

Endothelial Heparan Sulfate Is Necessary but Not Sufficient for Control of Vascular Smooth Muscle Cell Growth

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The state of the endothelial cell (EC) determines the nature of its control of vascular smooth muscle cell (vSMC) biology. Conditioned medium from postconfluent ECs inhibits vSMC proliferation, whereas subconfluent conditioned medium from the same ECs has a stimulatory effect. We and others have identified confluent endothelial cells' production of heparan sulfate proteoglycans (HSPG) as critical to vSMC growth control. The question that arises is whether the stimulation that is observed with subconfluent cells is from (1) aberrant HSPG production, (2) elaboration of noninhibitory species of HSPG, or (3) production of other factors, such as mitogens, which counteract the inhibitory HSPG to stimulate vSMCs. We studied the relative effects of conditioned medium produced by both subconfluent and postconfluent EC cultures on vSMC growth. Conditioned medium was fractionated into nonproteoglycan (non-PG) and proteoglycan (PG) components by anion-exchange chromatography. The PG fractionation profile and the antiproliferative activity of the HSPGs isolated from both subconfluent and postconfluent EC-conditioned media were similar. However, the HSPG fraction alone could not approach the inhibitory potential of unfractionated conditioned medium from postconfluent EC cultures. Non-PG proteins produced by the endothelial cultures had no effect on vSMC growth on their own. Yet, when they were mixed together with HSPG fractions, from either subconfluent or postconfluent EC cultures, the full growth effects were returned. Non-PG protein fractions from postconfluent cultures with HSPG fractions gave maximal inhibition of vSMC growth, whereas non-PG protein fractions from subconfluent EC cultures with HSPG fractions produced the maximal stimulation. Thus, whereas the net stimulatory or inhibitory effect on vSMC growth of EC-conditioned medium is density dependent, this effect does not result from a difference in the antiproliferative heparan sulfate component but rather from non-PG proteins that interact with the heparan sulfates. *J. Cell. Physiol.* 184:93–100, 2000.

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A characteristic feature of the healthy arterial wall is that the intimal endothelial cells (EC) and the underlying vascular smooth muscle cells (vSMC) are in a quiescent growth state. As long as the endothelium is healthy and intact, blood flow continues smoothly without thrombosis, platelet activation, adhesion, or aggregation; leukocytes glide over the arterial surface without adhering or diapedesing; and the arterial lumen remains patent with a limited intima. If the endothelium is damaged (Fishman et al., 1975; Schwartz et al., 1975; Davies and Hagen, 1993; Rubanyi, 1993), as may occur following vascular manipulation with angioplasty, implantation of endovascular devices, or vascular bypass grafting, the full range of biological phenomena are altered, culminating in stimulation of vSMCs migration, proliferation, and accumulation within the

intima. Stimulation of growth continues until the endothelial layer is restored, and if the processes progress

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sufficiently the lesions that form produce a set of accelerated arteriopathies, termed restenosis, that require clinical reintervention in 30 to 50% of cases.

The centrality of endothelial integrity stems not simply from the maintenance of an intact physical barrier, but from the retention of a delicate homeostatic biochemical equilibrium created by the balanced secretion of agents that can stimulate or inhibit each of the cellular events involved in the vascular response to injury. A natural endothelial cell product with great regulatory potential, heparan sulfate, inhibits not only thrombosis and accumulation of inflammatory cells but vascular smooth muscle cell proliferation and neointimal thickening as well (Clowes and Karnovsky, 1977; Castellot et al., 1981; Edelman et al., 1990). Postconfluent ECs themselves (Van Buul-Wortelboer et al., 1986; Fillinger et al., 1993), or their conditioned medium, inhibit vSMC growth (Castellot et al., 1981, 1982; Dodge et al., 1993; Nugent et al., 1993) in a manner dependent on endothelial-derived heparin/heparan sulfate proteoglycans (HSPG) (Castellot et al., 1987; Scott-Burden and Buhler, 1988). Addition of purified heparin or endothelial-derived HSPG to vSMC cultures inhibited cell growth *in vitro* (Castellot et al., 1981; Scott-Burden and Buhler, 1988; Au et al., 1993) and neointimal proliferation in small animal models of vascular disease (Clowes and Karnovsky, 1977; Guyton et al., 1980; Lindner et al., 1992; Völker et al., 1995).

It has been hypothesized that the *in vivo* effect (Pukac et al., 1991; Bingley et al., 1998) stems, in a major part, from the ability of this drug to restore the biochemical control lost with denudation of the endothelium. Indeed, the perivascular implantation of ECs grown in gelfoam wrapped around the injured vessel was especially effective in this regard, whereas mutant Chinese hamster ovarian cells deficient in HSPG had no effect on neointimal growth (Nathan et al., 1995). Local delivery of heparin alone is less effective in inhibiting neointima formation than is perivascular implantation of intact endothelial cells (Nathan et al., 1995; Nugent et al., 1999), indicating that other factors besides heparin are required for maximal inhibition. Moreover, heparin has failed to control intimal hyperplasia in complex animal models of vascular injury (Hanke et al., 1992; Geary et al., 1995; Hardhammer et al., 1996) as well as in clinical trials (Ellis et al., 1989; Faxon et al., 1994; Brack et al., 1995; Cairns et al., 1996). At the present time, the mechanism of heparin-like compounds in maintaining vascular homeostasis is still unclear.

The role of heparin-like molecules becomes all the more interesting when one considers that vSMCs can be stimulated by subconfluent ECs in coculture conditions (Gajdusek et al., 1980; Davies and Kerr, 1982; Graham et al., 1991; Fillinger et al., 1993) or by conditioned medium by subconfluent EC (Gajdusek et al., 1980; Castellot et al., 1981). The difference between the actions of subconfluent and postconfluent EC on vSMC growth was believed to be centered on the production of HSPG. In this study we sought to determine whether lack of inhibition of subconfluent EC-conditioned medium arises from (1) aberrant HSPG production, (2) elaboration of noninhibitory species of HSPG, or (3) production of nonproteoglycan (non-PG) factors, such as mitogens, which stimulate vSMCs. We now report

that both subconfluent and postconfluent EC-conditioned medium produce HSPGs that are inhibitory for vSMC growth. However, subconfluent and postconfluent EC cultures differ in their non-PG protein products such that the net stimulatory or inhibitory effect of the EC-conditioned medium on vSMC growth arises from the complex interaction of non-PG proteins with heparan sulfate.

MATERIALS AND METHODS

Cell culture

Endothelial cells isolated from bovine aortas by collagenase dispersion method (Wong and Gotlieb, 1984) were used in the second to sixth passages after harvest. Vascular smooth muscle cells were cultured from bovine aortas by the explant method (Koo and Gotlieb, 1989) and used only in the second to fifth passages after harvest. All cultures were grown in DMEM supplemented with 5% calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and glutamine (100 mmol/ml). The culture medium was changed every 48 h. All cultures were incubated in a humidified atmosphere of 10% CO₂ at 37°C. Cells were passaged by trypsinization. All cell culture reagents were obtained from GIBCO (Grand Island, NY).

Preparation of EC-conditioned and radiolabeled media

In preparation for conditioning, cultures of endothelial cells in DMEM containing 5% calf serum were established as subconfluent (1×10^6 cells/100-mm dish) or maintained for 3 days postconfluency (4×10^6 cells/100-mm dish). The cells were then washed and incubated for 30 min in serum-free DMEM at 37°C, followed by a 24-h incubation in serum-free DMEM (phenol-red free, GIBCO) supplemented with penicillin, streptomycin, and glutamine. Metabolic labeling was performed utilizing Na₂³⁵SO₄ (New England Nuclear, Boston, MA), at a concentration of 50 µCi/ml. Conditioned medium was centrifuged to remove cellular debris and either used immediately or frozen for use at a later time. Initial experiments were performed to demonstrate that fresh and frozen conditioned media gave equivalent results before the frozen media was used routinely. To optimize proteoglycan recovery, urea (Mallinckrodt Biochemicals, Paris, KY) was added to a final concentration of 1 M.

Cell numbers were determined by a Coulter counter (Coulter Electronics Limited, Miami, FL) after trypsin dislodgment, and maintained as equivalent between the two density conditions.

Conditioned media analysis

Separation of conditioned medium components by chromatography. To prepare the conditioned medium for chromatography and to remove unincorporated radiolabel, the conditioned medium was dialyzed for 48 h against TRIS ± urea buffer (20 mM TRIS [pH 8.0] ± 1 M urea) using Spectra/Por 6 membranes (MWCO 8,000; Spectrum, Houston, TX). In some studies the conditioned medium was passed through a Sephadex G-50M gel-filtration column (Pharmacia Biotech, Piscataway, NJ) to remove the unincorporated radiolabel and to equilibrate the medium in the TRIS buffer. The equilibrated conditioned medium from

either method was subsequently loaded at 1 ml/min onto a Source 30Q anion-exchange column (Pharmacia Biotech). The bound material was separated into its nonproteoglycan and proteoglycan components based on its elution from the column with a linear gradient of 0 to 2 M NaCl in TRIS buffer, at 1 ml/min and collected in an automated fraction collector. All chromatography was performed using a FPLC system equipped with a conductivity meter and UV monitor, set at 280 nm, under computer control using the Director software package (Pharmacia Biotech).

Protein and proteoglycan isolation and characterization. To determine whether a protein component was important for biological activity conditioned medium or chromatography fractions were subjected to various protein digestions. The effects of three different forms of protein digestion were examined: (1) a 2-h incubation with proteinase K (2.5 U) immobilized to agarose beads under agitation, at 37°C, followed by removal of the beads by centrifugation; (2) heating at 56°C for 1 h; or (3) boiling for 10 min. Enzymatic digestion of chromatography fractions for glycosaminoglycans (GAG) analysis was performed with heparinase (Hep I), heparitinase I (Hep III), or chondroitinase AC-I at working concentrations of 0.1, 0.1, and 0.5 U/ml, respectively. Samples and enzymes were incubated in a buffer containing 1 mM calcium acetate and 20 mM sodium acetate, at pH 7.0 for heparinase/heparitinase I or pH 7.4 for chondroitinase AC-I, for 3 h at 37°C. Chondroitinase ABC was used at 1.0 U/ml, and incubated in a buffer containing 40 mM sodium acetate, pH 8.0, for 3 h at 37°C. All enzymes were obtained from Seikagaku America Inc. (Rockville, MD). Enzyme activity was destroyed by boiling the samples for 10 min. The digested samples were assessed in the vSMC growth assay.

Dimethylmethylene blue glycosaminoglycan quantification assay. The amount of glycosaminoglycan in the conditioned media was determined by a modification of the dimethylmethylene blue (DMB) assay (Farndale et al., 1986). Dimethylmethylene blue (16 mg DMB [Sigma-Aldrich, St. Louis, MO] in 1 L ddH₂O containing 2.37 g NaCl, 3.04 g glycine, 0.77 ml HCl) was prepared and stored at room temperature. To determine the total amount of GAG in the samples, 700 μ l DMB was added to a 500- μ l sample in a cuvet and mixed; the rate of absorbance was immediately read at 525 nm, which was determined to be the optimal adsorption of the DMB solution used. The assay was calibrated by use of reagent blanks and standards containing up to 5 μ g/ml of heparan sulfate. Because protein can interfere with the binding of the DMB to GAG, bovine serum albumin (BSA) was added to the standards. The amount of BSA added was equivalent to the amount of total protein in the samples as determined by a protein assay (Bio-Rad Laboratories, Richmond, CA). A double-beam spectrophotometer was used (Perkin-Elmer, Norwalk, CT).

To determine the amount of heparan sulfate in the samples, conditioned media or fractions were digested with heparinase/heparitinase I solution for 3 h as described earlier. The reduction of reactivity with DMB was measured by reference to a heparan sulfate calibration curve. Enzyme activity was verified by digesting known amounts of heparan sulfate or mixtures of

heparan sulfate and chondroitin sulfate. The amount of total GAG or heparan sulfate was normalized to the amount in micrograms per 1×10^6 endothelial cells.

Isolation and identification of proteoglycans. Proteoglycans were purified from the conditioned medium by anion-exchange chromatography. To prepare the conditioned medium for chromatography and to remove unincorporated radiolabel, the conditioned medium was dialyzed for 48 h against TRIS-urea buffer (20 mM TRIS, pH 8.0, 1 M urea) using Spectra/Por 6 membranes (MWCO 8,000). The dialysate was loaded at 1 ml/min onto a Source 30Q and equilibrated in TRIS-urea buffer. The bound radioactivity was eluted from the column with a linear gradient of 0 to 2 M NaCl in TRIS-urea buffer, at 1 ml/min, and collected as 0.5-ml fractions.

The radiolabel content of each fraction was assessed utilizing liquid scintillation counting using Ultima Gold scintillation cocktail (Packard Instruments, Meriden, CT). Fractions containing high levels of incorporated radiolabel eluting at high salt concentrations were dialyzed against distilled water utilizing Spectra/Por Dispodialyzers (MWCO 8,000; Spectrum) and subjected to enzymatic digestion with heparinase/heparitinase I, chondroitinase AC-I, or chondroitinase ABC, as described earlier. After digestion for 3 h at 37°C, the respective samples (along with controls containing no enzyme) were analyzed to determine the identity of the GAG utilizing a modification of a previously described method (Rapraeger and Yeaman, 1989). Briefly, samples were applied to ZetaProbe nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA) on a vacuum-supported dot-blotting apparatus (Schleicher and Shuell, Keene, NH). The membranes were washed with TBS (20 mM TRIS, pH 8.0, 0.15 M NaCl) followed by washes with TBS plus Tween-20 (Bio-Rad). The membranes were again washed with TBS, followed by distilled water, and subsequently dried. The amount of bound radiation was quantified utilizing liquid scintillation counting. The presence of proteoglycan-rich compounds was confirmed by comparing the differences in bound radioactivity between control samples and those digested with each lyase.

Separation of conditioned medium for use in growth assay. Endothelial cell-conditioned medium was equilibrated in 20 mM TRIS, pH 8.0, by chromatography on Sephadex G-50M column (Pharmacia Biotech) and subsequently applied to Source 30Q column (Pharmacia Biotech) series. The column was washed with this buffer for five-column volumes, then eluted with a salt gradient from 0 to 2 M NaCl in the same buffer. To avoid any possible protein denaturation, urea was not used in samples that were to be tested in the growth assays. Fractions for growth assays were desalted and equilibrated in DMEM by chromatography on Sephadex G-50M column and sterilized by a 0.22- μ m Millipore filter.

To ensure that the proteoglycan fractions did not contain any possible heparin-binding proteins, the proteoglycan fractions obtained from the Source 30Q column in some studies were equilibrated in 0.01 M TRIS and 0.15 M NaCl, pH 7.0, and loaded onto a Heparin-5PW TSK-GEL column (TosoHaas, Montgomeryville, PA). The unretarded eluant containing the purified proteoglycans was equilibrated in DMEM by chroma-

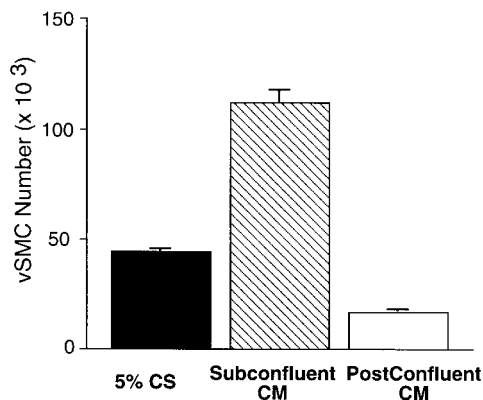


Fig. 1. Effects of subconfluent and postconfluent EC-conditioned medium on vSMC numbers. vSMC were plated at 5,000 cells/well in 5% calf serum. After a 72-h period of growth arrest in 0.1% calf serum, the vSMC were incubated in 5% calf serum with or without the addition of EC-conditioned medium containing 30 $\mu\text{g}/\text{ml}$ of total protein. vSMC were counted after 3 days incubation.

tography on a Sephadex G-50M column and sterilized by a 0.22- μm Millipore filter.

Smooth muscle cell growth assay. The biological activity of EC-conditioned medium components was tested in a growth assay that measured vSMC cell number. vSMCs were plated at 5,000 cells/well in 24-well plates in DMEM containing 5% calf serum. The plating medium was replaced 24 h later with DMEM containing 0.1% serum and the vSMCs were growth arrested for 72 h. Samples of unfractonated conditioned medium or fractionated conditioned medium were added to DMEM containing calf serum (to a final concentration of 5% serum). Nonproteoglycan protein, proteoglycan, and combinations of these fractions were analyzed. DMEM containing 5% calf serum was used as a control medium. The number of vSMCs in some of the wells was counted at this time (day 0) to determine the starting cell number. After 3 days of incubation the vSMC were trypsinized and in the wells were counted with a Coulter counter. Each experimental condition was performed in triplicate.

Statistical analysis

The percentage growth for each group of samples is expressed as the mean \pm SE for at least three experiments. Cell numbers were compared by one-way analysis of variances. Sample means were compared using Fisher's least-significant difference test. Significance was assumed at $P < 0.05$.

RESULTS

Effects of endothelial-conditioned media on vSMC growth

Growth of serum-starved vSMCs was stimulated by 2.5-fold by conditioned medium from subconfluent endothelial cell cultures compared with vSMCs grown in 5% calf serum ($11.2 \pm 0.54 \times 10^4$ vs. $4.4 \pm 0.14 \times 10^4$, $P < 0.05$) (Fig. 1). In contrast, vSMC growth was inhibited by 2.6-fold in the presence of conditioned medium from postconfluent ECs ($1.7 \pm 0.10 \times 10^4$, $P < 0.05$) (Fig. 1). EC-conditioned medium from postconfluent and subconfluent cultures were chromatographed

on Source 30Q columns and fractionated with a linear salt gradient into two main groups: (1) nonproteoglycan proteins (0.05–0.72 M NaCl) and (2) proteoglycans (0.80–1.02 M NaCl) (Fig. 2). The protein elution profiles at 280 nm were similar for both postconfluent and subconfluent EC-conditioned media (Figs. 2A and 2B). Two large ^{35}S -label peaks were seen at low salt concentrations that corresponded to nonproteoglycan proteins. Since any free, unincorporated radiolabeled was removed by either dialysis or gel-filtration chromatography prior to loading on the Source 30Q, the radioactive counts come from labeled protein. Two small ^{35}S -labeled peaks were obtained with the higher salt concentrations that correspond to the proteoglycan fractions (Fig. 2). Enzyme digestion studies showed that the first small peak was mainly comprised of heparan sulfate, whereas the later small peak was principally composed of chondroitin sulfate and a small amount of dermatan sulfate (Figs. 2C and 2D).

Subconfluent and postconfluent endothelial-conditioned media contained similar amounts of total protein, total GAG, and heparan sulfate (Table 1).

Smooth muscle cell growth assay

Conditioned medium collected from postconfluent or subconfluent EC cultures had a differential effect on vSMC growth (Figs. 1 and 3). Postconfluent EC-conditioned medium inhibited vSMC growth by $61.2 \pm 2.9\%$, whereas subconfluent EC-conditioned medium stimulated vSMC growth by $149.4 \pm 6.8\%$ (Fig. 3). The PG fractions of the conditioned medium isolated from either postconfluent or subconfluent cultures were equivalent at blocking vSMC proliferation: 32.4 ± 2.3 and $32.0 \pm 3.9\%$, respectively, when used at a concentration of 10 $\mu\text{g}/\text{ml}$ (Fig. 3). Only heparan sulfate side chains were responsible for the inhibition of the vSMCs. Treatment of the PG fractions with heparinase/heparitinase I, but not chondroitinase ABC, prevented the inhibition of vSMCs (Table 2). It was only when the nonproteoglycan fractions from postconfluent EC-conditioned medium were added to vSMCs in the presence of the PG fractions (from either postconfluent or subconfluent ECs) that maximal inhibition was achieved and at a level similar to complete conditioned medium from postconfluent ECs (Fig. 3A). Similarly, the addition of the non-PG fractions from subconfluent medium with the PG fraction (from either postconfluent or subconfluent ECs) stimulated vSMC growth to a level similar to complete subconfluent EC-conditioned medium (Fig. 3B). To ensure that the PG fractions were not contaminated with possible heparin-binding factors, the PG fractions were loaded onto a heparin-Sepharose column. The unretarded eluant contained the purified proteoglycans and possessed the same biological activities as the PG fractions obtained from the Source 30Q column (data not shown). This indicated that the PG fractions from the Source 30Q column were not contaminated with any heparin-binding factors.

The factors responsible for the biological activity in the nonproteoglycan fractions are proteins, since their activity was abolished by proteinase K or heat pretreatment, leaving only the level of inhibition obtained by the HSPG component alone (Table 2). Moreover, the non-PG fractions obtained from either postconfluent or subconfluent medium had no effect on vSMC growth

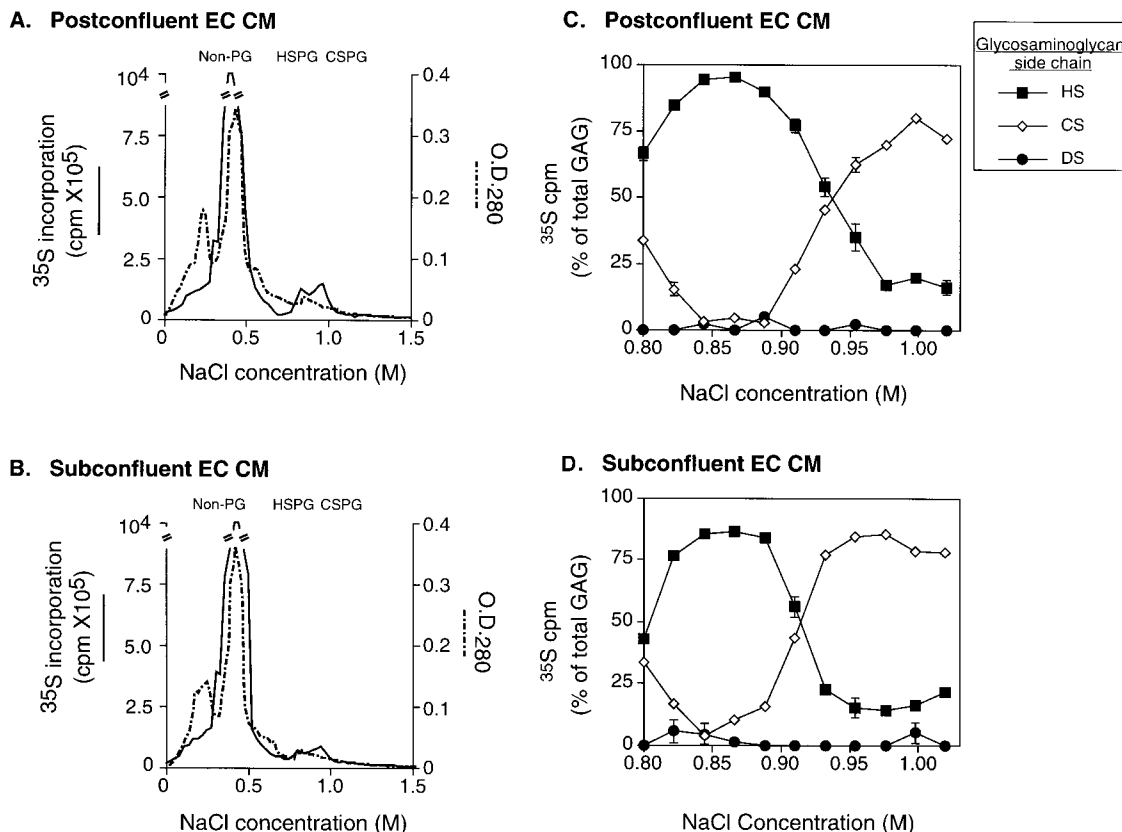


Fig. 2. Ion-exchange chromatograph of EC-conditioned medium. Postconfluent (A) and subconfluent (B) EC cultures were incubated for 24 h in the presence of ^{35}S and the radiolabeled conditioned medium was fractionated on Source 30Q as described in Materials and Methods. The proteoglycan fractions were digested by lyases to

determine the glycosaminoglycan content of the side chains as being either heparan sulfate (HS), chondroitin sulfate (CS), or dermatan sulfate (DS) (C and D). The radioactive counts in (C) and (D) represent the percentages from the total GAG content in the PG fractions obtained from the Source 30Q column.

TABLE 1. Amount of protein, glycosaminoglycans, and heparan sulfate in the endothelial-conditioned medium¹

Experimental substance	Subconfluent ECCM ($\mu\text{g}/10^6$ cells)	Postconfluent ECCM ($\mu\text{g}/10^6$ cells)
Total protein	171.2 ± 14.0	153.8 ± 14.6
Total GAG	3.53 ± 0.43	3.75 ± 0.50
Heparan sulfate	1.27 ± 0.19	1.41 ± 0.18

¹Total protein in the endothelial-conditioned medium (ECCM) was determined by the Bio-Rad Protein Assay. Total glycosaminoglycan (GAG) was measured by the dimethylmethylene blue (DMB) assay and the amount of heparan sulfate was determined by the DMB assay after heparinase/heparitinase I digestion as described in Materials and Methods.

when added in isolation (Fig. 3). Positively charged compounds in the conditioned medium that passed unretarded through the anionic Source 30Q column had no biological effect on vSMC growth, either separately or in combination with the HSPG fractions (data not shown). No material eluted out of the column at salt concentrations of greater than 1.06 M. Whether EC-conditioned medium will stimulate or inhibit vSMC growth is dependent on protein(s) in the non-PG fractions; addition of HS is necessary for this activity.

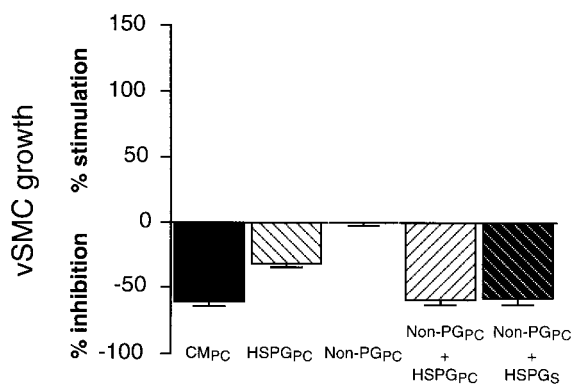
DISCUSSION

Although the density-dependent regulation of vSMC by ECs has been documented, the precise nature of this

switch has not been defined. It is not clear, for example, whether conditioned medium from postconfluent endothelial cultures inhibits vSMCs (Castellot et al., 1981, 1982; Nugent et al., 1993), and whether exponentially growing ECs produce a stimulatory effect (Gajdusek et al., 1980; Castellot et al., 1981), because the latter lack compounds that have the potential to inhibit proliferation or because a more complex regulatory mechanism is in place. In this study we have shown that both subconfluent and postconfluent EC cultures produce and secrete HSPG, which are equally inhibitory to vSMC growth. What determines whether the endothelial cell-conditioned medium will be stimulatory or inhibitory to vSMC growth are non-PG proteins produced by the endothelial cells that interact with the HSPGs. This sophisticated mode of growth control provides a flexible and dynamic environment for EC-vSMC growth regulation.

Anion-exchange chromatography was used to separate conditioned medium into nonproteoglycan and proteoglycan fractions. Although subconfluent fetal pulmonary endothelial cells can produce a vSMC-inhibitory HSPG (Benitz et al., 1990), it was generally believed that adult aortic endothelial cells in subconfluent conditions could not manufacture this critical compound (Castellot et al., 1981). We now document

A. Effects of Postconfluent EC CM Fractions



B. Effects of Subconfluent EC CM Fractions

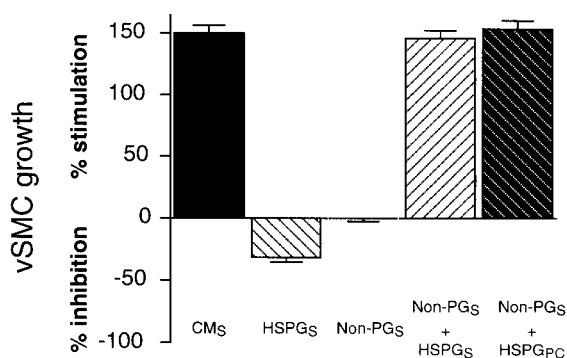


Fig. 3. Effects of EC-conditioned medium and its fractions on vSMC growth. Postconfluent (A) or subconfluent (B) EC-conditioned medium (CM) was separated into non-PG protein and PG fractions and added either separately or in combination to serum-starved vSMC and counted 3 days later as described in Materials and Methods. In some conditions the non-PG fraction from postconfluent EC (Non-PG_{PC}) was added to the HSPG fraction from subconfluent EC (HSPG_S) or the non-PG fraction from subconfluent EC (Non-PG_S) was added to the HSPG fraction from postconfluent EC (HSPG_{PC}). The bars represent the percentage stimulation or inhibition compared to vSMC grown in 5% calf serum.

that both subconfluent and postconfluent ECs isolated from bovine aortas produced and secrete HSPGs equally inhibitory to vSMC growth. The inhibition arose from the activity of the heparan sulfate side chains as the inhibitory effect was abolished when the samples were pretreated with heparinase/heparitinase, but not chondroitinase. At the same time, however, the inhibition caused by the HSPG fractions represented only approximately 47% of the maximal inhibitory capability of unfractionated postconfluent EC-conditioned medium ($61.2 \pm 2.9\%$). This clearly indicates that other components in the non-PG fractions must be involved for maximal inhibition by postconfluent EC-conditioned medium and stimulation by subconfluent EC-conditioned medium.

Neither the non-PG fractions from postconfluent nor subconfluent EC-conditioned media had any effect on vSMC growth on their own. However, when the non-PG fractions from postconfluent ECs were added to the

TABLE 2. Effects of denaturing endothelial cell-conditioned medium fractions on vSMC growth activity¹

Experimental substance	Percentage vSMC stimulation	
	Subconfluent	Postconfluent
Section I		
Conditioned Medium (complete)	142.4 ± 5.6	-59.1 ± 4.2
Non-PG only	3.3 ± 2.8	-3.0 ± 3.4
PG only	-23.7 ± 3.2	-26.2 ± 3.5
Non-PG + PG	136.6 ± 6.1	-56.3 ± 4.6
Section II		
Proteinase K (non-PG) + PG	-24.2 ± 2.7	-28.1 ± 5.7
56°C, 60 min (non-PG) + PG	-26.4 ± 3.1	-29.2 ± 4.8
100°C, 10 min (non-PG) + PG	-22.4 ± 2.9	-24.9 ± 3.7
Section III		
Heparinase/heparitinase (PG) + non-PG	10.1 ± 2.8	14.3 ± 3.9
Chondroitinase ABC (PG) + non-PG	138.3 ± 7.3	-54.4 ± 4.1
Boiled control (PG) + non-PG	140.7 ± 6.5	-56.8 ± 5.3

¹The nature of the compounds from EC-conditioned media required for the activity on vSMC growth was determined by degrading/denaturing experiments. Section I shows the results obtained with untreated conditioned medium or fractions added separately or together to vSMCs. Section II shows the results of non-PG fractions degraded by proteinase K or heat added to vSMC cultures in the presence of untreated PG fraction. To determine the type of GAG side chains required for the PG activity (Section III), the PG fractions were digested with heparinase/heparitinase I or chondroitinase ABC. Enzymes were inactivated by boiling the samples. Untreated PG fractions were boiled as well to serve as a control. The treated PG fractions were incubated with the vSMCs in the presence of untreated non-PG fractions and compared with untreated fractions. vSMC were counted 3 days after the addition of the fractions.

HSPG fractions, from either subconfluent or postconfluent conditioned medium, maximal inhibition was seen, comparable to that of unfractionated postconfluent EC-conditioned medium. Similarly, when the non-PG fractions from subconfluent ECs were added to the HSPG fractions, from either subconfluent or postconfluent conditioned medium, maximal vSMC growth stimulation occurred to a level similar to that of unfractionated subconfluent EC-conditioned medium. Exposure to proteinase K and heat treatment verified the protein composition of the non-PG fractions. These treatments denatured the non-PG protein fractions so that they could no longer interact with the HSPG fractions, leaving only the inhibitory contribution of heparan sulfate.

How can one reconcile all of these observations into a single coherent model in which ECs of any cell density produce inhibitory HSPG but in which the overall effect on vSMC proliferation is so profoundly different? One possible scenario is that the non-PG fraction enhances an inhibitory activity of the HSPG of postconfluent cells, and that the HSPG enhances a mitogenic potential of the non-PG protein fractions from subconfluent ECs. The interaction of heparin and heparin-like compounds with the protein(s) is complex and supports this hypothesis. The mitogenic effect on vSMC growth by the non-PG proteins from the subconfluent EC-conditioned medium could, for example, be derived from an interaction of the HSPGs with heparin-binding growth factors, such as basic fibroblast growth factor. The heparan sulfate side chains may bind to the factor, causing it to undergo a conformational change, thereby increasing its affinity with the cell surface receptors

(Rapraeger et al., 1991; Yayon et al., 1991). Alternatively, the heparan sulfate side chains may stabilize the interaction of the factor with its receptor (Nugent and Edelman, 1992).

The ability of the non-PG protein fraction from post-confluent EC-conditioned medium to augment the inhibitory capacity of HSPG on vSMC growth may be from the activation of a repressor molecule. When HSPG binds to this protein, the complex might (1) directly inhibit vSMC growth; (2) repress the actions of a mitogen by binding to it and inactivating it, or blocking the receptor-binding site for the mitogen; or (3) the complex may induce vSMC death by apoptosis. One possible candidate is transforming growth factor-beta (TGF- β), which is secreted by postconfluent endothelial cells in an inactive form. Heparin-like molecules have been shown to release the active TGF- β from the latent complex (McCaffrey et al., 1989). Continued experimentation, hopefully, will reveal the full extent of this sophisticated form of autocrine, paracrine, and endocrine regulation.

A low molecular weight nonproteoglycan factor (Dodge et al., 1993) and nitric oxide (Joly et al., 1992; Scott-Burden and Vanhoutte, 1993) have also been shown to be responsible for vSMC inhibition. Because of the extremely short half-life of nitric oxide, we assume that none was present in our conditioned medium and hence nitric oxide could not be involved in vSMC inhibition in our system.

Vascular SMC growth is central to the pathobiology of injury-induced intimal hyperplasia and restenosis (Ross, 1993). Heparin is a potent inhibitor of vSMC growth in vitro and in rat models (Clowes and Karnovsky, 1977; Hoover et al., 1980; Castellot et al., 1982, 1984; Clowes and Clowes, 1986; Edelman et al., 1990). Unfortunately, clinical trials of heparin in coronary balloon angioplasty have failed to demonstrate clinical benefit (Ellis et al., 1989; Laskey et al., 1990; Faxon et al., 1994). Possible explanations for this failure include relative differences in dose regimes, route and method of application, and duration of treatment, as well as the questionable applicability of animal models to human atherosclerotic lesions. Clinically, angioplasty is performed on diseased arteries, whereas most animal models involved balloon injury on previously normal arteries. Thus heparin treatment alone may be an insufficient form of therapy in such complex clinical lesions.

The central hypothesis of this work is that heparin-like compounds are necessary, but not sufficient, to control proliferative vascular diseases. Previous work from this laboratory has demonstrated that the entire endothelial cell is a far greater regulator of vasoproliferative disease than is a single agent alone (Nathan et al., 1995). As demonstrated in this study the key regulatory compounds responsible for vSMC growth stimulation or inhibition are contained in the non-PG fractions of the endothelial-conditioned medium. Both subconfluent and postconfluent endothelial-conditioned media contained inhibitory HSPG. Maximal vSMC growth inhibition occurred when the HSPG was mixed with the non-PG fractions derived from postconfluent endothelial-conditioned medium, whereas maximal vSMC growth stimulation occurred when the HSPG was combined with the non-PG fractions derived from subconfluent endothelial-conditioned

medium. Thus the difference of growth activity of conditioned medium derived from subconfluent and postconfluent endothelial cells is the result, not of a difference in HSPG production, but rather of the interaction of HSPG with different non-PG proteins made by these cells under different cell densities. The identity of the crucial non-PG proteins in the postconfluent and subconfluent EC-conditioned media is currently being determined. It may be feasible that, once identified, these compounds may be used clinically to prevent restenosis. At the time of vascular intervention, the proinhibitory compounds, together with HSPG, can be administered intravenously or delivered locally by embedding them in polymer-release devices or transfecting them into cells where they can be continuously released at the site of injury.

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