Rapid Release of Intracellular Galectin-3 from Breast Carcinoma Cells by Fetuin

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ABSTRACT

Galectin-3, a β-galactoside binding protein, plays a significant role in cell to extracellular matrix interactions. Despite its extracellular expression, the precise physiological mechanisms that trigger its release from the intracellular milieu have not been characterized. The present analyses were, therefore, done to identify the extracellular matrix proteins with propensity to induce the release of intracellular galectin-3 from breast carcinoma cells. Our studies demonstrate that fetuin, a serum glycoprotein that is abundant in the fetal serum, is capable of inducing the rapid release (~1 min) of intracellular galectin-3 from the cells. The mechanism by which galectin-3 is rapidly released appears to be novel and does not depend on changes in intracellular calcium levels. We also report that fetuin-expressing breast carcinoma cells in serumless medium adhere and spread well on microtiter wells in the presence of fetuin and divalent ions in a carbohydrate-dependent manner. The data suggest that fetuin is a natural modulator of galectin-3 secretion/release and that the secreted galectin-3 modulates the activity of cell surface receptors for extracellular matrix proteins.

INTRODUCTION

Galectins are a growing family of carbohydrate-binding proteins that share affinity for β galactosides and significant sequence homology in their carbohydrate-binding domains (1–3). Galectin-3 is expressed in the nucleus, in the cytoplasm, and on the cell surface of most epithelial cells and can be secreted into the extracellular matrix (4). On the cell surface, galectin-3 plays critical roles in cell-cell or cell-extracellular matrix interactions. It has been shown to mediate homotypic aggregation that may be responsible for tumor emboli (5, 6). It has also been shown to be responsible for rapid adhesion of breast carcinoma cells to ECM1 proteins such as collagen IV, laminin, and elastin (7, 8).

Galectin-3 like many other cytosolic proteins, such as thioredoxin (9), interleukin-1β (10), and acidic and basic fibroblast growth factor (11), can traverse the plasma membrane and yet lacks signal peptides necessary for secretion via the classical secretory pathway. The mechanisms by which galectin-3 and other proteins that lack signal peptides are secreted via the nonclassical pathway have yet to be elucidated. Galectin-3 may be concentrated in secretory vesicles that are concentrated in the membrane domains (12). It has been shown that NH2-terminal domain of galectin-3 is critical for its secretion and is the driving force that localizes it in the secretory vesicles (13, 14, 15). How galectin-3 moves from these vesicles into the ECM or the mechanism that triggers the exocytosis of these vesicles is the gap in our current knowledge.

It has been demonstrated that whereas secretion of galectin-3 is normal in medium that contains serum, it is dramatically reduced in serumless medium (4). In the present study, we have exposed breast carcinoma cells to different ECM proteins in order to identify the ECM proteins likely to elicit the release/secretion of galectin-3. Our studies demonstrate that fetuin, the serum glycoprotein abundant in fetal blood, if added to serumless medium in concentrations similar to that in medium supplemented with 10% fetal bovine serum, is capable of releasing intracellular galectin-3 rapidly from breast carcinoma cells. Fetuin was previously shown to bind to various tumor cells and to induce cell aggregation by binding to lectin-like molecules (6). More recently, it was demonstrated that insect cells that express galectin-3 on their surfaces undergo homotypic aggregation in the presence of asialofetuin or fetuin (5). It is, therefore, possible that fetuin can interact with cell surface lectins and that this is sufficient signal to trigger the release of galectin-3 and possibly other members of the family. Galectin-3 is released rapidly from intracellular domains in a dose-dependent manner. Changes in intracellular calcium ion concentration do not influence the release that appears to be mediated by a novel mechanism. The data further suggest that the galectin-3 released by the cells is responsible for the rapid adhesion and spreading of breast carcinoma cells to the substrata.

MATERIALS AND METHODS

Human breast epithelial cell lines MDA-MB-435; BT-549; and 11-9-1-4, which is a galectin-3-transfected BT-549, were kindly donated to us by Dr. Avraham Raz, Karmanos Cancer Institute, Detroit, MI. All of the cell lines were cultured in DMEM/F12 (Sigma) supplemented with 100 μg/ml penicillin-streptomycin, 2.5 μg/ml Fungizone, 20 ng/ml epidermal growth factor, 98 ng/ml cholaer toxin, 10% heat-inactivated fetal bovine serum, 2 mM glutamine and nonessential amino acids. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Human ECM was purchased from Collaborative Research and all of the other biochemicals from Sigma Chemical, unless otherwise stated.

Rapid Release of Galectin-3 from Cells. To assay for galectin-3 release, the cells were removed from the culture flasks by trypsinization and washed twice in serumless DMEM/F12 medium. After the last wash, the cells were left standing in suspension in the centrifuge tubes for at least 10 min. The cells were then counted using a hemocytometer and 500,000 cells in approximately 20 μl of serumless medium added to siliconized Eppendorf tubes containing 100 μl of serumless DMEM/F12 with the various additives. In the first experiment, the additives were: (a) fibronectin (2 mg/ml); (b) collagen IV (2 mg/ml); (c) human ECM (2 mg/ml); (d) TG (100 μM); (e) lactose (100 μM); (f) 10% serum; and (g) 0.25% fetuin. The samples were incubated for 10 min at room temperature and centrifuged to pellet the cells, and the supernatant from each tube (20 μl) was assayed for galectin-3 by Western blot as described previously (6). The galectin-3 release assay was repeated with different doses of fetuin (0–1%) and with 0.25% fetuin to obtain a time-course of release.

The Source of Released Galectin-3. To demonstrate that the secreted fetuin is from intracellular domains and not from the cell surface, cell surface proteins were labeled with biotin, and the labeled proteins were then chased after treatment of the cells with fetuin. Briefly, cells in 75-cm2 culture dishes were washed with serumless medium five times and then with PBS twice. The cells were then incubated with 2.6 mM NHS-Biotin (Biorad) for 30 min at room temperature with occasional swirling. The unreacted biotin was removed and the flask was washed thoroughly with PBS, and then trypsinized. Trypsin was inactivated by the addition of complete medium containing serum, and the cells were centrifuged. The cells were washed twice with serumless medium and then divided into two Eppendorf tubes (500,000 cells/tube) in 100 μl of serumless medium without (control) or with 0.25% fetuin. The cells were then incubated for 30 min at 37°C and centrifuged; 20 μl of the supernatant were taken from each tube (conditioned medium) and applied to SDS-PAGE gel.
The cell pellets were lysed in lysis buffer in the presence of protease inhibitors, and the membrane fractions were subjected to SDS-PAGE. The gels were blotted onto nitrocellulose and incubated with avidin-peroxidase followed by chemiluminescence reagents as described previously (7).

**Effect of Fetuin on Intracellular Calcium Levels in Breast Cancer Cell Lines.** Cells were trypsinized and washed in Krebs-Ringer-HEPES buffer (118.5 mM NaCl, 4.74 mM KCl, 1.18 mM MgSO₄, 1.18 mM KH₂PO₄, 2.54 mM CaCl₂, 24.9 mM NaHCO₃, 10 mM glucose, and 0.03 mM EDTA). They were then loaded with 4 μM of fura-2/AM in the same buffer for 45 min. At the end of the incubation, the cells were washed and placed in a cuvette with stirrer, and fluorescence measurements were made using a SPEX dual wavelength (AR-CM) fluorometer. After about 100 s of stabilization, fetuin was added to a final concentration of 0.25% and changes in intracellular calcium ion concentrations were monitored.

**Effects of Calcium Ionophore A23187 and Thapsigargin in the Rapid Release of Galectin-3 from Breast Carcinoma Cells.** To determine whether agents that increase intracellular calcium could induce the rapid release of galectin-3, the assay was done in the absence or presence of 5 μM of A23187 in serumless DMEM/F12 containing 1 mM CaCl₂. The assay was also done in the presence of 5 μM of A23187 and 0.25% fetuin. Thapsigargin (1 μg/ml), a known inhibitor of the endoplasmic reticulum Ca²⁺ ATPase was also tested in the assay by itself and in the presence of 0.25% fetuin.

**Role for the Released Galectin-3 in Cell Spreading and Adhesion.** We have previously demonstrated that galectin-3-expressing cells adhere and spread rapidly to ECM proteins compared with galectin-3 null-expressing cells (7, 16). In all of these assays, however, medium containing 10% serum was used. We, therefore, questioned whether galectin-3 release by fetuin was sufficient for this rapid adhesion and spreading. The cells were trypsinized and washed in serumless medium as described above. They were then allowed to adhere to tissue culture microtiter wells in the presence or absence of 0.25% fetuin in serumless DMEM/F12 containing Ca²⁺ ions. The adhesion assay was also done in the presence of 0.25% fetuin and 100 mM TDG. As a negative control (lack of rapid adhesion and spreading), BT-549 cells that do not express galectin-3 were also allowed to adhere in the presence of 0.25% fetuin. The cells were allowed to adhere for 30 min and then were photographed by a digital camera, and the images analyzed by adobe photoshop.

**Adhesion of Breast Carcinoma Cells to Elastin in the Absence and Presence of Fetuin.** To further implicate galectin-3 in cell-to-ECM interactions, we questioned whether the fetuin-induced rapid release of the lectin from the cells could ligate galectin-3 producing cells to elastin. Elastin (40 mg) was treated with anhydrous hydrazine and 1% hydrazine sulfate for 5 h at 80°C until all of the elastin was dissolved. The solution was then diluted with PBS to a final concentration of 2 mg/ml. The wells of a microtiter plate were then coated with the solubilized elastin for 1 h at 37°C. Nonspecific sites were blocked with 2% BSA, and cells were added to the wells in DMEM/F12 serumless medium without divalent ions and with or without 0.25% Fetuin. The cells were allowed to adhere for 12 h, and the nonadherent cells were washed off in the serumless medium. The adhered cells were photographed, and the number of cells adhered were estimated by the methylene blue assay (17).

**RESULTS**

Serumless medium in our hands consistently fail to show secreted galectin-3, at least when the blots were exposed to X-ray films in less than 10 min, as we routinely did in this report. As shown in Fig. 1A, only fetuin and DMEM/F12 medium supplemented with 10% fetal bovine serum (complete) was able to trigger the release of galectin-3 into the medium. Fetuin was used at a concentration of 0.25% (2.5 mg/ml), which is comparable to the concentration of fetuin in the complete DMEM/F12 medium. From the level of galectin-3 released in both cases, the data suggest that fetuin was the ingredient in DMEM/F12-10% FBS that was responsible for the release of the lectin. Galectin-3 was also released from MDA-MB-435 and 11-9-1-4 by human fetuin, α2HS glycoprotein (2 mg/ml) (data not shown). It is evident from Fig. 1B that the release of galectin-3 is dependent on the dose of fetuin used. Whereas the concentration of fetuin in the fetal blood can be as high as 20 mg/ml, the level drops to approximately 0.6 mg/ml in the adult (18). According to the dose-response data, fetuin levels in both fetus and adult are able to induce the release of galectin-3. The present data suggest that one possible function of fetuin in vivo is to trigger the release of intracellular galectin-3, at least in tumor cells. The induction of galectin-3 release by fetuin is very rapid, taking place within 1 min of exposure of cells to fetuin (Fig. 1C). Prechilling the cells at 4°C prior to adding fetuin did not affect the release of galectin-3 (data not shown), which demonstrates that the release was not affected by temperature. Inclusion of methylamine or propylamine in the fetuin medium did not change the level of galectin-3 released (data not shown), which implies that the pathway is independent of the endocytosis/exocytosis (4).

**The Source of Galectin-3 Released into the Medium.** It can be argued that galectin-3 that is rapidly secreted into the medium on fetuin stimulation is cell-surface bound and is not from the intracellular milieu. We, therefore, biotinylated the surface proteins of the breast carcinoma BT-549 clone 11-9-1-4 cells. As evidenced in Fig. 2, all of the label was retained in the cell membrane (Fig. 2, Lanes 1 and 2). The conditioned medium in neither the control (Fig. 2, Lane 3) nor the fetuin-treated (Fig. 2, Lane 4) tubes revealed any protein band after the normal 1–10-min exposure of X-ray film to the immunoblot membrane. Overnight exposure of the film revealed faint bands in

![Image](https://example.com/image.png)
both the control and fetuin-treated conditioned medium. Moreover, all of these bands were above M, 40,000 (data not shown).

**Intracellular Calcium Ion Concentration in Fetuin-induced Galectin-3 Release.** To investigate the mechanism(s) by which fetuin affects the release of galectin-3, we analyzed intracellular calcium concentration in the breast carcinoma cells before and after the addition of fetuin. Increases in intracellular calcium ion concentration have been shown to stimulate galectin-3 secretion (4, 12). As can be seen in Fig. 3A, fetuin raised the intracellular calcium in 11-9-1-4 transiently by about 30%. However, in the MDA-MB-435 cell line, the addition of fetuin actually reduced intracellular calcium levels (Fig. 3A). We also challenged the cells with agents that are known to increase intracellular Ca$^{2+}$ ion concentration in the absence of 0.25% fetuin. Galectin-3 was released from both 11-9-1-4 and MDA-MB-435 carcinoma cells in the presence of fetuin (Fig. 3B, Lanes 1 and 6, respectively). The inclusion of A23187 in serumless medium (Fig. 3B, Lanes 2 and 7) failed to release galectin-3. The inclusion of A23187/fetuin (Fig. 3B, Lanes 3 and 8) in the serumless medium triggered the release of galectin-3 in levels comparable with controls. Similarly, thapsigargin, the inhibitor of endoplasmic reticulum ATPase, by itself failed to trigger the release of galectin-3 (Lanes 4 and 9) and did not enhance the release in the presence of fetuin (Lanes 5 and 10). Taken together, the data suggest that fetuin-induced release of galectin-3 is independent of changes in intracellular calcium.

**Rapid Cell Spreading and Adhesion Modulated by Galectin-3 Released from Cells by Fetuin.** We show here that the 11-9-1-4 breast carcinoma cells spread and adhere to microtiter wells very rapidly in serumless medium containing fetuin (Fig. 4A) compared with adhesion in the absence of fetuin (Fig. 4B). The rapid adhesion and spreading in the presence of fetuin was slowed down considerably in the presence of 100 mM TDG (Fig. 4C). The parental BT-549 cells that do not express galectin-3 failed to spread and adhere in the presence of fetuin (Fig. 4D), requiring an overnight incubation to display cell spreading. The data demonstrate that fast spreading and adhesion to tissue culture plates requires intracellular galectin-3 released rapidly by fetuin. The failure of parental BT-549 to spread and adhere quickly suggests that galectin-3 and not galectin-1 is the relevant lectin. The parental BT-549 cells express high levels of galectin-1 (16).

**Breast Carcinoma Cells Adhere to Elastin in the Presence of Fetuin.** We have previously demonstrated that the interaction of breast carcinoma cells with elastin is heavily dependent on galectin-3 expression (8). In fact galectin-3 binds specifically to elastin and is associated with tropoelastin in breast carcinoma cells, which suggests that this interaction is physiologically relevant (8). The BT-549 (galectin-3 null-expressing cells) adhered poorly to elastin in the absence of 0.25% fetuin. Galectin-3 was released from both 11-9-1-4 and MDA-MB-435 carcinoma cells in the presence of fetuin (Fig. 3B, Lanes 1 and 6, respectively). The inclusion of A23187 in serumless medium (Fig. 3B, Lanes 2 and 7) failed to release galectin-3. The inclusion of A23187/fetuin (Fig. 3B, Lanes 3 and 8) in the serumless medium triggered the release of galectin-3 in levels comparable with controls. Similarly, thapsigargin, the inhibitor of endoplasmic reticulum ATPase, by itself failed to trigger the release of galectin-3 (Lanes 4 and 9) and did not enhance the release in the presence of fetuin (Lanes 5 and 10). Taken together, the data suggest that fetuin-induced release of galectin-3 is independent of changes in intracellular calcium.
was significantly better when compared with adhesion in the absence of fetuin. The interaction of cells with glycans such as laminin and collagen IV, which have polylactosamine residues, may trigger the release of more galectin-3 from intracellular stores. The data suggest that galectin-3 released by fetuin may be directly used to ligate the breast carcinoma cells to elastin-rich tissues such as the lungs.

**DISCUSSION**

In this report, we have demonstrated that fetuin, a serum glycoprotein, is capable of eliciting the rapid release of galectin-3 from breast carcinoma cells in a novel fashion. The data address a fundamental problem in biology, namely the role of fetuin and galectin-3 in cell growth regulation. The elucidation of the nonclassical pathway of secretion of galectin-3 is critical in order to understand its precise physiological role in cell-to-ECM interactions. Galectins, particularly galectin-1 and -3, have long been suspected of regulating the adhesion of a wide variety of cell types to ECM (19). We have previously demonstrated that galectin-3 plays a crucial role in the plating and cloning efficiencies of breast carcinoma cells. The cells that express galectin-3 interact more efficiently with substrata such as laminin, collagen IV, elastin, and soft agar compared with galectin-3 null-expressing cells (7, 8, 20). It is well documented that the galectin-3 gene is involved in tumorigenesis (6, 21, 22) and metastasis particularly of the breast and colon carcinomas (16, 23). More recently, it was reported that the sera of patients with breast cancer, gastrointestinal cancer, lung cancer, ovarian cancer, melanoma, and non-Hodgkin’s lymphoma had significantly higher levels of galectin-3, compared with sera of normal subjects (24). Moreover, galectin-3 concentrations in sera from patients with metastatic disease were higher than in sera from patients with localized tumors (24). This study suggests that circulating galectin-3 plays a role in tumor progression and that fetuin could be a player in the secretion of galectin-3 into the sera.

The rapid release of galectin-3 on contact of the cells with fetuin and not other ECM proteins is interesting because fetuin and its desialylated form (asialofetuin) interact strongly with galectin-3 via the CRD of the lectin. Normally galectin-3 is expressed on the cell surface presumably bound via its CRD to glycans containing polylactosamine residues such as lysosomal-associated membrane proteins (25). Galectin-3 molecules can also interact with each other via their NH2-domains, freeing extra CRD domains to interact with other glycoproteins such as laminins in ECM. We, therefore, suspected that the interaction of cells with glycans such as laminin and collagen IV, which have polylactosamine residues, may trigger the release of more galectin-3 from intracellular stores. The present data demonstrate that this is not the case for any of the glycans examined except fetuin. It is, therefore, apparent that the pathway by which fetuin triggers the release of galectin-3 from the intracellular stores is novel. Previous studies demonstrated clearly that serum is essential for galectin-3 secretion. Apart from fetuin, which is the major serum protein in bovine fetal blood, serum contains numerous proteins. In as much as the present study does not rule out all the other serum proteins in the process of galectin-3 release from intracellular stores, fetuin appears to be the critical factor in serum for the externalization of galectin-3 and hopefully other members of the family.

There are a number of models that have been suggested for galectin-3 secretion. For example, it has been proposed that before secretion, galectin-3 accumulates at sites on the cytoplasmic side of plasma membranes (12–14). This step of accumulation is rate limiting and can be up-regulated by heat shock and calcium ionophores (4, 13, 26, 27). The next step in galectin-3 secretion appears to be evagination of plasma membrane, a process that requires NH2-terminal domains of the protein (15). Finally, the process consists of the pinching off of evaginating plasma membrane domains and the release of galectin-3 from the externalized vesicles. However, as has been noted by others, some galectin-3 molecules may be released from plasma membrane domains directly into the extracellular medium (15). It is this pathway that appears to be supported by our data, because we are defining a process that takes place extremely rapidly (within minutes) and is mediated by fetuin. The molecular mechanisms of this pathway may involve other proteins such as chaperons. Our data clearly demonstrate that galectin-3 is from the cytoplasmic domains and not from the cell surface. The fetuin may induce the secretion of galectin-3/chaperon complex, thereby modulating the last stages of the externalization process and not the rate-limiting step.

On the basis of the data, we propose that the released galectin-3 is immediately recruited to modulate cell spreading and adhesion to the substratum. Galectin-3 may do this by interacting with and activating cell surface adhesion molecules and cytoskeleton elements via its CRD domains because this interaction is abrogated by TDG. Cells that lack galectin-3 but express galectin-1, such as BT-549 breast carci-
noma, lack this rapid cellular adhesion and spreading, as observed previously (7). Interestingly, fetuin has been implicated in cell spreading, stretching, and adhesion in other cell systems (18). The rapid cellular adhesion and spreading that is catalyzed by fetuin and galectin-3 may well explain cell-growth-promoting activities of fetuin (18). The cells that have the capacity to adhere and spread quickly to substrata obviously will have a growth advantage over those that spread and adhere more slowly. We recently demonstrated that the interaction of breast carcinoma cells with elastin could be directly linked to galectin-3 expression because exogenously supplied galectin-3 was able to ligate these cells to elastin. Galectin-3 expressing cells interacted well and proliferated on elastin fibers, but only in the presence of complete medium containing serum (8). We now show that fetuin-mediated release of galectin-3 in serumless DMEM/F12 is sufficient to ligate galectin-3-expressing cells to elastin whereas galectin-3 null-expressing cells are not ligated. The ligation occurred over a 12-h period, because a critical galectin-3 concentration has to be achieved for the adhesion to occur.

It can be argued that the fetuin-induced galectin-3 release is not necessary for in vitro cell growth and differentiation, because fetuin-deficient mice are fertile and mature normally (28). This suggests that there are other proteins or growth factors apart from fetuin that may trigger the rapid release of galectin-3. Alternatively, rapid release of galectin-3 may be relevant only in tumor cells, in which it confers a growth advantage. Similarly, galectin-3 null mutant mice are viable with no abnormalities (29). In this case, other members of the galectin family, such as galectin-5 are likely to substitute for galectin-3. Decrease or lack of tumorigenicity or metastatic potential in either fetuin- or galectin-3-deficient mice would be an interesting observation.

In summary, fetuin can induce a very rapid release of galectin-3 from breast carcinoma cells. This release takes place within 1 min and is necessary for the activation and modulation of cell surface receptors for ECM proteins. The released galectin-3 can also be used to ligate breast carcinoma cells to elastin rich tissues such as lungs during the metastatic dissemination of breast cancer. The novel pathway by which galectin-3 is released is independent of changes in intracellular calcium and temperature. The released galectin-3 can also be used to ligate breast carcinoma to extracellular matrix proteins is modulated by galectin-3. Invasion Metastasis, 17: 101–112, 1997.


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