GROWTH REGULATION BY HEPARIN IN THE VASCULAR WALL

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ABSTRACT

Vascular smooth muscle proliferation follows upon endothelial injury, and is thought to be an early component in the pathogenesis of atherosclerosis, and a possible noxious consequence of vascular surgery.

We have shown that heparin suppresses vascular smooth muscle proliferation in vivo and in vitro. The inhibitory effect is specific for heparin, and not other anions, and is not related to the antithrombin III binding activity of heparin. It is dependent on the size of the molecule, (hexasaccharides or smaller being ineffective), and O-sulfation, but not N-sulfation.

We have further shown that endothelial cells in vitro produce a similar inhibitory activity, which is probably due to heparin, and that this activity requires an endoglycosidase for its release from the endothelial cell surface.

These results implicate heparin in the growth regulation of vascular smooth muscle, both in physiological and pathological circumstances.
DECLARATION

This is to state that: —

a) the published papers submitted in respect of the candidature of Morris John Karnovsky for the degree of Doctor of Science in Medicine, represents work carried out under my direction, and with my active participation, in the Unit of Cell Biology and Experimental Pathology Harvard Medical School, Boston, Mass., U.S.A. of which Unit I am the Director. Details in respect to each publication are attached.

b) none of the work submitted has, is or will be submitted elsewhere to any university for a degree.

c) the information used in the thesis has been obtained whilst the candidate was an employee of Harvard University, and the work was supported by research grants from the National Institutes of Health, U.S.P.H.S. awarded to the candidate as Principal Investigator.

Morris John Karnovsky
10th day of June, 1983
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GROWTH REGULATION BY HEPARIN OF VASCULAR SMOOTH MUSCLE

Vascular smooth muscle (myointimal) proliferation is a common and important sequela of endothelial injury. Indeed, one of the modern theories of the pathogenesis of atherosclerosis involves myointimal proliferation as an crucial initial step in the formation of the lesion. Furthermore, it appears that myointimal proliferation not infrequently follows vascular surgery, and may contribute to surgical failures by compromising lumenal patency.

We present here a series of papers which demonstrate that heparin prevents vascular smooth muscle cell proliferation, in vivo and in vitro. These observations have important pharmacological and therapeutic implications and raise interesting questions as to the role of heparins and heparans in the (patho)biology of the vessel wall, and the role of these macromolecules in the regulation of cell growth in physiological and pathological conditions.

Paper I. Presents an experimental in vivo model for producing myointimal proliferation in the carotid artery of the rat, following endothelial injury caused by dessication. This "air-drying" injury has the advantages of damaging only the endothelium, and not the underlying smooth muscle, and of being reproducible, and quantitative. It shows the relationship between endothelial injury, platelet deposition and degranulation, and the subsequent myointimal proliferation. It forms the basis for our subsequent in vivo studies, and has been successfully utilized in a number of other laboratories.

Paper II. Because Paper I showed a relationship between platelet deposition and degranulation following endothelial injury, and because platelets are thought to release potent mitogen(s) for vascular smooth muscle, we tested to see if we could prevent vascular smooth muscle proliferation following endothelial injury in vivo by using antiplatelet drugs. Despite the fact that platelet-rich plasma from aspirin-, reserpine-, and fluobiprofen-treated rats showed markedly decreased platelet aggregation in response to collagen, and normal or slightly decreased aggregation in response to ADP or thrombin, as compared to platelets from control animals, none of these drugs when infused in vivo affected the degree of myointimal thickening, the morphology of the lesions, nor the extent of platelet deposition on the injured arterial wall.

Similarly, we were able to show (Papers III and IV) that in rats made hyperlipemic and hypercholesterolemic, there was no effect on the degree of myointimal proliferation. Superimposed on the basic proliferative lesion
found in normolipemic animals, was the presence of foam cells in the intima. In long-term hypercholesterolemic animals the foam cell lesions persisted but the regression of the myointimal proliferation was the same as in normolipemic controls.

Because the clotting sequence is no doubt activated in arterial injury, and because thrombin had been shown to be a mitogen, at least for some cell types, we thought it would be of interest to see if activation of the clotting sequence, and the generation of thrombin, was in any way related to the smooth muscle cell proliferation. In Paper V we tested the effect of heparin, which destroys thrombin by activation of antithrombin III, on the degree of myointimal proliferation. We found that myointimal proliferation was almost completely suppressed. There was no effect of the heparin on the number of circulating platelets, on the number of platelets deposited at the site of the lesion, their degranulation and structure, or on the rate of endothelial regeneration.

The next question asked was whether the antiproliferative activity of heparin was truly related to its anticoagulant activity. Commercial heparin is a mixture containing fractions that bind and activate antithrombin, and are therefore anticoagulant in activity, as well as fractions that are non-antithrombin binding, and are thus non-anticoagulant. Therefore, in Paper VI, the experiments were repeated with anticoagulant and non-anticoagulant heparin, separated by affinity chromatography with antithrombin as the adsorbant. It was clear that nonanticoagulant heparin was just as effective in preventing myointimal proliferation as was anticoagulant heparin.

Although our original hypothesis was wrong, the fact that a non-anticoagulant heparin was a potent antiproliferative agent, was clearly of interest. In order to explore the subtleties of the system further, we turned to in vitro systems. The test cells, bovine aortic endothelial and smooth muscle cells, and rat aortic smooth muscle cells were first grown in vitro, and then growth arrested. They were then released from growth arrest with either serum or platelet-extract — the latter containing platelet-derived growth factors (mitogens) —, and the effects of adding exogenous heparin, or other related compounds, were assayed in terms of increase in cell number over defined time periods.

In Paper VII we showed that heparin markedly inhibited the growth of vascular smooth muscle, and that anticoagulant heparin and non-anticoagulant heparin were both effective. Other anionic macromolecules (sulfated glycosaminoglycans), such as chondroitin sulfate, dermatan sulfate etc., were ineffective. We also showed that heparin interacts with the cell surface, that interaction with platelet growth factors in the culture media is not responsible for the heparin inhibitory effect, and that the mechanism of action is not mediated through an interaction with thrombin. Thus we concluded that heparin has some direct regulatory effect on smooth muscle cell growth.

In Paper VIII we extended these studies. We showed that heparin is inhibitory for vascular smooth cells, but not for endothelial cells. (In unpublished studies we have tested a large number of cell types: vascular
smooth muscle cells are at least 100 fold more sensitive to heparin than are other cell types). Anticoagulant and non-anticoagulant heparin were equally effective as inhibitors of vascular smooth muscle cell proliferation in vitro. Once again, the inhibitory effect was specific for heparin: highly purified heparin sulfates, dermatan sulfate, chondroitin ABC sulfates, and hyaluronic acid, at comparable doses were barely effective.

In order to establish what size of heparin molecule would have inhibitory effects, fragments of heparin were made by limit digest with nitrous acid and separated by chromatography. The detailed results are given in a forthcoming publication, but are summarized in Paper X. It was found that an inhibitory effect required an hexasaccharide or larger. Maximum inhibition was found in the dodecamer to 20- saccharide size range.

Chemical modification of the heparin molecule showed that O-sulfation was necessary for antiproliferative activity: however, N-sulfation was not. For instance, O-sulfated, N-desulfated, N-acetylated heparin was potent (Paper X).

The importance of the active heparin fragments and the active chemically-modified heparins is that they have lost anticoagulant activity, but have retained high antiproliferative activity; thus these compounds may have clinical usage in preventing myointimal proliferation, eg. after vascular surgery, without the dangers of causing bleeding.

In Paper X it is also reported that Scatchard analysis of $S^{35}$-labelled and $H^-$-labelled heparin - binding to vascular smooth muscle cells shows high affinity binding with a $K_d$ of $2 \times 10^{-7} M$, with $2 \times 10^7$ sites per cell. Furthermore, in work to be published we show that the molecule is internalized by a receptor-mediated process. As given in preliminary form in Paper X, DNA synthesis is inhibited in a marked and immediate fashion, down to 20 percent of normal within three hours. RNA and protein synthesis are only affected late. Flow microfluorimetry shows decreased entry of cells into $S_I$, and eventually the cells end up arrested in $G_I$. The mechanism(s) whereby heparin growth arrests vascular smooth muscle cells is currently under investigation in our laboratory.

The pharmacologic effects of heparin detailed above are of some interest, and of potential clinical importance. However, it was of moment to see whether a similar growth regulating system might be operative in situ in the vascular wall, utilizing endogenous heparin.

From our in vivo studies (Papers I and II) we had the impression that the vascular smooth muscle (myointimal) proliferation observed following endothelial injury ceased when re-endothelialization of the injured area occurred. Was it possible that endothelial cells had some sort of growth regulatory influence on the growth of vascular smooth muscle cells? We therefore examined the possible effect of endothelial cell secretions on vascular smooth muscle cell growth, in vitro.

In Paper VIII we show that conditioned media from confluent primary cultures of endothelial cells inhibited the growth of vascular smooth cells, released from growth arrest with serum.
Was this effect specific for the endothelial cell as producer cell, and the vascular smooth muscle cell as the target cell? In both cases the answer is in the affirmative (Paper VIII): only conditioned medium from endothelial cells was inhibitory, and only vascular smooth muscle cells were inhibited.

What is the chemical nature of the inhibitory activity? The biochemical properties of the medium conditioned by endothelial cells, in relation to the antiproliferative activity, were established. (Paper IX). The activity was heat stable, protease insensitive, and was also insensitive to hyaluronidase and chondroitin sulfate ABC lyase. However, a highly purified flavobacterial heparinase, which had no protease activity, and which did not degrade chondroitin sulfates, dermatan sulfate or hyaluronic acid, almost completely abolished the inhibitory activity in the endothelial cell conditioned medium. This strongly suggested that the inhibitory activity was due to heparin. A glycosaminoglycan fraction of the medium was also inhibitory, and the activity was heparinase-sensitive. It was, therefore, concluded that endothelial cells produce heparin, or a heparin-like molecule, which was capable of inhibiting the proliferation of vascular smooth muscle.

In Paper IX we show the role of a new type of endoglycosidase (heparitinase) in the release of the heparin from the surface of endothelial cells. In our previous experiments it had become evident to us that the release of inhibitory activity (heparin) from endothelial cells always required the presence of a small amount of serum. This suggested the possibility that a component of serum derived from platelets was involved. This idea was made more cogent by the fact that platelet-free plasma did not release the activity. One candidate for a platelet component was a new type of endoglycosidase (heparitinase) present in the lysosomes of platelets. This had been recently characterized by our colleague, Dr. Robert D. Rosenberg, and his collaborators. (Oosta, G.M., Favreau, L.V., Beeler, D.L. and Rosenberg, R.D. Purification and properties of human platelet heparitinase. J. Biol. Chem. 257:11249-11255, 1982). The enzyme is present in other cells and tissues, as well as platelets.

As shown in Paper IX, treatment of endothelial cells with this heparitinase in buffer or platelet-free plasma effectively released the inhibitory activity. This enzyme differs from the bacterial enzyme in that it cleaves only glucuronosyl-glucosamine linkages, whereas the bacterial enzyme cleaves randomly at the relatively common glucosamine-iduronate bonds within sulfated regions. As a consequence the platelet-derived enzyme differs from the bacterial enzyme in that it yields, from heparin, on the average, dodecasaccharides, which are, as we have shown, inhibitory, whereas the bacterial enzyme yields disaccharides, which are inactive. Thus, the activity released by the platelet-derived heparitinase was completely abolished by subsequent treatment with the bacterial enzyme. We take these results to indicate that platelet-derived heparitinase activity may be necessary, in vivo, for the release of the activity from endothelial cells, and they explain the serum requirement for release of activity found in vitro.
In Paper X we summarize our observations to date.

Coda

The above observations raise interesting questions as to the possible role of heparin or heparin-like compounds in growth regulation in the vascular wall. We have shown that exogenous heparin suppresses the growth of vascular smooth muscle cells in vivo and in vitro, and the endothelial cells produce a similar inhibitory activity in vitro, presumably heparin.

In the normal, uninjured artery, endothelial cells might serve as a source of heparin-like substances. Experiments are now on-going to establish whether smooth muscle cells might play a similar role. Sulfated glycosaminoglycans are found in the arterial wall in both the intima and the media, and are strategically positioned to prevent proliferation. It is possible that smooth muscle and, or, endothelial cells produce the endoglycosidase, as the enzyme has been found in the lysosomes of several cell types (L.V. Favreau and R.D. Rosenberg, personal communication). We would surmise that the enzyme is secreted into the extracellular milieu, where it could function to release antiproliferative heparin-like components from endothelial or other cells.

Upon endothelial injury, platelets would adhere and secrete their products, including platelet factor IV and mitogens. The presence of mitogens alone would not be sufficient to stimulate smooth muscle proliferation: the endogenous antiproliferative heparin must be overcome. This hypothesis is based on our observations that in vitro, even in the presence of large amounts of serum, or platelet extract, heparin in low doses suppresses a proliferative response (see Papers VII and VIII). Suppression by exogenous heparin also occurs under in vivo conditions (see Papers V and VI). There are several possible reasons as to why, after endothelial injury, in the absence of exogenous heparin, proliferation does occur. These are, inter alia:–

(a) damage or loss of the endothelium removes a major source of heparin-like molecules;

(b) endoglycosidase from platelets adhering to the injured area might cleave heparin-like substances into inhibitory fragments, but these could be washed away since the injury has removed the physical barrier to hydraulic flux provided by the endothelium;

(c) the local concentration of mitogens may be so high that smooth muscle cells can proliferate despite the presence of the complex carbohydrates; and

(d) platelet factor 4 could inactive heparin-like molecules.

Once the suppressing action of heparin is negated, the smooth muscle cells can migrate into the intima and proliferate. However, as soon as the damaged area is re-endothelialized, smooth muscle cell growth ceases (Paper I). This could be due to the presence of heparin-like substances released by the endothelial cells behind the regenerating front. The observations of Wight et al. (Wight, T.N., Curwen, K.D., and Minick, C.R. (1982) Am. J. Pathol. in press) that substantial amounts of heparan sulfates accumulate behind the regenerating endothelial front support this suggestion.
The cellular and molecular mechanisms of growth regulation in blood vessels are undoubtedly more complicated than the simple model presented above. However, alterations in the biosynthesis of heparin-like molecules by endothelial cells or other cells, changes in the production or release of endoglycosidases by platelets, smooth muscle cells, or endothelial cells, and alterations in the response of smooth muscle cells to these complex carbohydrates could, in part, be responsible for the initiation of the atherosclerotic process.

Lastly, one would like to know the precise biochemical mechanism whereby heparin produces these antiproliferative effects on vascular smooth muscle. Such an investigation is underway.
LIST OF PAPERS SUBMITTED


Endothelial Regeneration in the Rat Carotid Artery and the Significance of Endothelial Denudation in the Pathogenesis of Myointimal Thickening

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A new model was developed to study endothelial regeneration and the effects of endothelial denudation in large arteries. Complete endothelial loss was produced in a sharply defined, unbranched segment of the rat common carotid artery by brief drying with a gentle steam of air along the lumen of the vessel. Platelets became attached to the denuded surface, but no polymorphonuclear or mononuclear leukocytic adherence or infiltration was observed. Regeneration occurred by the ingrowth, from each end of the denuded segment, of sheets of rapidly dividing endothelial cells. Endothelial replacement was complete by 7 to 10 days after drying. It was also noted that, by 14 days after drying, a striking myointimal thickening developed in the central region of the denuded segment. This central region was the last area to be covered with new endothelium; while still denuded, the region showed edema and signs of cellular proliferation in the innermost medial layer. The myointimal thickening consisted of smooth muscle-like cells in a fibroelastic stroma in the deeper zones, and a layer of rounded, relatively undifferentiated cells in the superficial zones. Progressive regression and condensation of the thickening was observed between 14 days and 3 months.

In this model, the clear demarcation and completeness of endothelial denudation in the arterial segment allow study of the over-all process of endothelial replacement, avoiding the confusion imposed by islands of residual endothelium or contributions from branch vessels. The results suggest that the new endothelium in this model is derived from each end of the segment. In addition, because the method of inducing denudation does not cause significant damage to the underlying media (as shown by the absence of inflammatory cell attachment or infiltration), the model offers a unique opportunity to study the relationship between endothelial loss and changes in the vessel wall. The correlation between the duration of endothelial denudation and the extent of myointimal thickening suggests that sustained insudation of certain, as yet undefined, factors from the lumen may provoke cellular proliferation in the arterial wall. Further investigation of this model should provide information relevant to the pathogenesis of the fibromuscular thickenings of human atherosclerosis.

Additional key words: Atherosclerosis, Smooth muscle cells, Medial edema, Platelets, Scanning electron microscopy, Transmission electron microscopy, Autoradiography.

The origin of the cells responsible for endothelial regeneration following arterial injury is disputed. Although early studies of experimentally induced endothelial lesions indicated that replacement occurs by slow ingrowth of endothelial cells from the periphery of the denuded area (5, 6, 32, 33), later work suggested that blood leukocytes were the source of the new cells (1, 9, 27, 42). More recently, studies of regeneration following injury with a balloon-tip catheter have led to the proposal that redifferentiation of underlying smooth muscle results in the formation of new endothelium (40). Data obtained with this model also suggested that myointimal proliferation occurs in response to loss of the endothelial layer (35, 41), although it is difficult to exclude the possibility that this proliferation was due to medial damage caused by distention with the balloon. The relationship of such myointimal proliferation to that seen in atheromatous lesions has been reviewed by Ross and Glomset (35).

In the present study, we have developed a new model to examine endothelial regeneration in large arteries. The advantages of this model are: (1) endothelial denudation is complete in a defined segment of the vessel; (2) the segment has no side branches from which endothelium could be derived; and (3) injury to the media at the time of denudation is minimal, as shown by an absence of inflammatory cells in the area. Our findings indicate that regeneration can be attributed to rapid endothelial ingrowth from the ends of the injured segment of vessel; in addition, we have obtained clearer evidence that massive intimal thickening can result from endothelial denudation.
MATERIALS AND METHODS

One hundred six male Sprague-Dawley rats (Charles River Breeding Laboratories, North Wilmington, Massachusetts), weighing 250 to 350 gm., were used in this study. Animals were anesthetized with ether and the right common carotid artery was exposed for a distance of 2.5 to 3.0 cm. in the neck. Two ligatures of 4-0 nylon were tightly tied around the artery, separated by a distance of approximately 1.5 cm.: no branches were present along this segment of the vessel. A 30-gauge needle attached to a syringe filled with phosphate-buffered saline was inserted into either the upper or lower end of the isolated segment. After filling the artery with saline, the needle was passed along the lumen, avoiding contact with the vessel wall until the far end of the isolated segment was reached. At this point the needle was allowed to penetrate the vessel wall briefly and was withdrawn back along the lumen to a point just inside the initial point of entry (Fig. 1). All blood was rinsed from inside the isolated segment with saline, following which the syringe was replaced by a regulated source of compressed air. A gentle stream of air (25 ml. per minute) was passed along the lumen of the vessel for 3 minutes to produce drying injury of the endothelium (Fig. 1); this procedure was adapted from the technique used by Ryan, Grobety, and Majno (36) to study injury of peritoneal mesothelium. The segment was then refilled with saline prior to removal of the needle from the vessel. The ligatures were cut with iris scissors and removed. Bleeding stopped after 2 to 3 minutes of gentle pressure at the needle holes with a swab dampened with saline. The skin was closed with 7.5-mm. metal clips and pressure at the needle holes with a swab dampened with saline, the needle was passed along the lumen, avoiding contact with the vessel wall until the far end of the isolated segment was reached. At this point the needle was allowed to penetrate the vessel wall briefly and was withdrawn back along the lumen to a point just inside the initial point of entry (Fig. 1). All blood was rinsed from inside the isolated segment with saline, following which the syringe was replaced by a regulated source of compressed air. A gentle stream of air (25 ml. per minute) was passed along the lumen of the vessel for 3 minutes to produce drying injury of the endothelium (Fig. 1); this procedure was adapted from the technique used by Ryan, Grobety, and Majno (36) to study injury of peritoneal mesothelium. The segment was then refilled with saline prior to removal of the needle from the vessel. The ligatures were cut with iris scissors and removed. Bleeding stopped after 2 to 3 minutes of gentle pressure at the needle holes with a swab dampened with saline. The skin was closed with 7.5-mm. metal clips and washed with 70 per cent alcohol and Mercurochrome.

Animals were sacrificed at various times after surgery, ranging from 1 hour to 3 months. Only five animals subsequently developed thrombosis in the dried segment; these were discarded.

EN FACE SILVER STAINING

The technique of Poole, Sanders, and Florey (32) for staining of intercellular junctions was used to examine regrowth of endothelium at various times after injury. Animals were anesthetized with ether and received intravenous injections of heparin (Liquaemin; Organon, Inc., West Orange, New Jersey). 100 U.S.P. units per 100 gm. of body weight. The abdomen was opened and a retrograde cannula was inserted into the abdominal aorta. After cutting both jugular veins, the animal was subjected to perfusion successively of 100 ml. of a 5 per cent solution of glucose followed by 0.25 per cent silver nitrate (13 ml.), 5 per cent glucose (30 ml.), 3 per cent cobaltous bromide and 1 per cent ammonium bromide (80 ml.), 5 per cent glucose (30 ml.), and 4 per cent formalin (50 ml.). Perfusion was continued with formalin at a pressure of 130 cm. water for 30 minutes. Both carotid arteries were dissected free for a length of approximately 4.5 cm., at least 0.5 cm. beyond the tie points at each end of the dried segment. Each vessel was opened along its length and pinned on dental wax with entomologic pins. Preparations were cleared in glycerol or 1.4-dioxane (both reagent grade: Fisher Scientific Company, Fair Lawn, New Jersey).

Scanning and Transmission Electron Microscopy

Animals were anesthetized with ether and received intravenous injections of 100 U.S.P. units of heparin per 100 gm. body weight. A retrograde cannula was inserted into the abdominal aorta, and both jugular veins were opened to allow flow. Blood was washed from the animal by brief perfusion with Hanks' balanced salt solution (Microbiological Associates, Inc., Bethesda, Maryland), pH 7.3, at room temperature. Perfusion was continued with a mixture of 1 per cent paraformaldehyde (Matheson, Coleman and Bell, East Rutherford, New Jersey) and 1.25 per cent glutaraldehyde (Polysciences, Inc., Warrington, Pennsylvania) in 0.1 m cacodylate buffer (pH 7.3) (Karnovsky's fixative (21), diluted 1:3) at a pressure of 130 cm. water for 15 minutes. The left and right common carotid arteries were removed intact and fixed overnight in 1:1 diluted Karnovsky's fixative (2 per cent paraformaldehyde and 2.5 per cent glutaraldehyde). Each vessel was washed with 0.1 m cacodylate buffer (pH 7.4) by flushing the lumen with a 26-gauge needle on a syringe and four changes of buffer solution before postfixation in 2 per cent osmium tetroxide in 0.1 m cacodylate buffer for 2 hours at 4°C. After washing in buffer, as above, and dehydration in acetone, each vessel was sliced lengthwise in half. One half was left intact and processed for scanning electron microscopy. After drying in a Samdri PVT-3 CO2 critical point drying apparatus (Biodynamics Research Corporation, Rockville, Maryland), the preparation was attached to a metal stub with double sided tape, shadowed with palladium-gold (40:60) in a Ladd vacuum evaporator (Ladd Research Industries, Burlington, Vermont), and examined with an ETEC scanning electron microscope (ETEC Corporation, Hayward, California). The remaining half of each vessel was processed for light and electron microscopy. The complete length was cut into sequential pieces and embedded in Epon 812. Thick sections (2 μm) for light microscopy were cut with glass knives on a Porter-Blum MT-2 ultramicrotome (Ivan Sorval, Inc., Norwalk, Connecticut) and stained with 0.1 per cent toluidine blue and 0.1 per cent sodium borate. Light photomicroscopy was performed using a Zeiss photomicroscope (Carl Zeiss, Inc., New York, New York) with Kodak Panatomic-X film. Thin sections were cut with a diamond knife on an LKB Ultratome III (LKB Instruments, Inc., Bromma, Sweden), mounted on 200-mesh grids, stained with uranyl acetate and lead citrate, and examined at 60 kv. with a Philips 200 electron microscope (Philips Electronic Instruments, Mount Vernon, New York).

Autoradiographic Studies

At times ranging from 3 hours to 2 months after drying of the carotid artery, nine rats received intravenous injections of tritiated thymidine (0.75 μCi per gm. body weight [specific activity, 2 Ci per mmole]; New England Nuclear Corporation, Boston, Massachusetts). One hour later, the animals were killed and the carotid arteries were removed, opened lengthwise, and pinned on dental wax before fixation with 1:1 diluted Karnovsky's fixative. Each preparation was then attached to a slide, endothelium uppermost, with Kaiser's glycerol jelly. En
face autoradiography was performed on these vessels by dipping each slide into 70 per cent Ilford L-4 emulsion (Ilford Ltd., Essex, England) and drying vertically for 30 minutes. After incubation for 27 days, the preparations were treated for 6 minutes in Kodak D-19 developer (15.5 per cent), rinsed briefly in distilled water, and fixed for 8 minutes in 30 per cent sodium thiosulfate.

Another group of three rats was sacrificed 1 hour after a dose of tritiated thymidine at 5, 7, or 10 days after drying. The animals were heparinized and fixed by perfusion with paraformaldehyde-glutaraldehyde as above. Pieces of the carotid arteries were embedded in Epon 812, sectioned for light and electron microscopy, and left unstained. Thick sections on precleaned glass slides were dipped into 50 per cent Ilford L-4 emulsion and dried for 1 hour on a cold metal plate (34). Thin sections on 200-mesh grids were coated with 50 per cent Ilford L-4 emulsion by the loop technique of Hay and Revel (15). After 21 days, light microscopic autoradiographs were developed as above and then air-dried and stained for 30 seconds with 0.1 per cent toluidine blue. Also after 21 days, electron microscopic autoradiographs were developed for 3 minutes in D-19 developer, rinsed in distilled water, and fixed for 1 minute in sodium thiosulfate; the sections were then treated for 10 minutes in 0.1 N sodium hydroxide before staining with uranyl acetate and lead citrate.

RESULTS

En Face Observations of Endothelial Regeneration

Silver Staining. One hour after drying of the carotid artery, the interendothelial silver lines were thickened and irregular. At 3 hours, disruption was more advanced and some areas showed endothelial loss. By 12 hours, the dried segment was totally denuded of endothelium except for a small patch of cells sometimes found between the needle insertion hole and the proximal ligature site. Presumably, this patch of cells was protected from the stream of air by a small drop of saline between the needle and the ligature (Fig. 1). Between 2 and 4 days after drying, a sheet of elongated endothelial cells could be seen spreading upward from the proximal ligature site toward an identical sheet spreading downward from the distal ligature site (Fig. 2). At no stage could cellular outlines be discerned on the bare intervening area. The edges of these advancing sheets of endothelium became confluent near the central region of the dried segment between 7 and 10 days after the injury. By 1 month, the appearance of the silver lines within the segment was indistinguishable from normal.

Scanning Electron Microscopy. The time course and pattern of endothelial loss and regrowth found with silver staining were confirmed by scanning electron microscopy. By 12 hours, the dried area was covered with a carpet of platelets; no endothelial cells were present. Commencing at 2 days after drying, the platelet carpet was progressively replaced from each end by a sheet of endothelium (Figs. 3 and 4). These sheets were in continuity with normal endothelium beyond the ligature sites and, although each spreading edge showed an irregular outline, the cells maintained contact with one another. Isolated cells were not seen on the denuded area. The cells composing the endothelial sheets were elongated along the vessel length, showed nuclear humps, and displayed multiple microvilli (particularly...
over the trailing ends of the cells) (Fig. 5). In addition, at the margins between cells, irregular holes were often found (Fig. 5); these resembled the intercellular "pores" sometimes detected in normal endothelium (Fig. 6). (These pores, the significance of which is unknown, are not to be confused with the much smaller true capillary pores described by physiologists (22).) Polymorphonuclear leukocytes were found at, or adjacent to, the sites directly injured by the ligatures (Fig. 7), but were not seen on the dried area at any stage. After confluence of the endothelial edges between 7 and 10 days, the cells slowly flattened and became normal in appearance by 1 month.

**Endothelial Regeneration and Intimal Thickening in Sectioned Material**

*Histology.* Light microscopy of thick Epon sections (Fig. 8) confirmed the loss of endothelium in the dried segment, the presence of a thin layer of platelets on the denuded surface, and the progressive ingrowth of new endothelium from each end. This new endothelium usually consisted of a single layer of flattened cells (Fig. 7).
but sometimes showed double layering close to the spreading edge. At the ligature sites, there was rupture of the elastic laminae, leaving an ulcer-like crater with a base covered at first with a thin layer of fibrin and infiltrated with polymorphonuclear leukocytes and macrophages, but soon (within 2 to 3 days) covered by endothelium. Apart from the immediate vicinity of the ligature sites, polymorphonuclear leukocytes were never seen along the dried segment, either attached to the surface or infiltrating the media. By 24 hours after drying, the denuded segment developed edema of the innermost medial zone (Fig. 8B). This edema disappeared only when the surface was reendothelialized and was present for at least 7 days in the central region of the dried segment; mitoses were prominent in this residual edematous zone of the media at the end of the 1st week. Normally, the endothelium sits almost directly upon the internal elastic lamina (Fig. 8A). However, by 14 days after drying, massive intimal thickening (to approximately 3 times the thickness of the media) was present.
Fig. 6. Scanning electron micrograph of normal rat carotid artery endothelium. The intercellular borders between individual endothelial cells are characterized by a row of small, irregular projections and sometimes show small "pores" (arrows). Note microvilli (V). X1,100.

mainly in the central region of the denuded segment (Figs. 8D and 9). This thickening consisted of large numbers of cells in a fibroelastic stroma. At later stages, there was a progressive decrease in the number of cells in the thickened zone, along with condensation of the stroma (Fig. 8E and F). By 3 months, the intima was approximately one-third the thickness of the media (Fig. 8G). No such intimal thickening developed in sham-operated animals in which ligatures were applied to the carotid artery for 10 minutes but no drying was performed.

Transmission Electron Microscopy. Examination of thin sections of the denuded area showed that platelets were attached to a thin layer of loosely fibrillar material on the surface of the internal elastic lamina (Figs. 10 and 11). The platelets displayed various degrees of degranulation. No fibrin was detected. The internal elastic lamina appeared normal. At the spreading edge of the endothelial sheet, the cytoplasm was flattened against the surface (Fig. 10) and often extended as a thin veil beyond the cell body (Fig. 11); no platelets were trapped beneath the endothelium. Near the spreading edge, two layers of endothelial cells were sometimes seen. Between adjacent cells, junctional complexes like those in normal carotid endothelium were found (Fig. 10). The new endothelial cells contained many ribosomes, some rough endoplasmic reticulum, scattered microtubules, and abundant microfilaments, but no lysosomal granules. The microfilaments were commonly arranged in dense bundles along the underside of the cell (Fig. 11). Pinocytotic vesicles were present at the cell margins, and microvilli were found at the trailing ends of the cells (Fig. 10).

At the end of the 1st week, many of the cells in the edematous innermost zone of the media were packed with ribosomes and contained relatively few microfilaments (see Fig. 18); conventional smooth muscle cells with well developed bundles of microfilaments and dense bodies were in a minority in this region. At 14 days, the massively thickened intima (see Fig. 9) was packed with cells separated by a fibroelastic stroma (Figs. 12 and 13); collagen fibers and elastin were particularly dense in the deeper zones, i.e., near the internal elastic lamina (Fig. 12). The deep cells were elongated and showed predominantly smooth muscle-like features, with prominent muscles bundles (Fig. 12). In contrast, cells closer to the luminal surface were more rounded and contained large amounts of rough endoplasmic reticulum and relatively few microfilaments (Fig. 13). By 3 months, the intima consisted of a very dense fibroelastic stroma containing mature smooth muscle cells.

Autoradiographic Studies

The pattern of tritiated thymidine labeling in en face preparations at various stages after drying of the carotid artery is shown in Figure 14. Control preparations from normal rats sacrificed 1 hour after the administration of tritiated thymidine showed labeling in only 0.2 to 0.3 per cent of endothelial cells. No increase occurred in the labeling of the endothelium at the ends of the dried segment until 2 days after drying. At this stage, there was extensive labeling in the proximal remnant endothelial cells (between the needle insertion hole and the proximal ligature site) and in a small area above the
Fig. 8. Composite histologic figure illustrating the changes occurring in the wall of the carotid artery after endothelial denudation. To aid comparison of intimal thickness at each stage, the elastic laminae of the media are aligned. The vessel lumen is at the right of each picture. A. Normal vessel showing flat endothelium. B. Central area of the segment, 4 days after denudation, showing loss of endothelium and prominent edema of the innermost zone of the media. C. Newly covered area, 7 days after denudation, showing plump endothelial cells and resolution of the medial edema. D. Central area of the segment, 14 days after denudation, showing the massive extent of myointimal thickening. E. Central area, at 17 days, showing a slight decrease in the thickness of the myointimal layer. F and G. Central area, at 1 and 3 months, respectively, showing progressive condensation of thickened zone. x320.

Fig. 9. Histologic section of myointimal thickening at 14 days after endothelial denudation. Note the elastic fibers between cells of the deeper areas and the somewhat rounded appearance of the cells composing the more superficial zone next to the vessel lumen (top). Electron micrographs of thin sections from this block of tissue are shown in Figure 12 (deep zone) and Figure 13 (superficial zone). x410.

distal tie (Fig. 15). By 7 days, a high proportion (approximately 25 per cent) of cells in the central region was labeled (Fig. 16), with little or no labeling apparent at the ends of the segment. At 14 days, labeling had returned to normal levels.

The presence of endothelial labeling, in cells at or near the advancing edge, was confirmed in sectioned material (Fig. 17). At the end of the 1st week after drying, labeling was also present in cells of the innermost zone of the media, almost exclusively in the edematous central part of the dried segment. These labeled medial cells were packed with ribosomes and rough endoplasmic reticulum but contained relatively few microfilaments (Fig. 18).

DISCUSSION

The earliest experiments on arterial endothelial injury suggested that repair was dependent upon endothelial ingrowth from the periphery of the induced lesion (32, and resolution of the medial edema. D. Central area of the segment, 14 days after denudation, showing the massive extent of myointimal thickening. E. Central area, at 17 days, showing a slight decrease in the thickness of the myointimal layer. F and G. Central area, at 1 and 3 months, respectively, showing progressive condensation of thickened zone. x320.
but the severity of the lesion and the slowness of regeneration (up to 7 months) led others to question the pertinence of the model used. Florey et al. (5, 6) found that Dacron grafts in the abdominal aorta of baboons were more rapidly covered (in less than 10 weeks), apparently from endothelial ingrowth from the ends of the graft and from small vascular channels that developed openings at scattered points along the lumen. On the other hand, studies by other workers suggested that such new endothelium was derived from blood-borne cells (1, 9, 27, 42). Ghani and Tibbs (9) supported this view on the basis of light microscopy of Terylene grafts inserted into dog aortas. DeBakey's group (27, 42) originally came to the same conclusion in studies of the endothelialization of Dacron hubs suspended in the aortic lumen of pigs. However, these workers revised this opinion when they found that the new endothelium which lined impermeable Silastic vascular prostheses appeared to derive from endothelium at the ends of the implant (10). Using a model in which a balloon catheter was inserted, inflated, and withdrawn from the iliac artery of rabbits, Baumgartner and Spaet (1) reported the adhesion of platelets, granulocytes, and mononuclear cells to the denuded surface and suggested that monocytes may form the new endothelium. However, Spaet, Stemerman, and Lejneiks (40) more recently proposed that smooth muscle cells redifferentiate into endothelium in this system. The conflicting results obtained by different groups studying this problem may relate to inherent difficulties of the models used. Thus, the balloon-tip catheter model does not allow denudation of a clearly delineated segment of vessel, and the denudation obtained is often incomplete (35, 41). Furthermore, as indicated by the studies by Stemerman and Ross (41), it appears that significant medial damage is inflicted by the inflated catheter tip, resulting in local adhesion and infiltration of leukocytes in the injured area. It is difficult to isolate the effects of this nonspecific damage on endothelial regeneration or subsequent intimal thickening. In addition, the use of arterial segments with branch vessels may complicate studies of regrowth patterns because endothelium can grow into the damaged area from the mouths of these branches.

These considerations prompted us to develop a new model for the study of endothelial regeneration. A sharply defined length of rat common carotid artery with no side branches was subjected to brief drying with a gentle stream of air along the lumen. Such preparations, approximately 1.5 cm. in length, could then be examined in toto for histology and transmission and scanning electron microscopy, as well as allowing en face preparations of the entire area for silver staining and autoradiography. We found that, soon after drying, the endothelium disappeared completely from the area and was replaced by a carpet of platelets. There was no adhesion or infiltration of inflammatory cells. By 48 hours after drying, the remaining endothelium at the ends of the denuded surface showed signs of proliferation. This resulted in the development of an advancing sheet of endothelium which grew over the denuded area from

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**Fig. 11.** Electron micrograph of a thin endothelial cell veil (E) at the growing edge 4 days after denudation. Note internal elastic lamina (EL), platelets (P), and basally located intracellular bundles of microfilaments (arrow). x10,500.
Fig. 12. Electron micrograph of the deep zone of myointimal thickening shown in Figure 9. Note internal elastic lamina (EL) in the lower right corner, elongated smooth muscle-like cells containing microfilament bundles with dense bodies (arrows), and intercellular elastic tissue (EL). ×7,200.
Fig. 13. Electron micrograph of the superficial zone of myointimal thickening shown in Figure 9. The luminal surface is just beyond the upper left corner. The cells in this region are more rounded and contain far fewer microfilaments than do cells in the deeper zone (seen in Fig. 12). In addition, there are smaller amounts of intercellular elastic tissue (El). × 7,200.
ENDOTHELIAL REGENERATION AND MYOINTIMAL THICKENING

Fig. 14. En face autoradiographic labeling with tritiated thymidine of surface cells at various stages after endothelial denudation. As indicated, the abscissa represents the segment of carotid artery along which autoradiographic counts were performed; note the position of the ligature ("tie") sites. At each time, the bars indicate the number of labeled nuclei counted in successive ×430 microscopic fields along the length of the vessel. Note heavy labeling at each end of the denuded segment at 2 and 4 days and in the central region at 7 days.

Fig. 15. En face autoradiographic labeling of endothelial cells immediately above distal ligature site (L), 2 days after endothelial denudation. At this stage, no labeling is seen in the denuded area (D) to the left of the ligature site. ×200.

Fig. 16. En face autoradiographic labeling of cells in the central region of the previously denuded segment, 7 days after drying. ×850.

Fig. 17. Electron microscopic autoradiograph of an advancing edge 7 days after injury, showing silver grains over the endothelial nucleus. ×6,250.

Fig. 18. Electron microscopic autoradiograph of labeled cell in the innermost zone of the media 7 days after endothelial denudation. The cytoplasm of this cell contains many ribosomes but few microfilaments. ×16,300.

either end of the segment, displacing platelets from the surface. By 7 to 10 days after drying, the two growing edges of actively dividing endothelium had met in the center of the denuded surface and had stopped division by 2 weeks. By 1 month, the endothelial layer appeared to be normal. These findings strongly suggest that the new endothelium in such lesions is derived from endothelial cells at the periphery of the denuded region, although an origin from blood mononuclear cells has not yet been formally excluded. Kinetic studies to clarify this point are in progress.

In these experiments, we also observed the development of striking thickening of the intimal layer between the endothelium and the internal elastic lamina. The greatest thickening occurred in the central region of the segment, that is, in the last area to be recovered by the ingrowth of endothelium. The thickening was maximal at 14 days after denudation, achieving a width approximately 3 times that of the normal vessel wall. After 14
days, the thickness of the intima decreased gradually until, at 3 months after denudation, it was approxi-
mately one-third the width of the media. At 14 days, the
deeper part of this new layer was largely composed of
smooth muscle-like cells separated by collagen and
elastic fibers. The luminal part was made up of rela-
tively undifferentiated cells densely filled with ribo-
somes and very few microfilaments. The number of these
undifferentiated cells decreased rapidly and were rarely
seen at 1 month after injury. By 3 months, the new layer
was highly condensed and was composed of smooth
muscle cells, collagen, and elastic tissue.

The origin of this new myointimal layer is not clear. It
occurred to the greatest degree in the area longest
exposed to plasma protein insudation (as shown by
medial edema) and to platelet adherence. At the end of
the 1st week after denudation, the inner edematous zone
of the media showed many relatively undifferentiated
cells that became labeled with tritiated thymidine:
mitoses also were common in this area. The origin of
these undifferentiated cells, and of the new cells in the
thickened intima at 14 days, has not yet been estab-
lished; in other studies, similar cells have been presumed
to derive from medial smooth muscle (35). It is possible
that stimulatory plasma or platelet factors leak into the
arterial wall, where they trigger cells to proliferate and
migrate into the intima via fenestrations in the elastic
laminae. Such proliferation may be analogous to that of
fibroblasts in granulation tissue, the stimulatory mecha-
nism for which has also not been identified.

Smooth muscle cells are the predominant cellular
component in the focal fibromuscular intimal lesions in
human atherosclerosis (8, 14, 25) and in similar plaques
induced experimentally in animals (7, 29-31, 38, 41, 44-46), including the present model. It has been pro-
posed, as recently reviewed by Ross and Glomset (35),
that such lesions develop as a result of focal proliferation
of smooth muscle cells, followed by intracellular and
extracellular lipid deposition. Other workers have sug-
gested that the primary and crucial event in atherogene-
sis is lipid deposition with or without subsequent intimal
thickening (39, 43). It has been noted, however, that
these two processes can occur independently in most
species (4). Duncan (3) suggested that increased pene-
tration of the plasma proteins into the vessel wall could
result from focal alteration in the permeability proper-
ties of the endothelial lining, presumably due to some
form of injury. Recent studies indicate that, in regions of
increased hemodynamic shearing stress, there is in-
creased histamine synthesis in the endothelial layer and
an increase in vascular permeability (16) which would
allow penetration of plasma factors into the vessel wall.
It has also been demonstrated that sites of endothelial
damage have been associated with a local accumulation
of tracer molecules (such as Evans blue or radioactively
labeled albumin (2, 7, 20, 28, 38)) or subsequent intimal
thickening (7, 12, 26, 31, 35, 41, 46), or both (11, 46). In
addition, experiments by Ross and Glomset (35) demon-
strated the in vitro stimulation of smooth muscle prolif-
eration by low molecular weight plasma lipoproteins.
More recent work by Harker et al. (13) has shown that a
platelet-derived factor caused similar proliferation in
smooth muscle cultures. In related experiments on
induced homocystinuria in baboons, focal loss of endo-
thelium, coupled with the appearance of atheromatous
lesions, was observed (13). Similarly, in man, homocys-
tinuria is associated with myoproliferative intimal le-
sions with intracellular and extracellular lipid deposition
(19, 23, 24). The primary event in this disease is believed
to be focal loss of endothelium, without apparent medial
damage (19, 23, 24). In situations of endothelial damage
induced by hypertension (2, 17, 18) or by "shear forces";
(4, 16) at bifurcations, "intimal pads" characterized by
fibromuscular proliferation are frequently seen with or
without subsequent lipid deposition (4). Flaherty et al.
(4) have noted that the type of lesion varied not only
with age and species but also from vessel to vessel; inci-
dently, these workers pointed out that the common carotid
artery shows relatively little deposition.

In our model, endothelial denudation resulted in
myointimal thickening without lipid deposition in a
localized segment of the common carotid artery in the
rat, an animal not predisposed to arteriosclerosis. This
localized hyperplasia occurred most strikingly in regions
that were exposed to medial edema and surface platelet
adhesion for the longest period of time. It is important to
take that the thickening occurred in response to endo-
thelial denudation rather than to direct physical damage
to the media, that relatively undifferentiated cells were
the only medial cells showing signs of active prolifera-
tion, and that regression of the new myointimal layer
was observed over a period of 3 months. We are currently
using the model to determine the origin and fate of the
cells in the thickened zones and the factors involved in
the persistence of such lesions.

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Absence of Enhanced Intimal Thickening in the Response of the Carotid Arterial Wall to Endothelial Injury in Hypercholesterolemic Rats

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Young male Sprague-Dawley rats fed a high cholesterol, thyroid-suppressive diet were subjected to drying injury of carotid artery endothelium; animals were sacrificed at various times up to 3 months after injury, and the vessels were examined by light, scanning, and transmission electron microscopy. The diet induced marked elevation of serum cholesterol mainly present in lipoproteins of density <1.063. The morphology and degree of intimal thickening in the injured carotids of such animals were compared with the changes found in control groups of normolipemic rats. In the control groups, endothelium was completely regenerated between 7 and 14 days; intimal thickening was present at 14 days and at later stages and contained smooth muscle cells without lipid. In the cholesterol-fed animals, endothelial regeneration and intimal thickening occurred as in the controls with the following additional features: in the zone of intimal thickening in the injured segment, lipid was present in smooth muscle cells and, at later stages, in the extracellular matrix; undifferentiated mononuclear cells were also noted in the thickened intima and, at 3 months, were found adhering to normal and regenerated endothelium. However, no differences were found between control and hypercholesterolemic rats with respect to the degree of intimal thickening within the injured segment; enhancement of the smooth muscle proliferative response was not evident in the hypercholesterolemic rats. Our findings suggest that this form of hypercholesterolemia and its associated hyperlipoproteinemia may not be directly responsible for rat smooth muscle proliferation following endothelial denudation. They also indicate that hyperlipemia does not necessarily cause persistence of myointimal hyperplasia in arteries.

Additional key words: Arteriosclerosis, Lipoproteins, Smooth muscle cell.

Smooth muscle hyperplasia appears to be the principal event leading to intimal thickening following arterial injury (4, 12, 15, 22, 29, 32, 34, 35, 39, 45). However, the growth factors responsible for stimulating smooth muscle cell proliferation have not been clearly identified. Various lipoproteins and platelet factors have been reported to have mitogenic activity for smooth muscle cells in vitro, but not unequivocally in vivo (11, 31, 36, 37). Several in vivo studies have examined the response to endothelial injury in arteries of animals fed a high cholesterol diet. Such diets induce high serum levels of cholesterol mainly present in lipoproteins of D < 1.063 (26, 27, 40, 42). Using a balloon catheter technique, Nam et al. (32) noted enhanced aortic intimal thickening in hypercholesterolemic swine. On the other hand, Hardin, Minick, and Murphy (19) found that hypercholesterolemia did not increase the number of proliferative intimal lesions in rabbit arteries injured by repeated injections of horse serum.

In the present study, we have examined the effect of a high cholesterol diet upon the the smooth muscle proliferative response in the air-dried rat carotid artery (12). In this model, the endothelium is dried by infusing a stream of air along a segment of rat common carotid artery; this results in rapid loss of endothelium in the dried zone. A carpet of platelets quickly covers the denuded area, but no leukocytes are present. Endothelial ingrowth occurs from normal endothelium at the ends of the injured zone, and endothelial replacement is complete between 7 and 10 days after drying. The most striking change in such arteries is noted at 2 weeks after injury: marked myointimal thickening develops in the central region of the injured segment, i.e., in the area which was denuded of endothelium for the longest period of time. The advantages of this technique for studying the role of endothelial loss in intimal thickening are as follows: endothelial loss is complete and occurs over a clearly defined area; the injured segment has no side branches from which endothelium can be derived so that endothelium is regenerated only from sources of normal
endothelium at the ends of the injured segment; the initial injury is limited to the endothelium, with no direct effects detectable upon cells in the media; the lesion is not complicated by infiltration with leukocytes or the development of thrombosis; and the resultant myointimal thickening is highly reproducible. We were particularly interested in using this technique to determine whether the altered blood lipid levels in hypercholesterolemic rats would be associated with exacerbation or unusual persistence of intimal smooth muscle proliferation. Because normolipemic rats show a peak proliferative response at 2 weeks, followed by a progressive decrease in intimal thickness over the ensuing weeks (12), we concentrated upon the changes found in the hyperlipemic animals during the first 3 months after injury. This avoided confusion with the potential long term complications of a high cholesterol diet, such as thrombosis and intramural necrosis, hemorrhage, and calcification (32).

MATERIALS AND METHODS

Animals and Diet

Seventy-four male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., North Wilmington, Massachusetts) weighing 250 to 350 gm. (3 to 4 months old) were used (Table 1). Rats became hypercholesterolemic on a diet (9, 10, 33) containing 4 per cent cholesterol, 1 per cent cholic acid, and 0.5 per cent 2-thiouracil (Sigma Chemical Company, St. Louis, Missouri) mixed with standard stock diet (Purina rat chow, Ralston Purina Company, St. Louis, Missouri) and pelleted at the New England Regional Primate Center, Southboro, Massachusetts. Normal control animals were fed the standard stock diet. Hypothyroid control animals were fed a diet containing 1 per cent cholic acid and 0.5 per cent 2-thiouracil mixed with standard stock diet. Normal control animals gained weight at the rate of 25 gm. per week whereas hypothyroid control and cholesterol-fed animals maintained their weight at a constant level throughout the course of the experiment; the growth curves were similar to those previously published (10).

Lipid and Lipoprotein Analysis

Blood samples were obtained 1 week after commencing the high cholesterol diet and at the time of sacrifice; the blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA) (1 mg. per ml.) and plasma was obtained by centrifugation of the samples at 900 × g for 30 minutes. Total plasma cholesterol and triglyceride levels (expressed in milligrams per 100 milliliters ± standard error) were measured using the Technicon AutoAnalyzer II (Technicon Instruments Corporation, Tarrytown, New York). For lipoprotein studies, plasma was spun in a Beckman preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, California) at varying densities. Density adjustments were made with solid KBr according to standard techniques (24). Plasma from control rats was spun sequentially at D 1.006, D 1.040, D 1.063, and D 1.21. The supernatants were refloated through salt solutions of their own density, isolated, and dialyzed extensively against buffer A (0.15 M NaCl and 0.3 mM EDTA, pH 7.4). Lipoproteins of D <1.006, D 1.006 to D 1.040, and D 1.063 to D 1.21 were isolated and characterized. The turbid hyperlipemic plasma obtained from rats fed the cholesterol rich diet was spun at D 1.063. Under these conditions, the turbid material floated and the isolated supernatant was refloated through a salt solution, D 1.063, reisolated, and dialyzed extensively against buffer A.

Aliquots of whole plasma and isolated lipoproteins from normal and hyperlipemic animals were subjected to agarose electrophoresis at pH 8.6 using the Millipore Panagel electrophoresis system (Millipore Biomedica, Acton, Massachusetts). The lipoproteins were stained with Sudan black.

Experimental Procedure

Air injury of the right carotid artery was performed as described by Fishman, Ryan, and Karmovsky (12) 10 to 14 days after commencing the diets. Animals were sacrificed at each of the following times after injury (Table 1): 2 days, 7 days, 14 days, 1 month, 2 months, and 3 months. Perfusion fixation was performed (12), and both common carotid arteries were removed and fixed for an additional 4 hours in 2 per cent paraformaldehyde and 2.5 per cent glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The arteries were then processed for light microscopy, scanning electron microscopy, and transmission electron microscopy (12). Carotids used for oil red O preparations were cut on a model CTD International Harris cryostat (International Equipment Company, Needham, Massachusetts). Carotids processed for light microscopy and transmission electron microscopy were dehydrated through alcohol after postfixation in 2 per cent osmium tetroxide for 2 hours at room temperature; they were then embedded in paraffin or Epon 812 for longitudinal or seriated cross-sectioning along their entire length. Paraffin sections (6 μm.) were stained with hematoxylin and eosin; Epon sections (2 μm.) were stained with toluidine blue. Maximal plaque thickness (distance from luminal surface to internal elastic lamina) and adjacent media thickness (distance from internal elastic lamina to the most external elastic lamina) were measured with an ocular graticule. Plaque size was expressed as a ratio of plaque to media thickness ± the standard error.

RESULTS

Lipid and Lipoprotein Analysis

Rats on the high cholesterol diet developed marked hypercholesterolemia. At 1 week, the mean plasma cholesterol level in such rats was 910 ± 79 mg. per 100
controls (56 ± 10 mg. per 100 ml., 0.1 < value slightly higher than that found in the normal hypercholesterolemic rats was 79 ± 12 mg. per 100 ml., a hypothyroid control 109 ± 8 mg. per 100 ml., p < 0.001); at sacrifice, the value was 1047 ± 132 mg. per 100 ml.). Lipoprotein electrophoresis showed that normocholesterolemic rat plasma from normal and hypothyroid controls had α-bands and a faint β-band; hypercholesterolemic rat plasma had a normal α-region with a prominent β-band (Fig. 1).

The lipoproteins of D < 1.006, D 1.006 to 1.040, and D 1.063 to 1.21 isolated from the normal control animals had on lipoprotein electrophoresis pre-β-, β-, and α-mobility and had cholesterol to triglyceride ratios of 0.06, 0.47, and 30, respectively. In view of the prominent β-band on lipoprotein electrophoresis hypercholesterolemic plasma was spun at density 1.063. The resultant D < 1.063 fraction migrated as a single β-band and had a cholesterol to triglyceride ratio of approximately 60.

**LIGHT MICROSCOPY**

**Control Animals.** The changes found in the air-dried right carotid arteries of the control normolipemic group of rats were identical with those found by Fishman, Ryan, and Karnovsky (12). At 2 days after endothelial injury, a layer of platelets covered the denuded zone. There was no evidence of cell death in the media, and no polymorphonuclear leukocytes were present in the injured carotids except where the ligatures had been applied. Marked interstitial edema was present in the innermost layer of the media beneath the platelet covering and persisted wherever endothelium was absent. The regenerating endothelial layer grew from normal endothelium at the ends of the injured zone and advanced progressively towards the center of the denuded segment. By 7 days, only a small central area of denudation remained; by 14 days, new endothelium covered the entire zone of injury and appeared normal. Intimal thickening in the midportion of the injured segment developed under the newly regenerated endothelium between 7 and 14 days. Portions of carotid sectioned longitudinally often showed thickening on one side of the artery and minimal or no thickening on the other when both sides of the artery happened to be included in the same section; this asymmetric thickening was confirmed in seriated cross-sections. Injured carotids examined at later stages had variable intimal thickening composed of cells and progressively increasing amounts of extracellular material which stained with toluidine blue; mitoses were seen in the media and intima up to 2 weeks after carotid injury but not afterwards. No differences were seen between normal and hypothyroid control animals at 2 weeks and 1 month. Over the 3-month period, the uninjured left carotid remained unchanged, and in neither right nor left carotids were leukocytes or lipid seen.

**Hypercholesterolemic Animals.** At 2 days and 7 days, injured carotids from hypercholesterolemic rats resembled the controls in respect to the presence of platelets and medial edema in the denuded area. In addition, the rate of endothelial regeneration appeared unchanged. However, unlike the controls, hypercholesterolemic animals had lipid in the media, as shown by staining with oil red O. By 2 weeks, the endothelial layer was reestablished and covered a thickened intima. The intima in hypercholesterolemic animals differed from controls only by the presence of intracellular vacuoles which stained with osmium and oil red O; mitotic figures were observed in cells of the media and intima at 7 days and 2 weeks after injury but not at later times (Fig. 2A and B). Evaluation of rats at 2 weeks, 1 month, 2 months, and 3 months demonstrated considerable variability in the degree of intimal thickening. The mean maximal plaque size (expressed as a ratio of maximal intimal thickness to adjacent medial thickness) was 1.9 at 2 weeks (the equivalent ratio in the normal control animals being 1.4 and hypothyroid controls 1.7); 1.3 at 1 month (normal control 1.1 and hypothyroid control 1.4); 1.0 at 2 months (normal control 1.3); and 1.0 at 3 months (normal control 1.7) (Fig. 3). The maximal plaque size progressively diminished in the cholesterol-fed animals. The extent of longitudinal and circumferential spread of the zone of intimal thickening in such rats, although variable, did not differ significantly from the controls. Plaques examined 1 month and 2 months after injury demonstrated increasing amounts of intracellular lipid (Fig. 2C and D). At 3 months, extracellular crystalline structures also became evident at the base of the thickened intima and in the underlying media (Fig. 2E and F). These accumulations of extracellular lipid were not present in the media proximal or distal to the zone of intimal thickening. Of particular note was the appearance of mononuclear cells adherent to the endothelium of the plaque and in the left carotid artery at 3 months. Such cells sometimes appeared to invade the intima (Fig. 4). Except for the adherent mononuclear cells at 3 months, the left carotids of hypercholesterolemic rats were normal in appearance; no lipid could be demonstrated in the media by oil red O stain, although

![Fig. 1. Lipoprotein electrophoresis of plasma from normal control (left) and cholesterol-fed rats (right). Note the prominent β-band in the plasma of cholesterol-fed animals. The bars mark the origin.](image)
Fig. 2. Composite figure showing histologic changes occurring in the carotid artery of hypercholesterolemic rats after endothelial denudation. The vessel lumen is at the top of each photograph. A, central area of segment, 14 days after denudation, showing marked intimal thickening with lipid-containing cells. B, Higher power view of A showing mitosis. C and D, Central area, at 1 month, showing foam cells in intima and media. E and F, Central area, at 3 months, showing mononuclear cells adhering to the surface, foam cells, and crystalline structures in the intima and media. Note progressive decrease in the thickness of the intima. A, C, and E, ×600; B, D, and F, ×1,500.
occasional small vacuoles were observed in the vessel wall in Epon-embedded material.

Scanning Electron Microscopy

Control Animals. The scanning electron microscopy observations made in control animals at all six stages confirmed the observations made by light microscopy and the scanning electron microscopy results of the previous study (12). The endothelial growing edge seen at 2 days and 7 days advanced steadily and was in continuity with normal endothelium at the ends of the injured segment. The denuded segment was covered with a dense carpet of normal platelets but no leukocytes. By 2 weeks the endothelium was completely regenerated and, thereafter, resembled the normal uninjured endothelium of the left carotid artery.

Hypercholesterolemic Animals. Changes seen in the carotids from hypercholesterolemic animals were the same as those in the normolipemic animals, with two additional features. First, small spherical smooth structures, 0.5 to 1.5 μm. in diameter, were observed adhering to the endothelium of injured and uninjured carotid endothelium after 2 weeks; the nature of these structures is uncertain although they may be lipid particles (Fig. 5A) (46). Second, at 3 months but not before, leukocytes were found attached to the endothelium of both carotids (Figs. 8 and 9) and appeared to be migrating into the intima between adjacent endothelial cells (Fig. 10). These undifferentiated cells contained lysosome-like granules and occasional phagocytic vacuoles; presumably, they were monocytes. In the right carotid, similar cells, sometimes containing lipid, were present in the zone of intimal thickening, intermingled with mature smooth muscle cells (Fig. 8). Also at 3 months, the

Transmission Electron Microscopy

Control Animals. Transmission electron microscopy confirmed the light microscopic findings at each stage. At 2 days and 7 days after injury, a layer of flattened, degranulated platelets was present on the intimal surface. Interstitial edema, but no evidence of cell death, was present in the underlying media. As described previously (12), the advancing endothelial edge consisted of cells containing a prominent band of microfilaments near the abluminal surface, abundant rough endoplasmic reticulum, and relatively fewer pinocytotic vesicles than usually found in endothelium. The proliferating endothelium appeared to grow under and displace platelets as it moved. At 2 weeks, as shown also by scanning electron microscopy, the endothelium formed a complete layer over the thickened intima and resembled the left carotid endothelium. At 2 weeks, the thickened intimal zone contained a mixture of undifferentiated mononuclear cells, and cells with many of the characteristics of smooth muscle cells, i.e., a limiting basement membrane, micropinocytotic vesicles, and bundles of cytoplasmic microfilaments. At later stages, all of the intimal cells appeared to be mature smooth muscle cells; no undifferentiated mononuclear cells were observed after 2 weeks. The stroma showed progressively increasing amounts of collagen and elastin. Fibrin was never seen on the surface or in the intima.

Hypercholesterolemic Animals. Injured hypercholesterolemic rat carotids developed the same ultrastructural changes as normal rats except for findings previously noted by light microscopy: accumulations of intracellular lipid vacuoles and extracellular lipid crystals, and adherent and invading mononuclear cells. At 2 days and 7 days, the platelets and endothelial cells appeared normal whereas many of the medial smooth muscle cells contained lipid vacuoles. At 2 weeks, smooth muscle cells and undifferentiated mononuclear cells (Fig. 6), sometimes containing lipid vacuoles (Fig. 7), were observed in the thickened intima and in the media; occasionally, intracellular lipid vacuoles were seen in endothelium. At 3 months, undifferentiated mononuclear cells were seen attached to the endothelium of both carotids (Figs. 8 and 9) and appeared to be migrating into the intima between adjacent endothelial cells (Fig. 10). These undifferentiated cells contained lysosome-like granules and occasional phagocytic vacuoles; presumably, they were monocytes. In the right carotid, similar cells, sometimes containing lipid, were present in the zone of intimal thickening, intermingled with mature smooth muscle cells (Fig. 8). Also at 3 months, the
Fig. 5. Scanning electron micrographs of the luminal surface of carotids from cholesterol-fed rats at 3 months after injury. A, Mononuclear cells adherent to endothelium in the central segment of the injured right carotid. The smaller, smooth particles may be lipid remnants. B, Mononuclear cell with cell processes extending into intima between adjacent endothelial cells of uninjured left carotid. A, $\times 4,000$; B, $\times 12,500$.

crystalline extracellular material noted by light microscopy at the base of the plaque and in the media appeared as large empty spaces in transmission electron microscopy. As noted in controls, elastin and collagen formed much of the extracellular material; here again, fibrin was not seen.

DISCUSSION

A consistent finding in studies of arterial injury in animals and man has been the development of intimal thickening due to smooth muscle proliferation at sites of endothelial damage (4, 12, 15, 22, 39, 45). However, there has been little clarification of the process by which smooth muscle cells in the media are induced to proliferate. To study this problem, several groups have resorted to cell and organ culture of smooth muscle cells and intima-media strips in an attempt to identify growth-promoting factors. Myasnikov and Block (31) and Florentin et al. (13) have reported that hyperlipemic serum stimulated cellular outgrowth from intima-media strips. Fisher-Dzoga, Chen, and Wissler (11) found that lipoproteins with $D_1 1.019$ to 1.063 from hyperlipemic monkeys accelerated cellular outgrowth from medial explants whereas the same fraction from normolipemic animals did not affect the growth rate. On the other hand, Ross and Glomset (36) have identified both low density and high density lipoproteins from normolipemic animals as growth factors in primate smooth muscle cell cultures. Further work by this group has shown that platelets and insulin may be important contributors to the pool of necessary growth factors (37, 47). Other investigators have identified platelet products (25) and thrombin (6) as growth factors for fibroblasts.

Although a number of potential growth factors have been identified in vitro there is little evidence that any of them specifically provokes smooth muscle proliferation in vivo. In the present study we have examined the effect of a high cholesterol diet upon myointimal proliferation in the air-dried rat carotid artery model. Hypercholesterolemic and control rats both developed centrally located zones of intimal thickening in the air-injured segment. The hypercholesterolemic animals showed progressive accumulation of intracellular and extracellular lipid in the plaques, and, at later stages, the presence of mononuclear cells adherent to the endothelial layer and invading the intima. However, we found that, over the course of 3 months, the high cholesterol diet did not significantly affect the arterial smooth muscle proliferative response; that is, no differences were noted in the number of medial and intimal mitoses or in the maximal plaque size in hypercholesterolemic versus normocholesterolemic rats. Thus, in this model, cholesterol feeding and the attendant hypercholesterolemia and hyperlipoproteinemia did not enhance or prolong smooth muscle proliferation.

A number of investigators have examined the effects of high cholesterol diets on the arterial response to endothelial injury in vivo. In general, however, these studies have not focussed attention upon the problem of smooth muscle proliferation. Nam et al. (32) found that the combination of aortic endothelial injury using the balloon catheter technique and cholesterol feeding in swine produced thicker zones of intimal proliferation by 3 months than endothelial injury alone. After 3 months this effect was even more pronounced. In addition, these animals showed extensive lipid-filled lesions with necro-
Fig. 6. Transmission electron micrograph of intimal thickening in central segment of right carotid of a hypercholesterolemic rat 2 weeks after injury. Note the undifferentiated mononuclear cell (M) lacking the basement membrane and dense zones of myofilaments characteristic of mature smooth muscle cells (SM). Unlike controls, smooth muscle cells in cholesterol-fed animals show large accumulations of intracellular lipid in vacuoles (V). ×8,000.

Fig. 7. Transmission electron micrograph of undifferentiated mononuclear cell (M) found in the myointimal thickening of a hypercholesterolemic rat 2 weeks after injury. Note the intracellular accumulation of lipid. ×10,000.
Fig. 8. Transmission electron micrograph of intimal thickening in the carotid of a cholesterol-fed rat at 3 months. Note particularly the presence of mononuclear cells (M) in the intima and on the luminal face adherent to the endothelium (E). These cells are found in the myointimal thickening of right carotids of control animals at 2 weeks, but not at 3 months, after injury. Note smooth muscle cells (SM) massively engorged with lipid vacuoles. x9,100.
sis, hemorrhage, calcification, and superimposed thrombosis. These long term results may not be due to a direct growth-promoting effect of cholesterol and lipoproteins on the smooth muscle cell; rather, they may represent a secondary effect due to lipid-induced endothelial injury, perhaps of an inflammatory type, with subsequent exposure of smooth muscle to other growth-promoting factors. In contrast, Hardin, Minick, and Murphy (19) studied a model of endothelial injury produced by repeated injections of horse serum into rabbits fed a normal or high cholesterol diet and found the same number of proliferative lesions in each group. Interestingly, Wissler et al. (49) found that coronary proliferative lesions were less common in hypertensive rats on a cholesterol-rich diet than in hypertensive rats on a low cholesterol diet. In other studies on the effect of a high cholesterol diet on arteries subjected to endothelial injury (7, 14, 35, 38, 48), the accumulation of intracellular and extracellular lipid in the thickened intima has been reported, but no data were provided concerning the rate or the extent of the smooth muscle proliferative response.

In our studies, the development of intimal thickening appeared to be related to the length of time that the media was exposed. We have found that if the carotid segment subjected to injury is too short (1 cm. or less) intimal thickening does not occur; in such cases, endothelial replacement is complete within 5 to 7 days after injury (A. W. Clowes, G. B. Ryan, J. L. Breslow, and M. J. Karnovsky, unpublished results). This suggests that in this model, mitogenic factors take several days to activate myointimal proliferation. Although hypercholesterolemia did not appear to stimulate growth, other factors that could leak into the vessel wall during the period of endothelial loss and stimulate growth are thrombin and platelet degradation products (6, 25, 37). Thrombin has growth-promoting activity for fibroblasts in culture (6). However, if clotting factors were responsible for stimulating smooth muscle proliferation in the injured carotid artery, we might expect to find significant accumulations of fibrin on the denuded surface. This was not seen. On the other hand, platelets were observed to accumulate on the injured carotid surface, and in view of recent in vitro work (37), they may be responsible for instigating the smooth muscle hyperplastic response. In support of this hypothesis, certain drugs which inhibit platelet aggregation also appear to prevent intimal thickening after endothelial injury (23).

In agreement with others (12, 17, 34, 45), we have found that smooth muscle cells predominate in the thickened intima. Additional undifferentiated mononuclear cells lacking smooth muscle cell characteristics were present in the plaques of normal and hypercholesterolemic animals at 2 weeks; such cells may be undifferentiated smooth muscle cells (12, 17, 18). However, mononuclear cells were also seen in the intima at later stages in hypercholesterolemic animals, but not controls. Because such cells resembled the monocytes (3, 28, 46)

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**Fig. 9.** Transmission electron micrograph showing mononuclear cells (M) adherent to endothelium (E) and invading the intima of an uninjured left carotid from a hypercholesterolemic animal at 3 months. Lumen (L) is at top and internal elastic lamina (IEL) at bottom of photograph. ×9,100.
attached to the endothelial surface in these animals, they may be bone marrow-derived mononuclear phagocytes. In support of this, Spraragen et al. (41) have shown that exogenous radioactively labeled macrophages, when injected intravenously into hyperlipemic rabbits, not only adhere to the endothelium but also invade the intima.

The accumulation of intracellular lipid and, at later stages, extracellular lipid crystals in the air-dried carotid artery distinguished the injured carotids of hypercholesterolemic rats from those of control animals. The origin of this lipid is unclear, although it is probably due to continued insudation from the blood (21, 44). Significant endogenous cell synthesis of cholesterol is unlikely in view of work by Breslow et al. (5) showing that the \( D < 1.063 \) lipoproteins from hypercholesterolemic rat plasma is a potent suppressor of cholesterol biosynthesis in rat hepatocytes and fibroblasts in culture (reference 5 and A. W. Clowes, G. B. Ryan, J. L. Breslow, and M. J. Karnovsky, unpublished results). Cell death may make a contribution to the extracellular lipid deposits; that smooth muscle cells become foam cells and then lyse as cholesterol crystals form liberating lipid into the extracellular tissues is an hypothesis that has had some support (8, 16, 20, 43). The extracellular crystals seen at 3 months resemble the crystals of cholesterol and...
cholesterol ester in subcutaneous implants studied by Abdulla, Adams, and Morgan (1). They reported that in rats, subcutaneous implants of cholesterol, free fatty acids, and satu-rated and mono-unsaturated cholesterol esters were slowly resorbed and induced a macrophage response, followed by fibroblastic activity and collagen deposition. In our study the presence of adherent monocytes and intimal macrophages at 3 months may signify the onset of a similar sclerogenic response to lipid crystals in the arterial wall; continued influx of the monocytes into the intima through endothelial gaps may also be the result of a low grade, chronic endothelial injury in part caused by the hypercholesterolemic plasma (2, 30). Such a response might lead to renewed smooth muscle cell stimulation and intimal thickening in animals examined after 3 months; long term experiments are now in progress to examine this hypothesis.

In conclusion, although cholesterol feeding has been implicated in the formation of proliferative lesions in rats (9, 10, 49) we found, on the contrary, that hypercholesterolemia did not exacerbate or prolong the intimal hyperplastic response in rats subjected to carotid artery endothelial injury. This implies that the association of hypercholesterolemia with arterial intimal thickening may result from a secondary tissue response to the intimal deposition of lipids rather than a direct mitogenic effect of lipoproteins of D < 1.063 or cholesterol on smooth muscle cells.

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INTIMAL THICKENING IN HYPERCHOLESTEROLEMIC RATS


Regression of Myointimal Thickening following Carotid Endothelial Injury and Development of Aortic Foam Cell Lesions in Long Term Hypercholesterolemic Rats

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In an earlier report (Clowes AW, Ryan GB, Breslow JL, Karnovsky MJ: Lab Invest 35:6, 1976) we demonstrated that cholesterol feeding of rats led to hypercholesterolemia but no increase in smooth muscle cell (SMC) proliferation in right carotid arteries subjected to a standard endothelial injury when compared with normolipemic control animals. We have now examined these plaques at 6 months and 1 year after injury. In control animals, the carotid intimal thickening regressed to a relatively small, acellular, fibrous scar; there was no evidence of renewed endothelial injury and secondary SMC proliferation. Regression of the intimal thickening in the injured carotids of cholesterol-fed animals proceeded exactly as in control animals except for the accumulation of lipid. Unlike control animals, cholesterol-fed rats developed aortic intimal lesions containing extracellular lipid crystals and lipid-laden macrophages derived from mononuclear phagocytes in the blood. In addition to the lack of continued intimal SMC proliferation in the injured carotid, in the face of severe hypercholesterolemia the intima of the aorta did not contain mature SMC, or SMC-derived collagen and elastin. There was also no evidence of increased permeability to Evans blue, injected intravascularly. These findings suggest that hypercholesterolemia in the rat does not produce chronic endothelial injury, development of proliferative fibrous plaques, or enhancement of established SMC lesions.

Additional key words: Smooth muscle cell, Arteriosclerosis.

In a previous report (4) we addressed ourselves to the proposition that cholesterol feeding of rats might lead to enhanced intimal thickening in carotid arteries subjected to endothelial injury; the associated hypercholesterolemia and hyperlipoproteinemia might be expected to stimulate increased smooth muscle cell proliferation because in vitro studies had shown low density lipoproteins to be mitogens for smooth muscle cells (7, 22). Increased thickening of the intima was not observed in the injured carotids of hypercholesterolemic animals, and, therefore, we concluded that in this model hypercholesterolemia and the associated hyperlipoproteinemia induced by cholesterol feeding were not important growth factors for rat smooth muscle cells. However, because monocytic cells were observed invading the intima of injured and uninjured arteries in hypercholesterolemic animals, we surmised that cholesterol feeding might induce chronic arterial endothelial injury leading to recrudescence of intimal cellular proliferation at a later stage. In support of this hypothesis Nam et al. (19) have shown that 3 months or more after injury the aortas of cholesterol-fed swine show complex lesions containing not only massive intimal thickening and lipid accumulations but also hemorrhage, thrombosis, necrosis, and calcification.

In the current study, the original injury was produced by infusing a stream of air along the lumen of an isolated 1.5-cm. segment of right common carotid artery. As was shown previously (4, 8), after blood flow was restored the desiccated endothelium was washed away and was replaced by a carpet of platelets. Endothelial regeneration proceeded from normal sources of endothelium at the ends of the injured segment and was complete between 7 and 14 days. In the central zone of the injured artery, marked intimal thickening due to cholesterol feeding were not important growth factors for rat smooth muscle cells. However, because monocytic cells were observed invading the intima of injured and uninjured arteries in hypercholesterolemic animals, we surmised that cholesterol feeding might induce chronic arterial endothelial injury leading to recrudescence of intimal cellular proliferation at a later stage. In support of this hypothesis Nam et al. (19) have shown that 3 months or more after injury the aortas of cholesterol-fed swine show complex lesions containing not only massive intimal thickening and lipid accumulations but also hemorrhage, thrombosis, necrosis, and calcification.

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In this long term study we have examined the right carotid arteries of hypercholesterolemic rats 6 months and 1 year after endothelial injury. To assess the devel-
opment of arteriosclerotic lesions, the changes in the intima of the uninjured left carotid artery and the thoracic aorta have been compared in ultrastructural studies with the changes in the intima of the injured right carotid artery. Permeability changes have been assessed using the dye Evans blue as a tracer for albumin (20). Our data demonstrate that during long term exposure to hypercholesterolemia, injury-induced proliferative lesions in the carotid artery regress, whereas aortic foam cell lesions, devoid of a proliferative component, develop.

MATERIALS AND METHODS

Animals and Diet

Thirty-nine male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., North Wilmington, Massachusetts) weighing between 300 and 350 gm. were used. Experimental animals became hypercholesterolemic on a diet containing 4 per cent cholesterol, 1 per cent cholic acid, and 0.9 per cent 2-thiouracil (Sigma Chemical Company, St. Louis, Missouri) mixed with standard stock diet (Purina rat chow, Ralston Purina Company, St. Louis, Missouri) and pelleted at the New England Primate Center, Southborough, Massachusetts. Hypothyroid control animals received a diet containing 1 per cent cholic acid and 0.5 per cent 2-thiouracil; normal control animals were fed the stock diet. As noted previously (4), normal control rats gained weight steadily during the period of observation whereas hypothyroid control and cholesterol-fed rats maintained their weights unchanged.

Lipid and Lipoprotein Analysis

Blood samples in ethylenediaminetetraacetic acid (1 mg. per ml.) were obtained before starting the diet and 7 days after and at the time of sacrifice. Plasma cholesterol, triglyceride, and lipoprotein determinations were made as described previously (4).

Experimental Procedure

Right carotid endothelial injury was performed as described by Fishman, Ryan, and Karnovsky (8). In brief, after 10 days on the diet animals were anesthetized with ether and the right common carotid artery was exposed through a longitudinal incision. The endo-arterial injury was induced in an isolated 1.5-cm. segment of common carotid artery by an infusion of air through a needle cannula at 40 to 50 ml. per minute for 3.5 minutes. The wound was closed with skin clips after blood flow was reestablished and hemostasis obtained. Animals were sacrificed at 6 months and 12 months, after an overnight fast (Table 1). Three hours before sacrifice, blood samples were taken and Evans blue dye as a tracer for albumin (60 mg. per kg.). Perfusion fixation was performed through an abdominal aortic cannula as described by Fishman, Ryan, and Karnovsky (8). After 10 minutes of perfusion with 1 per cent paraformaldehyde and 1.25 per cent glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, the carotid arteries and thoracic aortas were dissected free and examined immediately for blue stain. These vessels were excised and placed in 2 per cent paraformaldehyde and 2.5 per cent glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 4 hours. Subsequently, the vessels were washed with buffer, postfixed with 2 per cent aqueous osmium tetroxide for 2 hours, and stained with uranyl acetate en bloc. Vessels were then dehydrated through graded solutions of alcohol and processed for light, scanning, and transmission electron microscopy as described earlier (4, 8). Carotid arteries and aorta were embedded in Epon 812 for longitudinal or seriated cross-sectioning at 500-µm. stages (2-µm. sections). The plaque size in the injured right carotid was measured as described earlier (4) by taking the ratio of the intimal thickness (luminal surface to internal elastic lamina) to the adjacent media thickness (internal elastic lamina to most external elastic lamina). The maximal plaque size was taken as an index of intimal cell proliferation.

RESULTS

Lipid and Lipoprotein Analysis

Rats developed marked hypercholesterolemia after 1 week of cholesterol feeding, as noted earlier (4). At sacrifice cholesterol-fed animals had mean plasma cholesterol levels of 1085 ± 84 mg. per 100 ml. (normolipemic control, 92 ± 11 mg. per 100 ml.; hypothyroid control, 143 ± 11 mg. per 100 ml.) and mean triglyceride levels of 50 ± 11 mg. per 100 ml. (normolipemic controls, 148 ± 15 mg. per 100 ml.; hypothyroid controls, 53 ± 15 mg. per 100 ml.). Lipoprotein electrophoresis was unchanged from previous work (4); Normocholesterolemic rat plasma had a-bands and a faint pre-β-band whereas hypercholesterolemic plasma had a normal a-region and a prominent β-band.

Permeability Studies

Neither control nor cholesterol-fed rats showed Evans blue stain in the injured right carotid artery, the left carotid artery, or the thoracic aorta at 6 months or 12 months.

Light Microscopy

Control Animals. The air-dried right carotid arteries of normal and hypothyroid control animals examined at 6 months and 12 months after injury showed residual intimal thickening composed of two or three layers of cells and large amounts of toluidine-stained extracellular material (Fig. 1A). As observed earlier (4), the inti-
Immunohistochemical findings were consistent with the morphological changes seen in cryostat sections. The maximal plaque size at 6 months was 0.9 (hypothyroid control, 0.7) and at 12 months was 0.8 (Fig. 2); these values do not differ significantly from similar measurements at 3 months (4). Uninjured right and left carotid arteries and aortas were unchanged in appearance; no spontaneous intimal thickening or accumulation of surface mononuclear cells was observed (Fig. 1C).

Hypercholesterolemic Animals. At 6 months, the thickening intimas of injured carotid arteries of cholesterol-fed rats resembled those examined at 3 months and were composed of lipid-containing cells, extracellular lipid crystals, and large amounts of toluidine-stained extracellular material (Fig. 1B). At 12 months, the number of lipid-containing cells was diminished; at this stage the intimal thickening in hypercholesterolemic and control injured carotid arteries was similar in size. In hypercholesterolemic rats, the maximal plaque size was 0.7 at 6 months and 1.0 at 12 months and did not differ significantly from control animals (Fig. 2). These measurements were not significantly different from measurements made at 3 months (1.0) (4). Mitotic figures, previously seen at 2 weeks after injury, were not present in the thickened intima at 6 or 12 months. There was no evidence of necrosis, hemorrhage, calcification, or thrombosis. The endothelial cells of the carotid arteries and the aorta contained small lipid vacuoles and occasional adherent mononuclear cells (4). However, uninjured carotid arteries did not develop lipid accumulations or intimal thickening. Thoracic aortas from hypercholesterolemic animals showed striking changes in the intima and first layer of the media (Fig. 1D) at 12 months and occasionally at 6 months. Intimal thickening due to large foam cells and extracellular lipid crystals was prominent. In addition, large numbers of mononuclear cells were adherent to the endothelium at sites of foam cell accumulation. The majority of the adherent cells contained little or no lipid; however,
occasional adherent cells approximately twice the size of the others and containing many lipid vacuoles were noted. These aortic lesions lacked the large amounts of toluidine-stained extracellular material seen in the injured right carotid arteries. In one 6-month rat with the highest cholesterol measured (1905 mg. per 100 ml.), an enormous plaque (maximal plaque size 7.0) containing actively dividing smooth muscle cells was found (Fig. 3). However, the aorta from this animal contained only foam cells.

SCANNING ELECTRON MICROSCOPY

Control Animals. The scanning electron microscopy (SEM) observations in control animals at 6 and 12 months confirmed the observations made by light microscopy and the SEM results at 3 months (4). In addition, the central part of many endothelial cells was noted to protrude and to be irregular. The appearance of carotid and aortic endothelium was similar in normal and hypothyroid control rats.

Hypercholesterolemic Animals. The overall appearance of arterial endothelium in hypercholesterolemic rats was similar to that of control animals, but with several important exceptions (Figs. 4A and B and 5A): (1) All of the vascular endothelium in hypercholesterolemic rats contained multiple small protuberances previously noted to be intracellular vacuoles by light microscopy. (2) The luminal surface of the injured and uninjured carotids had only occasional adherent cells; however, the aortas demonstrated tremendous numbers of cells adherent to focal raised lesions made up of endothelium draped over bulbous structures (foam cells). There appeared to be two types of adherent cell; the more numerous smaller cells were approximately 4 to 6 μm. in diameter whereas the less common cells (1 to 2 per cent of adherent cells) measured approximately 7 to 12 μm. in diameter (Fig. 5B). No difference in function could be demonstrated for these cells (3). Finally, the amorphous particles, measuring 0.5 to 1.5 μm. in diameter, were present, as noted earlier (4). There was no evidence of thrombosis or endothelial cell loss.

TRANSMISSION ELECTRON MICROSCOPY

Control Animals. Observations by transmission electron microscopy confirmed the results of SEM and light microscopy at 6 months and 1 year. The intimal thickening of the injured right carotid was sparsely populated with cells and was composed largely of elastin and collagen. An unbroken endothelial layer formed the luminal surface. Associated with the endothelium was a single underlying layer of mature smooth muscle cells. No other cell types or lipid were noted. Uninjured carotid arteries and aorta had only a simple endothelial lining.

Hypercholesterolemic Animals. By transmission electron microscopy, the injured right carotid artery in hypercholesterolemic animals appeared remarkably similar to that in control animals. The intimal thickening contained proteoglycan, elastin, collagen and lipid, and very few cells. The surface endothelial layer was complete and showed no evidence of injury. Unlike control arteries, small lipid vacuoles were present in the endothelium and large intracellular and extracellular lipid.
Fig. 4. A and B. Scanning electron micrographs of the luminal surface of thoracic aortas in 12-month hypercholesterolemic rats. A, Low power view shows thoracic aorta at the origin of the innominate artery; note cobblestone appearance of surface and adherent cells. B, Lower power view shows foam cell lesions around the orifice of the intercostal artery. Figure 4A, ×90; B, ×400.

Fig. 5. A and D. Scanning electron micrographs of lesions in thoracic aortas from 12-month hypercholesterolemic rats. A, Discrete foam cell lesion is shown in ascending thoracic aorta. B, High power view demonstrates two types of adherent cells, amorphous particles, and prominent endothelial vacuoles. Figure 5A, ×300; B, ×2,400.
accumulations were present in the thickened intima and media. In the intima, mature smooth muscle cells and undifferentiated mononuclear cells were noted as in previous experiments (4). By comparison, the lesions found in the aortic arch contained massive lipid-containing mononuclear cells with little surrounding extracellular elastin and collagen. In every instance an overlying attenuated endothelial layer could be distinguished (Fig. 6). Mononuclear cells, occasionally replete with lipid (Figs. 7 and 8), were adherent to the luminal surface of the endothelium and appeared to invade the intima. No mature smooth muscle cells could be identified in these aortic lesions.

DISCUSSION

In short term experiments (4), we demonstrated that hypercholesterolemia and hyperlipoproteinemia in the rat did not directly affect smooth muscle cell hyperplasia in the intima of injured carotid arteries; we suggested that in long term experiments cholesterol feeding might produce chronic endothelial injury leading to renewed intimal cellular hyperplasia and possibly the development of proliferative lesions.

Our long term experiments indicate that significant smooth muscle hyperplasia did not recur in arteries of hypercholesterolemic animals subjected to endothelial injury, except in one case. Instead, the arterial intima became slowly and progressively more fibrotic and less cellular. Furthermore, no chronic endothelial damage was noted. Hardin, Minick, and Murphy (14) have described similar results in a rabbit model of coronary artery endothelial injury induced by immunologic means. They reported that injured rabbits, fed either a normal or a high cholesterol diet, developed the same number of coronary artery intimal fibromuscular lesions; they did not quantitate the size of these lesions. These results are in striking contrast to those of Nam et al. (19); they observed continued intimal thickening and the formation of complex lesions containing lipid, calcification, hemorrhage, necrosis, and thrombosis in the balloon-injured aortas of swine that were fed cholesterol for 3 months or more. The differences in these results may be ascribed to variations in permeability of normal arterial endothelium in different animals, the location of the initial endothelial injury, and the endothelial response to hypercholesterolemia. For example, Fried-

Fig. 6. Transmission electron micrograph demonstrating intimal foam cells (F) in the thoracic aorta of a 6-month hypercholesterolemic rat. Note continuous layer of endothelium (E) at luminal surface and absence of mature smooth muscle cells in the intima. Lumen (L) is at top. ×7,400.
Man and Byers (9) showed that Evans blue stained normal rabbit aorta in discrete locations but did not stain normal rat aorta. They also showed that the proliferative lesions occurred at the sites of staining in cholesterol-fed rabbits. Similarly, Packham et al. (20) and Fry (10) have shown blue stain and intimal cellular proliferation at vessel orifices in normal pigs and dogs. Fry (10) has emphasized that increased endothelial permeability to Evans blue may be found at sites of unstable stress (flow dividers such as vessel orifices) but not in locations of moderate and continuous stress (straight arterial segments). The results in the pig aorta model may be explained as follows: the marked intimal thickening is the result of mechanical injury to an endothelial layer that even in its natural state around the vessel orifices is permeable to albumin; furthermore, following balloon injury, the hypercholesterolemia may retard endothelial regeneration at sites of perturbed blood flow if, as in the baboon (22), hypercholesterolemia is actually injurious to endothelium. Lacking a proper endothelial barrier, the aorta would then be exposed continuously to an influx of albumin and other blood components. In contrast, normal rat arterial endothelium in the common carotid artery is not permeable to albumin, is not subjected to unstable stress, and appears to be unaffected by hypercholesterolemia. Hence, after an initial injury, carotid endothelium in cholesterol-fed rats would be expected to regenerate unimpeded and to reestablish a stable, relatively impermeable barrier. We have shown that in the first 2 weeks after right common carotid endothelial injury, but not later, Evans blue staining occurs in areas still lacking endothelium (A. W. Clowes and M. J. Karnovsky, unpublished observations); and Björkerud and Bondjers (3) have reported that in rabbit arteries subjected to extensive injury, Evans blue staining is present at sites of delayed endothelial regeneration. We conclude that there has been further injury and perhaps loss of endothelium in the previously injured right carotid, Evans blue staining would have been present. As well as the species differences in endothelium, there may also be differences in smooth muscle cell behavior in response to hypercholesterolemia. For example, hyperlipemic low density lipoproteins have been reported to be mitogenic for rabbit smooth muscle cells (7). These lipoproteins might not be mitogenic for rat smooth cells as suggested in our earlier report (4). This possibility is supported by the recent observations that rat fibroblasts and probably rat smooth muscle cell membranes lack high affinity receptors for low density lipoproteins (J. L. Breslow, unpublished observations); these receptors have been demonstrated on human fibroblast and smooth muscle cell membranes (12) and may be present on cell membranes of other animal species.

Apart from the regression of injury-induced proliferative lesions, we have observed the development of foam cell lesions in the aortas of rats fed cholesterol for 6 months and 1 year. These lesions contained large lipid-filled cells and extracellular cholesterol crystals and were often associated with adherent luminal mononuclear cells. Characteristic smooth muscle cells and their matrix of proteoglycans (29), collagen, and elastin were notably absent in these lesions; thus, foam cells in these lesions were probably macrophages and were derived from luminal mononuclear cells. This conclusion is in agreement with the work of others (1, 2, 18, 25) and in particular Sprarragen et al. (23).

There has been some discrepancy between the descriptions of the lesions found in rats on atherogenic diets. Although Page and Brown (21) tried a variety of dietary manipulations, they were unable to induce atherosclerosis in the rat; some studies have reported only foam cell lesions (2, 26) whereas others have observed primarily proliferative lesions (6, 13, 17, 27, 28, 30). For the production of atherosclerosis in rats, emphasis has been placed on age, obesity, hypertension, and source of noncholesterol dietary fat. For example, Wexler, Anthony, and Kittinger (28) have demonstrated that proliferative intimal lesions occur spontaneously in retired breeder rats and Koletsky (16) has developed an obese, hyperphagic, hypertensive, and arteriosclerotic rat by cross-breeding. Our results demonstrate that the foam cell plaque is the dominant lesion in rat aortic arteriosclerosis induced by feeding cholesterol, thiouracil, and choleric acid; foam cell plaque develops as a result of the
Fig. 8. Transmission electron micrograph demonstrating mononuclear cell (M) without lipid vacuoles inserted between two adjacent endothelial cells (E) in the thoracic aorta from a hypercholesterolemic rat at 6 months. This cell corresponds in size to the smaller nuclear cell (N) without lipid vacuoles inserted between two adherent cells (Fig. 5) seen by SEM. Arrows mark the limits of the endothelial cells. Lumen (L) is at the top. ×18,200.

migration of lipid-containing mononuclear cells into the intima. In view of their initial size, these tissue macrophages must continue to accumulate lipid; as suggested by others (5, 11, 15, 24) cholesterol may be liberated into extracellular compartments by cell death and rupture.

We conclude that cholesterol feeding in hypothyroid rats produces marked hypercholesterolemia which, in time, leads to the accumulation of lipid in arterial walls. The lipid deposition is associated with the migration of mononuclear cells into the intima to form the foam cell lesion. The absence of smooth muscle cells, smooth muscle cell excretory products, or increased endothelial permeability suggests that hypercholesterolemia does not produce the chronic endothelial injury needed for the formation of the proliferative fibrous plaque. Furthermore, the regression and scarring of smooth muscle cell intimal thickening in arteries previously subjected to endothelial injury indicates that hypercholesterolemia in the rat does not enhance or sustain established proliferative fibrous lesions.

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Failure of Certain Antiplatelet Drugs to Affect Myointimal Thickening following Arterial Endothelial Injury in the Rat

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The effect of aspirin, reserpine, and flurbiprofen on in vivo platelet function and intimal smooth muscle cell hyperplasia in rat carotid arteries subjected to endothelial injury was investigated and related to the effect of these drugs on in vitro platelet aggregation. Endothelial injury was achieved by infusing air briefly into a segment of right common carotid artery. Beginning before or after surgery, experimental animals were given sufficient drug to suppress platelet aggregation in vitro in response to collagen, adenosine diphosphate, or thrombin. The carotid arteries were fixed by perfusion at 5 and 14 days after injury and examined by light, scanning electron, and transmission electron microscopy for platelet activity and intimal smooth muscle cell proliferation in the denuded segment. Platelets in platelet-rich plasma from control animals aggregated in response to collagen, adenosine diphosphate, and thrombin; platelets from aspirin-, flurbiprofen- and reserpine-treated rats showed markedly diminished aggregation in response to collagen and normal or slightly diminished aggregation in response to ADP and thrombin. At 5 days, platelets from control animals formed a dense layer in the denuded segment; at 14 days, marked intimal thickening due to smooth muscle cell hyperplasia was observed. In experimental animals, the platelets were morphologically identical with controls and covered the denuded segment; serotonin granules were missing in platelets of reserpine-treated rats. Intimal thickening at 14 days was the same as controls. We conclude that in the rat no correlation may be made between the effect of aspirin, reserpine, and flurbiprofen on in vitro platelet aggregation and the effect of these drugs on the function of platelets on an arterial wall denuded of endothelium, as judged by morphology; furthermore, even when these drugs are used in sufficient dose to inhibit in vitro aggregation of platelets, myointimal thickening is not inhibited.

Additional key words: Smooth muscle cell, Pseudoendothelium, Reserpine, Aspirin, Flurbiprofen, Dipyridamole.
thickening due to smooth muscle cell hyperplasia develops in the central part of the injured segment under the regenerating endothelium and is maximal at 14 days.

In this study we examine in rats the effect of antiplatelet drugs on the formation of the platelet layer in the denuded artery and the development of the thickened intima. These results are correlated with the in vitro response of platelets from drug-treated animals to collagen, adenosine diphosphate (ADP), and thrombin.

METHODS AND MATERIALS

Eighty-one male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts), weighing between 150 and 250 gm., were fed standard rat chow and kept in cages (four in each cage).

EXPERIMENTAL PROCEDURE

Air injury of the right common carotid artery was performed as described previously (17). In brief, animals were anesthetized with sodium pentobarbital (Nembutal sodium, Abbott Laboratories, North Chicago, Illinois) and each rat was given an intraperitoneal injection of 5 mg. per 100 gm. of body weight. Through a longitudinal neck incision, a 1.5-cm. segment of right common carotid artery isolated by ligatures was infused with air through a 30-gauge needle cannula at 40 to 50 ml. per minute for 3.5 minutes. Blood flow was reestablished and hemostasis obtained before the wound was closed. In the first group of animals, the antiplatelet drugs were administered 26 hours and 2 hours before carotid injury; a second group of animals was started on drug treatment beginning 24 hours after carotid injury. The animals tolerated the drug treatment better if the drugs were begun after carotid injury. Twenty-four hours after the first dose an effect of the drugs on platelet function could be demonstrated in vitro. The last dose of drug was given approximately 18 hours before sacrifice. Animals were sacrificed at 5 and 14 days after surgery (Table 1). After satisfactory anesthesia was obtained, 4.5 ml. of blood was drawn into a plastic syringe containing 0.5 ml. of 3.8 per cent sodium citrate through a sterile, disposable 18-gauge needle inserted retrograde into the abdominal aorta just below the renal arteries. Blood was then obtained for measurement of the whole blood clotting time and clot retraction. Through the same needle cannula 100 units of heparin (Elkins-Sinn, Inc., Cherry Hill, New Jersey) per 100 gm. of body weight was injected. The jugular veins were divided and perfusion was begun through the needle with Hanks’ balanced salt solution (Microbiological Associates, Inc., Bethesda, Maryland), pH 7.4, for 30 seconds followed by 1 per cent paraformaldehyde (Matheson, Coleman, and Bell, Norwood, Ohio) and 1.25 per cent glutaraldehyde (Taab Laboratories, Reading, England) in 0.1 m cacodylate buffer, pH 7.4, as described previously (17). Both common carotid arteries were excised and fixed by immersion for an additional 4 hours in 2 per cent paraformaldehyde and 2.5 per cent glutaraldehyde in 0.1 m cacodylate buffer, pH 7.4. The vessels were washed in buffer, postfixed for 2 hours in 2 per cent aqueous osmium tetroxide, and stained in 2 per cent aqueous uranyl acetate en bloc. They were then dehydrated through graded solutions of alcohol and embedded in Epon 812 for longitudinal or serial cross-sectioning. Epon sections (2 μm.) were stained with toluidine blue. Preparative methods for transmission and scanning (SEM) electron microscopy have been described earlier (9, 17). Earlier work (9) with the carotid injury model has demonstrated that when arteries are cross-sectioned at 500-μm. stages the maximal amount of intimal thickening occurs in almost every case in the center of the injured segment and correlates with the total amount of intimal smooth muscle cell hyperplasia. Hence, maximal plaque size was used as an indicator of smooth muscle proliferation and was recorded as the maximal ratio of the plaque thickness (distance from luminal surface to internal elastic lamina) to the adjacent media thickness (distance from internal elastic lamina) to the most external elastic lamina) (I to M ratio) as measured with an ocular graticule (Fig. 1).

IN VITRO PLATELET STUDIES

Platelet-rich plasma (PRP) was prepared in plastic test tubes by centrifuging anticoagulated blood at 150 x g for 25 minutes at room temperature. Platelet-poor plasma was obtained by centrifuging blood at 2000 x g for 15 minutes at room temperature. The platelet count of the PRP was measured in a hemocytometer and adjusted to 500,000 platelets per cubic millimeter with platelet-poor plasma. Platelet aggregation studies were performed in a platelet aggregometer (model 300, Chrono-Log Corporation, Broomall, Pennsylvania) by adding ADP (Sigma Chemical Company, St. Louis, Missouri), collagen, or thrombin (Sigma Chemical Company) to cuvettes containing 500 μl. of PRP. The extent of aggregation was expressed as percentage of control and was measured for concentrations of ADP, collagen, or thrombin that produced submaximal aggregation of control platelets.

CLOTTING TIMES, CLOT RETRACTION, AND MESENTERIC BLEEDING TIMES

Clotting times were measured on whole blood by the method of Lee and White (27). The clot retraction of dilute whole blood clots was measured as described by Taylor and Müller-Eberhard (47). Mesenteric bleeding times were measured as described by Hovig et al. (22) in a separate group of 16 rats that were not used for other studies.

ASPIRIN

Aspirin powder (Sigma Chemical Company) (20 to 200 mg. per kg. of body weight) was administered daily by stomach tube to rats lightly anesthetized with ether. Rats that were given doses greater than 200 mg. per kg.
of body weight per day died within several days. Aspirin-treated control rats were intubated daily under anesthesia. There was no difference in weight gain for aspirin-treated and control rats.

**Reserpine**

Reserpine (Serpasil, CIBA Pharmaceutical Company, Summit, New Jersey) (0.4 to 0.8 mg. per kg. of body weight) was diluted in phosphate-buffered saline, pH 7.4, and was given to the rats daily by subcutaneous injection. Within 1 day animals treated with reserpine developed ptosis of the eyelid, lassitude, diarrhea, and weight loss. Animals subjected to doses of 1.0 mg. per kg. of body weight per day or greater died within 1 week. Reserpine-treated control rats received daily subcutaneous injections of phosphate-buffered saline.

**Flurbiprofen**

Flurbiprofen (Upjohn Company, Kalamazoo, Michigan) was dissolved in batches of 50 mg. in several drops of 0.1 N NaOH and diluted with Hanks' balanced solution, pH 7.4, to a concentration of 4 mg. per ml. The pH was not adjusted and was approximately 8. Flurbiprofen (1 to 10 mg. per kg. of body weight) was injected subcutaneously every day; up to 10 mg. per kg. of body weight per day was given without adverse effect. Flurbiprofen-treated control rats received daily subcutaneous injections of Hanks' solution. There was no difference in weight gain for flurbiprofen-treated and control rats.

**RESULTS**

**In Vitro Platelet Aggregation Studies**

The platelet counts of control and experimental animals did not differ significantly (Table 2). Platelets of control animals aggregated reversibly in response to 1 μM ADP, 1 μg. of collagen, and 0.3 National Institutes of Health units of thrombin; no secondary wave of aggregation was noted (28). Platelets from animals treated with a range of doses of aspirin, flurbiprofen, and reserpine showed markedly decreased aggregation in response to collagen; platelets from aspirin-treated and reserpine-treated rats showed slightly decreased aggregation in response to ADP whereas only platelets from aspirin-treated rats showed decreased response to thrombin (Table 2).

**Clotting Times, Clot Retraction, and Mesenteric Bleeding Times**

The clotting times and clot retraction of dilute whole blood clots were not significantly different for control and drug-treated animals. The mesenteric bleeding times of aspirin-treated and reserpine-treated rats were slightly prolonged over controls when assayed at 18 hours (Table 3).

**Light Microscopy**

**Control Animals.** The observations at 5 and 14 days confirmed the results of previous studies in rats (9, 17). At 5 days, the proximal and distal endothelial growing edges had advanced over the ligature sites and had partly covered the injury zone. A thin layer of platelets was visible in the residual central zone of denudation.
Table 3. Clotting Times, Clot Retraction, and Mesenteric Bleeding Times

<table>
<thead>
<tr>
<th>Drug group</th>
<th>Clotting time* (mL/min)</th>
<th>Clot retraction% (10)</th>
<th>Mesenteric bleeding time/ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.9 ± 0.1 (9)</td>
<td>85 ± 2 (4)</td>
<td>192 ± 8 (4)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>3.8 ± 0.4 (6)</td>
<td>90 ± 3 (4)</td>
<td>206 ± 17 (4)</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>4.6 ± 0.4 (10)</td>
<td>86 ± 5 (5)</td>
<td>191 ± 38 (4)</td>
</tr>
<tr>
<td>Reserpine</td>
<td>0.8 ± 0.4 (10)</td>
<td>83 ± 5 (4)</td>
<td>525 ± 143 (4)</td>
</tr>
</tbody>
</table>

* Clotting time in minutes by the method of Lee and White (27) ± standard error.
% Clot retraction as percentage of initial volume ± standard error at 1/2 hours after clotting as described by Taylor and Müller-Eberhard (47).
/ Mesenteric bleeding time in seconds as described by Hovig et al. (22) ± standard error.
* Number of rats in each group is shown in parentheses.
* p < 0.05; other differences are not significant.

adherent to the internal elastic lamina (Fig. 2A). Edema was present in the inner layers of the media. Occasional single cells were present passing through clefts in the internal elastic lamina into the intima under the platelet layer. At 14 days, in some animals, endothelial regeneration was complete over the markedly thickened intima (9, 17) (Fig. 2B). All carotids showed intimal thickening. Of particular note in two-thirds of controls was the lack of complete endothelial covering in the central part of the plaque. In place of normal endothelium, pseudoendothelium (41) was present and it appeared to be enmeshed in the intima except for limited surface exposure to the lumen of the vessel (Fig. 3A and B). In every instance the pseudoendothelium was present at the point of maximal intimal thickening. Occasional mononuclear cells were seen adhering to endothelium and pseudoendothelium. There were no changes in the uninjured left carotid artery.

**Drug-Treated Animals.** At 5 days, the right carotid arteries of both groups of animals treated with aspirin, flurbiprofen, and reserpine showed no difference from control injured carotids (Fig. 2C). Partial endothelial regeneration had occurred and the remaining central area of denudation was covered with a thin layer of platelets. Occasional microthrombi were seen in the denuded carotids of flurbiprofen-treated rats. At 14 days, all right carotid arteries except one from an aspirin-treated rat (group 2) showed intimal thickening. The maximal plaque size (I to M ratio) for groups 1 and 2 together was as pronounced as in control animals (control, 1.5; aspirin, 1.3; flurbiprofen, 1.7; reserpine, 1.5) (Table 4). There were no significant differences between the plaque size of animals in group 1 (drug treatment started before surgery) and in group 2 (drug treatment started after surgery) except for the flurbiprofen-treated rats (group 1, 0.9; group 2, 2.3, p = 0.05). However, the difference between each flurbiprofen group and the respective control group was not significant. In most animals, the endothelial layer was incomplete and was substituted for by a pseudoendothelial layer (Fig. 2D). Left carotid arteries from experimental animals appeared normal at all stages.

**Scanning Electron Microscopy**

**Control Animals.** The SEM confirmed observations made by light microscopy and contributed the following additional information: (1) at 5 days, two layers of platelets could be distinguished—one composed of platelets which were spread and tightly adherent to the underlying substructure and the other platelets round or slightly spread overlying or inserted between the first (Fig. 4A); (2) at 5 days, the endothelial growing edge was advancing toward the center of the injury zone as a broad front in continuity with proximal and distal endothelium; (3) at 14 days, the endothelium was completely regenerated in some arteries and resembled normal uninjured endothelium; (4) in two-thirds of the control rats, pseudoendothelium could be distinguished; whereas normal endothelium tended to be smooth, fusiform, and elongated in the direction of blood flow, the pseudoendothelial cells were slightly ruffled, irregular or polygonal in outline, and were not organized in relationship to the blood flow (Fig. 5A and B). The junctions between these cells appeared to be open and made up of multiple interdigitizing processes from neighboring cells. Individual platelets, microthrombi, fibrin, and debris were found in the crevices between pseudoendothelial cells. No platelets were seen adhering to the actual surface of the pseudoendothelium. Leukocytes adherent to the endothelium were also present in the unhealed central zone.

**Drug-Treated Animals.** By SEM, experimental injured carotids at 5 days resembled controls, except for the presence of microthrombi in the right carotids of flurbiprofen-treated rats (Fig. 6A and B). The number of platelets and their appearance was unchanged (Fig. 4B); the extent of the ingrowth of the endothelial growing edges was the same for experimental and control animals. At 14 days, endothelium had not regenerated over the central part of the injured segment in most drug-treated rats. Instead, pseudoendothelium was present as in controls.

**Transmission Electron Microscopy**

**Control Animals.** The findings by light microscopy and SEM were confirmed by transmission electron microscopy. In addition, at 5 days a range of platelet forms could be identified. Degranulated platelets were stretched thinly over most of the denuded surface; in places, adherent platelets retained their granules. Other rounded forms were in contact with the first layer (Fig. 7A). The platelets appeared to interact with the loose fibrillar material overlying the internal elastic lamina. Occasional cells were seen migrating through the internal elastic lamina; these cells had smooth muscle cell characteristics (17, 20) (Fig. 8). At 14 days, the cell layer overlying the thickened intima had the appearance of normal endothelium with occasional Weibel-Palade bodies (50) and tightly opposed cell borders. As noted before (9, 17), the cells in the intima were either undifferentiated or mature smooth muscle cells and were surrounded by collagen and elastin. In some arteries, pseudoendothelium could be distinguished from normal endothelium (41) (Fig. 9A and B). In par-
ticular, pseudoendothelial cells were ensconced in a matrix of collagen and elastin, had a band of myofilaments under a flat luminal surface, and formed complex but interdigitating, loosely apposed cell borders. Normal endothelial cells were spread over the luminal surface of the intima, had basilar bands of myofilaments sometimes stretching diagonally across the cell and forming dense specializations with the basal attachments, and had junctions composed of tightly apposed membranes. No Weibel-Palade bodies were found in pseudoendothelium. As was also shown by SEM, no transition forms between normal endothelium and pseudoendothelium were seen. Adjacent cells had some or all of the characteristics of mature smooth muscle cells. Presumably, the pseudoendothelium derives from underlying smooth muscle cells (41).

**Drug-Treated Animals.** At 5 days, platelets of drug-treated animals showed the same range of forms as controls (Fig. 7B and C). Serotonin granules (11, 48) were not identified in reserpine-treated rats. At 14 days, pseudoendothelium and intimal smooth muscle cells were observed as in controls.

### DISCUSSION

Early after arterial endothelial injury, platelets adhere to the denuded wall; later, over a period of time, intimal smooth muscle cell hyperplasia occurs. Recent work has suggested that the two events are related and that platelet degradation may produce growth factors specific for smooth muscle cells (19, 21, 29, 38, 39). Based on this theory, one possible method for inhibiting smooth muscle cell proliferation after arterial injury might be to administer drugs that inhibit platelet aggregation to collagen and other components of the arterial wall. Numerous *in vitro* studies have shown that platelets taken from animals treated with antiplatelet

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**Fig. 2.** Histologic sections of injured right carotid arteries from control and drug-treated animals: A, zone of denudation in control carotid at 5 days after injury showing thin intimal layer of platelets and medial edema; B, central zone in control carotid at 14 days demonstrating marked intimal proliferation; C, denuded carotid from reserpine-treated rat at 5 days; note single intimal cell under platelets; D, intimal thickening at 14 days in carotid of flurbiprofen-treated rat. Figure 2A and C, x615; B and D, x320.
In this study we have attempted to relate the *in vitro* activity of platelets from rats treated with aspirin, flurbiprofen, or reserpine to *in vivo* deposition of similar platelets on the exposed carotid artery subendothelial layer. These drugs were employed because of their known long acting effect on platelets *in vivo* (3, 31, 42, 51, 55) and their inhibition of collagen-induced platelet aggregation *in vitro*; reserpine was used not only because it inhibits platelet aggregation but also because it selectively depletes platelets of one factor, serotonin (42). Our results demonstrated that although collagen-induced aggregation was markedly inhibited *in vitro* there was no alteration in the *in vivo* pattern of platelet interaction with the carotid arterial subendothelial layer; however, the slight prolongation of the mesenteric bleeding time for aspirin-treated and reserpine-treated rats does suggest diminished platelet activity *in vivo* in these animals (13). We also found that at 14 days after injury there was no decrease in intimal thickening or inhibition of smooth muscle cell proliferation in the drug-treated groups. The intimal thickening in the injured arteries of rats treated with flurbiprofen before surgery was decreased with respect to controls. However, the large biologic variability in plaque size in control and drug-treated groups rendered this difference not significant. We have found in other work that when treatment is successful the intimal thickening of the experimental group is unequivocally diminished with respect to controls (8). The failure of endothelium to regenerate by 14 days and the development of pseudoendothelium (41) probably derived from smooth muscle cells were unexpected additional results in both control

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**Table 4. Maximal Plaque Size (I to M Ratio) in Injured Right Carotid Arteries at 14 Days**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Group 1*</th>
<th>Group 2*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.8 ± 0.6 (4)</td>
<td>1.3 ± 0.4 (6)</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Aspirin 20-200 mg/kg/da.</td>
<td>1.5 ± 0.5 (6)</td>
<td>1.1 ± 0.4 (5)</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Flurbiprofen 1-10 mg/kg/da.</td>
<td>0.9 ± 0.4 (4)</td>
<td>2.3 ± 0.3 (6)</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Reserpine 0.4-0.8 mg/kg/da.</td>
<td>1.6 ± 0.4 (4)</td>
<td>1.5 ± 0.2 (6)</td>
<td>1.5 ± 0.2</td>
</tr>
</tbody>
</table>

* Animals in group 1 received drug 26 hours and 2 hours before carotid injury and were sacrificed 14 days following injury.  
  * Animals in group 2 received drug beginning 24 hours after surgery and were sacrificed at 14 days.  
  * Mean maximal plaque size (intima to media ratio) at 14 days ± standard error. The differences are not significant for \( p = 0.05 \).
  
* Number of animals in each group is shown in parentheses.

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Drugs aggregate less well in the presence of collagen than platelets from normal animals; however, it is not at all clear that platelet function *in vivo* at the surface of an injured artery is inhibited in the same way by these drugs. Thus, if platelets do liberate factors mitogenic for smooth muscle cells, it is uncertain whether these drugs could inhibit the intimal thickening due to smooth muscle cell hyperplasia that follows upon endothelial injury. The antiplatelet drug, dipyridamole, has been shown to inhibit intimal smooth muscle cell hyperplasia in baboons (21); however, platelet aggregation *in vitro* was not inhibited at the concentration of drug achieved *in vivo*.
and drug-treated rats. They are perhaps related to the stress of frequent traumatic stimuli since previous results in unmanipulated animals (9, 17) with the same model showed complete endothelial regeneration at 14 days.

Platelets and Subendothelium

Baumgartner et al. (2-4) and Stemerman (44) have shown that platelets adhere to microfibrils and collagen in the subendothelial layer but not to elastin. Huang, Lagunoff, and Benditt (23) have demonstrated that when platelets interact with glomerular basal lamina they adhere and spread but do not aggregate or degranulate. Our results at 5 days are in agreement with these findings, although some of the fully spread platelets seemed to lack granules. The lack of visible granules may have been a consequence of the spreading and the resultant decrease in cross-sectional area. In the drug-treated animals, although platelet morphology appeared to be unchanged in the injured carotid (except for the reserpine-treated rats), nevertheless platelet degranulation and turnover may have been affected. As yet no methods have been developed to measure the kinetics of platelet turnover in the area of endothelial denudation.

In rabbit arteries injured by the application of a ligature Ts'ao (49) found that platelets adhered to injured endothelium and degranulated; if the rabbits were treated with aspirin, platelet adherence still occurred but without degranulation, and platelet aggregates were more loosely constructed than in normals. Platelets from aspirin-fed rabbits aggregated in vitro in the presence of ADP and thrombin but not collagen. Weiss, Tschopp, and Baumgartner (52) have examined the in vitro interaction of human platelets with the subendothelial layer of the rabbit aorta and have reported that although the adhesion of platelets from patients given aspirin was normal, there were virtually no platelet thrombi present; Baumgartner and Muggli (3) reported that platelets from aspirin-treated rabbits showed normal adhesion to denuded rabbit aorta in vitro and in vivo but fewer microthrombi. On the other hand, Cazeneve et al. (7), using washed, chromium-labeled platelets from aspirin-fed rabbits, showed reduced platelet adhesion in vitro to the damaged intimal surface of everted segments of rabbit thoracic aorta. They point out that the use of citrated platelet preparations as employed by Baumgartner and Muggli reduces the amount of normal platelet adhesion to the level of aspirin-treated platelet adhesion. In other studies of arterial thrombosis, Renaud and Godu (36) reported inhibition of experimentally induced thrombosis in rats fed aspirin, and Danese, Voleti, and Weiss (10) have shown a similar effect in dogs. Our results indicate that even if aspirin did acutely reduce the adhesion of platelets to carotid subendothelium following injury, over a period of time the aspirin effect was not sufficient to diminish grossly platelet deposition.

Reserpine (15, 34, 40, 42, 43, 55) and flurbiprofen (12, 31) both have been used to inhibit platelet aggregation in vitro and to a limited extent in vivo models. These drugs seem to act by different pathways, although diminished collagen-induced aggregation is the end result. Aspirin interacts with platelets by acetylating the platelet membrane (1) and by inhibiting the platelet release reaction through suppression of prostaglandin synthesis (25, 32, 53, 54); although the mechanism by which platelet aggregation is inhibited by reserpine is unclear reserpine is known to deplete the platelets of their serotonin stores sequestered in the osmiophilic granules by preventing serotonin uptake at the platelet membrane (34, 43). Reserpine also inhibits thrombus formation in vivo (55). Flurbiprofen does not bind to the platelet (31); it too may act by inhibiting prostaglandin synthesis. Our data suggest that although both reserpine and flurbiprofen are potent inhibitors of collagen-induced platelet aggregation neither drug is potent enough to diminish platelet-arterial wall interaction in vivo.

Intimal Smooth Muscle Cell Hyperplasia

We have shown that myointimal thickening following arterial endothelial injury in the rat is not inhibited by the administration of aspirin, flurbiprofen, or reserpine. Our results support those of H. R. Baumgartner and R. K. Studer (personal communication) who found that aspirin and dipyridamole did not suppress myointimal

![Scanning electron micrographs demonstrating the appearance of platelets adherent to the luminal surface of denuded right carotid arteries from control (A) and reserpine-treated (B) rats at 5 days. Note the dense packing of the platelets in both micrographs. X5,000.](image)
Fig. 5. Scanning electron micrographs illustrating the differences between endothelial and pseudoendothelial cells in the injured right carotid of reserpine-treated rats: A, endothelial growing edge of 14 days (elongated cells aligned in the direction of blood flow) adjacent to pseudoendothelium (cells with polygonal outlines); B, higher power view of pseudoendothelium at 14 days. Figure 5A, ×2,100; B, ×4,000.

Fig. 6. Scanning electron micrographs of the luminal surface of the injured right carotid segment in flurbiprofen-treated rats at 5 days: A, low power view showing microthrombi between regenerating endothelial cells; B, high power view of microthrombus containing platelets and strands of fibrin. Figure 6A, ×800; B, ×5,000.

proliferation in injured rabbit aortas. Baumgartner and Studer report that sulfinpyrazone produced some inhibition of intimal smooth muscle cell hyperplasia which was not statistically significant when compared to appropriate controls. Harker et al. (21) have reported that the antiplatelet drug dipyridamole can affect platelet survival time and intimal thickening; *in vitro* this drug has antiplatelet aggregation effects but only at concentrations not normally attained *in vivo* (21). They have reported that chronic endothelial injury and decreased platelet survival time may be produced by infusing homocystine intravenously into baboons and that the
Fig. 7. Transmission electron micrographs of platelets in the denuded segment of injured right carotid arteries at 5 days from control (A), flurbiprofen-treated (B), and reserpine-treated (C) rats. Note osmiophilic (serotonin) granules (S) present in platelets from control and flurbiprofen-treated rats but absent in platelets from reserpine-treated animals. Empty vacuole (arrow) may be a serotonin-depleted granule. Platelets are adherent to microfibrils (M) and amorphous material overlying internal elastic lamina (IEL). Figure 7A, ×27,000; B, ×27,800; C, ×36,000.
resultant intimal smooth muscle cell hyperplasia may be suppressed and the platelet survival time returned to normal by the oral administration of dipyridamole. Also, Didisheim (14) has reported inhibition of arterial thrombosis in the rat with this drug. Using a method for continuous intravenous infusion of dipyridamole, we attempted to reproduce Harker's results in the rat. However, the drug precipitated about the catheter tip, and continuous infusion of dipyridamole at 20 mg. per kg. of body weight per day into a gastrostomy produced no change in in vitro or in vivo platelet function or intimal thickening at 14 days. In view of the short half-life of the drug in rats (5) and its paradoxical effects of rat platelets in some hands (33) it is possible that our negative results may reflect some intrinsic difference in platelet function in the two animals.

Although the work of Harker et al. (21) is supported by the in vivo studies of Friedman et al. (19) and Moore et al. (29) and the in vitro studies of Rutherford and Ross (39), Ross et al. (38), and Kohler and Lipton (26), our results and those of Baumgartner and Studer are in disagreement. In part, the discrepancies may be due to the difference in animals and methods used for suppressing platelet function as judged by conventional criteria. However, until methods are developed for assessing the turnover of platelets and the accumulation of mitogenic factors in the arterial wall, only inferential conclusions can be drawn about the relationship of the drug experiments in vivo to the growth factor experiments in vitro. It is possible that the agent responsible for stimulating smooth muscle cell proliferation in vivo is not as yet certain and might be shown in time to be a pool of factors generated possibly from plasma or platelets or both at the site of endothelial denudation.

In summary, we have shown a distinct lack of correlation between in vitro platelet aggregation and in vivo platelet interaction with arterial subendothelium in rats treated with a range of doses of aspirin, reserpine, and flurbiprofen. On the assumption that such drugs might suppress the release of growth factors from platelets, we measured the amount of intimal thickening in the rat carotid artery injury model and in drug-treated animals and found no change in intimal smooth muscle cell proliferation.

Fig. 8. Transmission electron micrograph of modified smooth muscle cell (SMC) in transit through fenestra in internal elastic lamina (IEL) of denuded carotid from aspirin-treated rat at 5 days. Note layer of platelets adherent to fibrillar material overlying smooth muscle cell. Lumen (L) of vessel is at the top. ×15,000.
Fig. 9. Transmission electron micrographs of endothelium (A) and pseudoendothelium (B) in injured carotid artery at 14 days. A, endothelial cell (E) spread over intima containing mature smooth muscle cell (SMC); note basilar myofilaments (MF); B, pseudoendothelial cell (PE) inserted in intima among smooth muscle cells (SMC) with limited membrane surface exposed to lumen (L); note myofilaments (MF) under luminal surface. Figure 9A, ×9,500; B, ×9,900.
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Suppression by heparin of smooth muscle cell proliferation in injured arteries

INTIMAL smooth muscle cell (SMC) proliferation dominates the early phase of healing after arterial endothelial injury1-3; what growth factors are responsible for this mitotic activity of an otherwise quiescent cell is not known. SMC growth in vivo is enhanced by platelet factors, insulin and lipoproteins4-11 and, according to some reports, can be diminished in vivo with various antiplatelet agents. Harker et al.4 have reported decreased myointimal thickening in homocystaenamic baboons treated with dipyrindamole, and Friedman et al.11 and Moore et al.12 have reported suppression of SMC proliferation in the injured arteries of rabbits injected with anti-platelet serum. Intimal thickening was not diminished, however, in the injured carotid arteries of rats given various anti-platelet drugs13. Because the clotting system is inextricably linked to arterial injury and because thrombin is used to generate the platelet growth factor and may itself be a mitogen14,15, we speculated that heparin might inhibit myointimal thickening. We report here the use of heparin to suppress intimal SMC hyperplasia in a rat model of arterial endothelial injury and suggest possible mechanisms for the heparin effect.

Endothelial injury was achieved by infusing air into isolated segments of right carotid arteries of 45 young male Sprague-Dawley rats (250-300 g, Charles River Breeding Laboratories) as described earlier1,2. This procedure desiccated the endothelium. After blood flow was re-established and haemostasis obtained, the wound was irrigated with Betadine solution. Using a sterile technique, a silastic catheter was inserted into the left jugular vein and connected to an infusion system as described by Steiger et al.17. Initially all rats were infused with lactated Ringer's solution (Abbott) at 0.91 ml h−1 using a syringe pump. After 24 h, experimental rats were switched to an infusion of heparin (from pig intestinal mucosa, Elkins-Sinn). Control rats were infused continuously with Ringer's solution. After 0.91 ml h−1 of saline had been collected for clotting times and platelet counts, Animals were killed at 5, 10 and 14 d by perfusion fixation1-12 after blood had been collected for clotting times7 and platelet counts. Carotid arteries were further fixed and processed as described earlier1-4 for light, scanning (SEM) and transmission electron microscopy (TEM).

Clotting times were prolonged in a predictable fashion; as noted before14, a linear relationship existed between the logarithm of the clotting time and the heparin dose. Heparin doses of 50 or 100 U kg−1 h−1 given intravenously yielded clotting times of 12.5 and 36 min (control 4.4 min). Platelet counts were not affected by heparin administration (control: 1.28 ± 11 × 106 (+s.e.); 50 U kg−1 h−1: 1.38 ± 0.04 × 106; 100 U kg−1 h−1: 1.38 ± 0.11 × 106).

In earlier studies on uncatheterised control animals1-3, we showed that after carotid injury the desiccated endothelium rapidly sloughed and was supplanted by a carpet of platelets. Endothelial regeneration proceeded from normal endothelium at the ends of the injured segment and was complete by 14 d; between 5 and 14 d smooth muscle cells migrated through the internal elastic lamina and proliferated in the intima. The intimal thickness was maximal at 14 d.

The right carotid arteries of catheterised control animals at 5 d demonstrated partial endothelial regeneration and a dense monolayer of platelets in the residual central zone; in place of endothelium and platelets, modified smooth muscle cells lined the luminal surface. A possible explanation for this failure of endothelial regeneration is that in stressed animals or after long arterial injuries, rapidly growing SMC can temporarily form a luminal surface and impede endothelial regeneration. At 10 d, all injured carotids showed marked intimal thickening due to SMC proliferation; at 14 d, the intimal thickening was even more pronounced. The injured right carotids of heparin-treated rats exhibited the same features as catheterised controls at 5 d with partial endothelial regeneration at the ends and a dense carpet of platelets in the middle of the injured segment; the platelets in heparin-treated rats were morphologically (SEM and TEM) similar to controls. At 14 d, a small central residual zone of denudation covered by platelets remained and at 14 d endothelial regeneration was complete as in uncatheterised controls. At 10 d, there was only slight evidence of intimal thickening which nevertheless was not significantly different from controls. But, at 14 d, because of increased intimal thickening in control carotids, the maximum ratio of intima to media (I/M) in carotids from heparin-treated rats was diminished significantly (Fig. 1 and Table 1).

Table 1 Myointimal thickening at 14 d

<table>
<thead>
<tr>
<th>Condition</th>
<th>I/M ± s.e.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.9 ± 0.5 (5)</td>
<td></td>
</tr>
<tr>
<td>Heparin (50 U kg⁻¹ h⁻¹)</td>
<td>0.4 ± 0.2, P = 0.02 (5)</td>
<td></td>
</tr>
<tr>
<td>Heparin (100 U kg⁻¹ h⁻¹)</td>
<td>0.6 ± 0.1, P = 0.005 (10)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.5 ± 0.1, P = 0.0007 (15)</td>
<td></td>
</tr>
</tbody>
</table>

I/M ± s.e. was measured in right carotid arteries, 14 d after injury, which were cross sectioned at 500-μm stages along their entire length at 5. Because the thickened intima at 14 d is almost entirely composed of smooth muscle cells4-12 (Fig. 1), the maximum I/M ratio reflects the amount of SMC proliferation and is measured by using an ocular gridule by taking the ratio of the maximum intimal thickness (distance from the luminal surface to the internal elastic lamina) to the adjacent media thickness (distance from the internal elastic lamina to the most external elastic lamina). Numbers of rats in each group are shown in parentheses.

maximum I/M ratios for high and low dose heparin were similar and both were significantly different from control values. At most, one to three SMC layers were seen at this stage in the intima under the endothelium. The normal rate of endothelial regeneration in heparin-treated animals may have been related to the absence of luminal SMC.

These results indicate that heparin in doses sufficient to prolong clotting markedly suppressed intimal SMC proliferation after arterial endothelial injury even though endothelial regeneration (mitosis and migration) progressed unimpaired. Heparin did not seem to inhibit platelet-subendothelium interaction in the injured carotid.

This suppression of SMC proliferation may be the result of one or more effects of heparin because heparin is known to have many biological activities. Heparin may act directly on the SMC. Alternatively, because of its charge, heparin may bind and inactivate platelet growth factors generated by platelet activity on the denuded arterial wall; this is particularly plausible in view of Ross's in vitro results demonstrating that the SMC platelet mitogen is a basic protein. Heparin may achieve several effects indirectly through its action on the clotting system. Thrombin and the other serine proteases of the clotting system (XII, XI, IX, X and plasmin) are undoubtedly slowly inactivating the clotting proteases; this process is known to have many biological activities. Heparin may act in particular, may generate mitogens from the adherent platelets as suggested by in vitro work. Antithrombin III normally regulates the level of proteolytic activity by slowly inactivating the clotting proteases; this process is almost instantaneous in the presence of heparin. Thus, heparin through antithrombin III may affect the degradation of platelets and generation of platelet SMC growth factors by thrombin, and the direct activity of the clotting proteases on SMC. Arterial SMC proliferation may be just one example of the general phenomenon of wound healing suppressible by heparin; previous studies of wounds in skin, bowel, artery and bone indicate that heparin can prevent scarring if given in doses large enough to produce a continuous and significant state of anticoagulation

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Inhibition of Rat Arterial Smooth Muscle Cell Proliferation by Heparin

In Vivo Studies with Anticoagulant and Nonanticoagulant Heparin

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SUMMARY Heparin inhibits the proliferation of intimal smooth muscle cells which occurs after denudation of endothelium by air-drying injury in the rat carotid artery. We determined (1) whether the antiproliferative effect of heparin is secondary to effects on platelet adherence to subendothelium or endothelial regeneration and (2) whether the antiproliferative and anticoagulant activities of heparin are related. Morphometric observations by scanning electron microscopy showed that heparin did not alter platelet adherence 5 days after arterial injury and had little or no effect on endothelial regeneration at 5 and 10 days. To study the relationship between the antiproliferative and anticoagulant effects, we fractionated heparin by affinity chromatography on antithrombin-Sepharose into purified anticoagulant and nonanticoagulant fractions. These heparin fractions were administered to rats in doses which were equivalent either in terms of anticoagulant activity or in terms of mass to the dosage of unfractionated heparin known to inhibit myointimal growth. Additionally, some rats received nonanticoagulant heparin at a dose which was greater in terms of mass than the highest dose of unfractionated heparin which could be administered without inducing fatal hemorrhage. Inhibition of myointimal growth, determined by morphometric analysis of total plaque volume 2 weeks after arterial injury, correlated with total mass of heparin administered but not with anticoagulant activity. Nonanticoagulant heparin given at high dose caused 77% inhibition of myointimal growth (P < 0.02 vs. controls). Heparin inhibition of arterial smooth muscle cell proliferation does not appear to be mediated either by effects on other cells at the level of the arterial wall or by antithrombin. This study should direct attention toward a potential growth regulatory role for arterial glycosaminoglycans.


IN A RECENT study on intimal smooth muscle cell proliferation in the rat carotid artery denuded of endothelium, we reported that intravenous heparin in doses large enough to cause continuous anticoagulation markedly inhibited myointimal proliferation (Clowes and Karnovsky, 1977). Several hypotheses for the mechanism of this antiproliferative effect of heparin have been considered. First, heparin may have little direct effect on smooth muscle cells in vivo, but may change platelet function (Zucker, 1974) or endothelial regeneration. From the Department of Pathology, Harvard Medical School, Boston, and Sidney Farber Cancer Institute and Beth Israel Hospital, Boston, Massachusetts.

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the antiproliferative effect probably is not related to changes in the humoral clotting system.

Methods

Preparation of Experimental Animals

We studied 69 male Sprague-Dawley rats (CD strain, Charles River Breeding Laboratories) weighing between 250 and 320 g. Arterial air-drying injury was induced using the technique of Fishman et al. (1975) modified with a higher air flow rate and longer drying time to ensure complete endothelial denudation. Briefly, the rat was anesthetized with intraperitoneal pentobarbital (Nembutal, Abbott Laboratories, 50 mg/kg body weight) and the right common carotid artery was exposed and ligated at two points 1.5 cm apart. A 30-gauge hypodermic needle was inserted into the proximal end of the segment. An exit hole was punctured at the distal end of the segment either by passing the same needle down the lumen and out through the wall or by puncturing with an additional needle from outside. After the lumen had been rinsed with phosphate-buffered saline, dry (compressed) air was allowed to flow through the segment for 3.5 minutes at a rate of 50–60 ml/min. Ligatures then were removed and hemostasis was obtained by pressure.

Within minutes to a few hours after reestablishment of blood flow in the arterial segment, desiccated endothelial cells detach from the arterial wall and are swept away. To monitor the completeness of endothelial denudation between the ligature sites, perfusion silver staining (Fishman et al., 1975) was performed 24 hours after operation in nine rats taken from various cohorts during the course of the study.

The intravenous infusion technique was the same as that described previously (Clowes and Karnovsky, 1977), except that the swivel connector was found to be unnecessary. A Silastic catheter was placed in the left jugular vein and passed through the skin at the back of the neck beneath a shoulder saddle, where it connected to an external catheter of Tygon tubing. The external catheter was protected by a stainless steel flexible coil which was anchored 14 or more inches above the animal. The animal's cage was turned daily to relieve any torque in the coil caused by the animal's net rotational movement. A syringe pump delivered fluid at a rate of 0.91 ml/min. Ringer's lactate (Abbott) was infused into control rats and served as diluent for the heparin solutions.

Preparation of Heparin Fractions

Human thrombin and human antithrombin were isolated in physically homogeneous form by methods previously reported (Rosenberg and Damus, 1973). The anticoagulant potency of mucopolysaccharide fractions was estimated by quantifying their ability to accelerate the interaction of antithrombin with thrombin and comparing the extent of enzyme neutralization to that attained with a heparin standard of known USP potency (Lam et al., 1976).

The concentration of antithrombin was determined by absorbance measurements at 280 nm assuming an extinction coefficient of 6.5. Mucopolysaccharide concentrations were estimated colorimetrically by assay of uronic acid at 530 nm according to the carbazole method of Bitter and Muir (1962). The relationship between this parameter and the dry weight of heparin fractions was determined experimentally.

A single lot of commercial heparin (Upjohn, lot 082ED) was used to prepare the heparin fractions. Heparin was mixed with a 2-fold molar excess of antithrombin to form complexes in a buffer consisting of 0.15 m NaCl in 0.01 m Tris-HCl at pH 7.5 and 24°C. Thereafter, heparin bound to inhibitor as well as uncomplexed mucopolysaccharide were isolated free of antithrombin by techniques analogous to those reported previously (Rosenberg et al., 1978; Jordan et al., 1979). The heparin that bound to antithrombin, called purified anticoagulant heparin, exhibited a specific anticoagulant activity of 317–352 USP units/mg. The mucopolysaccharide that did not complex with antithrombin, termed nonanticoagulant heparin, had a specific anticoagulant activity of 17–18 USP units/mg.

Chemical differences between anticoagulant and nonanticoagulant heparin are subtle. Analysis of low molecular weight fractions (~6000 daltons) has demonstrated that anticoagulant heparin contains 1.1 more residues of glucuronic acid and 1.5 fewer residues of N-sulfated glucosamine than nonanticoagulant heparin (Rosenberg et al., 1978). Recent studies have demonstrated the unique presence in anticoagulant heparin of a tetrasaccharide sequence with the unusual features of nonsulfated iduronic and glucuronic acid residues and an N-acetylated glucosamine (Rosenberg and Lam, 1979).

Platelet Deposition and Endothelial Regeneration

The inner surface of the right carotid artery was studied by scanning electron microscopy at 5 and 10 days after air-drying injury in 34 rats. Arteries from 10 heparin-treated and 9 control rats were fixed at 5 days, and from 9 heparin-treated and 6 control rats were fixed at 10 days. Only unfractioanted heparin was given to these animals. To avoid hemorrhage, heparin administration was not begun until 24 hours after the operation. The initial infusion rate was at least 50 units/kg body weight per hour, and this was increased to 100 units/kg body weight per hour on the 6th postoperative day. The minimal regimen had been shown previously to inhibit myointimal proliferation effectively (Clowes and Karnovsky, 1977). For the last 5 rats in each group fixed at 5 days and the last 3 rats in each group fixed at 10 days, whose arteries were studied, heparin was administered according to the schedule...
for unfractionated heparin shown in Table 1. Two commercial brands of porcine intestinal heparin (Elkin-Sinn and Upjohn) were used at various times, both of which were equally effective in inhibiting rat arterial smooth muscle cell proliferation in vitro (data not shown).

After blood had been drawn for Lee-White clotting times, hematocrits, and platelet counts (hematocytometer method), rats were fixed by retrograde perfusion via the abdominal aorta at 120 mm Hg pressure with 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer.

Excised tissues, including right and left common carotid arteries, were further fixed by immersion in 2% paraformaldehyde and 2.5% glutaraldehyde for 2-4 hours. Arteries were postfixed in osmium tetroxide and dehydrated using alcohols. They were sectioned at the midpoint, cut longitudinally, critical point dried in CO2 using a Samdri PVT-3 (Tousimis Research Corporation), and coated with gold-palladium using a Technics Hummer sputter-coating device (Technics). All observations were made on an ETEC Autoscan scanning electron microscope (ETEC Corporation) on coded randomly sequenced specimens by a single observer (J. R. Guyton).

To quantify platelet adherence to the arterial wall at 5 days, three fields from each artery selected at low magnification were photographed at 3000×. The photographs were coded and shuffled, and platelets were counted visually, marking those counted on a plastic overlay. Eight photographs were counted twice, showing good reproducibility with an intraclass correlation coefficient of 0.97 (Snedecor and Cochran, 1956).

Endothelial regeneration was quantified in 5- and 10-day specimens in the following manner. The specimens were oriented at a right angle to the electron beam. An average location for the advancing endothelial edge, which was often irregular around the circumference of the artery, was estimated visually. The sum of lengths of endothelial regrowth from the two ends divided by the total length of the original ligated segment, as measured on the microscope viewing screen, was taken to represent fractional endothelial regeneration.

### Table 1 Schedules of Heparin Administration in the Plaque Volume Study

<table>
<thead>
<tr>
<th>Heparin fraction infused</th>
<th>Dose schedule</th>
<th>Days post-op</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2-4</td>
</tr>
<tr>
<td>None (7)*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unfractionated (UF) (7)</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.39</td>
</tr>
<tr>
<td>Anticoagulant (AC) (4)</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.39</td>
</tr>
<tr>
<td>Nonanticoagulant (NAC1)</td>
<td>0</td>
<td>0.78</td>
</tr>
<tr>
<td>Nonanticoagulant (NAC2)</td>
<td>0.78</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* Numbers in parentheses = numbers of rats.
† The dosage schedule for the group receiving unfractionated heparin is given in terms of both anticoagulant activity and mass.

### Effect of Heparin Fractions on Plaque Volume

Twenty-six rats were divided into two control and three experimental groups, as shown in Table 1. Control rats received either no heparin or unfractionated commercial heparin (Upjohn) according to a progressive schedule. Gradual onset of heparin administration postoperatively was necessary to reduce the incidence of hemorrhage. Two experimental groups received purified anticoagulant or nonanticoagulant heparin in dosages which matched the control unfractionated heparin infusion in terms of anticoagulant activity (for those receiving purified anticoagulant heparin) or mass (for those receiving nonanticoagulant heparin). In order to study the response to a mass dose greater than that of control infusion, a third experimental group received a relatively high constant dose of nonanticoagulant heparin beginning immediately after the operation.

Myointimal growth was determined 2 weeks after air-drying carotid injury, because previous work (Fishman et al., 1975) showed that plaque size was maximal at this time. Blood for tests was drawn and animals were fixed by perfusion as described earlier. Carotids were sectioned midway between the original needle puncture sites and embedded in Epon 812. Two-micron sections were cut perpendicular to the vessel axis at 500-µm intervals to a depth of 5 mm in each half of the original carotid segment, thus yielding 22 sections for plaque volume analysis. Because the plaque tended to grow largest in the midportion of the denuded segment and because of shrinkage of tissue during processing, essentially all of the intimal plaque volume was contained within this distance.

Sections were stained with toluidine blue, then projected at approximately 200-fold magnification onto bond quality typing paper which varied in weight per unit area by ± 1%. The outlines of intima and media were traced, and the paper was cut and weighed. Repeated area determinations for individual samples of intima and media from 20 sections showed good reproducibility, with an intraclass correlation coefficient of 1.00. The total weight of
tracings of intima was divided by the total weight of tracings of media to give an index of plaque volume in each animal.

**Autopsies**

After perfusion fixation, viscera in all rats were inspected grossly for abnormality. In 21 representative animals, paraffin sections of various organs were stained with hematoxylin and eosin and with alcian blue and neutral red.

**Statistical Methods**

Reproducibility of morphometric technique was shown by the intraclass correlation coefficient, which yields values near 1.00 when the variance among replicated measurements is small compared to the variance among measurements on different specimens (Snedecor and Cochran, 1956). To demonstrate the lack of difference between heparinized and control groups in endothelial regeneration and platelet adherence, Student's t-test with confidence intervals for the differences between means was used. Analysis of variance was used to assess differences in plaque volume, with data from each animal transformed to the fourth root to give comparable variances within groups. Transformation to a root was more suitable than either the use of untransformed data or log transformation, because the variance could be expected to increase with increasing values, yet values near zero (i.e., almost no migration of smooth muscle cells into the intima) were possible and did occur. Scheffe analysis was chosen for a posteriori comparison of results, because it corrects for multiplicity of comparisons.

![Figure 1](image-url) Scanning electron micrograph showing platelets adherent to arterial wall 5 days after air-drying injury and 4 days after institution of heparin therapy, in a healthy animal without bleeding. Bar = 5 μm. 5800×.
HEPARIN INHIBITION OF ARTERIAL MYOINTIMAL GROWTH/Guyton et al. 629

(more conservatively than other methods) and at the same time allows combinations of groups to be compared (Brownlee, 1965).

Results

Completeness of Endothelial Denudation

Silver stains performed the day after air-drying carotid injury showed 98–100% endothelial denudation within the ligature marks in all nine rats. Three rats showed 100% denudation; in the other six a small tag of endothelium remained intact at the end of the segment next to a ligature mark, where it presumably was protected from drying by moisture trapped in crevices or beneath the needle tip.

Weight Gain, Blood Findings, Histology

There were no significant differences among experimental and control groups for weight gain (average was 21 g), platelet counts, or hematocrits. Routine hematoxylin and eosin staining of various organs showed no unusual findings. Alcian blue applied to the paraffin sections stained mucus, bone marrow cell granules, aortic media in all animals, and bony matrix variably. In one rat given high-dose nonanticoagulant heparin, alcian blue staining was seen in scattered large cells in the spleen.

Platelet Adherence

Figure 1 is a typical scanning electron micrograph of adherent platelets, from which visual counting was performed. Six rats which remained healthy after operation and heparinization gave results that were sharply different from those of three rats which bled massively and were moribund at the time of fixation. The former group showed a mean (±SEM) of 199,000 ± 14,000 platelets per square millimeter adherent to the arterial wall, which is not significantly different from the mean of 216,000 ± 13,000 found in unheparinized controls (Fig. 2). The three rats which bled had a range of platelet adherence from 10,000 to 55,000 per square millimeter (not shown in Fig. 2). This latter group could not be considered representative of rats which were submitted to studies lasting longer than 5 days, because in the longer studies rats which showed neck swelling and pallor at 5 days always died when heparin dose was increased.

Endothelial Regeneration

A typical view of endothelium regenerating as a continuous sheet of cells is shown in Figure 3. Heparin appeared to have little effect on the fraction of denuded surface reinvested by endothelium ± 5 and 10 days (Fig. 4). Heparin-treated animals had slightly less endothelial regeneration at 5 days, but the differences between heparin-treated and control groups were not significant at either time period.

Anticoagulant Activity and Myointimal Thickening

Clotting times were prolonged to approximately 2.5 times control values after administration of either unfractionated heparin or the anticoagulant fraction (Fig. 5). Two of the eight rats given nonanticoagulant heparin had clotting times slightly outside the normal range (7.5 and 7.8 minutes vs. a range of 3.7 to 5.8 minutes in control rats), but the mean log clotting times of nonanticoagulant heparin groups were not significantly different from controls.

Figure 6 shows light micrographs of myointimal thickening in a control rat and a rat given nonanticoagulant heparin. Determinations of intima-media volume ratios (Fig. 7 A and B) clearly showed inhibition of myointimal thickening in the high-dose nonanticoagulant heparin group (77% reduction in plaque size, $P = 0.02$ vs. controls) as well as in the unfractionated heparin group (64% reduction in plaque size, $P = 0.02$ vs. controls). There was also a significant difference between the group given the anticoagulant heparin fraction and the high-dose nonanticoagulant heparin group ($P = 0.04$). Scheffé analysis also allows a posteriori comparisons of combinations of groups; results are given in the legend to Figure 7.

Figure 7a presents data on intima-media volume ratios in individual arteries, plotted against the total cumulative dose of heparin in terms of mass. A negative correlation between mass dose of heparin and plaque growth is evident on visual inspection.

As shown in Figure 7b, the unfractionated heparin group tended to show greater inhibition of
plaque growth than the nonanticoagulant heparin group given the same mass dose (NAC1) and less inhibition than the group given a higher dose of nonanticoagulant heparin (NAC2). However, these trends did not reach statistical significance.

Discussion

Our results confirm earlier work (Clowes and Karnovsky, 1977), in demonstrating an inhibitory effect of heparin on intimal smooth muscle cell proliferation, which can account for as much as 77% inhibition of growth in terms of plaque volume. With the same progressive dosage regime used to demonstrate this effect, heparin had little or no effect on platelet adherence or endothelial regeneration. Experiments with heparin fractions differing in their binding to antithrombin showed that the antiproliferative effect of heparin is not related to its anticoagulant activity.

Role of Platelets

It is likely that platelets did play a role in initiating smooth muscle cell proliferation in this study, as has been demonstrated in other models of arterial endothelial denudation (Moore et al., 1976; Friedman et al., 1977). Heparin conceivably could interfere with platelet-mediated activation of smooth muscle cells either by inhibiting platelet attachment to subendothelium and granule release or by preventing platelet release products from exerting their effects on the smooth muscle cells. There are several reasons to believe that the antiproliferative effect of heparin in vivo is not mediated through the former mechanism—i.e., de-
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Figure 4 Endothelial regeneration in control and heparin-treated rats assessed by scanning electron microscopy 5 and 10 days after air-drying injury to the right carotid artery. Mean ± SEM are shown. Numbers of rats in each group are in parentheses. Means for the heparin-treated groups (\( \bar{x}_h \)) and control (\( x_c \)) and 95% confidence intervals (C.I.) for the difference between means were as follows: At 5 days, \( \bar{x}_h = 0.43, x_c = 0.54, \text{C.I.} = -0.28 \) to +0.08. At 10 days, \( \bar{x}_h = 0.72, x_c = 0.73, \text{C.I.} = -0.22 \) to +0.21.

Figure 5 Whole blood clotting times after 2 weeks of continuous infusion of various forms of heparin. The scale is logarithmic, and geometric means ± SEM are shown. Groups are as in Table 1. UF = unfractionated heparin, AC = anticoagulant heparin, NAC1 = nonanticoagulant heparin at equivalent mass dose, NAC2 = high-dose nonanticoagulant heparin. Using Student’s t-test with correction for unequal variances, a significant difference from control was found for UF (P = 0.003), but not for AC (P = 0.07) or any other group. Filled circles represent anticoagulated groups; unfilled circles represent nonanticoagulated groups.

Figure 6 Appearance of intimal plaque near the midpoint of injured arterial segment. A: Control rat. Arrow indicates internal elastic lamina. B: Rat given high-dose nonanticoagulant heparin. Arrow indicates internal elastic lamina. Toluidine blue, 490X; bar = 20 \( \mu \text{m} \).

rangement of platelet function. First, heparin inhibits smooth muscle cell growth in vitro in the presence of platelet products (R.L. Hoover, R.D. Rosenberg, and M.J. Karnovsky, unpublished observation). Second, heparin was not administered in this study until 24 hours after carotid air-drying injury, with the exception of the high-dose nonanticoagulant heparin group. Endothelial desquamation and platelet adherence are actually seen within minutes after air-drying injury to the rat carotid artery and reestablishment of blood flow. Complete coverage of the denuded surface and degremination of platelets follow within a few hours (unpublished observations). Subsequent turnover of platelets at the arterial surface probably is relatively slow, as shown by Groves and co-workers (1979) after balloon catheter endothelial denudation in the rabbit. Friedman (1977) has reported that severe thrombocytopenia initiated 1 day after endothelial denudation in the rabbit aorta does not inhibit the smooth muscle cell proliferative response. This fact suggests that platelets may trigger the smooth muscle cell response, but are not necessary to sustain it. Together, all these observations suggest that the major antiproliferative effect of heparin is likely to occur at some step subsequent to platelet degranulation.

In the absence of massive hemorrhage, heparin did not appear to affect platelet adherence to the arterial wall, observed 5 days after endothelial denudation and 4 days after the onset of heparin administration. This is in accord with the finding of Essien and co-workers (1978) that heparin did not
Figure 7  Intima-media volume ratios 2 weeks after air-drying carotid injury in rats continuously infused with various forms of heparin. A: Intima-media volume ratios for individual rats are shown plotted against total cumulative mass dose of heparin administered. Filled circles represent anticoagulated rats, which received either purified anticoagulant heparin (84 mg/kg) or unfractionated heparin (177 mg/kg). These two groups had equal mean log clotting times (Fig. 5). Unfilled circles represent nonanticoagulated rats that received either no heparin or nonanticoagulant heparin. Inspection shows that inhibition of plaque growth correlates with mass dose of heparin and not with anticoagulant activity. B: Groups of rats are as shown in Table 1 and Figure 5. Numbers of rats in groups are shown in parentheses. Means ± SEM were obtained after transformation of individual data to the fourth root. P values for significant differences between groups, corrected for multiplicity of comparisons by the Scheffé method, are as follows: Control vs. UF, P = 0.02; control vs. NAC2, P = 0.02; AC vs. NAC2, P = 0.04; control vs. NAC1 + NAC2, P = 0.03; control + AC vs. NAC1 + NAC2, P = 0.01; control + AC vs. UF + NAC1 + NAC2, P = 0.002.

Effectiveness of Nonanticoagulant Heparin

The most important conclusion of this study is that inhibition of smooth muscle cell proliferation by heparin does not require anticoagulant activity. Our in vitro data support this conclusion, and it is also consistent with the studies of Lippman and Mathews (1977) on L-M cells in tissue culture. They demonstrated a lack of correlation between anticoagulant and antiproliferative effects of various types of heparin. It may be inferred that thrombin, despite its potent mitogenic effect in fibroblast tissue culture (Chen and Buchanan, 1975), probably does not play a major or necessary role in the smooth muscle cell proliferative response. A modulating role for thrombin is not ruled out by the data, particularly in view of the trend toward a greater nonproliferative effect of unfractionated heparin as opposed to nonanticoagulant heparin at the same mass dose (NAC1 group in Fig. 7).

The data from this in vivo study do not answer the question of whether the purified anticoagulant heparin fraction might inhibit myointimal proliferation as effectively as the nonanticoagulant heparin fraction. Recent in vitro work in our laboratory suggests that, in fact, the two fractions have equal antiproliferative potency (R.L. Hoover, R.D. Rosenberg, and M.J. Kamovsky, unpublished observation). The maximum mass dose of purified anticoagulant heparin that could be administered safely in vivo appears to be below the threshold necessary for a distinct antiproliferative effect, as shown in Figure 7b.

Although heparin is a mast cell product and has not been found in the intima or media of arteries, other glycosaminoglycans, including the closely related compound, heparan sulfate, are present at high concentrations (Smith, 1973). Some of these glycosaminoglycans may be inhibitory for smooth muscle cell growth, as suggested by Eisenstein and colleagues (1979). Wasteson and co-workers (1977) have demonstrated that platelets contain an enzyme that specifically degrades heparin and heparan sulfate. It is possible that this platelet enzyme might act as a growth factor in concert with the well-known small cationic polypeptide growth factor (Ross and Vogel, 1978). Whatever the initial stimulus for medial smooth muscle cell activation, we may speculate that those cells which migrate to the intima might be exposed to an environment...
which lacks the usual inhibitory influence of certain medial glycosaminoglycans on cell division and therefore might continue to proliferate for some time after the initial stimulus (perhaps platelet factors) has subsided. Exogenous heparin administration in our study may have acted to restore the physiological inhibitory influence.

An alternative hypothesis for the action of heparin, that it may bind the cationic platelet-derived growth factor before it can contact smooth muscle cells, is considered doubtful. For reasons discussed above, it seems likely that the greatest quantity of platelet-derived growth factor would have reached the surface of smooth muscle cells during the first 24 hours, before heparin was given in most of our experiments. Also, in vitro work suggests that contact between heparin and the cell surface is a critical step in the antiproliferative effect (R.L. Hoover, R.D. Rosenberg, and M.J. Karonovsky, unpublished observation).

**Possible Effects of Heparin on Atherosclerosis and Other Conditions**

Studies on the inhibition of dietary atherosclerosis by heparin or heparin-like compounds have yielded variable results (Hess, 1964). Those with positive results have generally regarded plasma lipid changes due to heparin as the ameliorating factor in the atherosclerotic process. Interestingly, two recent studies with positive results [Besterman (1970) and Grossman et al. (1971)] utilized heparin-like compounds chosen for their lack of anticoagulant activity—a sulfated polysaccharide from seaweed and a heparan sulfate by-product from the commercial manufacture of beef lung heparin, respectively.

It would be dubious to propose nonanticoagulant heparin as a treatment for atherosclerosis. In certain clinical circumstances, however, intimal smooth muscle cell proliferation may occur much more rapidly than in ordinary atherosclerosis and thus be amenable to pharmacological inhibition. This situation may occur in some patients after saphenous vein coronary artery bypass grafting (Kern et al., 1972; Lawrie et al., 1976) or after arterial embolectomy by Fogarty balloon catheter (Chidi and DePalma, 1978).

This study suggests that heparin inhibition of arterial smooth muscle proliferation occurs through a direct process and is not mediated by effects on other cells or by antithrombin. Thus the antiproliferative effect of heparin should direct attention to the potential growth regulatory role for arterial glycosaminoglycans, but not toward such a role for the humoral clotting system.

**Acknowledgments**

We gratefully acknowledge the advice of Dr. Raymond K. Neff on statistical methods and the efforts of Bernard Corrow in the design and manufacture of the infusion apparatus, Robert Collazzo and Steven Greenberg in technical assistance, Robert Rubin in photography, and Kay Congrove in manuscript preparation.

**References**


Rosenberg RD, Lam L (1979) Correlation between structure and function of heparin. Proc Natl Acad Sci USA 76: 1218-1222


Inhibition of Rat Arterial Smooth Muscle Cell Proliferation by Heparin

II. In Vitro Studies

R.L. Hoover, R. Rosenberg, W. Haering, and M.J. Karnovsky

SUMMARY We studied in vitro the effects of heparin on the growth of rat aortic smooth muscle cells. Measurements of growth were monitored by [3H]thymidine uptake and changes in cell number over a period of 3 days. Our results show that heparin—highly anticoagulant or nonanticoagulant—significantly inhibits growth of smooth muscle cells. We also show that this is a highly specific interaction with regard to molecule and cell type: i.e., other polyanions, except for a low molecular weight dextran sulfate, do not have the same effect on growth, and not all cells are inhibited by heparin; e.g., endothelial cell growth actually is enhanced. After removing antithrombin from our media, we carried out experiments which show that heparin is effective even though thrombin, a potent mitogenic agent, is still present and active. We also found that passing the platelet extract over a heparin column did not remove all of the mitogenic activity of the platelet preparation. Both experiments indicate an inhibitory role for the heparin molecule, per se. Our results support the findings of a recent paper (Guyton et al., 1980) showing that heparin can limit the size of myointimal plaques in rats after carotid injuries by inhibiting smooth muscle cell proliferation. Circ Res 47: 578-583, 1980

THE proliferation of smooth muscle cells in blood vessels after endothelial injury contributes to the formation of a myointimal plaque which may play an important role in atherosclerosis (Ross and Glomset, 1973). Regulation of this proliferation, therefore, may help prevent the formation of atherosclerotic plaques or at least reduce their size. Clowes and Karnovsky (1977) have shown that if heparin is administered to rats whose carotid arteries have been injured in order to produce a myointimal plaque, the size of the myointimal thickening is dramatically reduced. In a recent paper, Guyton et al. (1980) have shown that the effect of heparin on the injured arterial wall is primarily to inhibit smooth muscle cell growth, and that this effect is not related to anticoagulant activity. Molecules similar to heparin also have inhibited the growth of a variety of cells in tissue culture (Goto et al., 1979; Lippman and Mathews, 1977). The mechanism by which heparin and heparin-like molecules affect cell proliferation is not known. It is possible that because of the high affinity for antithrombin, anticoagulant properties may be involved. It also has been shown that heparin binds to the surface of cells (Hiebert and Jacques, 1976), and this may alter permeability to ions necessary for growth, change conformation of molecules to which it binds (Villanueva and Danishefsky, 1977), or affect cell volume (Norman and Norrby, 1971). Since heparin is a highly charged molecule, it also could be interacting with other charged molecules which affect growth. For example, Ross et al. (1974) have shown that the addition of material released from platelets enhances cell growth; therefore, it is possible that the antiproliferative action of heparin is caused when it binds to these factors and prevents their interaction with the cell surface.

In this paper we examine, in vitro, the effects of heparin on rat aortic smooth muscle cell growth. We consider whether there is a specificity with regard to the molecule and to the cell type, in particular, smooth muscle cells, and whether interaction with antithrombin plays a role. We also investigate what role the interaction with a platelet extract may play and whether the mechanism of action is mediated through the cell surface. Our results support the findings of the in vivo study in an earlier paper (Guyton et al., 1980) which show that heparin inhibits smooth muscle cell growth and limits the size of myointimal plaques.

Methods

Isolation and Culture of Smooth Muscle Cells

Smooth muscle cells are isolated from aorta of Sprague-Dawley (Charles River, CD strain) rats by carefully stripping pieces of the intima and inner media and incubating in RPMI-1640 media supplemented with 20% fetal calf serum (FCS) plus penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin (0.25 µg/ml). After 1-2 weeks in culture, the smooth muscle cells migrate out of the tissue and begin to proliferate. Enzymes are not...
used initially to separate the cells. Once confluent, the cells are passaged and incubated in a special medium in which D-valine has been substituted for L-valine. Based on our own experiments and those of Gilbert and Migeon (1975), fibroblasts will not grow but smooth muscle cells will. These cells have been examined by electron microscopy and appear identical to the vascular smooth muscle cells described by others (Gimbrone and Cotran, 1975; Jones et al., 1979), i.e., numerous myofilament bundles and vesicles near the surface membrane. After this, the cells are maintained in RPMI-1640 medium + 20% FCS and passaged every 3-4 days. Cultures beyond the 15th passage are not used.

Growth Measurements

Cells are plated sparsely at 30,000-50,000 cells/ml in growth medium in 35-mm Petri dishes or Costar cluster dishes (#3524). After 24 hours at subconfluency, the cells are washed and RPMI-1640 medium and 0.4% serum are added to arrest cell growth. The cells then are incubated for another 24-48 hours. At the end of this incubation, the medium is replaced by the experimental solutions prepared in RPMI-1640 medium + 0.4% FCS. Growth is measured after 1, 2, and 3 days by direct cell counts and uptake of [3H]thymidine, as outlined by Chen and Buchanan (1975). These same procedures were used to look at the growth of calf aortic endothelium and BHK fibroblasts, the only difference being that the BHK cells were incubated with Dulbecco’s modified essential medium (DMEM) supplemented with 0.1% FCS, as the serum requirements for BHK cells are lower than for smooth muscle and endothelium. In these experiments, as with those of the smooth muscle cells, the endothelium and the BHK cells were tested at subconfluency.

All data have been subjected to a two-way analysis of variance for the purpose of determining statistical significance between the test groups. The analyses compared the growth values of the controls to those of the experimental groups. The analyses take into account not only experimental manipulation but also the time element; i.e., each value is compared to measurements made at the same time and then compared with the data from the other time points. In all experiments, we found that time was not a factor and that significance depended only on treatment to cells. All significant values have a P value of at least 0.01.

Preparation of Platelet Extract

Platelets are isolated by the methods of Tollefsen et al. (1974) from Sprague-Dawley rats. The withdrawn blood is added to a solution of ethylenediaminetetraacetic acid (5 mM final conc.) and centrifuged for 3 minutes at 1400 g. The supernatant is removed and spun at 2250 g for 15 minutes. The pellet of this centrifugation is suspended in phosphate-buffered saline (pH 6.5) containing no magnesium or calcium but including 5.5 mM glucose and 5 mg/ml bovine serum albumin. The suspension is centrifuged at 120 g for 10 minutes, and the pellet containing the leukocytes and erythrocytes is discarded. The platelet suspension then is washed 2x in the above-described phosphate saline and resuspended at a concentration 10⁶/ml in RPMI-1640 containing no serum. Under these conditions, according to Tollefsen et al. (1974), the platelets do not release their granules, as monitored by the absence of serotonin in the medium. The growth factor(s) then is released by freezing and thawing 6x. This solution is centrifuged for 20 minutes at 2250 g and sterilized by filtration through a Millipore filter, 0.22 μm pore size, which also removes any remaining particulate matter. In all experiments using this extract, 5 ml were added to 95 ml of medium.

Preparation of Purified Anticoagulant and Non-anticoagulant Heparins

Commercial heparin is separated into two major fractions based on anticoagulant activity by passing the material over a Sepharose-antithrombin column. The procedure is that described by Lam et al. (1976) and is outlined extensively by Guyton et al. (1980). In summary, the anticoagulant fraction which has a high affinity for antithrombin stays on the column while the nonanticoagulant fraction of low affinity passes through.

Results

Figure 1 shows results of a typical experiment involving controls (RPMI-1640 medium + 0.4% FCS), platelet extract, platelet extract plus heparin,
and 20% FCS, and provides the parameters for all subsequent experiments. The presence of heparin reduces the cell number, whereas growth in 20% FCS produces the greatest number of cells. We also found that $[^{3}H]$thymidine uptake mimicked these results and was used in conjunction with the data-monitoring change in cell number. In both methods, due to the variability in the cultures with increasing passage number, size of inoculum, differences in serum batches, etc., the data have been based on controls with RPMI-1640 + 0.4% FCS.

The addition of heparin to smooth muscle cultures inhibits growth (Table 1). As the concentration is increased from 5 μg/ml up to 20 μg/ml, the inhibitory effect increases. All values are significantly different from one another ($P < 0.01$) except at 10 and 15 μg/ml. All subsequent experiments were carried out using heparin at a concentration of 10 μg/ml because this corresponds to the in vivo situation, where this concentration is achieved readily.

Heparin can be separated into anticoagulant and non-anticoagulant fractions. When these are tested in our system, they produce similar effects on growth (Table 2). In each case, growth is inhibited by 50–60%. Because of variability in cultures, growth has been normalized to control conditions. Also, in all experiments, samples were tested with RPMI-1640 medium plus 0.4% FCS.

We have also considered whether heparin affects other cells besides smooth muscle. We have tested bovine aortic endothelium and BHK fibroblasts and found contrasting results (Table 3). In the case of the fibroblasts, heparin inhibited growth to the same extent as the smooth muscle, but with endothelium, there is no inhibitory effect—rather, the heparin significantly stimulates growth. Platelet extract alone had no significant effect on the uptake of $[^{3}H]$thymidine by endothelium.

Thrombin has been shown to be mitogenic for several cell types in vitro (Chen and Buchanan, 1975; Bohjanpelto, 1977) and, in preliminary experiments, it enhanced $[^{3}H]$thymidine uptake in our smooth muscle system. Therefore, by adding heparin, which binds to antithrombin and complexes with thrombin, we may be inhibiting growth because the thrombin is now inactive. However, two different experiments indicate that this is not the case (Table 4). First, if serum is depleted of antithrombin by passing over a heparin column and tested for growth with and without heparin, inhibition occurs only in the heparin cultures. In this case, thrombin still should be active. Second, the addition of antithrombin, which would complex the thrombin, to the system does not enhance the heparin.

**Table 1 Growth of Smooth Muscle Cells in the Presence of Heparin as Monitored by $[^{3}H]$Thymidine Uptake**

<table>
<thead>
<tr>
<th>Cell growth with following heparin concentration</th>
<th>5 μg/ml</th>
<th>10 μg/ml</th>
<th>15 μg/ml</th>
<th>20 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative growth values</td>
<td>0.79 ± 0.13</td>
<td>0.63 ± 0.18</td>
<td>0.63 ± 0.01</td>
<td>0.52 ± 0.07</td>
</tr>
</tbody>
</table>

Values (means ± SE) are expressed as a fraction of controls containing RPMI-1640 medium plus 0.4% FCS and rat platelet extract (5%). Number of measurements for each concentration of heparin, $n = 18$, which represents at least three separate experiments (as per experiment ≥ 6).

**Table 2 $[^{3}H]$Thymidine Uptake of Smooth Muscle Cells When Grown in the Presence of Various Glycosaminoglycans**

<table>
<thead>
<tr>
<th>Glycosaminoglycan</th>
<th>Relative growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
</tr>
<tr>
<td>—Without platelet extract</td>
<td>0.56 ± 0.09*</td>
</tr>
<tr>
<td>Nonanticoagulant-heparin</td>
<td>0.63 ± 0.07*</td>
</tr>
<tr>
<td>Anticoagulant heparin</td>
<td>0.60 ± 0.04*</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>1.07 ± 0.05†</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>1.06 ± 0.04†</td>
</tr>
<tr>
<td>Low molecular wt* dextran sulfate</td>
<td>0.61 ± 0.04*</td>
</tr>
<tr>
<td>High molecular wt† dextran sulfate</td>
<td>0.99 ± 0.07†</td>
</tr>
<tr>
<td>Protamine sulfate*</td>
<td>0.99 ± 0.05†</td>
</tr>
</tbody>
</table>

All values are based on controls containing 5% platelet extract, concentrations are 10 μg/ml for all compounds in RPMI-1640 medium + 0.4% FCS.

* Values which are significantly different from controls, $P < 0.01$.

Number of experiments for each glycosaminoglycan is at least 3, representing 18 measurements.

† Values which are not significantly different from controls, $P > 0.10$.

$n \geq 18$, representing at least three separate experiments.

‡ Standards obtained from Dr. Anthony Cifonelli, University of Chicago.

§ Obtained from Dr. P. Petrcezak, Riker Lab., Minneapolis, MN.

‖ Obtained from Sigma Chemical Co., St. Louis, MO.

**Table 3 The Effects of Heparin (10 μg/ml) on Growth of Calf Aortic Endothelium and BHK Fibroblasts**

<table>
<thead>
<tr>
<th></th>
<th>BHK</th>
<th>Endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>No platelet extract</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Platelet extract (5%)</td>
<td>1.00 ± 0.08*</td>
<td>0.90 ± 0.11*</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.63 ± 0.07†</td>
<td>1.44 ± 0.06†</td>
</tr>
<tr>
<td>Heparin and platelet extract</td>
<td>0.63 ± 0.07†</td>
<td>0.92 ± 0.14*</td>
</tr>
</tbody>
</table>

Basic medium for BHK cells was DMEM + 0.1% FCS and for endothelium, RPMI + 0.4% FCS.

Number of experiments is at least 3 with a total of 18 measurements for each cell type and condition.

† Values significantly different from controls, $P < 0.01$.

* Values not significantly different from controls, $P > 0.10$. 

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Table 4 The Effects on Growth of the Addition of Antithrombin (0.1 μg/ml) to Cultures of Smooth Muscle Grow with and without Heparin (10 μg/ml)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Growth Rate (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI + 0.4% FCS + platelet extract (PE)</td>
<td>1.00</td>
</tr>
<tr>
<td>RPMI + 0.4% FCS + PE + antithrombin</td>
<td>0.97 ± 0.09*</td>
</tr>
<tr>
<td>RPMI + 0.4% FCS + PE + antithrombin</td>
<td>0.77 ± 0.07†</td>
</tr>
<tr>
<td>RPMI + 0.4% FCS + PE + heparin</td>
<td>0.71 ± 0.08†</td>
</tr>
</tbody>
</table>

Fetal calf serum had been passed through a heparin-Sepharose column (equilibrated with 0.15 M NaCl/0.01 M Tris-HCl, pH 7.5) to remove the antithrombin.
* Values not significantly different from controls, P ≥ 0.10, n ≥ 18 representing at least three different experiments with six or more measurements/experiment.
† Values significantly different from controls, P ≤ 0.01.

Discussion

In a previous paper, Clowes and Karnovsky (1977) have shown that heparin inhibits the size of plaques formed in a rat carotid artery that has been injured. It also has been shown that the heparin effect is not related to its anticoagulant properties (Guyton et al., 1980). In this study, we have shown:
1. that these same heparins will inhibit the proliferation of smooth muscle cells in vitro;
2. that the only other polyanion to have an effect was a small dextran sulfate similar to heparin in charge and degree of sulfation;
3. that there is an inhibitory effect on fibroblasts similar to heparin in charge and degree of sulfation;
4. that the mechanism of action is not mediated through an interaction with antithrombin;
5. that interaction with platelet growth factors is not solely responsible for the heparin effect; and
6. that there is probably an interaction of the heparin with the cell surface.

Factors isolated from platelets can cause proliferation of several cell types (Rutherford and Ross, 1976). In this paper, we show that this stimulation is also true for growth of rat smooth muscle cells. It would follow from this, that interfering with these factors could result in an inhibition of growth; therefore, heparin might be inhibiting proliferation by complexing with the platelet growth factors. Our results show that after the platelet extract is passed through a column to remove heparin-binding molecules and added back to cell cultures, growth still continues. This indicates that heparin does not bind tightly to all factors necessary for growth and that the inhibition is not mediated merely through a depletion of these factors. However, the results do not rule out the possibility that heparin might be binding to the cell surface and preventing access to receptors. In fact, cells preincubated with heparin before addition of the platelet extract exhibit an inhibition, suggesting the above mechanism may be

Table 5 The Effect on Growth of Smooth Muscle Cells using Platelet Extract (PE) Passed over a Sepharose-Heparin Column

<table>
<thead>
<tr>
<th>Condition</th>
<th>Growth Rate (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI + 0.4% FCS</td>
<td>1.00</td>
</tr>
<tr>
<td>RPMI + 0.4% FCS + 5% platelet extract</td>
<td>1.44 ± 0.03*</td>
</tr>
<tr>
<td>RPMI + 0.4% FCS + 5% platelet extract from heparin column</td>
<td>1.40 ± 0.06*</td>
</tr>
</tbody>
</table>

Because the platelet extract was diluted after passage through the heparin-Sepharose column, the control PE was diluted to the same extent with the equilibration buffer, 0.15 M NaCl/0.01 M Tris-HCl, pH 7.5.
* Values significantly different from controls, P ≤ 0.01, n ≥ 18, representing at least three separate experiments with six measurements/experiment.

Table 6 The Effects of Preincubation of Heparin (10 μg/ml) on Growth of Smooth Muscle Cells as Monitored by Cell Number

<table>
<thead>
<tr>
<th>Condition</th>
<th>Growth Rate (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI + 0.4% FCS + PE</td>
<td>1.00</td>
</tr>
<tr>
<td>RPMI + 0.4% FCS</td>
<td>0.74 ± 0.01*</td>
</tr>
<tr>
<td>RPMI + 0.4% FCS + PE preincubated 1 hr</td>
<td>0.88 ± 0.07*</td>
</tr>
<tr>
<td>RPMI + 0.4% FCS + PE preincubated 24 hr</td>
<td>0.68 ± 0.06*</td>
</tr>
<tr>
<td>with heparin (10 μg/ml)</td>
<td></td>
</tr>
</tbody>
</table>

Values are based on a comparison between controls with platelet extract (PE) which had been incubated concurrently with each of the experimental conditions.
* Values significantly different from controls, P ≤ 0.01. Number of measurements for each condition is at least 18, representing three separate experiments with six measurements each.
true. In preliminary experiments, however, we have found that incubating the cells first with platelet extract and then adding heparin (from 1-12 hours later) inhibits growth. Furthermore, the in vivo studies of Clowes and Karmovsky (1977) and Guyton et al. (1980) show that heparin, when administered to animals beginning 24 hours after carotid injuries, inhibits the size of the myointimal thickening. This suggests that the heparin and the platelet growth factor(s) bind quickly and prime the cells for division (Pledger et al., 1977); yet, the addition of heparin to cultures that had been incubated with platelet extract still inhibited growth.

The results in Figure 1 also indicate that the inhibition caused by heparin occurs in the first 24 hours and that no growth occurs after that point. At approximately the same time, growth of cells in 0.4% fetal calf serum (with and without platelet extract) also subsides. Cells in 20% fetal calf serum, however, continue to grow until confluence, or to at least 48 hours. The addition of heparin to cultures with 20% serum does not affect growth during the first 24-48 hours, at which time a steady decline in growth rate begins (John Castellot, personal communication). Although we do not know the reason for this difference in the kinetics of the heparin effects, we suggest that it may be related to the interactions between heparin and the serum components, and at present are carrying out experiments to test this possibility.

Antithrombin binds very easily to heparin (Rosenberg, 1977) which, in turn, combines with thrombin to inhibit proteolytic activity. It is possible that this interaction has an effect on smooth muscle proliferation because of the known mitogenic properties of thrombin (Chen and Buchanan, 1975; Carney et al., 1978). However, our results, in which serum was depleted of antithrombin and growth continued, indicate that this is not the case. In the absence of antithrombin (active thrombin), heparin limited cell growth, and with the reintroduction of antithrombin into heparin-treated cultures, there was no enhanced inhibition. Apparently, the action of heparin is not mediated through thrombin inactivation.

We also know that, even though we use the term "depleted" with respect to antithrombin, a very small amount of antithrombin remains after the heparin column treatment—by our calculations, about 1%. This measurement is based on the neutralization of thrombin as outlined by Damon and Rosenberg (1976). Briefly, the sample is incubated with thrombin and the activity quantified by measuring the time needed to clot a fibrinogen solution. If one considers there are approximately 250 µg of antithrombin per ml at the start, then the final concentration would be 2.5 µg/ml. This is then used at a concentration of 0.4% in our media, giving a final concentration of 10 ng/ml; however, because of molecular size, antithrombin does not bind to heparin at a ratio of 1:1, rather 3:1. This means that the heparin-antithrombin complex would account for about 3-4 ng of heparin and would have essentially no effect in our experiments. Furthermore, the concentration of the antithrombin is so low in our medium and the binding constants such, that there is little chance of interaction with heparin. There is always the possibility that other molecules besides antithrombin are removed, but even if it were true, this does not affect the growth responses to heparin or platelet extract.

The specificity of the heparin molecule is demonstrated by the fact that all other polyanions tested have no effect, except a small dextran sulfate. It is interesting to note that this compound is similar in molecular weight and sulfation to heparin, whereas the others are not. The lack of inhibition by these other molecules also indicates that an interaction due to charge per se between the cell and heparin or between growth factors and heparin is probably not the mechanism involved in this effect. If it were, then the other molecules of similar charge would show inhibition. Apparently, the secondary structure or charge distribution of the molecules plays an important role. Goto et al. (1977) also have indicated that the specific structure of polyanions may play a part in determining saturation density.

The inhibition of growth by heparin in our experiments appears specific for smooth muscle cells and BHK fibroblasts because, in contrast to this, the growth of calf aortic endothelium is stimulated in the presence of heparin. These differential growth effects may be very important in the formation of atherosclerotic plaques because, once endothelium covers the smooth muscle cells, proliferation decreases (Schwartz et al., 1978). The effects of heparin, therefore, are amplified by increasing the growth of the endothelium and inhibiting that of smooth muscle which, as a result, reduce the size of the plaques. Indeed, we have found in preliminary experiments that conditioned media from endothelial cultures inhibit the growth of smooth muscle cells, suggesting a regulatory role for endothelium (Castellot et al., 1979).

This heparin-induced increase of endothelial growth differs from the results in the in vivo study (Guyton et al., 1980), which showed no endothelial enhancement. Clowes and Karmovsky (1977) similarly had found no endothelial growth increase and suggested that endothelial regeneration requires migration and mitosis; therefore, in the in vivo situation, migration may have been affected, restricting endothelial overgrowth, whereas in in vitro experiments, no such restrictions occurred.

The interaction of heparin with the cell surface probably initiates the inhibitory effects observed. Evidence for this comes from our data which show that if the cells were preincubated in heparin for as short a time as 1 hour and were washed before...
addition of the growth extract, inhibition occurred. In addition, we have found, in preliminary experiments, that heparin bound to Sepharose beads (Jordan et al., 1979) causes an inhibition of growth, although the response is not as great as when the heparin is added directly. We also have found recently that radiolabeled heparin binds readily to the cell surface of smooth muscle cells. At present we are considering whether there are specific receptors on the cell surface for heparin and whether there are any changes in morphology and physiology of the cells after binding of heparin.

Acknowledgments

We would like to acknowledge Dr. Raymond Neff for his assistance with the statistical analyses. We also want to thank Kay Cosgrove for her help in preparation of the manuscript and Betsy Renshaw for her technical assistance.

References


Cultured Endothelial Cells Produce a Heparinlike Inhibitor of Smooth Muscle Cell Growth

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Abstract Using cultured cells from bovine and rat aortas, we have examined the possibility that endothelial cells might regulate the growth of vascular smooth muscle cells. Conditioned medium from confluent bovine aortic endothelial cells inhibited the proliferation of growth-arrested smooth muscle cells. Conditioned medium from exponential endothelial cells, and from exponential or confluent smooth muscle cells and fibroblasts, did not inhibit smooth muscle cell growth. Conditioned medium from confluent endothelial cells did not inhibit the growth of endothelial cells or fibroblasts. In addition to the apparent specificity of both the producer and target cell, the inhibitory activity was heat stable and not affected by proteases. It was sensitive to Flavobacterium heparinase but not to hyaluronidase or chondroitin sulfate ABC lyase. It thus appears to be a heparinlike substance. Two other lines of evidence support this conclusion. First, a crude isolate of glycosaminoglycans (TCA-soluble, ethanol-precipitable material) from endothelial cell-conditioned medium reconstituted in 20% serum inhibited smooth muscle cell growth; glycosaminoglycans isolated from unconditioned medium (i.e., 0.4% serum) had no effect on smooth muscle cell growth. No inhibition was seen if the glycosaminoglycan preparation was treated with heparinase. Second, exogenous heparin, heparan sulfate, chondroitin sulfate B (dermatan sulfate), chondroitin sulfate ABC, and hyaluronic acid were added to 20% serum and tested for their ability to inhibit smooth muscle cell growth. Heparin inhibited growth at concentrations as low as 10 ng/ml. Other glycosaminoglycans had no effect at doses up to 10 μg/ml. Anticoagulant and non-anticoagulant heparin were equally effective at inhibiting smooth muscle cell growth, as they were in vivo following endothelial injury (Clowes and Karnovsky. Nature (Lond). 265:625-626, 1977; Guyton et al. Circ. Res. 46:625-634, 1980), and in vitro following exposure of smooth muscle cells to platelet extract (Hoover et al. Circ. Res. 47:578-583, 1980). We suggest that vascular endothelial cells may secrete a heparinlike substance in vivo which may regulate the growth of underlying smooth muscle cells.

A characteristic feature of the normal, healthy arterial wall is that the intimal endothelial cells form a continuous quiescent monolayer, and the underlying medial smooth muscle cells also remain in a quiescent growth state. If the endothelium is damaged, smooth muscle cell proliferation occurs until the endothelium regenerates (9, 29). The regulation of cell growth in the vascular wall is poorly understood. Ross (15, 28) and others (10, 25) have shown that platelet-derived growth factors are required for smooth muscle cell growth in vivo and in vitro. Recently, conditioned medium from macrophages has been found to stimulate smooth muscle cells (23). Gajdusek et al. (11) have shown that endothelial cells produce a factor which stimulates the growth of smooth muscle cells. Eisenstein et al. (8) have found that extracts from the inner arterial wall can be fractionated to produce both stimulators and inhibitors of smooth muscle cell growth.

We present evidence demonstrating that cultured endothelial cells produce both positive and negative effectors of smooth muscle cell growth. The inhibitory activity appears to be heparin or a heparinlike molecule. These results suggest a possible role for heparin in the regulation of vascular smooth muscle cell growth in vivo.
MATERIALS AND METHODS

Purification of Heparinase

*Flavobacterium heparinum* were either grown as described by Linker and Hovingh (22) or were provided by Dr. R. Langer (Massachusetts Institute of Technology, Boston, Mass.). The cells were sonicated at a cell protein concentration of ~8 mg/ml for 12 min with a Branson Sonifier set at 125 W. The sonicate was centrifuged at 20,000 g at 4°C for 30 min. The resulting supernatant was treated with protease at a level of 15 mg/ml and then exhaustively dialyzed against distilled water.

The crude preparation was subsequently chromatographed on hydroxylapatite and cellulose-phosphate as outlined by Linker and Hovingh (22). We purified the enzyme further by concentrating with ultrafiltration, and then by filtration on Sephacryl S-200 equilibrated in 0.5 M NaCl in 0.01 M Tris-HCl, pH 7.5. The elution profile exhibited a major protein peak at a molecular weight of ~60,000 daltons, as well as several minor protein peaks of lower molecular size. Direct analysis of the first peak with respect to heparin-cleaving potency (22) revealed a constant specific activity of ~3,000 U/mg across the initial two-thirds of the profile. The remaining two components of lower molecular size possessed essentially no glycosaminoglycan (GAG)-cleaving ability. The final enzyme preparation exhibited no capacity to degrade chondroitin sulfate, dermatan sulfate, or hyaluronic acid when employed at concentrations as high as ~200 mg/ml (24).

There was no detectable protease activity in this preparation as measured by the radiolabeled casein assay (17).

Isolation of Bovine Aortic Heparan Sulfate

Glycosaminoglycans were obtained from blood vessels by extensive proteolytic digestion of the intima and media of calf aortas (26). Heparan sulfate was isolated free of other GAGs by chromatography on DEAE-Sephadex A-25 and Sepharose 4B (30). The resulting product was identified by the two-dimensional electrophoretic system of Hata and Nagai (16) in conjunction with appropriate standards. The nature of the heparan sulfate was confirmed by its sensitivity to digestion with bacterial heparinase and its resistance to the action of chondroitin ABC and testicular hyaluronidase (24).

Cell Culture

Bovine aortic endothelial cells (BAEC) were isolated from freshly slaughtered calves, as previously described (2, 13, 19). The cells obtained from one aorta are grown in a single 75-cm² tissue culture flask in RPMI-1640 medium containing 20% fetal calf serum (FCS), 4 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin (0.25 μg/ml), and gentamicin (50 μg/ml) at 37°C in a humidified, 10% CO₂ atmosphere. At confluence, >95% of the cells are endothelial, as determined by their distinctive morphology and the presence of hyaluronidase when employed at concentrations as high as ~200 mg/ml (24). There was no detectable protease activity in this preparation as measured by the radiolabeled casein assay (17).

Growth Arrest of SMC

Sparsely plated rat or calf SMC were washed and placed in RPMI + 0.4% FCS or DMEM + 0.2% CS for 72 h. Flow microfluorimetry (32) and [3H]thymidine uptake into DNA were used to determine that the cells were arrested in G₀/G₁.

Inhibition Assay

To assay for inhibitory activity, 6 x 10⁴ cells were plated into 16-mm multiwell plates and growth-arrested as described above. Control cultures were released from the G₀ block by placing them in RPMI + 20% FCS. Other cultures were placed in the 1:1 mixture of CM and RPMI + FCS (final concentration FCS 20%). Cell numbers were measured in duplicate samples at daily intervals by washing the cells once in a trypsin-EDTA solution (Grand Island Biological Co., Grand Island, N. Y.), then innoculating the cells for several minutes in trypsin-EDTA solution to dislodge the cells. The dislodged cells were collected and counted in a Coulter counter. Trypsinized cultures were routinely checked by direct microscopic examination to ascertain that the trypsinization procedure had not lysed the cells (as determined by the presence of cell debris) and to ensure that all cells were removed from the multwell. The cells were not fed during the experiment.

Mitogenesis Assay

To assay for mitogenic activity, exponential BAEC-CM was fractionated and concentrated 50-fold using Amicon XM100 and UM10 filters (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). A concentrated fraction was mixed 1:1 with RPMI + FCS (final concentration FCS 1%) and added to growth-arrested rat SMC. In some control experiments, cells were exposed to a 1:1 mixture of 50-fold concentrated RPMI + 0.4% FCS and RPMI + FCS (final concentration FCS 18%). In other control experiments, cells received RPMI + 20% FCS. Cell numbers were measured in duplicate samples at daily intervals using a Coulter counter as described above.

Chemicals

Chondroitin sulfate ABC lyase and Streptomyces hyaluronidase were purchased from Miles Laboratories Inc., Miles Research Products (Elkhart, Ind.). Unless otherwise stated, heparin was obtained from Upjohn Co., Agricultural Prods. (Kalamazoo, Mich.) or Elkins-Sinn, Inc. (Cherry Hill, N. J.); other GAGs were kindly supplied by Drs. M. Mathews and J. Cifonelli (University of Chicago). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Growth Arrest of SMC

SMC were plated and exposed to low serum as described in Materials and Methods. We used flow microfluorimetry and [3H]thymidine uptake into DNA to determine the cell cycle distribution of exponentially growing and serum-deprived SMC (32). The results will be published in detail elsewhere. In brief, >90% of the growth-arrested rat SMC cells have the G₀ DNA content. These cells are most likely arrested at the same point in the cell cycle (G₀), because growth-arrested cells placed in fresh complete medium undergo a synchronous round of DNA synthesis, with the earliest cells beginning S phase 16-18 h after the addition of serum. The peak of DNA synthesis occurs ~28 h after release. Flow microfluorimetry confirms the time course of this release from G₀. Calf SMC behaved similarly, except that the onset of S phase began 12-14 h after release, and peaked at ~20 h.

Effect of Confluent Primary BAEC-CM on SMC

Rat and calf SMC were growth-arrested as described in Materials and Methods. Control cultures were released from the G₀ block by replacing the low serum medium with RPMI

+ 20% FCS. Other cultures were exposed to the 1:1 mixture of confluent primary BAEC-CM and RPMI + FCS (final concentration FCS 20%). Cell growth after exposure to CM is presented in Fig. 1.

Rat SMC exposed to CM go through one to one and a half doublings at the same rate as control cells. After this initial growth, rat SMC stopped proliferating. Control cultures reach confluence at day 4 or 5 under these conditions. If the CM was replaced with normal growth medium at day 4 or 5, the cells proliferated to confluence.

Calf SMC exposed to CM also go through one or two rounds of division at about the same rate as control cells. At this point (usually day 3), the cells in CM often began to round up and slowly detach. Very few cells remained by day 5. Once the cells began to round up, replacing the CM with normal growth medium did not reverse this effect. In approximately one-third of the experiments, the calf SMC did not round up, but their growth plateaued, as shown in Fig. 1. The inhibitory effect was reversible, as the cells in these experiments proliferated to confluence if the CM was replaced with normal growth medium.

Direct observation of cells undergoing mitosis in CM-exposed rat and calf SMC cultures revealed that both daughter cells remained attached and spread normally after cytokinesis. Neither visual inspection nor Coulter counting of the medium indicated the presence of detached cells in CM-treated rat SMC and calf SMC cultures in which the cells did not round up. Flow microfluorimetry data indicate that both CM-treated and control SMC were arrested mainly in G1. (G1) at the endpoint of the experiment (data not shown). CM-treated SMC did not stain with trypan blue at any time during the course of the experiment. Finally, the possibility that the detachment of calf SMC is related to something present in the serum is suggested by experiments in which CM was mixed 1:1 with lower concentrations of FCS (final FCS concentrations, 10% and 5%). In these experiments, growth of control calf SMC (i.e., no CM) is reduced to three to four doublings in 10% FCS and two to three doublings in 5% FCS. CM-treated calf SMC did not go through even one doubling, and they did not round up and detach. Because of the potential difficulties in quantifying results of experiments in which calf SMC detached, subsequent data on calf SMC in this paper are based on experiments in which calf SMC did not round up and detach.

To test the possibility that simple nutrient depletion was causing growth inhibition, some experiments were done using a 1:1 mixture of CM and RPMI containing twice the normal nutrient concentration + FCS (final concentration FCS 20%). The results were the same as those in Fig. 1.

**Specificity of the CM Effect**

CM was collected from exponential and confluent cultures of BAEC, rat and calf SMC, and BHK cells as described in Materials and Methods. The volume of medium used for conditioning was 1 ml/10⁶ cells. All cell types tested for their responsiveness to CM were plated as described in Materials and Methods, except that BHK cells were arrested using 0.2% CS for 60 h. Rat SMC used in these experiments were second or third passage; calf SMC were first or second passage. BAEC were first through fifth passage, except that the confluent BAEC-CM was always collected from primary cultures. Cell numbers in duplicate cultures were measured at daily intervals after exposing cells to CM. The data are presented in Table 1 as the maximum percent inhibition. We obtain this figure by first subtracting the starting cell number (i.e., the cell number at the time the cells are released from G0) from the cell number at day 5, when growth has essentially ceased in both control and CM-exposed cells, and any growth inhibition was therefore maximal. The net growth in control cells is set at 100%, and the net growth in CM-treated cells is expressed as a percentage of the control. The difference is the maximum percent inhibition. We used this method of expressing our data to (a) compare more readily growth inhibition in experiments using different cell types or different types of CM, (b) to compare more easily the effect of various physical and biochemical treatments on the inhibitory activity, and (c) to calculate an ED₅₀ for heparin and other substances with inhibitory activity.

Confluent, primary BAEC-CM preferentially inhibited rat and calf SMC; BHK and BAEC were much less affected (Table 1). Other CM did not substantially inhibit SMC growth. It should be pointed out that when exponential cells are used in 0.4% serum they begin the transition from proliferation to quiescence. We cannot rule out the possibility that a growth factor(s) secreted by truly exponential cells is lost or masked during this transition. With this reservation in mind, the data demonstrate an apparent specificity of the inhibitory activity for both the producer and the target cell.

**Serum Requirements for Release and Effectiveness of the Inhibitory Activity**

When we allowed confluent primary BAEC to condition serum-free medium for 48 h, the inhibitory activity decreased to 10–20% of the activity found in CM containing 0.4% serum.
The overall metabolic rate of confluent BAEC in serum-free medium as measured by the rate of protein synthesis was decreased by only 30% (data not shown). This suggests that production of the inhibitory activity by BAEC depends to a large extent on the presence of serum. FCS and CS were equally effective in releasing the inhibitory activity.

If growth-arrested calf SMC were exposed to a 1:1 mixture of BAEC-CM and RPMI + FCS (final FCS concentration, 20%), the growth inhibition shown in Fig. 1 occurred. However, if growth-arrested calf SMC were exposed to a 1:1 mixture of BAEC-CM and DMEM + CS (final CS concentration, 10%), much less inhibition was observed (Table II). Calf SMC are usually grown in DMEM + 10% CS; when grown in RPMI + 20% FCS, they grow at a slightly slower rate and reach 80-90% of the final cell density of calf SMC grown in DMEM + 10% CS. Mixing CS and FCS does not block the inhibitory activity (Table II). Growth-arrested calf SMC released into DMEM + 20% CS grow at the same rate as they do in DMEM + 10% CS; the inhibitory effect of BAEC-CM when the final CS concentration is 20% is similar to the effect when the final CS concentration is 10% (Table II). When Go calf SMC are exposed to RPMI + 10% FCS, they reach ~60% of the final cell density of calf SMC grown in DMEM + 10% CS. The inhibitory effect of BAEC-CM when the final FCS concentration is 10% is slightly greater than in 20% FCS because the cells do not go through even one doubling. These data suggest that BAEC-CM interacts with something present in greater concentrations in FCS than in CS to generate the inhibitory activity. However, we cannot rule out the possibility that substances present in CS compete with the SMC for the inhibitor. Similar experiments cannot be done with rat SMC because these cells do not grow well in CS. In summary, the release of inhibitory activity by BAEC requires the presence of a small amount of either FCS or CS; however, the inhibitory effect is greater in the presence of FCS than in CS.

Effect of Passage Number

CM from confluent BAEC at different passages was collected as described in Materials and Methods. It was tested for inhibitory activity on second or third passage rat SMC as described in Materials and Methods. The results are presented in Fig. 2A as the maximum percent inhibition. The data demonstrate that the inhibitory activity in confluent BAEC-CM decreases rapidly as the passage number of the BAEC increases.

Fig. 2B shows the results of experiments in which confluent primary BAEC-CM was tested on rat SMC at different passages. These data show that the inhibition by BAEC-CM decreases as the passage number of rat SMC increases. Calf SMC were always used between passages one and three because they tended to become pleomorphic at later passages. We have generally noted that third passage calf SMC were slightly less responsive to BAEC-CM (not shown).
Biochemical Characterization of the Inhibitory Activity

We subjected the CM obtained in 0.4% serum to a variety of treatments to characterize it. To test the heat sensitivity of the inhibitory activity, aliquots of CM were heated at 60°C for 30 min, or at 90°C for 5 min. CM treated this way lost <20% of its activity (Table III).

To determine whether the inhibitory activity was a protein(s), we treated it with 30 μg/ml trypsin for 2 h at 37°C. A tenfold excess of soybean trypsin inhibitor in a small volume was added 30 min before mixing the CM with RPMI + FCS (final concentration FCS 20%). Separate aliquots of CM were treated with 30 μg/ml chymotrypsin for 2 h at 37°C, or with 30 μg/ml subtilisin (Carlsberg) for 60 min at 37°C. A tenfold weight excess of chick egg white ovomucoid was added to the chymotrypsin-treated CM at least 30 min before mixing the CM with RPMI + FCS (final concentration FCS 20%). Subtilisin-treated CM was heated at 90°C for 5 min to kill the enzyme activity. Less than 10% of the inhibitory activity was lost after these protease treatments (Table III). To control for nonspecific protease or inhibitor effects, the same concentration of proteases and inhibitors (or boiled enzyme in the case of subtilisin) were mixed together for 30 min, then added to RPMI + 20% FCS or the 1:1 CM-RPMI + FCS (final concentration FCS 20%) mixture. The inhibitory activity was unaltered by these treatments.

BAEC-CM was incubated with 1 U/ml of chondroitin ABC lyase for 90 min at 37°C. Separate aliquots of BAEC-CM were treated with 15 U/ml hyaluronidase for 90 min at 37°C. The samples were then boiled for 5 min, a treatment which kills the enzyme activity but does not affect the inhibitory activity. These enzyme treatments resulted in little or no loss of activity (Table III). Boiled hyaluronidase or chondroitin ABC lyase added to RPMI + 20% FCS did not affect SMC growth.

When BAEC-CM was incubated with 10 U/ml purified Flavobacterium heparinase for 90 min at 37°C, 80% of the inhibitory activity was lost (Table III). The heparinase preparation has no detectable protease activity, even when tested in a sensitive assay system (17). In addition, the inhibitory activity in BAEC-CM was insensitive to digestion by a broad range of proteases, as described above. The heparinase is specific for a heparin or heparinlike substrate, in that it requires a 1→4 linkage between sugar residues and also requires sulfated iduronic acid or N-sulfated glucosamine as a substrate. Thus, it will not degrade chondroitin sulfates, dermatan sulfate, or hyaluronic acid. The inhibitory activity in BAEC-CM therefore appears to be heparin or a heparinlike molecule.

Two other lines of evidence support this conclusion. First, a crude isolate of glycosaminoglycans from BAEC-CM was prepared by the method of Dietrich and de Oca (7). The TCA-soluble, ethanol-precipitable material obtained was reconstituted in the starting volume of RPMI + 20% FCS. When rat or calf SMC were exposed to this medium, their growth was inhibited with the same kinetics displayed in Fig. 1, although the calf SMC did not round up and detach in these experiments. Treating the crude isolate of GAGs with heparinase destroyed the inhibitory activity. GAGs isolated in the same manner from unconditioned medium (i.e., RPMI + 0.4% FCS) had no effect on SMC growth.

The second approach was to examine the effect of exogenous GAGs on SMC growth. When chondroitin sulfate ABC, dermatan sulfate, hyaluronic acid, or two types of heparan sulfate

### Table III

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Call SMC</th>
<th>Rat SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>84 ± 8</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>60°C, 30 min</td>
<td>76 ± 9</td>
<td>53 ± 10</td>
</tr>
<tr>
<td>90°C, 5 min</td>
<td>93 ± 4</td>
<td>62 ± 5</td>
</tr>
<tr>
<td>Trypsin</td>
<td>82 ± 5</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>85</td>
<td>54</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>—</td>
<td>51</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>94 ± 4</td>
<td>50</td>
</tr>
<tr>
<td>Chondroitin S0 4 ABC lyase</td>
<td>88 ± 7</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>Heparinase</td>
<td>19</td>
<td>9 ± 6</td>
</tr>
</tbody>
</table>

CM from BAEC was subjected to the indicated treatments and then assayed for its ability to inhibit growth-arrested calf and rat SMC when mixed 1:1 with RPMI + FCS (final concentration 20% FCS). SD are given in those instances when the treatments were performed three or more times. In other cases, the treatments were performed twice.
were added to either growth-arrested or exponential cultures of rat SMC, no inhibition of growth occurred at concentrations up to 10 μg/ml (Fig. 3). At 100 μg/ml, slight inhibition (up to 20%) of growth-arrested cells was observed with these GAGs (Fig. 3). In contrast, heparin inhibited SMC growth by 50% at 1–2 μg/ml in growth-arrested SMC, and at 90–100 μg/ml in exponential SMC (Fig. 3). Anticoagulant and non-anticoagulant heparin (21) were equally effective in inhibiting SMC proliferation.

Note that growth-arrested SMC are nearly 100 times more sensitive to heparin than exponential SMC (Figs. 3 and 4). Although the ED₅₀ for heparin is rather variable from experiment to experiment, the 100-fold difference in sensitivity to heparin was very consistent.

We also examined the effect of heparin on BAEC growth. Exponential and growth-arrested BAEC were not inhibited by heparin at doses <100 μg/ml (Fig. 4). There was actually a slight stimulation of BAEC, which ranged from 10–30% in different experiments. This is in sharp contrast to the effect of heparin on SMC. Heparin did not affect the growth of either exponential or growth-arrested BHK cells at doses <100 μg/ml (data not shown).

**Mitogenic Activity in BAEC-CM**

When we attempted to fractionate BAEC-CM on Amicon filters, nearly all the inhibitory activity was lost. However, we noticed a small but consistent increase in cell number when cells were exposed to the indicated concentrations of heparin as described in Materials and Methods. In light of the recent observations by Gajdusek et al. (11) that BAEC secrete a SMC mitogen, we fractionated CM from exponential BAEC at passages five through seven. The mitogenic potential of these Amicon fractions was assayed on rat SMC as described in Materials and Methods. The >100,000-dalton fraction supported two rounds of SMC division, whereas the 1% FCS (final concentration) control supported less than one doubling (Fig. 5). The 10,000–100,000-dalton fraction had even greater mitogenic activity, but the background from this fraction of concentrated RPMI + 0.4% FCS was also higher and more variable. This is probably the result of concentrating the low molecular weight growth factors, e.g., platelet factors, known to be present in serum. The results from this fraction are thus more difficult to interpret. These results confirm the observations of Gajdusek et al. (11) on the production of an SMC mitogen by BAEC.

**DISCUSSION**

We have shown that cultured BAEC are capable of secreting both positive and negative effectors of SMC growth. The mitogen is probably similar to the one described by Gajdusek et al. (11). The inhibitor appears to be heparin or a heparinlike molecule. Using CM from confluent cultures of primary BAEC, we observed a slow-onset inhibition of proliferation of growth-arrested SMC (Fig. 1). The difference between our observations of an inhibitory activity in BAEC-CM and those of Gajdusek et al., in which a mitogenic activity dominates, are due likely to one or more of the following differences in the systems used: (a) we collected CM only from primary BAEC that have been confluent for at least a week, whereas Gajdusek et al. used later passage BAEC to collect CM; (b) we collected our CM in the presence of serum, whereas Gajdusek et al. used plasma-devoid serum; and (c) we used growth-arrested SMC to assay for inhibitory activity, whereas Gajdusek et al. used exponential SMC to assay for mitogenic activity.

The issue of cytotoxicity is difficult to address directly. Several observations suggest that the inhibitory effect of BAEC-CM is not strictly cytotoxic: (a) the rat SMC never detach, and the calf SMC detach only two-thirds of the time in experiments using 20% FCS; (b) experiments in which the final concentration of FCS was varied suggest that the detachment of calf SMC may be an artefact of serum concentrations; (c)
the medium does not contain detached cells (except in those calf SMC experiments in 20% FCS in which cells detach); (d) daughter cells are able to spread normally after cytokinesis; (e) flow microfluorimetry shows that both control and CM-exposed SMC have the same cell cycle distribution at the end of the experiment; (f) the CM-treated SMC never take up trypan blue; and (g) the BAEC-CM effect is reversible. Even if the growth inhibition is due to cytotoxicity, however, it is still important, because the inhibitory activity is specific for both producer and target cell (Table I). This type of specificity could be important in: (a) the normal vessel, in which this mechanism of SMC growth regulation may be involved in maintaining the SMC in a quiescent growth state; (b) the injured vessel, where the cessation of myointimal proliferation occurs after re-endothelialization of the damaged area (9); and (c) regulation of blood vessel morphogenesis during development and remodeling of the vasculature. The lack of inhibition of BAEC growth by BAEC-CM is corroborated by the observation that commercially-prepared heparin, while inhibiting SMC proliferation, did not inhibit BAEC (Fig. 4) or BHK cells.

Serum is required for production of the heparinlike inhibitor by BAEC. The nature of the serum requirement is not known, but at least two general possibilities exist: (a) serum provides a necessary biosynthetic precursor or cofactor for heparin synthesis; and (b) serum provides a substance that releases heparin from the endothelial cell surface. Because heparan sulfate has been shown to cause release of more heparan sulfate from the cell surface (4, 5), it is possible that the serum provides either heparan sulfate or an enzyme which liberates heparin. These possibilities remain to be tested.

The secretion of a heparinlike inhibitor of SMC by BAEC fits in well with observations made in this and other laboratories. Buonassisi (3), Gamse et al. (12), and Busch et al. (5) have shown that cultured BAEC secrete heparan sulfates (of which heparin may be considered a subclass). Indeed, about half of all the GAGs synthesized by BAEC are heparan sulfates secreted into the medium. In contrast, SMC and fibroblasts secrete only ~10% of their GAGs as heparan sulfates (12, 20, 31). In addition, Gamse et al. (12) found that the heparan secreted by BAEC was more heparinlike (i.e., more highly sulfated) than the heparan made by bovine aortic SMC.

The BAEC must be confluent before the inhibitory activity can be detected. Indeed, exponentially growing BAEC produce a SMC mitogen (Fig. 5). We suggest that the cessation of BAEC growth may be an important step in the regulation of vascular smooth muscle cell growth in vivo. This idea is supported by recent observations that in a nonregenerating (i.e., quiescent) vascular bed, heparan sulfates are the predominant cell-associated GAGs, whereas in an actively regenerating vascular bed, the amount of heparan sulfate is greatly diminished (1).

When purified GAGs were added to growth-arrested SMC cultures, heparin was by far the most potent inhibitor of growth (Fig. 3). Preliminary experiments (not shown) suggest that the degree of sulfation plays an important role in the antiproliferative effect of heparin. This idea is supported by the fact that the inhibitory activity of GAGs correlates with their degree of sulfation; heparin has 2.5–3 sulfate groups per hexosamine; lung heparan sulfate, dermatan sulfate, and chondroitin ABC sulfate have about one sulfate per hexosamine; aortic heparan sulfate and hyaluronic acid contain 0.3 and zero sulfates per hexosamine, respectively.

The effect of heparin on SMC proliferation in vivo has been studied in this laboratory for several years. Clowes and Kornovsky (6), using an air-dry model for vessel injury (9), showed that heparin markedly suppressed myointimal proliferation when infused in rats for 24 h after the injury. More recently, Guyton et al. (14), also using the air-dry model for vessel injury, found that heparin prevented myointimal proliferation, and that anticoagulant and non-anticoagulant heparin were equally effective. In a series of parallel studies, Hoover et al. (18) demonstrated that heparin blocked the stimulation of cultured rat SMC growth by platelet extract, and showed that the inhibition was probably not mediated via a direct interaction with platelet-derived growth factor. Again, anticoagulant and non-anticoagulant heparin were equally effective. The experiments using exogenous heparin and other GAGs reported here confirm and extend these previous studies.

Based on the in vivo and in vitro demonstrations that non-anticoagulant heparin is as potent as anticoagulant heparin in inhibiting SMC growth, it is possible that non-anticoagulant heparin might be useful in those clinical situations (balloon embolecomy, endarterectomy, vein grafting, etc.) in which suppression of myointimal proliferation is important to the patient, because the problem of increased bleeding that accompanies the use of anticoagulant heparin would be avoided. Synthetic, non-anticoagulant analogs of heparin which retain their SMC antiproliferative capacity might also be useful. The growth-arrested SMC assay would provide a good screening system for developing such compounds.

Experiments are now underway to examine the nature of the serum requirement for production and release of heparin from BAEC, the chemical structure of antiproliferative heparin, and the mechanism of heparin-induced inhibition of SMC proliferation. This system should prove useful in elucidating the molecular mechanisms involved in the regulation of vascular cell growth.

We are indebted to Dr. Richard Hoover for his advice throughout the course of this work. We thank Dr. James Marcum for providing us with bovine aortic heparan sulfate and anticoagulant and non-anticoagulant heparin. We thank Dr. Leonard Favreau for providing us with highly purified heparinase. We also thank Ms. Susan Byers for her assistance in preparation of the manuscript.

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Inhibition of Vascular Smooth Muscle Cell Growth by Endothelial Cell-derived Heparin

POSSIBLE ROLE OF A PLATELET ENDOGLYCOSIDASE*

(Received for publication, February 19, 1982)

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Bovine aortic endothelial cells release a heparin-like substance in the presence of 0.4% fetal calf serum. This substance inhibited the growth of smooth muscle cells in vitro by about 70%. Substitution of platelet-poor plasma for serum resulted in minimal liberation of inhibitory activity from the cells unless at least 10-fold higher concentrations of platelet-poor plasma were utilized. This suggested that a platelet product was involved in the release process. Therefore, we examined the ability of the platelet heparitinase described in the preceding communication to release heparin-like species from cultured endothelial cells. Our results show that when endothelial cells were exposed to serum-free medium containing 1 ng/ml of the purified platelet endoglycosidase, at least as much inhibitory activity was released as was obtained with 0.4% serum. Dose response experiments indicated that only 10 pg/ml of the enzyme were necessary to liberate 50% of the inhibitory activity from endothelial cells. The heparin-like nature of the inhibitory substance was demonstrated by its sensitivity to Flavobacterium heparinase. Utilizing appropriate controls, the release of heparin-like material by the endoglycosidase was shown to be enzyme-specific and was not due to artifacts of experimental manipulations. In addition, this enzyme did not convert prereleased material to an active component, but directly liberated the active heparin-like species from endothelial cells. A simple model describing the possible role of heparin-like components and the endoglycosidase in the normal and injured wall is presented.

The healthy arterial wall consists of the intimal endothelial cells which line the lumen, and the underlying medial smooth muscle cells. Both these cell types are in a quiescent growth state (1). In vivo and in vitro experiments indicate that endothelial cells may function as important regulators of smooth muscle cell growth within the vascular wall. However, little is known of the cellular and molecular mechanisms that govern this process.

Ross et al. (2), Harker et al. (3), and others (4, 5) have demonstrated that platelet-derived factors are required for smooth muscle cell growth in vivo and in vitro. Macrophages (6) and endothelial cells (7) have also been shown to produce mitogens for smooth muscle cells. Earlier work from our laboratory has demonstrated that exogenous heparin can almost completely suppress the in vivo myointimal smooth muscle cell proliferation that occurs after endothelial injury (8). We have found that nonanticoagulant heparin was as effective as anticoagulant active material in preventing myointimal hyperplasia (9). Hoover et al. (10) demonstrated that heparin blocked the stimulation of cultured aortic smooth muscle cell growth by platelet-derived growth factor. Nonanticoagulant fractions of heparin exhibited the same degree of potency as anticoagulantly active material. Furthermore, we have recently demonstrated that conditioned medium from bovine aortic endothelial cells inhibited the growth of aortic smooth muscle cells in vitro (11). This inhibitory component was identified as a heparin-like substance, which suggests a physiological role for the glycosaminoglycan within the vascular wall. We have previously demonstrated that low concentrations of serum are required to release this substance from bovine aortic endothelial cells (11). In this communication, we present data which suggests that the serum factor required for release of heparin-like components from the bovine aortic endothelial cell surface is the platelet heparitinase (endoglycosidase) described in the preceding communication (12), and propose a simple model for growth regulation in the vascular wall.

MATERIALS AND METHODS

Cell Culture—Endothelial cells were isolated from the thoracic segment of aortas of freshly slaughtered calves, as previously described (13, 14). The aortas were dissected free of fascia and incubated in a 1 mg/ml of collagenase solution for 20 min at 37 °C. The cell suspension obtained from one aorta was added to a single 75-cm² tissue culture flask that contained RPMI 1640 medium with fetal calf serum (final concentration, 20%), 4 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified 10% CO₂ atmosphere. After 1–3 h, the bulk of the cells had attached and were thoroughly rinsed with 4 ml of RPMI 1640 medium supplemented as described above. Subsequently, the medium was exchanged at 48-h intervals and the cultures were incubated at 37 °C in a humidified 10% CO₂ atmosphere. At confluence, more than 90% of the cells are of endothelial origin. This was verified by their distinctive morphology and the presence of Von Willebrand's antigen on the cell surface.

Smooth muscle cells were isolated from the aortas of Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA; CD strain) as previously described (15). The abdominal segment of the aorta was removed and the fascia cleaned away under a dissecting microscope. The aorta was cut longitudinally, and small pieces of media were carefully stripped from the vessel wall. Two or three strips with average dimensions of 2 X 2 mm were placed in 90-mm diameter tissue culture dishes that contained RPMI 1640 medium with fetal calf serum (final concentration, 20%), 100 units/
**Biological Role of Platelet Endoglycosidase**

ml of penicillin, and 100 μg/ml of streptomycin. Within 7 to 14 days, large patches of multilayered cells had migrated from the explants. Approximately 1 week later, smooth muscle cells from a 60-mm diameter tissue culture dish were briefly exposed to a 0.05% trypsin: 0.05% ethylenediaminetetraacetic acid solution collected from confluent aortic endothelial cells by brief trypsinization and washing, and 10 ml (approximately 1 ml/10^6 cells) of RPMI 1640 medium was added to cells in the control cultures. In some instances, the conditioned media were mixed with RPMI 1640 and fetal calf serum (final concentration, 20%). Daily estimates of cell numbers were obtained in duplicate by washing the culture cells and debranched by incubation at 37°C for 30 min. The plasma was centrifuged at 28,000 × g, dialyzed against 0.15 M NaCl, and stored prior to use.

**Platelet-poor Plasma**—Platelet-poor human plasma was generously provided by C. D. Stiles (Sidney Farber Cancer Institute, Boston, MA). It was prepared essentially as previously described (16). Briefly, citrated human plasma is centrifuged at 3,500 × g. The supernatant was harvested and defibrinated by heating at 65°C for 30 min. The plasma was centrifuged at 28,000 × g, dialyzed against 0.15 M NaCl, and stored prior to use.

**Conditioned Medium**—Five to seven days after reaching confluence, a 10^6 cell monolayer of platelet-poor aortic endothelial cells was subjected to further treatments prior to testing it on cells. In all cases, the conditioned medium was passed through a 0.22-μm filter at the end of the incubation period. Conditioned media containing either Flavobacterium heparinase or platelet heparanase was boiled for 5 min prior to testing. Subsequently, conditioned medium was mixed with RPMI 1640 medium and fetal calf serum (final serum concentration, 20%) before adding it to cells.

**Inhibition Assay**—To quantitate inhibitory activity, 6-8 × 10^4 rat smooth muscle cells were seeded in 100-mm diameter tissue culture dishes in RPMI 1640 medium and fetal calf serum (final serum concentration, 20%). The growth of smooth muscle cells (11). A small amount of fetal calf or fetal serum was required for release of the heparin-like material; in serum-free medium, little inhibitory activity was released from endothelial cells (Table I). Substituting 0.4% human platelet-poor plasma for 0.4% fetal calf serum resulted in only a small release of inhibitory activity above that released by RPMI 1640 alone (Table I). We tested the ability of platelet-poor human plasma to release inhibitory activity from endothelial cells (Fig. 1). Approximately 10 times as much platelet-poor plasma was required to release the same amount of inhibitory activity as 0.4% serum. These observations suggest that a platelet product is involved in releasing inhibitory activity from endothelial cells. However, to ensure that any differences observed between platelet-poor plasma and fetal calf serum were due to the absence of platelet products rather than the difference in species, human whole blood serum was prepared and tested for its ability to release inhibitory activity from confluent endothelial cells (Fig. 1). Human serum and fetal calf serum were equally effective at releasing inhibitory activity.

**RESULTS**

**Role of Platelet Products in Serum-mediated Release of Inhibitory Activity**—We have previously demonstrated that a heparin-like material released from confluent, primary aortic endothelial cells, and that this heparin-like material inhibited the growth of smooth muscle cells (11). A small amount of fetal calf or fetal serum was required for release of the heparin-like material; in serum-free medium, little inhibitory activity was released from endothelial cells (Table I).

<table>
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<tr>
<th>Treatment</th>
<th>Inhibition (±S.D.)</th>
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<tr>
<td>RPMI 1640 + 0.4% fetal calf serum</td>
<td>68 (±11)</td>
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<tr>
<td>RPMI 1640 alone</td>
<td>12 (±8)</td>
</tr>
<tr>
<td>RPMI 1640 + 0.4% platelet-poor plasma</td>
<td>15 (±24)</td>
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<tr>
<td>RPMI 1640 + 0.4% fetal calf serum conditioned medium then treated with Flavobacterium heparinase</td>
<td>15 (±28)</td>
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<tr>
<td>RPMI 1640 + 0.4% fetal calf serum; conditioned medium then boiled 5 min</td>
<td>73 (±29)</td>
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<tr>
<td>RPMI 1640 alone (pH 6.0)</td>
<td>10 (±24)</td>
</tr>
<tr>
<td>RPMI 1640 + 0.5 mg/ml bovine serum</td>
<td>7 (±25)</td>
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<tr>
<td>RPMI 1640 + 10 mg/ml bovine serum albumin</td>
<td>11 (±25)</td>
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**Enzymes—** Flavobacterium heparinase was purified by hydroxylapatite chromatography, cellulose phosphate chromatography, and Sephadex gel filtration, as previously described (11). The final preparation exhibited a heparin-cleaving potency of ~3000 units/ml and did not degrade hyaluronic acid, dermatan sulfate, or hyaluronic acid when employed at concentrations as high as 500 units/ml (11). Furthermore, there was no detectable protease activity in this preparation, as quantitated by the radiolabeled casein assay (17). Enzymatic activity of the Flavobacterium heparinase was destroyed by boiling for 5 min.

Heparitinase was purified from human platelets as described in the preceding communication (12). This enzyme is a lysosomal endoglycosidase which specifically degrades heparin or heparan sulfate. This enzyme did not degrade other glycosaminoglycans or proteins (12).

Enzymatic activity of the platelet heparitinase was destroyed by boiling for 5 min.

**Chemicals—** Heparin was obtained from The Upjohn Co. or Elkins-Sinn (Cherry Hill, NJ). All other chemicals were from Sigma.

**TABLE I**

<p>| Ability of different media to release inhibitory activity from bovine aortic endothelial cells |
| Confluent primary cultures of endothelial cells were incubated with the indicated media. In some instances, the conditioned media were subjected to further treatment. The resulting conditioned media were mixed with RPMI 1640 and fetal calf serum (final concentration, 20%) and tested for their ability to inhibit smooth muscle cell growth. For further details, see &quot;Materials and Methods&quot; and &quot;Results.&quot; |
| % inhibition = 1 - net growth in conditioned medium × 100 net growth in control medium |</p>
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Biological Role of Platelet Endoglycosidase Inhibition (±S.D.)

For further details, see "Materials and Methods" and "Results." For this reason, endothelial cells were exposed for 4 h at 37 °C to RPMI 1640 + 1 ng/ml of purified platelet heparitinase. During the conditioning period, the pH was maintained at 6.5, roughly the midpoint of the optimum pH range for the platelet endoglycosidase (12). When smooth muscle cells arrested in G_0 were exposed to a 1:1 mixture of this conditioned medium + RPMI 1640 and fetal calf serum (final serum concentration, 20%), significant inhibition of cell proliferation was observed (Table II). We have shown that at pH 6.5, RPMI 1640 alone liberates little inhibitory activity from the endothelial cells after 48 h of conditioning (Table I). To ensure that the lack of release in serum-free medium was not due to the absence of protein, we conditioned endothelial cells with RPMI 1640 containing up to 10 mg/ml of bovine serum albumin. No additional release of inhibitory activity was observed (Table I).

Several experiments were performed to demonstrate the heparin-like nature of the inhibitory substances liberated from the endothelial cells by platelet heparitinase and to ensure that this release process is enzyme-specific. We initially conditioned endothelial cells with medium containing platelet heparitinase as above, and then treated this conditioned medium with 10 units/ml of Flavobacterium heparinase for 60 min at 37 °C. This material was subsequently boiled for 5 min to destroy the enzymatic activities of Flavobacterium heparinase as well as platelet heparitinase, and passed through a 0.22-μm filter. Treating the platelet heparitinase-produced conditioned medium with Flavobacterium heparinase eliminated most of the inhibitory activity (Table II). Thus, it appears that this substance is heparin-like in nature. It was critical to rule out artifacts that might occur due to the boiling procedure. It should be noted that heparin and heparin-like molecules are not significantly altered by boiling. We demonstrated that the boiled 0.4% fetal calf serum-produced conditioned medium did not lose inhibitory activity (Table I). We have also found that boiled RPMI 1640, RPMI 1640 + fetal calf serum (final serum concentration, 0.4%), or RPMI 1640 + platelet-poor plasma (final concentration, 0.4%) did not inhibit smooth muscle cell growth when each was mixed 1:1 with RPMI 1640 + fetal calf serum (final serum concentration, 20%) (data not shown).

To show that heparin-like material was indeed present under those conditions in which inhibitory activity was not released, endothelial cells conditioned with serum-free RPMI 1640 or with RPMI 1640 + platelet-poor plasma (final concentration, 0.4%) were subsequently exposed to RPMI 1640 containing 1 ng/ml of platelet heparitinase. The results showed that the platelet heparitinase treatment released an equivalent amount of the inhibitory activity as would occur had the endothelial cells been initially exposed to the endoglycosidase (Table II). When these platelet heparitinase-produced conditioned media were incubated with Flavobacterium heparinase, 80% of the inhibitory activity was destroyed (Table II). It is possible that serum-free conditioned medium releases heparin-like substances, but that platelet heparitinase is required to convert it to an active form. We investigated this possibility by incubating serum-free conditioned medium with platelet endoglycosidase. Only a small increase in inhibitory activity occurred (Table II).

Amount of Platelet Heparitinase Required for Release of Inhibitory Activity—To determine the potency of the purified platelet heparitinase, we incubated replicate cultures of confluent, primary endothelial cells with various concentrations of the platelet endoglycosidase. Endothelial cells from three bovine aortas were pooled and plated into 80-mm^2 tissue culture dishes. The cells grew to confluence (2–3 × 10^6 cells/dish) and were allowed to remain in this state for 1 week. Subsequently, the cells were washed and incubated in 2.5 ml of RPMI 1640 (pH 6.5) containing platelet heparitinase at concentrations ranging from 100 fg/ml to 100 ng/ml for 4 h at 37 °C. The conditioned medium obtained was boiled for 5 min and passed through a 0.22-μm filter. It was mixed 1:1 with RPMI 1640 + fetal calf serum (final serum concentration, 20%), and the effect on smooth muscle cell growth was assayed. The results are shown in Fig. 2. From these data, we calculate that the concentration of platelet heparitinase required to release 50% of the inhibitory activity from the endothelial cells is approximately 10 pg/ml.
relatively rare glucuronsyl-glucosamine bonds outside the platelet heparitinase scissions heparin-like species at the relatively common glucosamine-iduron-berg, manuscript in preparation.

The inhibitory activity released from endothelial cells is unlikely to be due to a cytotoxic effect. The inhibitory activity was identified as a heparin-like molecule by its sensitivity to purified heparitinase. The inhibitory activity was liberated (Table II). Utilizing appropriate controls, we have shown that the material released is a heparin-like substance, and that the inhibitory effect is not an artifact of experimental manipulations. In addition, we have demonstrated that the liberation process is enzyme-specific. Furthermore, we have shown that the platelet heparitinase does not convert prereleased material to an active component, but directly liberates the active heparin-like species from the endothelial cells. Thus, our data suggest that the inhibitory activity emanating from endothelial cells is released from those cells by the platelet heparitinase present in serum. However, given the complexity of platelet products, we cannot exclude the possibility that other platelet components are also involved. Dose response experiments indicate that only 10 pg/ml of platelet heparitinase is necessary to liberate 50% of the inhibitory activity from the endothelial cells (Fig. 1). RPMI 1640 + 0.4% fetal calf serum could contain as much as 400 pg/ml of the endoglycosidase. This calculation is based upon the fact that 1 ml of serum contains the release products of about 5 x 10⁸ platelets, and that each platelet contains approximately 1,000 molecules of the 134,000 dalton enzyme. Of course, the yield of enzyme is unlikely to be 100%. Therefore, it is possible that with some serum batches, concentrations greater than 0.4% will be necessary to release sufficient heparin-like material to inhibit smooth muscle cell growth.

Based on the above observations, we would like to propose a simple model for the possible role of heparin-like components and endoglycosidases in the regulation of cell growth within normal and injured vessels. In the normal (uninjured) artery, intimal endothelial cells serve as a major source of heparin-like substances. These glycosaminoglycans are found within the extracellular matrix of the intima as well as the media and are ideally positioned to prevent proliferation of medial smooth muscle cells. The endoglycosidase has been detected in the lysosomal fraction of several types. Therefore, it is likely that smooth muscle cells and/or endothelial cells contain this enzyme. Given that lysosomal components can be transported into the external milieu, it is possible that endoglycosidases may be present within the vessel wall where they could function to release antiproliferative heparin-like components from the abluminal surface of the endothelial cells or other cells.

If intimal endothelial cells are injured or removed, circulating platelets would adhere to the exposed vessel wall, undergo aggregation and release their cellular contents. Therefore, it is likely that components present at the site of injury would include platelet-derived growth factor, platelet heparitinase, highly sulfated regions to yield a mixture of highly sulfated oligosaccharides with an average $M_r = 4,000$ (~12 residues).

We have shown that species of this size possess substantial inhibitory activity.

In this communication, we have attempted to identify the serum component responsible for the release of heparin-like substances from endothelial cells. Our data show that much less inhibitory activity was liberated by platelet-poor plasma than by serum (either human or fetal calf; Table I). Indeed, at least ten times as much platelet-poor plasma was required to release the same amount of inhibitory activity as was liberated with 0.4% serum. It is well known that small numbers of platelets undergo release reactions unless special precautions are taken during the blood collection process. Thus, it seemed likely that a platelet factor was required to release the inhibitory substance from endothelial cells. When these cells were incubated with RPMI 1640 containing platelet heparitinase, the inhibitory activity was liberated (Table II). Utilizing appropriate controls, we have shown that the material released is a heparin-like substance, and that the inhibitory effect is not an artifact of experimental manipulations. In addition, we have demonstrated that the liberation process is enzyme-specific. Furthermore, we have shown that the platelet heparitinase does not convert prereleased material to an active component, but directly liberates the active heparin-like species from the endothelial cells. Thus, our data suggest that the inhibitory activity emanating from endothelial cells is released from those cells by the platelet heparitinase present in serum. However, given the complexity of platelet products, we cannot exclude the possibility that other platelet components are also involved. Dose response experiments indicate that only 10 pg/ml of platelet heparitinase is necessary to liberate 50% of the inhibitory activity from the endothelial cells (Fig. 1). RPMI 1640 + 0.4% fetal calf serum could contain as much as 400 pg/ml of the endoglycosidase. This calculation is based upon the fact that 1 ml of serum contains the release products of about 5 x 10⁸ platelets, and that each platelet contains approximately 1,000 molecules of the 134,000 dalton enzyme (12). Of course, the yield of enzyme is unlikely to be 100%. Therefore, it is possible that with some serum batches, concentrations greater than 0.4% will be necessary to release sufficient heparin-like material to inhibit smooth muscle cell growth.

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2. L. V. Favreau and R. D. Rosenberg. unpublished data.
and platelet factor 4. The latter protein has a high affinity for heparin (23, 24) and penetrates into the media after endothelial cell denudation (30). Our data would suggest that the presence of mitogen may not be sufficient to stimulate smooth muscle cell migration and proliferation. Indeed, it seems likely that the inhibitory effect of heparin-like components must be overcome before smooth muscle cells can respond to the above stimuli. This supposition is based upon our observations that heparin is capable of suppressing smooth muscle growth in vitro (10, 11) even in the presence of large amounts of serum or platelet extract (up to 20%) as well as under in vivo conditions (9). There are at least four possible mechanisms for overcoming the inhibitory effect of this mucopolysaccharide: (a) damage or loss of the endothelium removes a major source of heparin-like molecules; (b) endoglycosidase from platelets adhering to the injured area might cleave heparin-like substances into inhibitory fragments, but these could be washed away since the injury has removed the physical barrier to hydraulic flux provided by the endothelium; (c) the local concentration of mitogens may be so high that smooth muscle cells can proliferate despite the presence of these complex carbohydrates; and (d) platelet factor 4 could inactivate heparin-like molecules. Once the suppressing action of heparin is negated, the smooth muscle cells can migrate into the intima and proliferate. However, as soon as the damaged area is re-endothelialized, smooth muscle cell growth ceases (23, 24). This could be due to the presence of heparin-like substances released by the endothelial cells behind the regenerating front. The observations of Wight et al. (25) that substantial amounts of heparan sulfates accumulate behind the regenerating endothelial front support this suggestion.

The cellular and molecular mechanisms of growth regulation in blood vessels are undoubtedly more complicated than the simple model presented above. However, alterations in the biosynthesis of heparin-like molecules by endothelial cells or other cells, changes in the production or release of endoglycosidases by platelets, smooth muscle cells, or endothelial cells, and alterations in the response of smooth muscle cells to these complex carbohydrates could, in part, be responsible for the initiation of the atherosclerotic process.

Acknowledgments—We gratefully acknowledge the excellent technical assistance of Kathleen Leppig. We thank Gladys Roth for her skillful assistance in preparation of the manuscript.

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AM DEEPLY appreciative of the signal honor paid me by the American Association of Pathologists in conferring on me the Rous-Whipple Award. In so doing, they honor implicitly my laboratory and my colleagues, with whom I had the good fortune and joy of fruitful collaboration.

Since about 1965, our laboratory has been interested in the pathobiology of blood vessels. Initially, having developed the use of exogenous peroxidases as ultrastructural tracers, and having introduced 3,3'-diaminobenzidine as a reagent for the ultrastructural cytochemical localization of peroxidases, we were urged by Lord Florey to undertake permeability studies on the microvasculature, using these techniques, in order to correlate the structural characteristics of small blood vessels with their permeability properties. We came up with the concept that it is the junctions in the microvasculature that are permeable to small hydrophilic molecules and represent the small pore system of Pappenheimer. This idea has, in fact, proved to be controversial, but the techniques we introduced have been rather useful in a variety of applications. We also documented that the blood-brain barrier in the cerebral microvasculature resides at the level of the endothelium, the junctions being "tight" and impermeable to ydrophilic tracers. Similarly, we showed that there is a blood-thymus barrier at the level of the apillary endothelial junctions. We have also been interested over the years in the problem of how polymorphonuclear leukocytes stick to the endothelium of small blood vessels during the acute inflammatory response. In brief, we have found that this sticking is enhanced by chemotactic agents and that the endothelium has high affinity receptors for chemotactic agents on the surface of the endothelial cells, the occupancy of which receptors by chemotactic agents apparently increases the stickiness of the endothelium for polymorphonuclear leukocytes.

More recently, we have become interested in possible endothelium-vascular smooth muscle cell regulatory interactions in larger vessels, which we think may have important implications in health, disease, and therapy.

Among the intriguing aspects of the normal, healthy blood vessel is that the intimal endothelial cells are nonthrombogenic and form a relatively quiescent, continuous monolayer, and the underlying medial smooth muscle cells also remain in a quiescent growth state. Little is known about the regulation of cell growth in the healthy vascular wall. As has become apparent in recent years, when the endothelium is damaged, medial smooth muscle cells migrate and proliferate into, and in, the intima, causing myointimal thickening. Ross and his colleagues have put forth the concept that the deposition of platelets in the area of endothelial damage leads to the release of platelet mitogens, which cause the migration and proliferation of the medial smooth muscle. This concept has received substantive support from the work of many other laboratories, including our own. Ross and others have shown that platelet-derived growth factors are required for the smooth muscle cell growth, both in vivo and in vitro.

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More recently, factors released from macrophages have been found to stimulate both endothelial and vascular smooth muscle cells, because macrophages are also found associated with the atherosclerotic lesion, they may also serve as a source of a mitogen for smooth muscle cell proliferation. In addition, it has been reported, and we have confirmed, that endothelial cells in vitro produce a factor that stimulates the growth of vascular smooth muscle cells. The in vivo function, if any, of this factor is at present unknown. Extracts of the arterial wall have been fractionated to produce both stimulators and inhibitors of endothelial cell and smooth muscle cell proliferation in vitro. These factors are at present largely uncharacterized.

Today I will present evidence that exogenous heparin is a negative effector of smooth muscle cell proliferation following endothelial injury in vivo, and also that cultured endothelial cells produce a negative effector of smooth muscle cell growth, which we believe is a heparin or a heparinlike molecule. We will suggest a possible role for heparin in the regulation of vascular smooth muscle cell growth in vivo, both in the normal and injured vessel.

**In Vivo Studies**

We first began some five years ago with a model for endothelial injury—the so-called “air-drying” model in the common carotid artery system of the rat. In this rather bizarre system, the contents of the carotid artery were evacuated, and a gentle stream of air was used to dessicate the endothelium. Then the blood flow was restored and the dessicated endothelium peeled off the underlying intima, leading to the deposition of platelets and, after several days, dramatic myointimal proliferation. The advantages of this system are that the injury is relatively gentle, and as far as we could establish, did not damage the underlying smooth muscle, but only the endothelium. Secondly, the myointimal proliferation produced was sharply defined, and the degree of smooth muscle cell proliferation and myointimal thickening could be quantitated both by morphometry and by autoradiographic methods. Thirdly, the contralateral carotid could serve as a control in the same animal. Lastly, because there are no branches in the segment of this vessel that we utilized, problems of regeneration of endothelium from branches could be obviated, because the endothelium regenertes solely from the uninjured distal and proximal segments of the vessel, allowing for easy quantitation of the rates of endothelial regeneration. Myointimal thickening was maximal at about 14 days after injury and could be most easily quantitated by measurement of intimal-medial ratios. We noticed that in this system, smooth muscle cell proliferation seemed to cease when the reendothelialization occurred, which is in accord with the experience of others.

Because the clotting sequence is activated in arterial injury, and because thrombin has been recently shown to be a mitogen, at least for some cell types, we thought it would be important to see whether the activation of the clotting sequence was in any way related to the smooth muscle cell proliferation. Therefore, we tested the effect of heparin, which prevents thrombin formation by activation of antithrombin, on the degree of smooth muscle cell proliferation following the “air-drying” injury. We found that with continuous intravenous infusion of heparin—the infusion being given by a catheter placed in the left jugular vein with the tip of the catheter lying in the superior vena cava—the smooth muscle cell proliferation following endothelial injury in the carotid artery was almost completely suppressed (Figure 1). At the same time, we found that there was no effect of the heparin on the number of circulating platelets and on the number deposited at the site of the lesion, or on their structure. There was also no detectable effect on the rate of reendothelialization of the denuded area.

The next question asked was whether the antiproliferative activity of the heparin was related to the anticoagulant activity of heparin. Commercial heparin is, as is well known, a mixture containing fractions.
that bind to antithrombin and are therefore anticoagulant in activity, as well as fractions that are non-antithrombin-binding, that is, nonanticoagulant. Therefore, the experiments were repeated with anticoagulant and nonanticoagulant heparin, separated by affinity chromatography with antithrombin as the adsorbant. It was clear that the nonanticoagulant heparin was just as effective in preventing smooth muscle proliferation following endothelial injury as was the anticoagulant heparin (Figure 2).

**In Vitro Studies: Effect of Exogenous Heparin on Smooth Muscle Cell Growth**

In order to explore the subtleties of the system further, we turned to *in vitro* experiments. The test cells (bovine aortic endothelial cells, bovine aortic smooth muscle cells, and rat aortic smooth muscle cells) were first grown *in vitro* and then growth-arrested by placement in low serum. They were then released from G₀, with 5–20% serum, and the effects of adding exogenous heparin or other related compounds on this stimulation of growth induced by serum after release from G₀ was measured by counting of cell number at various time intervals.

We first tested the effect of exogenous heparin on smooth muscle cells as compared with endothelium in both the exponential and growth-arrested states. It was found that whereas growth-arrested smooth muscle cells (bovine or rat) were almost completely inhibited in their response to serum after growth arrest by heparin (Figure 3), exponentially growing smooth muscle cells were much less inhibited. Furthermore, growth-arrested or exponentially growing bovine aortic endothelial cells showed no inhibition of growth in the presence of heparin (Figure 3). It seemed, therefore, that heparin was highly effective in inhibiting the growth of smooth muscle and had hardly any effect on endothelial cell proliferation. Thus, there was a relative specificity for the vascular smooth muscle cell: this was reinforced in that only huge doses of heparin suppressed the proliferation of several other cell types. Anticoagulant and nonanticoagulant heparin were equally effective as inhibitors of smooth muscle cell proliferation *in vitro*. We next compared the effects of polyanionic compounds on the inhibition of the growth of smooth muscle cells, and it was found that whereas heparin (anticoagulant or nonanticoagulant) was highly effective, heparan sulfate, dermatan sulfate, chondroitin ABC sulfates, and hyaluronic acid, at comparable doses, were hardly effective at all. The effectiveness of the compounds paralleled their degree of sulfation.

**Size-Effectiveness of Heparin Fragments**

The next step was to establish what size molecule of heparin was effective in producing this inhibitory
effect. Therefore, fragments of heparin were made by limit digest with nitrous acid and separated by chromatography. It was found\(^1\) that an inhibitory effect on the proliferation of smooth muscle cells required a fragment that was a hexasaccharide or larger in order to obtain a significant inhibition. Maximum inhibition was found in the dodecamer to 20-saccharide size range. Smaller fragments than hexasaccharides were ineffective.

**Modified Heparins**

We also have begun to look into the chemical properties of the heparin molecule that seem to be necessary for the antiproliferative effect.\(^1\) The aim in modifying the heparin was to see what chemical groups were responsible, and whether an antiproliferative, nonanticoagulant molecule could be obtained. So far, our experiments have indicated that O-sulfation is required for the antiproliferative effect, and that N-sulfation or some other group (neutral or negative) is required to block the positive charge of the amine. Thus, for instance, an O-sulfated, N-desulfated, N-reacetylated molecule is highly effective in its antiproliferative activities and is nonanticoagulant. Further work in this area is proceeding, because a nonanticoagulant, antiproliferative modified heparin may prove useful clinically.

**Heparin Binding**

In preliminary experiments, the kinetics of heparin binding to rat aortic smooth muscle cells have been examined.\(^1\) It appears that there are both saturable high-affinity and low-affinity sites on the smooth muscle cell for heparin, the former having a \(K_d\) of about \(2 \times 10^{-9}\) M, with about \(2 \times 10^4\) sites per cell, and the latter having a \(K_d\) of about \(1 \times 10^{-8}\) M, with about \(5-10 \times 10^5\) sites per cell. The biologic significance of heparin binding to these high or low-affinity binding sites, in terms of the antiproliferative effect, has not as yet been established.

**Effects on Cell Metabolism**

In regard to the metabolism of the smooth muscle cells under the influence of heparin, it has been found\(^1\) that at low doses—that is, below 100 \(\mu g\) per ml of heparin—there is marked and immediate inhibition of DNA synthesis down to 20% of normal in the smooth muscle cells. A similar inhibition of RNA metabolism is obtained but is not as marked. However, protein synthesis is not affected early, but only late, when it decreases slightly at doses of heparin of 100 \(\mu g\) per ml. Flow microfluorimetry shows a rapid effect with decrease entry of the cells into \(S_i\), and eventually most cells end up in \(G_i\) at the end of the inhibition experiment.

The pharmacologic effects detailed above are, I think, interesting; and it is thought that a nonanticoagulant, antiproliferative heparin analog might prove to be useful clinically in preventing vascular smooth muscle cell proliferation, which might follow vascular surgery.

**Endothelial Cell Effects on Vascular Smooth Muscle Cell Proliferation**

As mentioned before, it was our impression from a number of our studies involving the air-drying model in vivo that smooth muscle cells seem to cease to proliferate when reendothelialization occurs. One possibility was some form of regulatory influence of endothelial cells on smooth muscle cell proliferation, and vice versa. We have therefore examined the possible secretory effects of the endothelial cells in vitro, which might influence smooth muscle cell growth. As mentioned before, others\(^1\) have shown that endothelial cells secrete a mitogen for smooth muscle cells, and in experiments that I will not present today, we have confirmed that effect.\(^1\) Today, however, I want to show our evidence for production by endothelium of a negative effector of smooth muscle cell growth that appears to be heparin or a heparinlike molecule. It should be mentioned that the conditions necessary for the production by endothelial cells of the mitogen for vascular smooth muscle cells are quite specific, and different from those necessary for the production of the negative effector.

We conditioned medium from confluent, primary cultures of bovine aortic endothelial cells and studied the effect of this conditioned medium on the proliferation of smooth muscle cells released from \(G_0\) by serum. It was found that the conditioned medium had a profound inhibitory effect on the proliferation of smooth muscle cells\(^2\) (Figure 3).

We next examined whether this effect was specific for the endothelial cell as the producer cell and was specific for the vascular smooth muscle cell as the target cell. Testing conditioned media from a wide variety of cells, including vascular smooth muscle cells, we found that only confluent endothelial cell cultures produced a conditioned medium inhibitory to vascular smooth muscle cell proliferation.\(^3\) Exponentially grown endothelial cells produced the mitogen(s) that we have previously mentioned. Furthermore, it was found that only vascular smooth muscle cells responded in a highly sensitive way to the
inhibitory effect of the conditioned medium obtained from confluent endothelial cell cultures. It was also established that the production of the inhibitory factor or factors by confluent endothelial cell cultures only occurred in primary cultures or within three or four passages, being lost after subsequent passage.

Biochemical Properties of the Inhibitory Factor(s)

The biochemical properties of the conditioned medium in terms of inhibition of smooth muscle cell proliferative activity were then established. It was found that the activity was not abolished by heating, was protease-insensitive, and was also insensitive to hyaluronidase and chondroitin sulfate ABC lyase. However, a highly purified flavobacterial heparinase, which contained no protease activity, and which did not degrade chondroitin sulfate, dermatan sulfate, or hyaluronic acid, almost completely removed the inhibitory activity from the conditioned medium. A glycosaminoglycan fraction of the conditioned medium, that is, an ethanol-precipitated trichloracetic acid-soluble fraction, had strong inhibitory activity, but when this fraction was treated with the flavobacterial heparinase, the activity was almost completely abolished. It was, therefore, we think, reasonably concluded that the inhibitory activity produced by the endothelial cells was that of a heparin or heparin-like molecule. Several other groups have reported heparinase activity that endothelial cells in vitro produce heparan sulfates, of which heparin may be considered a subclass.

Role of Platelet-Derived Heparinase (Endoglycosidase)

It is apparent that the release of the inhibitory activity from the endothelial cells required in some way the presence of a small amount of serum; for instance, cells placed in platelet-poor plasma did not produce a conditioned medium that was inhibitory. It was therefore thought that some component of serum, possibly a platelet-derived component, was necessary for the secretion and/or release of the heparin activity by the endothelium.

Recently, Dr. Robert Rosenberg's laboratory has isolated and purified a highly potent and specific heparinase (endoglycosidase) from platelets as well as from other tissues. This enzyme, which is lysosomal in location, differs from the bacterial enzyme in that it yields, on the average, dodecasaccharides, whereas the bacterial enzyme yields, on the average, disaccharide fractions; the latter would be, as we have previously shown, inactive, whereas the former would be active in terms of antiproliferative effect.

Now, as mentioned before, serum in the conditioning medium was capable of releasing the antiproliferative activity from confluent endothelial cell cultures. Conditioning with serum-free medium, or with medium plus platelet-poor plasma, were both ineffective. However, if to either of these two systems purified platelet heparinase was added, a marked inhibitory activity was obtained, and if that conditioned medium containing the active material was then treated with the flavobacterial heparinase, the inhibitory activity was almost completely abolished. We take these results to indicate that platelet heparinase-like activity may well be necessary for the release of activity from endothelial cells, and they explain the previously established serum requirement for release of the inhibitory activity. The questions arise as to whether endothelial cells and/or vascular smooth muscle cells have a similar enzymatic activity in the normal, uninjured vessel wall, and whether the platelet-derived enzyme plays a role in vascular injury following the release of platelet granules after deposition at sites of vascular injury.

Summary

In summary, then, we have shown that exogenous heparin inhibits smooth muscle cell proliferation in vivo and in vitro, and that other classes of glycosaminoglycans do not so inhibit smooth muscle cell growth in vitro. Furthermore, anticoagulant and nonanticoagulant heparin seem to be equally effective at inhibiting smooth muscle cell growth, both in vivo and in vitro. For these effects, however, hexasaccharide or larger fragments are required, whereas di- or tetrasaccharide fragments are not inhibitory. Chemically modified heparins that have lost their anticoagulant activity can, under certain conditions, maintain potent antiproliferative activity. It seems that confluent primary cultures of endothelial cells secrete a heparinlike molecule that inhibits the proliferation of growth-arrested vascular smooth muscle cells. A platelet heparinase is capable of releasing this inhibitory activity (that is, heparin) from the endothelial cells at very low concentrations.

Speculations

We would speculate, therefore, that heparin, or similar compounds, may be involved in the intrinsic regulation of vascular smooth muscle cell growth in the healthy, uninjured vascular wall. In this regard it is interesting that it has been shown that growing vessels contain little heparin, whereas in differentiated quiescent vessels, considerable amounts of
hemin, or heparin-like substances, are present. Platelet-derived heparinase–heparin interaction may be important in the vascular smooth muscle cell proliferative response following endothelial injury. Perhaps heparinase from the adherent platelet is released and removes heparin bound to the smooth muscle cells, thus allowing the platelet mitogens to act. We have previously shown that heparin is effective as an inhibitor of vascular smooth muscle proliferation in vitro in the presence of large amounts of serum and/or platelet factors. The platelet heparinase would release bound heparin, possibly, of serum and/or platelet factors. The platelet heparinase has similar characteristics and can release active fragments. Experiments are under way to test the hypotheses.

We would postulate that some of the platelet heparinase is circulating in sufficient amounts to fulfill this activity or that the endothelial cells or the smooth muscle cells produce the heparinase, which has similar characteristics and can release active fragments of heparin. Experiments are under way to test these hypotheses.

It may be postulated that nonanticoagulant, anti-proliferative heparin derivatives might prove useful as selective antiproliferative agents in certain clinical conditions, such as postoperatively in vein grafts, endarterectomy, balloon embolectomy, and arteriovenous anastomosis, in which injury to the endothelium may possibly lead to myointimal proliferation and luminal occlusion.

It may well be that this vascular model is a model for embryonic and differentiation systems. It is intriguing to postulate that the control of growth in some areas and for some cells might be related to changes in the composition of the extracellular matrix.

Finally, I must add that one of the pleasures of being involved in this area of research has been the association with several highly creative coinvestigators. These include Jay A. Fishman and Graeme B. Ryan, who helped develop the air-drying model; Alex W. Clowes and John R. Guyton, who pursued the in vivo studies with anticoagulant and nonanticoagulant heparin; Richard L. Hoover, who initiated the in vitro studies, which were continued in depth by John J. Castellot, Jr. Our laboratory is greatly indebted to Dr. Robert D. Rosenberg, who has provided us with biochemical expertise, and without whose help we would not have been able to perform many of our experiments.

References

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