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 \(^{3}H\) 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 [³H]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 20% fetal calf serum (normal growth medium) to indicate the health and growth potential of the cells (see Fig. 1).

We have tested other sulfated polyanions, and none, with the exception of a small dextran sulfate (mol wt 25,000), inhibited growth like heparin (Table 2). The small dextran sulfate resembles heparin in the degree of sulfation and charge, whereas the others are sulfated to a lesser extent (R. Rosenberg, unpublished observations).

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 [³H]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 [³H]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 [³H]Thymidine Uptake

<table>
<thead>
<tr>
<th>Heparin concentration (µg/ml)</th>
<th>Relative growth values</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.79 ± 0.13</td>
</tr>
<tr>
<td>10</td>
<td>0.63 ± 0.18</td>
</tr>
<tr>
<td>15</td>
<td>0.63 ± 0.01</td>
</tr>
<tr>
<td>20</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 (n per experiment ≥ 6).

### Table 2 [³H]Thymidine Uptake of Smooth Muscle Cells When Grown in the Presence of Various Glycosaminoglycans

<table>
<thead>
<tr>
<th>Glycosaminoglycan</th>
<th>Relative growth values (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
</tr>
<tr>
<td>Without platelet extract</td>
<td>0.95 ± 0.05*</td>
</tr>
<tr>
<td>Nonanticoagulant-heparin</td>
<td>0.64 ± 0.07*</td>
</tr>
<tr>
<td>Anticoagulant heparin</td>
<td>0.61 ± 0.04*</td>
</tr>
<tr>
<td>Chondroitin sulfate‡</td>
<td>1.07 ± 0.08†</td>
</tr>
<tr>
<td>Dermatan sulfate§</td>
<td>1.08 ± 0.04†</td>
</tr>
<tr>
<td>Low molecular wgt dextran sulfate</td>
<td>0.61 ± 0.04*</td>
</tr>
<tr>
<td>High molecular wgt 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.
† n ≥ 18, representing at least three separate experiments.
‡ Standards obtained from Dr. Anthony Cifonelli, University of Chicago.
§ Obtained from Dr. P. Petracek, Riker Labs, Minneapolis, MN.
¶ Obtained from Sigma Chemical Co., St. Louis, MO.
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had been added to the medium from the beginning (Table 6). The results suggest that heparin need not be free in solution in order to inhibit growth, and that it may be binding to the cell surface and thus blocking access to receptors for the platelet extract.

**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 but not on endothelium, (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 4** The Effects on Growth of the Addition of Antithrombin (0.1 μg/ml) to Cultures of Smooth Muscle Grown with and without Heparin (10 μg/ml)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growth</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 + heparin + 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 separate experiments with six or more measurements/experiment.
‡ Values significantly different from controls, P ≤ 0.01.

It is possible that the heparin is binding to the cell surface and in doing so is interfering with the interaction between the platelet extract and the cell, thereby inhibiting growth. In an attempt to investigate this possibility, we preincubated the cells in heparin followed by two washes with Hanks' balanced salt solution before adding the platelet extract. If the preincubation is done for 1 hour, there is a slight inhibition, but by extending the preincubation time to 24 hours, the inhibitory effect increases, almost to the same extent as if heparin was passed over a heparin-Sepharose column and then tested for stimulatory effects on growth. Under these conditions, any molecule with a high affinity for heparin will be retained on the column; e.g., this is the same manner in which antithrombin was removed from the plasma. If the heparin column treatment removed growth factors, then a difference between the growth potential of the treated and untreated samples would be expected. As our results show, there is no inhibition with the treated sample and, statistically, it is the same as the untreated.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growth</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 + 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>Treatment</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI + 0.4% FCS + PE</td>
<td>1.00</td>
</tr>
<tr>
<td>RPMI + 0.4% FCS + PE preincubuted 1 hr with heparin (10 μg/ml)</td>
<td>0.74 ± 0.01*</td>
</tr>
<tr>
<td>RPMI + 0.4% FCS + PE preincubated 24 hr with heparin (10 μg/ml)</td>
<td>0.88 ± 0.07*</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, in the in vivo studies of Clowes and Karnovsky (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 confluency, 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 Damus 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 Karnovsky (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

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References

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