Galectin-3 Protects Human Breast Carcinoma Cells against Nitric Oxide-Induced Apoptosis

Implication of Galectin-3 Function during Metastasis

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Galectin-3 is a β-galactoside-binding protein which regulates many biological processes including cell adhesion, migration, cell growth, tumor progression, metastasis, and apoptosis. Although the exact function of galectin-3 in cancer development is unclear, galectin-3 expression is associated with neoplastic progression and metastatic potential. Since studies have suggested that tumor cell survival in microcirculation determines the metastatic outcome, we examined the effect of galectin-3 overexpression in human breast carcinoma cell survival using the liver ischemia/reperfusion metastasis model. While the majority of control cells died by hepatic ischemia/reoxygenation, nearly all of galectin-3 overexpressing cells survived. We showed that galectin-3 inhibits nitrogen free radical-mediated apoptosis, one of the major death pathways induced during hepatic ischemia/reperfusion. Galectin-3 inhibition of apoptosis involved protection of mitochondrial integrity, inhibition of cytochrome c release and caspase activation. Taking these results together with the previous observation that galectin-3 inhibits apoptosis induced by loss of cell adhesion, we propose that galectin-3 is a critical determinant for anchorage-independent and free radical-resistant cell survival during metastasis. *(Am J Pathol 2001, 159:1055–1060)*

Tumor cell metastasis is a complex process involving motility, invasion, and cell growth and survival. It was observed that the ability of tumor cells to colonize the liver was shown to be proportional to the number of tumor cells surviving within the sinusoid.[1,2] As tumor cells arrest in the hepatic sinusoid and terminal portal venules, microscopic infarcts develop, followed by re-establishment of blood flow. Ischemia/reperfusion results in oxygen and nitrogen radical formation that is toxic to the majority of the arrested tumor cells.[3,4] However, the survival rate of highly metastatic human carcinoma cells is 10-fold higher than that of weakly metastatic cells during hepatic ischemia and reperfusion.[3,4] Presently, the molecular mechanisms by which metastatic tumor cells become resistant to oxygen and nitrogen radicals during hepatic ischemia/reperfusion remain unclear.

Galectin-3 is a 30-kd protein member of the β-galactoside-binding family that is highly expressed in various human tumor cells.[5–9] *In vitro*, galectin-3 modulates a variety of biological processes such as cell adhesion, migration, cyst formation, secretion of cytokines, and pre-mRNA splicing.[10–15] Although the exact function of galectin-3 in cancer development is unclear, its expression is associated with neoplastic progression and metastatic potential[5–9] in head and neck,[10] thyroid,[16] gastric,[6] and colon cancers.[17]

Recently, we and others showed that galectin-3 protects cells against apoptosis.[18–21] Galectin-3 inhibits breast epithelial cell apoptosis induced by a variety of stimuli including staurosporine, cisplatin, and loss of cell adhesion. Similarly, galectin-3 prevents T-cell apoptosis induced by anti-Fas-antibody.[18] Inflammatory cells from galectin-3 deficient-mice were more prone to undergo apoptosis, re-emphasizing a galectin-3 role for apoptosis regulation.[22] In this study, we questioned whether galectin-3 protects tumor cells from oxidative stress-induced apoptosis during metastasis. To test this, we used a mouse ischemic liver tissue/human breast tumor cell co-culture system to examine whether galectin-3 protects human breast carcinoma cells from death during hepatic ischemia-reperfusion. We also investigated the effect of galectin-3 on nitric oxide-induced apoptosis, one of the

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major death pathways induced during hepatic ischemia-reperfusion.

Materials and Methods

Cell Culture

The human breast carcinoma cell line BT549 was obtained from Dr. E. W. Thompson, Vincent T. Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, DC. Galectin-3-transfected BT549 cell clones were previously established by introducing an expression vector containing human galectin 3 cDNA into BT549 parental cells.[19,20] Galectin-3-transfected BT549 and neo-resistant control vector-transfected BT549 cells are referred to as BT549Gal-3 and BT549neo, respectively. Cells on tissue culture dishes (Sarstedt, Newton, NC) were grown in DMEM/F-12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L L-glutamine and 0.5 μg/ml fungizone in a 95% air and 5% CO_2 incubator at 37°C.

Cell Labeling with Rd-Dx and Calcein AM

BT549Gal-3 and BT549neo cells were labeled with two fluorescing reagents, rhodamine B-isothiocyanate dextran (Rd-Dx; Sigma Chemical Co., St. Louis, MO), with maximum excitation/emission at 530/590 nm, and calcein AM, with maximum/emission at 485/530 nm. Rd-Dx was loaded into BT549 cells by electroporation (Cell-Porator, BRL, Gaithersburg, MD). Cells (5×10^6), suspended in 20 mg/ml Rd-Dx in phosphate-buffered saline (PBS) solution, received an electrical pulse (capacitance: 330 μF and 300 V; load resistance: high Ω; charge rate: fast). 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 (Molecular Probes, Eugene, OR) at a final concentration of 4 μmol/L for 30 minutes at 37°C.

Liver-BT549 Co-Culture

Athymic nude mice purchased from Harlan Sprague-Dawley Inc. (Frederick, MD) were anesthetized with a general inhalant as previously described.[4] A midline laparotomy incision was made under aseptic conditions to expose the liver. To induce ischemia, the portal vein and hepatic artery were clamped with a microvascular clamp for 3 minutes. The liver was excised and immediately placed in chilled DMEM/F-12 on ice and dissected into 1 to 3 mm fragments. The liver fragments were transferred into a 50-ml centrifuge tube and washed once by centrifugation at 400 $\times$ g for 5 minutes. The fragments were resuspended in 5 ml of the co-culture medium and loaded into a 55-ml Rotating Wall Vessel (RWV; Rotary Cell Culture System, Synthecon, Inc., Houston, TX). BT549 cells pre-labeled with Rd-Dx and calcein AM were then added to the RWV, and the 55-ml RWV chamber was completely filled with the liver fragment co-culture medium [a 1:1 mixture of HepatoZYME-SFM and DMEM/F-12 with 10% FBS and penicillin-streptomycin] to eliminate bubbles. An air pump was connected to the RWV for reoxygenation after liver ischemia. After co-culture, Rd-Dx and calcein AM-labeled cells were detected on a Nikon upright microscope (Nikon Microphot-FXL, Tokyo, Japan) equipped with epi-fluorescence using digital image capture by an 3CCD color video camera (Model DXC-930P, Sony, Tokyo, Japan) .

Mitochondria Staining

Cells were plated on a coverslip in a six-well plate. After 24 hours of apoptosis induction, the cells were incubated with media containing 250 μmol/L MitoTracker Red (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 15 minutes at 37°C. The coverslips were mounted onto glass plates using 0.1% phenylenediamine and 90% glycerol in PBS. Fluorescent staining of the mitochondrial membrane was examined with a Nikon Labophot microscope fitted with a digital video camera (Photometrics, Tucson, AZ).

Cytochrome c Release

Cells were harvested at 0, 24, and 48 hours following treatment with 200 μmol/L S-nitroso-*N*-acetylpenicillamine (SNAP; Sigma), resuspended in ice-cold lysis buffer (20 mmol/L Hepes-KOH, pH 7.5, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L sodium EDTA, 1 mmol/L sodium EGTA, 20 μl protease inhibitors (Sigma), 4 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride) containing 250 mmol/L sucrose, and incubated for 1 hour at 4°C. The lysates were passed through a 26 gauge syringe 15 times and then centrifuged at 10,000 rpm for 20 minutes at 4°C. The resulting supernatant was analyzed by immunoblot analysis using anti-cytochrome *c* antibody (ZYMED Laboratories Inc., CA) as previously described.[23]

DEVDase Activity

Cells were collected at 0, 24, 36, 48 and 60 hours after treatment with 200 μmol/L SNAP and lysed in caspase lysis buffer [50 mmol/L Tris buffer (pH 7.5), 0.03% Nonidet and 1 mmol/L DTT]. Nuclei were removed by low speed centrifugation (800 $\times$ g, 5 minutes), and the cytosolic fraction was incubated with 40 μmol/L DEVD-amc, 10 mmol/L HEPES (pH 7.5), 50 mmol/L NaCl, and 2.5 mmol/L DTT for 2 hours at 37°C. Fluromethylcoumarin fluorescence, released by DEVDase (caspase) activity, was measured using 380 nm excitation. A CCD device (Instapec IV; Oriel, Straford, CT) fitted with a monochromator was used to measure the fluorescence emission spectrum. The intensity at the optimum (~450 nm) was measured. DEVDase activity was normalized per micro-

(A)
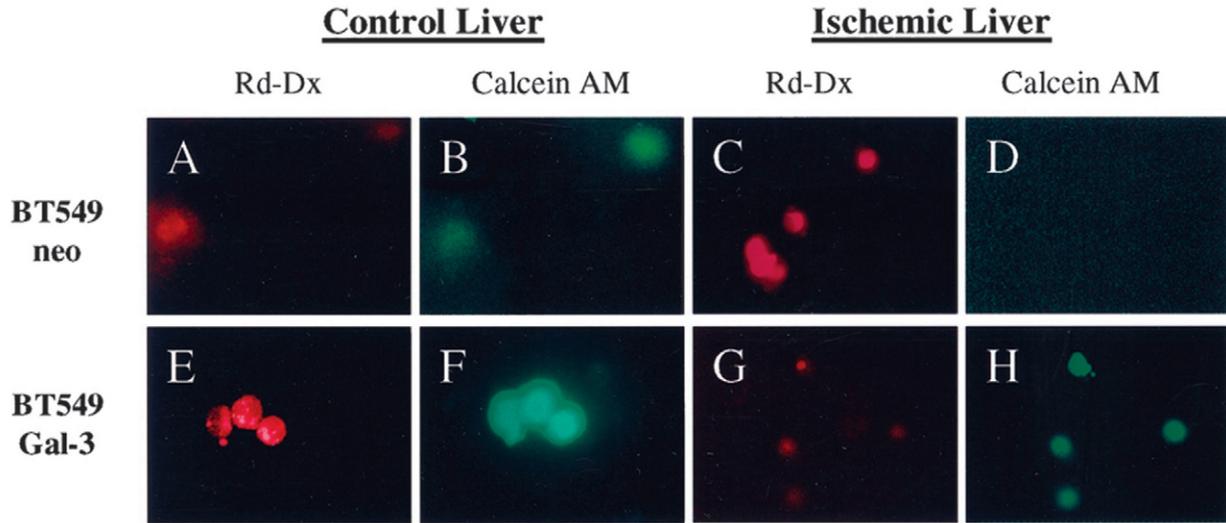
(B)

Cell line	Liver Fragments	Treatment	Viability(%)
BT549 neo	Control		97.2 ± 7.9
BT549 neo	Ischemia		61.6 ± 9.4
BT549 neo	Ischemia	NMMA	92.2 ± 6.1
BT549 Gal-3	Control		96.9 ± 9.8
BT549 Gal-3	Ischemia		100

Figure 1. Galectin-3 protects BT549 cell death induced by free radicals generated during hepatic ischemia/reperfusion. **A:** Rd-Dx and calcein AM-labeled BT549neo (**top panels**) and BT549Gal-3 (**bottom panels**) cells were co-cultured with the liver fragments isolated from control mouse liver (Control) or ischemic liver (Ischemic). Rd-Dx staining (**A, C, E, G**) and calcein AM staining (**B, D, F, H**) were detected with a maximum excitation/emission at 530/590 nm, and at 485/530 nm, respectively. **B:** After co-cultures of BT549neo or BT549Gal-3 cells with liver fragments, the numbers of cells containing Rd-Dx and calcein AM fluorescence were counted. The percentage of cell viability was determined by number of cells containing both Rd-Dx and calcein AM fluorescence/number of cells containing Rd-Dx fluorescence. For NMMA treatment, mice received injections with 20 mg of NMMA via tail vein 30 minutes before liver harvest. Co-cultures were incubated in the presence of 1 mmol/L NMMA. The assay was performed in triplicate and the results are presented as the mean ± SE.

gram of protein determined by BCA protein assay kit (Pierce, Rockford, IL).

Results

To study the role of galectin-3 in inhibiting human breast carcinoma cell death during liver ischemia/reperfusion, we cultured BT549Gal-3 or BT549neo cells in a rotating suspension culture system containing mouse liver fragments, a co-culture system previously shown to maintain the architecture and viability of the liver for at least 24 hours.[4] Hepatic ischemia was induced by clamping the hepatic arterial and portal vein, and an air pump was connected to the rotating suspension culture system to mimic the *in vivo* reoxygenation after liver ischemia. To distinguish human breast carcinoma cells from mouse liver cells, the human cells were labeled with Rd-Dx. The previous study[24] demonstrated that Rd-Dx is nontoxic,

does not leach out of cell cytoplasm, and retains its fluorescence until diluted by cell division. To distinguish viable cells after the co-culture period, the human cells were also prelabeled with calcein AM, since it produces fluorescence signal only in live cells with ATP-dependent cytoplasmic esterase activity. As shown in Figure 1, control mouse liver cells were not toxic to human breast carcinoma cells. When BT549neo cells were co-cultured with ischemic liver fragments, only 62% of the cells survived at 24 hours. In contrast, all of BT549Gal-3 cells survived after 24 hours of co-culture with ischemic liver fragments. These results imply that galectin-3 expression in human breast carcinoma cells protects against cell death during liver ischemia/reperfusion.

Previous studies showed that reactive nitrogen free radicals generated during liver ischemia/reperfusion are the major toxic molecules for the weakly metastatic colo-rectal cancer cells.[4] Here, we examined whether the
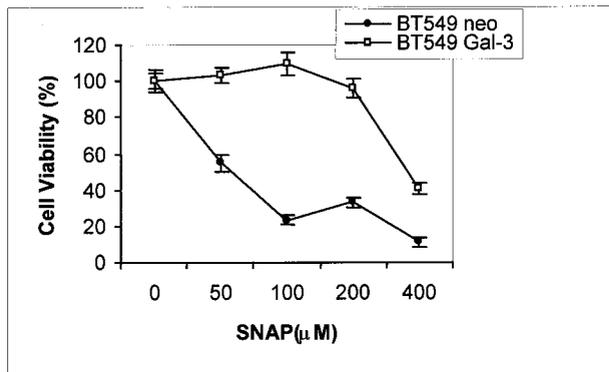
Figure 2. Galectin-3 inhibits SNAP-induced cell death in human breast epithelial cells. BT549neo and BT549Gal-3 cell cultures in the presence of 0, 50, 100, 200, or 400 μmol/L SNAP for 48 hours. The number of live cells was determined by trypan blue exclusion assay. The percentage of cell viability was normalized to the respective untreated cells. All experiments were performed in triplicate and the error bars represent the SD.

A.

B.

Figure 3. Galectin-3 protects mitochondria integrity and prevents cytochrome *c* release. **A:** BT549neo (**a, b**) and BT549Gal-3 (**c, d**) cells were plated on coverslips in six-well plates. Cells were treated with 0 (**a, c**) or 200 μmol/L SNAP (**b, d**) for 24 hours and stained with a fluorescent probe, MitoTracker Red. The mitochondria staining was examined with a Nikon Labophot microscope fitted with a digital video camera. **B:** Immunoblot analysis of cytosolic cytochrome *c* in BT549neo and BT549Gal-3 cells following 200 μmol/L SNAP treatment for 0, 24, or 48 hours.

same molecular species are responsible for killing BT540neo cells. To this end, mice were pretreated by a tail vein injection with 20 mg of N^G-monomethyl-L-arginine (NMMA), a NO synthase inhibitor, for 30 minutes before ischemic treatment. The isolated liver fragments were co-cultured with BT549neo cells in the presence of 1 mmol/L NMMA. The cell viability with NMMA treated ischemic liver fragments increased to the comparable levels of co-culture with control mouse liver fragments. These suggested that reactive nitrogen free radicals generated during hepatic liver ischemia/reperfusion were cytotoxic to human breast carcinoma BT549 cells similarly to colorectal cancer cells, and that galectin-3 protects BT549 cells against nitrogen free radical-induced cell death.

To further study galectin-3 inhibition of cell death induced by reactive nitrogen free radicals, we examined the role of galectin-3 in SNAP-induced cell death. SNAP is a NO donor known to induce cell death in many cell types including skeletal myoblasts, cortical neuronal cells, hepatocytes, and smooth muscle cells [25–27]. Substantial cytotoxicity was induced by SNAP in BT549neo cells (Figure 2). After 48 hours of treatment with 100 μmol/L SNAP, approximately 80% of BT549neo cells died, whereas no significant cytotoxicity was detected in BT549Gal-3 cells following up to 200 μmol/L SNAP treatment for 48 hours. This demonstrated that galectin-3 expression protects human breast epithelial cells from nitric oxide-induced cell death. Next we investigated whether galectin-3 regulates the nitric oxide-induced apoptotic pathway. It is now well established that mitochondria and caspases play central roles in apoptosis regulation [28,29]. Mitochondrial events critical for apoptosis include disruption of electron transport, loss of mitochondrial transmembrane potential (Ψ_m), and release of cytochrome *c*.[28,29] To examine whether galectin-3 protects mitochondria integrity, we stained BT549neo and BT549Gal-3 cells with MitoTracker Red which selectively stains mitochondria and serves as a marker for the mitochondrial membrane potential.[23] Twenty four hours of treatment with 200 μmol/L SNAP resulted in loss of mito-

chondria structure in ~90% of BT549neo cells. In contrast, more than 95% of the mitochondria in SNAP-treated BT549Gal-3 cells retained the fibrillar fluorescence pattern observed in the untreated cells, suggesting that galectin-3 overexpression protects cells against the loss of $\Delta\Psi_m$ (Figure 3A). As predicted from the loss of mitochondrial integrity (Figure 3A), immunoblot analysis of cytosolic cytochrome *c* showed that the level of cytochrome release from the mitochondria was elevated in BT549neo cells as compared with BT549Gal-3 cells following 200 μmol/L SNAP treatment (Figure 3B).

Cytosolic cytochrome *c* translocated from the mitochondria was shown to be critical for caspase activation, a group of cysteine proteases that initiate the apoptotic process.[30,31] To further examine the effect of galectin-3 on nitric oxide-induced apoptosis, we measured caspase activity using the flurogenic substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-amc), a substrate for caspase-3, -6, -7, -8, and -10. Caspase (DEVDase) activity was determined by amc release from the tetrapeptide substrate Ac-DEVD-amc. While SNAP treatment induced DEVDase activity ~fourfold in BT549neo cells following SNAP treatment, there was no induction in BT549Gal-3 cells following

A.
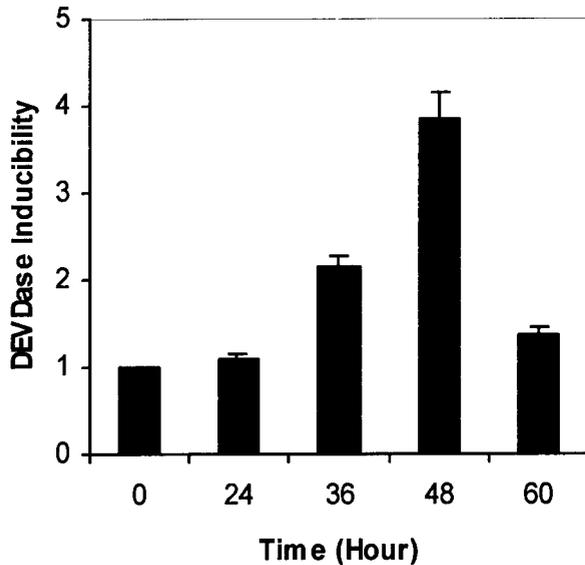
B.
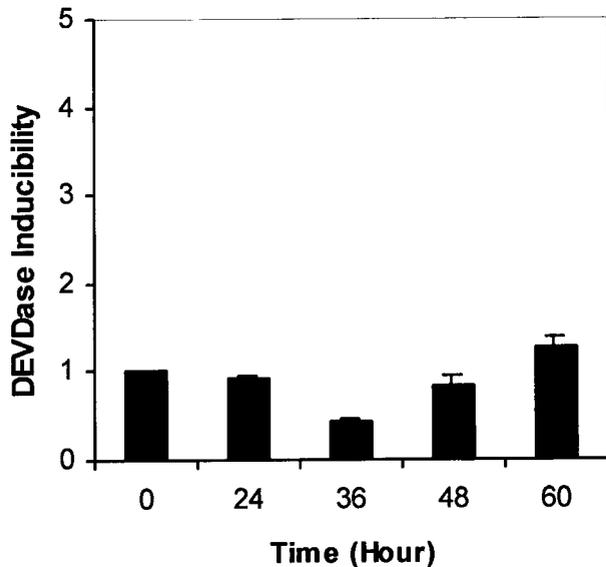
Figure 4. Galectin-3 inhibits DEVDase activity. BT549neo (**A**) and BT549Gal-3 (**B**) cells grown in 60-mm dishes were treated with 200 μmol/L SNAP. At indicated time points, the cells were washed with PBS and lysed with 200 μl caspase buffer as described in Materials and Methods. DEVDase activity in cytosol was determined by amc release from the tetrapeptide substrate Ac DEVD-amc, and DEVDase activity was normalized per μg protein. The DEVDase inducibility was normalized to the respective untreated cells. Three independent experiments were performed and the error bars represent SD of the mean of triplicates.

the same treatment (Figure 4). These results demonstrate that galectin-3 inhibition of apoptotic cell death involves protection of mitochondria integrity, prevention of cytochrome *c* release, and inhibition of caspase activation.

Discussion

Although recent advances in combined treatment modality including surgery, radio- and chemotherapy significantly improved the local control of breast cancer progression, most deaths from the disease still result from metastasis to the bone, lung, brain, and liver.[32] In liver metastasis, tumor cells enter the hepatic microcirculation mainly by size restriction, and are arrested in the hepatic sinusoids, where they may obstruct blood flow.[33–36] This appears to cause microscopic infarcts within the liver parenchyma located primarily near the capsule of the liver. *In vivo* and *in vitro* studies strongly suggest that the majority of tumor cells die in the microcirculation during reoxygenation of ischemic liver, mostly by reactive nitrogen free radicals.[4] The present study showed that galectin-3 may rescue human breast epithelial cells from nitrogen free radical-induced cell death during liver ischemia/reperfusion. Previously, we also demonstrated that galectin-3 inhibits apoptosis induced by loss of cell adhesion.[20] Taken together, we propose that galectin-3 is a critical determinant for anchorage-independent and free radical-resistant cell survival of disseminating cancer cells in the circulation during metastasis. This might explain why enhanced galectin-3 expression is often associated with the metastatic phenotype.[5–10,16,17]

Galectin-3 inhibits the intrinsic apoptotic pathway in breast epithelial cells induced by a variety of stimuli including nitric oxide, staurosporine, cisplatin, and loss of cell adhesion.[19–21] The ability of galectin-3 to protect cells against apoptosis induced by agents working through different mechanisms suggests that galectin-3 probably acts in a common central pathway of the apoptotic cascade, involving protection of mitochondrial integrity and caspase inhibition. Consistent with our study, Matarrese et al recently suggested that galectin-3 may be a mitochondrial-associated apoptotic regulator.[37] Thus, galectin-3 appears to be a potent inhibitor of mitochondria-mediated intrinsic apoptosis in breast epithelial cells. Interestingly, however, galectin-3 fails to prevent breast epithelial cell apoptosis induced by TRAIL (unpublished data), suggesting that galectin-3 may not be a general inhibitor of extrinsic apoptosis. Structurally, galectin-3 is composed of three distinct domains: an N-terminal leader domain of 12 amino acids containing serine/threonine phosphorylation sites, proline and glycine rich domain, and a globular C-terminal domain containing the carbohydrate recognition site.[5,38] Galectin-3 contains 4 amino acid residues (NWGR) that are conserved in the BH1 domain of the anti-apoptotic members of the bcl-2 gene family. This motif was shown to be critical for bcl-2 anti-apoptotic activity.[39] Similarly, substitution of the Gly residue with Ala in the NWGR motif of galectin-3 abrogates its anti-apoptotic function.[19,20] Galectin-3 may interact with apoptosis-regulating gene products through the NWGR motif and thus replace or mimic bcl-2 activity. Alternatively, galectin-3 may interact with bcl-2 family members and modulate their activity. The molecular mechanisms by which galectin-3 regulates intrinsic and extrinsic apoptosis are now under investigation.

Acknowledgments

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