Abnormal Expression of Perlecan Proteoglycan in Metastatic Melanomas

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Abstract

Abnormal expression of proteoglycans has been implicated in cancer and metastasis primarily because these macromolecules are involved in the control of cell growth and matrix assembly. In this report, we have investigated the expression and immunolocalization of perlecan, a major heparan sulfate proteoglycan of basement membranes and pericellular matrices, in human metastatic melanomas. Twenty-six of the 27 tumor samples showed a significant increase (up to 15-fold) in the perlecan mRNA levels when compared with normal tissue. This change correlated with a vast deposition of perlecan protein core in the pericellular matrix of metastatic melanomas. Furthermore, we have established a relationship between perlecan expression in cloned melanoma cells (70W) stimulated with neurotrophins and their increased invasiveness. Interestingly, perlecan mRNA levels were up-regulated within 10 min of neurotrophin stimulation, indicating that perlecan is an early response gene. This up-regulation also occurred prior to heparanase production, suggesting that perlecan expression and its regulation might play a pivotal role in the initial onset of invasion.

Introduction

The vascular basement membrane is the first barrier tumor cells need to cross to colonize distant organs. The major components of this extracellular matrix include type IV collagen, laminin, entactin, and perlecan. During tumor invasion, the basement membrane becomes disorganized through three major steps: changes in the expression of the constituents, alterations in their assembly, and enzymatic degradation of the matrix. In this cascade of events, the role of perlecan, the major heparan sulfate proteoglycan of basement membrane, has remained undefined. Perlecan is known to be involved in cell migration, adhesion, and proliferation. Such diversity of functions is probably a consequence of its chimeric structure of five distinct domains, with homologies to the low-density lipoprotein-receptor, laminin, epidermal growth factor, and the neural cell adhesion molecule N-CAM. Perlecan is a proteoglycan widely expressed in all basement membranes, in particular in the subendothelium of blood vessels, where it would hold a key position to influence extravasation of malignant cells. Several reports have pointed to qualitative and quantitative changes in perlecan expression linked to the neoplastic phenotype. Degradation of the heparan sulfate chains following the release of degradative enzymes such as heparanase has been well described in the progression to malignancy. In addition, tumor cytokines that influence the turnover and biosynthesis of proteoglycans in host cells are known to bind perlecan and can act as a reservoir of growth factors to stimulate tumor growth and angiogenesis.

Materials and Methods

Northern Blotting. RNA from invasive melanomas and metastatic cell lines was prepared using the Tri-Reagent-LS procedure (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's recommendations. We have analyzed 27 metastases: 15 to the brain, 10 to lymph nodes, 1 to the axillary lymph nodes, and 1 to the stomach. As control tissues, we tested 8 skin and 2 brain samples, as well as one primary melanoma of the foot. Metastatic melanoma cells were treated with either NTs NGF or NT-3 (2 nM for 10 min in serum-free conditions at 37°C) and cells were collected for RNA preparation. A 20-µg aliquot of total RNA was separated on a 0.75% denaturing agarose gel containing formaldehyde and transferred to a nylon membrane. The blots were hybridized at 42°C in 50% formamide with an α-32P-labeled polymerase chain reaction-generated probe spanning perlecan cDNA between 11,225 and 12,725 base pairs (4). The membranes were washed under stringent conditions in 0.5X standard saline-citrate-0.1% sodium dodecyl sulfate at 65°C for 1 h and exposed as indicated. For quantitation, RNA slot blots were performed. A 15-µg aliquot of total RNA was applied to nitrocellulose using the minifold slot-blot system (Schleicher and Schuell, Inc., Keene, NH) and air dried. Hybridization and washing were performed as described above. Quantitation was determined by measuring light absorbance in a densitometer, and normalization was made on the amount of total RNA.

Immunohistochemistry. Frozen sections of surgically obtained tissues were reacted with monoclonal antibody 7B5 in a 1:10,000 dilution of ascites medium in PBS. The bound antibodies were reacted with biotinylated horse anti-mouse secondary antibodies (Vector Laboratories, Burlingame, CA). All reagents were diluted in PBS and incubated with the sections for 60 min, followed by two washes in PBS. The sections were subsequently incubated with avidin and biotinylated horseradish peroxidase for 10 min, followed by an 8-min incubation with the peroxidase substrate. Sections were counterstained with hematoxylin.

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**Western Blotting.** Cultures of metastatic melanoma cells were treated with either NTs NGF or NT-3 and the medium was collected at various times after stimulation. Serial dilutions of media were applied to nitrocellulose using the minifold slot-blot system (Schleicher and Schuell, Inc.) and air dried. The membrane was blocked with 4% Carnation nonfat milk in PBST and incubated with a monoclonal antibody directed against domain III of perlecan (5). After three washes in PBST-0.1% milk, the membrane was incubated with an antiserum IgG labeled with horseradish peroxidase and then was washed again in PBST-0.1% milk. The chemiluminescent reagent was prepared according to the manufacturer’s instructions (Dupont-NEN, Wilmington, DE) and incubated with the membrane for 1 min at room temperature. Exposure of the film varied from 10 to 60 s.

**Results and Discussion**

**Elevated Expression of Perlecan in Invasive Melanomas.** We have investigated tumor samples from patients with metastatic melanomas to determine the levels of perlecan message compared with those of normal tissue or primary tumors from the same patient. Fig. 1 shows a typical Northern blotting analysis of a primary melanoma (Lane 1) and the metastasis of the same patient (Lane 2). Perlecan was increased about 5-fold in the metastatic sample compared to the primary tumor, when normalized on β-actin levels. A single transcript of ~14.5 kilobase was detected in all samples tested. In addition, reverse transcriptase-polymerase chain reaction analysis of the perlecan transcript detected the expected fragment (not shown), indicating no significant alternatively spliced variant of perlecan in these tumors. Most of the metastatic tumors we examined (26/27) were found to express perlecan at higher levels than the normal or primary samples. Quantitation of RNA slot blots by densitometric analysis indicated up to a 15-fold increase in perlecan expression in the invasive tumors when compared with the normal tissue (skin, brain) or primary counterparts. Only one tumor tissue expressed perlecan at a level lower than that of the control. Table 1 indicates the relative values obtained after quantitation for the population of tumors we analyzed.

**Immunolocalization of Perlecan in Invasive Melanomas.** Using a monoclonal antibody directed against the laminin-like domain III of perlecan (5), we localized perlecan in frozen sections of tumor biopsies. Perlecan was widely distributed in the pericellular matrix of the tumor from two separate cases: one amelanotic (Fig. 2C) and one melanotic (Fig. 2D). Strong immunoreactivity was detected particularly around the melanoma cells (arrowheads) and the sites of vascular proliferation (arrows). Perlecan was also present in the dermal/epidermal junction of the skin (Fig. 2A, arrowheads) and in the subendothelial basement membrane of the microvessels in the brain cortex and the dermis (Fig. 2, A and B, arrows). Control sections showed no reactivity when the primary perlecan antibody was omitted (Fig. 2, E and F). There are numerous cell-matrix interactions which occur in the multistep process leading to metastasis (2). These include the ability of the tumor cells to attach to the subendothelial basement membrane of microvessels and migrate through the matrix into the bloodstream. Several components of the matrix are affected during this process, and this is the first report of an increase in the protein core of the proteoglycan. Disruption of the balance between the different components of the basement membrane could participate in its destabilization and facilitate migration of tumor cells through the vascular wall and interstitial stroma. This elevated expression of perlecan in invasive tumors may also represent a marker for a more aggressive phenotype.

**Perlecan Expression Is Elevated in Melanoma Cell Lines Treated with NTs.** We then attempted to reproduce in vitro the results obtained in the tumor samples by investigating two melanoma cell lines of high-metastatic and low metastatic potential. The 70W melanoma cells of the MeWo cell line have been characterized for their ability to develop cerebral metastases (8, 9). When treated with NTs, such as NGF, these cells invaded a Transwell filter coated with a reconstituted basement membrane, thereby mimicking advanced stages of melanoma metastasis (10, 12). Furthermore, the 70W cells invade a Transwell filter as efficiently, whether it is coated with purified mouse perlecan or Matrigel, an extract of the EHS tumor (10). Here we examined melanoma cell lines of the MeWo series for perlecan expression to determine any association with invasion following treatment with two different NTs, NGF and NT-3. The incubation of 70W cells with either NT resulted in a dramatic increase in perlecan mRNA (Fig. 3A, Lanes 5 and 6), whereas the level of perlecan message in unstimulated 70W was barely detectable (Lane 4). These data correlated well with the degree of invasion of the 70W cells on stimulation with cytokines (10). The increase in perlecan message occurred within a 10-min induction, suggesting that perlecan may be an early player in the invasion process. In contrast, perlecan expression was not modulated in the parental MeWo cells (Lanes 1-3).

The NT-induced up-regulation of perlecan was also detected at the protein level. A serial dilution of the medium from unstimulated 70W
cells or NT-treated cells was immunoreacted with an anti-perlecan monoclonal antibody directed against domain III of the perlecan protein core (Fig. 3B). Perlecan protein increased as early as 30 min (not shown) and enhanced secretion persisted for at least 24 h (Fig. 3B, Lanes 3 and 5). These data also indicate that perlecan is up-regulated prior to the induction of heparanase activity, which appears only after 24 h of NT stimulation (10).

To produce a tumor at a secondary site, interactions of the tumor cells with their environment is crucial and the destabilization of the basement membrane is one of the major events in this process. The increase in perlecan expression that we detect in the invasive tumors is very significant, but why should a gene-encoding basement membrane perlecan be induced as an early response gene? Signal transduction in response to NTs indicates association of the p75-NT receptor with a NGF-stimulated purine analogue-sensitive protein kinase, which could modulate expression of genes involved in invasion (12). Similarly, perlecan is known to bind growth and angiogenic factors through its heparan sulfate chains (13) and allows their storage within the basement membrane. Local proteolytic activity by tumor cells leading to the release of glycosaminoglycan chains/growth factor complexes, in conjunction with changes in the sulfation pattern of the chains, is bound to influence the autocrine growth of the tumor and neovascularization (3, 6, 14).

Perlecan also has the ability to self-associate (15) and interact with type IV collagen (16), laminin (17), and entactin (18, 19). With the homologies to the low-density lipoprotein receptor, laminin, and N-CAM in its cDNA (4) and gene organization (20), the release of high amounts of perlecan in the basement membrane could lead to dramatic changes in the molecular architecture of this matrix. Gain of additional adhesion and matrix binding sites might be decisive in the invasion process. Recently, the N-CAM and laminin-globular subdomains of perlecan have been implicated in cell adhesion and spreading via a β1 integrin receptor interaction (21), suggesting a putative role of this region of perlecan in adhesion. In addition, alternatively spliced variants of perlecan, lacking domain IV and V of the molecule, have been identified in Caenorhabditis elegans development,
causing disruption in muscle cell-body wall interactions (22). Although not clear which function the different domains of perlecans play in the invasive process, the results presented here indicate that enrichment in basement membrane perlecans is associated with the metastatic potential of human melanoma cells. Elevated expression of perlecans in invasive melanomas may represent a marker for a more aggressive phenotype and correlate with our in vitro data. Further investigations are necessary to determine how perlecans affects invasion of tumor cells and which domains of the molecule may be implicated in this process.
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