

Effect of controlled adventitial heparin delivery on smooth muscle cell proliferation following endothelial injury

(atherosclerosis/angioplasty/polymer-based controlled delivery/vascular injury/vascular surgery)

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ABSTRACT Continuous intravenous infusion of heparin suppresses smooth muscle cell proliferation in rats after endothelial injury but may lead to hemorrhage and other complications. The anticoagulant property has been removed from chemically modified heparin without loss of antiproliferative effect but use of such compounds is still limited. In this study ethylene-vinyl acetate copolymer matrices containing standard and modified heparin were placed adjacent to rat carotid arteries at the time of balloon denudation. After 14 days arterial occlusion by smooth muscle cell proliferation was defined. Matrix delivery of both heparin compounds effectively diminished this proliferation in comparison to controls without producing systemic anticoagulation or side effects. In addition, this mode of therapy appeared more effective than the administration of the same agents by either intravenous pumps or heparin/polymer matrices placed in a subcutaneous site distant from the injured carotid artery. Thus, heparin's inhibition of smooth muscle cell proliferation after vascular injury might be most effective within the microenvironment of the injured vessel wall, and the accelerated atherosclerosis or restenosis that often follows angioplasty and other vascular interventions might best be treated with site-specific therapy.

Intimal smooth muscle cell (SMC) proliferation naturally follows controlled injury of the arterial endothelium in experimental animals (1, 2). Continuous intravenous (i.v.) infusions of heparin significantly reduce this cellular response independent of the drug's antithrombotic effect (3). Chemically modified, non-anticoagulant (NAC) heparin is just as effective on a mass basis (4), and there is no effect on platelet number, morphology, or function (2). Hence, heparin may be a possible therapeutic agent of great potential in states of accelerated atherosclerosis, especially after the endothelial injury that accompanies procedures such as angioplasty and vascular surgery. There are, however, problems with available means of heparin administration. Heparin is highly charged, has a molecular mass of 12,000–18,000 Da, and possesses a dose-dependent half-life of ≈ 1 –5 hr (5). Because of its size and polarity it crosses membranes poorly and therapeutic levels can only be maintained with restrictive and potentially complicating means such as continuous infusions through indwelling i.v. access. In addition, doses used for systemic anticoagulant (AC) heparinization are accompanied by the potential for hemorrhage, electrolyte shifts, and thrombocytopenia in the acute setting and osteoporosis and alopecia over longer periods of time (5). Chemically modified heparin compounds have reduced AC properties but bleeding still remains a problem in some cases (6). Alternative therapies or routes of heparin administration have been sought and, though some have succeeded in prolonging the dosing

interval, none has removed the need for and risks of systemic therapy. Oral dosing is limited by the inability of the intact compound to pass through the gastric mucosa. Degradation of the compound within the stomach produces smaller oligomer segments that can be absorbed and detected in the bloodstream (7). These small fragments unfortunately are tri- and tetramer sequences (7), oligomers that have been shown not to inhibit SMC proliferation in tissue culture (8). Transdermal heparin delivery has not been performed successfully and although subcutaneous (s.c.) administration (9), and even intrapulmonary (10) administration, are efficacious at achieving systemic anticoagulation, they do not remove or reduce the hemorrhagic complications. Heparin has been immobilized to a variety of medical devices to provide nonthrombogenic surfaces (11). Yet, bonding techniques suffer from variable desorption of the heparin and often utilize toxic binding agents (12).

Early clinical trials with systemic heparinization and accelerated atherosclerosis have been inconsistent and few studies have focused on the potential effects of the drug on SMC proliferation. We are now investigating the antiproliferative effects and AC potential of the controlled local release of heparin from polymeric matrices placed at the adventitial surface of injured arterial segments *in vivo*.

MATERIALS AND METHODS

Materials. AC heparin (Choay heparin 1453, 12,000–18,000 Da, U.S.P. 160 units/mg) and NAC heparin (Choay heparin 1772, 5000–8000 Da, U.S.P. 10 units/mg) were kindly supplied by Jean Choay (Choay Institute, Paris) and reported to have identical, 80%, antiproliferative activity in tissue culture (13). Ethylene-vinyl acetate copolymer (EVAc, 40% vinyl acetate), manufactured under the product name ELVAX-40P (DuPont), was washed in distilled water and 95% alcohol to remove impurities. Osmotic minipumps with a reservoir volume of 2.2 ml and lot-dependent flow rates were obtained from Alza. A no. 2 French Fogarty catheter was used to induce endothelial injury (American Edwards Laboratories, Santa Ana, CA). Male Sprague-Dawley rats (300–500 g) were obtained from Charles River Breeding Laboratories. Azure A and sodium nembutol were obtained from Fischer and Evans blue was from Sigma.

Matrix Fabrication. EVAc was dissolved in dichloromethane to a concentration (wt/vol) of 10%. Dry powdered heparin was sieved to particle sizes of $< 180 \mu\text{m}$ and added to the EVAc solution. Drug:polymer ratios were computed to achieve a final ratio (wt/wt) of 15%, 33%, or 50%. The

Abbreviations: aPTT, activated partial thromboplastin time; AC, anticoagulant; EVAc, ethylene-vinyl acetate copolymer; NAC, non-anticoagulant; SMC, smooth muscle cell.

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drug/polymer suspension was poured into precooled glass molds resting on dry ice. Once hardened the matrices were placed at -20°C for 2 days and then placed under house vacuum (600 mtorr; 1 torr = 133 Pa) for another 2 days. The resultant matrix was a homogeneous dispersion of heparin within EVAc (14). Smaller pellets were cut from the larger slabs to specific sizes and weights for determination of release kinetics *in vitro* and biological effect *in vivo*. *In vitro* release studies demonstrated a burst of drug release from the surface of matrices during the first 24–36 hr. This was undesirable *in vivo* and was obviated by coating the matrix pellets with a layer of EVAc and restraining release through a hole bored through the coating. Previous investigations have shown that matrices treated in this fashion demonstrate kinetics that approach zero order (15). The coating was applied by placing a 20-gauge needle 1 cm into the center of the face of the matrix pellet and then immersing the pellet in a solution of EVAc for 5 sec. As the pellets were withdrawn from the solution they were spun slowly to allow for uniform coating. This entire process was repeated two additional times. As a final step in sterilization prior to experimentation the matrices were removed from the needles and placed under ultraviolet light for 30 min on each face.

Release Kinetics. Preliminary *in vitro* release kinetics were defined for five flat slab-shaped matrices of different initial AC heparin concentrations or "loading" [15%, 30%, or 50% heparin:EVAc (wt/wt)] and for five slab matrices coated with plain EVAc with a hole drilled into one face (15% and 30% loading). The matrices were placed in 2 ml of buffered saline which was changed on a scheduled basis. The amount of heparin released into each sample was determined using the azure A assay (16), which relies on the metachromatic shift of the absorbance of a sample from 560 to 620 nm linearly related to the amount of heparin in the sample. To compare data at different loadings, drug release was normalized to the amount of drug available for release. This was accompanied by dividing the value obtained for the amount of heparin present at a given point in time by the initial amount of drug incorporated within the matrix. Thus, the ordinate in Fig. 1 is represented as the cumulative percent of total drug incorporated released at any given point in time.

SMC Proliferation. Endothelial denudation of the left common carotid artery in the rats was performed with a 2F balloon catheter (2). Rats were anesthetized with intraperitoneal sodium nembutol (0.05 mg/g of body weight). A midline incision exposed the distal left common and external carotid arteries. The balloon catheter was introduced into the external carotid artery and passed three times with the balloon distended sufficiently with air to generate slight resistance. Upon removal of the catheter the external carotid artery was ligated. The traditional i.v. route of heparin administration was then contrasted to controlled heparin release from implanted matrices.

Osmotic pumps were placed in a pocket made in the neck of the rat and a silastic catheter extended from the pump to the right internal jugular vein. AC and NAC heparins were dissolved in 2 ml of lactated Ringer's solution and delivered at 0.3 mg/kg of body weight per hr. Control animals received lactated Ringer's solution infusion. At the experiment's completion the pump system was retrieved for verification of catheter placement and patency. No aggregation was noted within the pumps, all catheters remained patent, and at the conclusion of the experiments residual volumes were all <0.3 ml.

In other groups of animals EVAc matrices containing no drug (control), standard AC heparin, or NAC heparin were placed in a subcutaneous pocket over the animal's dorsal lumbar region or adjacent to the injured artery. Muscles and fascia surrounding the artery were sutured closed to ensure pellet immobilization.

In a separate experiment a dose-response curve was generated for the matrix release of NAC heparin. Twelve rats were implanted with NAC-bearing matrices of different net weight so as to deliver different dosages of heparin over the 14-day period. The variation in net effect was then plotted against dose. A dose-response experiment was not performed for the AC heparin as substantial inhibition of SMC proliferation was noted at small heparin doses. It would have been difficult to deliver the drug over a range less than that dose or to detect a significant difference in effect on SMCs at higher doses.

In all animal experiments activated partial thromboplastin times (aPTTs) were determined within the first 24–36 hr after the procedure and at day 14. On the 14th postoperative day animals were euthanized and perfused clear retrograde via the abdominal aorta with lactated Ringer's solution followed by fixation with modified Ito-Karnovsky's fixative (17). One hour prior to fixation animals received an i.v. bolus of Evans blue in lactated Ringer's solution (60 mg/kg). Representative sections from the central blue region of the left common carotid artery were processed and examined by light microscopy. Photographs of all arterial sections were obtained and the percent of luminal occlusion was calculated for each arterial segment using computerized digital planimetry. The amount of occlusion noted with therapy was subtracted from the value recorded in the control state and then divided by the control value. This number served as an index of smooth muscle cell proliferation inhibition (Fig. 1).

Statistics. All data are presented as the mean \pm the standard error about the mean. The statistical significance of differences in luminal occlusion and aPTTs was determined using nonpaired *t* test for groups of unequal sample sizes. Data line fits were established using a linear regression model. These were all performed using standard routines available on our laboratory computer.

RESULTS

Release Kinetics. Uncoated matrices exhibit first-order release kinetics with the bulk of the drug eliminated in the first 24–48 hr. As expected, at the higher drug loadings heparin was released more rapidly and to a greater extent. Preliminary experiments performed with uncoated matrices releasing AC heparin at rates that coincided with the pump delivery rates of 0.3 mg/kg per hr led to oozing of blood adjacent to the site of implantation. Diminution in dose and the application of the impermeable coat with a single aperture removed this problem by eliminating the initial burst of release sustaining overall release (Fig. 1). Here too the

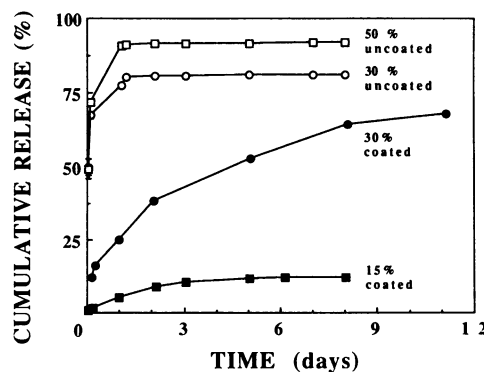


FIG. 1. Heparin release kinetics are presented for EVAc matrices with different loadings and coatings. The cumulative percent released is the running sum of the ratio of heparin released to heparin initially embedded within the matrix. Error bars about the mean are present but in most cases are smaller than the data point symbols.

amount released was a function of the initial loading, with the most protracted release observed from the coated 15% loaded EVAc matrices. The coated matrices chosen for the animal experiments, therefore, provided near zero-order release kinetics at estimated release rates of 0.05 and 0.10 mg/kg per hr of AC heparin and NAC heparin, respectively. At 14 days 7.6 ± 1.1 mg of AC heparin and 19.0 ± 2.4 mg of NAC heparin had been released. In contrast, the i.v. pumps set at 0.3 mg/kg per hr supplied 39.6 ± 2.3 mg of AC heparin and 48.2 ± 2.0 mg of NAC heparin to the animals by the 14-day experiment culmination point.

SMC Proliferation. The largest effects on inhibition of SMC proliferation were seen with periaadventitial matrix delivery (Fig. 2). In the six control animals receiving lactated Ringer's solution from indwelling pumps, $52.2\% \pm 4.2\%$ of the normal luminal area was encroached upon by SMCs. Similarly, the eight control animals with blank EVAc matrices implanted adjacent to the injured carotid artery exhibited occlusions of $55.9\% \pm 4.3\%$. When AC heparin was delivered periaadventitially luminal occlusion was reduced to $9.4\% \pm 2.6\%$ ($n = 8$, $P < 0.005$ compared with matched control). In contrast, $16.8\% \pm 4.3\%$ ($n = 5$, $P < 0.001$) of the lumen remained occluded when the AC heparin was infused i.v. and $28.0\% \pm 2.6\%$ ($n = 4$, $P < 0.05$) remained occluded when the heparin was administered to a dorsal s.c. site from a matrix release. NAC heparin yielded no statistically significant reduction in occlusion when administered i.v. ($46.4\% \pm 3.9\%$, $n = 5$, $P =$ not significant) or s.c. ($45.0\% \pm 2\%$, $n = 5$, $P =$ not significant) but was almost efficacious as AC heparin was released periaadventitially ($17.7\% \pm 3.7\%$, $n = 10$, $P < 0.002$). Microscopic examination of the stained sections revealed symmetric reductions in the SMC response and no increased effect on the vessel wall closest to the matrices. In addition, a linear inverse dose-response was observed for NAC heparin ($R^2 = 0.645$) with luminal occlusion decreasing as the dose delivered increased (Fig. 3).

Finally, only animals receiving AC heparin i.v. experienced systemic anticoagulation with a measurable change in the aPTT (Table 1). Neither the local matrix delivery of either heparin compound, in s.c. or periaadventitial positions, nor the i.v. infusion of NAC heparin had any discernable alteration in clotting function. None of the animals in any groups suffered from excessive bleeding.

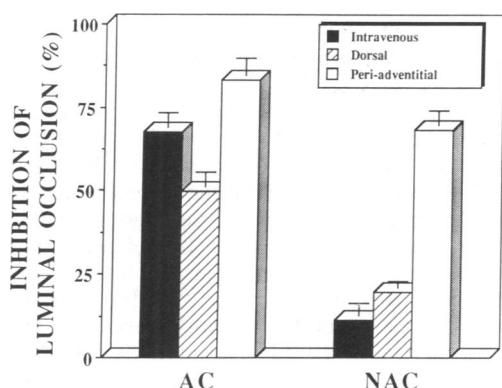


FIG. 2. The ability of AC heparin (AC) and NAC heparin (NAC) to inhibit SMC proliferation and occlusion of the carotid arterial lumen after balloon injury is displayed. A value of 100% represents suppression of all of the anticipated cellular proliferation. Dark bars illustrate data for animals who received i.v. pump therapy, stippled bars display data for animals who had heparin/EVAc matrices implanted s.c. in the dorsal region, and clear bars depict the results from rats with heparin/EVAc matrices implanted periaadventitially.

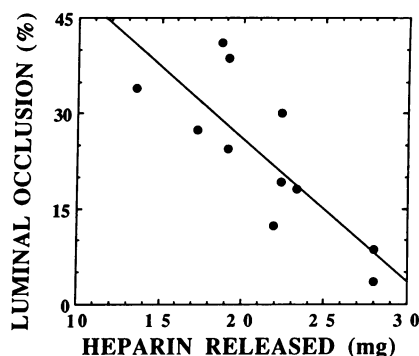


FIG. 3. Luminal occlusion of balloon deendothelialized carotid arteries in 12 rats 14 days after injury and periaadventitial EVAc/NAC heparin therapy is plotted against the amount of drug (mg) estimated to have been released in that time. An inverse linear dose-response with a slope of -2.3 and a multiple R^2 of 0.645 was observed.

DISCUSSION

The recent enthusiasm for the use of interventions such as angioplasty, intravascular stents, and laser angioplasty has been tempered by the high failure rate that accompanies these procedures. Abrupt vessel closure has been reported in 2–12% (18) of angioplasties and at 3 months 25–35% of the treated arteries have restenosed (19). This latter phenomenon appears to be a rapid recapitulation of the events that led to the development of the initial lesion—i.e., SMC proliferation within the intima of the involved vessel (20). A number of agents have been reported to inhibit this proliferation, including heparin (3), immunosuppressants such as cyclosporin (21), calcium channel blockers (22), and, most recently, captopril and other angiotensin-converting enzyme inhibitors (23). Though heparin, aspirin, and calcium channel blockers are routinely used in the post-angioplasty setting, preliminary trials examining the effects of these agents have revealed no significant effects on restenosis rates (24, 25). One potential reason these agents fail to fully suppress the vascular response following these procedures is that for many of these agents the *in vivo* effects on SMCs have only been observed at supraphysiologic doses. Captopril, for example, was administered to rats at an equivalent dose almost 100 times the dose that might be used in the clinical setting. Such dosing is restrictive in its potential for inducing hypotension and renovascular effects. Heparin has been administered to rats and rabbits who have been exposed to controlled endothelial injury without substantial hemorrhagic complications. Yet, limitations are imposed by the AC nature of these compounds and in the clinical setting when such medications and doses are used there is the potential for thrombocytopenia, bleeding, electrolyte shifts, and depletion of bone mineral (5). Chemical modification of heparin has reduced the AC properties of the drug, but currently available NAC heparin still possesses the potential for bleeding and other systemic complications (6).

Table 1. Measured aPTT (sec)

Group	i.v.	Matrix	
		Carotid	Dorsal
Control	16.2 ± 0.1 (6)	16.5 ± 0.4 (8)	15.0 ± 0.1 (5)
NAC hep.	18.4 ± 0.6 (5)	15.0 ± 0.4 (10)	17.5 ± 0.5 (5)
AC hep.	$40.0 \pm 11.8^*$ (5)	15.3 ± 0.1 (8)	17.0 ± 1.0 (4)

Data are presented as the average \pm SE, with the number of animals given in parentheses. The statistical significance was compared with the corresponding controls; except where noted (asterisk), $P =$ not significant for all comparisons. hep., Heparin. * $P < 0.0005$.

In this paper we have shown that local controlled release of heparin inhibits SMC proliferation within blood vessels following injury to the endothelium of that blood vessel: for AC heparin without the need for systemic anti-coagulation; for AC heparin when administered from a site distant from the injured vessel; and for some types of heparin in a manner more efficient than in systemic administration. NAC heparin, though equally as effective as the AC heparin at suppressing SMC proliferation in tissue culture (13), was virtually ineffective when administered *i.v.* or *s.c.* and only achieved a therapeutic response when delivered from matrices in the periadventitial position. As the dose of periadventitally administered NAC heparin was increased the effect on SMC proliferation rose, such that at the highest dose tested NAC heparin inhibited SMC proliferation to an equal extent as AC heparin, albeit at five times the equivalent dose. Verification of this dose-response arises when we use it to estimate the amount of NAC heparin that would be required to achieve the inhibition of luminal occlusion seen in the first NAC experiment where the matrix size and heparin dose were held constant. In those animals NAC/EVAc matrices placed periadventitally reduced luminal occlusion of the injured artery to 27.1%. Our line fit would imply that this would be observed if the average matrix heparin dose was 23.3 mg; 19.5 mg was estimated to have been administered.

The different *in vivo* SMC response from two drugs with identical abilities to inhibit SMC proliferation in tissue culture (13) highlights the complexity of bridging the gap between tissue culture and *in vivo* systems. Drug absorption, distribution, metabolism, and excretion all affect a compound's biological response after administration. Rapid elimination from the vascular space might have been of particular importance for the NAC heparin, which is half the size of its AC counterpart. At least two factors might be responsible for the differential influence of the different modes of administration: local drug dose and the delivery of drug directly to the site of desired action. The proximity of the matrix to the artery virtually assures that elevated doses of heparin will be found at the arterial wall with periadventitial implants. Yet, these doses are far lower than heparin doses currently used in the clinic. Previous experiments have shown that drug release from EVAc matrices *in vivo* identically mirrored release *in vitro* (26). Hence, if we use the *in vitro* release rates and assume that all of the drug released from the matrices was applied directly to the artery, then at the highest observed rate of heparin release, 0.8 mg/day (150 units/mg), about 5 units/hr would be delivered to the vessel wall. If we scale up for use in human arteries we need consider only that the difference in blood vessel diameter is ≈ 10 -fold. Because we specifically do not want to achieve systemic effects, body weight does not enter into our calculations. Thus, the maximum amount of heparin human arteries would be exposed to may be no higher than 50 units/hr and systemic levels would be undetectable. This is in marked contrast to the 1000–1500 units/hr (160–240 mg/day) *i.v.* infusions currently use clinically.

The presence of atherosclerotic plaque will increase the vessel wall thickness and the distance a compound must traverse to cross the wall. This might decrease the amount of heparin available to different parts of the diseased vessel. Yet, it is possible that the application of drugs to the adventitia reaches the most crucial segments of the wall while *i.v.* or *s.c.* administration does not. Intimal SMCs migrate from the media (27) and in some studies have been shown to arise from *vasa vasorum* that course through the adventitia (28). Local application of heparin to these target surfaces might produce an enhanced effect as compared to systemic or even intraluminal administration. This might explain why heparin bonded to and slowly releasing from implanted intravascular devices might reduce thrombogenicity sur-

rounding these implants but not local SMC hyperplasia. In addition, these implants are subjected to the rapid and turbulent flows seen in small arteries diseased with irregular stenoses. As such, the time during which the mural elements are exposed to the drug is markedly reduced. The clinical implications of such an observation are that the anastomotic atherosclerosis that plagues vascular surgery might be reduced by drug delivered from the exterior of the anastomosis, not just the interior, and that an implant embedded within the wall of an artery might provide adjunct pharmacotherapy to other concomitant forms of vascular interventions.

The inhibition of proliferation noted when matrices with AC heparin were placed in a *s.c.* site distant from the injury is in concert with previous reports of SMC inhibition with systemic heparin administration. The significant finding in our study is that the effects were observed at markedly reduced doses. Thus, it may be possible to administer certain modulators of SMC growth *s.c.* and reserve perivascular administration for specific drugs and clinical conditions. The differential inhibition of SMC proliferation with the various modes of administration is important not only in the mode of delivery that might be used but also in the drugs we examine. For example, the NAC heparin used in these experiments had no effect when infused from indwelling pumps or from matrices implanted in a site distant from the injured artery. Yet, periadventitial administration was effective. It is possible that potentially potent drugs have been examined in the traditional way and discarded as inhibitors of SMC proliferation because they do not act systemically, do not act in a similar fashion over different dosage ranges, or are degraded before they achieve their effects. The use of local drug-releasing implants can avoid some of these pitfalls.

Polymer-based drug delivery systems might make such implants possible. They are simple, small, and inexpensive and offer the potential for quantifiable sustained and controlled drug release (14). Elevated local concentrations can be sustained from these matrices without significant systemic levels. Because drug is incorporated in its dry state, shelf life is increased. This is especially important for agents unstable in solution, rapidly degraded, prone to precipitation, or of limited solubility. We and others have used a number of polymer materials in various device formulations to study a variety of disparate disease states (29). Any inert and biocompatible device releasing drug locally and in a sustained fashion would have sufficed. We chose the EVAc matrix because this polymer is a highly biocompatible material already approved by the Food and Drug Administration for biomedical device use, is known to provide the release kinetics we desired, and is not erodible, thereby allowing for verification of position and local tissue reaction at the conclusion of the experiment.

Matrix-controlled release systems are composed of a skeleton supporting structure of polymer material surrounding pockets of dry powdered drug connected by pores. The material is impermeable to all but the lowest molecular mass (100–300 Da) compounds, and for the larger molecular mass compounds, such as heparin, to be released they must traverse this complex network. This entails the absorption and entry of a fluid to solubilize the dry drug and then diffusion of drug through the network of pockets and channels. Individual and shared characteristics of the device fabrication technique, the embedded drug, and the surrounding polymer material determine the form of the network and the pattern of drug release. Modification of these characteristics can produce a range of devices of different sizes, shapes, water absorptivity, matrix porosity, tortuosity, etc., that ultimately produce different release kinetics. Adjusting the loading of the drug by varying the polymer:drug ratio changes the structure of the internal network. Pockets of drug are fewer in number with reduced interconnections. As a

result, as depicted in Fig. 1, the efficiency of release is diminished as some drug is trapped forever within the matrix. Coating the matrices retarded heparin release still further because the rapid dissolution and release of surface drug or drug residing within superficial pores was prevented. This produced uniform near zero-order release and avoided any of the bleeding that was noted when the larger boluses of AC heparin were released from uncoated matrices. Finally, we have shown that implantation of these matrices for up to 7 months elicited no local tissue reaction (30) and release rates *in vivo* that matched release rates *in vitro* (26).

In summary, these data support the view that heparin's inhibition of SMC proliferation after vascular injury might be a local phenomenon and the accelerated atherosclerosis or restenosis that often follows angioplasty and other vascular interventions might be regulated by local, site-specific therapy. Such an approach might remove the inherent problems with systemic administration of potent antiproliferative agents. Further investigations of periadventitial events in the vascular response to injury may contribute to our understanding of the mechanisms of accelerated atherosclerosis. The use of polymer-based drug delivery systems provides a means of controlling local drug levels in an efficient manner and a more effective means of examining the effects of various compounds in the control of SMC proliferation following vascular injury. Continued development of this type of therapeutic modality may allow better use of newer interventional therapies for atherosclerosis and play a role in the general management of accelerated atherosclerotic states.

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