Heparanase Induces Vascular Endothelial Growth Factor Expression: Correlation with p38 Phosphorylation Levels and Src Activation

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Abstract

Heparanase is an endo-β-α-glucuronidase involved in cleavage of heparan sulfate moieties and hence participates in extracellular matrix (ECM) degradation and remodeling. Traditionally, heparanase activity was correlated with the metastatic potential of a variety of tumor-derived cell types. Cloning of the heparanase gene indicated that heparanase expression is up-regulated in a variety of primary human tumors. In some cases, heparanase up-regulation correlated with increased tumor vascularity, an angiogenic feature that could be recapitulated in a number of in vitro and in vivo models. The mechanism by which heparanase enhances angiogenic responses is not entirely clear but is thought to be mediated primarily by release of ECM-resident angiogenic growth factors such as basic fibroblast growth factor and vascular endothelial growth factor (VEGF). Here, we examined the possibility that heparanase directly participates in VEGF gene regulation. We provide evidence that heparanase overexpression in human embryonic kidney 293, MDA-MB-435 human breast carcinoma, and rat C6 glioma cells resulted in a 3- to 6-fold increase in VEGF protein and mRNA levels, which correlated with elevation of p38 phosphorylation. Moreover, heparanase down-regulation in B16 mouse melanoma cells by a specific siRNA vector was accompanied by a decrease in VEGF and p38 phosphorylation levels, suggesting that VEGF gene expression is regulated by endogenous heparanase. Interestingly, a specific p38 inhibitor did not attenuate VEGF up-regulation by heparanase whereas Src inhibitors completely abrogated this effect. These results indicate, for the first time, that heparanase is actively involved in the regulation of VEGF gene expression, mediated by activation of Src family members. (Cancer Res 2006; 66(3): 1455-63)

Introduction

Heparanase is a mammalian endo-glucuronidase responsible for heparan sulfate degradation, yielding heparan sulfate fragments with appreciated size (5-10 kDa) and biological potency (1, 2). Heparan sulfate is a major constituent of the extracellular matrix (ECM) and heparan sulfate–degrading activity is thought to play a decisive role in fundamental biological processes associated with remodeling of the ECM, such as angiogenesis and cancer metastasis. Heparanase activity has long been correlated with the metastatic potential of tumor-derived cells (3–5). A proof of concept for this notion has recently been provided by applying siRNA and ribozyme technologies, clearly implicating heparanase-mediated heparan sulfate cleavage as a critical requisite for metastatic spread (6). Moreover, up-regulation of heparanase mRNA and protein expression has been documented in a variety of primary human tumor biopsies whereas adjacent normal-looking tissue did not exhibit detectable levels of heparanase. Heparanase up-regulation correlates with reduced postoperative survival of colorectal (7), pancreatic (8), bladder (9), gastric (10), and cervical (11) cancer patients. Similarly, heparanase up-regulation correlates with increased lymph node and distant metastasis (9, 10, 12–16), and with microvessel density (9, 12, 17, 18), providing a strong clinical support for the prometastatic and proangiogenic features of heparanase. The angiogenic potency of heparanase was confirmed in several in vitro and in vivo model systems, including wound healing (19, 20), tumor xenografts (21), Matrigel plug assay (19), and tubelike structure formation (22). Moreover, microvessel density was significantly reduced in tumor xenografts developed by anti-heparanase ribozyme–transfected Eb lymphoma cells (6), further implicating heparanase as an angiogenic mediator. The molecular mechanism by which heparanase facilitates angiogenic responses is not entirely clear but is thought to be mediated primarily by the release of heparan sulfate–bound growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF; refs. 23, 24).

Recently, we have shown that exogenous addition of latent heparanase stimulates Akt-dependent endothelial cell invasion and migration that seem to be independent of heparanase enzymatic activity (22). Nonenzymatic functions of heparanase also include enhanced adhesion of glioma (21), lymphoma (21, 25), and T cells (26), mediated by β1-integrin and correlated with Akt, Pyk2, and extracellular signal–regulated kinase (ERK) activation (21, 26). The ability of heparanase to function in a nonenzymatic manner and to elicit signal transduction cascades led us to hypothesize that its potent angiogenic feature observed under clinical and experimental settings is not due solely to the release of heparan sulfate–bound growth factors but also to the regulation of angiogenic growth factor expression. We provide evidence that heparanase overexpression in stably transfected human MDA-MB-435 breast carcinoma, rat C6 glioma, and human embryonic kidney 293 cells, or its exogenous addition, results in a marked elevation of VEGF expression levels that correlated with enhanced p38 phosphorylation. Moreover, heparanase down-regulation in...
B16 melanoma cells by a specific siRNA vector was accompanied by a comparable decrease in VEGF and p38 phosphorylation levels, suggesting that endogenous heparanase regulates VEGF gene expression. Interestingly, a specific p38 inhibitor did not attenuate VEGF up-regulation by heparanase whereas Src inhibitors completely abrogated this effect. These results indicate, for the first time, that heparanase is actively involved in the regulation of VEGF gene expression via activation of Src family members.

Materials and Methods

Antibodies and reagents. The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-ERK-2, anti-phospho-ERK, anti-p38, anti-Src, anti-VEGF, and anti- phosphotyrosine. Monoclonal antibodies to phospho-p38 (Thr180/Tyr182) and phospho-Src (Tyr416) were purchased from Cell Signaling (Beverly, MA), antiactin antibody was purchased from Sigma (St. Louis, MO), and anti-p120SHAM was purchased from Becton Dickinson (Palo Alto, CA). Antiheparanase 1453 and 733 antibodies have previously been characterized (27). Antimouse platelet/endothelial cell adhesion molecule 1 (PECAM-1; CD31) polyclonal antibody was kindly provided by Dr. Joseph A. Madri (Yale University, New Haven, CT). The selective p38 (SB 203580) and Src (PP1, PP2, and Src inhibitor 1) inhibitors were purchased from Calbiochem (San Diego, CA) and were dissolved in DMSO as stock solutions. DMSO was added to the cell culture as a control. Matrigel was kindly provided by Dr. Hynda Kleinman (NIDR, NIH, Bethesda, MD) and soluble Fli receptor was from R&D Systems (Minneapolis, MN).

Heparanase gene constructs. Membrane-targeted heparanase gene construct, generated by introducing the platelet-derived growth factor (PDGF) receptor transmembrane domain at the heparanase COOH terminus, a nonsecreted heparanase form generated by deletion of 15 amino acids mapped at the NH2 terminus of the 50-kDa heparanase subunit, and heparanase mutated at glutamic acid residues 225 and 343 that comprise the enzyme active site, yielding an inactive enzyme, were previously described (25, 27, 28). The pDNA3-Hpa plasmid containing the full-length human cDNA was used as the PCR template for generating heparanase gene construct lacking its signal peptide. Primers 5′-GGATTC-GGCATCATGACGACGAGTGCCATC-3′ (forward) and 5′-CTTCTA-GAGATGGAACGAGCTTGGATT-3′ (reverse) were used and included EcoRI and XbaI sites, respectively, enabling cloning in-frame into the pDNA3.1/myc-HisA vector at the EcoRI/XbaI cloning site. Heparanase encoded by this construct includes amino acids Gin36–His353 followed by the Myc-His tag at the protein COOH terminus.

Cell culture and transfecion. HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA) and rat C6 glioma cells were kindly provided by Dr. Eli Keshet (The Hebrew University School of Medicine, Jerusalem, Israel). Mouse B16-BL6 melanoma and human breast carcinoma MDA-MB-435 cells were described elsewhere (6, 29). Cells were cultured in DMEM supplemented with glutamine, pyruvate, antibiotics, and 10% FCS in a humidified atmosphere containing 8% CO2 at 37°C. For stable transfection, cells were transfected with the pSecTag 2 vector containing the full-length heparanase cDNA, kindly provided by Dr. Hua-Quan Miao (ImClone Systems Inc, New York, NY; ref. 30), using the FuGene reagent according to the instructions of the manufacturer (Roche, Mannheim, Germany), selected with Zeocin (Invitrogen, Carlsbad, CA) for 3 weeks, expanded, and pooled. Human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Neomi Lanir (Rambam Medical Center, Haifa, Israel) and were cultured in gelatin-coated flasks essentially as described (22). For growth curves, HUVECs (1.5 × 104) were incubated with medium conditioned by control Vo– or heparanase-transfected MDA-435 cells in the absence or presence of soluble FliI (100 ng/mL). Cells were dissociated by trypsinization and counted every day for 7 days using a Coulter counter and cell numbers were further confirmed by counting with a hemacytometer. Wild-type (WT) and glutamic acid residues 225 and 343 mutated recombinant heparanase proteins were purified from the conditioned medium of transfected 293 cells essentially as described (21).

Cell lysates, immunoprecipitation, and protein blotting. Cell cultures were incubated for 20 hours under serum-free conditions, pretreated with 1 mmol/L orthovovanadate for 10 minutes at 37°C, washed twice with ice-cold PBS containing 1 mmol/L orthovovanadate, and scraped into lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L orthovovanadate, 1 mmol/L phenylmethylsulfonyl fluoride] containing a cocktail of proteinase inhibitors (Roche). Total cellular protein concentration was determined by the bicinchoninic acid assay according to the instructions of the manufacturer (Pierce, Rockford, IL). Thirty micrograms of cellular protein were fractionated on SDS polyacylamide gel and immunoblotting was done as described (21, 22, 27, 31). Immunoprecipitation was carried out essentially as described (28). Briefly, 100 μg of cellular protein were brought to a volume of 1 ml in buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, and 0.5% NP40, incubated with the appropriate antibody (anti-phosphotyrosine or anti-p120SHAM) for 2 hours on ice, followed by incubation with purothin A/G-Sepharose (30 minutes on ice). Beads were washed twice with the same buffer supplemented with 5% sucrros. Sample buffer was added and, after boiling, samples were subjected to gel electrophoresis and immunodetection as described above.

Src kinase assay. Cell lysates (200 μg) were immunoprecipitated with anti-Src antibodies (sc-19), followed by incubation with 30 mmol/L ATP and 10 μCi [γ-32P]ATP in a kinase buffer [25 mmol/L HEPES (pH 7.4), 10 mmol/L MgCl2, 10 mmol/L MnCl2, 10% glycerol] for 30 minutes at 30°C. Samples were then subjected to gel electrophoresis; gels were fixed (30 minutes, 25% isopropanol + 10% acetic acid) and fluorographed (30 minutes; Amplify, Amersham, Buckinghamshire, United Kingdom), followed by drying and autoradiography.

PCR analysis. Total RNA was extracted with TRIzol (Life Technologies, Rockville, MD) and RNA (1 μg) was amplified using one-step PCR amplification kit according to the instructions of the manufacturer (ABGene, Epsom, United Kingdom). The PCR primer sets included 5′-TGACCCGTACCACACTGGTTGATCATA 3′ (forward) and 5′-CTAGAAG-CATTGGCTGGAGGATGAGGAG 3′ (reverse) for actin, and 5′-GGGATCCGAGAAGACATCTGCTCGCTGCT-3′ (forward) and GGTTGACCC-TCACCGCCTGGCCTGGCT-3′ (reverse) for VEGF.

Cell migration assay. Control Vo– and heparanase-transfected MDA-435 cells were allowed to grow for 2 days on tissue culture plates and were then scraped with the wide end of a 1-mL tip (time 0). Plates were washed twice with PBS to remove detached cells, incubated with complete growth medium in the absence or presence of the indicated inhibitor, and cell migration into the wounded empty space was examined over 2 days as described (21). Cell migration on fibronectin-coated insert filters was performed essentially as described (22).

Matrigel plugs assay. The Matrigel plug assay was done as previously described (19). Briefly, 6-week-old male severe combined immunodeficient (SCID) mice (n = 5; Harlan, Jerusalem, Israel) were injected s.c. at the lateral abdominal area with 0.5 mL of Matrigel premixed on ice with 1 × 106 control Vo– or heparanase-transfected MDA-435 cells. Matrigel plugs were removed 7 days postimplantation, photographed, fixed in formalin, and subjected to histologic analysis.

Tumorigenicity. Cells from exponential cultures of control Vo– or heparanase-transfected MDA-435 cells were detached by trypsin, washed with PBS, and brought to a concentration of 5 × 106/mL. Cell suspension (5 × 106/0.1 mL) was inoculated s.c. at the right flank of 5-week-old female SCID mice (n = 5). Xenograft size was determined twice a week by externally measuring tumors in two dimensions using a caliper. Tumor volume (V) was determined by the equation V = L × W2 × 0.5, where L is the length and W the width of the xenograft. At the end of the experiment, mice were sacrificed and xenografts were resected, weighted, and fixed in formalin. Paraffin-embedded 5-μm sections were stained with H&E or Mason's trichrome or immunostained with anti-heparanase, anti-PECAM-1, or anti-phospho-p38 antibodies using the Envision kit according to the instructions of the manufacturer (DAKO, Glostrup, Denmark) and as described (21, 27). All animal experiments were approved by the Animal Care Committee of Technion (Haifa, Israel).

Heparanase activity assay. Preparation of ECM-coated 35-mm dishes and determination of heparanase activity were done as described in detail.
Figure 1. Heparanase overexpression enhances p38 phosphorylation. A to C, heparanase activity. Heparanase cDNA was stably transfected into MDA-435 (A), C6 glioma (B), and HEK293 (C) cells. Control Vo– (■) and heparanase-transfected (▲) cells (1 × 10⁶) were subjected to three cycles of freeze/thaw and cell lysates were incubated (18 hours, 37 °C, pH 6.0) with 35S-labeled ECM. Sulfate-labeled degradation fragments released into the incubation medium were subjected to gel filtration on Sepharose 6B as described in Materials and Methods. D to F, immunoblot analysis. Lysate samples from control Vo– and heparanase-transfected MDA-435 (D), C6 glioma (E), and HEK293 cells (F) were subjected to immunoblotting with anti-heparanase (Hepa, top panels), anti-phospho-p38 (p-p38, second panels), anti-p38 (third panels), anti-phospho ERK (p-ERK, fourth panels), and anti-ERK (bottom panels) antibodies.

Statistics. Data are presented as mean ± SE. Statistical significance was analyzed by two-tailed Student’s t test. P < 0.05 was considered as significant.

Results

Heparanase overexpression induces p38 phosphorylation levels. We have previously noted elevated phosphorylation levels of Akt, ERK, and Pyk2 on heparanase addition or overexpression in stably transfected cells (21, 22, 26). To further explore the ability of heparanase to activate signaling cascades, heparanase was stably transfected into MDA-435 human breast carcinoma cells and high levels of expression were confirmed by heparanase activity assay (Fig. 1A) and immunoblotting (Fig. 1D, top). Next, we examined the phosphorylation levels of mitogen-activated protein kinase (MAPK) family members by subjecting the cell lysates to immunoblotting with antibodies directed against phosphorylated p38, ERK, and c-Jun NH2-terminal kinase (JNK). The phosphorylation levels of p38 were noticeably increased in heparanase-overexpressing cells (Fig. 1D, Hepa, second panel) whereas ERK (fourth panel) and JNK (data not shown) phosphorylation seemed to be unchanged. To verify these results further, heparanase-transfected rat C6 glioma (Fig. 1B and E) and HEK293 (Fig. 1C and F) cells were similarly analyzed. As was noted for MDA-435 cells, p38 phosphorylation was markedly induced in C6 (Fig. 1E, second panel) and 293 (Fig. 1F, second panel) cells whereas ERK phosphorylation was unaffected (C6) or even reduced (293) in response to heparanase overexpression (Fig. 1D-E, fourth panel). Densitometry analysis of three independent experiments revealed over 20-fold (C6 and 293) and 4-fold (MDA-435) increase of p38 phosphorylation levels in heparanase-transfected cells (Supplementary Fig. S1A-C).

Heparanase increases VEGF expression levels. The p38 MAPK module is involved in diverse biological functions, such as the regulation of cell motility, gene transcription, and chromatin remodeling, and is activated in response to different stimuli such as inflammatory cytokines and environmental stresses including hypoxic conditions (33). Moreover, p38 is implicated in hypoxia-inducible factor–dependent (34) and –independent (35) VEGF up-regulation in tumor cells. The potent angiogenic capacity of heparanase and enhanced p38 phosphorylation noted on its overexpression (Fig. 1) led us to examine VEGF expression levels in heparanase-overexpressing cells. To this end, total cell lysates of control Vo– or heparanase-transfected cells (Hepa) were subjected to immunoblotting with anti-VEGF (Fig. 2, top) or antiactin (Fig. 2, bottom) antibodies. We observed enhanced VEGF expression levels in MDA-435 (Fig. 2A), C6 (data not shown), and HEK293 (Fig. 2B) cells in response to heparanase transfection. Densitometry analysis of three independent experiments revealed 7-, 4.5-, and 3.3-fold increase of VEGF levels in heparanase-transfected MDA-435, C6, and HEK293 cells, respectively (Supplementary Fig. S2A-C). Enhanced VEGF expression was further examined by reverse transcription-PCR (RT-PCR) analysis (Fig. 2C and D), revealing 6- and 3-fold increase of VEGF mRNA in heparanase-transfected MDA-435 and HEK293 cells, respectively, comparable with VEGF induction at the protein level (Fig. 2, top).

We next examined whether elevated levels of VEGF found in cell lysates correlate with VEGF secretion into the culture medium. To this end, primary HUVECs (1.5 × 10⁵) were incubated with...
medium conditioned by control Vo– or heparanase-transfected MDA-435 cells, and the cell number was determined over 7 days (Fig. 3). A 40% increase in cell number was observed following 5 days of incubation of HUVEC with medium conditioned by heparanase-transfected cells (435–Hepa) compared with cells grown in medium conditioned by control cells (435–Vo). Importantly, the observed increase in cell proliferation is largely attributed to VEGF as indicated by its inhibition in the presence of soluble Flt1 (435–Hepa + sFlt1), a specific inhibitor of VEGF. Similarly, the decline in cell number observed on day 7 was prevented in HUVEC cultured in medium conditioned by control Vo cells (Supplementary Fig. S3A). The reduced VEGF expression in cells transfected with anti-heparanase siRNA vector produced tumors expressing MDA-435 cells (435–Hepa) versus HUVEC cultured in medium conditioned by control cells (435–Vo, Vo).

Induction of VEGF noted on heparanase overexpression was further examined by taking the opposite approach (i.e., down-regulation of heparanase expression). We have recently used siRNA technology to inhibit heparanase expression and showed that cells transfected with anti-heparanase siRNA vector produced tumors that were less metastatic and less vascularized than tumors produced by control cells (6). Transfection of B16-BL6 mouse melanoma cells with the anti-heparanase siRNA vector resulted in nearly 75% inhibition of heparanase expression as determined by RT-PCR analysis (Fig. 3C, top). The reduced heparanase expression correlated with a comparable decrease in VEGF levels (Fig. 3C, third panel). Densitometry analysis of three independent experiments revealed a 2-fold decrease in VEGF levels in response to heparanase down-regulation (Fig. 3D). Moreover, heparanase down-regulation also correlated with a marked inhibition of p38 phosphorylation (Fig. 3C, fifth panel), further supporting the involvement of heparanase in p38 activation and correlating p38 phosphorylation with VEGF expression. Furthermore, these results imply that endogenous heparanase is intimately involved in VEGF gene regulation, providing a molecular basis for its proangiogenic function.

Heparanase facilitates MDA-435 xenograft growth and angiogenesis. To further substantiate our in vitro results correlating heparanase with VEGF expression, we next used two in vivo angiogenic models. Gross examination of transplanted Matrigel embedded with heparanase-transfected MDA-435 cells revealed plugs that appeared more reddish and vascularized than Matrigel embedded with control Vo cells (Supplementary Fig. S3A). H&E staining of Matrigel plug sections clearly revealed increased vascularization of Matrigel plugs embedded with heparanase-overexpressing cells versus Matrigel embedded with control Vo cells (Supplementary Fig. S3B, HE-E, two left panels). Higher vascular density was further confirmed by immunostaining for PECAM-1 (Supplementary Fig. S3B, CD31, two right panels), an endothelial cell marker (38). Remarkably, whereas control Vo cells were largely confined within the Matrigel plug, MDA-435 cells overexpressing heparanase seemed to be highly invasive and efficiently repopulated the dermis (Supplementary Fig. S3B, top two left panels) and even the epidermis (Supplementary Fig. S3B, third and fourth panels), nicely combining the proinvasive and proangiogenic features of heparanase.

We next examined heparanase contribution to tumor growth and vascularization in a xenograft model (Fig. 4). Implantation of heparanase-transfected MDA-435 cells ($5 \times 10^9$) into SCID mice produced small tumors that did not differ from tumors produced by control Vo cells until day 41 following implantation (Fig. 4A). Subsequently, tumor xenografts produced by heparanase-overexpressing MDA-435 cells assumed a higher growth rate and generated increasingly bigger tumors than control xenografts. At the end of the experiment on day 62, xenografts developed by heparanase-transfected cells were 3.5-fold bigger in volume ($188 \pm 43$ versus $690 \pm 206$ mm$^3$; Fig. 4A) and 2-fold bigger by weight (0.55 ± 0.2 versus 1.0 ± 0.16 g; Fig. 4A, inset), differences that are statistically significant ($P = 0.036$). In addition, xenograft architecture appeared to be different as revealed by histologic examination of sections derived from control Vo– versus heparanase-overexpressing tumors (Fig. 4B, top) and an enhanced collagen deposition by these tumors as indicated by Mason's trichrome staining (Fig. 4B, bottom), in agreement with the in vitro studies (Fig. 1). Importantly, vascular density was markedly increased in xenografts produced by heparanase-overexpressing MDA–435 cells (Fig. 4C, top). Counting blood vessels in 10 random microscopic fields in three different xenografts yielded a 3-fold increase in vessel density in tumors produced by heparanase-overexpressing cells versus control cells (11 ± 1.3 versus 30 ± 2.8; Fig. 4C, bottom), differences that are statistically highly significant ($P = 0.0003$). RT-PCR analysis further supported VEGF mRNA induction in xenografts produced by heparanase-overexpressing cells versus control cells (Fig. 4D), comparable in magnitude with VEGF up-regulation noted in these cells in vitro (Fig. 2A and C). Thus, VEGF induction in response to heparanase overexpression supports endothelial cell proliferation and survival in vivo (Fig. 3A and B) and likely mediates Matrigel plug and tumor (Fig. 4C) vascularization in vivo.
VEGF up-regulation requires heparanase secretion but not enzymatic activity. To better understand the mechanism by which heparanase promotes protein phosphorylation and expression, we examined the ability of nonsecreted forms of heparanase to induce VEGF expression and p38 phosphorylation. We have recently reported that deletion of 15 amino acids (Δ15) residing at the NH₂ terminus of the 50-kDa heparanase subunit yielded a protein variant that failed to get secreted, was resistant to proteolytic processing, and lacked heparanase enzymatic activity (28). In addition, we generated heparanase mutant devoid of its signal peptide (amino acids 1-35; ΔSP). Subjecting lysate prepared from 293 cells stably expressing the ΔSP gene construct to immunoblot analysis revealed that this protein variant lacks a typical glycosylation pattern (32) as judged by its low molecular weight (Fig. 5B, top, arrow) compared with the WT enzyme, indicating, as expected, that the ΔSP protein was not directed to the endoplasmic reticulum/ Golgi apparatus and, hence, most likely remained in the cytoplasm rather than the lysosome; was not subjected to processing (Fig. 5B, top); and exhibited no enzymatic activity (Fig. 5A). Immunoblot analysis of medium conditioned by 293 cells stably expressing the heparanase variants confirmed that both the Δ15 and ΔSP mutants failed to be secreted (Fig. 5B, second panel). Interestingly, VEGF up-regulation (Fig. 5B, third panel) and enhanced p38 phosphorylation (Fig. 5B, fifth panel) were restricted to cells expressing the secreted form of heparanase (Fig. 5B, WT) whereas nonsecreted heparanase failed to elicit these effects (Fig. 5B, Δ15, ΔSP). In other experiments, heparanase was targeted to the cell membrane by introducing the PDGF receptor transmembrane domain at the heparanase COOH terminus (27). No change in VEGF expression or p38 phosphorylation levels was noted in 293 cells expressing the membrane-targeted heparanase (data not shown). These results suggest that heparanase exerts its effect from outside the cell, possibly through binding to heparan sulfate and/or putative receptor(s) on the cell surface. Indeed, enhanced p38 phosphorylation (Fig. 5C, top) and VEGF induction (Fig. 5C, third panel) were readily detected following exogenous addition of purified recombinant heparanase to MDA-435 (Fig. 5C, left) and 293 (Fig. 5C, right) cells in a time-dependent manner (Supplementary Fig. S4).

We have previously shown that exogenously added latent heparanase rapidly interacts with primary human fibroblasts, as well as with tumor derived cells, followed by internalization and processing into a highly active enzyme, collectively defined as heparanase uptake (27, 31, 39). Although efficient processing is not likely to occur within 5 to 15 minutes following heparanase addition, the possible involvement of heparanase enzymatic activity in cellular activation and signaling cannot be ruled out. To verify this aspect, we used heparanase in which glutamic acid residues 225 and 343 that comprise the enzyme active site (40) were point mutated, yielding an inactive enzyme (25). Addition of purified inactive heparanase to 293 cells resulted in an enhanced p38 phosphorylation (Fig. 5D, top) and VEGF up-regulation (Fig. 5D, third panel) comparable in magnitude to WT heparanase (Fig. 5C, right), clearly implying that enzymatic activity is not required for heparanase mediated stimulation of VEGF expression and p38 phosphorylation.

VEGF up-regulation by heparanase is mediated by Src. Although implicated in VEGF regulation, the causative role of p38 phosphorylation in VEGF induction by heparanase remains to be
shown. To this end, MDA-435 breast carcinoma (Fig. 6A, top) and C6 glioma (Fig. 6A, bottom) cells stably overexpressing heparanase were incubated without (Con) or with SB 203580, a specific inhibitor of p38 phosphorylation, and VEGF levels were then evaluated by immunoblotting. Surprisingly, SB 203580 had no effect on VEGF expression levels (Fig. 6A, right), suggesting that these two variables are not related. By examining several other protein kinase inhibitors, we noted a significant inhibition of VEGF expression in transfected MDA-435 cells treated with PP2, a potent inhibitor of Src (Fig. 6A, left). Similar results were observed with PP2-treated, heparanase-transfected 293 cells (Supplementary Fig. S5A, left). To verify this finding further, 293 cells were left untreated as control (Con) or stimulated with exogenous heparanase (1 μg/mL) in the absence (Hepa) or presence of two additional Src inhibitors (Src inh I and PP1). Whereas VEGF expression was markedly induced by the addition of exogenous heparanase (Supplementary Fig. S5A, right, Hepa), both Src inhibitors abrogated this effect (Supplementary Fig. S5, right, Src inh I, PP1). These findings imply that VEGF up-regulation in response to heparanase overexpression or exogenous addition may be due to Src activation. We examined Src activation by using an antibody directed against the phosphorylated state of Tyr416, an autophosphorylation site within the catalytic domain of Src which is highly phosphorylated in activated oncogenic Src and is thus regarded a valid indication for Src kinase activity (41). A 6-fold increase in phospho-Src levels was observed in heparanase-transfected 293 cells (Fig. 6B, left) or in response to exogenous addition of recombinant heparanase (Fig. 6B, right). Moreover, inactive heparanase mutant efficiently stimulated Src phosphorylation (Fig. 6B, right), similar to p38 and VEGF induction by the inactive enzyme (Fig. 5D). By using Src kinase assay, we noted a significant increase in the phosphorylation levels of a protein with molecular weight of about 120 kDa (Fig. 6C). We suspected that this protein is p120cat, a member of the catenin family originally identified as a Src substrate (42). We employed immunoprecipitation analysis and confirmed that p120cat is highly phosphorylated on tyrosine residues in cells that overexpress heparanase (Fig. 6D) and that p120cat phosphorylation was markedly reduced in response to PP2 treatment (Fig. 6D), confirming that indeed Src is responsible for p120cat phosphorylation. Thus, it seems that up-regulation of VEGF is brought about by heparanase-mediated Src activation, an oncogene previously implicated in VEGF gene regulation (43–45). We next questioned whether enhanced p38 phosphorylation noted in response to heparanase overexpression or exogenous addition is also due to Src activation. As was noted previously, addition of heparanase significantly stimulated p38 phosphorylation and the p38 inhibitor SB 203580 abrogated this effect (Supplementary Figure 4).
Figure 5. VEGF up-regulation and p38 activation by exogenously added heparanase. A, heparanase enzymatic activity. Control Vo (●) and 293 cells stably expressing WT (■) or ΔSP (▲) heparanase were examined for heparanase activity as described in Materials and Methods and in the legend of Fig. 1. Note lack of heparanase enzymatic activity on deletion of its signal peptide. B, VEGF up-regulation and p38 activation requires secreted heparanase. Cell lysates (Ly., top panel) and conditioned medium (Med., second panel) of control Vo and 293 cells stably transfected with WT, Δ15, and ΔSP heparanase gene constructs, were immunoblotted with anti-heparanase (top two panels), anti-VEGF (third panel), antiactin (fourth panel), anti-phospho-p38 (fifth panel), and anti-p38 (bottom panels) antibodies. C, VEGF up-regulation and p38 activation in response to added heparanase. MDA-435 (left) and 293 (right) cells were left untreated or incubated (30 minutes, 37°C) with the indicated concentrations (µg/mL) of purified recombinant 65-kDa heparanase. Cell lysates were immunoblotted with anti-phospho-p38 (top panels), anti-p38 (second panels), anti-VEGF (third panels), and antiactin (bottom panels) antibodies. D, inactive heparanase. 293 cells were incubated (30 minutes, 37°C) with the indicated concentrations (µg/mL) of point-mutated inactive recombinant heparanase. Cell lysates were immunoblotted with anti-phospho-p38 (top), anti-p38 (second panel), anti-VEGF (third panel), and antiactin (bottom) antibodies.

Discussion

Heparanase up-regulation has been documented in an increasing number of human tumor biopsies, correlating with metastatic spread and survival of cancer patients. Although providing a strong clinical support for its prometastatic function, the significance of heparanase in the primary tumor is largely unknown. In several malignancies, heparanase up-regulation correlates with increased microvessel density (7, 9, 11, 12, 17, 18), supporting a proangiogenic function of this enzyme and providing an important function of heparanase in tumor progression. The mechanism by which heparanase facilitates the angiogenic response is not entirely clear but is thought to involve the release of ECM-resident proangiogenic growth factors.

The results presented in this study suggest that VEGF up-regulation is another mechanism by which the proangiogenic function of heparanase is brought about. VEGF induction was noted in several cell lines in response to heparanase overexpression or exogenous addition in vitro (Figs. 2 and 5). The enhanced VEGF secretion was noted to promote endothelial cell proliferation and survival (Fig. 3). Moreover, elevated levels of VEGF in MDA-435 breast carcinoma cells likely facilitate Matrigel plug (Supplementary Fig. S4) and tumor xenograft (Fig. 4) angiogenesis in vivo. In fact, xenograft development seemed to be similar for control Vo– and heparanase-transfected MDA-435 cells until day 41 (Fig. 4). Subsequently, heparanase-overexpressing xenografts assumed a much higher growth rate, possibly due to activation of an angiogenic switch known to be critically important for tumor expansion and most often mediated by VEGF up-regulation (46, 47). Importantly, heparanase down-regulation by applying anti-heparanase siRNA vector was
accompanied by a comparable decrease in VEGF expression levels (Fig. 3), further strengthening the notion that endogenous heparanase is intimately involved in VEGF regulation. Thus, heparanase accelerates angiogenic responses directly by stimulating Akt-dependent endothelial cell migration and invasion (22) and indirectly by VEGF up-regulation in tumor cells (Figs. 2–4), or by releasing ECM-resident angiogenic mediators (19).

The mechanism by which exogenous heparanase activates endothelial cell has been shown to be heparan sulfate independent, augmented by heparin, and involves no enzymatic activity (22), suggesting that this effect is mediated by as yet unidentified heparanase receptor (22). It seems that the same holds true also for p38 and VEGF induction by heparanase. Clearly, inactive heparanase was able to activate p38 phosphorylation and to induce VEGF expression with a magnitude comparable to that of the active enzyme (Fig. 5D) whereas the two nonsecreted heparanase variants failed to do so (Fig. 5B). Interestingly, a p38 inhibitor had no effect on VEGF induction by heparanase (Fig. 6), suggesting that p38 activation and VEGF induction are not related. An inhibitor of p38, nonetheless, efficiently inhibited the promigratory phenotype noted on heparanase overexpression (Supplementary Fig. S6; refs. 21, 25, 26), in agreement with previous reports correlating the p38 module with cellular motility (48).

The ability of Src inhibitors (PP1, PP2, and Src inhibitor I) to prevent VEGF up-regulation in heparanase-overexpressing cells (Fig. 6A; Supplementary Fig. S5) suggests that Src is another yet unrecognized downstream component activated by heparanase.

Src activation in response to heparanase overexpression or exogenous addition was further confirmed by examining Src phosphorylation on Tyr416, as well as by a kinase assay (Fig. 6). We identified the 120-kDa protein emerging in the Src kinase assay as p120cat, originally identified as a Src substrate (42). It should be noted, however, that the anti-phospho-Src (Tyr416) antibody also recognizes equivalent sites within other members of the Src family. Altogether, it seems that heparanase may affect several key components essential for tumor progression, among which are enhanced VEGF levels resulting in increased vessel density and, possibly, vessel maturation (20); activation of Src oncogene family members; acceleration of tumor fibrosis (Fig. 4); and possibly Akt activation and cell survival noted in endothelial and several tumor-derived cells (21, 22). A schematic model summarizing the main results of the current study is presented in Supplementary Fig. S7. We suggest a model in which secreted heparanase binds to and activates heparanase-binding protein that is capable of initiating signal transduction, resulting in p38 and Src activation. The latter is responsible for VEGF up-regulation and p120cat tyrosine phosphorylation. Activated p38, Src, and, possibly, tyrosine phosphorylated p120cat participate in heparanase-stimulated cell motility. A key issue in understanding these nonenzymatic functions of heparanase is the identification of heparanase-binding proteins and perhaps heparanase receptor that mediate such diverse effects. These studies are currently in progress.

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References


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