Galectin-3 Cleavage: A Novel Surrogate Marker for Matrix Metalloproteinase Activity in Growing Breast Cancers

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Abstract

Failed therapies directed against matrix metalloproteinases (MMP) in cancer patients may be attributed, in part, to lack of diagnostic tools to differentiate between pro-MMPs and active MMPs, which indicate whether a treatment is efficacious or not. Because galectin-3 is cleavable in vitro by MMPs, we have developed differential antibodies recognizing its cleaved and noncleaved forms and tested their clinical utilization as a surrogate diagnostic marker for the presence of active MMPs in growing breast cancers. Wild-type and cleavage-resistant galectin-3 were constructed and expressed in galectin-3–null human breast carcinoma cells (BT-549). Tumorigenic and angiogenic potential of the clones was studied by injections into nude mice. MMP-2, MMP-9, full-length, and cleaved galectin-3 were localized in the xenografts by immunohistochemical analysis of paraffin-embedded sections using specific antibodies. Activities of MMP-2/9 were corroborated by in situ zymography on frozen tissue sections. Galectin-3 cleavage was shown in vivo by differential antibody staining and colocalized with predicted active MMPs both in mouse xenografts and human breast cancer specimens. In situ zymography validated these results. In addition, BT-549 cells harboring noncleavable galectin-3 showed reduced tumor growth and angiogenesis compared with the wild-type. We conclude that galectin-3 cleavage is an active process during tumor progression and could be used as a simple, rapid, and reliable surrogate marker for the activities of MMPs in growing breast cancers.

Introduction

Matrix metalloproteinases (MMP) are a family of zinc-dependent proteases that can be divided into five groups based on substrate specificity. They remodel extracellular matrix (ECM) components and cleave a broad range of cell surface proteins, resulting in substrate degradation in areas of cell-matrix contact, thus affecting various cellular activities. Certain aspects of the involvement of MMPs in tumor metastasis, such as angiogenesis, invasion, and establishment of metastatic foci, have received extensive attention, resulting in an overwhelming amount of data concerning critical roles of MMPs in cancer (1). The gelatinases MMP-2 and MMP-9, which specifically degrade collagen IV, are important for initiation and development of tumor vascularization (2, 3). Dependency of tumor angiogenesis on the activity of these MMPs renders this step a likely target of synthetic MMP inhibitors.

The search for MMP inhibitors with possible anticancer efficacy is a nearly three-decade endeavor and an ideal effective inhibitor is yet to be found [reviewed by Lindsey (4) and Zucker et al. (5)]. Possible reasons for this failure include broad MMP subtype selectivity and toxicity as well as the diversity of MMP biology. MMPs have been shown to enhance angiogenesis by recruiting pericytes (6), releasing ECM-bound angiogenic growth factors (7), exposing cryptic proangiogenic integrins binding sites (8, 9), and cleaving endothelial cell-cell adhesion molecules (8, 10). MMPs can also contribute negatively to angiogenesis through the generation of endogenous angiogenesis inhibitors by proteolytic cleavage of collagen and plasminogen and by modulating cell receptor signaling by cleaving off their ligand-binding domains (11, 12). MMPs are synthesized as inactive proenzymes, which are activated by proteolytic cleavage of the propeptide domain (13). However, to date, there is no simple diagnostic tool to distinguish between active and nonactive MMPs in vivo. To search for an anticancer inhibitor, which must possess selectivity against the MMP subtype critically important in relation to temporal progression of metastasis as well as with degredation of the matrix, the foremost requirement is to be able to differentiate between the active and the proactive form of MMP in the tissue. Two techniques are currently being used to evaluate MMP activity in tumors: MMP targeting probes for in vivo imaging (14) and in situ zymography (15–17). The results obtained from in vivo imaging have yet to be validated by biochemical or functional methodologies (14). In situ zymography is difficult to analyze due to imprecise localization, broad range of targets, and need of adequate controls to validate specificity and efficacy. Moreover, it is only applicable to fresh frozen specimens. In the present article, we provide evidence that cleavage of galectin-3 can be used as a novel surrogate diagnostic marker for the activity of MMP-2/9 in cancer tissues, which can be easily analyzed by differential immunostaining on paraffin-embedded specimens as well as fresh frozen sections providing a wider range and ease of use.

Galectin-3, a ~30-kDa chimera carbohydrate-binding protein belonging to the galectin gene family is composed of three distinct structural motifs, an amino terminal domain consisting of 12 amino acid residues, preceding an amino terminal half of collagen-like sequence containing Pro-Gly-Tyr tandem repeat, and a sugar-binding carboxy terminal half (18–20). The collagen-like domain of galectin-3 is susceptible to rapid and efficient cleavage by MMPs (enzyme/substrate, 1/10–100), in particular MMP-2, MMP-9, and membrane type 1-MMP at the Ala⁶²-Tyr⁶³ peptide bond, resulting in the generation of a ~22 kDa cleaved product (19). Although the NH₂-terminal region of human galectin-3 contains five Ala-Tyr repeats, the Ala⁶²-Tyr⁶³ peptide bond is the only site that is sensitive to MMP-2/9 cleavage (19). The Ala⁶²-Tyr⁶³ is
followed by histidine, whereas the other sites are followed by proline. This suggests that structure around the MMP cleavage site may be important in determining its availability for enzymatic degradation. Galectin-3 is implicated in different cell lineages at different developmental and pathologic stages and is involved in cell growth, apoptosis resistance, adhesion, differentiation, inflammation, transformation, angiogenesis, invasion, and metastasis (21, 22). Galectin-3, although present in the cytoplasm, nucleus, and the cell surface, is also secreted into the ECM, where it binds to the ECM proteins laminin, fibronectin, and collagen IV. An additional band of ~22 kDa was observed in the cell lysates and a ~27 kDa from the conditioned medium from the three-dimensional co-cultures of epithelial and endothelial cells (23). The 22-kDa polypeptide is also observed in the conditioned medium from various cell lines occasionally. It has been presumed that the active form of MMP may be responsible for the cleavage of galectin-3 in the extracellular environment. No experimental evidence was provided thus far.

In this article, we show for the first time the cleavage of galectin-3 by MMPs in vivo and its colocalization with active MMPs. It is expected that this study will assist in monitoring MMP activity in cancer tissues and eventually in predicting the efficacy of MMP inhibitors in vivo. In addition, the approach described here may aid in the diagnosis and prognosis of the diseases involving MMP activity due to its ease of use, reliability, and cost effectiveness.

Materials and Methods

Cell lines, antibodies, and recombinant enzymes. The human breast cancer cell line BT-549 was a gift from Dr. Eric W. Thompson (St. Vincent's Institute of Medical Research and University of Melbourne, Melbourne, Australia). The cells were maintained in DMEM (Invitrogen Corporation) containing 10% FCS, essential and nonessential amino acids (Invitrogen), vitamins, and antibiotics (Mediatech Cellgro, Inc.). 11-9-1-4 is a clone obtained by the transfection of wild-type galectin-3 in BT-549 as described earlier (24). MCF10DCIS.com cells were developed at the Karmanos Cancer Institute (25) and maintained in DMEM/F12 (1:1) with 5% horse serum, 0.029 mol/L sodium bicarbonate, and 10 mmol/L HEPES. All cells were maintained in a humidified chamber with 95% air and 5% CO2 at 37°C. The cells were grown to near confluence and detached from the monolayer with 0.25% trypsin and 2 mmol/L EDTA for 1 to 2 min at 37°C. The use of cell lines was approved by the Human Investigation Committee, Wayne State University, Detroit, MI. A monoclonal antibody specific for full-length galectin-3 was isolated from the hybridoma TIB166 clone (American Type Culture Collection). A custom-made polyclonal antibody (anti-hL31) was prepared against the whole molecule, which recognized the full-length as well as fragments of galectin-3 (19, 26). Human recombinant pro–MMP-2 and pro–MMP-9 were expressed in HEla cells infected with the appropriate recombinant vaccinia viruses and purified to homogeneity, as previously described (27). The zymogens were activated by incubation with 1 mmol/L p-aminophenylmercuric acetate in a buffer containing 0.02% Brij-35, 5 mmol/L Tris-HCl (pH 7.5), 0.15 mmol/L NaCl, and 5 mmol/L CaCl2 at 37°C for 30 min. Anti-CD34 antibodies were from Cell Sciences (Canton, MA). anti-MMP-9 and anti–MMP-2 antibodies were from Oncogene.

Site-directed mutagenesis. To generate various point mutations on galectin-3 human cDNA, Quick Change Site-Directed Mutagenesis kit (Stratagene) was used by using the primer pairs sense 5′-CTGGTTGGGAG-GGGTCAAGCAG-3′ and antisense 5′-CCTGGTAGCCCTCCCCCAG-CAG-3′ for A33G; and sense 5′-GGCTACTAGGCAGGCTGCAG-3′ and antisense 5′-GCCTAGCTAGGGTGAACGCCC-3′ for H64P. Briefly, pGEX-6P-2 vector containing human wild-type galectin-3 cDNA fused with glutathione S-transferase (GST) was used as a template for PCR to generate A33G and H64P point mutations. After amplification, the template DNA was cleaved with Dpn-1 restriction enzyme and transformed into Escherichia coli XL1-Blue supercompetent cells. Recombinant pGEX-6P-2 /gal-3 mutant plasmids were purified and sequenced at the Macromolecular Core Facility of Wayne State University. Double mutations including both A33G and H64P were generated using the A64P primer pair on plasmid containing the A33G mutation.

Protein purification and cleavage by MMP-2 and MMP-9. The mutant and wild-type galectin-3 proteins were isolated as GST fusion proteins using the manufacturer's instructions (GE Healthcare Biosciences Corp.). Briefly, E. coli containing the desired plasmid was grown to log phase, and protein expression was induced by adding 0.1 mmol/L isopropylthio-β-d-galactopyranoside. After 4 h, the bacteria were centrifuged and the pellet was sonicated in 1× PBS. After solubilization of the proteins with 1% Triton X-100, the extract was centrifuged and the supernatant was incubated with slurry of glutathione Sepharose 4B with gentle agitation to bind the fusion protein to the slurry. Galectin-3 was separated from the fusion protein by incubation with PreScission Protease (GE Healthcare Biosciences Corp.) and isolated by centrifugation.

The purified protein was incubated with activated recombinant MMP-2 and MMP-9 at a molar ratio of 1:10 for 30 min, separated on a 12.5% polyacrylamide gel, and analyzed by Western blot analysis using polyclonal anti–galectin-3 antibodies to detect full-length as well as cleaved fractions of galectin-3.

Stable transfection of galectin-3 mutants. To analyze the biological significance of these substitutions in the context of galectin-3–mediated functions, the coding sequence of galectin-3 containing mutations at A33G, H64P, and A33G/H64P was removed from the pGEX-6P-2 vector by restriction digestion and placed into pCNC10 expression plasmid containing the cytomegalovirus early promoter and a dominant selection marker, G418 (24). The orientation of the insert was determined by restriction mapping and transfection in the non–galectin-3–expressing nontumorigenic breast cancer cell line BT-499 with either the control (pCNC10 vector) or the pCNC10-mutated galectin-3 construct by Lipofectamin reagent (Life Technologies, Inc.) according to the manufacturer's protocol. After 48 h, 500 µg/mL G418 (Invitrogen) were added to the cultures for 14 days to obtain stable transfected clones. Single cell clones were expanded, and galectin-3 expression was determined by Western blot analysis. From each transfection, the clone with highest galectin-3 expression was selected. The resulting clones were given the nomenclature of M33, M64, M33+64, vector, and 11-9-1-4 for A33G, H64P, A33G/H64P vector alone, and wild-type galectin-3 transfections, respectively.

Western blot analysis. Cells (1×106) were suspended in 100-mm Petri dishes. The cells were trypsinized, lysed, and equivalent numbers of cells (1×106) or equal amounts of total protein were subjected to SDS-PAGE and Western blot analysis with a 1:500 dilution of TIB166 or 1:2,000 dilution of anti–galectin-3 polyclonal antibody. Blots were also immunoreacted with a 1:5,000 dilution of antibunbin mouse polyclonal antibody (Santa Cruz Biotechnology, Inc.) to normalize for protein loading.

Tumor growth in nude mice. Cells (2×106) suspended in Matrigel were injected into NCR nu/nu mice, obtained from Taconic into the mammary fat pad region s.c. on both sides in two groups of 6 mice each, respectively. Tumor growth was measured twice a week, and the tumor volumes were calculated using the following formula: volume = length × width/2. The xenografts were harvested at 35 or 56 days as described. The tumors were weighed, fixed with 10% buffered formalin, and processed for immunohistochemical staining. As the BT-549 cells transfected with galectin-3 formed undifferentiated tumors in nude mice, MCF10DCIS.com xenografts and human in ductal carcinoma in situ (DCIS) were used to analyze if differential distribution of galectin-3 could also be detected in differentiated tumors. The DCIS xenografts were obtained similarly by injecting MCF10DCIS.com cells (25, 28). After 28 days, the xenografts were harvested, and half of the tumor was fixed with buffered formalin whereas the other half was fixed with 2-methylbutane in liquid nitrogen. The human DCIS serial sections were obtained from Karmanos
Cancer Institute tissue core. The infiltrating ductal carcinoma sections were part of a breast cancer progression tissue array (BR480) from U.S. Biomax. The animal experiments were performed according to the guidelines provided by the Animal Investigation Committee, Wayne State University.

**Immunohistochemical analysis.** Four-micrometer tissue sections were deparaffinized, rehydrated, and microwaved on high twice for 5 min each in 1 mMol/L sodium citrate buffer (pH 6.0). The sections were washed thrice in PBS and blocked with Super Block (Skytek Laboratories) for 10 min. Sequential sections were incubated with primary antibodies (anti-CD34, anti-galectin-3 monoclonal, anti–galectin-3 polyclonal, and anti–MMP-2 and anti–MMP-9) at 4°C overnight at the suitable dilution. The sections were washed thrice for 10 min each in PBS and linked with the appropriate host secondary antibodies (Vector Laboratories). The secondary antibodies were tagged with Avidin-biotinylated horseradish peroxidase, colorized with 3’3’-diaminobenzidine, and counterstained with hematoxylin. Visualization and documentation were accomplished with an OLYMPUS BX40 microscope supporting a Sony DXC-979MD 3CCD video camera and stored with the M5+ microcomputer imaging device (Interfocus).

**In situ zymography.** In situ zymography was performed on the fresh frozen DCIS xenografts as described by Mook et al. (29). In brief, 8-μm-thick cryosections were air dried, rehydrated with PBS for 5 min, and overlaid with a solution of 50 μg/ml fluorescein-labeled gelatin (DQ gelatin; Molecular Probes), 1% w/v low-melting-temperature agarose (BioWhittaker Molecular Applications), and 5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) in PBS in the presence or absence of 2 mmol/L EDTA, and incubated on ice for 15 min followed by incubation in a humidified chamber at 37°C for 2 h. Protease-catalyzed hydrolysis of the heavily labeled and totally quenched DQ Gelatin releases the intramolecular cross-linking, yielding brightly fluorescent peptide, which was visualized by using imaging microscope.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay.** Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was performed to visualize the fragmented DNA directly by fluorescence microscopy in paraffin-embedded sections using DeadEnd Fluorometric TUNEL system (Promega). Briefly, the paraffin sections were deparaffinized and permeabilized with proteinase K. Fluorescein 12-dUTP was then catalytically incorporated into the 3’-hydroxyl ends, which are exposed in fragmented DNA of the apoptotic cells using the enzyme TdT. The sections were then counterstained with propidium iodide, which, in contrast to fluorescein-12-UTP, stains both apoptotic and nonapoptotic cells.

**Statistical analysis.** The experiments conducted to measure growth of the tumors were repeated twice with multiple animals. We used one-way ANOVA with Tukey’s multiple comparison test to calculate the statistical significance when the number of readings was the same and Dunnett’s multiple comparison test using Prism software in the experiments where the number of readings was not the same. All statistical tests were two sided, and P values of <0.05 were considered statistically significant.

**Results**

**Cleavage of galectin-3 mutants by MMP-2 and MMP-9.** First, we identified the amino acids that were important in determining the susceptibility of galectin-3 to MMPs cleavage and mutated them. Five galectin-3 mutants A63G, A62V, Y63H, Y63P, and H64P were generated by amino acid substitution around the MMP cleavage site. Only the two mutants, Y63P and H64P, showed resistance to cleavage at the Ala62-Tyr63 site, while exposing a new cleavage site producing a ~27-kDa polypeptide. NH2-terminal sequencing revealed that the 27-kDa product displays an NH2 terminus starting with Ala63, indicating that the alternate cleavage site occurs at the Gly52-Ala53 peptide bond. We performed amino acid substitutions at this new site and obtained clones displaying A33G or H64P substitutions alone or in combination. Recombinant proteins were incubated with purified active MMP-2 or MMP-9 for 15 and 60 min, respectively (Fig. 1). Wild-type (WT) and A33G galectin-3 produced a polypeptide of ~22 kDa, consistent with cleavage at Ala62-Tyr63. The H64P mutant produced a ~27-kDa polypeptide upon incubation with MMP-2, indicating that it is cleaved at Gly52-Ala53. Interestingly, MMP-9 did not cleave the H64P mutant. Double mutation at A33G and H64P showed complete resistance to cleavage by both MMP-2 and MMP-9. The cleavage products were not seen when galectin-3 was incubated alone or with pro-MMPs.

**Expression of galectin-3 in the mutant clones.** Galectin-3 was detected in the total cell lysates (Fig. 2A) of all the BT-549 clones transfected with wild-type and mutant galectin-3. Galectin-3 expression was also seen in the nucleus and cell surface (data not shown). However, secretion of full-length as well as the ~27-kDa polypeptide was detected only in 11-9-1-4 (Fig. 2A).

**Tumorigenicity of mutant clones.** Cells (2 × 106) from each clone were injected in the mammary fat pad region of nude mice to study the effect of galectin-3 mutation on tumor take and growth. By 5 weeks, there was ~60% tumor take in the wild-type–transfected cell clone (11-9-1-4) and only 10%, 3%, 6%, and 23% in M33-, M64-, M33+64-, and vector-transfected cells, respectively (average of two experiments). Average tumor volume in tumor-bearing mice of the representative experiment through day 35 is depicted in Fig. 2B. Due to increased tumor burden, mice injected with 11-9-1-4 cells were sacrificed after 5 weeks; other groups were sacrificed after 8 weeks. The average tumor weight at the time of sacrifice in 11-9-1-4 was 1.7 ± 1.44 g and 0.7 ± 0.07, 0.6 ± 1.13, 0.05 ± 0.03, and 0.5 ± 0.98 g with P values of 0.020, 0.018, 0.0006, and 0.011, respectively, for vector, M33, M64, and M33+64.

**Figure 1.** Cleavage of recombinant galectin-3 by MMP-2 and MMP-9. One microgram of protein was incubated at 37°C for the indicated time with activated or proenzyme and separated on a 15% polyacrylamide gel.
Immunohistochemical analysis of xenografts. Tumor specimens were sectioned and stained with anti-CD34 antibody to visualize angiogenesis (Fig. 3A). The 5-week tumor of the 11-9-1-4 cell clone depicted many fully formed blood vessels with lumens (top right), whereas vector and M33+64 mutant cells' tumor of 8 weeks showed a few endothelial precursor cells (fibrocytes) that were positive for CD34, indicating the slow initiation of angiogenesis (top left and bottom right). No tumors could be obtained from M64 mutant cell clone. No blood vessels or precursor cells were seen in M33 xenograft even after 8 weeks (bottom left).

The xenografts obtained from vector alone, M33, and M33+64 mutant cell clones (Fig. 3B, top left, bottom right, and bottom left) showed a very high incidence of apoptosis using TUNEL assay, whereas no significant apoptosis could be detected in BT-549 wild-type 11-9-1-4 tumors (Fig. 3B, top left).

Galectin-3 cleavage as surrogate marker for MMP activity in tumor xenografts. Anti-MMP-2/9 and anti–galectin-3 monoclonal and polyclonal antibodies were used to visualize whether cleaved galectin-3 could be identified in the xenografts (Fig. 4). In 11-9-1-4 xenograft, full-length galectin-3 (using monoclonal antibody) is present in tumor cells localized in the periphery of the tumor mass (arrow), whereas cleaved galectin-3 (using polyclonal antibody) as well as MMP-9 are localized in tumor cells present in the center of the xenograft (arrowheads). M33+64 xenograft, on the other hand, did not show differences in the distribution of galectin-3 using either monoclonal or polyclonal antibody. Because the mutant galectin-3 is resistant to cleavage, there was no indication of its cleavage despite of the presence of MMP-9 throughout the section. MMP-2 also showed a staining pattern similar to MMP-9 (data not shown). Immunostaining with mouse, rat, and rabbit IgG as negative controls for MMP-2/MMP-9, monoclonal, and polyclonal galectin-3, respectively, did not show any staining (data not shown).

Galectin-3 cleavage as surrogate marker for MMP activity in DCIS xenografts and human breast cancer tissue. In DCIS xenografts, intact galectin-3 was localized mainly in the cytoplasm of epithelial cells in focally intense clusters (Fig. 5, top left) as recognized by the monoclonal antibody. Some cells in the stroma
also stained positive with this antibody (arrow). Cleaved galectin-3 (top right) and MMP-9 (bottom left) was distributed throughout the ducts and the stroma. *In situ* zymography on fresh frozen DCIS xenograft sections showed no gelatinolytic activity in the epithelial ducts, whereas positive activity was seen in the stroma (Fig. 5, bottom middle), which was abrogated when incubated with EDTA, a nonspecific MMP inhibitor (bottom right).

In the human DCIS, full-length galectin-3 was more intensely localized toward the luminal side, and many cells on the stromal side of the duct were devoid of the protein (Fig. 6A, left). Cleaved protein (middle) and MMP-9 (right) were distributed uniformly in all the cells, indicating that many cells adjacent to stroma have cleaved galectin-3 and active MMP-2 and MMP-9. In the infiltrating ductal carcinoma, the presence of full-length galectin-3 was seen in the ductules that still maintained a normal morphology (Fig. 6B, left) and a positive reactivity for the cleaved galectin-3 in normal ductules as well in the invasive cells and stroma (middle). MMP-9, on the other hand, was not expressed in the normal ductules but localized in the stroma and clusters of invasive cells (colocalizing with the cleaved galectin-3; right), implying that it is in the active form.

**Discussion**

The strong causal relationship between MMPs overexpression and a wide range of tumorigenic events, including early carcinogenesis, tumor growth, tumor invasion, angiogenesis, and metastasis, makes them attractive therapeutic targets. Consequently,
several broad range inhibitors (MMPI) advanced to phase III clinical trials in patients with advanced cancer. Unfortunately, the trials failed to reach their end points of increased survival (5, 30, 31), probably due to lack of adequate target validation and identification of in vivo substrate(s), among other factors. Indeed, to date, no diagnostic marker is available to distinguish between latent and active MMP(s) to monitor the response of tumor MMP response to treatment. Our results show that cleaved galectin-3 colocalized with active MMP-2 and MMP-9 and, therefore, could be used as a novel diagnostic marker for MMP activity. In paraffin-embedded DCIS.com xenograft, epithelial cells stained for intact galectin-3, whereas the stromal cells stained only for cleaved galectin-3, where it is localized after its secretion and cleavage. This hypothesis was validated using in situ zymography on fresh frozen tissue. Whereas total MMP-2/9 showed positive staining in epithelial and stromal cells, the active MMPs were identified only in the stroma by in situ zymography and colocalized with cleaved galectin-3, attesting to the validity of the use of galectin-3 cleavage as a surrogate marker for the activities of MMPs. The localization of MMP activity in the stroma of the xenografts confirms the earlier observation of Stuelton et al. (17) in CA1A xenografts and cocultures of fibroblasts and CA1A cells. The differential staining of full-length versus cleaved galectin-3 could also predict the activity of MMP in human DCIS and infiltrating ductal carcinoma. Many cells adjacent to the stroma exhibited the presence of cleaved galectin-3 and probably active MMP-2 and MMP-9 in DCIS, indicating their possible invasive phenotype. Consistent with this observation, the invasive cell clusters and stroma were positive for cleaved galectin-3 and active MMP-9 in the infiltrating ductal carcinoma, whereas the ductules that maintained a normal morphology expressed only full-length protein detected by monoclonal as well polyclonal anti–galectin-3 antibodies.

The diverse effects of MMP-2 and MMP-9 cleavage on many proteins have been reported, e.g., MMP-9 cleaves the proangiogenic cytokine interleukin-8, increasing its activity 10-fold, as well as degrading and inactivating the angiogenic inhibitor platelet factor-4 (32). On the other hand, MMP-2 cleaves the fibroblast growth factor (FGF) receptor 1 (FGFR1), releasing the soluble ectodomain of FGFR1 that can still bind FGF's but lacks signaling capacity (33). Proteolytic processing of some ECM substrates, such as laminin 5, exposes cryptic epitopes (34, 35) and new molecules with properties that are distinct from their precursor protein (36). In vitro cleavage of galectin-3 by MMP-2/MMP-9 resulting in a ~22-kDa product has been reported, but its occurrence in vivo and its biological significance have yet to be elucidated.

It was reported that loss of its NH2-terminus 62 amino acids leads to increased binding of the ~22-kDa fragment to endothelial cells (23) and laminin (26). Injection of the cleaved galectin-3 peptide containing 108 to 250 amino acids into mice bearing MDA-MB-435 tumors resulted in loss of tumor growth and metastasis because of competitive inhibition of carbohydrate binding (37). It was suggested that loss of the NH2 terminus reduces self-association of galectin-3 and, thereby, abrogates the biological properties dependent on such association e.g., formation of tumor cell emboli in vivo and hemagglutination in vitro (26). Yang et al. (38), however, showed that only the COOH terminus of galectin-3 could self-associate in the absence of its saccharide ligand, raising more questions about the role of the collagen-like domain in galectin-3 and how its interaction

Figure 5. Distribution of full-length and cleaved galectin-3 in MCF10DCIS.com xenograft. A, paraffin-embedded; B, fresh-frozen. A, monoclonal anti–galectin-3 antibody (left) shows mainly epithelial and some stromal labeling (arrows); middle, polyclonal anti–galectin-3 antibody shows some epithelial (arrow) and strong stromal labeling (wider arrows); right, anti–MMP-9 antibody showing epithelial (arrow) and strong stromal (wider arrow) labeling. B, in situ zymography in the fresh-frozen section (left) showing stromal activity (green), nuclei were stained with DAPI (blue); right, in situ zymography in the presence of 20 mmol/L EDTA. Bar, 100 μm.
with MMP-2/9 affects biological functions of galectin-3. To answer these questions, we created cleavage-resistant galectin-3. The mutations rendered the recombinant protein resistant to cleavage at that particular site but the protein cleaved at the remaining site.

Single or double mutations at the MMP cleavage sites did not alter cellular distribution of galectin-3 but inhibited its secretion. Intracellular galectin-3 regulates pathways including mRNA splicing reactions, cell growth, cell cycle, and apoptosis ([38–40]; Figure 6).

**Figure 6.** Distribution of full-length and cleaved galectin-3 in human breast cancer. A, DCIS; B, infiltrating ductal carcinoma. In human DCIS (A), full-length galectin-3 is concentrated more toward the lumen of the duct; many cells toward the stromal end are devoid of the protein (mAb; arrow). The cleaved galectin-3 fragments (pAb) as well as MMP-9 could be seen in all cells. In infiltrating ductal carcinoma (B), full-length galectin-3 is seen in ductules that maintained a normal morphology (arrow) and in some invasive cells (mAb); cleaved galectin-3 (pAb) and MMP-9 are seen in invasive cell clusters and stroma (arrows). Bottom panels in A and B represent box in the top panels. Bar, 50 (top) and 200 μm (bottom).
reviewed by Liu et al. ([41]), whereas extracellular galectin-3 modulates cellular adhesion and signaling, immune response, angiogenesis, and tumorigenesis (21, 42–46) by binding to cell surface glycoproteins, such as integrin subunits (12, 26) or to ECM glycoproteins, such as laminin, fibronectin, and collagen IV (21, 47).

We have shown earlier that overexpression of galectin-3 in nontumorigenic breast or colon cancer cell lines induced tumorigenicity and metastasis, whereas its suppression resulted in loss of tumorigenicity and metastasis (24, 48, 49). When the clones were injected in nude mice, the wild-type clone showed a rapid increase in tumor volume >35 days, but cleavage-resistant clones also showed many more inability of these cells to induce blood vessel formation. The galectin-3 mutant clones showed lack of angiogenesis and induction of apoptosis in the xenografts. It was proposed earlier that secreted galectin-3 binds to cell surface receptors on endothelial cells, inducing their migration and morphogenesis leading to angiogenesis (50). Absence of secreted protein in the mutated clones may be the reason for inability of these cells to induce blood vessel formation. The xenografts from cleavage-resistant cells also showed many more apoptotic cells compared with BT-549 11-9-1-4 cells. Although the cellular localization of galectin-3 was not affected in mutant clones, on the cell surface however, the mutant proteins could not be cleaved and affect processes such as chemoinvasion, chemotaxis, tumor growth, and angiogenesis. It is possible that the cleaved fragment is responsible for the tumorigenic potential of the cells. Once the surface protein is cleaved, the carbohydrate-binding domain remains attached to the surface receptor, and the cleaved product is released into the ECM, where it may either interact with other extracellular proteins or may be internalized and interact with various signal transduction pathways.

To summarize, we report here that galectin-3 is cleaved in vivo by MMPs and this phenomenon could be used to distinguish between active and latent MMPs in the tumor, which could affect decision regarding therapeutic strategies and anti-MMP drugs efficacy.

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