Heparin Inhibits Mesangial Cell Proliferation in Habu-Venom–Induced Glomerular Injury

ALINE K. COFFEY, PhD, and
MORRIS J. KARNOVSKY, MBBCh, DSc

From the Department of Pathology, Harvard Medical School, Boston, Massachusetts

The authors have investigated the ability of anticoagulant heparin and nonanticoagulant heparin to inhibit mesangial-cell proliferation after the administration of habu (*Trimeresurus flavorivids*) snake venom to rats. Rats given injected habu venom exhibited glomerular capillary cystic lesions 6 to 24 hours later, and marked mesangial proliferation was noted within the cyst after 3 days. At 7 days 87% of these lesions (nodules) contained primarily mesangial cells embedded in a dense matrix and fibrin. A decrease in the frequency of nodules and the persistence of cysts indicate effective antiproliferative treatment. When anticoagulant heparin treatment extended from 18 hours after venom administration until sacrifice at 7 days, the percentage of nodules was reduced to 40%. Nonanticoagulant heparins resulted in some, but inconsistent, inhibition of mesangial-cell proliferation. The mechanism of the antiproliferative action of heparin on mesangial cells is not known but may be similar to that for vascular smooth muscle growth regulation. The authors suggest that endogenous heparin in the glomerular basement membrane and mesangial matrix may exert an antiproliferative effect under normal conditions. Loss of this inhibition due to glomerular damage might be reversed by the addition of exogenous heparin. (Am J Pathol 1985, 120:248–255)

PROLIFERATION of glomerular mesangial cells is associated with a number of renal syndromes including diffuse proliferative glomerulonephritis, membranoproliferative glomerulonephritis, poststreptococcal glomerulonephritis, diabetic glomerulosclerosis, Henoch-Schonlein purpura, and hemolytic-uremic syndrome. Diffuse mesangial hypercellularity (focal and segmental glomerulosclerosis) is the most characteristic feature exhibited by a subgroup of children with idiopathic nephrotic syndrome who are unresponsive to standard therapy with corticosteroids.[1–4] The causes of mesangial proliferation and its role in glomerular disease processes is not well understood. Animal models of mesangial proliferation have been produced by various agents, including habu snake venom,[5] nickel subsulfide,[6] porcine thyroglobulin,[7] monocrotaline,[8] *Saccharomyces cerevisiae*,[9] and *Corynebacterium parvum*[10]; and proliferation is also seen in Masugi nephritis.[11]

The mesangial lesion produced by injection of habu snake venom was evaluated in this study. The habu venom model has features in common with diffuse mesangial hypercellularity in that glomeruli exhibit extensive mesangial proliferation, synechiae,[12] and fusion of foot processes, but no crescent formation or glomerular basement membrane deposits.[13] The lesion caused by habu venom has been well characterized as a ballooning of glomerular capillaries into a large cyst within 6–24 hours after venom administration, followed by proliferation of mesangial-like cells within the cysts beginning on Day 3.[13,14] Early investigations indicated that lysis of mesangial cells is involved in the development of capillary ballooning[5,13]; however, Cattell and Bradfield[14] have reported endothelial disruption but no mesangial lysis. Bradfield and co-workers,[15] have demonstrated with autoradiographic studies that the hypercellularity in the habu snake venom model is due to proliferation of local mesangial cells and not, as some have suggested, from mononuclear cells which invade the cyst shortly after venom administration. The separation in time of cyst formation from cell proliferation allows confirmation that the initial administration of venom has been effective in causing mesangiolysis. If the cystic formations remain after further treatment

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Dr. Coffey's current address is Department of Anatomy, Dartmouth Medical School, Hanover, NH 03755.

Address reprint requests to Dr. Morris J. Karnovsky, Department of Pathology, Harvard Medical School, 25 Shattuck St., Boston, MA 02115.

efforts to prevent the proliferative phase, then the effects of antimesangial proliferative drugs, for instance, can be assessed. The administration of the venom results in a focal lesion involving 1-5% of the glomeruli; the confined, segmental area within which the proliferation occurs highlights the presence or absence of proliferation.

The regulation of mesangial cell proliferation is poorly understood. Vascular smooth-muscle-cell proliferation, however, has been extensively investigated in this laboratory. We have demonstrated that vascular smooth muscle cell proliferation, which occurs in early healing after arterial endothelial injury, is specifically inhibited *in vivo*[16] and *in vitro*[17,18] by both anticoagulant and nonanticoagulant heparin species. The similarities between smooth-muscle cells and mesangial cells are marked including their origin,[19] morphology,[20,21] and contractility.[22] On the basis of these similarities and the observation that heparin also inhibited *in vitro* mesangial-cell proliferation (paper submitted), we examined the ability of anticoagulant heparin and nonanticoagulant heparins to inhibit the mesangial-cell proliferation seen in the cysts formed as a result of habu snake venom injection. Anticoagulant heparin clearly inhibited mesangial-cell proliferation in this model. The effects of nonanticoagulant heparin were equivocal.

Materials and Methods

Production of Habu Cystic Lesion

Female Lewis rats, weighing approximately 200 g each, were used.[1] Lyophilized venom from the habu pit viper *Trimeresurus flavorivids*, (from the Japan Snake Institute) was dissolved in physiologic saline at a concentration of 1 mg/ml. After ether anesthesia, a single dose of venom, 3 mg/kg, was administered to each rat via the femoral vein. The combined effects of reducing the dose from the previously reported 4 mg/kg[14] to 3 mg/kg and maintaining the animal body temperature for a few hours after venom injection resulted in 95% survival in our study, as compared with the 60% survival previously noted.[14] The effectiveness of the venom in producing glomerular capillary cysts was determined for all animals by removal under ether anesthesia of the right kidney 24 hour after venom administration. These kidneys were immediately fixed by immersion in cacodylate buffered 2% glutaraldehyde plus 1% sucrose and cut into slices 1-3 mm thick. After 6 hours of immersion fixation, the slices were prepared for light and transmission electron microscopy and evaluated for the presence of capillary cysts. The route of injection used (femoral vein) resulted in the development of segmental cystic glomerular lesions in every animal, whereas Cattell and Bradfield[14] reported only 70% effectiveness when injections were made into the tail vein. Animals that received habu venom were treated with anticoagulant heparin or nonanticoagulant heparin or remained untreated and were then sacrificed at 7 or 10 days after venom injection. The remaining (left) kidney was perfusion-fixed for evaluation. Five animals were evaluated in each treatment group (Table 1).

Heparin Administration

Heparin sodium was injected subcutaneously every 12 hours, beginning 18, 48, or 72 hours after the habu venom injection (see Table 1 for experimental groups). Heparin was not administered earlier than 18 hours because of the hemorrhagic effects of habu venom. Various doses of heparin were checked to identify a dose which would give full but not excessive anticoagulation. Administration of 250 U/kg, 1.25 mg/kg heparin sodium (Elkins-Sinn, Inc.) resulted in increased clotting time of 17-22 minutes when tested at 4 and 12 hours after heparin injection, a level of 20 minutes clotting time being considered fully anticoagulated, 4-7 minutes being the normal clotting time.

A nonanticoagulant heparin derivative, Choay 1452 (MW 12,000-15,000), produced by ion exchange chromatography, was administered every 12 hours subcutaneously (0.05 ml, 5 mg/ml), beginning 18 hours after venom injection. These animals exhibited normal clotting times. A low-molecular-weight (4500 daltons) molecule produced from heparin by controlled depolymerization (Choay 797) was administered to another group twice a day subcutaneously (0.05 ml; 5

Table 1—Experimental Protocol

Treatment group	Number of animals	Habu injected (day)	Treatment time	Sacrifice day
Habu	5	0	None	7
Habu + heparin beginning at 18 hours	5	0	18 hours → 7 days	7
Habu + heparin beginning at 48 hours	5	0	48 hours → 7 days	7
Habu + heparin beginning at 72 hours	5	0	72 hours → 7 days	7
Habu + heparin ending at 72 hours	5	0	18 hours → 3 days	10
Habu + Choay heparin 797 beginning at 18 hours	5	0	18 hours → 7 days	7
Habu + Choay heparin 1452 beginning at 18 hours	5	0	18 hours → 7 days	7

mg/ml), beginning 18 hours after venom injection. These animals exhibited clotting times of 20–30 minutes. This derivative of heparin is antithrombotic by interaction with Factor Xa.

Renal Perfusion Fixation

The remaining kidney was fixed by vascular perfusion at 7 or 10 days after the injection of habu venom. Each rat was anesthetized with ether and secured to a dissecting table. A laparotomy was performed, and the viscera were reflected for exposure of the left kidney. In order to maintain blood flow to the kidneys, and thus prevent ischemic shut down, we performed perfusion in the following manner. A loose ligature was placed around the abdominal aorta above the level of the renal vessels, and a bulldog clamp was used for occlusion of the aorta just below the renal vessels. The abdominal aorta was cannulated with a 20-gauge needle below the bulldog clamp. Retrograde perfusion of the kidneys was achieved by first cutting the inferior vena cava, followed immediately by simultaneous removal of the bulldog clamp and initiation of perfusion at 140 mm Hg. Immediately after onset of perfusion the abdominal aorta ligature above the renal vessels was tied. After 1–2 minutes of flushing with a 0.1 M cacodylate buffer, pH 7.4, containing 1% sucrose, the kidneys were perfusion-fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 3–4 minutes.

Preparation for Light- and Electron-Microscopic Evaluation

The kidneys were removed from the body after perfusion and immersed in 0.1 M cacodylate-buffered 2% glutaraldehyde. They were cut across their width into 1–3-mm-thick slices and prepared for light and transmission electron microscopy. Slices were rinsed in 0.1 M cacodylate buffer and postfixed in cacodylate-buffered 2% osmium tetroxide for 1 hour and then stained for 1 hour *en bloc* in 2% aqueous uranyl acetate and embedded in Spurr medium. Thick sections were stained with toluidine blue and evaluated and photographed with the use of a Zeiss light microscope. Thin sections approximately 60 nm were poststained with uranyl acetate and lead citrate[23] and examined with an AEI electron microscope.

Evaluation of Lesions

Four of the slices approximately equidistant from each other were used for evaluation of each kidney. Each slice was cut in half through the length of the papilla and embedded in such a way that sections could be taken along this edge. With this procedure many glomeruli per section were evaluated, and cortical versus juxtamedullary glomeruli could be distinguished. Thick sections were cut 1–2 μ thick, and every 12th section was stained and mounted for evaluation. In this way a particular glomerulus could be evaluated in two to four sections, which provided samples of the entire glomerulus. This was important because of the segmental nature of the habu lesion and our observation that a cyst might have some areas without mesangial cells while the majority of the cyst contained many mesangial cells. The sections were mounted in order, and the slides were numbered consecutively to allow for an accurate count of total glomeruli because they each appear in a number of sections.

Glomeruli were scored as severely affected when obvious segmental ballooning of the capillary loop was evident. Such lesions were further scored as 1) cysts, containing blood cells (erythrocytes, polymorphonuclear leukocytes), fibrin, one or two mesangial-like cells, and/or debris (Figure 1A), or 2) nodules, containing many mesangial-like cells (Figure 1B). Small nodules were also evident in the glomeruli of these habu-treated animals, generally identifiable when they contained 6 or 7 mesangial-like cells. However, a cyst of comparable size could not be distinguished from the somewhat larger capillary loops found regularly in glomeruli of control rats. Therefore, only severely affected glomeruli were evaluated in these studies of the effect of heparin administration on mesangial-cell proliferation. Data are expressed as numbers of nodules, numbers of cysts, and statistical evaluation made on the percentage of nodules. Because the variance of the populations differed, the test used to evaluate the difference between treatment groups and the habu-treated controls was a test due to Cochran[24] based on the Behren's and Fisher test. The percentage of glomeruli exhibiting severe lesions was also determined.

Results

Glomerular Lesion Produced by Habu Venom

The early morphologic changes in the glomerulus produced by habu venom were determined in rat kidneys perfusion-fixed 15, 30, and 60 minutes and 1 and 4 hours after venom injection. Our observations of the initial effects are substantially the same as those noted by Cattell and co-workers.[14,15] They include the early (15 minutes after venom) appearance of many platelets and free granules in the glomerular capillary lumen, followed at 1 hour later by blebbing of glomerular capillary endothelial and epithelial cell bodies, detachment of fenestrated portions of endothelial cells from the glo-

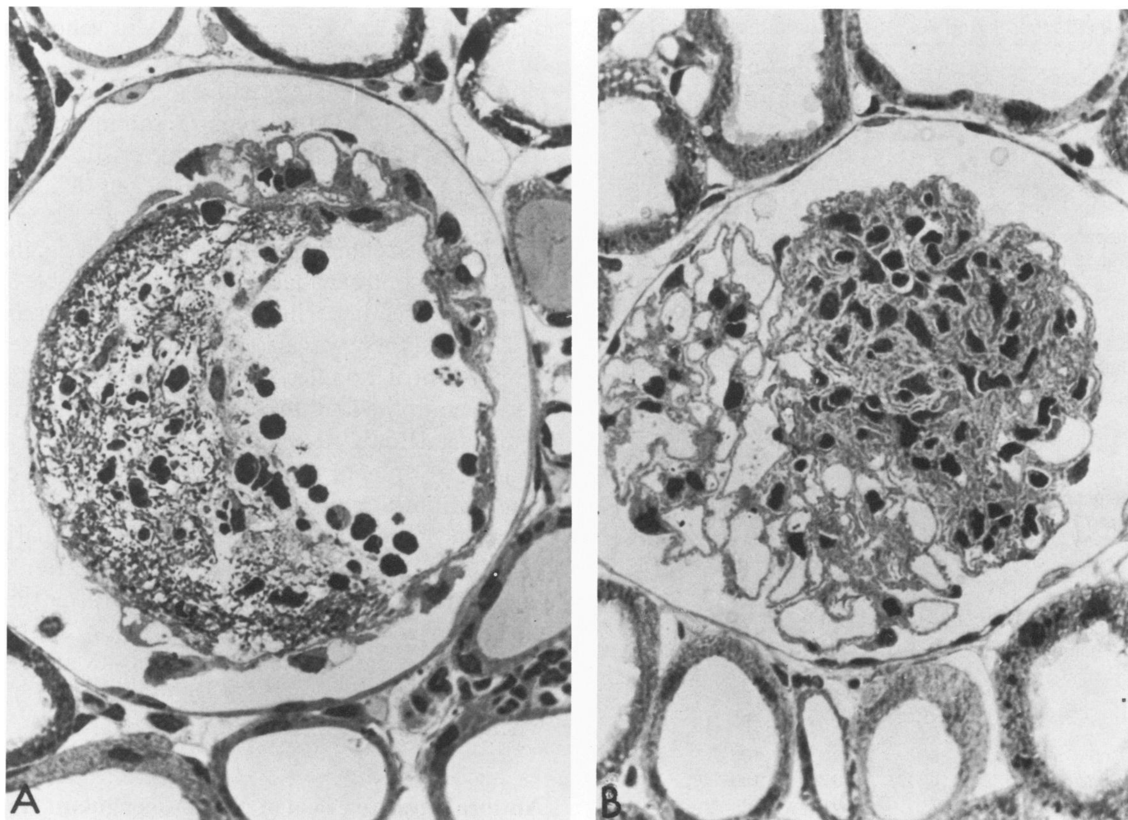

Figure 1A—Light micrograph of a glomerular cyst from a kidney of a rat that 7 days earlier was given habu snake venom and treated with heparin from 18 hours until 7 days. The cyst has empty areas, red blood cells, fibrin, and debris, but no mesangial-like cells. (×500) **B**—Light micrograph of glomerular nodule from the kidney of a rat given habu snake venom 7 days earlier. The nodule contains mesangial-like cells embedded in a dense matrix, and fibrin. (×500)

merular basement membrane, the presence of erythrocytes, polymorphonuclear leukocytes, platelets, and fibrin in the capillary loops, and decreased density of the mesangial matrix. Few if any mesangial-like cells were evident in the cysts until about 72 hours. Mesangial cells exhibited extension of cytoplasmic processes into the capillary lumen, but there was, as previously noted,[14,15] no evidence of mesangial-cell necrosis. These data therefore do not support previous reports of mesangial-cell lysis.[5,13] The glomerular basement membrane appeared to be undisrupted, continuing to line the cyst.

The constancy of lesion production and nature of the lesion was evaluated at 24 hours and at 7 or 10 days for each animal (Table 2). At 24 hours the kidneys from the majority of the habu-treated animals in this study exhibited severe segmental glomerular lesions in 1–7% of the glomeruli. One animal exhibited severe lesions in 18% of the glomeruli at both 24 hours and 7 days. This is likely to be the result of a previously reported differential effect of habu venom which can result in from 0 to 30% affected glomeruli.[14] At this time the lesions were large cystic formations which appeared to involve one-fourth to three-fourths of the glomerulus (similar to Figure 1A). Involvement of capillary loops adjacent to the already formed cyst was also noted at this time. Capillary endothelial cells were noted to be in the process of detaching from one side of the glomerular basement membrane, and the lumen of this capillary loop was becoming a part of the cyst. At the time of sacrifice at 7 or 10 days after venom injection, severe segmental glomerular lesions were noted in the other kidney of all the animals (Figure 1B). Also, the proportion of affected glomeruli remained at 1–7% after 7–10 days. At 7 days most of the lesions contained numerous cells which exhibited characteristics of mesangial cells such as spindle-shaped, irregular cell processes and moderate amounts of rough endoplasmic reticulum, Golgi apparatus, and mitochondria.[25–27] Also, the cells were embedded in electron-dense, matrix-like material and fibrin, did not form intercellular bridges or connections, and were organized in swirllike configurations. Some affected glomeruli were observed to have formed adhesions to the parietal epithelium of Bowman's capsule (synechiae) similar to those seen in Masugi nephritis[11,12] and in diffuse mesangial hyper-

Table 2—Incidence of Nodules and Cysts in Individual Rats

Treatment	Nodules*	Cysts*	% Nodules	% Cysts
Habu	10	3	77	23
	20	4	83	17
	8	1	89	11
	8	0	100	0
	21	2	91	9
Habu + heparin beginning at 18 hours	11	60	16	84
	40	19	68	32
	7	16	30	70
	9	7	56	44
	6	10	38	62
Habu + heparin beginning at 48 hours	20	14	59	41
	12	12	50	50
	12	13	48	52
	11	11	50	50
	13	6	68	32
Habu + heparin beginning at 72 hours	14	3	82	18
	6	4	60	40
	13	2	87	13
	17	18	48	52
	67	102	40	60
Habu + heparin ending at 72 hours	11	4	73	27
	17	1	94	6
	6	0	100	0
	24	3	89	11
	13	1	93	7
Habu + Choay heparin 797 beginning at 18 hours	38	0	100	0
	10	0	100	0
	7	9	44	56
	19	20	49	51
	22	4	85	15
Habu + Choay heparin 1452 beginning at 18 hours	13	2	87	13
	33	11	75	25
	13	2	87	13
	26	9	74	26
	15	1	94	6

* At 7 days after venom injection except where otherwise stated.

cellularity.[1] We also observed that habu venom results in lesion formation to the same extent in cortical and juxtamedullary glomeruli. In habu-treated rats with no further treatment until sacrifice 7 days later, 87% of the lesions were nodular and 13% were cystic (Table 3).

Antiproliferative Effect of Heparin

Because anticoagulant and nonanticoagulant heparin effectively inhibited growth of vascular smooth muscle cells both *in vivo*[16,28] and *in vitro*,[17,18] as well as mesangial cells *in vitro* (paper submitted), we tested the ability of heparins to prevent cell proliferation within the habu cystic lesion. The total incidence of lesions, cystic and nodular, was not significantly changed by the administration of anticoagulant heparin (1.25 mg/kg of heparin). This was determined by comparison of the percent affected glomeruli in the kidney removed at 24 hours after venom injection with the second kidney removed from the same animal at 7 days. Thus, heparin did not appear to be creating cysts in venom-treated rats. Heparin given in control rats had no effects on glomeruli. Although the relative proportion of nodules to cysts was changed by heparin administration, this treatment did not change the characteristics of nodules or cysts. When heparin treatment began 18 hours after venom administration and continued until sacrifice on Day 7, the percentage of nodules decreased significantly from 87% (control habu animals) to 40% (Table 3) (Figure 1A). When heparin treatment was begun after 48 or 72 hours, there was also a significant decrease in the proportion of nodules to cysts (Table 3). Beginning treatment at the later times (48 and 72 hours) resulted in an overall lower percentage of nodules. However, beginning treatment at 48 hours resulted in more consistent antiproliferative effect noted when individual animals are compared (Table 2). When heparin was given only from 18 hours until 72 hours after venom injection and sacrifice occurred 1 week after the completion of treatment, no reduction was seen in the proportion of nodules (Table 3). Thus, even if heparin promoted cyst formation, these proceeded to nodules when the heparin was withdrawn.

Antiproliferative Effect of Nonanticoagulant Heparins

Choay 1452 heparin and Choay 797 heparin have also been noted to have an antiproliferative effect on growth of vascular smooth-muscle cells and mesangial cells *in vitro* (manuscript submitted). We tested the ability of 1.25 mg/kg of these heparin derivatives to prevent mesangial proliferation within the habu cystic lesion. Treatment with Choay 797 heparin derivative resulted in no antiproliferative effect in three animals but was as effective as heparin in two other animals (Table 2).

Table 3—Prevention of Mesangial Proliferation

Treatment	Nodules	Cysts	% Nodules
Habu	67	10	87
Habu + heparin beginning at 18 hours	73	112	40*
Habu + heparin beginning at 48 hours	68	52	57†
Habu + heparin beginning at 72 hours	117	129	48‡
Habu + heparin ending at 72 hours	61	9	87
Habu + Choay heparin 797 beginning at 18 hours	75	33	69
Habu + Choay heparin 1452 beginning at 18 hours	100	25	80

Behrens–Fisher comparison of two samples when standard deviations differ.
* $P < 0.01$.
† $P < 0.02$.
‡ $P < 0.05$.

Treatment with Choay 1452 heparin resulted in slight inhibition in only two animals (Table 2). Neither of these results was statistically significant.

Discussion

We have found that treatment with anticoagulant heparin results in a dramatic decrease in mesangial cell proliferation in rats given habu snake venom. The antiproliferative effect is highly significant if treatment is begun at 18 hours after venom administration and is increasingly less effective with initiation of treatment at later times. Treatment only between 18 hours and 72 hours, the latter being when proliferation is first observed in this model, is ineffective in the prevention of mesangial proliferation. These data indicate that the stimulus to mesangial proliferation is present in the habu-treated animal from at least 18 hours until sometime after 72 hours, and also suggests that the inhibition is reversible, and that the heparin must be continuously present for an inhibitory effect. This latter point would be in accord with our observations on vascular smooth-muscle cells.[1,19,31] Our studies showed that a nonanticoagulant, nonantithrombotic derivative of heparin, Choay heparin 797, very effectively decreased mesangial proliferation in some animals. The lack of consistent inhibition of proliferation with this heparin may be due to its having an average molecular weight approximately one quarter that of heparin and due to the fact that only equimolar rather than equiweight doses were administered because of the scarcity of this material.

Both the nonanticoagulant derivatives of heparin, Choay 1452 and Choay 797, are effectively antiproliferative for vascular smooth-muscle cells and mesangial cells *in vitro* (manuscript submitted). The relative lack of effect *in vivo*, as compared with anticoagulant heparin, may be due to an increased rate of clearance or modulation of the cells in culture, so that they become responsive to the antiproliferative effect of heparin. In this regard, Olson[29] found that a low-molecular-weight nonanticoagulant heparin had no protective effect *in vivo* after reduction of renal mass, whereas anticoagulant-heparin was protective. Nonanticoagulant heparin (similar in properties to Choay 1452) clearly inhibited vascular smooth proliferation *in vivo*.[28] It is possible that mesangial cells respond differently from vascular smooth-muscle cells *in vivo*, because of inherent differences. Further experiments to clarify these similarities and differences are underway.

The antiproliferative effects of heparin on mesangial cells *in vivo* has not been previously reported; however, protective effects of heparin on renal vascular tissue have been noted. Administration of heparin to hypertensive rats[30] or rats subjected to a decrease in renal mass[29] resulted in decreased fibrinoid renal vascular lesion formation. Each of these investigations postulated the mechanism of protection to be related to the anticoagulant action of heparin. The present study does not clarify the theory that the protective role of heparin in the habu model is solely due to its anticoagulant effects, because nonanticoagulent heparin also effectively decreased mesangial cell proliferation in some, but not all, animals.

Our results reinforce the commonality of mesangial cells and vascular smooth-muscle cells. Previous work has delineated the similarities in origin,[19] morphologic features,[20,21] and contractility.[22] Data from this laboratory revealed that both vascular smooth-muscle cells and mesangial cells *in vitro* (but not endothelial cells) are extremely sensitive to anticoagulant heparin and nonanticoagulant heparin.[16–18,28] Our studies also indicate that heparin causes an arrest in the growth cycle in late G1 or at the G1-S interface.[31] Heparin thus appears to be an antiprogression factor rather than an anticompetence factor, *vis-a-vis* the cell cycle.[31]

Cattell[32] invokes platelets or their products as the stimulus to mesangial-cell proliferation in the habu snake venom model. Supportive of this evidence is the observation that platelet-derived growth factor[33] and monocyte products[34] stimulate mesangial-cell proliferation *in vitro*. Antiplatelet serum was effective in the prevention of mesangial-cell proliferation after cyst development.[32] However, the data indicating that the antiplatelet agent dipyridamole also effectively prevents mesangial-cell proliferation within the cysts are less convincing.[35] Earlier studies in this laboratory indicated that the mechanism by which heparin inhibits vascular smooth-muscle cell proliferation *in vivo* does not involve inhibition of platelet adherence and degranulation.[28,36] Because heparin administration in our study was begun considerably after the early appearance of platelets, it appears unlikely that the mechanism of heparin action involves an inactivation of platelets. Fibrin has been suggested as the stimulus to cell proliferation in Masugi nephritis.[37,38] The mechanism of action of heparin in our study is unlikely to involve an effect on fibrin, because fibrin is found within both nodules and cysts even in heparin-treated animals exhibiting highly significant inhibition of mesangial proliferation. Similarly, Thomson[39] has found that defibrination prevented crescent formation but did not prevent mesangial or endothelial proliferation.

Although elements present at the site of development of renal disease—phagocytes, fibrin, and platelets—may be responsible for inducing mesangial proliferation, our results do not indicate that the site of action of heparin involves any of these targets. A possible

mechanism in the habu model involves the replacement of endogenous heparan sulfate. Evidence that endothelial cells in culture produce a heparin like molecule which inhibits growth of vascular smooth muscle[18] may mean that vascular endothelial cells produce a heparin like substance that regulates the proliferation of nearby smooth-muscle cells. By analogy, the glomerular endothelium may play a similar role in regulation of mesangial cell division. In addition, glomerular epithelial cells in culture produce a heparinlike molecule which markedly suppresses mesangial cell proliferation (manuscript submitted). This speculation is made more attractive by the presence of heparan sulfate (the class of glycosaminoglycan to which heparin belongs) in both the glomerular basement membrane and mesangial matrix,[40] known to be produced by glomerular epithelial cells and possibly by endothelial cells but not by mesangial cells.[41] Loss of the glomerular basement membrane anionic sites, especially heparan sulfate, occurs in at least two models of glomerular injury which exhibit mesangial cell proliferation, ie, in Masugi nephritis[42] and in rats given injections of the lymphocytes from minimal-change nephropathy patients.[43] The endothelial and epithelial damage noted in the animals that received injected habu snake venom and Masugi nephritis could be responsible for decreased heparan sulfate levels. A possible relationship between loss of glomerular basement membrane heparan sulfate sites and mesangial proliferation is also supported by the correlation of the short half-life of heparan sulfate in the glomerular basement membrane (3–4 days)[44] and the observation that the mesangial proliferation first appears 3 days after habu venom injection.

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The animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education and Welfare publication [NIH] 78-23, revised 1978).

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