Role of Galectin-3 in Breast Cancer Metastasis

Involvement of Nitric Oxide

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We investigated the role of galectin-3 in metastasis of human breast carcinoma BT549 cells using the experimental liver metastasis model. Underlying mechanisms were then elucidated using the liver/tumor coculture and cell culture systems. After intrasplenic injection, galectin-3 cDNA transfected BT549 cells (BT549gal-3 wt) formed metastatic colonies in the liver, while galectin-3 null BT549 cells (BT549par) did not, demonstrating that galectin-3 enhances metastatic potential. More than 90% of BT549gal-3 wt cells survived after 24 hours-co-culture with the liver fragments isolated following ischemia treatment. In contrast, more than half of BT549par cells showed metabolic death following co-culture with the liver fragments. When the liver from inducible nitric oxide synthase (iNOS) knockout mice was used, no cytotoxicity to BT549par cells was observed. Thus, iNOS exerts cytotoxicity on BT549par cells and galectin-3 can protect against iNOS-induced cytotoxicity. BT549gal-3 wt also exhibited enhanced survival against peroxynitrite (up to 400 μmol/L) in vitro. A single mutation in the NWRG motif of galectin-3 obliterated both metastatic capability and cell survival, indicating that the antiapoptotic function of galectin-3 is involved in enhanced metastasis. In conclusion, galectin-3 enhances the metastatic potential of BT549 cells through resistance to the products of iNOS, possibly through its bcl-2-like antiapoptotic function.


Although advances have been made in breast cancer treatment, most deaths of patients are still from metastasis. Therefore, a better understanding of the mechanisms of tumor metastasis is important to develop more effective therapeutic strategies. Metastasis is a complicated process involving invasion into the neighboring tissues, intravasation, arrest within the microcirculation, extravasation, and finally proliferation within the metastatic site. During these processes, the arrest of tumor cells within the microcirculation is made by size restriction.¹ As a result of tumor cell arrest, blood flow stops and the local area suffers from ischemia (12 to 24 hours), followed by reoxygenation. This phenomenon is called an ischemia-reperfusion injury which causes the production of toxic reactive nitrogen and oxygen species such as nitric oxide (NO), superoxide anion (O₂⁻), and the reaction product of NO and O₂⁻, peroxynitrite (ONOO⁻). One of the critical factors for metastasis appears to be the survival of tumor cells against these toxic molecules. Metastatic potential has been shown to correlate with resistance to NO, O₂⁻, and ONOO⁻.² The molecular mechanisms conferring resistance to highly metastatic tumor cells remain unclear.

Galectin-3 is a M₉ 31 kd carbohydrate-binding protein with affinity for β-galactoside.³⁴ This protein is involved in diverse biological processes such as cell-cell and cell-matrix interactions,⁵–⁷ induction of pre-mRNA splicing,⁸ cell proliferation,⁹ cell cycle regulation,⁶,¹⁰ angiogenesis,¹¹ and more importantly, tumorigenesis¹²–¹⁴ and metastasis.⁴,¹² In a variety of primary and metastatic tumor cells, the level of galectin-3 is elevated compared to that of normal tissues,¹² although this has not been a consistent observation.⁴ Recently, it was shown that the metastatic potential of colon cancer cell lines directly correlated with the expression of galectin-3,¹⁵ supporting the role of galectin-3 in enhancing metastasis. However, the mechanisms by which galectin-3 enhances metastasis are yet to be elucidated.

Growing evidence has shown that galectin-3 protects against apoptotic cell death in response to a variety of apoptosis-inducing stimuli including staurosporine, anti-Fas antibody, cisplatin, tumor necrosis factor-α, menadione, and loss of cell adhesion.⁶,⁷,¹⁶–¹⁸ We hypothesized that galectin-3 enhances metastasis of tumor cells by functioning as a survival factor against the cytotoxic reactive nitrogen and oxygen species generated when they are arrested within the microcirculation. In this study, we show that galectin-3 enhances the metastatic potential of human breast carcinoma BT549 cells using the experimental liver metastatic model. We also show that galectin-3 protects BT549 cells from the cytotoxic effect of the liver fragments previously exposed to ischemia treatment in a co-culture system, as well as of peroxynitrite treat-
ment in cell culture. A single amino acid mutation in the NWGR motif obliterates the metastatic potential enhancement and cytoprotective effect of galectin-3.

Materials and Methods

Cell Culture

The human breast carcinoma BT549 (BT549gal-3wt) and its stable clones expressing the human galectin-3 proteins, wild-type (BT549gal-3wt, clone 11914, reference 13) and mutant (BT549gal-3mt, clone 5, reference 17), were obtained from Dr. A. Raz (Wayne State University, Detroit, MI). BT549gal has a galectin-3 null phenotype and shows nontumorigenic in athymic nude mice. The galectin-3 mutant contains an alanine residue instead of a glycine residue at position 182. All cell lines were grown in Dulbecco’s modified Eagle medium/nutrient mixture F-12 (DMEM/F-12, Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/L L-glutamine in a 95% air and 5% CO₂ incubator at 37°C.

Animals

Athymic nude (Crl:CD-1-nuBR) and C57BL/6 mice (C57BL/6NCrlBR) were purchased from Charles River (Wilmington, MA). Mice deficient in inducible nitric oxide synthase (iNOS) were generated by the gene targeted disruption method as described19 and backcrossed onto the mice C57BL/6 background through ten generations. All mice were female and 10 to 14 weeks old. Animal protocol was approved by Institutional Animal Care and Use Committee (Protocol no. 0101142).

Experimental Liver Metastasis Model

Experimental liver metastasis was performed according to the procedure described previously.20 Briefly, groups of five athymic nude mice were anesthetized by intraperitoneal injection of 2,2,2-tribromoethanol (200 mg/kg) and the abdomen was aseptically opened. The portal and hepatic artery were clamped separately with a hemostat for 3 and 20 minutes, respectively, in athymic nude and C57BL/6 mice.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for iNOS mRNA

Total RNA was isolated from approximately 100 mg of liver tissue using RNA-STAT (Tel-test, Friendswood, TX), treated with 10 U/µl of RNase-free DNase ( Sigma, St. Louis, MO). Reverse transcription of 1 µg of total RNA to cDNA was carried out using a GenAmp RNA PCR Core Kit (Perkin-Elmer, Foster City, CA) in 20 µl volume, according to the manufacturer’s protocol. The final cDNA samples (2 µl) were added into a PCR reaction mixture (48 µl) containing iNOS primers (sense, ACAACACGGAACCTACCAAGCTC; antisense, GATGTTGTACGGCTGTTGT-GTCA). Composition of the reaction mixture was 0.2 µmol/L 5’ primer, 0.2 µmol/L 3’ primer, 50 mmol/L KCl, 10 mmol/L Tris-HCl pH 9.0, 1.5 mmol/L MgCl₂, 200 µmol/L each dNTP, and 0.5 U/reaction Taq DNA polymerase (Perkin Elmer). PCR was performed with 30 cycles (94°C, 1 minute; 60°C, 30 seconds; 72°C, 40 seconds), with initial incubation at 94°C for 3 minutes, and final extension at 72°C for 3 minutes. Ten µl of the amplified products were resolved by 2% agarose gel electrophoresis and visualized with ethidium bromide staining.

Cell Labeling with Fluorescence Dye

For the measurement of cell viability, BT549 cells were labeled with two fluorescing reagents, rhodamine B-isothiocyanate dextran 10s (Rd-Dx; Sigma), with maximum excitation/emission at 530/590 nm, and calcein-AM (Molecular Probes, Eugene, OR), with maximum/emission at 485/530 nm according to the method described previously with slight modifications.2 Briefly, cells (5 × 10⁵) were suspended in 20 mg/ml Rd-Dx in PBS, and received two electrical pulses (capacitance: 330 µF and 300 V; load resistance: high Ω; charge rate: fast) using Celloporator Electroporation System I (BRL, Gaithersburg, MD). The Rd-Dx-loaded cells were incubated for 16 hours at 37°C in complete tissue culture medium, and then adherent cells were recovered by trypsin. The cells were suspended in PBS and incubated with calcein-AM at a final concentration of 4 µmol/L for 30 minutes at 37°C.

Induction of Liver Ischemia

To induce liver ischemia, mice were anesthetized by intraperitoneal injection of 2,2,2-tribromoethanol (200 mg/kg) and the abdomen was aseptically opened. The portal vein and hepatic artery were clamped separately with a hemostat for 3 and 20 minutes, respectively, in athymic nude and C57BL/6 mice.

Mouse Liver/Tumor Cell Co-Culture

The effect of liver tissue on human breast carcinoma cells was studied using a co-culture method.20 Briefly, cells pre-labeled with Rd-Dx and calcein AM were added to a 55-ml rotating wall vessel (RWV, Synthecon, Houston, TX), which had been filled with the co-culture medium (a 1:1 mixture of HepatoZYME-SFM (Life Technologies) and DMEM/F-12). Liver was dissected into 1- to 3-mm fragments, washed once with ice-cold PBS, and then loaded into the RWV. The RWV was connected to a rotator unit equipped with an air pump to provide the RWV chamber with air. The whole co-culture system was placed in a 37°C-incubator with 5% CO₂ and rotated at 15 rpm for 24 hours. Following a period of co-culture, tumor cells were observed under a fluorescence microscope (Nikon Microphot-FXL, Tokyo, Japan).
Survival of BT549 Cells with Peroxynitrite Treatment

BT549 cells were plated and grown for 2 days to obtain 70 to 80% confluency before treating with peroxynitrite (Alexis, San Diego, CA). Cell viability was assessed by a Trypan Blue dye exclusion method. Briefly, both floating and attached cells were collected by centrifugation at 200 g for 3 minutes. Trypan Blue solution (Life Technologies) was added to the cells in equal volume. At least 300 cells were counted using hemacytometer after 15 minutes of incubation at room temperature and survival fraction was calculated.

Results

Galectin-3 Enhances the Metastatic Potential of BT549 Cells

The human breast carcinoma BT549 cell line has been widely used to investigate the function of galectin-3 since it does not express this protein. Introduction of human galectin-3 cDNA into BT549 cells results in acquisition of a tumorigenic phenotype. However, the effect of galectin-3 transfection on metastatic potential has not been tested in the BT549 cell line. Therefore, we investigated whether galectin-3 transfected BT549 cells (BT549gal-3 wt) can produce liver colonization after intrasplenic injection into athymic nude mice. While none of the animals injected with BT549gal-3 mt cells developed tumors in the spleen or liver (Figure 1A, B, E), all of the mice injected with BT549gal-3 wt cells developed tumors in both the spleen and liver (Figure 1, A, D, G). These results indicate that the galectin-3 protein can enhance the metastatic potential of BT549 cells. This agrees with previous results showing that galectin-3 expression correlates with the metastatic potential of human colon cancer cell lines.

Galectin-3 contains a four amino acid motif (NWGR) that is highly conserved in the BH1 domain of the bcl-2 gene family. The BH1 domain is responsible for the antiapoptotic activity of bcl-2 and galectin-3 proteins. We tested whether the NWGR motif plays a role in metastasis. Mice injected with BT549gal-3 mt cells did not develop tumors in either the spleen or the liver (Figure 1, A, C, F). Since the mutant galectin-3 is different from the wild-type protein in only one amino acid residue (glycine to alanine in 182 position) that is located in the NWGR motif, this result points to the importance of the antiapoptotic NWGR motif of galectin-3 in promoting metastasis.

Galectin-3 Inhibits Ischemia-Reperfusion Mediated Cytotoxicity

Based on previous results, we hypothesized that the promotion of metastatic potential in galectin-3 expressing BT549gal-3 wt cells is associated with resistance against the cytotoxic reactive oxygen and nitrogen radicals which are formed during ischemia-reperfusion. To determine the role of galectin-3 in enhancing tumor cell viability during the liver ischemia-reperfusion period, we cultured BT549 cells in a rotating suspension culture system con-
taining the mouse liver fragments with or without ischemia induction. The reperfusion of the liver fragments is ensured by providing air into the culture vessel with positive pressure using the compressor attached to the device. This co-culture system has great advantages in simulating the in vivo experiments, since the architecture and viability of the liver can be maintained for at least 24 hours. 20 To distinguish tumor cells from mouse liver cells, the BT549 cells were dual-labeled with Rd-Dx and calcein-AM. The latter produces a fluorescence signal only in live cells with ATP-dependent cytoplasmic esterase activity.

As shown in Figure 2A, BT549 tumor cells in the same field were observed under the fluorescence microscope. The red-colored Rd-Dx (top panels) and green-colored calcein-AM (bottom panels) serve as the cell and viability marker, respectively. After co-culturing with the normal liver fragments for 24 hours, all three types of the Rd-Dx-labeled BT549 cells showed the green fluorescence of calcein-AM at the same positions, indicating that BT549 cells maintain their metabolic integrity under the co-culture system for at least 24 hours. The overall survival fractions were 85.0% to 96.0% (Figure 2B). When BT549par cells were co-cultured with the liver fragments that had been exposed to ischemia in vivo, more than half of Rd-Dx-labeled cells lost the calcein-AM fluorescence (pointed by the arrowheads in Figure 2A), which means that BT549par cells are killed by the liver fragments stimulated with ischemia. On the other hand, BT549gal-3 wt cells were not killed by the ischemia-stimulated liver fragments. The galectin-3-induced enhanced survival of BT549 cells was completely obliterated with a single mutation in the NWGR motif. These results indicate that galectin-3 may contribute to the survival of BT549gal-3 wt cells in the presence of these toxic chemicals produced by the liver during the ischemia-reperfusion injury, and that the NWGR motif of the protein is involved in the cell survival.

Ischemia-Reperfusion Induces iNOS Gene Expression in the Liver

Jessup and colleagues reported that tumor cells induce ischemia in microscopic regions of the host liver during the first 24 hours after arrest in the hepatic sinusoids. 2 Based on this, we postulated that ischemia induced during tumor cell arrest in hepatic sinusoids leads to the formation of toxic reactive oxygen and nitrogen species by increasing iNOS expression during reoxygenation. Thus, we examined whether ischemia-reperfusion of the liver activates iNOS gene expression. Figure 3 shows that the level of iNOS mRNA was significantly increased in the liver 2 hours after ischemia-reperfusion injury, while iNOS gene expression in the normal liver was not significantly increased. This is similar to our previous studies using a model of partial hepatic ischemia-reperfusion where iNOS mRNA can be detected at 3 hours following reperfusion in the ischemic but not non-ischemic lobe. 22

NO Is Involved in Ischemia-Reperfusion-Mediated Cytotoxicity

To prove the involvement of iNOS in tumor cell death, we adopted the co-culture system using the liver fragments from iNOS knockout (iNOS−/−) mice. As shown in Figure 4A and B, the ischemia-induced liver from iNOS knockout mice did not cause cytotoxicity to BT549par, BT549gal-3 mt, and BT549gal-3 wt cells. These results and Figure 2 indicate that NO plays an important role in ischemia-reperfusion-mediated cytotoxicity. Galectin-3 probably protects tumor cells from NO and/or ONOO−-mediated cytotoxicity during ischemia-reperfusion.

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**Figure 2.** Survival of BT549 tumor cells after the co-culture with the liver fragments from C57BL/6 mice. BT549 cells labeled with the fluorescent dyes were co-cultured with the liver fragments from a control mouse (normal liver) and a mouse induced with liver ischemia (ischemic liver). A: Viability of BT549 cells was determined by observation under a fluorescence microscope after 24 hours. Representative images of BT549 cells were shown. Arrowheads point out the dead cells. B: Survival fractions of BT549 cells are shown as the mean ± SE from the cell counts of 20 fields from two mice.

**Figure 3.** Effect of ischemia-reperfusion on iNOS mRNA expression in nude mice liver. Ethidium bromide stained 2% agarose gel shows RT-PCR products made with RNA from a control liver (normal liver) and a liver after 2 hours of ischemia-reperfusion injury (ischemic liver). Arrows show the locations of iNOS and GAPDH. The GAPDH gene was used as an internal control.
Galectin-3 Inhibits Peroxynitrite-Mediated Cytotoxicity

Although the data presented here and those of others suggest that the reactive oxygen and nitrogen species are responsible for tumor cell death after they arrest in the liver sinusoid and terminal portal vein, the identity of the chemicals that kill the tumor cells is yet to be determined. Peroxynitrite, a product of NO and O$_2$/$H_2O_2$, is thought to contribute to the pathophysiology of many diseases associated with inflammation and ischemia-reperfusion injury. We tested whether galectin-3 can inhibit the ONOO$^-$/H$_2O_2$-mediated cytotoxicity in BT549 cells. Figure 5 shows that the treatment of BT549par and BT549gal-3 mt cells with ONOO$^-$ resulted in cell death in dose- and time-dependent manners, while BT549gal-3 wt cells were resistant to ONOO$^-$. For example, approximately 10% of BT549par and BT549gal-3 mt cells were resistant to ONOO$^-$ for 12 hours, while more than 90% of BT549gal-3 wt cells survived under the same conditions. These results suggest that galectin-3 could protect BT549 cells from cytotoxicity of ONOO$^-$ and the NWGR motif in the COOH-terminus of galectin-3 is involved in its protective mechanism.

Discussion

The goal of the present study was to investigate the underlying mechanisms by which galectin-3 enhances metastasis of human breast cancer cells. Specifically, we studied the role of galectin-3 in enhancing the survival of breast cancer cells against NO and ONOO$^-$ by the experimental hepatic ischemia-reperfusion injury or direct treatment. From the data presented in this report, we can provide some insights to the important questions regarding the pathogenic role of galectin-3 in metastasis. The first and fundamental question would be the correlation between the level of galectin-3 and metastatic potential of tumor cells. Two approaches have been taken to answer this question. The first approach was to compare the level of the galectin-3 protein and mRNA in the normal tissue and tumor with different stages using immunohistochemistry and/or in situ hybridization. Based on this approach, the role of galectin-3 appears to be controversial. Correlation was observed in melanoma, fibrosarcoma, and prostate carcinoma, and some hematopoietic malignancies, whereas inverse correlation was observed in thyroid and breast carcinoma. Furthermore, results in colon carcinoma have not been consistent. The second approach employs the experimental liver metastasis model used in this study. Bresalier and colleagues have previously reported correlation between the level of galectin-3 and metastatic potential using human colon cancer cell lines. According to this report, the down-regulation of galectin-3 using galectin-3 cDNA in antisense orientation results in marked decrease in the metastatic...
potential of highly metastatic HM7 cells, while the up-regulation of this protein enhances the metastatic potentials of low metastatic LS174T cells. Data from our study also shows similar results using a human breast carcinoma cell line (Figure 1). While these data provide direct evidence for the role of galectin-3 in metastasis, it is still premature to generalize on the importance of galectin-3 in metastasis of all types of tumor cells. The conflicting evidence suggests that the role of galectin-3 in metastasis might be tissue or cell type-dependent.

The next question addressed in this study is the underlying mechanisms by which galectin-3 enhances the metastatic potential of tumor cells. Our results show that galectin-3 enhances survival of BT549 cells and protects BT549 cells from toxicity associated with iNOS expression and ONOO⁻ (Figures 2, 4, 5). Previous studies demonstrated that hepatocytes and Kupffer cells produce the reactive nitrogen and oxygen species during hepatic ischemia and reperfusion. This oxidative stress probably leads to the apoptotic death of breast tumor cells by activating caspases. Recently, Wang and colleagues reported evidence that NO is a natural anti-metastatic molecule. In that study, the production of NO in the liver was observed immediately after injection of B16F1 melanoma cells via the portal vein. The injected tumor cells underwent apoptotic death within 4 to 24 hours and NO production and apoptotic death was blocked by the iNOS inhibitor, N₃-nitro-L-arginine methyl ester. However, NO can be either anti- or pro-apoptotic. Our previous data indicates that NO inhibits apoptosis in hepatocytes by suppressing caspase activity and activation. Interestingly, iNOS which can be induced by ischemia-reperfusion (Figure 3 and Reference 22) can induce hepatic injury as shown in studies comparing iNOS +/+ and iNOS −/− mice. This NO may either protect or injure even liver cells depending on the presence of oxidant such as O₂⁻. In general, it is thought that hepatocytes are resistant to NO, while tumor cells are sensitive to NO. These controversial functions of NO are beginning to be understood by a recent study, which shows that non-heme iron content in the cells is probably a key factor in determining the effect of NO on cell viability by regulating the chemical fate of NO. This is why hepatocytes that contain high levels of non-heme iron are resistant to NO toxicity. Data from this study shows that BT549gal-3 wt cells are resistant to the ischemia-induced liver fragments (Figure 2) suggesting that galectin-3 may enhance the metastatic potential of BT549 cells by protecting them from reactive nitrogen and oxygen species generated during the arrest of tumor cells in the hepatic microvasculature. Although the exact nature of the toxic chemicals that directly kill the tumor cells is still unclear, several evidences show that both NO and O₂⁻ are important in cytotoxicity of tumor cells. These reports showed that the cytotoxic effect of the ischemia-induced liver fragments and the hepatic sinusoidal endothelial cells was abolished in the presence of either N₃, monomethyl-L-arginine (NMMA, an iNOS inhibitor) or superoxide dismutase (a scavenger of superoxide anions), suggesting that the direct cytotoxic chemical may be ONOO⁻, an adduct of NO and O₂⁻. In this study, we demonstrate that galectin-3 transfected BT549gal-3 wt cells become resistant to peroxynitrite treatment (Figure 5). These results indicate that galectin-3 protects cells from peroxynitrite-induced cytotoxicity.

Finally, a question would arise about the mechanisms by which galectin-3 protects BT549 cells from the cytotoxicity mediated by reactive nitrogen and oxygen species. A possible protection mechanism has been suggested to be the inhibition of apoptosis induced by loss of cell adhesion by galectin-3. Our data shows that a single point mutation in the NWGR motif of galectin-3 results in the abrogation of its protective effect from the ischemia-induced liver (Figure 2), as well as ONOO⁻ treatment in cell culture (Figure 5). Furthermore, BT549gal-3 wt cells also lost tumorigenicity and metastatic potential in the experimental liver metastasis model (Figure 1). Since the NWGR motif of galectin-3 is critical for anti-apoptotic activity, these results suggest that the protective mechanisms might involve inhibition of apoptosis on NO and/or ONOO⁻, and this anti-apoptotic function directly affects the metastatic potential of BT549 cells. Presently, the detailed mechanism by which galectin-3 inhibits apoptosis of BT549 cells against these chemicals is yet to be elucidated. Using other apoptosis-inducing reagents such as cisplatin, TNF-α, and menadione, however, it has been shown that galectin-3 protects cells from apoptotic death by inhibiting the caspase pathway or by maintaining mitochondrial homeostasis.

In conclusion, we provide evidence that galectin-3 enhances the metastatic potential of human breast carcinoma BT549 cells by increasing resistance to the reactive nitrogen and oxygen species, such as NO and ONOO⁻, most likely through the bcl-2-like antiapoptotic function of galectin-3. Although we are far from understanding how galectin-3 protects cells from peroxynitrite-induced cytotoxicity and ischemia-reperfusion injury, this model will provide a framework for future studies.

References