Structural Determinants of the Capacity of Heparin to Inhibit the Proliferation of Vascular Smooth Muscle Cells. II. Evidence for a Pentasaccharide Sequence That Contains a 3-O-Sulfate Group

John J. Castellot, Jr.,* Jean Choay,‡ Jean-Claude Lormeau,‡ Maurice Petitou,‡ Edgar Sache,‡ and Morris J. Karnovsky*

*Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115; ‡Institute Choay, 75782 Paris, Cedex 16, France

Abstract. Earlier work from our laboratory demonstrated that heparin inhibited the proliferation of vascular smooth muscle cells in vivo and in vitro. Both anticoagulant and non-anticoagulant heparin species were equally effective as antiproliferative agents. Previous structure–function studies indicated that hexasaccharide and larger fragments retained antiproliferative activity, whereas tetra- and disaccharides were inactive. These experiments also suggested that both N- and O-sulfates of heparin were necessary for growth inhibitory capacity. In this paper, we have further analyzed the structural determinants of the antiproliferative activity of heparin. These experiments were done using synthetically prepared and therefore chemically defined heparin oligosaccharides. We present evidence that a pentasaccharide fragment retains antiproliferative activity, and that the 3-O-sulfate on the internal glucosamine residue is critical for growth inhibitory capacity of the pentasaccharide. We also show that heparins obtained from different manufacturers differ significantly in their ability to suppress smooth muscle cell proliferation.

THE proliferation of vascular smooth muscle cells (SMC)[1] after endothelial injury is thought to be a key step in the pathogenesis of arteriosclerosis (Ross and Glomset, 1973). Although much attention has been focused on factors that stimulate SMC proliferation (Schwartz et al., 1982), very little is known about the mechanisms involved in maintaining these cells in a quiescent growth state in healthy blood vessels, and in re-establishing the quiescent growth state of SMC after their proliferative response to intimal injury.

Earlier work from our laboratory demonstrated that heparin inhibited the proliferation of SMC in vivo and in vitro (Clowes and Karnovsky, 1977; Guyton et al., 1980; Hoover et al., 1980; Castellot et al., 1981). Both anticoagulant and non-anticoagulant heparin species were equally effective as SMC antiproliferative agents. These studies suggested both a pharmacological basis for the use of heparin after vessel injury, and a possible physiological role for heparin-like species as regulators of SMC growth in the vasculature.

Previous structure–function studies (Castellot et al., 1984) indicated that hexasaccharide and larger fragments retained antiproliferative activity, whereas tetra- and disaccharides were inactive against SMC growth. These experiments also indicated that both the N- and O-sulfates of heparin were necessary for antiproliferative activity, although the N-sulfates could be replaced with acetyl groups without significant loss of activity. Both the hexasaccharide fragment and the N-desulfated, re-N-acetylated heparin are devoid of anticoagulant activity (Kazatchkine et al., 1981; Oosta et al., 1981), thus providing a chemical basis for the difference between the antiproliferative and anticoagulant activities of this glycosaminoglycan.

In this paper, we have further analyzed the structural determinants of the antiproliferative effect of heparin. These experiments were done using synthetically prepared and therefore chemically defined heparin oligosaccharides. We present evidence that a pentasaccharide fragment retains antiproliferative activity, and that the 3-O-sulfate on the internal glucosamine residue is critical for antiproliferative capacity of the pentasaccharide. We also show that heparins obtained from different manufacturers differ significantly in their ability to inhibit SMC growth.

Materials and Methods

Cell Culture

All cells were cultured at 37°C in a humidified, 5% CO_2/95% air atmosphere. All growth media contained 4 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Smooth Muscle Cells

Rat aortic SMC from Sprague-Dawley rats (CD strain, Charles River Breeding Laboratories, Inc., Wilmington, MA) were isolated, cultured, and characterized as previously described (Hoover et al., 1980; Castellot et al., 1982). Briefly, the

1. *Abbreviations used in this paper:* FCS, fetal calf serum; SMC, smooth muscle cells.

© The Rockefeller University Press, 0021-9525/86/05/1979/06 $1.00

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 such strips were placed in 60-mm tissue culture dishes. Within 1–2 wk, SMC migrate from the explants; they were capable of being subcultured about a week after the first appearance of cells. They were grown in RPMI-1640 medium that contained 20% fetal calf serum (FCS).

Rat mesenteric artery SMC were isolated by enzymatic digestion as described by Larson et al. (1984). Briefly, the mesenteric artery was removed from Sprague Dawley rats and subjected to collagenase and elastase digestion for 25–30 min at 37°C. They were grown in RPMI medium that contained 20% FCS.

Calf aortic SMC were isolated from explants of bovine aortas as described by Ross (1971). They were grown in Dulbecco's modified Eagle's medium that contained 10% calf serum.

Cebus monkey aortic SMC were the generous gift of Dr. Richard Hoover (Harvard Medical School). They were obtained from primate aortas using the explant method of Ross (1971). They were grown in RPMI medium that contained 20% FCS.

All smooth muscle cell types were identified as SMC by the presence of (*a*) numerous myofilament bundles in the cytoplasm, (*b*) numerous vesicles near the plasma membrane, (*c*) SMC-specific myosin, as revealed by indirect immunofluorescence (Larson et al., 1984), and (*d*) characteristic "hillocks and valleys" appearance of confluent cultures.

Growth-arrest of Cells

To growth-arrest SMC, sparsely plated cultures were washed and placed in RPMI + 2% platelet-poor plasma or in RPMI + 0.4% FCS for 72 h. Flow microfluorimetry indicated that ~90% of the cells were arrested in $G_0(G_1)$.

Inhibition Assay

To assay for the antiproliferative activity of heparin, 5–8 × 10^3 SMC were plated into 16-mm multiwell plates in normal growth medium. After 24 h, they were growth arrested as described above. Control cultures were released from G_0 by placing them in RPMI + 20% FCS. Other cultures were exposed to RPMI + 20% FCS that contained heparin. Cell numbers were measured in duplicate samples using a Coulter counter. Trypsinized cultures were routinely checked by direct microscopic examination to ascertain that the trypsinization procedure had not lysed the cells and to ensure that all cells were removed from the multiwell. The cells were not fed during the experiment. The net growth of SMC in control cultures was obtained by subtracting the starting cell number (at the time the cells are released from G_0) from the cell number at the end of the experiment. The net growth of SMC in heparin-containing medium is computed in a similar fashion. The degree of inhibition is determined from the following relationship

$$\text{Percent inhibition} = \left(1 - \frac{\text{net growth in heparin}}{\text{net growth in controls}}\right) \times 100.$$

Preparation of Heparin Oligosaccharides

The synthesis and chemical characterization of the heparin derivatives and their effects on coagulation factors have been described elsewhere in great detail (Sache et al., 1982; Choay et al., 1983; Sinay et al., 1984; Petitou, 1984). The chemical structure of some of these fragments is shown in Fig. 1; the letters that designate the fragments and the residues are based on the nomenclature used in the above-mentioned reports. In addition to the synthetic heparins shown in Fig. 1, we also tested a higher molecular weight (7,000–11,000 D; this fraction still shows some polydispersity) non-anticoagulant heparin obtained by ion exchange chromatography, and two lower molecular weight heparin species (2,500 and 4,500 D), prepared by controlled depolymerization which have no activity against thrombin (Factor IIa) but have high Factor Xa-inhibiting activity. These two compounds are considered antithrombotic but have low anticoagulant activity.

Results

Antiproliferative Activity of a Heparin Pentasaccharide

In our previous structure–function studies, we had found that hexasaccharide fragments of heparin were moderately active, while tetra- and disaccharide species were totally inactive. These heparin fragments were prepared by nitrous acid cleavage followed by gel filtration of heparin. This procedure yields heparin fragments with even numbers of sugar residues, if we include the reducing end 2,5-anhydromannose; thus we could not determine the antiproliferative activity of a pentasaccharide species. The recent chemical synthesis of a pentasaccharide heparin fragment of known structure (Fig. 1, fragment *a*; Choay et al., 1983) made it possible to directly assay the activity of this oligosaccharide (Fig. 2). When SMC from several species and from two different types of arteries were exposed to pentasaccharide *a*, their growth was inhibited by an average of 46% at 100 μg/ml, 31% at 10 μg/ml, and 17% at 1 μg/ml. This is approximately the same degree of inhibition seen when these cells were treated with the hexasaccharide fragment. The activity of some of the larger heparin fragments as well as the lack of effect of smaller species is also shown for comparison.

We considered the possibility that the reason for the inactivity of the tetrasaccharide species in our earlier studies (Castellot et al., 1984) was that the method of preparation yielded a mixture of different tetrasaccharide fragments (including, as mentioned above, the 2,5-anhydromannose residue at the reducing end) rather than a single pure tetrasaccharide of known structure, and that other specific tetrasaccharide structures might possess some antiproliferative activity. To test this possibility, we synthesized the two tetrasaccharides shown in Fig. 1, one (fragment *c*) that lacked the nonreducing terminus residue of pentasaccharide *a*, and the other that lacked the terminal residue from the reducing end (fragment *d*). When these compounds were assayed for their antiproliferative activity (Fig. 3), they inhibited SMC growth by 18–22% at 100 μg/ml. In terms of potency, the tetrasaccharides were 20–100 times less active than pentasaccharide *a*. This is slightly more antiproliferative than the tetrasaccharide mix-

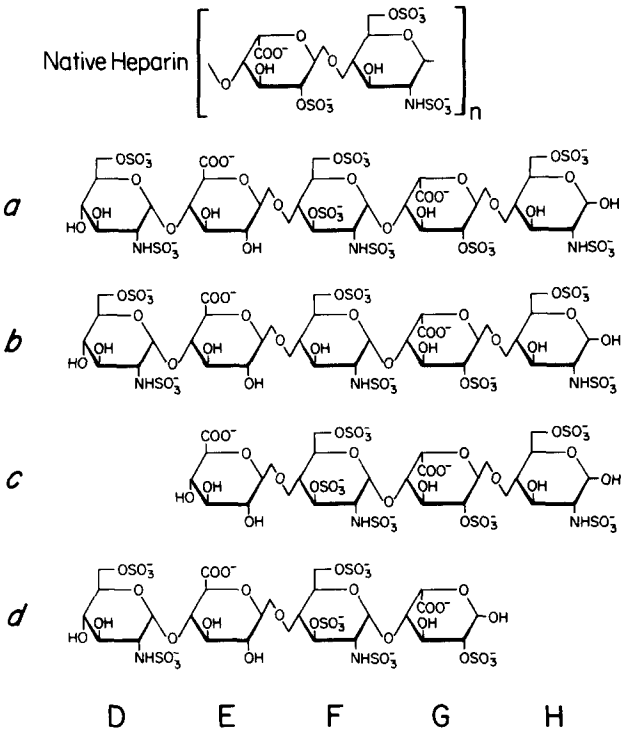

Figure 1. Chemical structure of some of the heparin derivatives used in this communication.

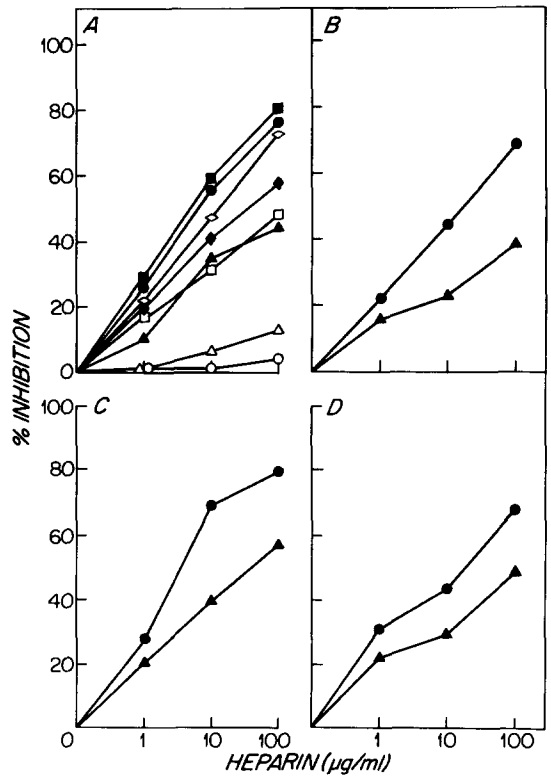

Figure 2. Effect of a chemically synthesized heparin pentasaccharide (*a*) on the proliferation of several SMC types. SMC from the rat aorta (*A*), Cebus monkey aorta (*B*), rat mesenteric artery (*C*), and calf aorta (*D*) were growth-arrested as described in Materials and Methods. These cultures were released from the G_0 block by placing them in RPMI + 20% FCS with or without the indicated concentrations of heparin or heparin derivatives. Cell number was determined after 5–7 d, at which time growth in both control and heparin-treated cultures had ceased. The commercial heparin used was from Laboratoire Choay, and had a molecular weight of ~15,000 (40–50 residues). ■, dodecasaccharide fragment; ●, commercial heparin; ◇, decasaccharide; ♦, octasaccharide; □, hexasaccharide; ▲, pentasaccharide *a*; △, tetrasaccharide; ○, disaccharide.

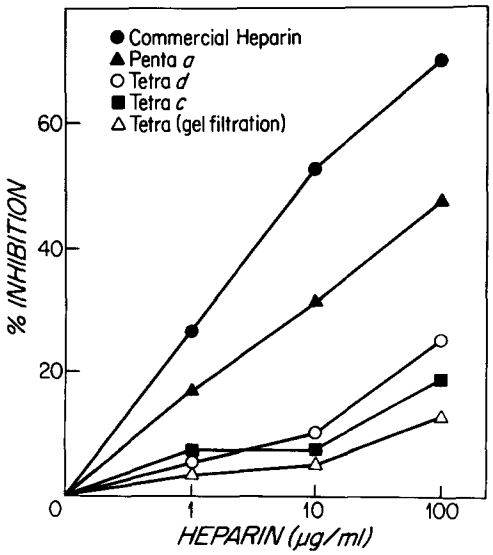

Figure 3. Effect of tetrasaccharides on SMC growth. Rat mesenteric artery SMC were growth-arrested and then exposed to RPMI + 20% FCS with or without the indicated heparin derivatives for 5–7 d, at which time cell number was determined. Tetrasaccharide *c* lacks the terminal sugar at the nonreducing end of pentasaccharide *a*. Tetrasaccharide *d* lacks the terminal residue from the reducing end of pentasaccharide *a*. A mixture of tetrasaccharides obtained by gel filtration (Oosta et al., 1981) was also tested. Virtually identical results were obtained when these heparin species were assayed on rat aortic and calf aortic SMC.

ture produced by gel filtration, but still substantially less activity than pentasaccharide *a* (Fig. 3). From the above data, we conclude that tetrasaccharide heparin fragments have a negligible capacity to inhibit SMC growth, while pentasaccharide *a* and larger species retain this ability.

Evidence for a Critical O-Sulfate in the Pentasaccharide

To determine if a particular structural feature was critical for maintaining the antiproliferative activity of pentasaccharide *a*, we synthesized (Choay et al., 1983) a novel pentasaccharide (Fig. 1, fragment *b*), which did not contain a 3-O-sulfate on residue F (the internal glucosamine). The resulting molecule could inhibit SMC proliferation by only 23% at 100 μg/ml (Fig. 4). In terms of potency, pentasaccharide *b* was ~20-fold less active than pentasaccharide *a*, which contained a 3-O-sulfate on residue F. These data suggest that the 3-O-sulfate on residue F is critical for conferring growth inhibitory activity.

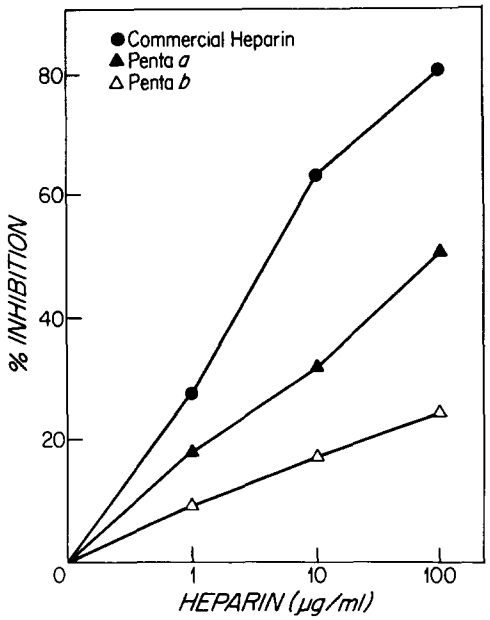

Figure 4. Role of the 3-O-sulfate on the internal glucosamine residue. A pentasaccharide lacking the 3-O-sulfate on the internal (F) glucosamine residue was synthesized (pentasaccharide *b*) and tested for its ability to inhibit the proliferation of rat mesenteric artery SMC. Essentially identical results were obtained using rat and calf aortic SMC.

Antiproliferative Activity of Non-anticoagulant Heparin Species

We had previously found that heparin, which was devoid of anticoagulant activity by virtue of its failure to bind to an

antithrombin III affinity column, was as potent and efficacious an antiproliferative agent as highly anticoagulant material (Castellot et al., 1981). Heparin inhibits a number of procoagulant proteases mainly by binding to antithrombin III and enhancing the effects of this inhibitor. With respect to this inhibition, the blood coagulation proteases may be classified into two types: Factor Xa type and Factor IIa (thrombin) type. Heparin would be defined as anticoagulant if it has high activity against thrombin. Some heparin species may have no activity against Factor IIa but have high Xa-inhibiting activity, and could be considered antithrombotic but non-anticoagulant. As the activity against thrombin derives from heparin binding to antithrombin III, this provides the rationale for the use of antithrombin III affinity column chromatography to produce non-anticoagulant heparin.

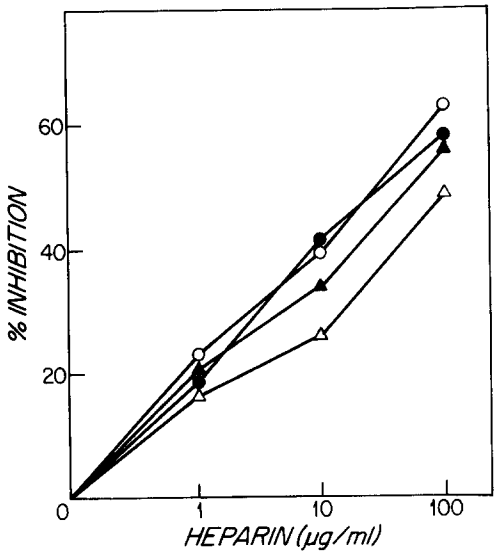

Figure 5. Effect of non-anticoagulant heparins on SMC proliferation. Rat aortic SMC were exposed to non-anticoagulant heparin species which were prepared in different ways and which differed in their abilities to accelerate the inhibition of Factors IIa and Xa (see Materials and Methods for references). The 7,000–11,000-D non-anticoagulant heparin (○) was prepared by ion exchange chromatography. The 4,500- (▲) and 2,500-D species (△) were prepared by controlled depolymerization and by extraction and fractionation of native heparin, respectively. Both of these compounds have little activity against thrombin (Factor IIa) and high activity against Factor Xa, and are thus considered antithrombotic but low anticoagulant. Similar results were obtained with rat mesenteric artery, calf aortic, and Cebus monkey aortic SMC. ●, commercial heparin.

Two heparin fragments with low anticoagulant activity were prepared and tested for their ability to inhibit SMC growth (Fig. 5): (*a*) a 4,500-D product derived from the controlled depolymerization of commercial heparin, and (*b*) a 2,500-D product also derived from controlled depolymerization of commercial heparin. These products have significant activity against Factor Xa (Yin et al., 1973) but almost no activity against Factor IIa (Larsen et al., 1978), and are antithrombotic. These were compared to a 7,000–11,000-D non-anticoagulant, non-antithrombotic heparin prepared by ion exchange chromatography (Sache et al., 1982). All of these low anticoagulant heparins possess substantially the same antiproliferative activity as anticoagulant species (Fig. 5).

Antiproliferative Capacity of Heparins from Different Sources

We had noted empirically that heparin from some sources yielded more consistent inhibition of SMC growth than others, especially at the lower doses. We therefore tested the ability of heparins obtained from ten different manufacturers to inhibit rat aortic SMC proliferation (Fig. 6). All the heparins tested were effective at 100 μg/ml, although Choay, Elkins-Sinn, and Upjohn heparins were somewhat more efficacious. At 10 μg/ml, the difference between these three producers and the others was more pronounced. At 1 μg/ml, a wide spread between the various sources was observed, with some heparins actually stimulating SMC growth slightly at this dose.

Discussion

In this paper, we have investigated some of the precise structural determinants of the ability of heparin to inhibit SMC proliferation. The basis for these studies is a series of obser-

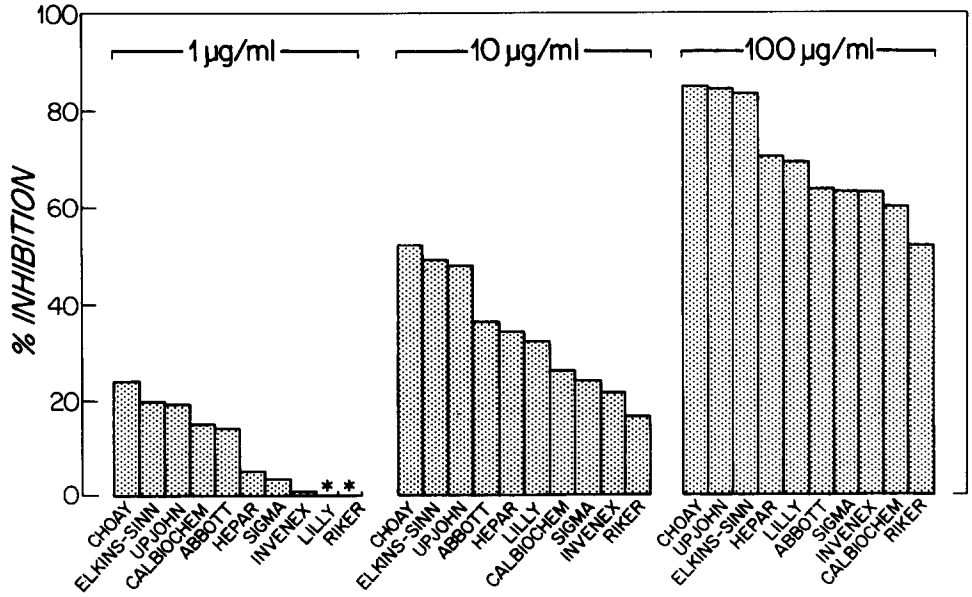

Figure 6. Effect of heparins obtained from different sources. Commercial grade heparins were obtained from ten suppliers and assayed for their ability to inhibit the growth of rat aortic SMC. Essentially the same results were obtained using rat mesenteric artery SMC. The asterisks (*) indicate that the Lilly and Riker heparins stimulated SMC growth slightly at the 1 μg/ml dose (12 and 19%, respectively).

vations made in our laboratory over the past several years, which demonstrate that heparin inhibited the growth of SMC in vivo and in vitro (Clowes and Karnovsky, 1977; Guyton et al., 1980; Hoover et al., 1980; Castellot et al., 1981). In our previous structure–function studies (Castellot et al., 1984), we used heparin fragments and chemically modified commercial heparin to determine that the smallest antiproliferative fragment was a hexasaccharide, and that both N- and O-sulfates were important for maintaining the growth inhibitory capacity of heparin. Several of the heparin fragments and modifications which retained antiproliferative activity had lost their anticoagulant activity, in particular the hexa- to dodecasaccharide species, and the N-desulfated, re-N-acetylated derivative. On the basis of these studies, we hypothesized that antiproliferative, non-anticoagulant heparin species might be clinically useful after vascular procedures that produce injury to the vessel wall. These would include vein grafts, angioplasties, arteriovenous shunts (for kidney dialysis), and endarterectomies. A significant number of these procedures, perhaps as high as 20%, appear to fail because of SMC hyperplasia (Whittemore et al., 1981).

The above structure–function experiments were done with compounds derived from commercial heparin, a very heterogeneous mixture of heparin glycosaminoglycans. The recent chemical synthesis of heparin oligosaccharides by Choay and co-workers (Choay et al., 1983; Sinay et al., 1984; Petitou, 1984) has allowed a more critical examination of the precise molecular determinants of the antiproliferative capacity. A pentasaccharide (Fig. 1, fragment *a*) was found to have approximately the same growth inhibitory effect as hexasaccharides. When a pentasaccharide (Fig. 1, fragment *b*) was synthesized which did not have the 3-O-sulfate moiety on the F residue, it was 20 times less potent than the pentasaccharide, which contained the 3-O-sulfate. We conclude from these data that a critical structure for antiproliferative heparin is a pentasaccharide fragment with a 3-O-sulfate group on the internal glucosamine residue. This conclusion is further supported by the inability of the two synthetic tetrasaccharide fragments (Fig. 1, *c* and *d*) to inhibit SMC growth, since these fragments also contain the 3-O-sulfate moiety and have the same charge density as pentasaccharide *a*. Our data do not rule out the possibility that other fragments with O-sulfate groups not in position 3, or in position 3 on residues other than F, can be active. The answer to this question awaits the chemical synthesis and bioassay of new heparin pentasaccharide fragments. It should be noted that the criticality of 3-O-sulfate groups in larger heparin fragments has not yet been established. Indeed, our studies indicate that higher molecular weight, low-affinity heparins, which do not contain 3-O-sulfate groups, are antiproliferative, which suggests that the active structure does not depend solely on the presence of the 3-O-sulfate group. It is also worth mentioning that the observation that pure, synthetic heparin fragments are antiproliferative makes it highly unlikely that the antiproliferative effect that we and others observe with native preparations is due to a contaminant in these heterogeneous mixtures.

Two other aspects of the antiproliferative nature of heparin were examined. First, heparins that were rendered low or non-anticoagulant were tested and found to possess antiproliferative activity. Neither of the low anticoagulant derivatives retained the capacity to inhibit Factor IIa (thrombin). We therefore may conclude that the anticoagulant and antiproliferative properties are clearly separate. It is unlikely that the growth inhibitory effect of heparin is mediated by kallikrein, since the ability of heparin to inhibit this protease depends upon the heparin–antithrombin III interaction (Burrowes et al., 1975; Vennerod and Laake, 1975). Since these low anticoagulant species have very low Xa-inhibitory activity, these data also rule out the possibility that the antiproliferative activity could be due to the interaction of heparin with Factor Xa or a similar type of enzyme.

The second aspect we considered was the confusing variability in the capacity of heparin to inhibit SMC growth in our laboratory as well as other laboratories. We had noticed that heparin from certain sources was more potent and more consistent than heparin from other suppliers. We obtained heparin from ten different producers and found that considerable differences existed between them, especially at the lower doses. The reasons for this are unclear, but it is apparent that to obtain the most consistent antiproliferative effect one must use heparin from Laboratoire Choay, Elkins-Sinn, or Upjohn.

The authors gratefully acknowledge the expert technical assistance of Mr. Benjamin Caleb and the photographic assistance of Mr. Robert Rubin.

This work was supported by National Institutes of Health grant HL-17747.

Received for publication 15 November 1985, and in revised form 31 January 1986.

References

Burrowes, C. E., F. M. Habal, and H. Z. Movat. 1975. The inhibition of human plasma kallikrein by antithrombin III. *Thromb. Res.* 7:175–183.

Castellot, J. J., M. L. Addonizio, R. D. Rosenberg, and M. J. Karnovsky. 1981. Cultured endothelial cells produce a heparin-like inhibitor of smooth muscle cell growth. *J. Cell Biol.* 90:372–379.

Castellot, J. J., D. L. Beeler, R. D. Rosenberg, and M. J. Karnovsky. 1984. Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells. *J. Cell. Physiol.* 120:315–320.

Castellot, J. J., L. V. Favreau, M. J. Karnovsky, and R. D. Rosenberg. 1982. Inhibition of smooth muscle cell growth by endothelial cell-derived heparin: possible role of a platelet endoglycosidase. *J. Biol. Chem.* 257:11256–11260.

Choay, J., M. Petitou, J.-C. Lormeau, P. Sinay, B. Casu, and G. Gatti. 1983. Structure-function relationships in heparin: a synthetic with high affinity for antithrombin III and eliciting high anti-factor Xa activity. *Biochem. Biophys. Res. Commun.* 116:492–499.

Clowes, A. W., and M. J. Karnovsky. 1977. Suppression by heparin of smooth muscle cell proliferation in injured arteries. *Nature (Lond.).* 265:625–626.

Guyton, J. R., R. D. Rosenberg, A. W. Clowes, and M. J. Karnovsky. 1980. Inhibition of rat arterial smooth muscle cell proliferation by heparin. I. In vivo studies with anticoagulant and non-anticoagulant heparin. *Circ. Res.* 46:625–634.

Hoover, R. L., R. D. Rosenberg, W. Haering, and M. J. Karnovsky. 1980. Inhibition of rat arterial smooth muscle cell proliferation by heparin. II. In vitro studies. *Circ. Res.* 47:578–583.

Kazatchkine, M. D., D. T. Fearon, D. D. Metcalfe, R. D. Rosenberg, and K. F. Austen. 1981. Structural determinants of the capacity of heparin to inhibit formation of the human amplification C3 convertase. *J. Clin. Invest.* 67:223–228.

Larsen, M. L., U. Abildgaard, A. M. Teien, and K. Gjesdal. 1978. Assay of plasma heparin using thrombin and the chromogenic substrate H-D-phe-pip-arg-pNA (S-2238). *Thromb. Res.* 13:285–288.

Larson, D. M., K. Fujiwara, R. W. Alexander, and M. A. Gimbrone. 1984. Myosin in cultured vascular smooth muscle cells: immunofluorescence and immunochemical studies of alterations in antigenic expression. *J. Cell. Biol.* 99:1582–1589.

Oosta, G. M., W. T. Gardener, D. L. Beeler, and R. D. Rosenberg. 1981. Multiple functional domains of the heparin molecule. *Proc. Natl. Acad. Sci. USA.* 78:829–833.

Petitou, M. 1984. Synthetic heparin fragments: new and efficient tools for the study of heparin and its interactions. *Nelle. Rev. Fr. Hematol.* 26:221–226.

Ross, R. 1971. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell Biol.* 50:172–186.

Ross, R., and J. A. Glomset. 1973. Atherosclerosis and the arterial smooth muscle cell. *Science (Wash. DC).* 180:1332–1339.

Sache, E., M. Maillard, H. Bertrand, M. Maman, M. Kunz, J. Choay, J. Fareed, and H. Messmore. 1982. Studies on a highly active anticoagulant fraction of high molecular weight isolated from porcine sodium heparin. *Thromb. Res.* 25:443–458.

Schwartz, S. M., C. M. Gajdusek, and G. K. Owens. 1982. Vessel growth control. *In* Pathobiology of the Endothelial Cell. H. L. Nossel and H. T. Vogel, editors. Academic Press, Inc., New York. 63–78.

Sinay, P., J. C. Jacquinet, M. Petitou, P. Duchaussoy, I. Lederman, J. Choay, and G. Torri. 1984. Total synthesis of a heparin pentasaccharide fragment having high affinity for antithrombin III. *Carbohydrate Res.* 132:C5–C9.

Vennerod, A. M., and K. Laake. 1975. Inhibition of purified plasma kallikrein by antithrombin III and heparin. *Thromb. Res.* 7:223–226.

Whittemore, A. D., A. W. Clowes, N. P. Couch, and J. A. Mannick. 1981. Secondary femoropopliteal reconstruction. *Ann. Surg.* 193:35–42.

Yin, E. T., S. Wessler, and J. V. Butler. 1973. Plasma heparin: a unique, practical, submicrogram-sensitive assay. *J. Lab. Clin. Med.* 81:298–310.