Inhibition of Heparanase-Mediated Degradation of Extracellular Matrix Heparan Sulfate by Non-anticoagulant Heparin Species

By Matia Bar-Ner, Amiram Eldor, Lina Wasserman, Yaacov Matzner, Irun R. Cohen, Zvi Fuks, and Israel Vlodavsky

Incubation of human platelets, human neutrophils, or highly metastatic mouse lymphoma cells with sulfate-labeled extracellular matrix (ECM) results in heparanase-mediated release of labeled heparan sulfate cleavage fragments (0.5 < K<sub>m</sub> < 0.85 on Sepharose 6B). This degradation was inhibited by native heparin both when brought about by intact cells or their released heparanase activity. Degradation of heparan sulfate in ECM may facilitate invasion of normal and malignant cells through basement membranes. The present study tested the heparanase inhibitory effect of nonanticoagulant species of heparin that might be of potential use in preventing heparanase-mediated extravasation of bloodborne cells. For this purpose, we prepared various species of low-sulfated or low-mol-wt heparins, all of which exhibited <7% of the anticoagulant activity of native heparin. N-sulfate groups of heparin were manifested by chemically modified heparins devoid of anticoagulant activity and some of which exhibited <7% of the anticoagulant activity of native heparin. N-sulfate groups of heparin are necessary for its heparanase inhibitory activity but can be substituted by an acetyl group provided that the O-sulfate groups are retained. O-sulfate groups could be removed provided that the N positions were resulfated. Total desulfation of heparanase-mediated degradation of ECM was inhibited by native heparin abolished its heparanase inhibitory activity. Heparan sulfate was a 25-fold less potent heparanase inhibitor than native heparin. Efficiency of low-mol-wt heparins to inhibit degradation of heparan sulfate in ECM decreased with their main molecular size, and a synthetic pentasaccharide, representing the binding site to antithrombin III, was devoid of inhibitory activity. Similar results were obtained with heparanase activities released from platelets, neutrophils, and lymphoma cells. We propose that heparanase inhibiting nonanticoagulant heparins may interfere with dissemination of bloodborne tumor cells and development of experimental autoimmune diseases.

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MATERIALS AND METHODS

Fibroblast growth factor (FGF) was kindly provided by Dr D. Gospodarowicz, University of California, San Francisco. Dulbecco’s medium conditioned by ESb lymphoma cells. Native heparin inhibited heparanase-mediated degradation of ECM by intact cells as well as by their respective cell-free enzyme preparations. The aims of the present study were (a) to evaluate the extent to which chemically modified or low-mol-wt heparins, that lack anticoagulation activity, retain the capacity of native heparin to inhibit heparanase-mediated release of HS fragments from subendothelial ECM; (b) to determine the minimal structural features of heparin required for inhibition of heparanase; and (c) to compare the inhibitory effect of native and modified heparins on heparanase elaborated by platelets, granulocytes, and metastatic lymphoma cells.

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modified Eagle's medium (DMEM-H-16), RPMI medium 1640, calf serum, fetal calf serum (FCS), penicillin, and streptomycin were obtained from Gibco (Grand Island, NY). Tryptsin-EDTA solution was obtained from Biological Industries (Beit-Haemek, Israel). Tissue culture dishes and flasks were obtained from Falcon Labware Division, Becton Dickinson (Oxnard, CA). Some of the ECM-coated tissue culture dishes were kindly provided by International Biotechnologies (IBT, Jerusalem). Triton X-100 and dextran T-40 were from Sigma Chemical (St Louis). Thrombin was from Sigma, and sodium heparin was from Diosynth (Oss, Holland). Low-mol-wt heparins were prepared as previously described.22-24 In brief, pyridinium heparin22 underwent exhaustive desulfation with dimethyl sulfoxide (Merck, Rahway, NJ) containing 10% water to yield totally desulfated heparin.22,24 N-desulfated heparin was obtained from the respective pyridinium salt22,24 with dimethyl sulfoxide containing 5% water. Totally desulfated heparin and N-desulfated heparin were N-acetylated with acetic anhydride,25 whereas resulfation of the free amino residues of the totally desulfated or N-desulfated heparin was performed with sulfuryl trifluoromethanesulfonic acid complex (Aldrich Chemical, Milwaukee) as described.22,23 Cephalin in the form of activated cephaloplastin reagent (Sigma) was used as a source for ESb heparanase activity.8 Preparation of sulfate-labeled ECM-coated dishes. Corneal endothelial cells were plated at an initial density of 4 x 10^4 cells/35-mm dish and maintained as described above except that 5% dextran T-40 was included in the growth medium.15,16 Na_2[35S]SO_4, was added twice (40 μCi/ml), on the third day after seeding when the cells were confluent, and 4 days afterwards, and the cultures were incubated with the label no medium change. Ten to 12 days after seeding, the cell layer was dissolved by exposure (3 minutes, 22°C) to a solution containing 0.5% (vol/vol) Triton X-100 and 0.025N NH_4OH in PBS followed by four washes in PBS, leaving the underlying ECM intact, firmly attached to the entire area of the tissue culture dish and free of cellular debris.4,14,15 Assay for heparanase activity. Heparanase activity assay using sulfate-labeled ECM as substrate was previously reported in studies with human platelets, human neutrophils, and ESb mouse lymphoma cells.14,15 In brief, cells or cell-free enzyme preparations were incubated (4 to 24 hours, 37°C, pH 6.2 to 6.5) with [35S]O_4-labeled ECM. Samples were then centrifuged (10,000 g, 5 minutes, 4°C), and 0.5-ml aliquots of the supernatants were applied for gel filtration on Sepharose 6B columns (0.7 x 35 cm) equilibrated with PBS containing 0.1% sodium azide. Fractions (0.2 ml) were collected at a flow rate of 5 ml/h and counted for radioactivity. The excluded volume (V_e) was marked by blue dextran, and the total included volume (V_t) was marked by phenol red. Similar gel filtration profiles (K_m values) were obtained by using the ECM produced by corneal or vascular endothelial cells, and whether the centrifuged media were subjected to gel filtration under dissociation conditions (4 mol/L of guanidine-HCl in 0.1 mol/L of sodium acetate pH 5.5) or eluted with PBS.4 Recoveries of labeled material applied to the columns ranged from 85% to 95% in different experiments. Each experiment was performed at least three times, and the variation in elution positions (K_m values) was <10%.

RESULTS

Modified and low-mol-wt heparins. Several modified heparins and low-mol-wt heparins of low anticoagulant activity were tested in the present study. The modified heparins were first either totally desulfated or N-desulfated. These heparins were then left with their N-position exposed or were further N-acetylated or N-resulted. Although the unmodified heparin had an anticoagulant activity of 250 U/mg, and the totally desulfated heparin was devoid of any anticoagulant activity, the other modified heparins (N-desulfated; N-desulfated and N-acetylated; totally desulfated and N-desulfated; totally desulfated and N-acetylated) exhibited <5% of the anticoagulant activity of native heparin (Fig 1 and Table 1). Two low-mol-wt heparins, one ranging from 1,500 to 8,000 daltons (main mol wt species 2,500 daltons) and the other ranging from 1,800 to 8,000 daltons (main mol wt species 4,500 daltons)12 as well as a synthetic
HEPARANASE INHIBITION BY MODIFIED HEPARINS

Fig 1. Dose dependence of coagulation inhibition activity of modified and low-mol-wt heparins. Activated partial thromboplastin time (APTT) was determined for various concentrations of standard heparin (I); totally desulfated heparin (II); N-desulfated and N-acetylated heparin (III); low-mol-wt heparin, 4,500-dalton main mol wt species (IV); or low-mol-wt heparin, 2,500-dalton main mol wt species (V). APTT was performed with activated cephaloplatin reagent, using an automatic coagulation analyzer. APTT clotting time in the absence of any heparin was 20 seconds. Each data point represents the mean of four determinations and the standard deviation did not exceed 12% of the time measured.

α-methyl-glycoside pentasaccharide2 were also tested. The anticoagulant activities of these low-mol-wt heparin species were 17.5, 21.5, and 10.5 U/mg, respectively. For some of these heparin species, we tested dose dependence of coagulation-inhibiting activity. As shown in Fig 1, some of the heparin derivatives retained low anticoagulant activity even at high concentrations, whereas the two species of low-mol-wt heparins effectively inhibited coagulation at high concentrations, despite their low anticoagulant activity at low concentrations.

Inhibition of platelet heparanase. Human platelets (2.7 × 10⁴ platelets/μL), washed and suspended in ACD saline were incubated (37°C, 18 hours, pH 6.2) with sulfate-labeled ECM without or with either native heparin, N-desulfated and N-acetylated heparin, or heparin that underwent total desulfation followed by N-resulfation. HS degradation products released from ECM into the incubation medium were analyzed by gel filtration. Platelets cleaved the HS proteoglycan side chains into low-mol-wt fragments which eluted from Sepharose 6B at Kₐₜₒ ≈ 0.7 (93% of the released radioactivity eluted in peak II, fractions 30 through 46). With native heparin (25 μg/mL) only high-mol-wt labeled material was released (Fig 2) (radioactivity eluted in the region of peak II, fractions 25 through 40 was 6.5% of that observed in the absence of heparin, similar to that obtained in the absence of heparanase activity). With 10 mg/mL of native heparin, the amount of radioactivity eluted in peak II (fractions 30 through 46) was decreased by 85%, and the residual 15% degradation fragments had a higher mol wt than in the absence of heparin (Kₐₜₒ ~ 0.5 as compared to Kₐₜₒ ~ 0.7). Totally desulfated heparin added at the same concentration as native heparin did not exhibit any inhibitory effect on the platelet heparanase, as indicated by elution of >90% of the released radioactivity in the form of low-mol-wt material (peak II), similar to that released without heparin (Fig 2A). In contrast, totally desulfated heparin that underwent resulfation at the N-position inhibited by 51% heparanase mediated release of low-mol-wt labeled material (Fig 2A). N-desulfated and N-acetylated heparin inhibited even more effectively the cleavage of HS side chains, and the amount of radioactivity eluted in peak II was 30% of that eluted by platelets without any heparin, indicating 70% inhibition in the release of heparanase degradation products (Fig 2A). With both these modified heparins, the size of released HS fragments was larger (Kₐₜₒ ~ 0.6) than that observed in the absence of any heparin. Although native heparin, N-desulfated and N-acetylated and totally desulfated and N-resulted heparins inhibited by 52%, 40%, and 31%, respectively, the total amount of radioactivity released from ECM by platelets, they inversely affected the amount of radioactivity eluted in the form of high-mol-wt material (Fig 2A).

The inhibitory effect of modified heparins was also demonstrated with the platelet heparanase in a cell-free preparation. In these experiments, platelet-depleted serum, obtained from thrombin-treated PRP, was incubated with sulfate-labeled ECM with or without the various heparins. Although native heparin (5.5 μg/mL) completely inhibited the release of low-mol-wt HS fragments, both N-desulfated and N-acetylated heparin and totally desulfated and N-resulted heparin, added at the same concentration, inhibited the release of low-mol-wt fragments by 50% to 60% (data not shown). Hence, the relative inhibitory effect of these modified heparins was similar when tested with either whole platelets or with their released heparanase.

Table 1. Inhibition of ESb Heparanase by Modified Heparins

<table>
<thead>
<tr>
<th>Heparin Species</th>
<th>Anticoagulant Activity (U/mg)</th>
<th>Radioactivity in Peak I (%)</th>
<th>Radioactivity in Peak II (%)</th>
<th>Inhibition of Heparanase Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No heparin</td>
<td>—</td>
<td>27</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>Unmodified heparin</td>
<td>250</td>
<td>83</td>
<td>13</td>
<td>100</td>
</tr>
<tr>
<td>Totally desulfated</td>
<td>0</td>
<td>24</td>
<td>73</td>
<td>0</td>
</tr>
<tr>
<td>N-Desulfated</td>
<td>16.5</td>
<td>31</td>
<td>66</td>
<td>7</td>
</tr>
<tr>
<td>Totally desulfated and N-resulted</td>
<td>11.5</td>
<td>80</td>
<td>15</td>
<td>96</td>
</tr>
<tr>
<td>N-Desulfated and N-acetylated</td>
<td>11.5</td>
<td>82</td>
<td>14</td>
<td>99</td>
</tr>
<tr>
<td>Totally desulfated and N-acetylated</td>
<td>11.5</td>
<td>82</td>
<td>14</td>
<td>99</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>ND</td>
<td>29</td>
<td>68</td>
<td>3</td>
</tr>
</tbody>
</table>

Serum-free medium conditioned by ESb cells was incubated with sulfate-labeled extracellular matrix (ECM) (37°C, 48 h, pH 6.2) without or with 5 μg/mL of native heparin, heparan sulfate, or modified heparins, as indicated. The incubation media were collected, and labeled degradation products were analyzed by gel filtration over Sepharose 6B. Percentage of radioactivity in peak I (Kᵥᵥ < 0.33, fractions 15–27) and in peak II (0.4 < Kᵥᵥ < 0.85, fractions 31–47) was determined in relation to the total radioactivity released from the matrix by the ESb-conditioned medium in the presence of the heparin species tested. Heparanase inhibition was considered 100% with 5 μg/mL unmodified heparin, and inhibition values for the other heparin species were determined in relation to native heparin.
The inhibitory effect of low-mol-wt heparins on platelet heparanase is shown in Fig 2B. Platelets (2.7 x 10⁷/mL) washed and suspended in ACD saline, were incubated with sulfate-labeled ECM without or with each of the low-mol-wt heparins. At 25 µg/mL, both preparations of low-mol-wt heparins (main mol wt species 2,500 and 4,500 daltons) inhibited heparanase-mediated release of labeled fragments from ECM, albeit to a lower extent than did native heparin (Fig 2B). At higher concentration (50 µg/mL) inhibition of the platelet heparanase by low-mol-wt heparins was similar to that exerted by full-size unmodified heparin (not shown). The synthetic pentasaccharide exhibited only mild inhibition (10% to 20%) at 50 µg/mL and none at all at 25 µg/mL (Fig 2B).

Inhibition of neutrophil heparanase. Incubation buffer containing heparanase activity was derived from human neutrophils (2.5 x 10⁶ cells/mL) kept on ice for 60 minutes. Incubation of this supernate with labeled subendothelial ECM resulted in release of low-mol-wt HS cleavage fragments which eluted with Kᵥ > 0.63 from Sepharose 6B. In contrast, proteolytic degradation products released by the neutrophil supernate from ECM in the presence of native heparin (10 µg/mL) eluted from Sepharose 6B as a broad peak next to Vᵥ. Under this condition, 13% of the total released radioactivity eluted with Kᵥ > 0.5 (Table 2), similar to that obtained without any heparanase activity (ie, pH 8). Inhibition of neutrophil heparanase by native and modified heparins had a pattern similar to that observed with platelets, yet lower concentrations were required for complete inhibition. Although totally desulfated heparin and the synthetic pentasaccharide failed to inhibit the neutrophil heparanase at 10 µg/mL, 86% inhibition of heparanase-mediated release of low-mol-wt labeled material was observed with the same concentration of N-desulfated and

<table>
<thead>
<tr>
<th>Heparin Species</th>
<th>Radioactivity in Peak I (of Total)</th>
<th>Inhibition of Heparanase Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No heparin</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Unmodified heparin</td>
<td>65.7</td>
<td>100</td>
</tr>
<tr>
<td>Totally desulfated and N-resulfated heparin</td>
<td>51</td>
<td>75</td>
</tr>
<tr>
<td>N-desulfated and N-acetylated heparin</td>
<td>57.7</td>
<td>86</td>
</tr>
<tr>
<td>Low-mol-wt, 2,500-dalton main species</td>
<td>63.9</td>
<td>95</td>
</tr>
<tr>
<td>Low-mol-wt, 4,500-dalton main species</td>
<td>66.4</td>
<td>99</td>
</tr>
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Supernates of freshly isolated neutrophils were incubated (37°C, pH 6.2, 18 hours) with sulfate-labeled extracellular matrix in the absence or presence of 10 µg/mL modified and low-mol-wt heparins, as indicated. The incubation media were collected, and labeled degradation products were analyzed by gel filtration over Sepharose 6B. Percentage of radioactivity eluted in peak I (Kᵥ < 0.33, fractions 15–27) and in peak II (0.5 < Kᵥ < 0.85, fractions 35–47) was determined in relation to total radioactivity released from the matrix. Heparanase inhibition was considered 100% with 10 µg/mL of native heparin (described in text), and inhibition values for the other species of heparin were determined in relation to native heparin.
N-acetylated heparin, 95% to 99% inhibition with the two low-mol-wt heparins and 75% inhibition with totally desulfated and N-resulfated heparin (Table 2). When these heparins were tested at a lower concentration (2 µg/mL) for inhibition of a more concentrated heparanase preparation (supernatant derived from 8 x 10^6 neutrophils per mL), however, only native heparin completely inhibited heparanase-mediated degradation of the ECM HS.

**Inhibition of ESb heparanase.** Serum-free medium conditioned by highly metastatic ESb mouse lymphoma cells was incubated with sulfate-labeled ECM in the presence of chemically modified heparins. The inhibitory effect of totally desulfated and N-resulfated heparin and of N-desulfated and N-acetylated heparin was, at 5 µg/mL, essentially similar to that of native heparin (Table 1). Similar results were obtained with native heparin and with N-desulfated and N-acetylated heparin at 2 µg/mL, whereas at 1 µg/mL release of low-mol-wt HS fragments was inhibited by 60% and 50%, respectively. In contrast, both N-desulfated heparin and totally desulfated and N-acetylated heparin failed to inhibit the enzyme, as demonstrated by the appearance of low-mol-wt (peak II) labeled products (Table 1). Hence, retention of O-sulfate groups is essential for the heparanase inhibitory activity of N-acetylated heparin. HS was a much less efficient inhibitor of heparanase than was native heparin or the other inhibitory modified heparins (Table 1). Complete inhibition of the ESb heparanase was not obtained even with a 20-fold higher concentration of HS (100 µg/mL) as compared with native heparin. Dermatan sulfate, at 50 µg/mL, completely inhibited release from ECM of low-mol-wt HS degradation fragments, whereas chondroitin sulfate failed to inhibit the ESb heparanase significantly at this concentration.

With the ESb enzyme, we also tested the effect of heparin fragments obtained through cleavage of commercial heparin and kindly provided by KabiVitrum AB (Stockholm). Preliminary results indicated that whereas disaccharides and trisaccharides were inactive as heparanase inhibitors at 50 µg/mL, oligosaccharides as small as tetrasaccharide inhibited release of low-mol-wt HS fragments from ECM, at this concentration. Decasaccharide, but not octasaccharide, however, exhibited a significant inhibition of the ESb heparanase already at 10 µg/mL (data not shown).

**DISCUSSION**

In the present study, we showed that heparin species of low mol wt as well as some chemically modified heparins inhibited degradation of HS in the subendothelial ECM by heparanase activities derived from various cell types. This inhibition depended on the dose of heparin, and its effectiveness was related to the main size and certain chemical features of the heparin molecules. The inhibition also depended on enzyme concentration, but for a given heparin species the inhibitory capacity did not depend on the cell source of the heparanase, whether normal or malignant. In an attempt to mimic the complexity of the situation in vivo, we used a bioassay in which heparanase activity is measured by its capacity to degrade HS proteoglycans embedded in the ECM deposited by cultured endothelial cells. Our results indicate that native and certain modified heparins inhibited the enzyme when expressed by whole cells incubated with this ECM, as well as crude preparations of enzyme released by cells and incubated with ECM. Native heparin was a more potent heparanase inhibitor than any of the modified heparins used. When these heparins were added at the minimal concentration sufficient for native heparin to inhibit the enzyme completely, only partial inhibition of heparanase was observed. When tested at higher concentrations, however, some chemically modified heparins completely inhibited degradation of the ECM HS. Similar results were obtained with the various cell types and heparanase preparations that were tested, whether cell associated or not.

Using the chemically modified heparin species, we were able to characterize some of the structural requirements for heparanase inhibition activity. Our results indicated that heparanase inhibition depended on the degree of sulfation of the heparin molecule, the position of sulfate groups, and the occupancy of the N-positions of the hexoseamines. Although totally desulfated heparin did not inhibit the enzyme, sulfation of the N-position reestablished its heparanase inhibitory activity, suggesting that a sulfate group at the O-position was not necessary for inhibitory effect provided that the N-position was sulfated. In contrast, N-acetylation of totally desulfated heparin did not result in heparanase inhibitory activity, indicating that under certain conditions the O-sulfate group was indeed required for enzyme inhibition. Partial desulfation of heparin that resulted in exposure of the N position, yet left the O-sulfates intact, caused complete loss of heparanase inhibitory activity. In this case however, if the desulfated N-position was substituted with the neutrally charged acetyl group, the modified heparin (N-desulfated and N-acetylated heparin) efficiently inhibited the three heparanases tested. Such heparin was also reported to inhibit melanoma heparanase.26 In this modified heparin, the O-sulfate group had an important role since N-acetylation of totally desulfated heparin yielded a noninhibitory species for heparanase activity. A neutral charge at the N-position and intact O-sulfate groups were also necessary for the expression of antiproliferative activity on vascular smooth muscle cells by nonanticoagulant heparin.11,12 HS was about a 25-fold less efficient inhibitor of heparanase than native heparin or some of the nonanticoagulant heparin species. This could be due in part to the observation that heparin is a poor substrate of the enzyme as compared with HS.7

The heparanase inhibitory activity of heparin and its anticoagulant activity appeared to be unrelated. Low-mol-wt heparins showing <10% of the native heparin anticoagulant activity and certain chemically modified heparin species with <5% of the anticoagulant activity, retained substantial heparanase inhibitory activity. In contrast, a synthetic homogeneous pentasaccharide, representing the binding site in heparin for antithrombin III, had a very small or no heparanase inhibitory effect, dissociating these two activities as well. Heparin possesses several biological activities that are not related to its anticoagulant activity. Modified nonanticoagulant heparins retained the ability to inhibit vascular smooth muscle cell proliferation, angiogenesis, neutrophil chemotax-
is, and delayed-type hypersensitivity, and were suggested to be of potential therapeutic use.11,15

Our studies with various types of normal bloodborne cells have demonstrated a correlation between expression of heparanase activity and the cell capacity to penetrate through blood vessel walls.1,4 Expression of heparanase appeared to be an early event in T lymphocyte activation, one that could be induced by antigen1,9 and possibly other signals. Likewise, as found by other researchers and ourselves, the metastatic potential of mouse melanoma, fibrosarcoma, and lymphoma sublines correlated with their ability to degrade the ECM by means of their heparanases.7,10 Although the functional significance of heparanase activity in various normal and malignant cells is not yet clear, this enzyme, in conjunction with other hydrolytic enzymes,25,27,28 is likely to play a role in the solubilization of extracellular matrices and basement membranes. In this respect, heparin-like molecules, either present on the endothelial cell surface11,30 and in subendothelial ECM19 or, as previously suggested,11 released in response to vascular injury, may modify the ability of cells to extravasate. Some indirect indications show that heparin and nonanticoagulant heparins may indeed affect lymphocyte traffic and inhibit the movement of cells through vessel walls. Administration of heparin prevented the disappearance of macrophages from peritoneal exsudates, following intraperitoneal injection of antigen into sensitized guinea pigs.31 Sy and colleagues reported that the inhibition of hapten-specific delayed-type hypersensitivity by nonanticoagulant heparins was associated with reduction of the leukocytic infiltrate and suggested that heparins may impair the emigration of inflammatory cells at the antigen challenge sites.15 We found that a daily injection of either native heparin or of heparanase inhibiting modified heparins markedly inhibited the progression of experimental autoimmune encephalomyelitis and adjuvant arthritis.16 Moreover, a similar treatment reduced the incidence of lung metastasis in tumor bearing experimental animals (manuscript in preparation). Likewise, Irimura and co-workers reported that preincubation of B16-BL6 melanoma cells with native and modified heparins greatly reduced the number of lung metastases following intravenous injection of the tumor cells to syngeneic mice.26

Exogenously administered heparanase inhibitors such as nonanticoagulant heparins might be of potential therapeutic use in preventing the extravasation of normal and malignant cells in certain pathological situations. They can also serve as an important tool in elucidating the involvement of heparanase in cell invasion and migration during various processes such as inflammation, autoimmunity, tissue repair, and tumor metastasis, and in determining which of the biologic effects exerted by heparin is related to its heparanase inhibitory activity. Our in vitro experiments clearly indicate an inhibitory effect of nonanticoagulant heparins on degradation of HS in a naturally produced subendothelial ECM.

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