubilize membrane-associated proteoglycans) followed by 2% SDS (to solubilize ECM proteoglycans). To assess ³⁵SO₄ incorporation into proteoglycans aliquots of the medium, nonionic detergent extracts, and SDS extracts were subjected to gel permeation chromatography on a TSK-4,000 SW column.

Column profiles from control cultures are shown in Fig. 1 (*A*, *C*, and *E*). As shown in Fig. 1 *A* the medium of control cultures contained a large radioactive peak that was eluted at fraction 13, a smaller shoulder in fractions 16–20, and a small peak at fraction 23. Chemical and enzymatic analysis revealed that the first peak was mostly chondroitin sulfate and dermatan sulfate proteoglycans, whereas the smaller shoulder was primarily free glycosaminoglycan chains (not shown). The small peak appearing at fraction 23 was unincorporated ³⁵SO₄. The elution profile of the CHAPS extract of the control cells showed a major peak that was eluted at fractions 10 and 11 (Fig. 1 *C*). We have shown that this material is a hydrophobic cell surface heparan sulfate proteoglycan (Hamati, H., and D. Carey, manuscript submitted for publication). The elution profile of SDS solubilized material

from control cells (Fig. 1 *E*) showed a major peak that was eluted at fraction 10. This peak contains basement mostly membrane heparan sulfate proteoglycans plus chondroitin sulfate proteoglycans that have been incorporated into ECM (Hamati, H., and D. Carey, manuscript submitted for publication).

Column profiles from cultures grown for 5 d in medium containing β-D-xyloside are also shown in Fig. 1 (*B*, *D*, and *F*). The amount of ³⁴SO₄-labeled proteoglycans appearing in the medium of β-D-xyloside-treated cells was reduced to 26% of the control value, while the radioactivity in the glycosaminoglycan peak was increased more than threefold (Fig. 1 *B*, Table I). As shown by results presented below, β-D-xyloside-treated cultures contain fewer cells than control cultures. When the ³⁵SO₄ incorporation data were normalized to cell numbers, incorporation of SO₄ into culture medium proteoglycans of β-D-xyloside-treated cells was found to be reduced to 38% of the control value, whereas ³⁵SO₄ incorporation into the medium glycosaminoglycans was increased by fivefold (Table I). The column profiles from CHAPS and SDS extracts of β-D-xyloside-treated cultures also showed a decrease in the amounts of heparan sulfate proteoglycans relative to the control cultures (Fig. 1, *D* and *F*). Quantitation revealed that SO₄ incorporation per culture into proteoglycans was reduced to 33 and 35% of the control, or 48 and 50% of control when normalized to cell number (Table I). Thus, β-D-xyloside treatment inhibited synthesis of secreted chondroitin and dermatan sulfate proteoglycans and to a lesser extent, cell-associated heparan sulfate proteoglycans. While β-D-xyloside treatment resulted in a large stimulation of glycosasminoglycan synthesis these glycosaminoglycans were found exclusively in the culture medium and did not become associated with the cells.

### β-D-Xyloside Inhibits ECM Protein Deposition in VSM Cell Cultures

In other studies inhibition of proteoglycan synthesis resulted in a decrease in the deposition of ECM (3, 31). We used indirect immunofluorescence staining with specific antibodies

*Table I. Effect of β-D-Xyloside on ³⁵SO₄ Incorporation into Proteoglycans and Glycosaminoglycan Synthesis*

| Species | cpm/culture | | | cpm/10⁵ cells | | |
|---|---|---|---|---|---|---|
| | −*xyl* | +*xyl* | +/− | −*xyl* | +*xyl* | +/− |
| Medium | | | | | | |
| Proteoglycans | 230,820 | 61,164 | 0.26 | 40,200 | 15,400 | 0.38 |
| Glycosamino-glycans | 279,876 | 954,576 | 3.4 | 48,800 | 241,800 | 5.0 |
| CHAPS-extracted proteoglycans | 104,160 | 34,110 | 0.33 | 18,100 | 8,600 | 0.48 |
| SDS-extracted proteoglycans | 98,490 | 33,990 | 0.35 | 17,200 | 8,600 | 0.50 |

VSM cells were grown in plastic dishes in DME-FCS without or with 1 mM β-D-xyloside for 5 d. During the last 24 h ³⁵SO₄ was present in the medium to label the proteoglycans. Parallel groups of cells were either harvested for biochemical analysis of radiolabeled proteoglycans, or trypsinized and counted to determine total cell numbers. ³⁵SO₄ incorporation in proteoglycans and glycosaminoglycans was determined by gel permeation HPLC and liquid scintillation counting (see Fig. 1).
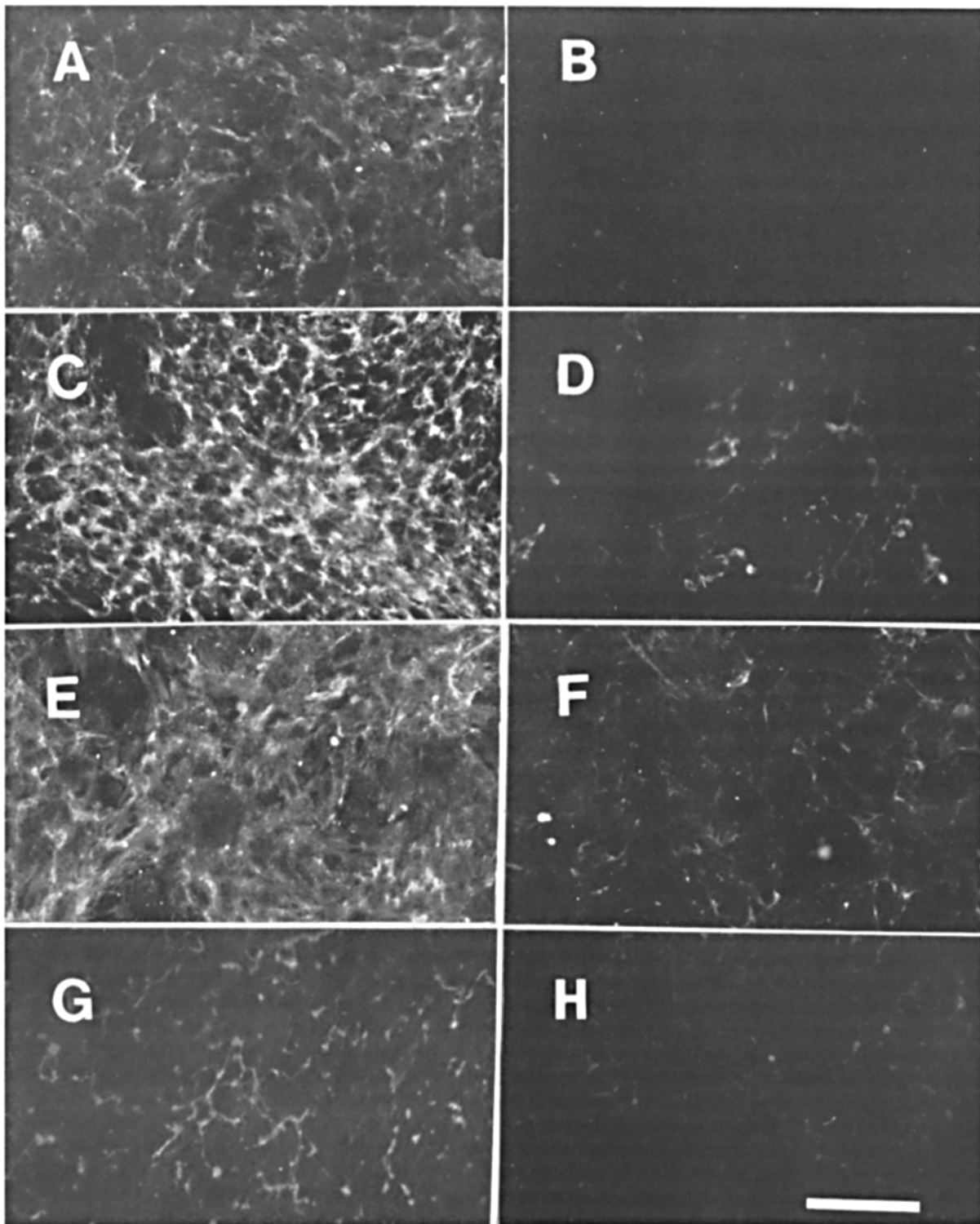
*Figure 2.* Effects of β-D-xyloside on accumulation of ECM proteins. VSM cell cultures were grown in DME-FCS without (*A, C, E,* and *G*) or with (*B, D, F,* and *H*) 1 mM β-D-xyloside. After 7 d the cells were processed for indirect immunofluorescent staining with anti–chondroitin sulfate antibodies (*A* and *B*), anti–fibronectin antibodies (*C* and *D*), anti–laminin antibodies (*E* and *F*), and anti–thrombospondin antibodies (*G* and *H*). Fields were photographed and printed under identical conditions. Bar, 100 μm.

to determine the effect of inhibition of proteoglycan synthesis on ECM protein deposition in VSM cell cultures. Control cultures stained with anti–chondroitin sulfate antibodies showed bright and specific fibrillar staining of the ECM (Fig. 2 *A*). As shown in Fig. 2 *B* staining by this antibody was nearly abolished by the drug treatment. Similar results were obtained after staining with antibodies to fibronectin, thrombospondin, and laminin (Fig. 2). A decrease in the deposi-
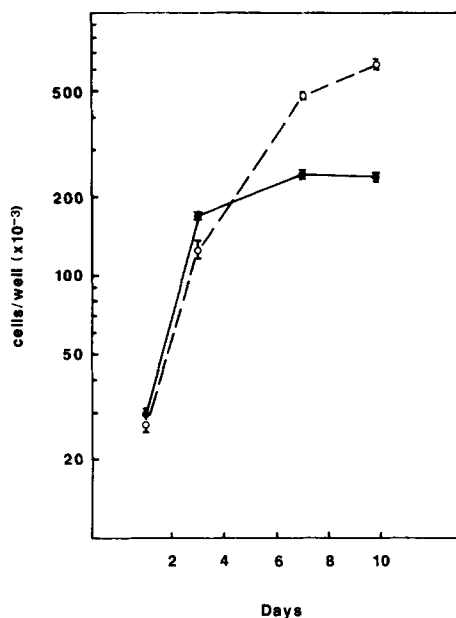
*Figure 3.* Effects of β-D-xyloside on VSM cell growth. VSM cells were seeded in four-well plates and grown in DME-FCS without (○) or with (●) 1 mM β-D-xyloside. Cells were trypsinized and counted after 24 h and every few days thereafter. Each point represents the mean ± SD of four cultures.

tion of ECM in β-D-xyloside–treated cultures was also observed by transmission electron miscroscopy (not shown). These results demonstrate that inhibition of proteoglycan synthesis leads to a decreased deposition of both proteoglycans and glycoproteins into ECM.

### Effects of β-D-Xyloside on VSM Cell Growth and Morphology

To assess the effects of β-D-xyloside treatment on VSM cell growth freshly trypsinized cells were seeded in plastic dishes at low density and maintained in DME-FCS with or without the drug. Cells were released from the dishes by trypsinization and cell numbers were determined every few days. The presence of the drug had no effect on cell attachment measured 24 h after seeding or initial (subconfluent) growth rates (Fig. 3). In these experiments the cells became confluent on day 4 after plating. As is typical for VSM cells in culture the control cells continued to proliferate after they reached confluence to form multilayers with cells growing on top of one another. This phenomenon can be seen by observing the cells with differential interference contrast microscopy (Fig. 4 *A*).

In contrast, proliferation of the β-D-xyloside–treated cells stopped when the cells became confluent and cell numbers remained nearly constant from day 4 after plating until day 10. As revealed by differential interference contrast microscopy these cells formed a monolayer and did not exhibit the typical multilayered growth (Fig. 4 *B*). Thus, β-D-xyloside treatment inhibits the postconfluent but not subconfluent growth of VSM cells.

In addition to these changes in the pattern of VSM cell growth, β-D-xyloside treatment also caused a change in the morphology of the VSM cells. As shown in Fig. 5 *A*, the control cells showed the typical spindle shape characteristic of

these cells. The drug-treated cells, in contrast, were rounded or cuboidal in shape (Fig. 5 *B*).

These effects of the drug on growth and morphology were reversible. When cultures that had been grown for 9 d in the presence of 1 mM β-D-xyloside were switched to normal medium, within 48 h the cells regained their spindle morphology and began to proliferate (not shown). In other experiments we showed that DMSO, which was used as a vehicle for the drug, and α-methylxyloside, an inactive xylose derivative, had no effects on the growth or morphology of VSM cells (data not shown).

### Effects of β-D-Xyloside on VSM Cell Growth Are Not Caused by the Increase in Medium Glycosaminoglycans

As shown above, inhibition of proteoglycan synthesis by β-D-xyloside also caused an increase in the amount of glycosaminoglycans secreted into the medium. Heparin (a glycosaminoglycan related to heparan sulfate) is a potent inhibitor of VSM cell proliferation (4, 5). To determine whether the glycosaminoglycans present in the medium of β-D-xyloside–treated culture were responsible for the inhibition of VSM cell growth, we purified the glycosaminoglycans from medium conditioned by β-D-xyloside–treated cultures and added them to the medium of cells growing in DME-FCS without β-D-xyloside. As shown in Table II, there was no detectable inhibition of growth caused by addition of the glycosaminoglycans.

### Effects of Exogenous ECM

Another effect of inhibition of proteoglycan synthesis by β-D-xyloside is inhibition of ECM deposition. If the lack of ECM is responsible for effects of β-D-xyloside on VSM growth and morphology, it should be possible to prevent these effects by providing the cells with exogenous ECM. For these experiments, VSM-ECM was obtained by growing cells to a high density and then removing the cells by extraction with 2% deoxycholate. This treatment does not remove the ECM deposited by the cells as shown by immunofluorescent staining with antibodies to chondroitin sulfate, fibronectin (Fig. 6), laminin, and basement membrane proteoglycans (not shown). That deoxycholate extraction removed the cells was demonstrated by lack of staining with anti–vimentin antibodies (not shown).

To examine effects of exogenous ECM on VSM cell growth, cells were seeded on plastic or ECM-coated dishes and grown in DME-FCS containing 1 mM β-D-xyloside for 7 d. As shown in Table III, the final density of cells grown on ECM-coated plates was increased significantly in comparison to cells on plastic (which had formed a confluent monolayer). In addition to this effect on growth, cells grown on ECM in the presence of β-D-xyloside displayed normal morphology. Fig. 5 compares the appearance of cells grown on plastic without (*a*) and with (*b*) 1 mM β-D-xyloside with cells grown on ECM with 1 mM β-D-xyloside (*c*). The drug-treated cells on ECM were spindle shaped and were indistinguishable from the control cells grown on plastic.

### Effects of β-D-Xyloside on α-Actin Expression

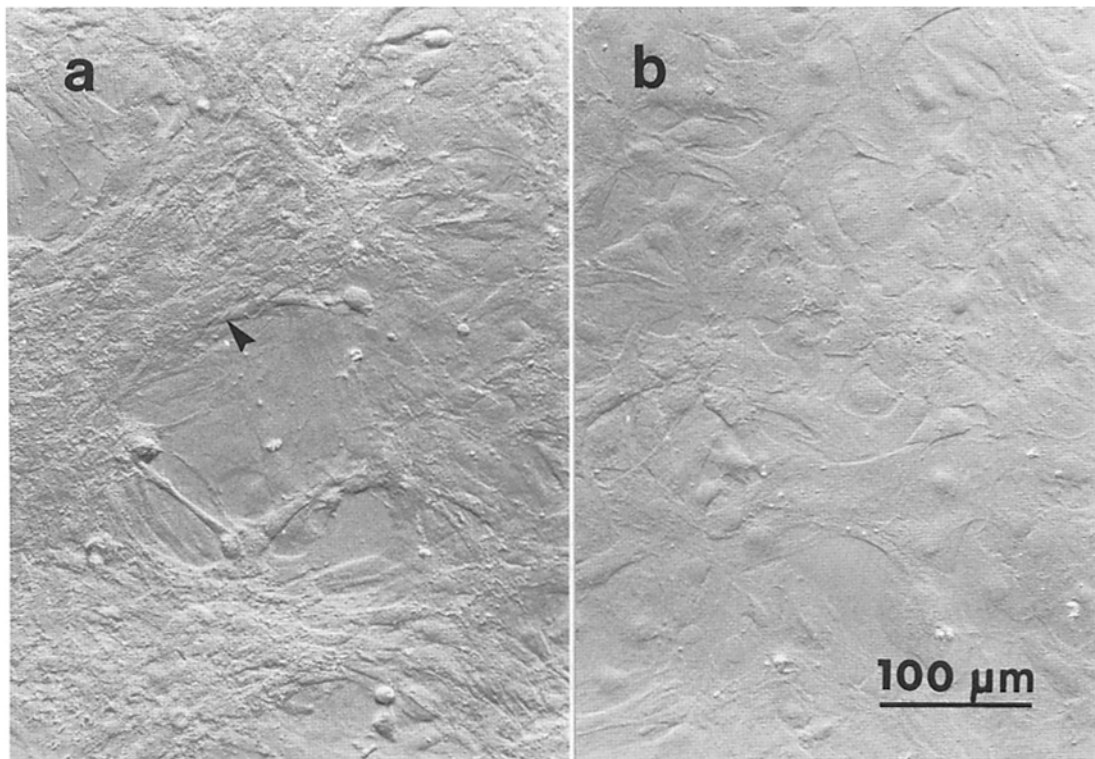The results presented above demonstrate that inhibition of proteoglycan synthesis affects the growth and morphology of

*Figure 4.* Effects of β-D-xyloside on VSM cell multilayered growth. VSM cells were grown in DME-FCS without (*a*) or with (*b*) 1 mM β-D-xyloside for 5 d. Cells were visualized by differential interference contrast microscopy. The arrow shows a thickened area where the VSM cells were beginning to grow on top of one another.

VSM cells; these effects appear to be due to the lack of ECM in these cultures. To determine whether β-D-xyloside treatment had any effect on the phenotypic differentiation of the VSM cells we examined the appearance of smooth muscle specific α-actin in the cells. α-Actin is the predominant actin subtype synthesized by differentiated VSM cells. It has been shown that VSM cells in culture stop producing α-actin when they are plated at subconfluent densities and are growing rapidly, but reexpress α-actin when they become postconfluent and quiescent (33). α-Actin expression was examined by staining with a specific monoclonal anti–smooth muscle α-actin antibody. The specificity of the antibody was demonstrated by showing that it did not stain rat liver or skeletal muscle actin but did stain rat aortic smooth muscle actin on Western blots (not shown).

Immunofluorescent staining of a postconfluent VSM cell culture grown for 10 d in DME-FCS is shown in Fig. 7 *A*. The antibody stained thick actin bundles that in most cells were arranged in parallel arrays along the long axis of the cells. When cells that had been grown for 10 d in medium containing 1 mM β-D-xyloside were stained with this antibody there was essentially no staining of actin bundles (Fig. 7 *B*). Some antibody staining could be seen but it was either diffusely distributed in the cytoplasm or present in small punctate spots throughout the cytoplasm. This effect of β-D-xyloside on α-actin filaments was reversible. Fig. 7 *C* and *D* shows anti–α-actin staining of cells that were grown for 7 d in medium containing β-D-xyloside and switched to medium lacking the drug 3 d before staining. These cells

contained bundles that stained brightly with the α-actin antibody, and were indistinguishable from the control cultures.

The immunofluorescence staining results could be explained if inhibition of proteoglycan synthesis by β-D-xyloside led to inhibition of α-actin synthesis. To determine whether α-actin synthesis was blocked, extracts of cells were separated on 10% polyacrylamide gels, transferred to nitrocellulose, and stained with anti–α-actin antibody. As shown in Fig. 8, when extracts from equal numbers of control and β-D-xyloside–treated cells were loaded on the gel, there was no visible difference in the amount of α-actin staining. Thus, β-D-xyloside treatment does not appear to alter the synthesis of α-actin, but interferes with its assembly into filaments.

## Discussion

The vascular wall is enriched in connective tissue (44). Previous studies have shown that a major component of this ECM is proteoglycans synthesized by VSM cells (1, 6, 42, 43, 44, 45). In the studies reported here we used β-D-xyloside, a specific inhibitor of proteoglycan synthesis, to examine the effects by inhibition of proteoglycan synthesis on the behavior of cultured VSM cells. Biochemical analysis showed that 1 mM β-D-xyloside inhibited proteoglycan synthesis by 50–62%, depending on the class of proteoglycan, and stimulated glycosaminoglycan synthesis fivefold. Inhibition of proteoglycan synthesis was also confirmed by immunofluorescent staining of cultures with anti–chondroitin sulfate antibodies. These observations are consistent with the mode of
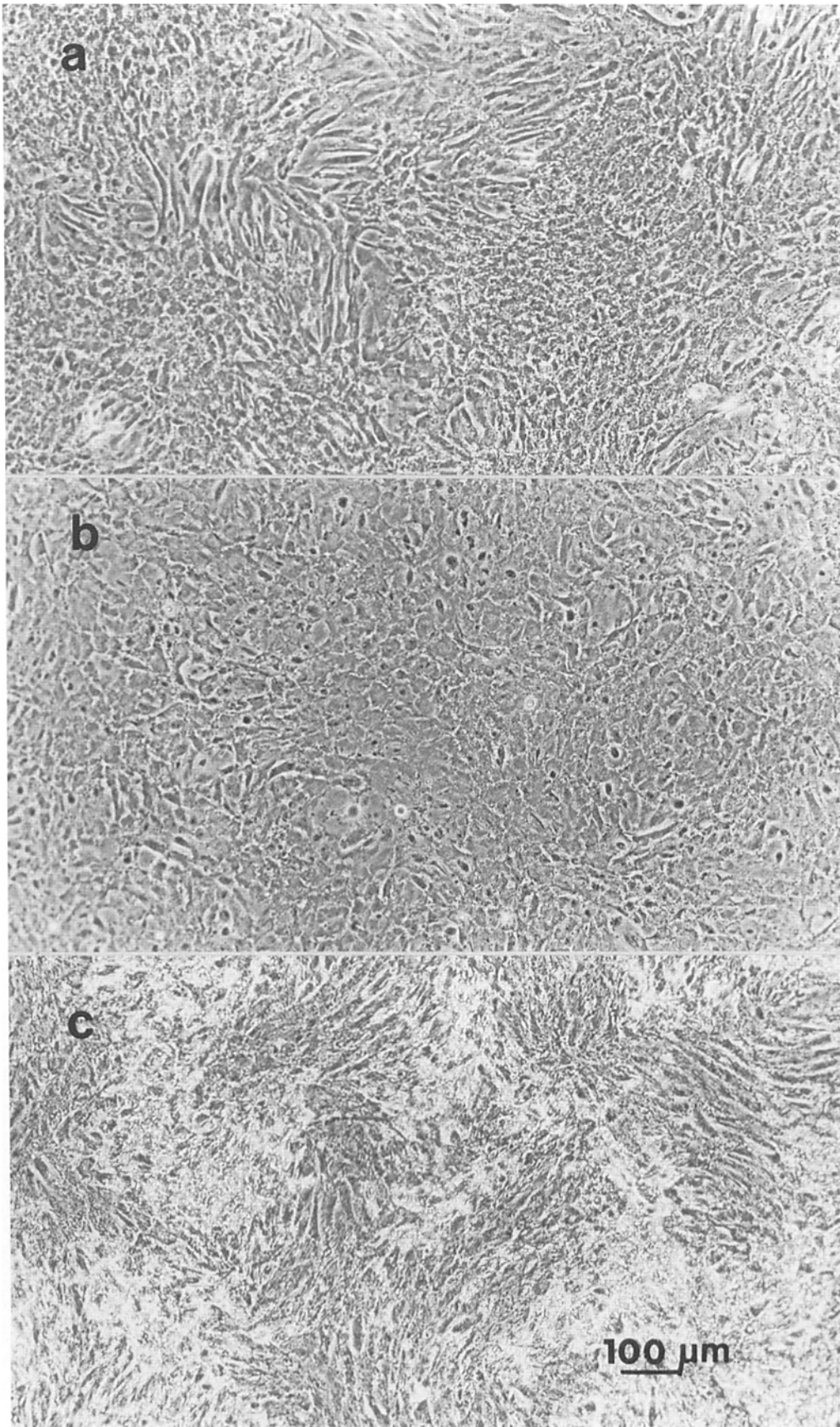
*Figure 5.* Effects of β-D-xyloside on VSM cell morphology. VSM cells were grown in DME-FCS for 7 d on plastic (*a* and *b*) or ECM-coated (*c*) dishes in the absence (*a*) or presence (*b* and *c*) of 1 mM β-D-xyloside. Cells were visualized by phase contrast microscopy.

## Table II. Effect of Secreted Glycosaminoglycans on VSM Cell Proliferation

| Additions* | Cells per well‡ | Percent control |
|---|---|---|
| None | 921,160 ± 13,680 | 100 |
| β-xyl GAG | 999,720 ± 20,800 | 109 |

* Glycosaminoglycans were partially purified by anion exchange chromatography from medium of β-D-xyloside–treated cells (β-xyl GAG) (see Material and Methods).
‡ Cells were seeded (5 × 10⁴ cells/cm²) in four-well plates (2 cm² growth area per well) and grown in DME-FCS with or without the purified glycosaminoglycans; after 7 d the cells were counted; the values shown are the mean ± SD of four cultures.

action of β-D-xyloside, which is to act as a primer for glycosaminoglycan chain initiation and compete with the core protein at the level of the first glycosyltransferase, which adds galactose to a xylose residue on the core protein. The drug

appears to be specific in its action. It has been demonstrated by immunological techniques that in chondrocytes β-D-xyloside does not affect core protein synthesis (38). Other studies with chondrocytes have shown that β-D-xyloside has only a slight effect on total protein synthesis as measured by [³H]serine incorporation (31).

Our studies indicated that inhibition of proteoglycan synthesis had profound effects on the phenotypic behavior of VSM cells. We observed an inhibition of postconfluent multilayered growth of the cells, an alteration of their morphology, and an inhibition of the polymerization of smooth muscle α-actin into filaments. These effects appeared to be due to decreased deposition of ECM proteins that occurred in β-D-xyloside–treated cultures. VSM cells grown on exogenous ECM-coated dishes in β-D-xyloside containing medium exhibited normal morphology and growth characteristics.

Several observations indicated that the alterations in cell behavior observed in this study are not attributable to toxicity of the drug. First, the extent of attachment of control cells
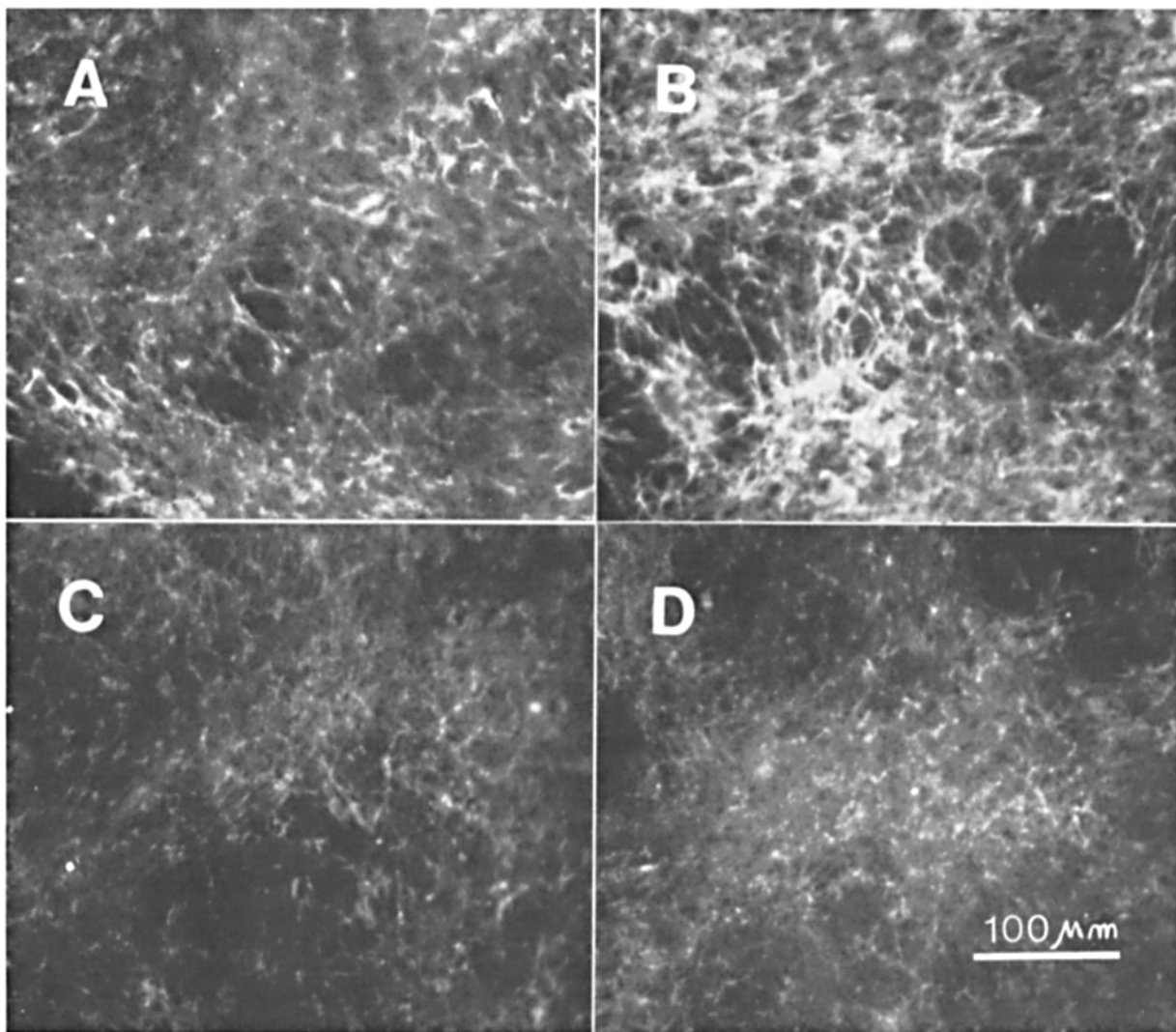


Figure 6. Immunostaining of ECM proteins in cultures before and after removal of the cells by detergent. VSM cells were grown in DME-FCS for 7 d. Control cultures (a and c) and cultures from which cells were removed by extraction with 2% deoxycholate (b and d) were processed for indirect immunofluorescent staining with anti–chondroitin sulfate antibodies (a and b) and anti–fibronectin antibodies (c and d). Fields were photographed and printed under identical conditions.

and β-D-xyloside–treated cells to plastic dishes was identical. Second, β-D-xyloside had no effect on subconfluent growth rates of the cells during the first 3 d after plating.

Third, in long term experiments with β-D-xyloside the cell numbers remained constant, indicating there was no appreciable cell death even after long exposure to the drug. Fourth, effects of β-D-xyloside on VSM growth and morphology were rapidly reversed by switching to medium lacking the drug. Fifth, the effects of β-D-xyloside on growth and morphology were prevented by growing cells on ECM-coated dishes.

Previous studies have shown that the glycosaminoglycan heparin is a potent inhibitor of VSM cell proliferation (4) and that the inhibition is caused by a specific structural variant of heparin (5). Our results demonstrated, however, that the glycosaminoglycans secreted by β-D-xyloside–treated cells do not possess this activity. When partially purified glycosaminoglycans from the medium of β-D-xyloside–treated cultures were added to cells grown in DME-FCS there was no effect on VSM proliferation or morphology. This occurred
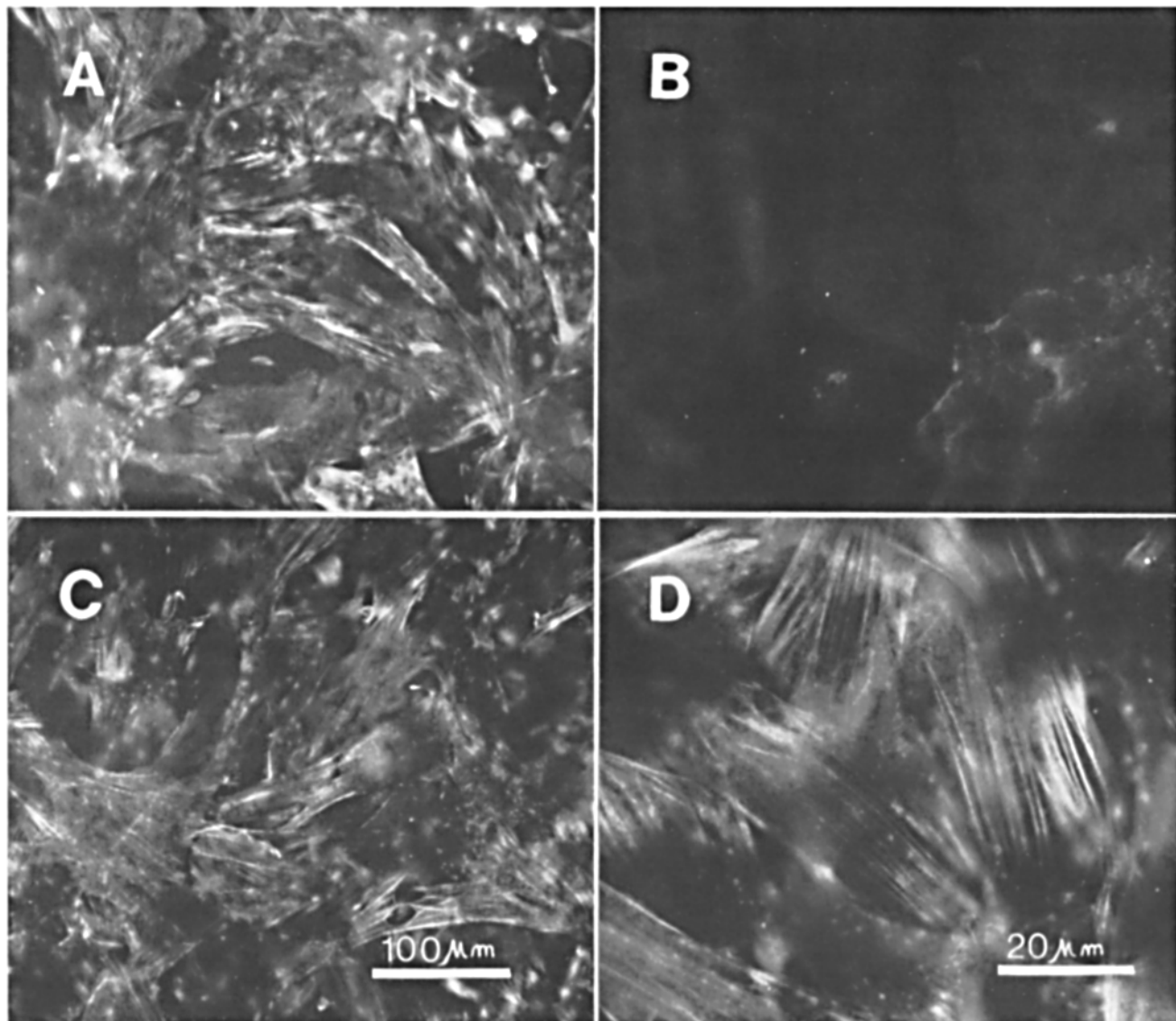


*Figure 7.* Effects of β-D-xyloside on α-actin distribution. VSM cells were grown in DME-FCS for 10 d without (a) or with (b) 1 mM β-D-xyloside or for 7 d in DME-FCS containing 1 mM β-D-xyloside followed by 3 d in DME-FCS alone (c and d). On day 10 the cells were processed for indirect immunofluorescent staining with smooth muscle–specific anti–α-actin antibodies. A-C were photographed and printed under identical conditions to demonstrate the difference in staining intensity. D is a high power view to show the fibrillar organization of the α-actin.
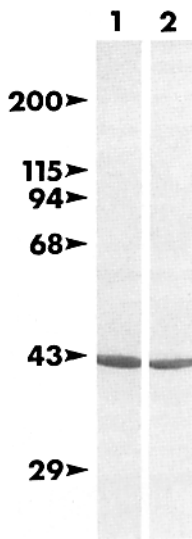
*Figure 8.* Effects of β-D-xyloside on the α-actin content of VSM cells. VSM cells were grown in DME-FCS for 10 d without (lane *1*) or with (lane 2) 1 mM β-D-xyloside. Parallel cultures were trypsinized and counted or extracted with 2% SDS for electrophoresis. Extracts from equal numbers of cells were loaded on a 10% polyacrylamide SDS slab gel. After electrophoresis the proteins were electrophoretically transferred to nitrocellulose and stained with anti-α-actin antibody. Numbers and arrows to the left indicate position of migration of molecular mass standards (in kilodaltons).

even when the glycosaminoglycans were added at four times their original concentration in the medium of drug-treated cells. In other experiments we have shown that our VSM cells are inhibited in their proliferation by commercial heparin or heparan sulfate proteoglycan purified from postconfluent VSM cell cultures (Hamati, H., and D. Carey, manuscript submitted for publication).

Our data indicated that an adequate supply of proteoglycan is necessary for deposition of VSM ECM. Extracellular matrix assembly is a highly cooperative process (25, 47). Proteoglycans are essential probably because of their ability to form complexes with other ECM molecules (19). Some arterial chondroitin sulfate proteoglycans are able to form large supramolecular complexes with hyaluronic acid in a manner similar to that of cartilage proteoglycans (42). In that tissue a specific region of the chondroitin sulfate proteoglycan core protein binds to hyaluronic acid, and the binding is stabilized by a glycoprotein called link protein (17). The large ($M_r$ = $10^8$) highly hydrated aggregates are important in determining the mechanical properties of cartilage and presumably the arterial wall. Other proteoglycans, especially heparan sulfate proteoglycans, bind via their glycosaminoglycan side chains to ECM proteins such as fibronectin, laminin, collagen, and thrombospondin (13, 19, 30, 32, 36, 39). Because of their multiple side chains the proteoglycans serve as multivalent cross-linkers in the ECM. Proteoglycans are also present on cell surfaces and have been proposed to act as receptors for collagen and fibronectin (26, 37).

Our data also indicated that lack of ECM resulted in altered morphology of VSM cells and a failure of organization of α-actin filament. A relationship between cytoskeletal organization and ECM contact has been observed in other cells. For example, corneal epithelial cells become columnar and reorganize their actin-rich cortical cytoskeleton when placed on collagen or basement membrane (15, 40). These observations are consistent with other experiments that have shown an apparent linkage between ECM and cytoskeletal components mediated by transmembrane receptors. It was shown that mesenchymal cells bind to fibronectin via a cell surface receptor known as the 140-K complex (7, 10, 21).

Biochemical and structural studies have suggested that the fibronectin receptor is a hydrophobic transmembrane protein (41). Other studies have shown that the 140-K complex colocalizes with fibronectin and microfilaments (10), and binds to talin (20), a cytoskeletal protein, thus suggesting a possible mechanism for fibronectin cytoskeletal interactions.

Our results demonstrated that inhibition of proteoglycan synthesis prevented the characteristic multilayered growth of VSM cells. Results with exogenous ECM suggested this is due to the lack of ECM in drug-treated culture. Previous studies have also demonstrated a relationship between ECM and VSM cell growth. It was shown that bovine aortic VSM cells grown on corneal endothelial-derived ECM proliferate in plasma-containing medium, whereas VSM cells grown on plastic proliferate only in the presence of serum (16). It has also been shown that rat VSM cells grown on bovine aortic endothelial cell–derived ECM have altered growth rates and final cell densities than cells grown on plastic (18). The mechanism by which cell growth is regulated by ECM is not well-understood. One possibility is that the ECM regulates growth by altering the shape of the cells. Changes in cell shape have been shown to alter the growth properties of cells (12), although the underlying mechanism is not known. In the case of postconfluent VSM cell growth, it is conceivable the ECM provides a three-dimensional adhesive scaffold that is needed for the cells to grow on the top of one another.

This relationship between ECM deposition and VSM cell growth and differentiation may have relevance to certain cardiovascular diseases. VSM proliferation is a major event in the development of artherosclerosis. VSM cells and ECM are major components of the fibrous plaques found in atherosclerotic vessels (35). Our data indicate that under certain conditions ECM production is obligatory for VSM cell proliferation. For these reasons, further investigations into the regulation of ECM production in vascular tissue and the mechanism of the effects of ECM on VSM cells are of obvious clinical importance.

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