Sustained Inhibition of Intimal Thickening

In Vitro and In Vivo Effects of Polymeric β-Cyclodextrin Sulfate

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Abstract

Intimal thickening after vascular injury may be modulated in part by heparin binding growth factors. We hypothesized that placement of a therapeutic polymer in the periadventitial space capable of tightly binding growth factors might alter the vascular response to injury. We first demonstrated that incubation of rat aortic smooth muscle cells with an insoluble, sulfated polymer of β-cyclodextrin (P-CDS) was associated with a dose-dependent inhibition of proliferation induced by fetal calf serum, fibroblast growth factor-2 (FGF-2), platelet-derived growth factor BB, or epidermal growth factor. Preincubation studies of P-CDS with FGF-2 revealed a very rapid removal of mitogenic activity. Using radiolabeled FGF-2 (0.25 μg/ml), we observed a very rapid association rate (0.34 ± 0.07 min^{-1}, $n = 4$) and a very slow dissociation rate ($3.3 \pm 0.2 \times 10^{-7}$ min^{-1}) at 37°C, suggesting a high affinity interaction. Using both Transwell and linear under-agarose assays, we demonstrated a significant inhibition of random migration (chemokinesis) by P-CDS. Unsulfated polymeric β-cyclodextrin (P-CD) had little if any of these effects, suggesting that the high negative charge density of P-CDS was important for the effects. Finally, rats undergoing carotid artery balloon injury were randomized to treatment with periadventitial P-CDS or no treatment, and were killed at 4 ($n = 20$), 14 ($n = 59$), and 88 d ($n = 14$). Morphometric analysis demonstrated significant and sustained inhibition of intimal thickening in P-CDS–treated rats at 14 ($P < 0.01$) and 88 d ($P < 0.05$) using absolute intimal area or intima/media area ratios. No inhibition was seen in a group of rats treated with P-CD. In P-CDS–treated rats, bromodeoxyuridine labeling studies revealed fewer labeled smooth muscle cells in the intima at 14 d ($P = 0.01$), while staining with Evans blue revealed enhanced late endothelial cell regrowth. Thus, periadventitially applied sulfated β-cyclodextrin polymer, which can tightly bind heparin binding growth factors, inhibits intimal thickening in vivo in a sustained fashion without using an additional delivery system. These studies suggest that cellular processes mediated by heparin binding growth factors may be modulated by P-CDS. (*J. Clin. Invest.* 1995. 96:2583–2592.) **Key words: angioplasty • smooth muscle cell • restenosis • fibroblast growth factor • migration**

Introduction

Intimal thickening is a common response to many forms of vascular injury such as angioplasty of an atherosclerotic artery (1, 2) or the arterialization of veins as grafts (3). Arterial wall remodeling after injury is thought to be mediated by a variety of factors, both blood-borne and locally produced. Some factors stimulate vascular smooth muscle cell (SMC)[1] proliferation and migration into the intima, followed by the accumulation of extracellular matrix (4–6). Heparin and related compounds have been shown to inhibit SMC proliferation and migration (7–13), alter deposition of matrix proteins from SMC (13, 14), and enhance endothelial regrowth after vascular injury (15–18). The ability to modulate cellular activity appears to be unrelated to anticoagulant activity (19–21). The usefulness of heparin in human vascular disease, however, is limited by its requirement for parental administration, its potential bleeding complications, and its higher dose requirements for modulating SMC activity compared with the dose required for anticoagulation. Heparin has been ineffective in recent clinical trials of angioplasty restenosis, possibly related to inadequate dosing regimens (22).

Soluble, monomeric, β-cyclodextrin tetradecasulfate (CDS) is a universal "mimic" of heparin, paralleling or exceeding the demonstrated cell modulating capabilities of heparin without significant anticoagulant properties (23, 24). It has a simple structure, with seven glucose units arranged in a ring and 14 sulfate groups attached, yielding a high negative charge density and significant biologic activity. It is known that heparin and related glycosaminoglycans, as well as CDS, bind avidly to a class of proteins known as heparin binding growth factors (HBGF, for review see reference 16) including fibroblast growth factors 1 and 2 (FGF-1, FGF-2) (25). It has been postulated that HBGF play a pivotal role in modulating SMC and endothelial cells after vascular injury. We have demonstrated that CDS inhibits SMC proliferation (23) and migration (26) and stimulates endothelial cell proliferation in vitro in combination with FGF-1 (27). In vivo, soluble CDS stimulates angiogenesis, whereas when administered in combination with a steroid, it is a potent inhibitor (15). In these and other biological activities, the efficacy of sulfated β-cyclodextrins depended on

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CDS, β-cyclodextrin tetradecasulfate; HBGF, heparin binding growth factor; LDH, lactic dehydrogenase; P-CD, polymeric β-cyclodextrin; P-CDS, polymeric β-cyclodextrin sulfate; PDGF-BB, platelet-derived growth factor BB; SMC, smooth muscle cell; VEGF, vascular endothelial cell growth factor.

a minimum required sulfate density of at least 10 sulfates per molecule. In animal models of vascular injury, CDS administered orally (23) or intravenously (24) inhibited intimal thickening, although somewhat larger doses were required. To circumvent potential problems with systemic administration, several investigators have used local, periadventitial drug delivery with heparin (12), dexamethasone (28), or antisense oligomers (29), which have all been demonstrated to inhibit intimal thickening but have required additional delivery vehicles.

To accomplish local therapy without the need for additional vehicles, we have developed a novel insoluble, sulfated, polymeric form of β-cyclodextrin, P-CDS. The polymer is applied to the adventitial side of the artery in the form of a dry, fine powder, which after hydration offers internal heparin-like surfaces capable of potentially adsorbing HBGF proteins like FGF-2 (25) or vascular endothelial cell growth factor (VEGF) (30). These sulfated β-cyclodextrin polymers with $>$ 10 sulfates per monomer have been shown to rapidly bind several classes of cationic molecules including dyes and growth factors with high capacity (25). We tested the effects of sulfated or unsulfated polymers of β-cyclodextrin on SMC proliferation and migration in vitro and determined whether placement of either polymer in the periadventitial space could provide sustained inhibition of intimal thickening after injury.

Methods

P-CDS. P-CDS was obtained as a gift from American Maize, Inc. (Hammonton, IN). It is supplied as a white, crystalline powder. Microscopically it is heterogeneous in size and shape, with particles ranging from $\sim$ 1 to 150 μm in size. The particles swell as they absorb water and take on a gelatinous consistency. Unsulfated β-cyclodextrin polymer (P-CD) was also supplied by American Maize, Inc. and had a very similar appearance. For in vitro experiments, the dry powdered polymer was weighed and added to the appropriate liquid, and the final volume was adjusted accordingly. For in vivo experiments, it was added directly to the vessel and drops of saline were added afterwards.

SMC proliferation assay. To determine the effect of P-CDS on SMC proliferation, rat aortic SMC, grown using an explant technique, were plated at 2,500 cells/well in 96-well microtiter plates with DME/F12 (JRH Biosciences, Lenexa, KS) with 10% FCS (Gibco, Grand Island, NY) at 37°C and allowed to attach overnight. The next day, the culture medium was exchanged for media containing 1% FCS and either recombinant human FGF-2 (gift from A. Protter, Calbiochem-Novabiochem, Mountain View, CA), recombinant human platelet-derived growth factor BB (PDGF-BB; R&D Systems, Minneapolis, MN), or recombinant human epidermal growth factor (R&D Systems) as the mitogenic stimulant. Control plates underwent similar exchange without the addition of P-CDS. During the last 48 h, [^{3}H]thymidine (100 μCi/ml, New England Nuclear, Boston, MA) was added. At the end of the assay, the cells were washed twice with cold PBS (Gibco), once with cold 5% trichloroacetic acid (Sigma Chemical Co., St. Louis, MO), and once with cold methanol (Sigma Chemical Co.). The DNA was then solubilized with 0.1 N NaOH, and the radioactivity was quantitated using scintillation fluid (Ecolite) and a beta counter. Background levels of proliferation were assessed by the addition of 1% FCS alone and were subtracted from each value. In some experiments, unsulfated cyclodextrin polymer was also evaluated in parallel.

Measurements of [^{3}H]thymidine incorporation alone may not take into account hypertrophic responses or accumulations of extracellular matrix proteins. Furthermore, proliferation studies with single mitogens may not reflect the more complex stimulation seen during vascular injury. To attempt to take some of these factors into account, we repeated the experiments above with 10% FCS, a more general, potent stimulant, and assessed the accumulation of total cell protein. After the 3 d of stimulation, cells were washed with PBS (Gibco), fixed with 10% formalin (Fisher Scientific, Pittsburgh, PA), stained with Naphthol blue-black (0.05%, Sigma Chemical Co.), and lysed with 0.1 N NaOH, and total cellular protein was quantitated by absorption at 630 nm (model MR5000; Dynatech Laboratories Inc., Chantilly, VA). Parallel wells had total protein quantitated after attachment to determine baseline total protein levels. The percent inhibition of protein accumulation was expressed relative to untreated cells, corrected for the amount of protein present at baseline.

Next we addressed whether the P-CDS needed to be physically present in the wells to inhibit protein accumulation or whether preincubation of the mitogen with the polymer would be sufficient to limit its effect. To study this question, a solution of FGF-2 (5 ng/ml) in culture medium was divided; one-half served as control, and P-CDS (1 mg/ml) was added to the other. Additional control solutions contained 0 or 1 ng/ml FGF-2 in medium. After varying periods of time (1 min to 3 d), aliquots of each were withdrawn, passed through a 0.22-μm filter, and incubated at 37°C until all samples had been collected. The absence of particulate P-CDS in the supernatants was confirmed microscopically. Then, supernatants with or without P-CDS preincubation were added to rat aortic SMC (plated at 2,500 cells/well as above) after overnight attachment. After 3 d, total protein accumulation was quantitated as described above.

Toxicity studies. To assess potential toxicity of P-CDS or P-CD, we evaluated the degree of lactic dehydrogenase (LDH) release by SMC incubated for 6 h in medium containing varying concentrations of either polymer in 1% FCS. Supernatants were harvested, cell debris was removed by centrifugation, and the level of LDH was measured using a colorimetric assay as described previously (26). In parallel wells, SMC at time 0 were lysed with Triton X-100, and the supernatants were assayed for LDH. The amount of LDH release was then expressed as a percentage of the total LDH in cell lysates.

Linear under-agarose migration assay. Random SMC migration was measured using a linear under-agarose migration assay (26). 3 ml of a solution containing P-CDS (1 mg/ml) and agarose (0.7 wt%; IBI, New Haven, CT) in DME/F12 with 10% FCS was added to fibronectin-coated chamber slides (Nunc, Naperville, IL) and permitted to solidify. The control group had a similar solution plated without P-CDS added. A 3-mm-wide band was removed from the center of the slide, forming a well into which cultured rat aortic SMC ($\sim 5 \times 10^5$ cells in 100 μl) were plated and incubated for 30 min to allow for attachment. A thin layer of 0.3% agarose gel was added to the well; 1 ml of DME/F12 with 10% FCS was then added to the entire plate. The distance of SMC migration was quantitated by photomicrography (magnification of 40) taken at 0, 24, 48, and 72 h.

Transwell migration assay. As a second assay to study the effects of P-CDS on migration, we utilized a Transwell assay (12 μm pore size; Costar Corp., Cambridge, MA), as previously described with minor modifications (26). Rat aortic SMC (1.5×10^4) in 0.5 ml volume DME/F12 with 10% FCS were added to each fibronectin-coated top well of a Transwell plate and permitted to attach. The lower chamber was filled with 1.5 ml of the same medium without cells. 12 h later, the medium was changed and replaced with new medium containing 10% FCS as the stimulant, with or without P-CDS. P-CDS was added to the upper, lower, or both chambers, while control wells received only medium. The effect of P-CD was also assessed in parallel wells. After an additional 12 h of migration, the cells were fixed with 10% neutral buffered formalin, washed twice with acetate buffer, twice with distilled water, and allowed to dry. The cells were stained for 30 min with toluidine blue and rinsed three times with distilled water. The cells on top of the filter were mechanically removed, leaving adherent to the filter only the cells that had migrated through the filter. The filters were examined by light microscopy (magnification of 100), and 10 fields were counted. The amount of baseline migration that had occurred during the initial 12-h attachment period was assessed in parallel wells. The number of cells migrating through at the end of the assay was corrected by subtracting baseline migration values.

Radiolabeled FGF-2 binding studies to P-CDS. To further investi-

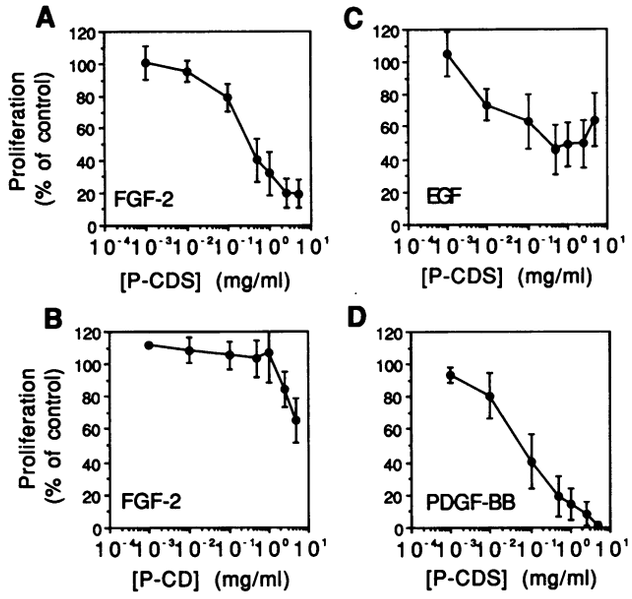

Figure 1. Effect of sulfation of P-CD on growth factor–induced SMC proliferation in vitro. Rat aortic SMC were stimulated for 3 d in medium containing 1% FCS plus FGF-2 (5 ng/ml, *A* and *B*), epidermal growth factor (5 ng/ml, *C*), or PDGF-BB (5 ng/ml, *D*) with or without the addition of varying amounts of P-CDS (*A, C,* and *D*) or P-CD (*B*). During the last 48 h, [^{3}H]thymidine was added to the medium. The degree of [^{3}H]thymidine incorporation is expressed as a percentage of control (n = 4, ±SEM).

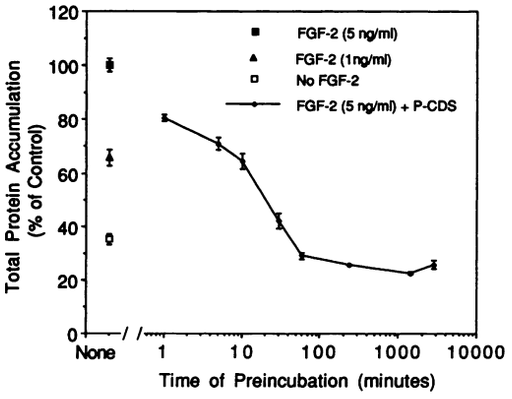

Figure 3. Effect of preincubation of FGF-2 with P-CDS on total protein accumulation: time course. SMC media supplemented with 5 ng/ml FGF-2 as a stimulant were preincubated for varying times with P-CDS (1 mg/ml) as described in Methods. After filtration to remove the P-CDS, the supernatants were used to stimulate growth-arrested SMC over 3 d; total cell protein was then assessed as described in Methods. In parallel, the accumulation of total cell protein was assessed for cells treated with medium supplemented with 0, 1, or 5 ng/ml of FGF-2 alone that had not been pretreated with P-CDS. The results are expressed as a percentage of the response demonstrated with 5 ng/ml FGF-2 in the absence of P-CDS (control).

gate the kinetics of binding of FGF-2 to P-CDS, we studied the ability of P-CDS to remove radiolabeled FGF-2 from a liquid suspension. FGF-2 was radiolabeled using Iodobeads (Pierce Chemical Co., Rockford, IL) and Na ^{125}I, and separated from unbound ^{125}I chromatographically (sp act, 2.3 × 10^6 cpm/μm). A 1.7-ml aliquot of a well-mixed suspension of P-CDS (10 mg/ml) in DME/F12 with 10% FCS was placed in each of three Eppendorf tubes (to perform the experiment in triplicate). FGF-2 (0.25 μg/ml) was added to each tube at 37°C. Aliquots (0.1 ml) were removed immediately (for determination of baseline values) and at various time points. To each sample 1 ml of cold PBS with BSA was added (3 mg/ml, Sigma Chemical Co.) and spun at 15,000 rpm for 2 min to allow for separation of P-CDS and supernatant. The supernatant and P-CDS pellet at time 0 underwent gamma counting to determine the total amount of FGF-2 present initially. In all other samples, after initial centrifugation, the supernatant was discarded, and the P-CDS pellet was washed twice in cold PBS/BSA and counted. The percentage of FGF-2 bound was calculated by dividing the counts per minute at

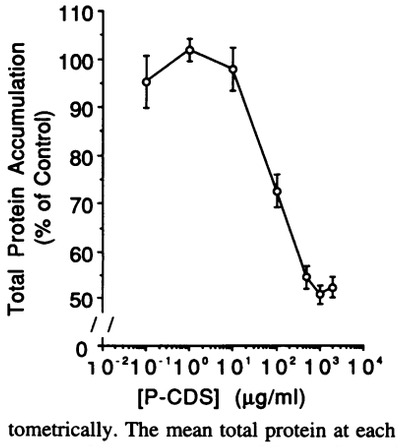

Figure 2. Effect of P-CDS on total protein accumulation by SMC. SMC were plated subconfluently in 96-well microtiter plates, allowed to attach overnight, and then allowed to proliferate for 3 d in medium containing 10% FCS in the presence of varying concentrations of P-CDS. The cells were washed, fixed, stained for total cell protein, and quantitated spectrophotometrically. The mean total protein at each concentration of P-CDS is expressed as a percentage of the total protein measured in untreated cells, corrected for background attachment levels. The data represent the mean of nine independent experiments performed at least in triplicate (±SEM).

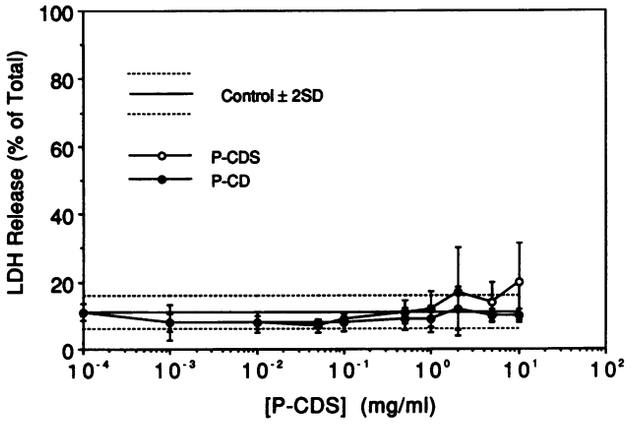

Figure 4. Toxicity assessment of P-CDS: release of LDH. Confluent monolayers of rat aortic SMC were incubated with varying concentrations of P-CDS or P-CD for 6 h, with control SMC receiving no polymer. Supernatants were then harvested, and the amount of LDH was quantitated using a colorimetric enzyme assay, as described. Values are expressed as a percentage of the total amount of LDH that was released in parallel wells lysed with Triton X-100 at time 0. The solid line and individual points represent the mean value in five independent experiments performed in triplicate. The dashed lines represent ±2 SD, or the normal range for control cells in this assay. The error bars represent ±1 SD for the five individual experiments.

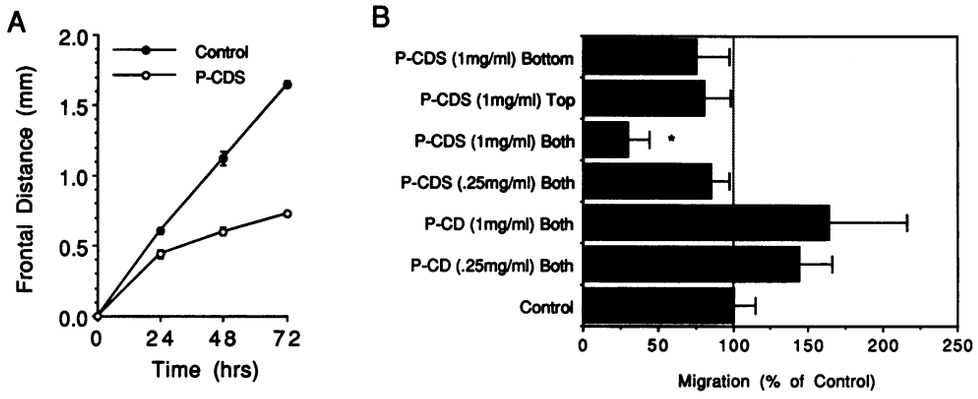

Figure 5. Effect of P-CDS on migration. (*A*) Linear under-agarose migration assay. Cultured rat aortic SMC plated in a central well scored in a 0.7% agarose block with or without 1 mg/ml P-CDS incorporated into the agarose. Results are expressed as distance migrated ±SEM for three experiments. (*B*) Transwell assays. Rat aortic SMC were plated on the top of fibronectin-coated Transwell membranes (12 μm pore size) for 12 h, enabling attachment. The media in the top and bottom chambers were then exchanged for medium containing 10% FCS with or without polymer at varying concentrations. After an additional 12 h, the filters were washed, fixed, stained, and 10 high powered field counted per filter. Parallels were fixed and similarly analyzed after attachment to correct for baseline migration before the addition of polymer. The amount of migration is represented as a percentage of cells having migrated through during the second 12 h in the presence of polymer relative to control cells not receiving polymer. * $P = 0.006$.

each time point by the sum of counts per minute in the supernatant and P-CDS pellet at time 0. Parallel binding experiments were performed using P-CD to address whether sulfation was important for growth factor binding activity.

To study the release of bound FGF-2 from P-CDS, we allowed a suspension of FGF-2 (0.25 μg/ml) and P-CDS (10 mg/ml) to incubate at 37°C for 1 h, to maximize binding. The P-CDS with bound FGF-2 was washed three times with 1 ml of cold PBS/BSA as above. A volume of 1.2 ml of standard cell culture media was added to the P-CDS and vortexed, and 0.1-ml aliquots of the suspension were removed at various time points from 0 to 72 h after vortexing. Each aliquot was washed three times with 1 ml of cold PBS/BSA as above and counted. The percent FGF-2 still bound to P-CDS was calculated by dividing the counts per minute at each time point by the counts per minute bound at time 0.

Rat carotid artery model of intimal thickening. To determine whether the beneficial biological and physical properties of P-CDS could impart significant therapeutic utility, we tested the ability of P-CDS to inhibit intimal thickening in a rat carotid artery injury model. 99 male Sprague-Dawley rats (350–400 grams; Charles River Breeding Labs, Kingston, MA) were anesthetized with intramuscular injection of ketamine (50 mg/kg; Aveco Co., Fort Dodge, IA), xylazine (5 mg/kg; Mobay Corp., Shawnee, KS), and acepromazine (1 mg/kg; Aveco Co.). The left common, external, and internal carotid arteries were exposed in the neck through a midline incision, and a 2F balloon embolectomy catheter (Baxter Healthcare Corp., Santa Ana, CA) was introduced into the external carotid artery. The catheter was positioned into the aorta, inflated, and withdrawn three times as described by Clowes et al. (4). The common carotid artery was gently stripped of adventitia, and animals were randomized to either treatment with 10 mg of dry P-CDS placed 0.5 cm from the bifurcation of the common carotid to 1.5 cm caudally along the common carotid or control which received nothing. To control for possible effects of the polymer in the adventitia, additional animals were treated with 10 mg of P-CD. After placement of polymer, one to two drops of normal saline were added to the area of treatment, producing a gel-like substance which could be manipulated to assure that the entire circumference of the artery had adequate contact with the polymer. Control rats also received periadventitial normal saline and similar artery manipulation. A 4-0 nylon suture was placed subcutaneously adjacent to the center of the carotid artery treatment or corresponding control area. After wound closure, animals were observed and housed after recovery from anesthesia. At 4 (P-CDS $n = 10$, control $n = 10$), 14 (P-CDS $n = 28$, P-CD $n = 6$, control $n = 27$), or 88 d (P-CDS $n = 10$, control $n = 8$) after injury, animals were anesthetized, and the carotid arteries were fixed by perfusion at 120 mmHg pressure with normal saline, followed by 10% formalin solution (J. T. Baker, Inc., Phillipsburg, NJ) via a large cannula placed retrograde in the abdominal aorta. The left cervical region was removed en bloc and gently dissected down to the common carotid artery, and the adjacent suture was identified. The sample was immersion-fixed for an additional 48 h in 10% formalin. The left and right carotid artery were cut into 0.5-cm sections and embedded in paraffin, and 5-μm-thick sections were mounted on glass slides. Representative hematoxylin and eosin–stained sections through the treated and corresponding control segments were analyzed using an automated image analysis system (SummaSKetch II Plus; Summagraphics Corp., Seymour, CT) to determine intima and media areas, and intima/media area ratio.

Bromodeoxyuridine (BrdU) SMC labeling and immunohistochemistry. To delineate proliferating SMC in the intima and media, BrdU (Boehringer Mannheim, Indianapolis, IN), a thymidine analogue, was administered intramuscularly (30 mg/kg) to a subgroup of rats at 17, 9, and 1 h before time of killing. Intracellular BrdU was identified immunocytochemically as described previously (31) using a mouse IgG anti-BrdU antibody (diluted to 10 μg/ml, Boehringer Mannheim) and detection with avidin–peroxidase complex (Vector Laboratories, Burlingame, CA) and 3,3-diaminobenzidine substrate (Sigma Chemical Co.). Sections were counterstained with hematoxylin. All slides included cross sections from both left and right carotid artery and small intestine. Each slide was checked for the presence of BrdU-positive cells in the small intestine (positive control) and the lack of BrdU-positive cells in the right carotid artery (negative control). Total and BrdU-positive SMC number in the intima and media were manually counted using standard light microscopy at a magnification of 400. The number of BrdU-positive SMC was divided by the total number of cells to obtain percent BrdU-positive SMC. The total SMC count was divided by the respective measured area to obtain SMC density in the intima and media.

Endothelial cell regrowth analysis. To determine the effects of P-CDS on endothelial cell regrowth, 30 min before time of killing, a subgroup of rats at 14 and 88 d received a 1.0 ml/kg intravenous injection of Evans blue solution in phosphate-buffered saline (10 wt%) to allow differentiation between arterial segments denuded of endothelium (blue) versus segments with intact endothelium (white). After perfusion fixation, the left carotid artery was exposed, and the distance from the bifurcation of the common carotid artery to the white-blue transition was measured. In three rats at 88 d (two control, one P-CDS), the Evans blue staining was determined to be technically inadequate, and these animals were excluded from the final analysis.

Statistical analysis. Grouped data for morphometric indices, SMC numbers, and endothelial cell regrowth length were compared using Student's two-tailed t test with a P value of < 0.05 considered significant. Unless otherwise specified, all data are provided as means±standard error of the mean.

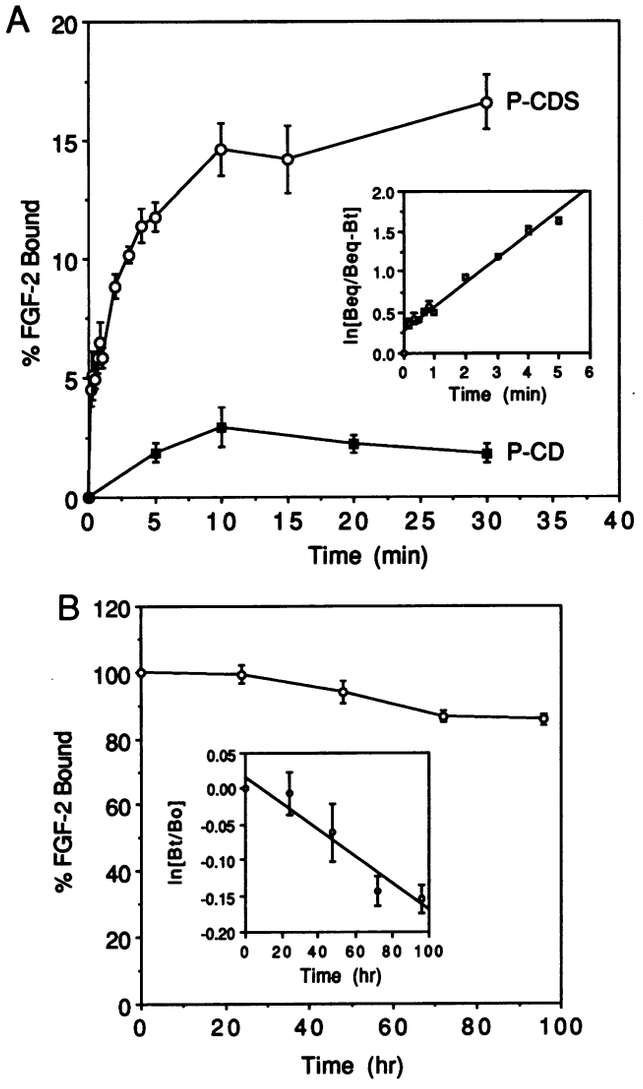

Figure 6. Kinetic analysis of the interaction of FGF-2 with P-CDS or P-CD: (*A*) on rate analysis. ^{125}I-labeled FGF-2 (0.25 μg/ml) was added to a liquid suspension of P-CDS (○) or P-CD (■) (each at 10 mg/ml) in DME/F12 and 10% FCS and vortexed. Aliquots of the FGF-2/polymer suspensions were removed at various time points and, after washing, the amount of labeled FGF-2 remaining bound to polymer was assessed by gamma counting. The inset shows a graphical transformation of the data for P-CDS which enables calculation of the observed on-rate from the slope of the line where *Beq* is the amount bound at equilibrium and *Bt* is the amount bound at time "t". (*B*) Off-rate analysis. The rate of release of ^{125}I-labeled FGF-2 from P-CDS was measured by adding DME/F12 and 10% FCS to P-CDS preincubated to saturation with ^{125}I-FGF-2, and then removing aliquots over time. At each point, the amount left bound to P-CDS after washing was assessed by gamma counting. The mean±SEM of three independent experiments performed in triplicate is shown. A graphical transformation of the data is shown in the inset which enables calculation of the off-rate using the slope of the line where *Bt* is the amount bound at time "t" and *Bo* is the amount bound at time "0".

Results

Effect of P-CDS on SMC proliferation. We first sought to determine the effect of P-CDS on proliferation using a [^{3}H]-thymidine incorporation assay. Using FGF-2 as the mitogen, we demonstrated a dose-dependent inhibition of proliferation by P-CDS in cultured rat aortic SMC (Fig. 1 *A*) with an IC_{50} of ~ 300 μg/ml and with the maximal degree of inhibition of ~ 80%. To determine if the inhibition was related to sulfation, we performed parallel experiments with P-CD. No inhibition was seen at concentrations ≤ 1 mg/ml, with minimal inhibition at very high doses (Fig. 1 *B*). We also tested whether inhibition of proliferation depended on the mitogen being used. We demonstrated that P-CDS can also inhibit proliferation by a non–heparin binding growth factor such as epidermal growth factor (Fig. 1 *C*) although the maximal inhibition was ~ 50% at 500 μg/ml. In contrast, P-CDS was quite effective in the inhibition of mitogenicity by PDGF-BB, an HBGF, with an IC_{50} of ~ 50 μg/ml and nearly complete inhibition at higher doses (Fig. 1 *D*).

Since cellular hypertrophy and/or the accumulation of extracellular matrix proteins can contribute to the development of intimal thickening in the absence of proliferation, we addressed the effect of P-CDS on both proliferation and the accumulation of protein, using a protein stain after 72 h of stimulation with 10% FCS, a potent stimulant. P-CDS inhibited the accumulation of protein in a dose-dependent fashion with a maximum degree of inhibition of ~ 50% at 1 mg/ml (Fig. 2).

Finally, in all of the preceding experiments, P-CDS was placed directly in the well with the SMC. Since both FGF-2 and PDGF are HBGFs, we hypothesized that P-CDS may have inhibited FGF-2– and PDGF-induced SMC proliferation by preventing access of the growth factors to their cognate receptors. This would also imply that the polymer itself would not necessarily have to be present in the well to have this effect. Therefore, we tested the ability of preincubation of P-CDS with FGF-2 to remove the stimulatory activity from media containing FGF-2. Media including FGF-2 (without FCS) were preincubated for varying periods of time with P-CDS, filtered (to remove P-CDS), and used as a stimulant in studies of total protein accumulation. As shown in Fig. 3, there was a very rapid removal of mitogenic activity with a significant reduction after only 1 min of exposure, 50% inhibition within 10 min, and complete removal of mitogenic activity by 60 min. Preincubation for as long as 3 d had a similar effect.

Toxicity studies. To exclude the possibility that direct toxicity and cell death were responsible for some of the above findings, we assessed the release of intracellular LDH as a marker of cell death. Over 6 h in 1% FCS, there was no significant increase in the release of LDH compared with baseline levels for P-CD or P-CDS at all concentrations up to 10 mg/ml. At the highest concentrations, P-CDS had slightly higher levels of LDH release than P-CD (Fig. 4).

Effect of P-CDS on migration. We next hypothesized that since migration of SMC may be stimulated by heparin binding migration factors in an autocrine or paracrine manner, placement of P-CDS particles near the migrating SMC might affect the rate or extent of random migration. To test this, we used a modification of a linear under-agarose assay. We incorporated P-CDS particles into the agarose, such that SMC migration would take place under agarose containing particles of P-CDS. The addition of P-CDS (1 mg/ml) to the agarose yielded an inhibitory effect on SMC migration (55±3% inhibition of mean frontal distance at 72 h) with a significant depression to less than one-half the mean migration velocity (n = 3) (Fig. 5 *A*).

We also used a Transwell assay system to determine if P-CDS had any chemoattractant properties or if it needed to be

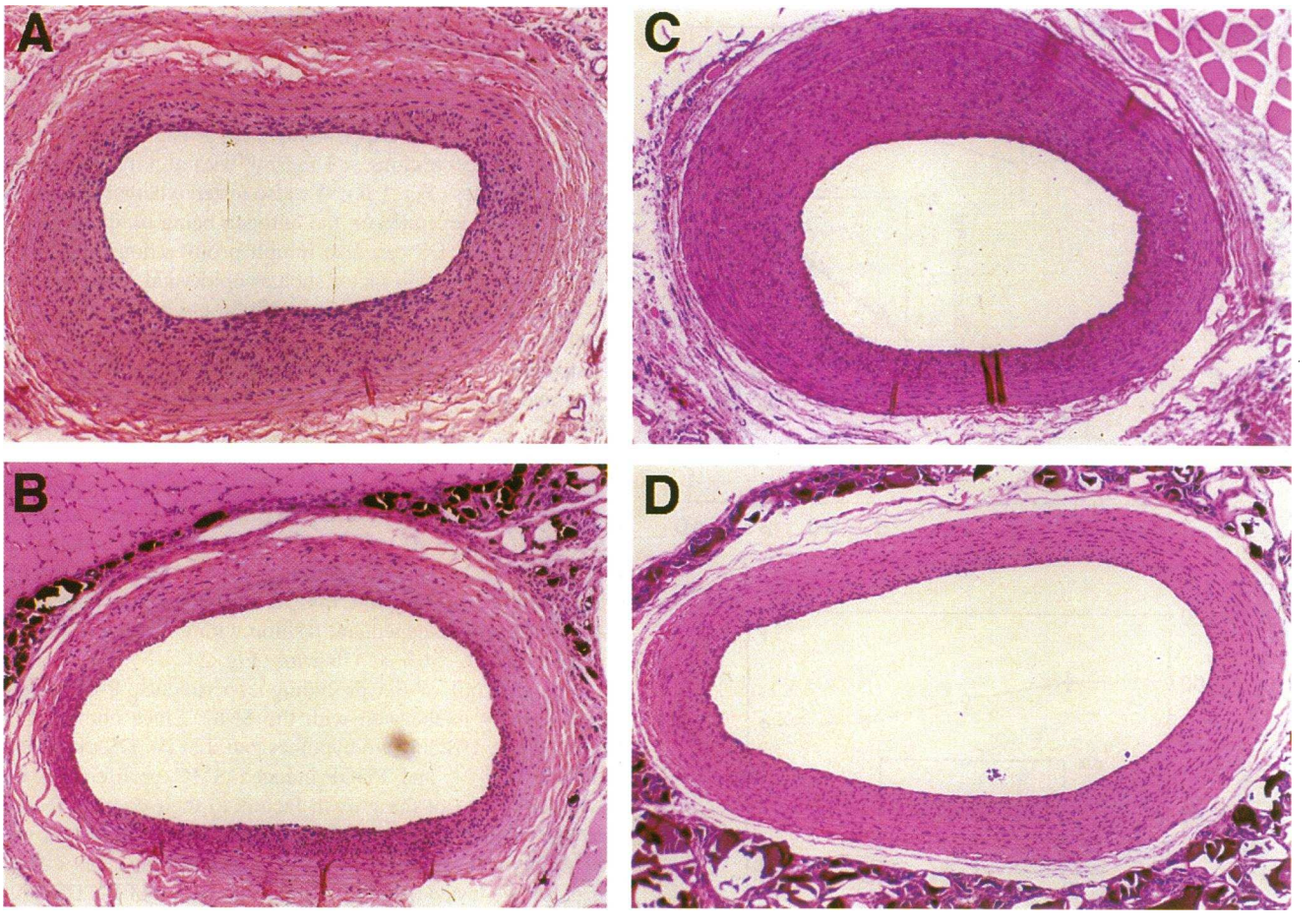

Figure 7. Effect of periadventitial administration of P-CDS in the rat carotid artery injury model. Representative cross sections stained with hematoxylin and eosin from injured left carotid artery in the control (*A* and *C*) and P-CDS (*B* and *D*) treated animals at 14 (*A* and *B*) and 88 d (*C* and *D*) after balloon denudation. The original magnification was 40. Note the dark purple color of the periadventitial P-CDS which avidly takes up hematoxylin at both time points (*B* and *D*).

in contact with the cells in order to have its effect. First, we observed a significant, dose-dependent inhibition of migration with P-CDS, but no effect with P-CD (Fig. 5 *B*). Second, we observed inhibition of migration to a similar extent if P-CDS was placed either in the upper or lower chamber (Fig. 5 *B*), although the effect was less than when the polymer was added above and below. Since the volume of the lower chamber (1.5 ml) was three times that of the higher chamber, although the concentration of polymer was the same, the lower chamber received three times more polymer. However, there was a similar degree of inhibition, suggesting a more potent effect when in close proximity to the SMC. When P-CDS was placed both above and below, the inhibitory effect was maximal.

Kinetics of FGF-2 binding to P-CDS. To define more precisely the kinetics of interaction between FGF-2 and P-CDS, we radiolabeled FGF-2 and performed on-rate and off-rate experiments. At an input concentration of 0.25 μg/ml at 37°C, we measured an observed on-rate of 0.34 ± 0.07 min^{-1} ($n = 4$), demonstrating the ability of P-CDS to rapidly sequester this growth factor, while little FGF-2 bound to unsulfated P-CD (Fig. 6 *A*). When labeled FGF-2 was preabsorbed to the P-CDS, we measured a dissociation rate constant of $3.3\pm0.2 \times 10^{-7}$ min^{-1} ($n = 4$), suggesting that desorption of bound material in the absence of surrounding growth factor would be only half complete in 14 d (Fig. 6 *B*).

Effects of P-CDS in the rat carotid artery model. Finally, we determined whether periadventitial delivery of P-CDS in vivo could limit the intimal thickening response to balloon injury in the rat carotid artery injury model. At 14 and 88 d after balloon injury, the P-CDS–treated animals demonstrated a substantial reduction in intimal thickening compared with the control or P-CD groups (Figs. 7–9). The P-CDS group demonstrated a significant reduction in cross sectional intimal area (0.072 ± 0.013 vs. 0.126 ± 0.016 mm^2, $P = 0.01$) and intima/media area ratio (0.55 ± 0.10 vs. 0.97 ± 0.10, $P = 0.005$) when compared with control at 14 d after injury (Fig. 8). The importance of the sulfation was demonstrated by the complete lack of effect of the unsulfated polymer (P-CD) to inhibit thickening at 14 d. At 88 d, there was continued inhibition of intimal thickening in the P-CDS group demonstrated by an approximately 50% reduction in both intimal area and intima/media area ratio ($P < 0.05$).

Histologic analysis of the adventitia in the P-CDS group at 4 d revealed a proliferative (as demonstrated by BrdU labeling), mixed fibroblastic and histiocytic response surrounding but not infiltrating the central regions of P-CDS particle aggregates. At

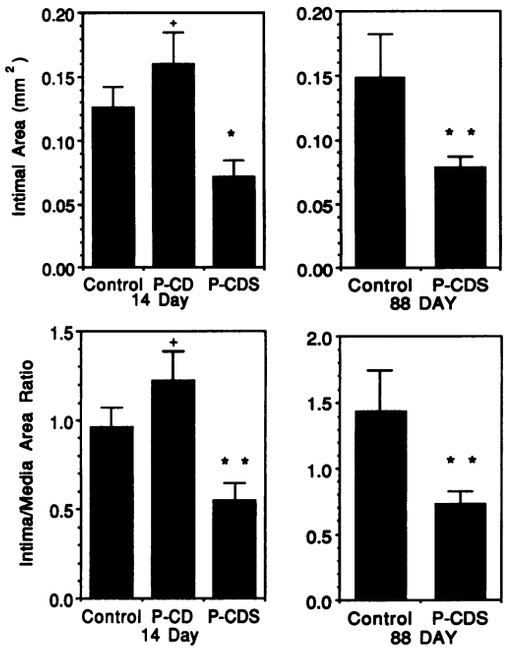

Figure 8. Effect of periadventitial therapy on intimal thickening: quantitative analysis. Grouped data for morphometric indices from control (n = 26), P-CD (n = 5), and P-CDS (n = 28) treated animals 14 d after injury and control (n = 6) and P-CDS (n = 8) treated animals 88 d after injury were compared using unpaired, two sided t tests. Data are provided as mean±SEM. * $P < 0.01$ vs. control, ** $P < 0.05$ vs. control, + P = NS vs. control.

14 d, there was infiltration of fibroblasts, macrophages, and multinucleated giant cells around individual P-CDS particles, with the formation of granulation tissue (Fig. 9, *F* and *J*). At 88 d, there was a minimal proliferative response to the polymer, and there appeared to be further organization of the fibroblastic and histiocytic response surrounding the P-CDS particles (Fig. 9 *H*). Of note, the sulfated polymer continued to retain the hematoxylin dye to a similar extent at 88 d, suggesting retention of sulfation for this period, in contrast to P-CD (unsulfated polymer) which demonstrated minimal staining with this positively charged stain (Fig. 9 *E*).

The effects of P-CDS on SMC migration and proliferation in vitro were not clearly mirrored in the BrdU labeling assay of the injured arteries. At 4 d, the optimal time to assess SMC migration in vivo in this model, there was a mild reduction in total SMC count in the intima that was not statistically significant. There was clearly no reduction in proliferation as measured by BrdU incorporation in the intima or media assessed at day 4 (Table I). However, at 14 d, the P-CDS group demonstrated a significant reduction in the number of BrdU-positive cells in the intima (79±11 vs. 144±23, P = 0.01, Fig. 9, *J* vs. *I*). We observed reductions of 32 and 26% in percent BrdU-positive SMC at 14 and 88 d, respectively, although these reductions did not reach statistical significance. There was also a trend toward reduced total SMC count in the P-CDS versus control group, respectively, at 14 and 88 d in the intima. At all time points, the P-CDS group demonstrated a tendency to increased intimal SMC density (SMC count per area). In the media, there were no differences at 14 and 88 d in any of the measured parameters.

Finally, after balloon injury, P-CDS led to enhanced endothelial regrowth. As seen in Fig. 10, measured endothelial regrowth at 14 d was almost twofold greater in the P-CDS group (P = 0.09). This effect was sustained in that by 88 d, the P-CDS group continued to have an increased amount of regrowth (13.0±0.7 vs. 9.2±0.7 mm, P = 0.005). When the results for all rats were analyzed, there was a modest negative correlation between extent of endothelial regrowth and degree of intimal thickening at 88 d (r^2 = 0.50).

Toxicity of P-CDS. Of the 99 rats randomized, there was no evidence of overt toxicity related to P-CDS. There were three perioperative deaths (P-CDS n = 1, P-CD n = 1, control n = 1) and one death before time of killing in each of the control and P-CD groups. There was no difference in the incidence of thrombosis in the balloon injured artery at time of killing between P-CDS (n = 1) and control (n = 2) rats.

Discussion

Based on extensive work in animal models of vascular injury, SMC proliferation and migration appear to be critical in the development of an exuberant neointima. In the ballooned rat carotid artery, for example, the first event is a burst of SMC proliferation in the media within 48 h, followed by penetration of migrating SMC through the internal elastic lamina into the neointima, where proliferation continues (4–6). Accumulation of SMC in the neointima reaches a maximum at 2 wk, and intimal thickening continues by the deposition of extracellular matrix, until a steady state is achieved at 3 mo (4–6). Several mechanisms have been proposed for the beneficial effects of heparin and related glycosaminoglycans to modulate the response to vascular injury. Unfractionated heparin, low molecular weight heparin, and acylated heparin fragments have been shown to have direct inhibitory effects on SMC proliferation and migration (7–13). Although the precise mechanism of action of these compounds is largely unknown, it is postulated that their ability to bind and modulate HBGF plays a pivotal role. For example, administration of FGF-2 at the site of injury leads to increased intimal thickening, but the addition of heparin inhibits its action (32). Transfection of FGF-1 directly into an arterial wall stimulated intimal thickening as well as angiogenesis in a porcine model (33). The complexing of heparin and heparin-like compounds with certain HBGFs has been shown to enhance their ability to stimulate endothelial cell regrowth in vitro (15–18), and early reestablishment of endothelium after vascular injury may lead to a reduction in intimal thickening. Alternately, heparin has the ability to enhance the activity of growth inhibitors such as transforming growth factor β by releasing it from its inactive complex amplifying its antiproliferative effects (34).

The rationale for testing the effects of sulfated β-cyclodextrins on the prevention of intimal thickening derives from their ability to mimic several important biological functions of heparin without effects on coagulation. Our group has demonstrated that orally administered soluble, monomeric CDS inhibits restenosis and intimal hyperplasia in a hypercholesterolemic rabbit angioplasty model (23). Reilly and colleagues (24) have shown recently inhibition of neointima formation in the rat carotid artery balloon injury model by continuous intravenous administration of the same soluble CDS. It has become apparent from these studies that although CDS has minimal effects on coagulation, the usefulness of CDS may be limited by the rather large

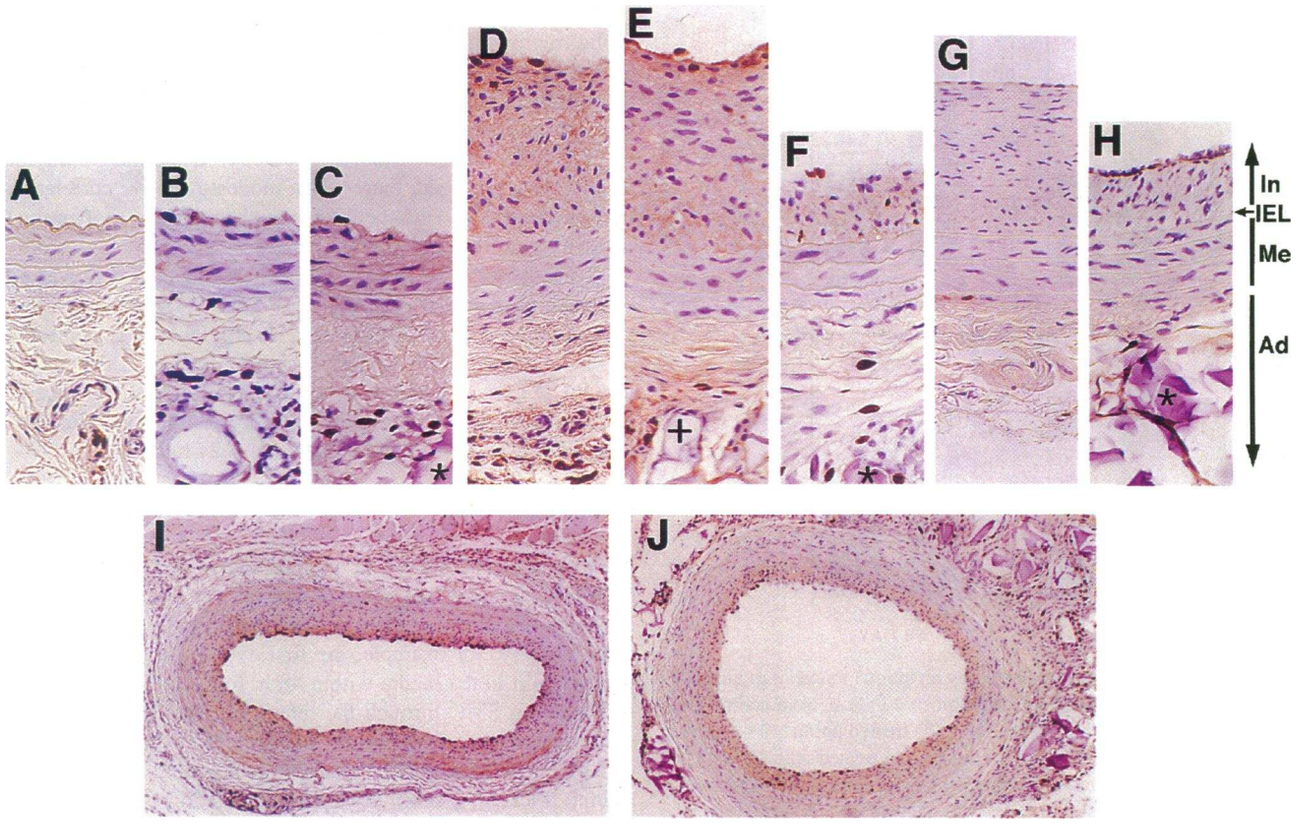

Figure 9. Effects of periadventitial P-CDS and unsulfated P-CD on intimal thickening and SMC proliferation: BrdU immunohistochemistry. Representative cross sections from uninjured right (*A*) or injured left (*B–H*) rat carotid arteries at 4 (*B* and *C*), 14 (*D–F*), or 88 (*G* and *H*) after injury. Rats were treated with no polymer (*B, D,* and *G*), unsulfated polymer (*E*), or sulfated polymer (*C, F,* and *H*). The locations of the intima (*In*), media (*Me*), and adventitia (*Ad*) are noted to the right. All sections are aligned such that all of the internal elastic laminae (*IEL*) are aligned. Sections were stained with an antibody to detect BrdU (dark brown) and counterstained with hematoxylin. The + (*E*) denotes the position of unsulfated polymer (P-CD) which takes up the purple hematoxylin poorly. The * (*C, F,* and *H*) denotes the location of sulfated polymer (P-CDS) which stains dark purple. Cells that were proliferating at the time of BrdU administration have brown nuclei. Some nonspecific nonnuclear brown staining is present. Full cross sections for a control artery (*I*) and a P-CDS–treated artery (*J*) at 14 d are also shown (original magnification of 40). Original magnification, *A–H,* ×400.

doses required for effectiveness. It is with these principles in mind that the use of the polymer, P-CDS, was investigated. We have demonstrated a dose-dependent inhibitory effect on both proliferation and migration in cultured rat aortic SMC treated with P-CDS. We have shown that in the rat carotid artery balloon injury model, periadventitial administration of P-CDS at the time of injury leads to a persistent inhibition of intimal thickening for at least 3 mo. The degree of inhibition of intimal

Table I. Immunohistochemical Analysis of BrdU Labeling of the Rat Carotid Artery after Balloon Injury

	4-d*			14-d			88-d		
	Control‡	P-CDS§		Control	P-CDS		Control	P-CDS	
Left carotid artery	(n = 9)	(n = 9)		(n = 12)	(n = 14)		(n = 6)	(n = 8)	
Intima									
Total SMC count	14±3‖	11±5	NS	852±145	603±63	P = 0.11	1230±273	823±106	P = 0.15
BrdU-positive SMC count¶	9±2	9±4	NS	144±23	79±11	P = 0.01	2±1	2±1	NS
Percent BrdU-positive SMCs	60.4±11.9	83.2±6.2	P = 0.17	20.2±4.5	13.7±1.7	P = 0.16	0.23±0.04	0.17±0.04	NS
SMC density index**	5548±966	6911±1877	NS	7658±390	8878±527	P = 0.08	8130±659	10242±775	P = 0.07
Media									
Total SMC count	266±12	253±23	NS	354±37	402±29	NS	335±34	367±23	NS
BrdU-positive SMC count	37±5	48±7	NS	3±1	5±1	NS	1±1	1±1	NS
Percent BrdU-positive SMCs	13.8±1.8	18.7±2.2	P = 0.11	1.1±0.6	1.4±0.4	NS	0.2±0.1	0.3±0.2	NS
SMC density index	2013±117	1747±122	NS	2590±156	2996±269	NS	3291±518	3247±334	NS

‖ Data reported as mean±SEM. * Time of examination after balloon injury. ‡ Injured rat carotid artery without pharmacologic agent. § Injured rat carotid artery treated with periadventitial placement of 10 mg of P-CDS. ** Total number of SMC per area in square millimeters. ¶ BrdU-positive SMC cells are those that have taken up BrdU, indicating recent DNA synthesis (i.e., proliferation).

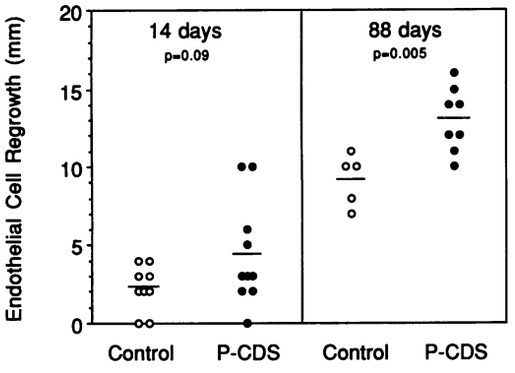

Figure 10. Effect of periadventitial P-CDS on endothelial regrowth. Before time of killing an intravenous injection of Evans blue solution was given to allow differentiation between arterial segments denuded of endothelium (blue) versus segments with intact endothelium (white) for animals at 14 and 88 d. The distance from the carotid bifurcation to the blue/white transition was measured.

thickening by P-CDS appears to be similar to that observed with locally delivered heparin or related derivatives from polymer matrix systems (12, 35). However, this study differs in that the polymer itself has the desired activity and remains intact for up to 3 mo, whereas previous studies were limited to 14 d of observations.

Much like heparin, the precise in vivo mechanism of action of P-CDS is unclear. We observed at 14 d after injury that, compared with controls, the number of proliferating SMC in the intima was significantly decreased in the P-CDS group and there was a trend toward fewer total intima SMC, without significantly reducing in the media the number of proliferating SMC or the percentage of proliferating SMC. Our findings are in concordance with those of Clowes and Clowes (9) who demonstrated that treatment with intravenous heparin after balloon injury led to a reduction in the total number of intimal SMC and the number of proliferating SMC in the intima; furthermore, this reduction was primarily due to a decreased number of migrating cells from the media to the intima and a decrease in intimal SMC growth fraction. P-CDS may affect SMC volume or extracellular matrix production, or both, inferred by the trend toward increased intimal SMC density in the P-CDS group. Since SMC numbers in the intima of P-CDS–treated carotid arteries were less, the increased SMC density was likely due to decreased extracellular matrix production, although a decrease in SMC volume cannot be excluded. Previous studies in the rat carotid model have shown that heparin decreases SMC production of collagen and elastin and reduces the volume density of intimal SMC which are closest to the lumen (13, 14). Finally, after balloon injury, P-CDS leads to enhanced endothelial regrowth, which inversely correlated with intimal thickening. Several HBGFs are mitogenic and chemokinetic for endothelial cells including FGF-1, FGF-2, and VEGF. VEGF is relatively selective in its mitogenic activity for endothelial cells (30). Although speculative, if VEGF were upregulated after injury, it could be sequestered and slowly released from P-CDS, perhaps accounting in part for the increased endothelial cell regrowth. Another possibility for the observed differences in vivo is the possibility that P-CDS in the periadventitial space modulates regulatory molecules generated in the adventitia in response to injury. Scott et al. (36) have noted both inflammatory and proliferative changes in the adventitia of porcine coronary arteries after balloon angioplasty, with increased expression of PDGF-A mRNA in the adventitia. Adventitial myofibroblast proliferation has also been documented in the same model (37), which could participate in maladaptive chronic remodeling with constriction which has been suggested to possibly play a role in restenosis. Whether part of the efficacy of P-CDS depends on modulation of adventitial growth and migration factors remains to be determined.

As pointed out recently by Peppas and Langer (38), the merger of knowledge in cell biology and material science may enable the generation of materials with specific medically useful properties. The utility of P-CDS, a prototype in a new class of therapeutic polymers, may extend beyond the prevention of intimal thickening. By virtue of its very high affinity and capacity to bind certain growth factors, it could be used to deliver slowly one or more of these factors adsorbed to it before implantation. Slow release of these growth factors could facilitate angiogenesis and/or wound healing as examples. Alterations in the polymer chemistry may also facilitate or retard its eventual absorption, enabling some control over the duration of action of these effects.

In conclusion, this study demonstrates that a sulfated, polymer of β-cyclodextrin is a potent and durable inhibitor of intimal thickening after vascular injury. The effectiveness of P-CDS may be related in part to its inhibition of SMC proliferation and migration and its stimulation of endothelial cell regrowth. The mechanism of its inhibitory action may involve the demonstrated ability of P-CDS to absorb HBGFs in vitro. The ease of local application of this polymer at the time of vascular surgery, especially at anastomoses where intimal thickening is a particular problem, may enable more durable results from vascular procedures.

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