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Modulation of Mesangial Cell Migration by Extracellular Matrix Components

Inhibition by Heparinlike Glycosaminoglycans

JETTIE M. PERSON, MD, DAVID H. LOVETT, MD, and GREGORY J. RAUGI, MD, PhD

From the Divisions of Dermatology and Nephrology, Department of Medicine, University of Washington, and the Veterans Administration Medical Center, Seattle, Washington

Extension of mesangial cells (MC) into the pericapillary space is a pathologic response seen in several forms of glomerulonephritis. This process may involve both cytoplasmic extension by MC and actual cellular migration. For investigation of whether extracellular matrix factors could modulate this process, the migratory responses of rat MC were quantitatively examined using a cell culture model. Denuding ("wounding") a portion of a confluent culture of MC was followed by migration of mesangial cells into the denuded area. The expected proliferative response to this treatment was blocked by irradiation. The migratory response began within 8 hours of wounding and continued for at least 80 hours. The MC migratory response was specifically inhibited in a dose-dependent and reversible manner by heparin and heparinlike glycosaminoglycans (GAGs). Chondroitin sulfates and hyaluronic acid did not significantly inhibit MC migration. Glomerular basement membrane heparinlike GAGs may normally prevent MC extension into the pericapillary space. Changes in the density or composition of these substances during glomerular inflammatory processes could permit the development of MC pericapillary extensions and thereby lead to further alterations in basement membrane integrity. (Am J Pathol 1988, 133: 609–614)

ALTERATIONS IN THE GLOMERULAR mesangium are observed in many forms of acute and chronic glomerulonephritis (GN). Although a diversity of individual disease processes may be involved, the pattern of response by the intrinsic mesangial cell (MC) population is primarily characterized by cellular proliferation and extracellular matrix expansion. In addition, extension of MC into the pericapillary space between the endothelium and the basement membrane is a prominent feature of the membranoproliferative forms of GN.[1–4] Extension of MC around the capillary loop accounts for the formation of the classical "tram track" noted on silver staining, which is the result of newly synthesized mesangial matrix. The juxtaposition of mesangial cells and glomerular basement membrane in these diseases could possibly result in further structural damage through the release of MC-derived lysosomal enzymes and neutral proteinases.[5,6] It is interesting to note that MC also surround the glomerular capillary loop during the metanephric stage of mammalian kidney development and this pattern is a common feature of the developed kidneys of lower vertebrates.[7–10] Thus, the pericapillary extension of MC seen in certain forms of glomerular disease may represent a form of structural or organizational dedifferentiation.

Little is presently known about the actual mechanism whereby glomerular capillary interposition occurs. While simple MC cytoplasmic extension is clearly involved,[3] it is likely that an element of cellular migration may play an important role. Observations of prolonged cultures of MC suggested that cellular migration is involved in the development of macroscopic "hillocks," which are composed of cells and

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Address reprint requests to Gregory J. Raugi, MD, PhD, Medical Service, Veterans Administration Medical Center, 1660 Columbian Way South, Seattle, WA 98108.

large accumulations of extracellular matrix (ECM) constituents.[11] Because of our interest in the regulation of MC function by ECM factors, we elected to quantitatively assess the migratory ability of cultured rat MC and to determine the effects of ECM factors on MC migration.

Sulfated glycosaminoglycans (GAGs), in particular heparan sulfate, are of special interest in glomerular pathophysiology. The anionic heparan sulfate is an important determinant of the glomerular basement membrane (GBM) charge-filtration barrier.[12,13] In addition, recent studies by Castellot et al[14] have indicated that heparanlike substances secreted by glomerular epithelial cells, as well as heparin, have anti-proliferative effects on MC. Heparin treatment decreases the extent of MC proliferation in the Habu snake venom model of nephritis.[15] Given these findings and the potential importance of GAGs in the modulation of intrinsic glomerular cell phenotypes, we chose a model system that would allow us to study the proliferation-independent migration of MC in a quantitative manner. In this report we show that MC are capable of migration and demonstrate that heparin and heparinlike GAGs specifically and reversibly inhibit this process.

Materials and Methods

Reagents

Anticoagulant heparin (derived from porcine intestine, 159 USP U/mg) was purchased from Lypho-Med. RD-heparin (average molecular weight 5000 d, 69 USP U/mg) was purchased from Calbiochem. Chondroitin sulfate A (chondroitin 4-sulfate derived from shark cartilage), chondroitin sulfate C (chondroitin 6-sulfate derived from whale cartilage), hyaluronic acid (derived from umbilical cord), and dextran sulfates (average molecular weights 5 kd and 500 kd) were purchased from Sigma Chemical Co., St. Louis, MO.

Mesangial Cell Culture and Characterization

MC were obtained from the blood-free, perfused kidneys of male Sprague-Dawley rats (150–200 g, Charles River Laboratories, Charles River, MA) using methods described previously.[6,11] In brief, primary cultures of MC were obtained from the outgrowths of collagenase-treated glomerular remnants.[6] Proliferating MC were maintained in complete growth medium consisting of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 20% heat-inactivated fetal calf serum (FCS), penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (300 μg/ml), insulin (10^{-6} M), transferrin (5 μg/ml), and selenous acid (5 ng/ml) in a 37 C, humidified 5% CO_2 atmosphere. Outgrowths of MC appeared as stellate cells and were clearly distinguishable from epithelial cells. Small MC colonies were picked out, replated, and grown to confluency. The cells were fed twice weekly and passed at a 1:6 split ratio. MC were used between the fifth and tenth passages for these studies.

Effect of Radiation on Cell Viability and Proliferation

Cells were plated at a density of 8000 cells per 9.6 sq cm dish and allowed to attach and proliferate for 24 hours. Cells were irradiated with 1500 R from a ^{137}Cs source and were further incubated in the presence or absence of heparin (100 μg/ml). Cells were counted electronically after trypsinization. Viability was assessed by trypan blue exclusion.

Cell Migration Assay

For cell migration experiments, MC were plated on 35-mm polystyrene gridded (2-mm squares) tissue culture dishes (LUX, Scientific Supply and Equipment, Seattle, WA) and maintained in complete medium. Before use in migration experiments, confluent cultures were growth arrested by gamma irradiation with 1500 rads from a ^{137}Cs source.

Migration was stimulated by cell layer "wounding." Briefly, a portion of a confluent culture was denuded of cells ("wounded") by scraping with a sterile razor blade. Approximately half the cells were removed, creating a linear wound edge through the middle of each dish. Fifteen 2-mm squares along the wound edge were identified and numbered for each plate. Each wound edge was examined microscopically to check the uniformity of the wound, to confirm its position relative to the grid markings, and to ensure that all of the MCs were removed from the grids to be evaluated. Cultures were then rinsed three times with sterile phosphate-buffered saline (PBS) to remove any nonadherent cells and fresh complete medium containing the various test materials was added.

In the heparin dose-response and specificity experiments, the MC were allowed to migrate for 24 hours in complete medium containing the test materials. Controls were maintained in complete medium. Thereafter, the cells were fixed in 3% glutaraldehyde and stained with toluidine blue.[16] The number of cells migrating beyond the linear wound edge in each 2-mm grid was counted; in some experiments, the greatest distance migrated by a cell was measured with an

ocular micrometer. Each treated culture was compared with simultaneous controls.

Experiments to test the reversibility of the heparin effect on cell migration were done as follows: MC were allowed to migrate in complete medium or in medium supplemented with heparin (100 μg/ml) for 38 hours. Plates were washed twice with PBS. One half of the heparin-containing cultures continued to be incubated in complete medium with heparin (100 μg/ml) and the other half were incubated in complete medium without heparin. Cell migration was assessed by counting the number of cells that had migrated into the wound at intervals over the next 48 hours and compared with controls that had incubated in heparin-free medium throughout.

Statistical Analysis

The number of cells migrating into each of 15 grids for 4 culture plates was averaged for each experiment. Group means were compared by analysis of variance. In the reversal experiment, the statistical analysis was performed after randomization of the culture plates into the reversed and heparin-treated groups to determine whether the extent of migration among plates was equivalent up to the point of experimental intervention. In dose–response experiments, results for each heparin concentration were compared with controls using the Tukey studentized range method.

Results

One thousand five hundred rads of gamma radiation completely inhibited MC proliferation in the presence or absence of heparin while leaving 99% of the cells viable for the course of the experiments (Figure 1). Differences in the numbers of cells observed in the wounded area of the cultures under experimental conditions cannot therefore be due to altered proliferative rates of MC.

Other authors[17,18] have evaluated cell migration primarily by measuring the distance migrated by the leading edge of the migrating cell sheet. We found this measurement difficult to perform in our experimental system because glomerular MC do not migrate as a sheet (as do endothelial cells) but as individual units, and local variations in cell migration along a single wound edge preclude unambiguous measurement. We compared the greatest-distance-migrated method with direct counting of the number of cells migrated and found that the former method consistently underestimated the percent inhibition of migration (Table 1). We analyzed the remainder of the experiments described below by counting the number of cells migrated in each 2-mm grid. Each plate contained 15 of the grids and 4 plates were used for each point.

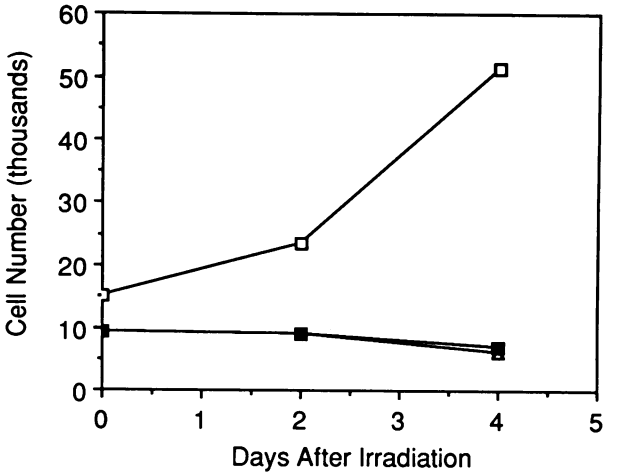

Figure 1—Effect of radiation on mesangial cell proliferation. Cells were grown and plated as described in Materials and Methods. On day 0 some cultures were treated with 1500 rads from a ^{137}Cs source and were further incubated in the presence or absence of heparin (100 μg/ml). Cell number and viability was assessed at the indicated times thereafter. Each point represents the average of triplicate counts on two dishes. □, no radiation. △, irradiated, no additions. ■, irradiated, plus heparin.

Figure 2 shows a representative photomicrograph of irradiated MC cultured for 72 hours after wounding in the presence or absence of 100 μg/ml heparin. Incubation with heparin results in a marked diminution in both the number of migrating MC as well as the greatest distance migrated by an individual cell.

The migration inhibition response to heparin is dose-dependent (Figure 3). At 100 μg/ml of heparin there is 75% inhibition of migration compared with controls. Concentrations of heparin as low as 0.1 μg/ml were sufficient to cause about 20% inhibition. Analysis of variance demonstrated a statistically significant trend ($P < 0.00005$) over the range of heparin concentrations tested. Controls were significantly different ($P < 0.05$) from 0.1 μg/ml heparin, as assessed by the Tukey studentized range method. The t value for contrasts in the group means was significant at $P < 0.00005$, showing a linear trend in migration inhibition as heparin concentration increased.

We performed additional experiments to determine if the antimigratory effect of heparin was reversible. Irradiated, wounded cultures were incubated in the presence or absence of 100 μg/ml heparin for 38 hours, resulting in significant inhibition of MC migration in heparin-treated cultures as compared to controls (Figure 4). After removal of the heparin-containing medium, cells began migrating into the wound at a rate closely comparable with the untreated controls. Heparin-treated MCs continued to migrate at a much reduced rate. The recovery of MC migration after

Table 1—Quantitative Methods for Analysis of MC Migration: Comparison of the Greatest-Distance-Migrated Method with the Grid-by-Grid Counting Method*

	Greatest distance migrated (measured micrometer divisions)			Grid-by-grid (counted number of cells migrated/2 mm)		
	Control	Heparin-treated	Percent of control	Control	Heparin-treated	Percent of control
Experiment 1	5.0 ± 1.8 (50)	3.1 ± 1.3 (44)	62	61.9 ± 33.1 (50)	23.8 ± 17.4 (44)	35
Experiment 2	4.0 ± 1.3 (59)	2.3 ± 1.4 (63)	58	50.6 ± 31.3 (58)	20.5 ± 17.0 (63)	40
Experiment 3	3.9 ± 2.0 (55)	2.0 ± 1.2 (39)	51	54.7 ± 23.6 (55)	8.5 ± 6.7 (40)	12
Experiment 4	2.7 ± 1.7 (46)	0.8 ± 1.0 (51)	30	21.6 ± 20.6 (48)	4.5 ± 6.7 (51)	21

* Experimental groups were incubated with 100 μg/ml heparin for 72 hours and compared with controls. Four or five plates with as many as 15 usable grids per plate were assessed for each experiment. The greatest distance migrated by any cell in a grid was measured with an ocular micrometer or the total number of cells migrating into the wound was counted. Data are expressed as means ± SD. Numbers in parentheses refer to the number of grids assessed.

removal of the heparin was highly significant ($P < 0.0005$) at each measured interval.

Further experiments compared the inhibitory effects of heparin on migration with other GAGs and sulfated dextrans (Table 2). At concentrations of 10 μg/ml, chondroitin 4-sulfate, chondroitin 6-sulfate, and hyaluronic acid had no significant effect on MC migration, a concentration at which heparin is highly active. Higher concentrations (100 μg/ml) of these substances induced only a modest inhibition in migration (about 70% of controls). In contrast, nonanticoagulant heparin and sulfated dextrans were highly effective in migration inhibition. Thus, the antimigratory effect on heparinlike molecules is independent of their anticoagulant activity.

Discussion

Cell-matrix interactions are important in both morphogenesis and the tissue response to injury. Whereas the influence of ECM factors on the functions of vascular endothelial cells and smooth muscle cells has been studied in detail, the potential importance of the ECM in modulation of MC function is just beginning to be appreciated.[19] MC pericapillary extension, or interposition, is a characteristic feature of membranoproliferative forms of GN. This is thought to result in part from direct extension of MC cytoplasm into the pericapillary (subendothelial) space of the glomerulus.[3] The concept that MC interposition is also the result of actual cellular migration, taken with the tendency of MC to aggregate and form macroscopic hillocks during prolonged cell culture,[11] led us to investigate whether MC were indeed capable of migration and if ECM components could modulate this activity.

We have shown that cultured MC are capable of proliferation-independent migration. MC migrate as individual units in analogy to vascular smooth muscle

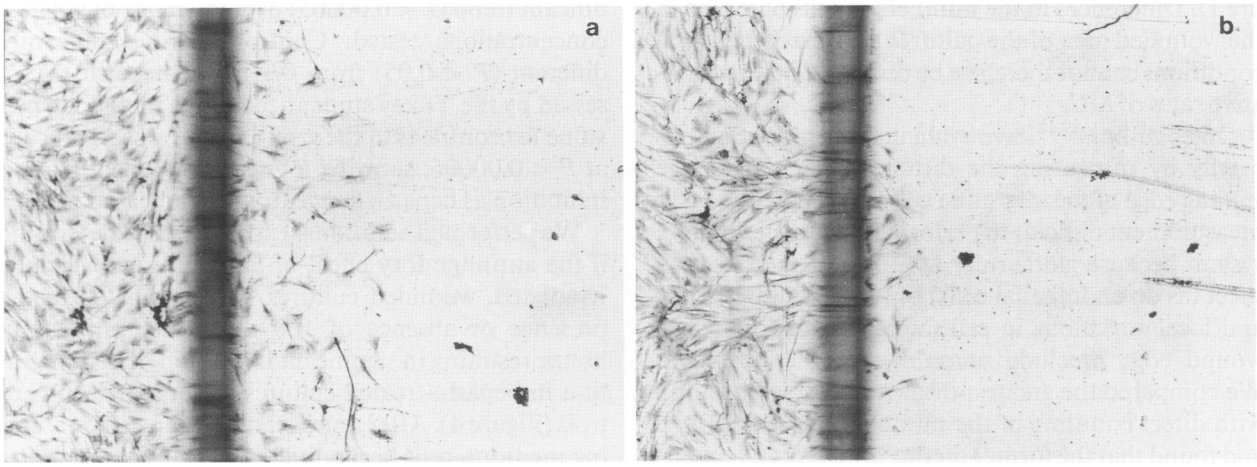

Figure 2—Inhibition of mesangial cell migration by heparin. The wound edge is marked with solid line. The cultures were irradiated, wounded, and treated with either control media (**a**) or media containing 100 μg/ml heparin (**b**) for 72 hours. The illustration shows one 2 × 2-mm grid section along the wound line. MC migration occurs as single cellular units. Quantitation was performed by counting the cells in each grid, as well as by measuring the greatest distance migrated (μ) by a single cell. Final magnification, ×475.

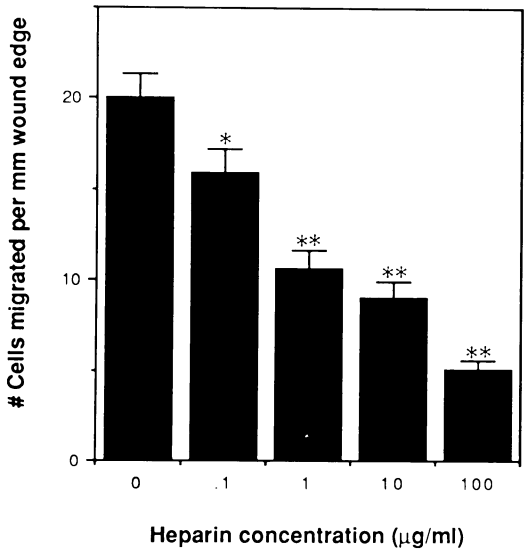

Figure 3—Dose–response effect of heparin on RMC. Cultures were irradiated, wounded, and treated with the indicated concentrations of heparin for 24 hours. Note that the heparin effect is dose-related. Data are expressed as means ± SEM. *$P < 0.05$; **$P < 0.01$.

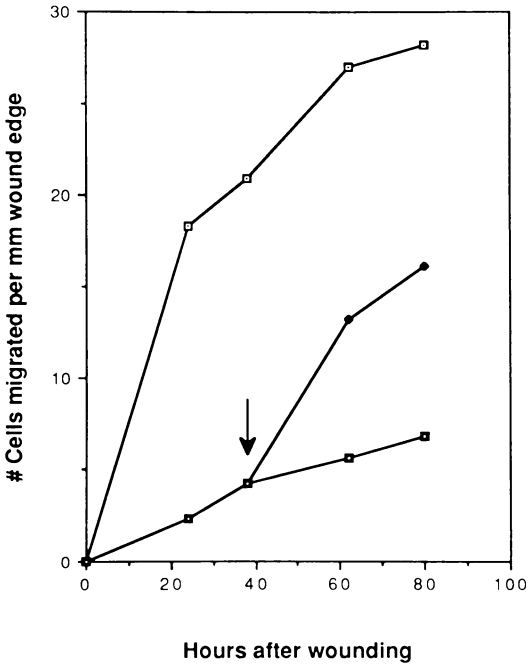

Figure 4—Reversibility of heparin inhibition of RMC migration. Data points shown are the means of duplicate dishes of cells. The cells were counted at daily intervals for control cultures (□), cultures treated continuously with 100 μg/ml heparin (■), and cultures treated for 38 hours with heparin, washed, and treated with control medium (arrow, ♦). Control cultures are significantly different from heparin-treated cultures ($P < 0.01$) at all time points after wounding. Heparin-treated cultures incubated in the absence of heparin after 38 hours of heparin treatment are significantly different from cultures continuously maintained in heparin ($P < 0.01$) at each time point after the change in culture conditions. Data points shown are from one representative experiment and represent the mean number of cells migrated in a total of 60 grids.

cells and fibroblasts. The inhibition of MC migration by heparin and heparinlike GAGs is dose-dependent, specific, and reversible. The anti-migratory effects of heparin are independent of its anticoagulant activity. The heparin dose–response curve for migration inhibition closely resembles that obtained by Castellot et al[14] for inhibition of mesangial cellular proliferation. In addition Majack and Clowes[17] observed a similar heparin dose–response curve for the inhibition of migration of cultured rat aortic smooth muscle cells. The GAG specificity and reversibility of MC migration inhibition are also similar and further underscore the close resemblance of vascular SMC to the intrinsic mesangial cell.

Heparin is a naturally occurring GAG with many biologic activities comparable with the carbohydrate moieties of heparan sulfate proteoglycan. In addition to the anti-proliferative and anti-migratory effects on vascular SMC described above, heparin or related GAGs modulate the secretory phenotypes of these cells.[20–22] The mechanisms by which heparin modulates cellular proliferation, migration, and protein secretory phenotypes remain unclear. Interaction with specific cell surface receptors, internalization via clathrin-coated pits, or alterations in the composition of the ECM may all be involved.[22,23]

Heparan sulfate proteoglycan is of critical importance in the maintenance of the glomerular charge-filtration barrier.[12,13] Our results, taken with those of Castellot et al,[14] expand the physiologic role of ECM heparinlike GAGs to include modulation of MC proliferation and migration. We hypothesize that glomerular disease processes involving MC pericapillary extension may result in part from an imbalance between the inhibitory actions of glomerular ECM heparan sulfate and the mitogenic, and potentially chemoattractant, effects of platelet and complement-derived

Table 2—Comparison of Related Glycosaminoglycans and Sulfated Dextrans on MC Migration*

	Percent control migration	
Substance added	10 μg/ml	100 μg/ml
None	100	100
Hyaluronic acid	102 ± 1	70 ± 2†
Chondroitin 4-sulfate	90 ± 6	78 ± 12†
Chondroitin 6-sulfate	89 ± 9	72 ± 14†
Heparin	44 ± 5†	31 ± 6†
RD-heparin	ND	28
Dextran sulfate (MW, 5000)	42 ± 8†	16 ± 5†
Dextran sulfate (MW, 500,000)	ND	19 ± 10†

* After irradiation and wounding the various substances were added at final concentrations of 10 and 100 μg/ml. Inhibition of cellular migration was assessed after 24 hours of culture and is expressed as the percent inhibition as compared with controls. Data given as mean ± SEM. Statistical significance is not assessed in the RD-heparin experiment because the data are derived from a single experiment. ND, not done.

† $P < 0.05$.

mediators. Using our quantitative model for MC migration such issues may now be approached directly.

References

1. Habib R, Kleinknecht C, Gubler MC, Levy M: Idiopathic membranoproliferative glomerulonephritis in children: A report of 105 cases. Clin Nephrol 1973, 1: 194–212
2. Davis BK, Cavallo T: Membranoproliferative glomerulonephritis. Am J Pathol 1976, 84:283–298
3. Churg J, Grishman E: Ultrastructure of glomerular disease: A review. Kidney Int 1975, 7:254–270
4. Sterzel RB, Lovett DH, Stein HD, Kashgarian M: The mesangium and glomerulonephritis. Klin Wochenschr 1982, 60:1077–1094
5. Lovett DH, Ryan JL, Kashgarian M, Sterzel RB: Lysosomal enzymes in glomerular cells of the rat. Am J Pathol 1982, 107:161–166
6. Lovett DH, Sterzel RB, Kashgarian M, Ryan JL: Neutral proteinase activity produced in vitro by cells of the glomerular mesangium. Kidney Int 1982, 23:342–347
7. Kazimierczak J: A study by scanning (SEM) and transmission (TEM) electron microscopy of the glomerular capillaries in developing rat kidney. Cell Tiss Res 1980, 212:241–255
8. Osathanondh V, Potter EL: Development of human kidney as shown by microdissection. V. Development of vascular pattern of glomerulus. Arch Pathol 1966, 82:403–411
9. Kuhn K, Sterzel RB, Stolte H, Reale E: Mesangial cells in different vertebrate kidneys: A thin-section and freeze-fracture study. Contrib Nephrol 1976, 2:9–16
10. Pak Poy RKF: Electron microscopy of the amphibian renal glomerulus. Austral J Exp Biol 1957, 35:583–594
11. Sterzel RB, Lovett DH, Foellmer HG, Perfetto M, Biemesdorfer D, Kashgarian M: Mesangial cell hillocks: nodular foci of exaggerated growth of cells and matrix in prolonged culture. Am J Pathol 1986, 125: 130–137
12. Kanwar YS, Farquar M: Anionic sites in the glomerular basement membrane. J Cell Biol 1979, 81:137–153
13. Kanwar YS, Jakubowski ML, Rosenzweig LJ: Distribution of sulfated glycosaminoglycans in the glomerular basement membrane and mesangial matrix. Eur J Cell Biol 1983, 31:290–295
14. Castellot JJ, Hoover RL, Harper PA, Karnovsky MJ: Heparin and glomerular epithelial cell-secreted heparinlike species inhibit mesangial cell proliferation. Am J Pathol 1985, 120:427–435
15. Coffey AK, Karnovsky MJ: Heparin inhibits mesangial cell proliferation in Habu-venom-induced glomerular injury. Am J Pathol 1985, 120:248–255
16. Raugi GJ, Lovett DH: Thrombospondin secretion by cultured human glomerular mesangial cells. Am J Pathol 1987, 129:364–372
17. Majack RA, Clowes AW: Inhibition of vascular smooth muscle cell migration by heparin-like glycosaminoglycans. J Cell Physiol 1984, 118:253–256
18. Castellot JJ, Favreau LV, Karnovsky MJ, Rosenberg RG: Inhibition of vascular smooth muscle cell growth by endothelial cell-derived heparin. J Biol Chem 1982, 257:11256–11260
19. Kashgarian M: Editorial: Mesangium and glomerular disease. Lab Invest 1985, 52:569–571
20. Majack RA, Bornstein P: Heparin and related glycosaminoglycans modulate the secretory phenotype of vascular smooth muscle cells. J Cell Biol 1984, 99:1688–1695
21. Majack RA, Bornstein P: Heparin regulates the collagen phenotype of vascular smooth muscle cells: Induced synthesis of an M_r 60,000 collagen. J Cell Biol 1985, 100:613–619
22. Majack RA, Cook SC, Bornstein P: Platelet-derived growth factor and heparin-like glycosaminoglycans regulate thrombospondin synthesis and deposition in the matrix by smooth muscle cells. J Cell Biol 1985, 101: 1059–1070
23. Castellot JJ, Wong K, Herman B, Albertini DF, Wright TC, Hoover RL, Karnovsky MJ: Binding and internalization of heparin by vascular smooth muscle cells. J Cell Biol 1983, 97(5,Pt2):430a.

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