Cell Cycle Arrest and Inhibition of Anoikis by Galectin-3 in Human Breast Epithelial Cells

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ABSTRACT

Galectin-3 is a member of a growing family of animal β-galactoside-binding proteins shown to be involved in cell growth, differentiation, apoptosis resistance, and tumor progression. In the present study, we investigated whether galectin-3 can protect against apoptosis induced by the loss of cell anchorage (anoikis). Because studies suggest that cellular sensitivity to anoikis is associated with cell cycle regulation, we examined the role of galectin-3 on cell cycle regulation. Although BT549 cells (human breast epithelial cells) undergo anoikis, galectin-3-overexpressing BT549 cells respond to the loss of cell adhesion by inducing G1 arrest without detectable cell death. Galectin-3-mediated G1 arrest involves down-regulation of G1-S cyclin levels (cyclin E and cyclin A) and up-regulation of their inhibitory protein levels (p21WAF1/CIP1 and p27KIP1). After the loss of cell anchorage, Rb protein becomes hypophosphorylated in galectin-3-overexpressing cells, as predicted from the flow cytometric analysis and immunoblot analysis of cyclins and their inhibitors. Interestingly, galectin-3 induces cyclin D1 expression (an early G1 cyclin) and associated kinase activity in the absence of cell anchorage. On the basis of these results, we propose that galectin-3 inhibition of anoikis involves cell cycle arrest at an anoikis-insensitive point (late G1) through modulation of gene expression and activities of cell cycle regulators. The present study suggests that galectin-3 may be a critical determinant for anchorage-independent cell survival of disseminating cancer cells in the circulation during metastasis.

INTRODUCTION

Cell-matrix interactions regulate many cellular responses including gene expression, differentiation, and cell survival (1–3). The ECM is a complex network of macromolecules that is composed of collagen, laminin, fibronectin, proteoglycans, and many soluble molecules including growth factors (4). The ECM of both the prostate and the lactating mammary gland undergo drastic involution upon removal of the appropriate trophic hormones (5). Because hormone ablation induces secretion of ECM-degrading enzymes that disrupt the interaction of epithelial cells with the ECM, it was hypothesized that the loss of cell-matrix interactions induces anoikis, a specific form of apoptosis (6).

Integrins are heterodimeric cell surface receptors that mediate cell adhesion to ECM (7, 8). Neutralizing antibodies against integrins induce cell detachment, followed by anoikis in epithelial cells, suggesting a role for integrin signaling in the regulation of anoikis (9). Integrin and growth factor receptor signals coregulate the turnover of phospholipids and play nonredundant roles in cell cycle progression (10, 11). Growth factor signaling triggers cell cycle progression from G0 to G1, whereas cell adhesion mediates the transition through the restriction point contained in late G1. Cyclin D1 expression and the activities of cyclin E-associated kinases, the functions of which are critical for G1-S transition, are shown to depend on cell adhesion (10, 11). This may explain why nonadherent cells fail to transit the cell cycle. Although normal epithelial cells require cell-extracellular matrix interactions for cell survival and growth, invasive and motile mesenchymal cells survive without these interactions and become arrested at the G1 stage of the cell cycle (6). This shows that anoikis sensitivity is associated with cell cycle regulation.

Because retaining anchorage-independent viability of disseminating cells in the circulation is critical for tumor cell metastasis, we investigated the role of galectin-3 in the cellular response to the loss of cell contact. The human galectin-3 is a M1 31,000 glycoprotein that belongs to a family of galactoside-binding proteins that are highly expressed in many human tumor cells including breast carcinoma cells (12–16). Structurally, galectin-3 is composed of two distinct domains: an NH2-terminal of 12 amino acids preceding a collagen-like sequence, and a globular COOH-terminal domain containing the carbohydrate-binding site (12, 17). Although galectin-3 is not a bcl-2 family member, it contains the same four amino acid motif (NWGR) conserved in the BH1 domain of the bcl-2 gene family. This motif is critical for bcl-2 antiapoptotic activity (18). Consistently, we and others have found that galectin-3 is a novel antiapoptotic gene product (19, 20) and showed that substitution of the Gly182 residue with Ala in the NWGR motif of galectin-3 abrogates its antiapoptotic activity (19). Thus, similar to the bcl-2 protein (18), the NWGR motif appears to be critical for the antiapoptotic function of galectin-3.

Although the exact function of galectin-3 is still debatable, galectin-3 expression has been associated with transforming and metastatic potentials (12–16). Ectopic expression of galectin-3 in the human breast cancer cell line BT549 resulted in the acquisition of a potent tumorigenic phenotype in nude mice (15, 21). We questioned whether oncogenic activity of galectin-3 involves anti-anoikis activity. Because cell cycle regulation is associated with anoikis (6, 9, 11), we have investigated the roles of galectin-3 on cell cycle regulation during anoikis. We report that galectin-3 prevents anoikis in human breast epithelial cells. We also present data that suggest that galectin-3-mediated inhibition of anoikis may result from its ability to induce cell cycle arrest at late G1 through expression modulation of cyclins and their inhibitors.

MATERIALS AND METHODS

Cell Line and Monolayer Culture Condition. The human breast cancer 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 established previously by introducing an expression vector containing human galectin-3 cDNA in either the sense or the antisense orientation into BT549 parental cells (19, 21). Galectin-3 antiapoptotic activity was characterized previously using three independent, galectin-3-expressing BT549 clones (11811, 11913, and 11914; Ref. 19). In the present study, we have also used these three clones. We present data mostly obtained using clone 11914 to avoid redundancy.
GALECTIN-3 PROTECTS AGAINST ANOIKIS

galactin-3-transfected BT549 clone 11914, antisense galactin-3-transfected BT549 clone 41421, and neo-resistant control vector-transfected BT549 (19) are referred to as BT549-Galwt, BT549-GalAS, and BT549neo, respectively. The mutant galactin-3-expressing BT549 in which the Gly182 of the NWG motif was substituted to Ala (Ref. 19) is referred to as BT549-GalAS. Cells on tissue culture dishes (Sarstedt, Newton, NC) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 0.5 μg/ml fungizone in a 95% air and 5% CO2 incubator at 37°C.

**Induction of Anoikis (Suspension Culture).** PolyHEMA (purchased from Aldrich Chemical Co., Madison, WI) was solubilized in methanol (50 mg/ml) and diluted in ethanol to a final concentration of 10 mg/ml. To prepare polyHEMA-coated dishes, 4 ml of polyHEMA solution were placed onto 100-mm Petri dishes and dried in a tissue culture hood. The polyHEMA coating was repeated twice, followed by three washes with PBS. Anoikis was induced by culturing 1.5 × 10^6 cells on polyHEMA-coated 100-mm dishes in a humidified 5% CO2 incubator supplemented as described above.

**Determination of Cell Cycle Distribution.** The percentage of cells in each cell cycle phase was determined by flow cytometry (PAS-II; Partec AG, Münster, Germany). Cells grown for 24 h in suspension (polyHEMA-coated dishes) were trypsinized, suspended in culture medium, centrifuged at 150 × g for 5 min, and fixed with 70% ethanol. The fixed cells were centrifuged, and the cell pellet was resuspended in Hoechst staining solution (polyHEMA-coated dishes) were trypsinized, suspended in culture medium, centrifuged at 150 × g for 5 min, and fixed with 70% ethanol. The fixed cells were centrifuged, and the cell pellet was resuspended in Hoechst staining solution at a concentration of 1 × 10^6 cells/ml and incubated for 3 min at room temperature. The staining solution consisted of 3 μg/ml Hoechst 33258 (Sigma Chemical Co., St. Louis, MO) in Tris buffer (2 mM MgCl2, 0.1% Triton X-100, 154 mM NaCl, and 100 mM Tris, pH 7.5). The stained cells (30,000) were analyzed by flow cytometry. Optical filtration included a UG-1 excitation filter, a 420-nm dichroic filter, and 435-nm long pass emission filter. Single-channel data were acquired and subsequently analyzed with a computer program (Phoenix Flow Systems, San Diego, CA).

**SDS-PAGE and Immunoblot Analysis.** Protein extracts were prepared by lysing 1 × 10^6 cells with 50 μl of 2× SDS sample buffer, and protein concentration was measured using BCA protein assay reagents (Pierce, Rockford, IL). Extracts were boiled for 10 min, chilled on ice, and subjected to SDS-PAGE analysis, followed by electrophoretic transfer to a nitrocellulose membrane. Expression levels were measured using the following antibodies: anti-PARP (C-2–10; Biomol Research Laboratory, Plymouth Meeting, PA), anti-galectin-3 (American Type Culture Collection, Rockville, MD), anti-human bcl-2 (DAKO A/S, Denmark), anti-cyclin D1 (Ab2; Oncogene Research, Cambridge, MA), anti-cyclin E (HE67; PharMingen, San Diego, CA), anti-cyclin A (BF683; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p15 (C-20; Santa Cruz Biotechnology), anti-p21^WAF1/CIP1 (Cal Biochem, San Diego, CA), anti-p27^KIP1 (M-197; Santa Cruz Biotechnology), and anti-Rb (SC4112; Santa Cruz Biotechnology).

**Cyclin D1 and Cyclin E-associated Kinase Assay.** Extracts were prepared by lysing the cells in ice-cold buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 25 mM NaF, 1 mM sodium vanadate, 1 mM EDTA, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 0.5% NP40, and 10 μg/ml leupeptin and aprotinin. After removing the precipitate by centrifugation at 14,000 rpm for 15 min at 4°C, the cyclin D1 and cyclin E complexes were collected using the anti-cyclin D1 or anti-cyclin E antibodies and protein G-Sepharose. The complex was washed three times with 1.0 ml of washing buffer (1% deoxycholate, 0.5% Tween 20, and 50 mM Tris-HCl, pH 7.5) and once with 1 ml of 50 mM Tris (pH 7.5) and 10 mM MgCl2. Kinase activity of the cyclin D1-immune complex was measured using 2.5 μM GST-pRb protein (purchased from Santa Cruz Biotechnology), and cyclin E complex using histone H1 protein (Boehringer Mannheim, Indianapolis, IN) as substrate in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM DTT, 0.5 mM NaF, 0.1 mM sodium orthovanadate, 5 μg/ml leupeptin, and [γ-32P]ATP (0.01–0.3 nCi/ml). After kinase reaction at 30°C for 30 min, samples were mixed with 2× SDS buffer and subjected to SDS-PAGE analysis. [γ-32P]ATP-labeled Rb and histone H1 proteins were visualized by autoradiography.

**RESULTS**

**Galectin-3 Inhibits Apoptosis and Induces G1 Arrest after Loss of Cell-Matrix Interactions.** Anoikis was induced by culturing cells in suspension using polyHEMA-coated tissue culture dishes, which prevent cell adhesion, as described in “Materials and Methods.” To determine whether galectin-3 inhibits anoikis, we examined the apoptosis-specific cleavage of PARP, an early event in apoptosis resulting from the activation of interleukin 1β-converting enzyme (caspase)/Ced-3 family members (22). As shown in Fig. 1A, proteolytic cleavage of PARP was readily detected in the parental control and BT549-GalAS cells, whereas it was significantly inhibited in BT549-Galwt cells, even after 3 days in suspension culture. Galectin-3 inhibition of anoikis was also confirmed by nuclear morphological analysis. The nuclei were stained using Hoechst 33258 after 48 h in suspension culture, as described previously (23). The parental control and the BT549-GalAS cells showed fragmented nuclei that were consistent with nuclear morphological changes in other apoptotic cells (23). In contrast, no significant changes in nuclear morphology could be observed in BT549-Galwt cells (data not shown).

Because cell cycle regulation is associated with sensitivity to anoikis (6, 9, 11), flow cytometric DNA analysis was performed to determine the cell cycle phase distribution of cells grown on monolayer or in suspension for 24 h. In exponentially growing monolayer cultures, the percentage of BT549neo in G1 phase was 46.8%, whereas only 26.2% of BT549-Galwt was in the G1 phase (Fig. 2, A and C). In suspension culture of BT549neo, the G1 population decreased to 25.4%, and the sub-G1 population increased from 7 to 9% for 3 days in suspension culture. The G2/M population decreased to 25.4%, and the sub-G1 population increased from 7 to 9% for 3 days in suspension culture.
25.4% (Fig. 2, A and B). Flow cytometric DNA analysis, morphological analysis, and PARP cleavage showed that BT549 cells underwent apoptotic cell death in response to the loss of cell-substrate interactions. In contrast, no sub-G₁ population of BT549-Gal wt was detected, regardless of the state of cell-substrate interactions, whereas a dramatic increase in the G₁ population (from 26.2 to 78.6%) was detected in BT549-Gal wt in the absence of cell-substrate interactions (Fig. 2, C and D). These results show that galectin-3 overexpression renders BT549 cells able to respond to the loss of cell-ECM contact by growth arrest at G₁ without detectable apoptotic cell death.

**Galectin-3 Down-Regulates Expression of Late G₁ and S phase Cyclins and Up-Regulates CDKIs.** After the loss of cell-substrate contacts, the G₁ population increase of BT549-Gal wt was accompanied by a 6.7-fold decrease in the proportion of cells in S phase, suggesting that galectin-3 overexpression inhibits G₁ to S phase progression. Cyclin E-associated kinases are activated late in G₁ phase, and their activities are thought to be critical for the progression to S phase (24–26). Cyclin A-associated kinase activities are then activated concomitantly with the onset of S phase and involved in maintenance of S phase (27–29). Therefore, we sought to determine whether expression of these cyclins is modulated by galectin-3 when cultured on monolayer or in suspension. As shown in Fig. 3, expression of cyclin E was down-regulated by galectin-3, especially after the loss of cell-substrate interactions. In monolayer culture, cyclin A expression was up-regulated by galectin-3, in agreement with a proportional increase in BT549-Gal wt in S phase compared with BT549neo. However, cyclin A expression was significantly down-regulated in BT549-Gal wt upon cell detachment, whereas the loss of cell-substrate interactions had no effect on cyclin A expression in BT549neo. These data (Fig. 3, A and B) suggest that galectin-3-mediated G₁ arrest in the absence of cell anchorage involves drastic down-regulation of cyclin E and cyclin A expression.

**Kinase activities of cyclin E and cyclin A complexes are regulated by CDKIs, including p21WAF1/CIP1 and p27KIP1 (30–32). Therefore, we also examined the effects of galectin-3 overexpression on CDKI expression with regard to cell-matrix interactions.** As shown in Fig. 3C, expression of both p21WAF1CIP1 and p27KIP1 was up-regulated by galectin-3 and further increased after cell-matrix detachment (Fig. 3C). Galectin-3 modulation of cyclin E and cyclin A as well as their inhibitors in suspension culture is consistent with cell cycle arrest at mid-G₁, as shown in flow cytometric analysis (Fig. 2D). However, the immunoblot analysis showed that galectin-3 down-regulated cyclin E and slightly up-regulated p21WAF1CIP1 and p27KIP1, even in proliferating monolayer culture. To assess the functional significance of changes in expression of cyclin E and its inhibitors, we measured cyclin E-associated kinase activity **in vitro** using histone H1 as sub-

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**Fig. 2. Galectin-3 induces G₁ arrest after the loss of cell-matrix interactions.** Flow cytometric cell cycle histograms of BT549neo (A and B) and BT549-Gal wt (C and D). The same numbers of cells were grown for 24 h on monolayer (A and C) or in suspension (B and D). The proportions of cells in each cell cycle phase are presented.
strate. Surprisingly, cyclin E-associated kinase activity was not drastically altered by galectin-3 on monolayer culture (Fig. 4A), whereas its activity is significantly down-regulated by galectin-3 in suspension culture. The cyclin E-associated kinase activity agrees with G1-S transition in monolayer culture of BT549-Gal wt and G1 arrest in suspension culture. In monolayer culture, it is not clear how cyclin E-associated kinases are activated in BT549-Gal wt cells with low levels of cyclin E protein as efficiently as in BT549neo (see “Discussion”).

To determine whether the in vivo kinase activities of cyclin complexes were modulated by galectin-3, we examined the status of Rb phosphorylation in monolayer or suspension cultures of three independent galectin-3-overexpressing BT549 clones. The Rb protein is hypophosphorylated in G0 and early G1 phases and binds to a series of cellular proteins including E2F transcription factor. Rb protein becomes hyperphosphorylated by D-type cyclin-dependent kinases and further phosphorylated by cyclin E- and A-dependent kinases (33, 34). The hyperphosphorylated pRb becomes inactive and is no longer capable of binding to its interacting molecules such as E2F. This hyperphosphorylated state is maintained through S, G2, and most of M phases. As shown in Fig. 4B, hyperphosphorylated Rb was dominant in three clones, indicating that most of the cells are in S to M phases when cultured on monolayer (also see Fig. 2C). In contrast, only hypophosphorylated Rb was detected in suspension cultures of BT549-Gal wt clones, showing that these cells fail to enter S phase, and cdk activities are down-regulated, as predicted by flow cytometric analysis and the immunoblot assay (Figs. 2 and 3). It should be noted that the Rb protein in the control cells was barely detectable, so the phosphorylation status of Rb protein in BT549neo was not determined.

Fig. 4. Galectin-3 down-regulates cyclin E-associated kinase activity, and the Rb protein becomes hypophosphorylated in BT549-Gal wt cells after the loss of cell-matrix interactions. A, cyclin E-complexes were immunoprecipitated from the cell extracts of BT549-Gal wt, BT549-Gal wt clone A5, BT549-Gal wt clone A3, or BT549-neo as indicated above each panel. Cells were cultured for 24 h on polyHEMA-coated plates (suspension) or on regular tissue culture plates (monolayer).

Fig. 3. Galectin-3 modulates expression of cyclins and their inhibitor proteins. Immunoblot analyses of galectin-3, cyclin A, cyclin D1 (A); cyclin E, β-actin (B); and p27, p21, and p15 (C). Protein samples were prepared from BT549-Gal wt, BT549-Gal m, or BT549-neo as indicated above each panel. Cells were cultured for 24 h on polyHEMA-coated plates (suspension) or on regular tissue culture plates (monolayer).
D-type cyclins, early G1 cyclins, requires cell adhesion (10, 11). To regulated by growth factors and cell anchorage. Expression of the Adhesion. Recent studies showed that cell cycle progression is co-

clin D1 expression was further enhanced in suspension cultures. In p19 INK4D was detectable, and expression of p15 INK4B was not signif-

antly altered by galectin-3, regardless of cell adhesion (Fig. 3C). We next questioned whether galectin-3 induction of cyclin D1 expression lead to increased kinase activity. We examined whether cyclin D1 in BT549-Gal wt cells was complexed to its functional partners (CDKs). The cyclin D1 complex was immunoprecipitated, and its associated kinase activity was measured using purified GST-pRb fusion protein as a substrate. The Rb fusion proteins of M, 46,000 were more efficiently phosphorylated by cyclin D1-associated kinases in BT549-Gal wt cell lysates compared with those in BT549-neo in the absence of cell anchorage (Fig. 5). Although in vitro kinase assay showed that galectin-3 induction of cyclin D1 lead to activation of cyclin D1-associated kinases, the phosphorylated form of Rb was not detected in BT549-Gal wt suspension culture (Fig. 4B). This is in agreement with previous reports that maintenance of hyperphosphorylation of Rb in vivo requires cyclin E and cyclin A-dependent kinase activities (33, 34).

Galectin-3 Up-Regulation of Cyclin D1 Is Independent of Cell Adhesion. Recent studies showed that cell cycle progression is co-regulated by growth factors and cell anchorage. Expression of the D-type cyclins, early G1 cyclins, requires cell adhesion (10, 11). To examine whether galectin-3 modulates the expression of the D-type cyclins, immunoblot analysis of cyclin Ds was performed. As shown in Fig. 3A, cyclin D1 expression was induced by galectin-3 when cultured on monolayer. Interestingly, galectin-3 up-regulation of cyclin D1 expression was further enhanced in suspension cultures. In BT549 cells, neither cyclin D2 nor D3 was detected, irrespective of galectin-3 expression levels (data not shown). This suggests that galectin-3 up-regulates cyclin D1 expression independently of cell-substrate interactions.

The D-type cyclins are often inhibited by a family of inhibitors including p15^INK4b, p16^INK4a, and p19^INK4b (35). When the levels of these inhibitors were examined in BT549 cells, neither p16^INK4a nor p19^INK4b was detectable, and expression of p15^INK4b was not significantly altered by galectin-3, regardless of cell adhesion (Fig. 3C). When cultured on polyHEMA-coated plates for 24 h. Kinase activity of the complexes was measured in the presence of [γ-32P]ATP using purified GST-pRb fusion protein as a substrate. Radioactivity incorporated into GST-pRb fusion protein was determined by scintillation counting. Kinase activities in BT549-Gal wt and BT549-Gal m were normalized to that in BT549-neo. In vitro kinase assay showed that a substitution of Gly^182 to Ala in the NWGR motif of galectin-3 abolished its ability to modulate kinase activity of the cyclin D1 and cyclin E complexes after ECM detachment (Fig. 5). These results reemphasize that the integrity of the NWGR motif in the BH-1-like domain of galectin-3 is a prerequisite for its antiapoptotic activity as well as its ability to modulate expression of cell cycle regulators.

DISCUSSION

Although survival of “stationary” epithelial cells depends on cell-cell and cell-matrix interactions, invasive and motile mesenchymal cells survive without these interactions (36). Cancer cells often acquire a “fibroblast-like” phenotype and become invasive. The present study showed that galectin-3 overexpression renders breast epithelial cells (BT549) able to respond to the loss of cell-ECM contact by growth arrest at G1 without detectable apoptotic cell death (anoikis), which resembles the response of fibroblast to loss of cell-ECM contact. Acquisition of anti-anoikis activity may be critical for anchorage-independent cancer cell survival in circulation during metastasis and might explain why galectin-3 expression is enhanced in some tumors with malignant phenotypes (12–16). A key step in the apoptosis cascade is the activation of members of the interleukin-1β converting enzyme/Ced-3 family (caspases; Ref. 37). This commit-

Fig. 5. Galectin-3 induces cyclin D1-associated kinase activity in the absence of cell anchorage. Cyclin D1 complexes were immunoprecipitated from the cell extracts of BT549-neo, BT549-Galwt, and BT549-Galm, clone 5 (GalA5) cultured on polyHEMA-coated plates for 24 h. Kinase activity of the complexes was measured in the presence of [γ-32P]ATP using purified GST-pRb fusion protein as a substrate. Radioactivity incorporated into GST-pRb fusion protein was determined by scintillation counting. Kinase activities in BT549-Gal wt and BT549-Gal m were cultured on polyHEMA-coated dishes for 24 h. Unlike in BT549-Gal wt cells, apoptosis-specific cleavage of PARP protein occurred in both BT549-Gal m cells and control cells (Fig. 1, B and C).

We next examined the significance of the NWGR motif of galectin-3 for cell cycle regulation after loss of cell-substrate interactions. As shown in Fig. 3, mutant galectin-3 failed to modulate expression of cyclin D1, cyclin E, cyclin A, p21^WAF1/CIP1, and p27^KIP1. In addition, in vitro kinase assay of the cyclin D1 and cyclin E complexes showed that a substitution of Gly^182 to Ala in the NWGR motif of galectin-3 abolished its ability to modulate kinase activity of the cyclin D1 and cyclin E complexes after ECM detachment (Fig. 5). These results reemphasize that the integrity of the NWGR motif in the BH-1-like domain of galectin-3 is a prerequisite for its antiapoptotic activity as well as its ability to modulate expression of cell cycle regulators.

Fig. 6. The NWGR motif in bcl-2 and galectin-3. The topologies of the human bcl-2 protein (adapted from Ref. 39) and galectin-3 protein are depicted. Both bcl-2 and galectin-3 contain a short NH₂-terminal amino acid se-

sequence that does not share homology among their family members.
Fig. 7. Model for galectin-3 regulation of the cell cycle and anoikis. Effects of galectin-3 overexpression on cell cycle regulation with or without cell contacts. Arrows, effects of galectin-3 overexpression on expression and/or activity of G1/S cyclins and their inhibitors. Galectin-3 induces expression/activity of cyclin D1 and cyclin A in monolayer culture, while it down-regulates cyclin E expression without affecting its associated kinase activity. After the loss of cell contacts, galectin-3 further induces cyclin D1 expression/activity and down-regulates cyclin E and cyclin A, resulting in G1 arrest without detectable apoptosis.

The present study suggests that galectin-3 regulates the cell cycle through modulation of the cell cycle regulating gene expression. Galectin-3 up-regulates cyclin A expression, in agreement with an increase in BT549-Galwt in S phase compared with BT549neo. This is consistent with a recent report that the cellular expression of galectin-3 is proliferation dependent (44). Galectin-3 does not alter cyclin E-associated kinase activity, although it down-regulates cyclin E protein expression in monolayer culture. The question is then how cyclin E-associated kinases are efficiently activated in these cells. Many isoforms of cyclin E are detected in breast cancer cells, and activities of these isoforms appear to be regulated by different mechanisms (45). It should be noted that although galectin-3 down-regulates the major cyclin E protein, the lower molecular weight cyclin E protein was induced by galectin-3 in monolayer culture (Fig. 3B). Galectin-3-induced low molecular weight cyclin E isoform may be efficiently activated in monolayer culture. Or, galectin-3 up-regulation of cyclin D1 may be critical for efficient activation of cyclin E. Cyclin E-associated kinase was previously suggested to be active after it is phosphorylated by cyclin D1-associated kinase (46). Lastly, galectin-3-induced p21\(^{WAF1/CIP1}\) and p27\(^{KIP1}\) may regulate cyclin E-associated kinase activities in a bidirectional manner. It was shown previously that the low levels of p21\(^{WAF1/CIP1}\) and p27\(^{KIP1}\) promote the assembly of active kinase complexes, whereas at higher concentrations, they inhibit activity (47). Thus, it is possible that galectin-3-induced p21\(^{WAF1/CIP1}\) and p27\(^{KIP1}\) in monolayer culture may function as adaptor proteins of cyclin E complexes, which are critical for G1-S transition, whereas further induction in suspension culture results in inhibition of kinase activity.

Cyclin D1 overexpression has been associated with a poor prognosis (48–52), dysregulation of the cell cycle, increased cell proliferation, increased EGF receptor expression, and p53 abnormalities in a transgenic model (53). Cyclin D1 expression was shown to depend on adhesion in epithelial cells. Interestingly, galectin-3 up-regulates cyclin D1 expression independent of anchorage. It is not clear whether galectin-3 induction of cyclin D1 is associated with anti-anoikis activity of galectin-3 and/or with its oncogenic activity. Mounting evidence suggests that apoptosis regulation is tightly linked to regulation of the cell cycle. Although apoptosis can be induced at any point of the cell cycle, sensitivity of apoptosis greatly differs, depending on cell cycle points. Our study suggests that galectin-3 inhibition of anoikis may be related to its ability to regulate critical decision points between cell cycle progression and induction of apoptosis. Galectin-3 induction of cyclin D1-associated kinase activity may help cells pass the apoptosis-sensitive point in early G1. Similarly, galectin-3 down-regulation of cyclin E and cyclin A expression may be involved in cell cycle arrest at an anoikis-resistant point as summarized in Fig. 7.

At present, it remains unclear how galectin-3 regulates expression of the cyclins and their inhibitors. Galectin-3 was shown to be a nuclear matrix protein that binds to RNA and single-stranded DNA (54) and identified as a splicing factor in cell-free splicing assay (55). Nuclear galectin-3 may directly modulate gene expression through regulation of transcription and/or mRNA splicing. Or, cytoplasmic galectin-3 may modulate the activity of signaling molecules (e.g., kinases, phosphatases, and proteinases), which, in turn, transduce signals to regulate a set of gene expression. Galectin-3-mediated changes in the levels of the cyclins and their inhibitors may result from changes in gene expression of the adhesion-related genes. Cell adhesion transduces biochemical signaling, including focal adhesion kinase activation that is critical for anoikis regulation as well as cell cycle transition (7, 56–58). Whether cell cycle regulating genes are primary or secondary effects of galectin-3 overexpression, the present study showed that galectin-3 inhibition of anoikis is associated with cell cycle regulation. However, it remains to be fully investigated whether cell cycle regulation is a prerequisite for galectin-3 regulation of anoikis.

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GALECTIN-3 PROTECTS AGAINST ANOIKIS


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