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Antiproliferative Effects of Novel, Nonanticoagulant Heparin Derivatives on Vascular Smooth Muscle Cells *In Vitro* and *In Vivo*

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The proliferation of vascular smooth muscle cells (VSMC) is strongly inhibited by whole heparin both* in vitro *and* in vivo. *To identify and characterize antiproliferative, but nonanticoagulant heparin derivatives, heparin fragments made by periodate treatment were produced and acylated with 2-, 4-, or 6-carbon chain lengths. In culture, the 4- and 6-carbon acylated compounds were more effective than whole heparin in inhibiting serum stimulated VSMC growth at equal mass or approximately equal mean molar concentrations. Further testing was performed in the rat carotid balloon injury model. Myointimal VSMC proliferation produced by balloon catheterization of rat carotid arteries was inhibited by the 4-carbon acylated compound as effectively as heparin at the same mass dose. Importantly, unlike heparin, the 4-carbon acylated compound had no anticoagulant effect* in vivo. *These experiments suggest nonanticoagulant, acylated heparin derivatives may have a pharmacologic role in preventing myointimal proliferative lesions that are responsible for failures of vascular surgeries and angioplasties. (Am J Pathol 1991, 139:1501–1509)

Atherosclerotic vascular disease is the major cause of morbidity and mortality in the United States. Vascular smooth muscle cells (VSMC) are a prominent cell type in atheromatous plaques, and VSMC growth leading to occlusive lesions is a major cause for long-term failure of vascular surgical procedures.[1] Heparin is a potent antiproliferative agent for VSMC. Thus heparin may have an important pharmacologic role in the prevention of accelerated atherosclerosis involving VSMC growth that accompanies procedures such as angioplasty and vascular surgery. Heparin is an anticoagulant, however, that acts by increasing the affinity of the protease inhibitor antithrombin III for the serine proteases important for coagulation,[2] and therefore, heparin treatment, because of its anticoagulant activity, can lead to hemorrhagic complications as well as the potential for electrolyte shifts and thrombocytopenia. Clinical failures due to VSMC proliferation, however, might be prevented by treating patients with nonanticoagulant, antiproliferative heparin compounds, thus avoiding the hemorrhagic complications. The purpose of these experiments was to test several acylated heparin derivatives for their ability to both inhibit VSMC proliferation *in vitro* and to inhibit the development of myointimal thickening seen *in vivo* after arterial injury. Their anticoagulant activities also were investigated *in vivo*.

Currently this laboratory is actively studying the pathobiology of accelerated atherosclerosis in the rat using a carotid artery endothelial injury model. In this system, massive smooth muscle cell myointimal hyperplasia occurs in areas denuded of endothelium. Treatment of the injured rats with whole heparin markedly suppresses the smooth muscle cell proliferation.[3] Heparin is required in the first 2 to 3 days after injury, and a short 3- to 5-day treatment is enough to confer a long-lasting impact on VSMC proliferation in the injured artery. In this model, heparin also inhibits the migration of VSMC but does not affect adherent platelet number, or endothelial regeneration.[3–5] Anticoagulant or nonanticoagulant heparin species, isolated by separation of binding and nonbinding fractions on an antithrombin III affinity column, are equally effective in abolishing the VSMC proliferation in this sys-

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tem; thus the antiproliferative activity of heparin is not dependent on its anticoagulant activity.[5] In other induced injury systems, heparin inhibits the pulmonary arterial thickening in response to hypoxemia[6] and mesangial cell proliferation after glomerular injury.[7]

In VSMC culture systems, both anticoagulant and nonanticoagulant heparin inhibit VSMC proliferation. Other glycosaminoglycans such as chondroitin sulfate, dermatan sulfate, or hyaluronic acid are not active.[8] A variety of cultured cell types such as cervical epithelium, glomerular mesangial cells, BALB/c 3T3 fibroblasts, and mouse L-M cells are also sensitive to heparin to varying degrees, suggesting a more general physiologic role for heparin/heparan sulfates in regulating cell growth.[8,9]

Heparin is composed of repeating disaccharide units of alternating glucosamine and uronic acid residues. Heparin is highly charged because of heterogenous modification of the saccharides by carboxylate groups and N- or O-linked sulfate groups. The carbons of the saccharide units are conventionally numbered 1 through 6, and hydroxyl groups or O-linked sulfate groups occur at the 3 and 6 positions of glucosamine and 2 and 3 positions of the uronic acids.[10] In the present experiments, chemical modifications of heparin have been made in an attempt to modulate its pharmacologic properties and particularly to abolish the anticoagulant activity while maintaining other types of pharmacologic activities. Whole heparin was periodate treated, and the resulting heparin fragments were subjected to acylation with 2-, 4-, or 6-carbon (C) chains at the hydroxyl or O-linked sulfate groups (O-positions) of both the glucosamine and uronic acid residues. The preparation of O-acylated compounds was undertaken having in mind the following questions: 1) Does this modification preserve or abolish the anticoagulant and antithrombotic properties, and 2) What is the influence of such modifications on the different pharmacologic activities, especially the antiproliferative activities. In this report we show that all the acylated heparin compounds inhibited VSMC proliferation in culture; the 4-C and 6-C compounds were more effective than whole heparin. *In vivo,* the 4-C acylated compound had no effect on clotting time and strongly inhibited VSMC growth in the rat carotid injury system.

Materials and Methods

Preparation and Characterization of Heparin Compounds

The whole heparin used for these studies and the subsequent modifications was the sodium salt derived form porcine mucosa with a molecular weight of 12,000 to 18,000 daltons and was isolated at the Institute Choay, Paris, France. Heparin fragments were obtained by periodate oxidation of the sodium salt of the pig mucosa heparin by solubilizing the heparin in water and cleaving with periodate oxidation at pH 5 at a concentration of heparin and sodium periodate of 2%. The solution then was dialyzed and β-elimination conducted in basic conditions (sodium hydroxide, final molarity 0.2 N) and fragments reduced by sodium borohydride. The product was recovered by alcohol precipitation (1.5 volumes of ethanol) after addition of NaCl (20 g/liter). These periodate-treated fragments average approximately 6000 molecular weight (range 3,600–11,000), and the sulfate/carboxylate ratio determined by conductimetry was 2.35. Periodate-treated heparin fragments then were acylated and the acylated derivatives were characterized.[11] Heparin fragments to be acylated were first converted into their tetrabutylammonium or tributylammonium salt by passage through a cation exchange column under acidic form, followed by neutralization with tetrabutylammonium hydroxide or tributylamine. The dried ammonium salt then was dissolved in dimethylformamide and acylated by the desired carboxylic acid anhydride in the presence of tributylamine and a catalytic amount of dimethylaminopyridine. The product was isolated by alcohol precipitation and converted into the sodium salt by passage through a cation exchange column under sodium form. We have checked by nuclear magnetic resonance (NMR) that acylation only takes place at the *O*-positions, ie, three or six of the glucosamine units and two or three of the uronic acid residues. An average of 1.5 acyl chains were introduced per disaccharide unit. This value was determined by ^{1}H-NMR and also by gas liquid chromatography after transesterification. The sulfate content and the sulfate/carboxylate ratio of the products were the same as in the starting material.

Cell Culture

Rat aortic VSMC from Sprague-Dawley rats (Charles River, Wellesley, MA; CD strain) were isolated, cultured, and characterized as previously described.[12] The abdominal aortae of 50- to 100-g male rats were harvested and the adventitia removed under a dissecting microscope. The aorta was cut longitudinally and pieces of media were taken from the vessel wall and placed under a small stainless steel mesh screen in 60-mm tissue culture dishes. Within 1 to 2 weeks, VSMC migrated from the explants, and were subcultured about a week after the first appearance of cells. They were grown in RPMI-1640 medium containing 20% fetal calf serum (FCS) and cultured in a humidified incubator at 37°C and 5% CO_2 atmosphere and were used from the fourth through 12th passages. Vascular smooth muscle cells phenotype was

confirmed by the presence of smooth-muscle-cell–specific actin in growth arrested cells as demonstrated by immunocytochemistry, and the characteristic 'hill and valley' appearance of confluent cultures.

Growth Assays

Before growth assays, VSMC were growth arrested by culturing cells for 48 hours in 0.4% FCS/RPMI medium. Vascular smooth muscle cells were released from G_o by addition of 20% FCS/RPMI with or without various doses of whole heparin or heparin derivatives. After 4 to 6 days, when serum stimulated cells began reaching confluence, VSMC were washed in Ca^{++} and Mg^{++} free Hank's Hepes, cells were released from the dish by trypsinization, and cell number was determined using a Coulter counter. Percent inhibition of proliferation was measured as [1 − (cell number in 20% FCS plus heparin/cell number in 20% FCS)] × 100.

Balloon Injury

Male Sprague-Dawley rats weighing from 300 to 350 g were anesthetized with an intraperitoneal injection of sodium pentobarbital at 50 mg/kg. The bifurcation of the left common carotid artery was exposed through a midline incision. A 2-Fr Fogarty balloon catheter was introduced through an external carotid artery arteriotomy. The catheter was passed three times with the balloon distended with a volume of air (0.4 to 0.6 cc) sufficient to generate moderate resistance. The catheter was withdrawn and the proximal external carotid ligated. After placement of intravenous pump tubing for heparin delivery into the right jugular vein, the wound was closed with a running nylon suture. All procedures were carried out by one investigator who was blind to the treatment group at the time of balloon injury and by subsequent pump placement.

Heparin Delivery

Heparin compounds were administered in lactated Ringer's solution at a dose of 0.3 mg/kg·hr using an implantable osmotic pump (Alza Corp, Palo Alto, CA), placed at the time of surgery. These osmotic pumps reliably deliver at a predictable rate over a period of 14 days. Control pumps were filled with sterile carrier solution. Pumps were placed in a beaker of lactated Ringer's solution at 37°C for 48 hours to assure that uniform pumping had begun and that the attached Silastic catheters were filled. After balloon catheterization, the pumps were implanted in a subcutaneous pocket over the dorsum of the rat's chest. Silastic tubing, attached to the pump, was passed through a subcutaneous tunnel, to the ventral neck, and placed in the right jugular vein using a cut-down technique. Satisfactory drug delivery was confirmed at time of harvest by determination of pump residual volume, intravenous catheter position, and patency.

Morphology and Morphometry

Injured carotids were harvested at 14 days. One hour before harvest animals received an intravenous injection of Evans blue dye at 60 mg/kg. Animals were anesthetized as for surgery and retrograde perfused with lactated Ringer's solution using a 22-gauge Silastic catheter placed in the abdominal aorta. The lactated Ringer's solution was suspended at a height sufficient to generate a pressure of 90 cm H_2O as measured at the catheter tip. Venous drainage was obtained through bilateral jugular venotomies, and the perfusion was carried out until the jugular effluent was clear. The animals then were perfused with 100 ml of a modification of Ito-Karnovsky fixative (4% paraformaldehyde, 0.05% glutaraldehyde, 15% saturated picric acid vol/vol). The neck then was opened and a 1-cm segment was taken from the central blue region of the left common carotid artery. Segments were further immersion fixed at 4°C for 1 hour.[13]

After fixation, specimens were extensively washed in 0.1 mol/l (molar) phosphate buffer at 4°C and stored under the same conditions until paraffin embedded. Five-micron sections were taken from the midportion of the arterial segment and stained with hematoxylin and eosin (H&E) for elastin using Verhoeff's method. Verhoeff-stained slides were projected onto a digitizing pad with a Leitz projecting microscope. The areas bound by the lumen, internal elastic lamina, and outer medial boundary were measured with a digital planimeter, and the intimal and medial areas were calculated. All measurements were made with the observer blind to treatment group. Cell number per unit area was determined by examining H&E-stained sections at 1000× magnification. Using an optical graticule, the number of nuclei per graticule area was counted in four distinct regions around the circumference of the intima. Two sections were counted from each carotid, for a total sampling of 14,800 μ^2.

Anticoagulation Assay

To compare the *in vivo* anticoagulant effects of whole heparin and the heparin derivatives, male rats weighing 350 to 400 g received an injection of 3.6 mg/kg of heparin or heparin derivative. Whole blood clotting times and ac-

tivated partial thromboplastin times were measured before injection, and at 30, 60, 120, and 180 minutes after injection. To determine clotting times, animals were tail bled into 0.6-mm internal diameter capillary tubes, and the tubes were broken every 30 seconds until a strand of clot was observed. Partial thromboplastin times were determined by applying sample blood into a diagnostic test cartridge and measuring cessation of blood flow by laser photometry using a 512 coagulation monitor (Ciba Corning Diagnostic Corp., Medfield, MA).

Results

Whole heparin and acylated heparin compounds were tested for their antiproliferative activity on cultured rat VSMC. Past experiments have shown that heparin is a potent inhibitor of smooth muscle cell proliferation. In the present experiments we tested a periodate-treated heparin fragment, and derivatives of this compound that were modified at the O-positions with acyl groups containing 2, 4, or 6 carbons, and compared their antiproliferative activity with the antiproliferative activity exhibited by whole heparin itself. Vascular smooth muscle cell growth assays demonstrated that native heparin, the periodate-treated compound, and acylated derivatives all have substantial antiproliferative activity (Figure 1). In general, the lowest dose of these compounds that was significantly inhibitory was 10 ug/ml. There were differences in the antiproliferative activities between the compounds, however. The periodate-treated compound was less effective than whole heparin at most doses tested. In contrast, the 4-C and 6-C acylated derivatives had higher antiproliferative activity than whole heparin, whereas the 2-C derivative was equally as effective as heparin.

We determined the antiproliferative activity of the heparin compounds if compared at approximately equal mean molarities, based on the average molecular weight of the different heparin compounds. As the mean molecular weight of the periodate-treated heparin and acylated derivatives (an average of 6000 MW) are approximately half the molecular weight of whole heparin (an average of 15,000 MW), we compared the inhibitory activity of 200 ug/ml of heparin (equal to a mean molarity of 13.3 μmol/l [micromolar]) *versus* the periodate-treated compound and acylated heparin derivatives at 100 μg/ml (equal to a mean molarity of 16.5 μmol/l) (Figure 2). At these doses, native heparin is significantly more inhibitory (58.8%) than the periodate-treated precursor (40.1%), and less inhibitory than the 4-C (74.1%) or 6-C (76.8%) acylated compounds. Similarly whole heparin at a dose of 100 μg/ml (equal to a mean molarity of 6.7 μmol/l) compared with the heparin derivatives at 50 μg/ml (equal to a mean

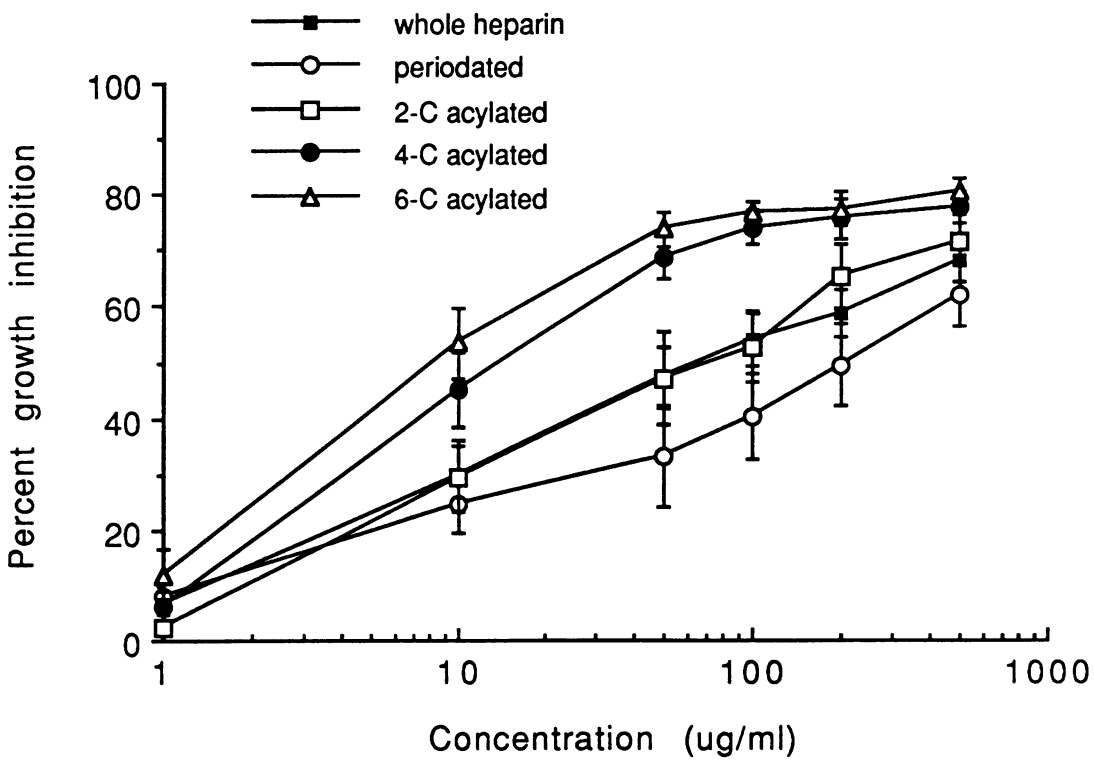

Figure 1. *Heparin and heparin derivatives inhibit cultured VSMC growth. Rat VSMC were growth arrested by culturing in 0.4% FCS/RPMI for 48 hours and were released from G_0 by addition of 20% FCS/RPMI with or without the indicated concentrations of whole heparin, the periodated heparin precursor, or the 2-C, 4-C, or 6-C acylated heparin derivatives. Cell number was determined after 4 to 6 days. Data are given as the percent inhibition compared to non-heparin treated control cultures and are the mean values from at least four experiments.*

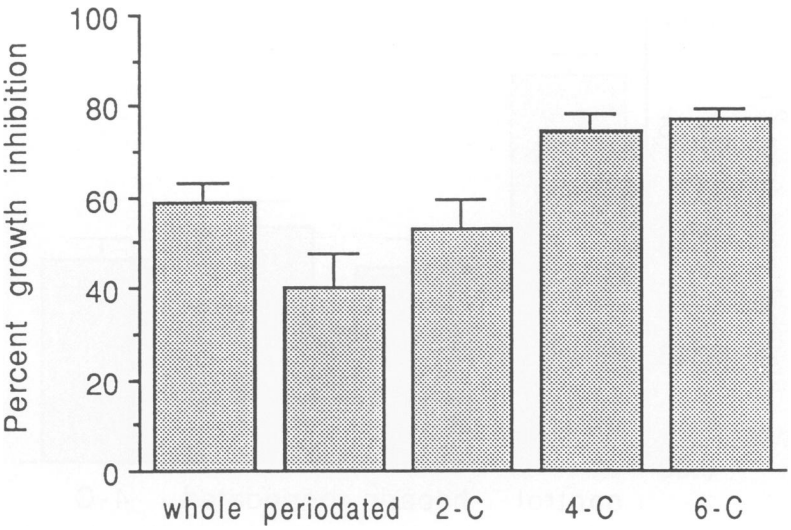

Figure 2. In vitro *growth inhibitory activity of approximately equal mean molar concentrations of heparin and heparin derivatives. Rat VSMC were growth arrested and treated with 20% FCS/RPMI with or without heparin or heparin derivatives as described in Figure 1. Percent growth inhibition is shown for approximately equal mean molar concentrations of whole heparin (200 μg/ml, equal to a mean molarity of 13.3 μM), the periodated heparin precursor (100 μg/ml), or the 2-C, 4-C, or 6-C acylated heparin derivatives (100 μg/ml, equal to a mean molarity of 16.5 μM). At these approximately equal mean molar concentrations, whole heparin and the 2-C acylated derivative are more inhibitory than the periodated heparin precursor ($P \leq 0.06$); and significantly less inhibitory than the 4-C or 6-C acylated derivatives ($P \leq 0.01$; Student's t-test).*

molarity of 8.3 μmol/l) compared with the heparin derivatives at 50 μg/ml (equal to a mean molarity of 89.3 μmol/l) had approximately 21% more antiproliferative activity than the periodated precursor and 17% less activity than the 4-C or 6-C acylated compounds. Thus these experiments show that the 4-C and 6-C acylated compounds were very effective at low doses, and were more effective than heparin even at approximately equal mean molar doses.

After determining the increased effectiveness of the 4-C and 6-C acylated compounds to inhibit VSMC *in vitro,* we compared whole heparin, the periodate-treated precursor, and the 4-C acylated compound *in vivo,* testing their ability to suppress the development of myointimal thickening after arterial injury. Rat carotid arteries were subjected to balloon injury and whole heparin, periodate-treated precursor, 4-C acylated compound or control carrier solution was delivered by pump infusion into the jugular vein. After 14 days, the carotids were perfusion fixed, harvested, sectioned, and stained (Figure 3). Intimal and medial areas were quantitated by digital planimetry. The development of myointimal thickening compared with non–heparin-treated controls was reduced approximately 50% by heparin, 47% by the 4-C acylated heparin derivative, and 40% by the periodate treated precursor (Figure 4). Although the data suggest that whole heparin and 4-C acylated derivative were more effective than the periodate-treated precursor, these differences did not reach statistical significance. Two separate preparations of the 4-C acylated compound were tested, and these were quite comparable in their ability to inhibit myointimal thickening. Medial areas were modestly decreased by the compounds, compared with control ballooned animals: whole heparin, 22%; 4-C acylated fragment, 28%; periodate-treated precursor, 20%. The ratio of intimal area to medial area, a

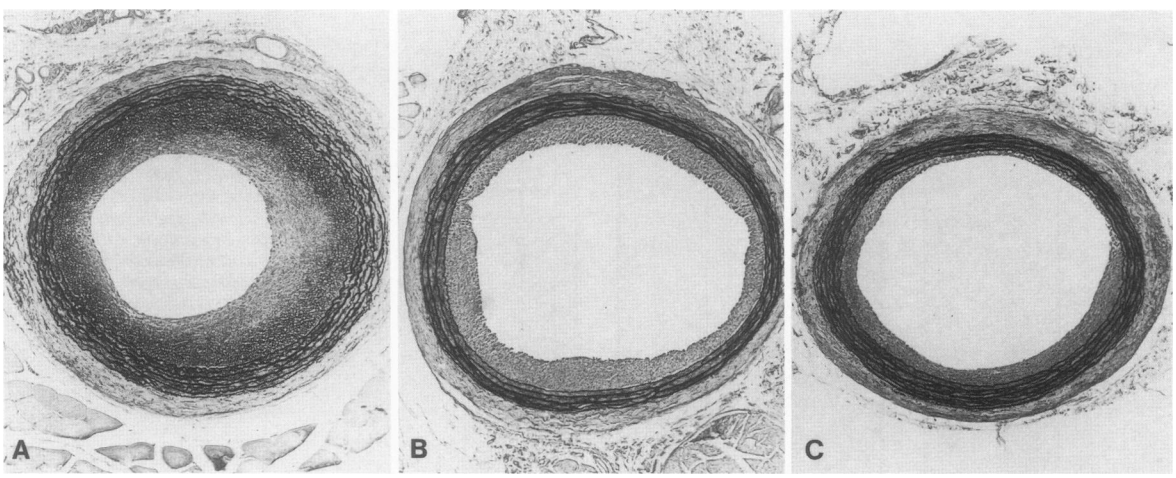

Figure 3. *Light photomicrographs of carotid arteries from balloon-injured rats at 14 days receiving: control saline solution* (**A**), *whole heparin* (**B**), *and 4-C acylated heparin derivative* (**C**). *Elastic lamina appear as darkly stained bands throughout the media (Verhoeff's elastin, ×100).*

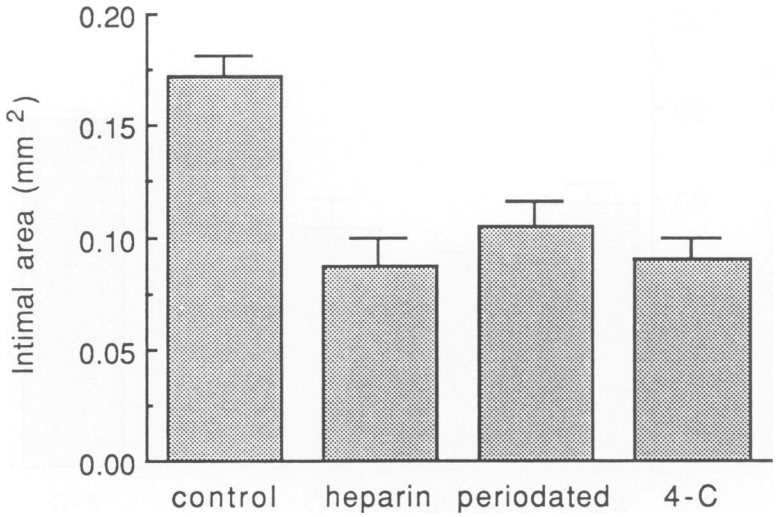

Figure 4. *Heparin and heparin derivatives inhibit intimal thickening in balloon injured rat carotid arteries. Rats were subjected to balloon injury of the left common carotid artery and treated by continuous iv infusion with saline (control, n = 10), or 0.3 mg/kg-hr of whole heparin (heparin, n = 5), periodated heparin fragment (periodated, n = 6), or the 4-C acylated heparin derivative (4-C, n = 9). After 14 days the carotids were harvested, sectioned, stained and the intimal areas were measured with a digital planimeter. Whole heparin, the periodated heparin fragment, and the 4-C acylated derivative all significantly inhibited myointimal thickening compared with control (P ≤ 0.001).*

measure of the myointimal proliferative response that controls for oblique sectioning, showed that, compared with control, heparin decreased the I/M ratio by 30%, and the 4-C acylated compound by 35% (Figure 5).

Because the reductions in intimal thickening may be due to changes in cell number or extracellular matrix or both, we assessed the intimal cell density in the control, heparin, and heparin-derivative–treated samples by determining cell number per unit area. Intimal cell number per square millimeter was 7950 ± 865 for saline-treated controls, 7680 ± 865 for whole heparin, 8060 ± 580 for periodate-treated precursor, and 8440 ± 1180 for the 4-C acylated compound. There was no statistical difference in cell number/unit area of the heparin or heparin derivative samples compared with control; thus the changes in intimal thickening in both heparin and the heparin derivative-treated samples are due to changes in cell number and not to extracellular matrix volume.

It was of particular interest to determine the *in vivo* anticoagulant activities of the 4-C acylated derivative and the periodated heparin fragment relative to whole heparin. The antiocoagulant activity of whole heparin, the periodated heparin fragment, and 4-C acylated heparin were tested as a bolus injection to insure a large dose of the heparin derivatives and thus more readily detect any anticoagulant effect of the compounds. Rats received a single dose of 3.6 mg/kg compound (an equivalent dose to 12 hours of continuous infusion in the antiproliferative experiments). Both whole blood clotting time and activated partial thromboplastin times were determined before the heparin injection, and at 30, 60, 120, and 180 minutes after the dose was given (Figure 6). Whole heparin prolonged the clotting time by 2.5- to 3-fold, whereas neither the 4-C acylated fragment nor periodated precursor had any anticoagulant activity. Similarly heparin prolonged the activated partial thromboplastin times by 5- to 10-fold over the first 2 hours, whereas the other two heparin derivatives again had no effect. The anticoagulant

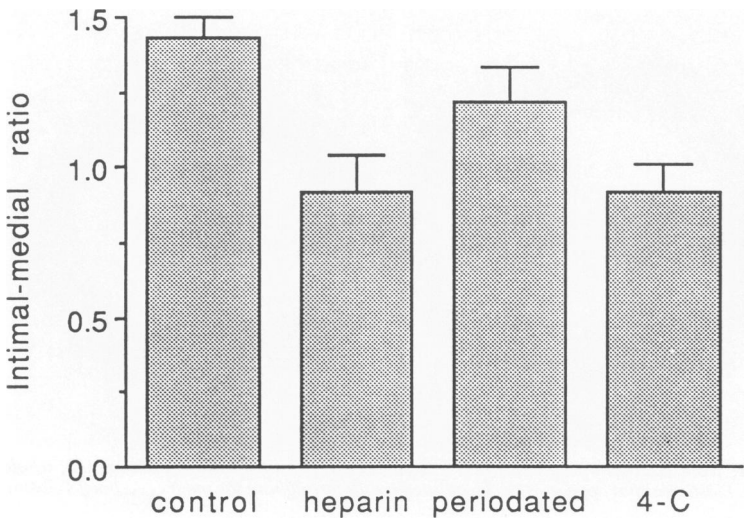

Figure 5. *Inhibitory activity of heparin and heparin derivatives on the intimal-medial ratio after balloon injury of the carotid artery. Balloon injured rats were treated with saline (control), or 0.3 mg/kg-hr of whole heparin (heparin), periodated heparin fragment (periodated), or the 4-C acylated heparin derivative (4-C), and the intimal and medial areas of the injured carotid arteries were measured by digital planimetry and the ratio of the two calculated. The intimal/medial ratio was significantly reduced compared to control by whole heparin (P ≤ 0.01) and by the 4-C acylated heparin derivative (P ≤ 0.001).*

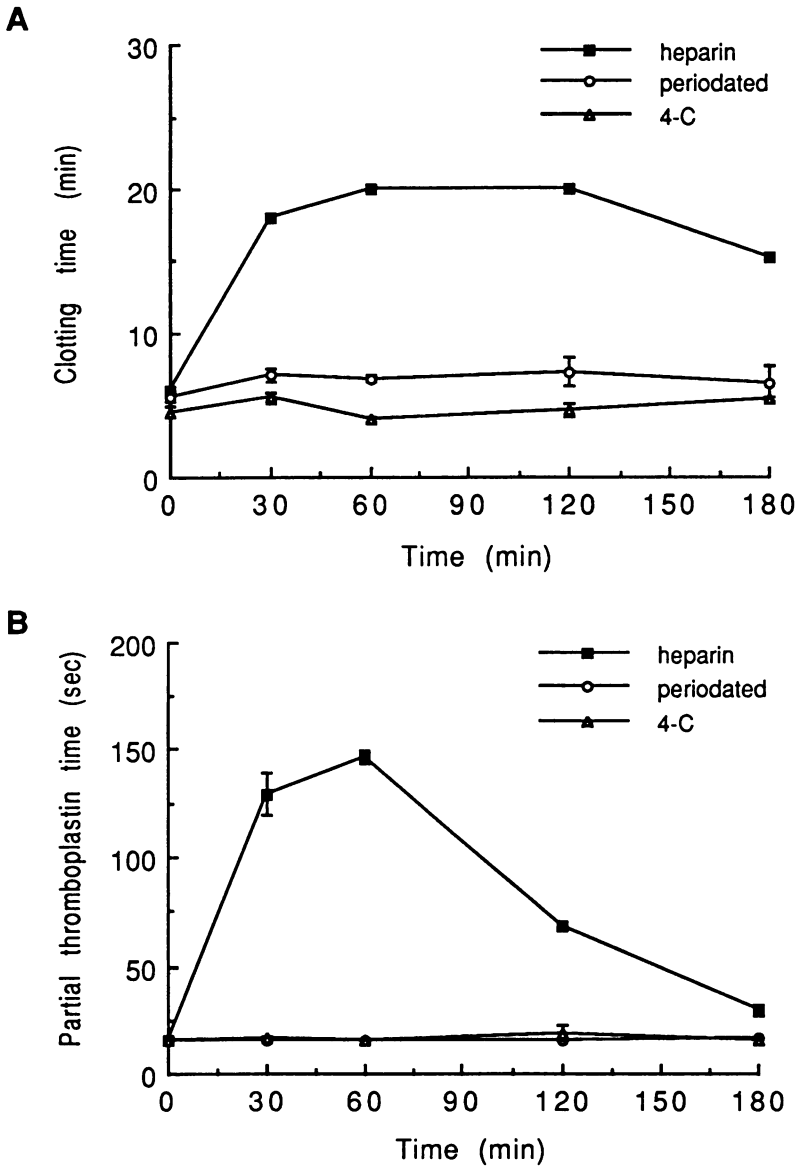

Figure 6. *Heparin but not the heparin derivatives elevate whole-blood clotting times and partial thromboplastin times. Rats were given a 3.6 mg/kg dose of whole heparin, periodate treated heparin derivative, or 4-C acylated heparin derivative. Whole blood clotting times* (A) *and activated partial thromboplastin times* (B) *were determined before injection (0), or 30, 60, 120, and 180 minutes after injection. Heparin significantly elevated both clotting time and partial thromboplastin times compared to treatment with the periodated heparin fragment or the 4-C acylated derivative* ($P \leq 0.001$).

activities of the heparin derivatives also were tested in the experimental animals receiving continuous infusion at 0.3 mg/kg·hr of either 4-C acylated derivative, or periodated precursor. These animals showed no elevation of whole blood clotting time at 14 days. We and others have repeatedly shown that administration of whole heparin results in elevation of whole blood clotting times from 1.5 to 2 times normal.[3,5,14] Thus neither the 4-C acylated fragment or periodate precursor had any anticoagulant activity as measured by *in vivo* assays of antithrombin III mediated anticoagulation.

Discussion

In this article we report that several low-molecular-weight, nonanticoagulant heparin derivatives were effective in inhibiting smooth muscle cell proliferation. For VSMC in culture, the acylated compounds were shown to be more effective than the periodated heparin fragment in inhibiting growth. Further the 2-C acylated fragment was as active as whole heparin, but was less effective than either the 4-C or 6-C acylated compounds in suppressing VSMC proliferation. Structure–function studies using chemically modified heparin compounds have identified several important structural features of heparin contributing to its growth inhibitory activity. The minimum oligosaccharide size needed for antiproliferative activity is a pentasaccharide; maximal antiproliferative activity is obtained with dodecasaccharide (~6500 MW) and larger fragments. In addition, *N*- or *O*-desulfated heparins are without effect, and *N*-desulfated *N*-reacylated compounds have antiproliferative activity.[15,16] In all cases, the

antiproliferative activity of heparin increases with increasing size and degree of sulfation. In the present experiments, an increase in potency of the acylated heparin derivatives was noted with increased carbon chain length. Acylation may increase the hydrophobicity of the fragments and may affect the binding affinity or increase the half-life of the compounds.

The results *in vitro* suggested that the 4-C and 6-C acylated compounds were potent antiproliferative agents; thus we tested the 4-C acylated compound in the rat carotid injury model. Our results showed, at the dose tested, whole heparin and the 4-C acylated compound strongly inhibited myointimal proliferation. The nonacylated periodate fragment inhibited the intimal thickening, although perhaps not as effectively as whole heparin or the 4-C acylated compound. The nonacylated periodate compound also was active *in vitro,* but to a lesser extent than whole heparin or the acylated derivatives. It has been demonstrated that the reduction in intimal area in heparin-treated animals after carotid injury at 14 days is largely due to a reduction in intimal smooth muscle cell number without a change in intimal smooth muscle cell density or extracellular matrix volume.[17,18] Our data confirm this observation for whole heparin and show the periodated precursor and 4-C acylated derivative-treated samples also had no change in cell number per unit area compared with controls. These results indicate that the reduction in intimal thickening by whole heparin and the heparin derivatives is due to diminished migration and proliferation of VSMC in the intima, rather than to changes in extracellular matrix volume.

Significantly the acylated compound as well as the periodate-treated precursor had no effect on blood clotting time or activated partial thromboplastin times, whereas the same dose of whole heparin resulted in marked elevation of the clotting and partial thromboplastin times. Furthermore studies have shown that this dose of whole heparin given by pump infusion significantly elevates clotting times up to 14 days after implant.[3,14] It has been shown, however, that doses of whole heparin that are not anticoagulant can inhibit mesangial[19] or myoinitmal proliferation.[20] The dose, time, and mode of delivery of heparin and heparin derivatives are important variables that need to be carefully assessed to determine the most effective approach.

There are several hundred thousand vascular surgical procedures performed annually in the United States, of which a significant number fail within 1 year because of smooth muscle cell proliferation.[21] For example, of the more that 200,000 aortocoronary and peripheral vascular bypass procedures using vein grafts performed annually, the leading cause of clinical failures is myointimal hyperplasia within the graft itself.[22] For heart transplant patients, the development of transplant atherosclerosis, in which the lesions are predominantly populated by VSMC, currently ranks as the leading cause of death in cardiac transplant recipients surviving beyond the first postoperative year.[23] Finally a large number of angioplasty procedures are performed each year; however, the success of these procedures is limited because up to 40% of these undergo restenosis, largely due to myointimal proliferation.[21,24] Thus in addition to the intrinsic biologic interest of characterizing the antiproliferative features of heparin in structural–functional terms, there is considerable interest in using heparin-derived species for preventing occlusion due to VSMC proliferation after vascular surgeries. Nonanticoagulant heparin derivatives of enhanced potency would be of significant clinical impact. Several nonanticoagulant heparins isolated by antithrombin III affinity chromatography or controlled depolymerization have been shown to have antiproliferative activity. The variable and expensive isolation procedures and antiproliferative activity of these compounds, however, have necessitated investigation and characterization of other, more effective nonanticoagulant heparins. In this report we have shown a potent inhibition of myointimal proliferation in an arterial injury model of accelerated atherosclerosis by a nonanticoagulant heparinlike compound. This compound has a direct effect on VSMC, as shown by inhibition of cell proliferation in culture. This or similar compounds may be an effective treatment for vascular surgical procedures where VSMC growth is a problem. Further research into the lowest possible dose, necessary time of delivery, and the effect of acylation on the half-life, binding ability, and oral administration will be of utmost interest to further define important aspects of this compound's activity.

Acknowledgments

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References

1. Ross R: The pathogenesis of atherosclerosis—An update. N Engl J Med 1986, 314:488–500
2. Rosenberg RD: Role of heparin and heparin-like molecules in thrombosis and atherosclerosis. Fed Proc 1985, 44:404–409
3. Clowes AW, Karnovsky MJ: Suppression by heparin of smooth muscle proliferation in injured arteries. Nature 1977, 265:625–626
4. Clowes AW, Clowes MM: Kinetics of cellular proliferation after arterial injury: Heparin inhibits rat smooth muscle mitogenesis and migration. Circ Res 1986, 58:839–845
5. Guyton JR, Rosenberg RD, Clowes AW, Karnovsky MJ: Inhibition of rat arterial smooth muscle cell proliferation by

heparin: I. In vivo studies with anticoagulant and non-anticoagulant heparin. Circ Res 1980, 46:625–634
6. Hales CA, Kradin RL, Branstetter RD, Zhu Y: Impairment of hypoxic pulmonary artery remodeling by heparin in mice. Am Rev Respir Dis 1983, 128:747–751
7. Castellot JJ, Hoover RL, Harper PA, Karnovsky MJ: Heparin and glomerular epithelial cell-secreted heparin-like species inhibit mesangial cell proliferation. Am J Pathol 1985, 120:427–435
8. Castellot JJ, Addonizio ML, Rosenberg RD, Karnovsky MJ: Cultured endothelial cells produce a heparin-like inhibitor of smooth muscle cell growth. J Cell Biol 1981, 90:372–379
9. Lippman M, Mathews MB: Heparins: Varying effects on cell proliferation in vitro and lack of correlation with anticoagulant activity. Fed Proc 1977, 36:55–59
10. Casu B: Structure of heparin and heparin fragments. Ann N Y Acad Sci 1989, 556:1–17
11. Petitou M, Coudert C, Level M, Lormeau JC, Zuber M, Simenel C, Fourneir JP: Selectively O-acylated glycosaminoglycan derivatives. (Submitted for publication)
12. Hoover RL, Rosenberg RD, Haering W, Karnovsky MJ: Inhibition of rat arterial smooth muscle cell proliferation by heparin. Circ Res 1980, 47:578–583
13. Somogyi P, Takagi A: A note on the use of picric acid-paraformaldehyde-glutaraldehyde fixative for correlated light and electron microscopic immunocytochemistry. Neuroscience 1982, 7:1779–1783
14. Hirsch G, Karnovsky MJ: Inhibition of vein graft intimal proliferative lesions in the rat by heparin. Am J Pathol (In press)
15. Castellot JJ, Choay J, Lormeau J, Petitou M, Sache E, Karnovsky MJ: Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cell: II. Evidence for a pentasaccharide sequence that contains a 3-0-sulfate group. J Cell Biol 1986, 102:1979–1984
16. Wright TC, Castellot JJ, Petitou M, Lormeau JC, Choay J, Karnovsky MJ: Structural determinants of heparin's growth inhibitory activity. J Biol Chem 1989, 264:1534–1542
17. Clowes AW, Clowes MM: Kinetics of cellular proliferation after arterial injury: II. Inhibition of smooth muscle cell growth by heparin. Lab Invest 1985, 52:611–616
18. Snow AD, Bolender RP, Wight TN, Clowes AW: Heparin modulates the composition of the extracellular matrix domain surrounding arterial smooth muscle cells. Am J Pathol 1990, 137:313–330
19. Diamond JR, Karnovsky MJ: Nonanticoagulant protective effect of heparin in chronic aminonucleoside nephrosis. Renal Physiol 1986, 9:366–374
20. Edelman ER, Adams DH, Karnovsky MJ: Effect of controlled adventitial heparin delivery on smooth muscle cell proliferation following endothelial injury. Proc Natl Acad Sci U S A 1990, 87:3773–3777
21. Ip JH, Fuster V, Badimon L, Badimon J, Taubman MB, Chesebro JH: Syndromes of accelerated atherosclerosis: Role of vascular injury and smooth muscle cell proliferation. J Am Coll Cardiol 1990, 15:1667–1687
22. Atkinson JB, Forman MB, Vaughn WF, Robinowitz M, McAllister HA, Virani R: Morphologic changes in long-term saphenous vein grafts. Chest 1985, 88:341–348
23. Chomette G, Auriol M, Cabrol C: Chronic rejection in human heart transplantation. J Heart Transpl 1988, 7:292–298
24. Beatt KJ, Serruys PW, Hughenholtz PG: Restenosis after coronary angioplasty: New standards for clinical studies. J Am Coll Cardiol 1990, 15:491–498