Heparin/heparan sulfate chelation inhibits control of vascular repair by tissue-engineered endothelial cells

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1Harvard-Massachusetts Institute of Technology Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge 02139; 2Cardiac Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston 02114; and 3Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

Han, Richard O., David S. Ettenson, Edward W. Y. Koo, and Elazer R. Edelman. Heparin/heparan sulfate chelation inhibits control of vascular repair by tissue-engineered endothelial cells. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2586–H2595, 1997.—The relative importance of heparin-like compounds in mediating vascular repair is unclear. We investigated how protamine, a chelator of heparin, affected endothelial cell inhibition of vascular smooth muscle cell growth and intimal hyperplasia. The 52% (P < 0.001) reduction in smooth muscle cell proliferation produced by postconfluent endothelial cell-conditioned medium was entirely reversed by pretreatment of medium with heparinase and heparitinase and was inhibited in a dose-dependent fashion by the coadministration of protamine. Pretreatment of conditioned medium with heparinase and heparitinase largely prevented protamine's mitogenic activity, suggesting that protamine affects growth by interacting with heparin-like compounds. Perivascular implantation of polymer-engrafted endothelial cells reduced neointima formation in denuded rat carotid arteries by 92% (P < 0.001) and cell proliferation, by 81% (P < 0.001). Coadministration of protamine abolished the inhibitory potential of the cell implants, resulting in a nearly twofold exacerbation of intimal hyperplasia compared with controls (P < 0.001). Thus heparin-like molecules are essential to the biochemical regulation of vascular repair provided by endothelial cells, and the continued routine clinical use of heparin chelators, like protamine, may be questionable.

vascular injury; restenosis; biopolymers; vascular smooth muscle cell growth

THE PATHOLOGICAL HALLMARK of accelerated arteriopathies that follow intravascular interventions is the formation of a neointima composed of hyperplastic vascular smooth muscle cells and their elaborated extracellular matrix. There is accumulating evidence that disruption of normal endothelial cell function is critical to the development of this neointima (14, 39). The intact endothelium serves as a regulator of many vascular phenomena and creates a milieu that maintains vascular smooth muscle cells in their normally quiescent state. Endothelial injury, as might be induced by angioplasty, disrupts vascular homeostasis, releasing smooth muscle cells from basal growth inhibition and allowing formation of a proliferative neointimal lesion. We have recently demonstrated that endothelial biochemical regulation of smooth muscle cell proliferation can be achieved without the need to restore the endothelial cell luminal monolayer. Endothelial cells engrafted onto Gelfoam biopolymeric matrices retained their viability, growth kinetics, immunologic markers, and biochemical secretory activity. Perivascular implantation of the Gelfoam-endothelial cell devices around balloon-denuded rat carotid arteries restored biochemical balance within the arterial wall and reduced intimal hyperplasia by 90% (33).

Endothelial cells produce numerous substances that could potentially mediate this regulatory effect. Heparin sulfate is one such product with known ability to inhibit vascular smooth muscle cell proliferation (6, 8, 10). Perivascular implantation of mutant Chinese hamster ovarian cells deficient in heparan sulfate proteoglycan secretion failed to prevent, and actually potentiated, neointima formation (33). The actions of endogenous heparin-like compounds, however, do not account for all endothelium-derived growth inhibition, and heparin administration alone is insufficient to achieve optimal growth control. Local delivery of heparin alone is much less effective in inhibiting neointima formation than is perivascular implantation of intact endothelial cells (33). Moreover, heparin has failed to control intimal hyperplasia in complex models of vascular injury, as well as in clinical trials (13, 27). Thus the relative importance of heparin-like compounds in maintaining vascular homeostasis is unclear. To determine whether endothelial cell control of vascular repair is actually dependent on the actions of heparin-like compounds, we examined how protamine, a chelator of heparin, affects the ability of endothelial cell grafts to inhibit neointima formation.

Protamines are a family of basic, arginine-rich proteins purified from fish sperm that reverse the anticoagulant effects of heparin by forming one-to-one pairings of cationic sites with anionic heparin sites (25). Protamine can antagonize the antiproliferative effects of heparin in a dose-dependent manner (11). We now demonstrate that chelation of heparin-like molecules abolishes endothelial cell-mediated growth inhibition. Whereas conditioned medium from postconfluent endothelial cells inhibits vascular smooth muscle cell growth, the coadministration of protamine entirely negates this antiproliferative effect. Systemic administration of protamine from polymer-based continuous release devices exacerbates intimal hyperplasia after balloon injury to rat carotid arteries and completely neutralizes the inhibitory potential of the polymeric endothelial cell implants. Thus heparin-like molecules appear essen-
tial to the biochemical regulation of vascular repair provided by endothelial cells.

METHODS

Effects of Endothelial Cell-Conditioned Medium and Protamine on Vascular Smooth Muscle Cells In Culture

Collection of conditioned medium. Bovine aortic endothelial cells were harvested by collagenase dispersion (4). More than 95% of the cells harvested in this manner were endothelial at confluence, as determined by their distinctive cobblestone morphology and the presence of positive immunostaining for von Willebrand's factor. Studies were performed with bovine aortic endothelial cells, as we have reproducibly harvested and cultured these cells with complete retention of their biochemical regulation of vascular smooth muscle cell proliferation (36). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum, 5.6 mmol/l glucose, 50 U/ml of penicillin, and 50 µg/ml of streptomycin. The medium was replaced every 48 h. Confluent cultures were subcultured with 0.05% trypsin and 0.02% EDTA, and cells were used at passage 3. All tissue culture reagents were obtained from GIBCO Laboratories. For collection of conditioned medium, endothelial cells were grown to confluence in 100-mm tissue culture dishes. Fresh medium (10 ml) was added 72 h after confluence was reached, and cells were harvested with 0.05% trypsin and 0.02% EDTA, and aliquots of the supernatants were stored at −20°C.

Proliferation of cultured smooth muscle cells. Bovine aortic smooth muscle cells (5 × 10^3/well, passage 3) were seeded into 12-well plates and incubated in 5% calf serum-DMEM for 24 h. The cells were washed with serum-free DMEM and then growth arrested through incubation in 0.1% calf serum-DMEM for 72 h. The medium was then replaced with 5% calf serum-DMEM or with equal volumes of 10% calf serum-DMEM and endothelial cell-conditioned medium in the absence or presence of various amounts of protamine sulfate (Sigma, St. Louis, MO). After 3 days the vascular smooth muscle cells were washed in Ca^2+ - and Mg^2+-free phosphate-buffered saline solution and removed from the trypsinization. Cell number was then determined using a Coulter counter.

In another set of experiments, growth-arrested vascular smooth muscle cells were stimulated by medium pretreated with heparinase and heparitinase. Endothelial cell-conditioned medium was preincubated with both 2 µl/ml heparinase and 2 µl/ml heparitinase (Seikagaku America, Ijamsville, MD) for 3 h at 37°C. After this time, the enzymes were inactivated by boiling for 10 min. The treated conditioned medium was mixed with equal volumes of 10% calf serum-DMEM in the absence or presence of 100 µg/ml protamine sulfate, and the resultant mixture was administered to vascular smooth muscle cells that were growth arrested as described above. As a control, 5% calf serum-DMEM was pretreated in a similar manner to the endothelial cell-conditioned medium. Cells were counted after 3 days, as described above.

Effects of Perivascular Endothelial Cell Implants and Protamine on Intimal Hyperplasia After Arterial Injury

Cell engraftment. Gelfoam (Upjohn, Kalamazoo, MI) has long been used as an implantable surgical sponge and more recently as a scaffolding for cell growth (7, 33). This material, isolated from porcine dermal gelatin, was cut into 2.5 × 1 × 0.3 cm pieces and hydrated by autoclaving for 10 min in Hanks' balanced salt solution (HBSS). On cooling, each block was placed in a 17 × 100 mm polypropylene tube containing 2 ml of an endothelial cell suspension in DMEM, supplemented with 5.6 mmol/l glucose and 10% calf serum (0.5 × 10^6 cells/ml). The culture tubes containing the blocks were gently agitated to disperse the cells and then incubated at 37°C for up to 17 days at a 45° angle in humidified 5% CO2-95% air. Growth medium was changed every 3–5 days. The number of cells attached to the Gelfoam blocks was determined every 2–4 days. The blocks were first washed four times with HBSS and then digested with collagenase (1 mg/ml). Cell viability was determined by trypan blue exclusion. The number of viable cells was counted using a hemocytometer. Gelfoam blocks were implanted around arteries only after the cell number per block had plateaued, suggesting confluence. Viable cell number plateaued at −1.4 × 10^6 cells/block. After engraftment onto Gelfoam blocks, the identity of the cells was confirmed by immunostaining for the endothelial marker von Willebrand's factor, as previously described in detail (12).

Protamine administration. Protamine was administered from subcutaneously implanted polymer-based controlled release devices (10, 11). Dry powdered protamine sulfate (1 mg) was added to a solution of ethylene-vinyl acetate copolymer (EVAc, DuPont, Wilmington, DE) dissolved in dichloromethane (15% wt/vol) to achieve a final ratio of 33% (wt/wt). The drug-polymer suspension was poured into precooled molds on dry ice, removed after hardening, and placed at −20°C and then under vacuum (600 mTorr) for 2 days each. The resultant matrix was a homogeneous dispersion of protamine sulfate within a porous network of EVAc. Smaller pellets were cut from the larger slabs using a no. 3 cork borer, and the pellets were then coated with two layers of EVAc. Drug release was restrained to emanate from an 18-gauge hole in the EVAc coating. With the use of such devices, protamine release has been extended for over 4 wk with near-zero-order kinetics at a dose of 821 µg·kg rat^-1·day^-1 (10, 11). Protamine-containing devices (18 animals) or blank controls (17 animals) were inserted into the abdominal subcutaneous space of 300- to 350-g male Sprague-Dawley rats (Charles River Breeding Laboratories, Kingston, MA). After 3–6 days of pretreatment, arterial injury was induced, and Gelfoam implants were applied to the perivascular space.

Arterial injury. Endothelial denudation of the left common carotid artery in the rats was performed with a 2-Fr Fogarty balloon catheter (Baxter Healthcare). Rats were anesthetized with intraperitoneal ketamine (0.04 mg/g body wt) and xylazine (0.02 mg/g body wt). A midline incision exposed the distal left common and external carotid arteries. An arteriotomy of the external carotid artery was performed with iris scissors, and the balloon catheter was then introduced and threaded into the proximal common carotid artery. The balloon was distended sufficiently with air to generate slight resistance and was passed three times along the entire length of the common carotid artery. On removal of the catheter, the external carotid artery was ligated just proximal to the arteriotomy. Gelfoam blocks containing either endothelial cells or no cells were then wrapped around the common carotid artery. Overlapping ends of the Gelfoam ensured that the blocks did not unravel or migrate over the course of the experiment (33). Rats were divided into four groups: 8 animals received both protamine and endothelial cell implants, 8 animals received endothelial cell implants alone (with blank EVAc devices), 10 animals received protamine alone (with blank Gelfoam blocks), and 9 animals received blank EVAc devices and blank Gelfoam blocks. All procedures were in accordance with institutional guidelines.
Tissue processing. On the 14th postoperative day, animals were euthanized and perfused clear via the left ventricle with Ringer lactate solution. The location of the Gelfoam implants was noted and the implants were recovered intact with the left common carotid arteries. The harvested arteries were cut into four equal segments, immersed in Carnoy’s fixative (60% methanol-30% chloroform-10% glacial acetic acid) for 4 h, and then transferred to ethanol. The segments were paraffin embedded and microtome sectioned. Eight to twelve 6-µm sections along the length of each segment were obtained and stained with hematoxylin-eosin or Verhoeff’s elastin stain. The intimal and medial areas were determined for each arterial segment by using computerized digital planimetry with a dedicated video microscope and customized software. Analyses were confirmed by visual inspection and were carried out by an investigator blinded to the nature of the specimens.

Immunocytochemistry. Cell proliferation was also followed over the entire duration of the experiment by injecting the thymidine analog 5-bromo-2’-deoxyuridine (BrdU; 50 mg/kg ip; NEN, Boston, MA) on postsurgery days 3 and 7 and 1 h before the animals were killed. Intracellular BrdU was identified immunocytochemically, as previously described (11). Sections were exposed to mouse immunoglobulin G (IgG) anti-BrdU antibody (Coulter Immunology, Hialeah, FL) diluted 1:100 for 60 min. Sections were subsequently exposed to biotinylated anti-mouse IgG and then to avidin-peroxidase complex (Vector Labs, Burlingame, CA). Antibody visualization was established after exposure to 3,3’-diaminobenzidine (Sigma) with hydrogen peroxide. Sections were counterstained with hematoxylin. Identification of proliferating cells was performed using computer-based microscopy and video image processing. The number of antibody-positive cells as a fraction of the total number of cells was determined by an investigator blinded to the nature of the specimens.

Statistics. Data are presented as means ± SE. Statistical comparisons were performed using analysis of variance. Sample means of the cell culture experiments were compared using Scheffé’s test. Sample means of the in vivo experiments were compared using Fisher’s protected least significant differences test. In all cases, data were rejected as not statistically significant if values of $P > 0.05$ were observed.

RESULTS

Effects of Endothelial Cell-Conditioned Medium and Protamine on Vascular Smooth Muscle Cell Growth In Vitro

Growth-arrested bovine aortic smooth muscle cells were released from $G_0$ phase by exposure to 5% calf serum, with or without conditioned medium collected from 3-day postconfluent bovine aortic endothelial cells, and in the presence or absence of various concentrations of protamine. As shown in Fig. 1, endothelial cell-conditioned medium (without protamine) inhibited growth of vascular smooth muscle cells by 52% after 3 days ($P < 0.001$). In the absence of endothelial cell-conditioned medium, protamine stimulated smooth muscle cell growth in a dose-dependent fashion (Fig. 2A). A protamine concentration of 30 µg/ml stimulated smooth muscle cell growth twofold ($P < 0.001$). Furthermore, protamine negated the growth-inhibitory effects of endothelial cell-conditioned medium in a dose-dependent manner (Fig. 2A). Increasing doses of protamine progressively reduced the growth inhibition achieved by endothelial cell-conditioned medium, and at a protamine concentration of 20 µg/ml, the antiproliferative effects of the endothelial cell-conditioned medium were completely overcome. At higher protamine concentrations, the combination of protamine plus endothelial cell-conditioned medium actually stimulated vascular smooth muscle cell growth more than protamine in the absence of endothelial cell-conditioned medium. The mitogenic effect of protamine plus endothelial cell-conditioned medium peaked at a protamine concentration of 100 µg/ml (2.3-fold stimulation vs. 100 µg/ml protamine in the absence of endothelial cell-conditioned medium, $P < 0.001$). The percent inhibition or stimulation achieved by endothelial cell-conditioned medium in the presence of increasing concentrations of protamine is shown in Fig. 2A, inset. At protamine concentrations $>120$ µg/ml, vascular smooth muscle cells displayed morphological evidence of cell toxicity and cell numbers decreased. At lower protamine concentrations ($\leq 120$ µg/ml), the vascular smooth muscle cells appeared healthy, without evidence of decreased viability.

To examine the mechanism by which protamine overcomes the growth-inhibitory potential of endothelial cell-conditioned medium, we performed a set of experiments using conditioned medium pretreated with heparinase and heparitinase I. Enzymatic digestion of heparin-like compounds in the endothelial cell-conditioned medium eliminated its inhibitory effect and actually yielded a medium that was moderately mitogenic for vascular smooth muscle cells (1.5-fold stimulation vs. control, $P < 0.001$, Fig. 2B). Of note, the degree of smooth muscle cell proliferation induced by heparinase- and heparitinase-pretreated endothelial cell-conditioned medium was less than one-half that induced by 100 µg/ml protamine plus untreated endothelial
endothelial cell-conditioned medium (6.7 × 10^4 ± 7 × 10^3 cells vs. 1.43 × 10^5 ± 2 × 10^3 cells, *P* < 0.001), suggesting that protamine may produce a mitogenic effect beyond simple neutralization of heparin-like compounds. Interestingly, enzymatic digestion of endothelial cell-derived heparan sulfate prevented the growth-stimulatory effects of the combination of protamine and endothelial cell-conditioned medium (Fig. 2B). Smooth muscle cells exposed to heparinase- and heparitinase-pretreated endothelial cell-conditioned medium plus 100 µg/ml protamine proliferated only slightly more than those cells exposed to 100 µg/ml protamine in the absence of conditioned medium (7.15 × 10^4 ± 6 × 10^3 cells vs. 6.0 × 10^4 ± 1 × 10^3 cells, *P* = 0.004) and 50% less than those cells exposed to untreated endothelial cell-conditioned medium plus 100 µg/ml protamine (1.43 × 10^5 ± 2 × 10^3 cells, *P* < 0.001). Furthermore, the addition of 100 µg/ml protamine to heparinase- and heparitinase-pretreated conditioned medium yielded no significant incremental growth of vascular smooth muscle cells [7.15 × 10^4 ± 6 × 10^3 cells in the presence of pretreated conditioned medium plus 100 µg/ml protamine vs. 6.7 × 10^4 ± 8 × 10^3 cells in the presence of pretreated conditioned medium without protamine, *P* = not significant (NS)].

**In Vivo Effects of Perivascular Endothelial Cell Implants and Protamine on Neointimal Hyperplasia**

The in vivo effects of perivascular endothelial cell implants and protamine on intimal hyperplasia (Figs. 3 and 4) correlated well with our in vitro data. In control rats (blank Gelfoam implants, blank EVAc devices), balloon denudation of the carotid artery induced neointima formation such that the ratio of the area of the tunica intima to the area of the tunica media (I/M) reached 0.61 ± 0.12 after 14 days (Fig. 4A). The addition of endothelial cell implants to the perivascular space successfully reduced intimal hyperplasia by 92% (I/M = 0.05 ± 0.01, *P* < 0.001 vs. controls). The continuous release of protamine sulfate from the abdominal subcutaneous space beginning several days before balloon injury and extending through 14 days postinjury increased intimal hyperplasia 1.95-fold compared with controls (I/M = 1.19 ± 0.07 vs. 0.61 ± 0.12 in controls, *P* < 0.001). Furthermore, protamine administration completely reversed the antihyperplastic effects of implanted perivascular endothelial cells. Animals receiving both protamine and perivascular endothelial cells experienced as much intimal hyperplasia as animals receiving protamine alone and more hyperplasia than control animals (I/M = 1.09 ± 0.10, *P* < 0.001 vs. endothelial cell implant without protamine; *P* < 0.001 vs. control).

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**Fig. 2.** A: effect of protamine on VSMC growth in presence of CS and EC-CM. Increasing doses of protamine were added to growth-arrested VSMCs stimulated either by addition of 5% CS alone or CS + EC-CM. VSMC number was determined after 3 days. Protamine stimulated growth of cultured BASMCs (A) and negated the growth inhibition caused by addition of EC-CM collected from 3-day postconfluent BAECs (B). Amount of protamine required to totally overcome inhibitory effect of EC-CM was 20 µg/ml. At higher protamine concentrations, protamine with EC-CM stimulated VSMC growth more than protamine without EC-CM (*P* < 0.001, paired *t*-test of CS with and without EC-CM). This mitogenic effect of protamine with EC-CM plateaued at protamine concentration of 100 µg/ml. Values are means ± SE. Inset: %inhibition or %stimulation of VSMC growth achieved by EC-CM in presence of increasing protamine concentrations vs. controls (5% CS alone). B: effect of heparinase-heparitinase pretreatment of EC-CM. Heparin-like compounds in EC-CM were digested by preincubating the medium with 2 µM heparinase and 2 µM heparitinase for 3 h at 37°C. Afterward the enzymes were inactivated by boiling for 10 min. As a control, 5% CS-DMEM was treated similarly. Treated EC-CM or treated 5% CS-DMEM was mixed with equal volumes of 10% CS-DMEM with or without 100 µg/ml protamine sulfate, and resultant mixture was used to stimulate growth-arrested VSMCs. VSMC number was determined after 3 days. Values (means ± SE) are %inhibition or %stimulation of VSMC growth achieved by treated EC-CM in presence or absence of 100 µg/ml protamine vs. controls (5% CS without EC-CM). For comparison, %inhibition or %stimulation achieved by untreated EC-CM in presence or absence of 100 µg/ml protamine is also shown. Heparinase-heparitinase pretreatment transformed EC-CM from growth inhibitory to mitogenic. Heparinase-heparitinase pretreatment also largely prevented the mitogenic effects of 100 µg/ml protamine added to EC-CM. *P* < 0.001, †*P* = 0.004 vs. control.
The effects of protamine and perivascular endothelial cell implants on cell proliferation were determined by BrdU incorporation (Fig. 4B). The percentage of BrdU-positive intimal cells was used as an index of proliferation. Perivascular implantation of endothelial cells reduced intimal cell proliferation by 81% compared with controls (3 ± 1% proliferation vs. 17 ± 2% in controls, P < 0.001). Concordant with its effects on neointima formation, protamine administration completely reversed the antiproliferative effects of the endothelial cell implants. Coadministration of protamine completely abolished the inhibitory effect of perivascular EC implants on neointima formation (D). Arrows point to the internal elastic laminae that separate tunicae intima from tunicae media. The intima stained with unusual intensity in rats administered protamine (B). This pattern may have resulted from protamine deposition in the vessel wall with subsequent binding of anionic dye to the polycation.

**DISCUSSION**

The contribution of the endothelium to preserving vascular homeostasis involves far more than its function as a barrier lining. The normal endothelium supports a milieu that maintains the blood vessel wall quiescent. Endothelial loss or injury alters this balance, initiating a coordinated sequence of events that culminates in smooth muscle cell proliferation, migration, and neointima formation. Smooth muscle cells continue to proliferate until the endothelium is restored (14, 39), and stimulation of reendothelialization reduces intimal hyperplasia (3). Our work with endothelial cell implants supports a biochemical mechanism underlying endothelial regulation of vascular homeostasis. Endothelial cells engrafted onto biopolymeric scaffolding and placed in the perivascular space nearly eliminated intimal hyperplasia after balloon-induced arterial injury without the need for recapitulation of the endothelial lining (Ref. 33 and Figs. 3 and 4). Transplantation of intact cells offered more potent regulation of the fibroproliferative response than the isolated infusion of a single endothelial cell product (33). We now show that, although this may be the case, chelation of heparin-related compounds with protamine completely reverses the benefit of the endothelial implants (Figs. 3 and 4). These observations support the notion that heparin-like compounds are essential, but not entirely sufficient, for control of vascular repair. Moreover, they call into question the
continued routine clinical use of protamine, which removes these critical elements from their position of growth regulation.

Endothelial Cell Implants and Heparin

It has been known for some time that endothelial cells secrete compounds that can influence all the major axes of vascular biology, including thrombosis and hemostasis, vasomotor tone, cell growth, and matrix remodeling. A good deal of the control exerted by endothelial cells stems from their production of heparin-like compounds, such as heparan sulfate (6, 8–10, 18, 36, 45). Furthermore, endothelium-derived heparan sulfate is more effective at inhibiting growth factor binding and mitogenesis than heparan sulfate secreted by vascular smooth muscle cells or fibroblasts (36). Yet heparan sulfate is only one of many growth-inhibitory products secreted by endothelial cells, and the relative importance of these products in vivo is unknown.

Endothelial cells engrafted onto biopolymeric matrices were three- to fivefold more effective in inhibiting intimal hyperplasia than the perivascular administration of heparin from hydrogel films, despite hydrogel release of heparin at twice the rate of heparan sulfate secretion from the endothelial cell implants (33). This observation suggests the role of other endothelium-derived products in achieving optimal growth control. Heparin administration has been unable to fully control intimal hyperplasia in complex models of vascular injury or to prevent restenosis after coronary angioplasty in clinical trials (13, 27). In higher animal species or after more sophisticated forms of arterial injury, the full effect of the endothelial cell may be required to prevent the force of intimal hyperplasia. Because heparin-like compounds do not mediate all of the endothelium's growth-regulatory effects, one could surmise that some degree of control over intimal hyperplasia would still be evident even after heparin-like compounds were neutralized.

This hypothesis was not supported by our data. Systemic protamine administration completely overcame the ability of endothelial cells to control the fibroproliferative response to arterial injury. Protamine entirely reversed the antiproliferative effects of endothelial cell-conditioned medium on cultured smooth muscle cells in a dose-dependent manner (Fig. 2) and abolished the inhibitory effects of perivascular endothelial cell implants on intimal hyperplasia (Figs. 3 and 4). If protamine sulfate achieves these effects by chelating heparin/heparan sulfate, it then follows that although heparin does not account for all of the growth-regulatory functions of the endothelial cell, this regulation cannot occur without functional heparin-like compounds present. In this manner heparin/heparan sulfate become crucial elements in controlling the vascular response to injury, and even the local presence of intact endothelial cells cannot overcome the reversing effects of protamine.
Heparin-Dependent Effects of Protamine

The major limitation in interpreting our data is that the precise mechanism(s) of protamine’s actions remains unclear. Yet we can at least surmise from our cell culture experiments that the inhibitory potential of endothelial cell-conditioned medium requires the presence of functional heparin-like compounds and that the effects of protamine depend on its interaction with heparin-like compounds. Endothelial cell-conditioned medium alone inhibited vascular smooth muscle cell growth by 52% (Fig. 1). Enzymatic digestion of heparin-like molecules in the endothelial cell-conditioned medium eliminated its inhibitory effects and actually yielded a medium that was moderately mitogenic (Fig. 2A-B). This later observation suggests that endothelial cells secrete non-heparin-like compounds that, either alone or in combination with a factor(s) in calf serum, stimulate smooth muscle cell growth. Indeed, endothelial cells, especially when in a proliferating, nonconfluent state, are able to produce numerous smooth muscle cell mitogens (e.g., basic fibroblast growth factor, platelet-derived growth factor, insulin-like growth factor I, interleukin-1, angiotensin II, and endothelin) (6, 16). Exponential endothelial cells (analogous to the situation in the local microenvironment after balloon injury) produce a conditioned medium that is predominantly growth stimulatory, whereas quiescent, confluent endothelial cells (as in our perivascular implants) produce a conditioned medium that is net inhibitory. Despite the overall antiproliferative effect of our postconfluent conditioned medium, the enzymatic digestion of a major growth inhibitor, such as heparin-like compounds, could unmask the effect of endothelium-derived mitogens.

Protamine not only reversed the growth-inhibitory effects of endothelial cell-conditioned medium. At higher protamine concentrations, the combination of protamine plus endothelial cell-conditioned medium stimulated smooth muscle cell proliferation more than protamine in the absence of conditioned medium and more than heparinase- and heparitinase-pretreated conditioned medium (Fig. 2A-B). Thus a combination of both high-dose protamine and endothelial cell-conditioned medium was required to yield maximal smooth muscle cell growth, and the degree of proliferation induced by this combination was greater than what would be expected from simple neutralization of heparan sulfate. Furthermore, enzymatic digestion of heparin-like compounds in the conditioned medium prevented the mitogenic effects of the combination of protamine and conditioned medium. Thus protamine’s ability to affect vascular smooth muscle cell growth is dependent on the presence of heparin-like compounds. It therefore is most plausible that protamine reverses the inhibitory potential of endothelial cell-conditioned medium by chelating heparin-like molecules and that the resulting protamine-heparan sulfate complex has a direct or indirect mitogenic effect on vascular smooth muscle cells. The possibilities that protamine directly stimulates vascular smooth muscle cell proliferation or complexes with non-heparin-like factors in a way that influences smooth muscle cell growth do not appear important in vitro. The observation that protamine stimulated vascular smooth muscle cell proliferation even in the absence of endothelial cell-conditioned medium was not unexpected, as vascular smooth muscle cells produce some heparin-like compounds that appear to inhibit their own growth in a paracrine or autocrine feedback loop (15).

We cannot exclude the possibility that the boiling used to inactivate the heparinase and heparitinase confounded our results, despite the use of controls. Differential heat inactivation of growth inhibitors and growth factors could have influenced vascular smooth muscle cell proliferation.

Protamine and Heparin-Binding Growth Factors

The molecular basis for protamine’s effects on cell growth are unknown. Heparin has a multitude of effects on vascular repair, and protamine may interfere with any one of them. One of the more obvious effects of endogenous heparin-like compounds is their regulation of vascular growth factors (9, 45). Protamine may inhibit soluble heparin-like compounds from blocking the binding of heparin-avid growth factors to their receptors and thereby promote growth factor activity. Alternatively, protamine might antagonize the activity of heparin-avid growth factors by inhibiting their binding to cell-associated or extracellular matrix-bound heparin-like receptors. Protamine may prevent heparan sulfate proteoglycan from sequestering growth factors and enhancing their binding to cell-surface receptors. Protamine appears to inhibit the mitogenic potential of acidic fibroblast growth factor, basic fibroblast growth factor (35), and platelet-derived growth factor (21), all of which display heparin avidity, by preventing their binding to cell-surface receptors. Protamine has also been reported to impede angiogenesis in vivo (41). Both acidic and basic fibroblast growth factor can promote endothelial regeneration (3). Indeed, administration of acidic fibroblast growth factor selectively stimulates endothelial regrowth after denuding arterial injury and minimizes intimal hyperplasia (3). It is thus conceivable that protamine administration impedes vascular repair by inhibiting the activities of these growth factors and consequently results in worsened intimal hyperplasia. Alternatively, protamine might stimulate vascular smooth muscle cell growth and neointima formation by potentiating other growth factors. For example, protamine has been reported to augment the mitogenic activity of epidermal growth factor, a growth factor with negligible heparin avidity (21, 22, 35).

Potential Non-Heparin Effects of Protamine

It is not entirely clear that the actions of protamine only result from the chelation of heparin and heparin-like compounds in vivo. Protamine might also affect the production or release of other endothelium-derived growth inhibitors, such as prostacyclin and endothelium-derived relaxing factor. Heparin administration
has been reported to stimulate group II phospholipase A2 activity and prostacyclin generation without increasing thromboxane levels (24, 32). Subsequent protamine administration appears to cause phospholipase A2 activity and prostacyclin synthesis to fall, whereas thromboxane release remains unchanged (32) or increases (29, 31). Thus protamine may produce elevation in the thromboxane-to-prostacyclin ratio, which in turn may yield a milieu that promotes vasoconstriction, platelet aggregation, and vascular smooth muscle cell growth. Others (34, 44), however, have observed stimulation of prostacyclin release by protamine administration, whereas still others (28) have found no significant effect on eicosanoid production.

Protamine and other basic polyamino acids rich in L-arginine, lysine, or ornithine have been shown to produce endothelium-dependent vasodilatation by inducing the generation of endothelium-derived relaxing factor (nitric oxide) (23, 37). However, prolonged exposure to such compounds results in refractoriness to stimulants of endothelium-mediated vasorelaxation (1, 23). To explain this paradoxical observation, Ignarro et al. (23) have proposed that such basic polyamino acids serve as partial substrates for the enzyme system that catalyzes the production of endothelium-derived relaxing factor, a process that involves the generation of nitric oxide from L-arginine. Chronic exposure to protamine and related compounds might lead to desensitization of nitric oxide synthase or other component(s) of this enzyme system and thus refractoriness to nitric oxide formation (23). Such inhibition of nitric oxide generation could lead not only to impairment of endothelium-dependent vasorelaxation but also to promotion of platelet aggregation, vascular smooth muscle cell proliferation, and neointima formation after vascular injury. Indeed, others (17, 19, 26, 30, 40, 43) have demonstrated that administration of nitric oxide or nitric oxide donors reduces intimal hyperplasia in animal models.

Another possible explanation for protamine's ability to reverse tissue-engineered endothelial cell inhibition of intimal hyperplasia is that protamine's polycationic properties may produce a nonspecific toxic effect on endothelial cells (1). We did observe evidence of vascular smooth muscle cell toxicity in cultures exposed to very high concentrations of protamine sulfate (>120 μg/ml), but at less extreme concentrations the cells appeared healthy. Moreover, the endothelial cells lining the Gelfoam scaffolds harvested from rats that received protamine displayed no histological evidence of toxicity in our experiments. Using scanning electron microscopy, others (23) have also seen no evidence of endothelial cell damage after prolonged exposure to basic polyamino acids. Furthermore, the ability of bradykinin to stimulate endothelium-mediated prostanoïd formation and vasorelaxation appears preserved despite prolonged exposure to high-dose basic polyamino acids, which argues against generalized endothelial damage (23).

Clinical Use of Protamine

Irrespective of mechanism, protamine sulfate, a commonly used clinical compound, exacerbates vascular injury. Protamine infusions are used to reverse systemic anticoagulation after cardiac catheterization and coronary artery bypass (25). Protamine is also used to slow the uptake of insulin from subcutaneous depots by decreasing its solubility at physiological pH. The sustained actions of protamine zinc insulin and neutral protamine Hagedorn insulin are the result of the addition of 1:3 and 1:8 ratios of protamine to insulin, respectively. The patients who routinely receive protamine, i.e., those with coronary artery disease and diabetes, are already at high risk for vasculoproliferative disease, particularly in the setting of concurrent vascular injury and manipulation. In addition to the data in this report, we have previously demonstrated that protamine administration by single intravenous bolus injection (as is typically performed to reverse systemic heparinization after cardiac catheterization or cardiopulmonary bypass) or by periodic subcutaneous injections as part of commonly used insulin preparations (as is routinely performed by diabetic patients) exacerbates proliferative lesions after vascular injury in rats (11). Diabetes appears to be an independent risk factor for restenosis after intracoronary interventions (5, 20), and diabetic patients may be a subset that has especially poor clinical outcomes after such interventions (38, 42). The mechanisms behind these observations are not well defined (2), but the possible role of protamine exposure in promoting vasculoproliferative disease in insulin-dependent diabetics, as well as others, needs further study.

The data we present suggest that the perivascular implantation of tissue-engineered endothelial cells may provide a useful model for studying the complex intercellular regulatory mechanisms that operate within the blood vessel wall. The potency of the endothelial cell as a mediator of vascular homeostasis may lie in its ability to secrete an array of products in a regulated manner as part of a sophisticated autocrine-paracrine network. An adequate understanding of the role of the endothelium in vascular pathobiology may not be possible unless experiments are performed with intact cells present. Tissue-engineered endothelial cells may also provide a novel means of combating restenosis after carotid endarterectomy and other vascular surgeries. The potential therapeutic efficacy, safety, and feasibility of such an approach requires further investigation.

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