A Role for Perlecan in the Suppression of Growth and Invasion in Fibrosarcoma Cells

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Abstract

Perlecan is a major heparan sulfate proteoglycan of basement membranes and cell surfaces. Because of its strategic location and ability to store and protect growth factors, perlecan has been implicated in the control of tumor cell growth and metastatic behavior. To test the role of perlecan in malignancy, we generated several stably transfected clones of HT-1080, a human fibrosarcoma cell line, harboring a perlecan cDNA in the antisense orientation. Surprisingly, clones with a reduced synthesis of perlecan mRNA and protein core grew faster, formed larger colonies in semisolid agar, and induced faster formation of s.c. tumors in nude mice than the wild-type cells. Their growth properties in vitro were independent of exogenous basic fibroblast growth factor. Reduction of perlecan expression was associated with three distinct properties typical of tumor cells with a more aggressive phenotype: enhanced migration through 8-μm-pore filter, increased invasion in Matrigel-coated filters, and heightened adhesiveness to type IV collagen substrata. These results thus provide the first evidence that perlecan may inhibit the growth and invasiveness of fibrosarcoma cells in a basic fibroblast growth factor-independent pathway and raise the possibility that perlecan may prevent the infiltration of host tissues in mesenchymal neoplasms.

Introduction

Perlecan is a large heparan sulfate proteoglycan (1, 2) that is expressed in virtually all vascularized tissues either as an intrinsic component of the basement membrane, the thin layer of connective tissue separating epithelial from mesenchymal cells, or in close association with the cell surfaces (3), where the binding is mediated by members of the integrin gene family (4–6). The strategic location of perlecan at the interface of tumor invasion immediately suggests that this gene product may be directly involved in the modulation of cell surface events known to be altered in the multistep process of invasion and metastasis (7).

The central role of cell-associated heparan sulfate proteoglycans was revealed by the finding that high-affinity receptor binding of bFGF(3) was abolished in mutant cell lines defective in their glycosaminoglycan metabolism (8) and in myoblasts depleted of sulfated glycosaminoglycans (9). Moreover, bone marrow heparan sulfate proteoglycans, including perlecan, bind growth factors and present them to hematopoietic progenitor cells (10). Highly O-sulfated oligosaccharide sequences are required for bFGF binding and receptor activation (11, 12). Of note, cell surface proteoglycans of the syndecan and glypicanc gene family failed to promote high-affinity binding of bFGF (13). Subsequently, it was shown that perlecan was directly involved in this coupling and that it acted as a low-affinity receptor and an angiogenic modulator (14).

An emerging body of evidence further supports the notion that perlecan is directly involved in promoting the growth and invasion of tumor cells through its ability to capture and store growth factors (15) within the basement membranes of various tissues (16) and within the tumor stroma (17). For example, in human melanomas, a predominance of heparan sulfate proteoglycans at the cell surface is a marker of a more aggressive behavior (18), and perlecan mRNA and protein levels are notably increased in metastatic melanomas (19). Purified perlecan enhances invasiveness of human melanoma cells (20), whereas contact with basement membrane perlecan augments the growth of transformed endothelial cells but suppresses that of normal endothelial cells (21). Stable overexpression of an antisense perlecan cDNA in NIH-3T3 cells, as well as in human metastatic melanomas, leads to reduced levels of perlecan and concurrent suppression of cellular responses to bFGF (22).

Large deposits of immunoreactive perlecan are present in the newly vascularized stroma of colon, breast, and prostate carcinomas (23). In tumor xenografts induced by s.c. injection of human prostate carcinoma PC3 cells into scid/scid mice, although perlecan was actively synthesized by the human tumor cells, it was deposited along newly formed blood vessels of murine origin (23). Hence, perlecan deposited by growing tumor cells may act as a scaffold upon which proliferating capillaries grow and eventually form functional blood vessels. These findings are in support of the original observations that heparan sulfate proteoglycans can store bFGF in an active form and protect it from proteolytic degradation (24, 25). The recent observation that bFGF binds to heparan sulfate chains located in the NH2-terminal domain of perlecan synthesized by human endothelial cells (26) reinforces the hypothesis that perlecan represents a major storage site for bFGF in the blood vessel wall.

We hypothesized that if perlecan promotes growth and angiogenesis, then its loss would result in growth suppression. To test this hypothesis, we generated several stably transfected clones of HT-1080, a human fibrosarcoma cell line, harboring a perlecan cDNA in the antisense orientation. Surprisingly, the results showed that the clones with a reduced synthesis of perlecan mRNA and protein core grew faster, formed larger colonies in semisolid agar, and induced faster tumor growths in nude mice than the wild-type cells and were independent of the exogenous addition of bFGF. Furthermore, reduction of perlecan expression was associated with three properties typical of tumor cells with a more aggressive phenotype: enhanced migration through an 8-μm-pore filter, increased invasion in Matrigel-coated filters, and heightened adhesiveness to type IV collagen substrata. These results, thus, provide the first evidence that perlecan can negatively affect the growth and invasive properties of fibrosarcoma cells in a bFGF-independent pathway and raise the possibility that, at
least in this mesenchymal neoplasm, perlecan may prevent the infiltration of host tissues.

Materials and Methods

Materials. DMEM, Medium 199, Dulbecco’s PBS, and glutamine were purchased from Mediatech, Inc. (Herndon, VA). Gentamicin, penicillin/streptomycin, insulin-transferrin-selenium, and Fungizone were obtained from Life Technologies, Inc. (Gaithersburg, MD). BSA and heparin sodium were obtained Fisher Scientific (Springfield, NJ). Fetal bovine serum (FBS) and FCS were obtained from HyClone Laboratories, Inc. (Logan, UT). Recombinant bFGF was obtained from Collaborative Biomedical Products (Bedford, MA). LeukoStat Stain Kit was purchased from Fisher Scientific. Matrigel was generated as described (27).

Construction of the Perlecan Antisense Vector and Generation of Stably Transfected Clones. Two constructs were generated: one spanning 622 bp in domains I-II (base pairs 91—712), and the other one spanning 1.1 kb (base pairs 11,764—12,899) and encompassing the end of domain IV and the beginning of domain V of perlecan protein core (2). The cDNAs were digested with EcoRI or Apal and KpnI and then ligated to the 3’ end of the human cytomegalovirus early gene promoter/enhancer in a eukaryotic expression vector pcDNA3 (Invitrogen). Proper orientation of the inserts was verified by DNA sequencing. About 10⁶ cells were transfected with 20 μg of DNA, and after 2 days in nonselective medium to allow expression of the transfected gene, the cells were passaged and cultured with neomycin analogue G418 (600 μg/ml). Independent colonies were isolated by ring cloning, transferred to microtiter wells, and expanded in the same G418 concentration. Individual clones were routinely grown in medium supplemented with 400 μg/ml G418.

Northern and Immunoblotting Analyses. Total RNA was prepared using the Tri-Reagent-LS procedure (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s recommendation. A 30-μl aliquot of total RNA was separated on a 1% denaturing agarose gel containing formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham). The blots were hybridized at 42°C overnight with 32P-labeled probes spanning the two perlecan cDNAs used for antisense construction (19). For quantification, the blots were stripped in a boiling solution of 0.1% SDS and then reprobed with 32P-labeled glyceraldehyde-3-phosphate-dehydrogenase. For immunoblotting, HT-1080 and antisense transfected cells were grown to subconfluence in DMEM with 10% calf serum. After incubation for 30 h in DMEM, medium and cells were collected separately. The medium was frozen at —20°C, and nuclei were stained in 0.1 M citric acid with 0.1% crystal violet and counted in a hemocytometer. Aliquots of media correspond to —2 X 10⁶, 1 X 10⁷, 5.5 X 10⁷, and 2.7 X 10⁸ cells were blotted on nitrocellulose and reacted with the monoclonal antibody 7BS, which recognizes specifically domain III of human perlecan (28). Detection by chemiluminescence was performed as described before (29).

Response to bFGF, Cell Proliferation Assays, and Growth in Soft Agar and in Nude Mice. Control murine NIH-3T3 cells, as well as wild-type, vector alone, and antisense-transfected HT-1080 cells (antisense expressing and nonexpressing), were seeded at a density of 5 X 10⁶ cells in a 48-well plate (Corning) in DMEM with 10% FBS. After 1 day, the medium was exchanged to serum-free medium, and following an additional day in culture, it was supplemented with either 1 ng/ml bFGF or with 1 ng/ml bFGF plus 1 μg/ml heparin. After a total of 28 h of incubation, a colorimetric cell number assay (Cell Titer 96; Promega) was performed in which the amount of the reaction product (Formazan) is time dependent and proportional to the number of viable cells (30). To each well, 30 μl of the MTS/phosphene methanol solution mixture were added and incubated at 37°C for 1 h. Quantification was performed by photometry at 490 nm. In vitro tumorigenicity was tested by growth in a soft-agar colony assay. Sixty-mm² dishes were covered with 2 ml of DMEM, 0.5% agar, and 10% FBS. The middle layer contained approximately 10⁶ cells in 1 ml of DMEM in 0.33% agar plus 10% FBS, whereas the top layer consisted of 1.5 ml of DMEM with 10% FBS. The cells were cultured for 21 days, stained with vital tetrazolium dye, fixed in 10% buffered formaldehyde, and photographed (31).

To test for growth in vivo, 16 NCR nude mice (males, 7—9 weeks old) were purchased from Taconic Farms. Three HT-1080 clonal cells (PA0, PA17, and PA31) and HUVECs were injected s.c. at a density of 3 X 10³ in 0.5 ml of Matrigel as described before (32). For each cell type, four animals received injections in the left groin, except for HUVECs, which were injected into the right side. Tumor volume was measured by a microcaliper at daily intervals for the 1st week and then every other day until the animals were sacrificed (day 18 postinjection).

Tumor Cell Migration, Invasion, and Attachment Assays. To investigate the ability of the stably transfected clone to migrate through a filter or to invade a biological barrier, we used a modified Boyden chamber as described previously (33). We used 24-well culture plates and cell culture inserts (8.0-μm pore size; Falcon). Approximately 2 X 10⁵ tumor cells in 500 μl of

Fig. 1. A, perlecan transfection construct; B, Northern blotting of transfected clones; C, Western blotting of transfected cells; D, schematic presentation of the construct harboring 1.1-kb perlecan cDNA in antisense orientation and including the signal peptide and the polyadenylation signal from the bovine growth hormone. CMV, human cytomegalovirus major immediate early promoter/enhancer region; fi ori, fi origin for rescue of single strand; neo, neomycin resistance marker; ColE1, ColE1 origin of replication from pUC19; amp, ampicillin-resistant gene; ori, origin of replication. B, autoradiogram of RNA blotting using either the perlecan antisense (Perlecan AS) vector or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as labeled probes. Lanes 1—9, individual stably transfected clones; Lane 10, wild-type human fibrosarcoma HT-1080 cells. C, Immuno-slit blot analyses of three stably transfected clones expressing various levels of antisense. PA 20, nonexpressing; PA 7 and PA 31, antisense expressing clones; WiDr, medium conditioned by colon cancer cells as positive control (+) or DMEM alone (—). Bottom, quantification by laser scanning densitometry and computer integration. Columns, mean of triplicate determinations, with SD <15% of the mean.
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Fig. 2. Reduction of perlecan expression leads to enhanced growth of fibrosarcoma cells in a bFGF-independent pathway. Growth curves of NIH-3T3 cells or various HT-1080 clones (as indicated) in the presence or absence of bFGF alone or in combination with heparin (A and B), proliferation assay (C), and soft-agar colony formation (D–G). A and B, approximately 5 x 10⁶ cells were seeded in a 48-well plate in either serum-free DMEM or DMEM with 1 ng/ml bFGF or 1 ng/ml bFGF plus 1 μg/ml heparin. C, cells were incubated in DMEM with 10% FBS, and a colorimetric assay was performed on days 1, 2, and 4. Fresh medium was added daily. D–G, the clones were seeded at a density of 10⁶ cells in a sandwich of soft agar (0.5% bottom layer and 0.3% top layer) and cultured for 3 weeks. D, PA20; E, PA7; F, PA17; G, PA31. Plates were stained with a vital tetrazolium dye, fixed, and photographed at the same magnification. Columns (A and B) and data points (C), mean values of three independent experiments run in duplicate; bars (A–C), SD. Scale bar, 500 μm.

serum-free DMEM were added to 8-μm tissue culture inserts, which had been positioned in the wells. For the invasion assay, a matrix barrier was formed by coating with diluted Matrigel at 5 mg/ml in serum-free DMEM. Inserts were coated with 50 μl of Matrigel solution and air-dried overnight in a laminar flow hood. Approximately 10⁶ cells suspended in DMEM-1% BSA were added to the upper filter. In both assays, the bottom chambers were filled with serum-free medium conditioned for 24 h by mouse melanoma M2 cells, which served as a chemoattractant. After either 6 h (for the migration assay) or 18 h
Results and Discussion

Reduced Perlecan Expression in Antisense Transfected Human Fibrosarcoma Cells. To investigate the role of perlecan in the growth and invasive properties of fibrosarcoma HT-1080 cells, we generated a number of clones harboring perlecan antisense vectors driven by the CMV promoter and carrying a neomycin resistance cassette as a positive selection marker. Of note, the 5′ antisense construct (see “Materials and Methods”) did not produce any stably transfected clones, although the same number of cells as in the 3′ construct were used. Fig. 1A is a schematic representation of the 3′ antisense construct, and Fig. 1, B and C, shows representative Northern and Western blottings of various clones. We selected three antisense-expressing cell lines, designated PA7, PA17, and PA31 (corresponding to Fig. 1B, Lanes 1, 3, and 9, respectively), that showed the expected transcript at 1.1 kb for detailed studies and a nonexpressing cell line (PA20; Fig. 1B, Lane 8) as a control. An additional HT-1080 clone, designated PA0, transfected with an insertless pcDNA3 vector (31) was also investigated. The intensity of perlecan antisense expression correlated well with a reduction in perlecan protein core biosynthesis, as detected by Western immunoblotting using a monoclonal antibody directed against domain III of human perlecan (Fig. 1C). The highest antisense-expressing cell line (PA31) demonstrated a reduction in perlecan synthesis by ~55%. In intermediate expressing cell lines such as PA7 and PA17 (data not shown), the perlecan released into the medium was reduced by ~35% and 40%, respectively.

Perlecan Reduction Leads to Growth Enhancement in a bFGF-Independent Manner. Because perlecan is known to be involved in cell adhesion and proliferation and inasmuch as this proteoglycan represents the low-affinity binding factor for bFGF (14), a reduction in perlecan expression would presumably result in an overall inhibition of bFGF binding and cell proliferation. Hence, we performed several growth experiments under different serum concentrations (Fig. 2C) or without added serum (data not shown), as well as in the presence of exogenous recombinant bFGF, with or without the cofactor heparin. As a positive control for bFGF activity, we used NIH-3T3 cells. As expected, the NIH-3T3 cells did respond well to bFGF, with a nearly 3-fold increase in cell number (Fig. 2A). The presence of exogenous heparin, known to stabilize bFGF-receptor complexes and bFGF dimerization (12, 34), only slightly increased the growth of NIH-3T3 cells. In contrast, both the control HT-1080 clones (PA20 and PA0) and the other perlecan antisense clones did not show any response to exogenous bFGF (Fig. 2, A and B). Moreover, no difference was seen when heparin was added at concentrations shown previously to favor bFGF activity (8, 9). Although the presence of exogenously added heparin or of endogenous cell surface heparan sulfate proteoglycan increases the affinity of bFGF for its receptor, a recent study using BALB/c3T3 fibroblasts depleted of endogenous heparan sulfate has shown that these proteoglycans are not absolutely required for binding, internalization, or stimulation of mitogenic activity (35).

Surprisingly, the clones with reduced levels of perlecan (PA7, PA17, and PA31) exhibited an enhanced growth rate, and this was independent of either bFGF or bFGF/heparin (Fig. 2, B and C). This enhanced replication rate in vitro was not caused by exogenous growth factors present in the serum, insofar as repeated growth studies in serum-free medium showed no difference in growth behavior apart from a general reduction in growth in both mock-transfected and antisense transfected cells (data not shown). The changes in growth kinetics were further confirmed by growth studies in the soft agar (Fig. 2, D-G). Wild-type HT-1080 (Fig. 2D) or mock-transfected cells (data not shown) generated only small colonies when compared to the stably expressing clones, which produced remarkably larger colonies (Fig. 2, E-G). Collectively, these data indicate that a reduction in perlecan expression leads to bFGF- and serum-independent growth of fibrosarcoma cells in both attached and suspended growth conditions.

Perlecan Reduction Leads to Faster Growth in Nude Mice and to Enhanced Migration, Invasion, and Adhesion in Vitro. To investigate further the characteristics of the fibrosarcoma cells with reduced perlecan gene expression, we injected the vector-transfected clone PA0 and two of the antisense expressing clones (PA17 and PA31) together with Matrigel s.c. into nude mice (32). As a further control, we used HUVECs. The results showed that within the first 8 days following injection, both PA17 and PA31 formed larger tumors than PA0 (Fig. 3). At later days, however, the growth rate of PA0 was much increased, whereas the HUVECs never produced tumors larger than 1 cm³ (data not shown). These results thus indicate that reduction of perlecan levels also contributes to enhanced tumor growth in vivo, at least in the initial stages of tumor development.

We next tested the various cell lines in migration and invasion assays using a modified Boyden chamber (33). Migration assays...
through a 8-μm-pore filter revealed that the cells with reduced perlecan expression showed a significant increase in transmigratory ability toward the M2 chemoattractant (Fig. 4). In addition, the reduction of perlecan gene expression correlated with a heightened ability to invade Matrigel (Fig. 5), a reconstituted murine matrix composed of various basement membrane constituents including laminin, type IV collagen, nidogen, and perlecan, as well as various growth factors (27). The PA7 and PA31 clones had the greatest invasive ability in the assay. These data would suggest that these transfectants have the ability to digest the Matrigel, probably by enhanced proteolytic ac-
tivity. The cells that migrated to the other side of the filter exhibited a change in cell shape, forming spindle-shaped cells that anastomosed with each other (data not shown).

Finally, we tested the stably transfected clones for their ability to attach to either plastic dishes alone or to purified type IV collagen-coated dishes. Tumor cell attachment to the surrounding matrix is one of the initial steps leading to invasion of host tissues (7). The results showed a greater ability of the clones expressing the antisense perlecan vector to attach and spread on both collagen-coated and native dishes (Fig. 6). Of note, the antisense perlecan clones exhibited 2–3-fold enhancement of cell attachment on plastic surfaces (Fig. 6A), but this effect was even more pronounced when the dishes were precoated with various concentrations of type IV collagen (Fig. 6, B–D). Hence, a reduction in perlecan proteoglycan expression leads to a promotion of cell attachment and suggests that perlecan, at least in these fibrosarcoma cells, is a counteradhesive protein. This is interesting because human perlecan can behave as both an adhesive and an antiadhesive protein for endothelial and bone marrow cells, respectively (36). Perlecan also inhibits mesangial cell adhesion to fibronectin (37) and is antiadhesive for polymorphonuclear granulocytes (38). Although antiadhesive for hematopoietic and fibrosarcoma cells, perlecan is still capable of binding granulocyte macrophage-colony stimulating factor and presenting it to the hematopoietic progenitor cells in a semisolid colony assay (36). These findings are in agreement with the present results and indicate that perlecan may have a dual function (either adhesive or counteradhesive), depending on the histogenetic origin or the cells. An alternate possibility is that perlecan may influence the cell surface expression of cell adhesion molecules such as integrins.

In conclusion, the results presented here indicate that the HT-1080 clones, genetically modified to produce reduced amounts of perlecan proteoglycan, have enhanced growth and heightened capacity to invade a biological basement membrane and to attach to a collagenous matrix. In this cellular context, thus, perlecan may play a role as an antiadhesive molecule. Further studies are needed to establish the precise role of perlecan in sarcomas and to determine whether this proteoglycan may play opposite roles in carcinomas and melanomas.
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