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Contrasting Effects of the Intermittent and Continuous Administration of Heparin in Experimental Restenosis

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Background Heparin inhibits proliferation of smooth muscle cells in culture and intimal hyperplasia in experimental animals but paradoxically exacerbates vascular injury in clinical trials. To determine whether the difference in the means by which heparin was administered explained the benefit in animals and aggravation in humans, we examined the vascular effects of a range of heparin treatments.

Methods and Results When laboratory rats were injected subcutaneously with heparin (55.5 IU, 1.0 mg/kg) per clinical trial protocols, intimal hyperplasia after arterial injury was exacerbated rather than alleviated. The intima to media area ratio was increased 22.5% with every-other-day injections and was increased 16.8% with daily injections. When the daily dose of heparin was increased to 7.2 mg/kg or when injections were initiated a week before injury, intimal hyperplasia was made even worse (52.2% and 59.9% above control). Twice-daily heparin, 7 and 17 hours apart, had no demonstrable effect one way or the other, and it was not until the heparin was administered at 12-hour intervals that intimal hyperplasia and cell proliferation were lessened (44.6% decrease). The greatest reduction in intimal hyperplasia was obtained when the heparin was administered continuously. The continuous osmotic pump intravenous infusion of heparin inhibited 62.5% of the expected proliferation, and perivascular polymeric device release of heparin blocked the response by 74.2%. While subcutaneous injections transiently increased activated partial thromboplastin time, neither mode of continuous delivery altered coagulation.

Conclusions We might reconsider the use of heparin in vascular diseases and not neglect this promising compound because of inappropriate extrapolation from the laboratory to clinical use. (Circulation. 1994;89:770-776.)

Key Words • atherosclerosis • pharmacokinetics • angioplasty

The rush toward innovative technologies for the treatment of atherosclerotic vascular diseases has been accompanied by a sobering complication: restenosis, the rapid recapitulation of initial cellular events in the 3 to 6 months after intervention.1-6 As the cellular events that govern this process are progressively elucidated, increasingly potent agents directed against these events have been identified in hopes of halting this process.7-29 Unfortunately, while many agents suppress cell growth in tissue culture, and a subset of these compounds reduce proliferation in animal models of vascular disease, no agent has been proven effective in inhibiting restenosis in humans.

The dichotomy between experimental benefit and clinical futility is no more evident than with heparin. Heparin is the gold standard for inhibitors of cultured smooth muscle cell growth and is unique in that a natural heparin-like compound may be central to reparative processes that serve as a first-line mechanism in the attempt of the body to limit accelerated atherosclerosis.30-31 Heparin markedly and rapidly inhibits DNA and RNA synthesis in growth-arrested cells released from Go block.32 Continuous intravenous infusion of heparin almost completely abolishes intimal smooth muscle cell proliferation in the injured carotid artery.6 However, studies initiated to determine whether the beneficial effects of heparin observed in laboratory animals and cell culture could be achieved in humans paradoxically exhibited exacerbation of vascular injury. For example, when patients were randomized to heparin or dextrose infusion over the first 18 to 24 hours after angioplasty, 41.2% of the heparinized patients and only 36.7% of the dextrose infusion patients had evidence for restenosis.18 Moreover, bleeding complications were twice as frequent in the heparinized group. Similarly, when heparin was administered as a single daily subcutaneous injection at 10 000 IU/d, patients fared so poorly that the trial was halted prematurely.19 Eighty-two percent of the patients who received heparin suffered restenosis, almost 2.5-fold more often than the 33% of the patients treated in standard fashion. Angina, myocardial infarction, and bleeding complications were virtually nonexistent in the control subjects but appeared in 76%, 18%, and 41% of the heparinized patients, respectively.

One interpretation of the results of these trials is that heparin is ineffective in treating accelerated atherosclerosis in humans. Alternatively, the marked difference in the way in which the drug was administered to obtain a beneficial effect in animals and a deleterious effect in humans might explain these observations. The antiproliferative effect of heparin requires that the drug be administered for at least 4 to 7 days after injury in a simple model, such as the endothelial denudation of an otherwise normal artery in healthy rats.33 Therapy for the complex human angioplastied lesion probably re-

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quires at least that duration of administration; anything less might not show an antiproliferative effect. To address this issue, we examined the intimal hyperplastic response to denuding arterial injury after a range of heparin treatments ranging from injections every other day to constant intravenous infusion. We examined two different doses of heparin as well, including the dose that has been used in clinical trials and the dose used in previous animal studies. We confirmed that constant infusion and continuous local perivascular release of heparin were each especially effective at inhibiting smooth muscle cell proliferation and intimal hyperplasia and that the benefit from perivascular release could still be observed at much lower doses than for intravenous infusion. We also demonstrated that the arterial lesion was made worse when rats were treated with the kinetics of heparin release that humans were exposed to in clinical trials.

These data argue for a reevaluation of the means by which heparin is used in accelerated vascular diseases and requires that we not neglect this promising compound because we have not extrapolated appropriately from the laboratory setting to clinical use.

Methods

Vascular Injury and Heparin Therapy

Sprague-Dawley rats (300 to 500 g; Charles River Breeding Laboratories, Kingston, Mass) were anesthetized with Nembutal 0.5 mg/kg. A midline incision exposed the carotid arteries, the left external carotid artery was isolated, and a 2F Fogarty balloon catheter (American Edwards Laboratories, Santa Ana, Calif) was inserted through an arteriotomy. The catheter was advanced to the aortic arch and pulled back with the balloon distended with sufficient air to generate slight resistance and denude the endothelium. Upon removal of the catheter, the external carotid artery was ligated. The contralateral artery underwent identical manipulation except for the introduction of the balloon catheter.

Starting immediately after injury, animals received subcutaneous injections of heparin (55.5 IU equivalent to \(=1.0 \text{ mg/kg} \)) once every other day (n = 7, QOD), once a day (n = 7, QD), twice a day spaced approximately 7 and 17 hours apart (n = 7, BID), or every 12 hours (n = 7, Q12). The heparin preparation was obtained from Choay Institute, Paris, France (Choay 1453; m w 12 000 to 18 000 d; USP, 160 U/mg) and has been shown to inhibit smooth muscle cell proliferation in culture\(^{31,27-40}\) and neointimal hyperplasia in a number of animal models of vascular injury.\(^{39-42}\) Separate groups of animals received heparin continuously from implanted osmotic minipumps with indwelling intravenous catheters (n = 7, PUMP) or from polymeric controlled release matrices residing in the perivascular space (n = 7, CR). Pump infusion was set at 0.3 mg/kg per hour corresponding to the effective dose previously documented to have a substantial inhibitory effect on intimal hyperplasia.\(^{3,8,36,41,42}\) Upon tissue harvest, the pumps were retrieved, and the veracity of release and verification of rates were determined by examining the heparin content within the pump reservoir residual volume. Polymer matrices were constructed from ethylene-vinyl acetate copolymer (EVAc) as previously described.\(^{41,44}\) Heparin was mixed with a solution of EVAc dissolved in dichloromethane (10% wt/vol) to achieve a final ratio of 33% wt/vol. The drug-polymer suspension was poured into precooled glass molds, removed after hardening, and placed at \(-20°C\) and then under vacuum (600 mm Hg) for 2 days each. The resultant matrix was a homogenous dispersion of drug within a porous network of EVAc.\(^{44}\) Smaller pellets were cut from the larger slabs and coated with six layers of EVAc. Drug release was restrained to emanate from a hole in the coating, and near zero-order kinetics were obtained in this fashion.\(^{41,45}\) Matrices were prereleased for 4 hours in sterile water to allow for any burst of release to occur and for linear release to commence. As with the pumps, matrices were retrieved at the time of tissue harvest, the heparin was extracted, and total amount released was determined using the Azure-A colorometric assay\(^{41,46,47}\) and compared with in vitro release rates from identical matrices. In this manner, we determined that the matrices were releasing heparin at 2.16±0.14 \(\mu\text{g/kg} \) per hour during the linear phase of drug release, almost 140-fold less heparin than what was administered intravenously.

The amount of heparin injected was calculated from the amount of heparin used in clinical trials that had shown exacerbation. In those trials, 10 000 to 12 500 IU of drug was injected or infused daily. Accordingly, we scaled down for animal weight and injected animals with 55.5 IU per injection (=1.0 mg/kg). In other animals, the amount of heparin injected subcutaneously was increased to the amount of drug that would be delivered over that period of time if infused from the pumps: 7.2 mg/kg per day. Finally, in an effort to determine whether preexisting drug levels might be beneficial, daily heparin injections were initiated a full week before the arterial injury was imposed. Four sets of control animals were used, including animals with no therapy after balloon injury, animals receiving injections of saline, animals in whom implanted pumps delivered Ringer's lactate at the identical rate to the volume delivery of heparin, and animals implanted with an EVAc matrix without heparin.

Tissue Processing and Analysis

On the 14th postoperative day, animals were euthanized and perfused clear via the left ventricle with Ringer's lactate solution followed by immersion fixation with Carnoy's fixative (60% methanol, 30% chloroform, 10% glacial acetic acid). The location of the implanted devices was noted, and the devices were recovered with the intact arteries. Both common carotid arteries were harvested and cut into three equal segments. The segments were paraffin embedded and microtome sectioned. Eight to 12 sections along the length of each segment were obtained and stained with hematoxylin and eosin or Verhoef's elastin stain. The intimal, medial, and adventitial areas, the intima to media area ratio, and the percent of luminal occlusion were calculated for each arterial segment using computerized digital planimetry with a dedicated videomicroscope and individualized software. The averages of all sections and segments were used for comparison. Edge detection software was further used to detect cell number within 8 to 32 sections per media or intima and when combined with area data used to determine cell density. All analyses were confirmed by visual inspection, and the accuracy of the system was verified with a series of matched manual cell density determinations.

Cell proliferation was followed by injecting the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU, Du Pont Corp) intraperitoneally, at 50 mg/kg, 3 and 7 days after surgery and 1 hour before the animals were killed. Intracellular BrdU was identified immunocytochemically as previously described using a mouse IgG anti-BrdU antibody diluted 1:50 (Coulter Immunology, Hialeah, Fla) and peroxidase labeling with avidin-peroxidase complex (Vector Laboratories, Burlingame, Calif) and 3,3'-diaminobenzidine (Sigma Chemical Co, St Louis, Mo). Sections were counterstained with methyl green or hematoxylin. Identification of proliferating cells was performed as described above for cell density measurements using computer-based microscopy and video image processing on multiple arterial sections. The number of antibody-positive cells was divided by the total number of cells in the section, and the values were reported as percent of total±SEM.
Fig 1. Bar graph: Intimal hyperplasia is exacerbated by intermittent injections of heparin and relieved by continuous heparin administration. When heparin was administered every other day (QOD, n=7), every day (QD, n=8), and twice daily spaced 7 and 17 hours apart (BID, n=7) the area of the injured intima exceeded that observed in the untreated controls. When heparin was administered at 12-hour intervals (Q12, n=8), the detrimental effect was removed, and a beneficial effect was observed. The most significant reduction in intimal hyperplasia arose when the drug was continuously infused intravenously from osmotic pumps (PUMP, n=7) or continuously released into the perivascular space from controlled release polymer matrices (CR, n=7).

**Statistics**

Data are presented as mean±SEM. Statistical comparisons were performed with nonpaired t test for groups of unequal sample sizes, and data were rejected as not significantly different if P values >.05 were observed.

**Results**

**Cell Proliferation and Intimal Hyperplasia**

The subcutaneous injection of heparin every other day, every day, and even twice daily spaced 7 and 17 hours apart exacerbated rather than alleviated intimal hyperplasia after arterial injury (Fig 1). Pretreatment of the animals with daily injections of heparin or the use of much higher doses of heparin akin to those used clinically had no benefit either and, if anything, were perhaps somewhat worse than the lower doses (Table). It was not until the heparin was administered at 12-hour intervals (Fig 1, Q12) that intimal hyperplasia and smooth muscle cell proliferation (Fig 2) were lessened. When the drug was released continuously from osmotic pumps or from polymeric controlled release devices within the perivascular space, the inhibitory effects were maximized (Figs 1 and 2). There was no difference in intimal hyperplasia between any of the control groups. Thus, the results from these four groups of animals were pooled.

**Coagulation and Thrombosis**

The subcutaneous injections of heparin elevated aPTT above control (Figs 3 and 4), but this effect was as variable and cyclical (Fig 3) as reported in the literature. Coagulation was not affected by the controlled release of heparin from polymer matrices or by the infusion of heparin from osmotic pumps. Though it has been speculated that this cyclical variation in coagulation might induce thrombosis, none of the retrieved arterial segments in any group exhibited evidence for gross or microscopic thrombosis.

**Discussion**

One of the paradoxes of the accelerated arteriopathies is their clinical resistance to agents with great potential for inhibition of the cellular and molecular events that should regulate these lesions. Many agents can suppress vascular smooth muscle cell proliferation in culture.
and/or in animal models of arterial disease, but none have done so in clinical trials. It is possible that the human lesion is much more complex and sophisticated than the animal lesion that one cannot extrapolate from observations in the latter to the former. Alternatively, it may well be that the means by which these agents have been used to demonstrate benefit in the one case have not been followed in the other. We believe that the latter is the case with heparin. We now show that while the continuous intravenous infusion or controlled perivascular release of heparin dramatically reduced both intimal hyperplasia (Fig 1) and smooth muscle cell proliferation (Fig 2), when the same drug was administered intermittently, lesion size and cell proliferation were increased. These findings are in concert with and do not contradict human trials. The data imply that just as a deleterious effect is obtained when animal studies are performed the way in which negative human trials were conducted, so too should we only expect a beneficial effect when the human trials are performed as the positive animal studies were conducted.

Subcutaneous Heparinization

Heparin has been proven effective for the prevention and propagation of venous thrombosis and as an adjunct to coronary arterial thrombolysis in the setting of acute myocardial infarction from coronary arterial thrombosis. In many instances, subcutaneous administration has replaced intravenous therapy.51-53 Yet, the enthusiasm for subcutaneous injections of heparin in this setting has been tempered by the realization that the pharmacokinetics of heparin administered in this fashion are highly erratic and variable.49,50 In part, this variability must arise because heparin is a heterogeneous mixture of sulfated glycosaminoglycans isolated from bovine lung or porcine gut. However, one must also be aware that this compound is very soluble and has complex clearance, with a dose-dependent serum half-life on the order of minutes.56 It is possible that heparin is simply rapidly and efficiently metabolized and cleared after intermittent administration.

Antiproliferative Heparin

While antithrombotic heparin is clearly antiproliferative, the antiproliferative effect of heparin is independent of its antithrombotic effect.8,37,41,57,58 In point of fact, heparin has a myriad of effects on the vascular wall and vascular cell growth in addition to effects on thrombin.59 Heparin directly inhibits vascular smooth muscle cell proliferation,8,30,31,50,61 diminishes extracellular matrix production,62 binds to a large number of growth factors,53-66 interacts with tissue plasminogen activator,67 inhibits the expression of various proto-oncogenes,68 alters complement activation and hypersensitivity,69 and affects lymphocyte trafficking.70 Thus, any, some, or all of these and other effects may account for why we observed a greater decrease in intimal hyperplasia (Fig 1) than what can be accounted for solely by inhibition of cell proliferation (Fig 2). Moreover, the multitude of effects that can potentially contribute to a decrease in the intimal mass implies that the dose and timing of drug administration necessary to inhibit proliferation may well be different than that required to alter coagulation alone. Indeed, the findings reported in this study confirm the independence of the ability of heparin to inhibit clotting and proliferation, as the most effective antiproliferative dosing did not alter coagulation, and the heparin dosing that did anticoagulate the animals had no beneficial effect on intimal hyperplasia.

That continuous administration of heparin was the optimum means of drug delivery is also not surprising. The initial demonstration of an antiproliferative effect was obtained with continuous intravenous heparin infusion for at least 4 to 7 days after injury and in simple models such as a single instance of endothelial denudation of an otherwise normal artery in healthy rats.36 When 350 IU/kg per day (=2.33 mg/kg per day) of heparin was administered subcutaneously once a day, no effect was observed on the air-desiccated femoral artery of rabbits maintained on a high-cholesterol diet.25 The intima to media ratio was, if anything, somewhat worse for the heparin-treated animals than comparable controls: 2.34±2.38 and 1.77±1.26, respectively.25 Clowes,71 in an accompanying editorial, discussed this report and compared it with a study of Buchwald et al72 wherein the subcutaneous administration of a low-molecular-weight heparin had a significant beneficial impact on healing of stented minipig coronary arteries. Clowes mentioned that the discrepancy be-
between these studies may have arisen from differences in the animal model used, the vascular bed in which the injury was applied, the form and nature of the induced injury, and the type of heparin used. The last concern is of particular importance, as the clearance of standard and modified heparins is dramatically different. Standard heparin has a dose-dependent serum half-life that plateaus at approximately 30 to 35 minutes for concentrations >1.6 mg/kg (≈250.0 IU/kg).46 Dawes et al46 examined the absorption of standard and modified heparins and showed that both the maximal plasma concentration (microgram per milliliter) and the AUC (area under the curve, μg/hr per milliliter) increased with decreasing molecular weight of the heparin studied. Moreover, absorption of modified lower-molecular-weight heparins from the site of subcutaneous injection was substantially more efficient and less variable than with the standard heparin. Peak plasma levels were obtained 2 to 3 hours after the subcutaneous injection of standard heparin and cleared to baseline within 7 hours of injection. We observed the same variability in effect and duration of action in our experiments with standard heparin. Error bars were wide, and the elevation in aPTT that was noted within 2 hours of injection was cleared to baseline within 6 hours (Fig 3). Thus, intermittent administration provides significant oscillations in the systemic thrombotic state, spanning a threefold to fourfold range, with marked anticoagulation in one part of the day and full recovery in the other. Finally, there exists the controversial notion of heparin rebound. There are a number of reports that venous thrombosis and arterial disease (eg, unstable angina) become unstable or are reactivated when heparin is removed.73 If this phenomenon occurs, it may represent progression of disease, loss of the therapeutic inhibitory effect with removal of the heparin, or true exacerbation by a rebound effect. Our observation that intermittent heparin therapy is deleterious may legitimately reflect the results of cyclical variations in clotting (Fig 4) or growth factor binding/displacement as well as periods of time without the beneficial effects of the presence of heparin.

Clinical Implications

In light of the above and the data we now report, it may not be that surprising that restenosis was worse when patients were injected subcutaneously with 10 000 IU of heparin once daily after angioplasty.19 While the mechanism of this exacerbation will require additional research and investigation, it is becoming increasingly clear that if we are to accept or reject the use of potentially potent vasoactive agents for the treatment of vasculoproliferative diseases, we should do so with the appropriate extrapolation of clinical use from experimental data. It is unjust to reject as inactive a compound with the proven track record of heparin on the basis of human trials that do not match animal studies. In this study, when the animal studies were performed to mirror clinical use, exacerbation of disease was observed. Perhaps if human trials were performed as the beneficial animal trials were conducted, ie, with continuous infusion or local administration, a positive effect might be observed.

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