The Degradation of Human Endothelial Cell-derived Perlecan and Release of Bound Basic Fibroblast Growth Factor by Stromelysin, Collagenase, Plasmin, and Heparanases*

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Perlecan is a major heparan sulfate proteoglycan (HSPG) that is localized to cell surfaces and within basement membranes. Its ability to interact with basic fibroblast growth factor (bFGF) suggests a central role in angiogenesis during development, wound healing, and tumor invasion. In the present study we investigated, using domain specific anti-perlecan monoclonal antibodies, the binding site of bFGF on human endothelial perlecan and its release by proteolytic and glycolytic enzymes. The heparan sulfate was removed from perlecan by heparitinase treatment, and the ~450-kDa protein core was digested with various proteases. Plasmin digestion resulted in a large fragment of ~300 kDa, whereas stromelysin and rat collagenase cleaved the protein core into smaller fragments. All three proteases removed immunoreactivity toward the anti-domain I antibody. We showed also that perlecan bound bFGF specifically by the heparan sulfate chains located on the amino-terminal domain I. Once bound, the growth factor was released very efficiently by stromelysin, rat collagenase, plasmin, heparitinase I, platelet extract, and heparin. Interestingly, heparitinase I, an enzyme with a substrate specificity for regions of heparan sulfate similar to those that bind bFGF, released only small amounts of bFGF. Our findings provide direct evidence that bFGF binds to heparan sulfate sequences attached to domain I and support the hypothesis that perlecan represents a major storage site for this growth factor in the blood vessel wall. Moreover, the concerted action of proteases that degrade the protein core and heparanases that remove the heparan sulfate may modulate the bioavailability of the growth factor.

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1 The abbreviations used are: HSPG, heparan sulfate proteoglycan; ECM, extracellular matrix; HS, heparan sulfate; PAGE, polyacrylamide gel electrophoresis; HUAEC, human umbilical arterial endothelial cell; FGF, fibroblast growth factor; bFGF, basic FGF; GlcNSO₃, N-sulfated glucosamine; 2-OSO₃, 2-sulfate; 6-OSO₃, 6-sulfate; ELISA, enzyme-linked immunosorbant assay; MMP, matrix metalloproteinase; ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); mAb, monoclonal antibody; TBS, Tris-buffered saline; RAM, rabbit anti-mouse IgG; TIMP-1, tissue inhibitor of metalloproteinases.
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The presence of repeating motifs (15, 16). Domain I is unique to perlecans and contains three serine-glycine-aspartic acid sequences that may act as glycosaminoglycan attachment sites (16). Since these HS chains are needed for perlecans to bind FGF, the integrity of domain I is important for anchoring the growth factor activity to the ECM. We reasoned that if this domain were degraded or released by proteases, it could provide a mechanism whereby anchored growth factor molecules could be liberated. It has also been suggested that domain I may be oriented toward the cell surface, which would facilitate its co-receptor activity (17). Domain II has sequence homology to the low density lipoprotein receptor, domain III has homology to the A chain of laminin, domain IV is composed of immunoglobulin-like repeats that demonstrate homology to the neural cell adhesion molecule, N-CAM, and domain V has regions that have similarity to epidermal growth factor and the globular domains of the laminin A chain (15).

The fact that bFGF binds a HSPG in the matrix and is released by enzymes such as plasmin, thrombin, heparanase, and collagenase has been known for a number of years. The identity of the HSPG responsible for this binding, however, was unknown until recently when it was shown that perlecans bound bFGF (8). The major goal of this paper was to investigate which enzymes would degrade the protein component of perlecans and whether these same enzymes could facilitate the release of the bound bFGF. This was facilitated by the use of anti-perlecans monoclonal antibodies, which were characterized with respect to their domain specificity. Plasmin, strepomysin, and rat collagenase significantly degraded the protein core, reduced the immunoreactivity toward domain I, and released significant amounts of the growth factor from the HSPG. Plasmin cleaved the protein core to leave a product that probably contained domain III, whereas strepomysin and rat collagenase degraded perlecans core protein into many fragments. Heparitinase I released bound bFGF, whereas heparitinase I did not, finding consistent with the substrate specificity of heparitinase I for highly sulfated regions of heparan sulfate. Platelet extract was the most efficient agent at releasing growth factor, and this may be due to the presence of many HS-degrading enzymes. This supports the hypothesis that degradation of platelets at sites of injury may be very effective at mobilizing growth factor from the matrix, thereby aiding in the wound healing process.

EXPERIMENTAL PROCEDURES

Materials—Heparin (H3149), Tris, Triton X-100, ABTS, human plasmin, and human thrombin were purchased from Sigma. Heparitinase I, heparitinase I (heparinase I), and chondroitinase ABC were from Seikagaku Corporation Co. All other chemicals were of analytical grade. Tissue culture plastic ware was from either Nunc or Corning. Medium 199 containing Earle’s salts was from Life Technologies, Inc. All solutions for endothelial cell culture were prepared using pyrogen-free Earle’s Salts containing 20% pyrogen-free fetal calf serum, 100 Ci/ml streptomycin sulfate, 100 μg/ml streptomycin sulfate, 100 μg/ml heparin, and 2% bovine brain extract prepared as described (27). Cells were passaged at a 1:3 split ratio after disaggregation with 0.125% trypsin, 0.02% EDTA and used between passages 6 and 10. Rat collagenase was purified from rat mammary carcinoma cells as described previously (24). For immunoblotting, calf collateral lysates were obtained by boiling seeded bacteria in SDS-PAGE sample buffer prior to electrophoresis.

Cell Culture—Primary cultures of human umbilical arterial endothelial cells (HUAEcs) were prepared from fresh umbilical cords delivered by caesarian section at Royal North Shore Hospital, Sydney, as described (25) using 0.1% bacterial collagenase. Cells were grown on tissue culture plastic precoated for 2 h at 37 °C with human fibronectin (10–13 μg/ml), purified from fresh plasma by affinity chromatography on gelatin-Sepharose as described (26). Culture medium was Medium 199 with Earle’s Salts containing 20% pyrogen-free fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 100 μg/ml heparin, and 2% bovine brain extract prepared as described (27). Cells were passaged at a 1:3 split ratio after disaggregation with 0.125% trypsin, 0.02% EDTA and used between passages 6 and 10. Rat collagenase was purified over amylase resin columns as before (24). For immunoblotting, calf collateral lysates were obtained by boiling seeded bacteria in SDS-PAGE sample buffer prior to electrophoresis.

Immunological Procedures—Native ECM ELISAs were performed as described previously (29) using biotin-conjugated rabbit anti-mouse IgG and peroxidase conjugated to streptavidin to enhance the signal. ABTS substrate reaction absorbance was read at 405 nm in a Bio-Rad plate reader. The reference wavelength was 490 nm. Plates coated with the various fusion proteins at 2 μg/ml were washed and incubated with freshly made 0.05% Tween 20, 1% bovine serum albumin, 5% lactose for 1 h at 20 °C under rocking. The plates were washed and allowed to dry at 20 °C under rocking. The plates were drained and allowed to dry at room temperature for 4 h. The plates were stored at room temperature both while in transit and until use. ELISAs performed on the fusion proteins used peroxidase conjugated directly to rabbit anti-mouse IgG instead of using biotin and streptavidin enhancement.

For immunoprecipitation, protein A-Sepharose was saturated with rabbit anti-mouse IgG (RAM) by incubating 1 ml of a 50% suspension with 200 μl of the antibody solution for 2 h at room temperature with rocking. The RAM-saturated protein A-Sepharose was washed with...
Tris-buffered saline (TBS) to remove unbound RAMB, and TBS was added to give a final volume of 1 ml (50% suspension). Samples (1 ml) of radiolabeled HUAEC conditioned medium were preheated by a 2-h incubation at room temperature with 100 μl of RAM-saturated protein A-Sepharose suspension. Simultaneously, 100-μl samples of RAM-saturated protein A-Sepharose suspension were incubated with 200 μl of purified monoclonal antibody (mAb) A76 at 100 μg/ml. The mAb was microcentrifuged, and the mAb-loaded protein A-Sepharose was washed twice with TBS and resuspended in the preheated medium. After a 2-h incubation at room temperature with shaking, the protein A-Sepharose was washed four times with TBS and resuspended in 50 μl of SDS sample buffer (10% (w/v) glycerol, 0.4% (w/v) SDS, 0.001% (w/v) bromophenol blue, 10 mM Tris, pH 6.8), boiled for 10 min, and microcentrifuged to pellet the protein A-Sepharose. The supernatant was electrophoresed on SDS-PAGE gels, dried, exposed to a phosphor screen (Molecular Dynamics) and imaged on a PhosphorImager (Molecular Dynamics) using ImageQuant software.

Immunoprecipitation of labeled bFGF-perlecan complexes was achieved using the same protocol as described above except that the HUAEC conditioned medium was not labeled and recombinant [35S]methionine was added to give a final activity of 0.2 μCi/μl of medium before precipitation with mAb-loaded protein A-Sepharose. Treatment of the labeled bFGF-perlecanc-mAb-protein A-Sepharose complexes with NaCl or heparin was performed at room temperature for 16 h. Treatment with degradative enzymes was for 16 h at 37°C (as described below). After all treatments, the samples were microcentrifuged, and the supernatants were removed, counted in a γ counter (LKB 1275 Multispect, Finland), and expressed as counts/min. The remaining pellet was washed twice with phosphate-buffered saline, 50 μl of SDS-PAGE sample buffer was added, and the samples were prepared for SDS-PAGE analysis. The amount of labeled bFGF released by the various treatments was expressed as a percentage of the amount bound in the initial immunoprecipitate, which was estimated by extraction of the immunoprecipitate with 6 ml urea, 0.2% SDS in phosphate-buffered saline. The values were corrected for the amount of [35S]labeled bFGF released by buffer alone by applying the following formula: corrected % = (uncorrected % - buffer only %) × 100/(100 - buffer only %).

Protease and Endoglycosidase Treatment—Digestions with heparitinase I, heparitinase II, or chondroitinase ABC were performed at 37°C for 16 h in 10 mM Hepes, 3 mM Ca(CH₃COO)_2, 0.1% Triton X-100, pH 7.0, 1 mM benzamidine, 1 mM e-amino caproic acid, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, as described by Lindblom et al. (30). Digestion with human plasmin, human thrombin, rat collagenase, or human stromelysin was performed using the same buffer without the addition of the protease inhibitors for 16 h at 37°C. The activity of these proteases was titrated against native matrix using the ELISA approach; a concentration of enzymes chosen was the minimum concentration that gave 100% of the degradative activity as demonstrated using the anti-domain V antibody A74. In the cases of heparitinase I and platelet extract, standard maximum concentrations were selected. The final concentrations of the various enzymes were as follows: heparitinase I and heparitinase II, 0.1 unit/ml; chondroitinase ABC, 0.5 units/ml; plasmin, 0.6 units/ml; thrombin, 1.2 units/ml; rat collagenase, 5 μg/ml; gelatinases, 1 μg/ml; and stromelysin, 2 μg/ml. Platelet extract was used at a final concentration of 90 μg/ml in enzyme buffer either with or without added protease inhibitors. For all enzyme digestions the aim was to perform exhaustive cleavage on the substrate in an attempt to demonstrate an absolute presence or absence of cleavage. Enzyme digestions of immunoprecipitated molecules were performed after the four washes with TBS in a volume of 50 μl and were terminated using SDS-PAGE sample buffer and boiling the samples for 10 min. Enzyme digestions of native ECM were also performed in a volume of 50 μl in the wells of the microtiter plates using the same conditions and final concentrations of enzymes. The plates were washed twice with phosphate-buffered saline prior to analysis by ELISA.

SDS-GE Electrophoresis and Immunoblotting—SDS-PAGE was performed using 4–15% polyacrylamide gradient gels as described by Laemmli (31). After electrophoresis, the gels were washed twice with 50 μg/ml of protein A-Sepharose (32). Nonreactive bands were detected using alkaline phosphatase-conjugated rabbit anti-mouse IgG as secondary antibody and 5-bromo-4-chloro-3- indolyl phosphate with nitro blue tetrazolium as substrate.

RESULTS

Characterization of Monoclonal Antibodies Specific for Perlecan Domains—We have isolated a number of monoclonal antibodies directed toward endothelial matrix. Four such mAbs (A71, A74, A76, and A81) were specific for perlecan. The SDS gel profile of the immunoprecipitate of [35S]methionine or [35S]sulfate-labeled HUAEC conditioned medium with A76 is shown in Fig. 1 (lanes 1 and 4, respectively). The immunoprecipitated molecule only just entered the 4–15% polyacrylamide gel, consistent with the expected size of perlecan. When [35S]methionine-labeled perlecan was digested with heparitinase I, the high molecular weight band disappeared (Fig. 1, lane 5), indicating that all the incorporated label was present as HS. When the [35S]methionine-labeled perlecan was digested with heparitinase I, the labeled band moved further into the gel (Fig. 1, lane 2), yielding an estimate of the molecular mass of the protein core of perlecan as 450 kDa. Chondroitinase ABC digestion had no effect on either the intensity or the mobility of either [35S]sulfate-labeled (Fig. 1, lane 6) or [35S]methionine-labeled endothelial cell-derived perlecan (Fig. 1, lane 3). Incubation with both heparitinase I and chondroitinase ABC gave the same results as those for heparitinase I alone (data not shown), supporting the suggestion that human endothelial perlecan contained only HS and no CS. The mobility of the protein core was unaffected by reduction with 10 mM dithiothreitol (data not shown). Results from immunoprecipitation experiments using any of four mAbs were identical (data not shown). The four antibodies reacted, by ELISA, with perlecan in native HUAEC matrix (data not shown). To determine further the specificity of the antibodies, they were screened by Western blotting and ELISA against recombinant fusion proteins encoding domains I, II, III, and V of the perlecan protein core. The fusion proteins were generated as bacterial maltose-
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To establish sites of proteolytic cleavage in the perlecan protein core we used a variety of proteases in combination with heparitinase I digestion. Heparitinase I degraded the perlecancore into several fragments of various molecular weights, some of which were seen with plasmin digestion but not with heparitinase I (data not shown). Thrombin in combination with heparitinase I had no effect on the molecular weight of the protein core (Fig. 3, lane 4). In contrast, stromelysin (MMP 3) in the presence of heparitinase I degraded the perlecan core into several fragments of various molecular weights with no evidence of a major cleavage product (Fig. 3, lane 6). This enzyme also cleaved the whole proteoglycan (in the absence of heparitinase), however the large molecular weight smear seen with plasmin digestion was not evident, suggesting that the digestion with stromelysin was more complete (data not shown). Incubation of the perlecan protein core with 0.5 μg/ml stromelysin showed incomplete degradation (data not shown). Incubation with rat collagenase also demonstrated cleavage of the protein core, although the digestion was not as complete, with a number of the smaller fragments being absent (Fig. 3, lane 8). A mixture of the two gelatinases (MMP 2 and MMP 9) had no effect on the size of the protein core (Fig. 3, lane 6) or the intact proteoglycan (data not shown). These findings indicate that perlecan has specific protease-sensitive sites in its protein core and that of the enzymes tested, plasmin, rat collagenase, and stromelysin (also known as a "proteoglycanase") were the most efficient at fragmenting the protein core. The activities of both rat collagenase and stromelysin toward the protein core of perlecan were completely inhibited when digested in the presence of a one molar excess (w/w) of the specific metalloproteinase inhibitor, TIMP-1 (data not shown). When the intact proteoglycan was incubated with platelet extract, in either the presence or absence of protease inhibitors, a protein core band identical in relative molecular mass to that obtained with heparitinase I was seen, suggesting that the major perlecan-degrading activity in platelet extract was that of a heparanase (data not shown).

Removal of Specific Perlecan Domains—The same major plasmin cleavage product was demonstrated when A71, A74, or A76 was used to immunoprecipitate the perlecan, suggesting that the generation of the 300-kDa band was not due to protection of the protein core by the antibody bound to a particular domain. The three smallest domains of perlecan are domain I (20 kDa), domain II (30 kDa), and domain V (80 kDa). Of note, the combined molecular mass of domains III (130 kDa) and IV (210 kDa) is 340 kDa, and the combined mass of domains IV and V is 310 kDa. Both of these values are close to the esti-
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more reliant on the tertiary structure of perlecan.

Binding of Basic FGF to Perlecan and Competition by Heparin—To establish whether bFGF bound perlecan under physiologic conditions, we added 125I-labeled bFGF to HUAEC conditioned medium and then immunoprecipitated the medium with mAb A76. Recombinant 125I-labeled bFGF (18 kDa) was added both in the presence and absence of heparin (100 μg/ml). In the absence of heparin, significant amounts of [125I]bFGF were co-immunoprecipitated (Fig. 5, compare lane 3 with non-specific control in lane 1). There was some evidence of the formation of dimers and trimers of bFGF, as bands at 36 and 54 kDa were also evident. When the conditioned medium also contained 100 μg/ml heparin, the intensity of the co-immunoprecipitated 18-kDa band was reduced almost to background levels, i.e. the levels produced by an irrelevant mouse IgG (Fig. 5, compare lane 4 with lane 2). The reduction of the binding of bFGF to perlecan in the presence of heparin suggested that the latter was competing with the HS chains of perlecan for bFGF binding. This was confirmed in experiments where heparin was capable of eluting bound growth factor from the proteoglycan (Fig. 6, lane 2). Results obtained from immunoprecipitation experiments using the anti-domain I mAb, A71, were the same as those results obtained using A76 (data not shown). These results indicate a specific interaction between the soluble perlecan and bFGF.

Enzymatic Release of bFGF Bound to Perlecan—Because plasmin, stromelysin, and rat collagenase digested the protein component of perlecan and were capable of reducing the immunoreactivity of domain I in the matrix, it was of interest to determine whether these proteases, as well as those enzymes that removed the HS from domain I, were capable of liberating bound bFGF. To test this hypothesis, the immunoprecipitated perlecan-bFGF complexes were used as the starting material. After incubation with the various enzymes the amount of complexed bFGF remaining was visualized by exposing the polyacrylamide gels to phosphor screens and analyzing them on a PhosphorImager. The amount of bFGF complexed to perlecan was reduced from control levels (Fig. 6, lanes 1, 7, and 10), to those obtained by incubation with heparin (Fig. 6, lane 2), with heparitinase I (Fig. 6, lane 4), platelet extract (Fig. 6, lane 6), stromelysin (Fig. 6, lane 8), and rat collagenase (Fig. 6, lane 11). The intensity of the band after incubation with heparinase I (Fig. 6, lane 3) or plasmin (Fig. 6, lane 9) was reduced but was still greater than background levels. Thrombin (Fig. 6, lane 5) did not reduce the intensity of the bFGF band. Of note was the finding that both rat collagenase and stromelysin reduced the molecular mass of the released bFGF by approximately 3 kDa.
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**TABLE I**

| Treatment                      | Released bFGF (percentage of total) |
|-------------------------------|-------------------------------------|
|                               | Uncorrected | Corrected² |
| Urea                          | 100         | 100        |
| Platelet extract (90 μg/ml)   | 90 ± 3.1    | 86 ± 4.1   |
| Stromelysin (2 μg/ml)         | 77 ± 2.3    | 69 ± 3.1   |
| Heparin (100 μg/ml)          | 74 ± 0.9    | 64 ± 1.2   |
| Heparitinase I (0.1 units/ml) | 67 ± 2.3    | 56 ± 3.0   |
| Plasmin (0.6 units/ml)        | 65 ± 1.5    | 53 ± 2.0   |
| Collagenase (5 μg/ml)         | 62 ± 3.2    | 50 ± 4.4   |
| Heparitinase I (0.1 units/ml) | 33 ± 1.2    | 10 ± 0.8   |
| Thrombin (1.2 units/ml)       | 30 ± 0.1    | 5 ± 0.1    |
| NaCl (5 μ)                    | 23 ± 2.1    | 0          |
| Buffer only                   | 26 ± 1.0    | 0          |

* Perlecan-[³⁵S]bFGF complexes were immunoprecipitated using mAb A76 and treated with the same combinations of enzymes under the same conditions as those used for the incubations shown in Figs. 3 and 4. Lanes 1, 8, and 10 are buffer only controls. In lanes 2–6, 8, and 9, the immunoprecipitated complexes were incubated with the treatments indicated by a + above the lane. Material remaining complexed to the protein A-Sepharose beads was collected by centrifugation, subjected to SDS-PAGE, and visualized with a PhosphorImager system. This figure is a composite of three minigels. Lanes 1–6 were run in the same experiment, whereas lanes 7–9 and lanes 10 and 11 were run in separate experiments. Lanes 1, 8, and 10 are the buffer only controls from their respective experiments. The relative positions of molecular mass standards (in kDa) are shown on the left. Top, the origin of the running gel.

FIG. 6. The release of bFGF from perlecan by enzymes. Perlecan-[³⁵S]bFGF complexes were immunoprecipitated using mAb A76 and treated with the same concentrations of enzymes under the same conditions as those used for the incubations shown in Figs. 3 and 4. Lanes 1, 8, and 10 are buffer only controls. In lanes 2–6, 8, and 9, the immunoprecipitated complexes were incubated with the treatments indicated by a + above the lane. Material remaining complexed to the protein A-Sepharose beads was collected by centrifugation, subjected to SDS-PAGE, and visualized with a PhosphorImager system. This figure is a composite of three minigels. Lanes 1–6 were run in the same experiment, whereas lanes 7–9 and lanes 10 and 11 were run in separate experiments. Lanes 1, 8, and 10 are the buffer only controls from their respective experiments. The relative positions of molecular mass standards (in kDa) are shown on the left. Top, the origin of the running gel.

FIG. 7. The presence of bFGF does not affect digestion of perlecan by heparinases. THS-labeled perlecan was immunoprecipitated as described for Fig. 1 using A76 antibodies. The immunoprecipitates were incubated with the treatments indicated by a + above the lane, incubated in SDS-PAGE buffer, electrophoresed through a 4–15% polyacrylamide SDS gel, stained with Alcian blue, dried, exposed to a phosphor screen, and analyzed with a Phosphor Imager. The intensity of the various bands was quantitated using ImageQuant software. The concentration of both heparitinase I and heparinase I used was 0.1 unit/ml. 0.5 μg of bFGF was added to the immunoprecipitate/enzyme mix in lanes 3 and 6. This figure is a composite of two minigels run on different days with their appropriate controls. The relative positions of molecular mass standards (in kDa) are shown on the left. Top, the origin of the running gel.

**DISCUSSION**

Perlecan derived from human endothelial cells contains HS and no chondroitin sulfate, as demonstrated by its sensitivity to heparinase I and its full resistance to chondroitinase ABC digestion. This is the most common form of the molecule (1), although some forms of the basement membrane proteoglycan have been described that contain chondroitin sulfate and heparan sulfate on the same core protein (33). We estimated the mass of the protein core of perlecan to be ~450 kDa, which was very similar to the published mass of 467 kDa deduced from cDNA cloning from human tumor and non-tumor cell lines (15, 16). The anti-perlecan monoclonal antibodies were characterized with respect to their domain specificity and used to study the effects of protease treatment on the various domains. This study was performed in view of the fact that perlecan was recently shown to bind bFGF (8) and to have the necessary HS sequences to cross-link with the high affinity receptors on the
cell surface. Since the HS is attached to domain I of perlecan (1), and since the removal of this domain by proteases would release a HS-growth factor complex, it was of interest to determine which proteases were effective at degrading the protein core and in particular which proteases removed domain I. It was of further interest to determine whether these same proteases were able to release bFGF that was bound to perlecan.

In the present study we demonstrated that stromelysin, rat collagenase, and plasmin reduced the immunoreactivity toward the domain I antibody and were also effective at releasing bound growth factor. Plasmin was previously shown to release bound bFGF from whole matrix preparations (6). It was not known, however, which component of the matrix functioned as the plasmin substrate or whether the serine protease released the growth factor indirectly by activating other latent proteases (34). We demonstrated here that plasmin cleaves immunopurified perlecan, leaving a major product with a molecular mass of ~300 kDa, which still contained domain III, thereby suggesting that the serine protease cleaved perlecan outside of this domain. In addition to the demonstration that plasmin cleaved perlecan, we demonstrated the involvement of metalloproteinases in perlecan degradation, as stromelysin (MMP 3) and rat collagenase (MMP 13) both degraded the basement membrane proteoglycan. MMP 3 was first described as a “proteoglycanase” because it degraded proteoglycans isolated from cartilage (35). More recently, it has been shown to cleave the aggregating cartilage proteoglycan, aggrecan, at a single site close to the N-terminal region of the G1 domain (36). In contrast, stromelysin cleaved perlecan at many sites, giving rise to fragments of various molecular weights. This enzyme was also very efficient at removing the immunoreactivity toward domains I, III, and V in HUAEC ECM and at liberating bound bFGF from perlecan. Collectively, the data suggest that the enzyme degraded the protein core at multiple locations. Rat collagenase also cleaved the protein core of perlecan, giving rise to a different digestion pattern, which showed no evidence of smaller fragments. The human homologue of rat collagenase has been cloned recently and termed collagenase-3. The human recombinant enzyme was found to degrade fibrillar type I collagen but not gelatin or casein (20). The work described in this paper demonstrates that the native rat enzyme has significant “perlecanase” activity. The cleavage of perlecan by stromelysin and rat collagenase was an attribute not shared by all metalloproteinases, as a mixture of the two gelatinases (MMP 2 and MMP 9) had no effect on core protein size or the release of bound growth factor.

The serine protease thrombin has been shown to release bFGF from native matrix (5). However, in our system, it had little effect on either the immunopurified protein core or the immunoreactivity of domains I and III of perlecan in matrix. Also, we showed that thrombin released very little of the growth factor from perlecan. Therefore, thrombin may have been removing bFGF from native matrix indirectly by degradation of the surrounding ECM, by activating ECM-bound proteases, or by stimulating heparanase activity (37). In contrast, heparinase I (heparinase IIII) and platelet extract released significant amounts of bFGF from perlecan, whereas heparinase I released a smaller amount. This is consistent with results obtained elsewhere using native ECM as the source of binding HSPG (4, 38). Platelet extract may contain more than one heparan/heparin degrading activity and has been used as a starting material to purify platelet heparitinase (39). The substrate specificity of the heparan sulfate lyases may explain why heparinase I released bFGF from perlecan at a higher rate than heparinase I. Heparinase I has a specificity for degrading highly sulfated, heparin-like regions that have a high proportion of di- or trisulfated disaccharides (40). These same regions have been shown to bind bFGF (10, 41) and have also been shown to inhibit bFGF-induced mitogenesis (14). When bFGF was present, however, our results showed that the growth factor did not interfere with the activity of either heparinase I or heparinase I. The reason why a small amount of the growth factor was released from perlecan by digestion with heparinase I yet the enzyme was capable, although less so than heparinase I, of degrading perlecan-HS in the presence of bFGF is unknown but may be due to differences between the two experimental systems (i.e. obtaining 125I-labeled bFGF-perlecan complexes and adding enzyme versus obtaining 35SO4-labeled perlecan and adding both enzyme and unlabeled bFGF). Heparinase I (heparinase IIII), on the other hand, preferentially degrades the less sulfated regions of HS, which are more common in “non-heparin” HS (42) and often separate bFGF binding sequences. Therefore, the action of heparinase I on perlecan-bFGF complexes was to liberate more efficiently bFGF bound to its HS binding sequence.

Bound bFGF can be released from heparin immobilized to Sepharose beads by treatment with 1.4–1.6 M NaCl (3, 9), and can be extracted from native matrix with 3 M NaCl (3). Our data demonstrate that 5 M NaCl does not remove significant quantities of the growth factor from perlecan, thereby suggesting that binding between perlecan and bFGF is very specific and avid. This apparent increase in avidity may be due to the fact that in our assay incubation mixture we have the whole proteoglycan bound to immunoglobulin, and incubation with 5 M NaCl may increase any hydrophobic attraction that may exist between bFGF and either the perlecan protein core or immunoglobulin. The previous use of 3 M NaCl to remove bound bFGF from matrix (3) may have been possible due to the presence of other HSPGs besides perlecan in the matrix that bind bFGF with a lower affinity. The finding that heparin removes bFGF from perlecan is likely due to the fact that in the competitive reaction mixture, perlecan was present in much smaller amounts when compared with the 100 µg/ml of competing glycosaminoglycan. Heparin has also been shown to be a competitive inhibitor of bFGF binding to matrix at concentrations 10 times lower than those used in these studies (3). We could not liberate significant amounts of bound bFGF with up to 15 µg/ml of “cold” growth factor. Due to economic constraints and the logistics of the assay (i.e. 50 µl volume of perlecan-growth factor complex attached to protein A-Sepharose beads) we were unable to go above this concentration. Taking the concentration of bFGF required for 50% displacement of labeled growth factor as in excess of 15 µg/ml, we calculated that the Kd had to be less than 800 nM, which is consistent with previous estimates for the affinity of bFGF for extracellular matrix and HSPGs (4, 43). We are planning to isolate perlecan in the absence of immunoglobulin and protein A-Sepharose and repeat these experiments.

The binding of bFGF to HSPGs in matrix and its release by incubation with heparin or digestion of the matrix with enzymes are not novel findings. However, in all of the previous experiments used to study bFGF-HSPG interactions, whole native matrix was used as the source of HSPG. Since the matrix may contain more than one HSPG as well as other proteoglycans, it was unknown which HSPG was responsible for the binding of the growth factor. Furthermore, these experiments were complicated by the presence in the matrix of endogenous proteases, heparanases, and other growth factors. Our data demonstrate directly that perlecan binds bFGF very tightly and that significant amounts are released by proteases that degrade the protein component of the proteoglycan, by heparan sulfate-degrading enzymes that remove the HS, or by...
incubation of the complex with a competitive ligand such as heparin. Because perlecan has been shown to possess the oligosaccharide sequences necessary to activate cell surface receptors (8), it has been assigned a co-receptor role. Therefore, growth factor bound to perlecan may be very relevant to processes involving cell growth and differentiation. The regulatory mechanisms involved in the synthesis/degradation of this proteoglycan may provide ways of controlling the bioactivity of the growth factor.

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REFERENCES
1. Iozzo, R. V., Cohen, I. R., Grassel, S., and Murdoch, A. D. (1994) Biochem. J. 302, 625–639
2. Vlodavsky, I., Bar-Shavit, R., Korner, G., and Fux, Z. (1993) in Molecular and Cellular Aspects of Basement Membranes (Roehrbach, D. H., and Timpl, R., eds) pp. 327-343, Academic Press, Inc., New York
3. Baird, A., and Ling, N. (1987) Biochem. Biophys. Res. Commun. 142, 428–435
4. Baskin, P., Doktor, S., Klagsbrun, M., Magnus Shvahn, C., Folkman, J., and Vlodavsky, I. (1989) Biochemistry 28, 1737–1743
5. Benezra, M., Vlodavsky, I., Ishai-Michaeli, R., Neufeld, G., and Barshavit, R. (1993) Blood 81, 3324–3331
6. Saksela, O., and Rifkin, D. B. (1990) J. Cell Biol. 110, 767–775
7. Saksela, O., Moscatelli, D., Sommer, A., and Rifkin, D. B. (1988) J. Cell Biol. 107, 743–751
8. Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G., and Yayon, A. (1984) Cell 30, 1005–1013
9. Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasae, J., and Klagsbrun, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2292–2296
10. Tyrrell, D. J., Ishihara, M., Rao, N., Horne, A., Kiefer, M. C., Stauber, G. B., Lam, L. H., and Stack, R. J. (1993) J. Biol. Chem. 268, 4684–4689
11. Turnbull, J. E., Fernig, D. G., Wilkinson, D. P., and Gallagher, J. T. (1992) J. Biol. Chem. 267, 10337–10341
12. Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K., and Kimata, K. (1992) Biochem. J. 285, 805–813
13. Schmidt, A., Yoshida, K., and Buddelke, E. (1992) J. Biol. Chem. 267, 19292–19297
14. Nugent, M. A., Karnovsky, M. J., and Edelman, E. R. (1993) Circ. Res. 73, 1051–1060
15. Murdoch, A. D., Dodge, G. R., Cohen, I., Tuan, R. S., and Iozzo, R. V. (1992) J. Biol. Chem. 267, 8544–8557
16. Kallunki, P., and Tryggvason, K. (1992) J. Cell Biol. 116, 559–571
17. Couchman, J. R., and Woods, A. (1993) in Cell Surface and Extracellular Glycoconjugates: Structure and Function (Roberts, D. D., and Mecham, R. P., eds) pp. 33–52, Academic Press, Inc., San Diego
18. Lyons, J. G., Nethery, A., O’Grady, R. L., and Harrop, P. J. (1989) Matrix 9, 7–16
19. Nethery, A., Lyons, J. G., and O’Grady, R. L. (1986) Anal. Biochem. 159, 390–395
20. Freije, J. M. P., Diez-Itza, I., Babina, M., Sanchez, L. M., Blasco, R., Tolivi, J., and Lopez-Otin, C. (1995). J. Biol. Chem. 269, 16768–16773
21. Graham, L. D., Mitchell, S. M., and Underwood, P. A. (1995) Biochem. Mol. Biol. Int. 37, 239–246
22. Underwood, P. A., and Bennett, F. A. (1989) J. Cell Biol. 130, 641–649
23. Underwood, P. A., Kelly, J. F., Harman, D. F., and McMullan, H. M. (1983) J. Immunol. 130, 33–45
24. Murdoch, A. D., Liu, B., Schwarting, R., Tuan, R. S., and Iozzo, R. V. (1994) J. Histochem. Cytochem. 42, 239–249
25. Weiss, J. R., Sun, B., and Rodgers, G. M. (1991) Thromb. Res. 61, 171–173
26. Ruoslahti, E., Hayman, E. G., Pierschbacher, M., and Engvall, E. (1982) Methods Enzymol. 82, 803–831
27. Madig, T., Cerundolo, J., Hisley, S., Kelley, P. R., and Forourd, R. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5674–5678
28. Gospodarowicz, D., and Lui, G. M. (1981) J. Cell. Physiol. 109, 69–81
29. Underwood, P. A., Dalton, B. A., Steele, J. G., Bennett, F. A., and Strike, P. (1992) J. Cell Sci. 102, 833–845
30. Lindblom, A., and Lars-Ake, F. (1990) Glycoconjugate J., 7, 545–562
31. Laemmli, U. K. (1970) Nature 227, 680–685
32. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
33. Danielson, K. G., Martinez-Hernandez, A., Hassell, J. R., and Iozzo, R. V. (1992) Matrix 11, 22–35
34. Menashi, S., Vlodavsky, I., Ishai-Michaeli, R., Legrand, Y., and Frizman, R. (1995) FEMS Lett. 361, 61–64
35. Solters, A., Reynolds, J. J., and Mekle, M. C. (1978) Biochem. J. 171, 493–496
36. Fosang, A. J., Neame, P. J., Hardingham, T. E., Murphy, G., and Hamilton, J. A. (1991) J. Biol. Chem. 266, 15579–15582
37. Benezra, M., Vlodavsky, I., and Bar-Shavit, R. (1992) Exp. Cell Res. 201, 208–215
38. Ishai-Michaeli, R., Eldor, A., and Vlodavsky, I. (1990) Cell Regul. 1, 833–842
39. Oosta, G. M., Favreau, L., Vlodavsky, I., and Rosenberg, R. D. (1982) J. Biol. Chem. 257, 11249–11255
40. Linhardt, R. J., Turnbull, J. E., Wang, H. M., Loganathan, D., and Gallagher, J. T. (1990) Biochemistry 29, 2611–2617
41. Walker, A., Turnbull, J. E., and Gallagher, J. T. (1994) J. Biol. Chem. 269, 931–935
42. Nader, H. B., Porciniotto, M. A., Tersario, I. L. S., Pinhail, M. A. S., Oliveira, F. W., Moraes, C. T., and Dietrich, C. P. (1990) J. Biol. Chem. 265, 16807–16813
43. Vignay, M., Olifer-Hatmann, M., Lavigne, M., Fayein, N., Jeanny, J. C., Laurent, M., and Courtis, Y. (1988) J. Cell. Physiol. 137, 321–328
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