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13. ABSTRACT (Maximum 200 Words) <p style="text-align: center;">The overall goal of this project is to understand the significance of the interactions between breast carcinoma cells and elastin. The central hypothesis states that expression of galectin-3 on the cell surface of breast carcinoma cells is critical for their interactions with elastin. Our data demonstrate that galectin-3 interacts and can be co-immunoprecipitated by antibodies to elastin from cell lysates of breast carcinoma cells. Cells which express galectin-3 on their cell surfaces adhere and proliferate on elastin fibers while non-expressers do not. In addition we have demonstrated that purified galectin-3 can enhance the adhesion of human breast carcinoma cells to elastin. We have also demonstrated that breast carcinoma cells which express galectin-3 on their surfaces adhere avidly to cryosectioned human lung tissue. The cells can be seen under light microscope specifically bound to lung elastin fibers. In comparison to bovine elastin fibers, the human lung elastin fibers appear to be more superior in their interactions with the breast carcinoma cells. We intend to develop this as a simple assay to evaluate the preferential growth of human breast cancer cells in lung extracellular matrix. In summary our data strongly suggest that the interaction of breast carcinoma cells with elastin plays a critical role in their growth and that galectin-3 is the molecule which modulates this interaction.</p>			
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PROGRESS REPORT.

Josiah Ochieng and Jacqueline Akech

INTRODUCTION

Galectin-3 is a multifunctional housekeeping lectin which has been implicated in several pathological conditions [1-4]. We have recently demonstrated that the expression of galectin-3 by breast carcinoma cells is critical for their interaction with elastin, a member of the extracellular matrix proteins [4]. The overall goal of this project is to demonstrate that the galectin-3 mediated cell/elastin interaction has direct consequences in the proliferative potential of breast carcinoma cells and not merely a trivial cell/extracellular matrix interaction. It is generally believed that relevant extracellular matrix proteins which are required for cellular growth are laminin, collagens, fibronectin proteoglycans and vitronectin. Very little attention has been given to elastin and so this proposal really challenges existing paradigms. These studies will go a long way in defining a specific role for galectin-3 in breast cancer metastasis.

The following are the research activities that have been carried out in the laboratory since the award notice was given in the early part of 1999. The first paper, from the laboratory was accepted and appeared in press in December of 1999. The second paper is now under editorial consideration in Cancer Research. The specific aims of the grant are: a) To establish the biological relevance of galectin-3 in breast carcinoma cells to elastin interactions and b) To Evaluate the Proliferative potential of elastin peptides on breast carcinoma cell. More specifically specific aim 1 was intended to be done through Tasks 1 and 2 as written in the statement of work.

Task 1. *To establish the domains in both galectin-3 and elastin responsible for the binding interactions.*

Galectin-3 is a chimeric molecule which consists of the Amino terminal domain, an R-domain which has the characteristic G-X-Y repeats common in collagen, and the carbohydrate binding domain at the Carboxy terminal end. We have demonstrated that matrix metalloproteinases can cleave galectin-3 into a ~9 kDa and a 22 kDa fragments [5]. Whereas the 22 kDa fragment retains the biological activity of the lectin (sugar binding property), the 9 kDa fragments does not. We have established that the carbohydrate recognition domain of galectin-3 is necessary for its interaction with elastin because lactose abrogates this binding activity [4]. We questioned whether galectin-3 interacts

with some glycoproteins which may be associated with elastin. We therefore heated elastin in hydrazine containing 1% hydrazine sulfate for 8 hours at 80°C. This treatment is capable of removing any sugar residues from elastin. Interestingly when elastin is treated with hydrazine in this manner it dissolves completely. When the dissolved elastin was diluted in PBS to a final concentration of 2 mg/ml, and the wells of a 96-well microtiter plate coated with this solution, galectin-3 was shown to bind to the elastin coated wells. Therefore the interaction of galectin-3 and elastin does not appear to involve carbohydrate moieties that may be associated with elastin.

We have recently observed that whereas the ~ 22 kDa binds to elastin as expected, its affinity for elastin is reduced relative to the intact galectin-3. This was rather interesting because we and others have shown that the ~22 kDa fragment has a higher affinity for glycans when compared to the intact galectin-3 molecule [6]. The data therefore means that the R-domain of galectin-3 may also be required for galectin-3/elastin interaction. We will continue to do more detailed biochemical studies of galectin-3/elastin interactions.

Motifs in Elastin which are responsible for the binding interactions. This proposed experiment posed greater technical difficulties than originally anticipated. The elastase easily dissolved elastin within 2 hours at 37°C. However unknown to us at the time that the grant was written, elastase efficiently hydrolyses elastin and not partially as we anticipated and so it was not possible to obtain sizable elastin fragments that we could separate on h.p.l.c as proposed. Since then as an alternative approach, we have resorted to synthesizing specific elastin peptides such as the repeating VPGVG and testing their binding interactions with galectin-3. We have done several binding studies with VPGVG. Whereas purified galectin-3 can show some binding to this fragment, the results are not consistent. Reproducible and strong binding interactions may require longer peptides which we are currently synthesizing. We have also established that the chemotactic domain of elastin VGVAPG interacts poorly with elastin.

The objective of aim # 1, was to prove unequivocally that galectin-3 interacts with elastin. We have demonstrated this. The tropoelastin that is secreted from breast epithelial cells can be co-precipitated with galectin-3 as depicted in Figure 1. The complex was initially immunoprecipitated with antibodies to galectin-3 (Figure 1A). The complex bound in protein A/G gel was dissolved in sample buffer, subjected to SDS/PAGE and transferred to immunoblot membrane. The membrane was then probed with antibodies to elastin. To confirm the results, we also immunoprecipitated the complex with antibodies to elastin and probed the membrane with antibodies to galectin-3 (Figure 1B)

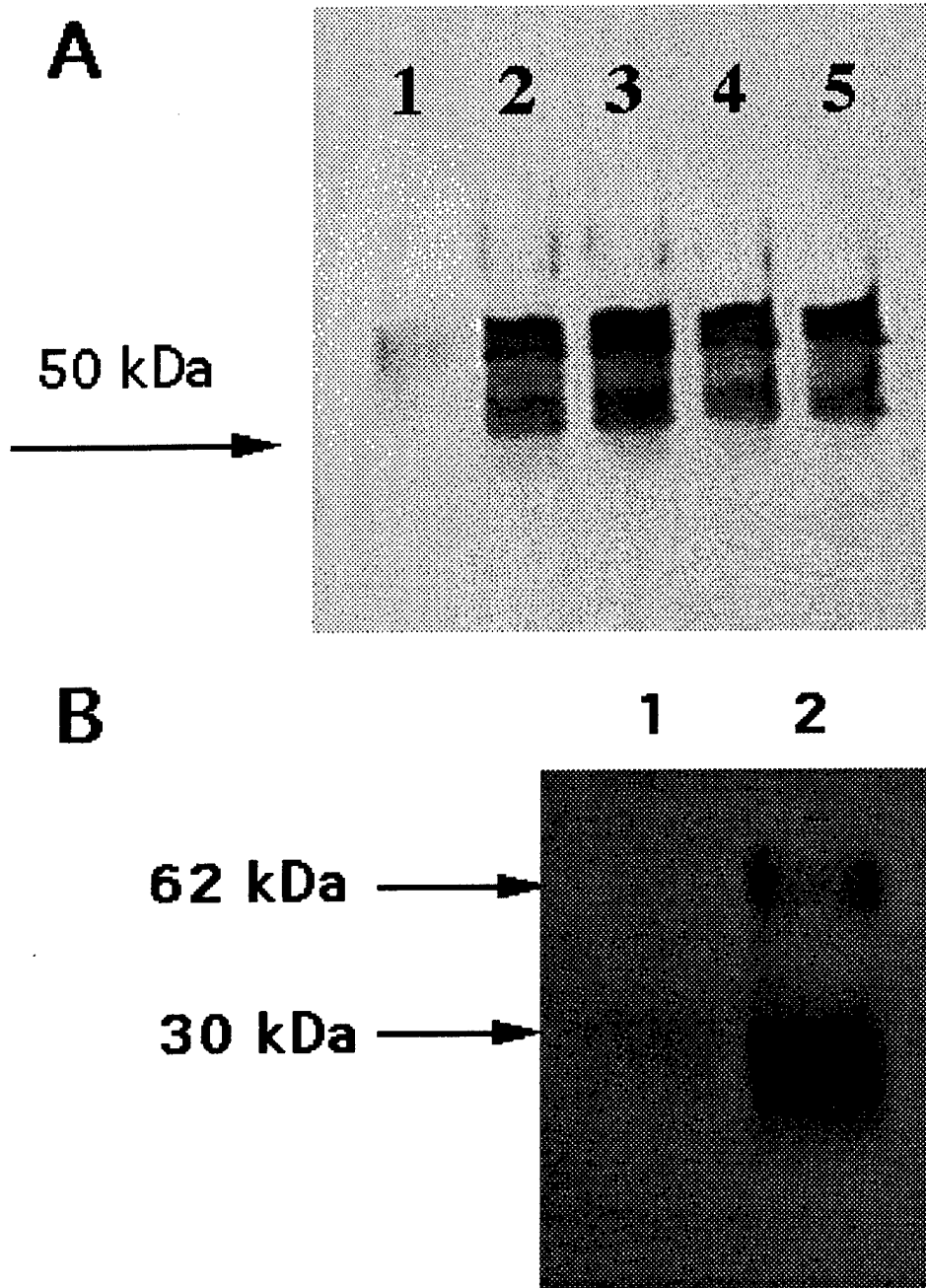


Figure 1. Association of Elastin and galectin-3. Galectin-3 expressing breast carcinoma cells were lysed and the lysate immunoprecipitated with either rabbit pre-immune serum (A, lane 1) or rabbit polyclonal antibodies to recombinant human galectin-3 (A, lanes 2-5). In lanes 2-5, the lysates were incubated with 0, 5, 10, and 20 μ g of recombinant galectin-3 for 2 h at 40C prior to the addition of anti-galectin-3 antibodies. The samples were then resolved by 10% reducing SDS-PAGE, blotted, and the membrane probed with mouse monoclonal antibodies to elastin. The bands represent tropoelastin ~62-67 kDa. The immunoprecipitation was also done with either non-immune mouse serum (B, lane 1) or mouse monoclonal anti-elastin (B, lane 2). In this case the membrane was probed with rat monoclonal anti-galectin-3. The galectin-3 band (30 kDa) as well as a band which most likely represents a galectin-3 dimer (~62 kDa) are represented in B, lane 2.

Task 2a To establish the contribution of cell surface galectin-3 to cell/elastin interactions (months 8-24). We have continued to establish the contribution of cell surface galectin-3 to cell/elastin interactions. Addition of purified galectin-3 to breast epithelial cells significantly enhances their interactions with elastin as shown in Figure 2. Therefore cell surface expressed galectin-3 is capable of ligating breast epithelial cells to elastin. We have now submitted a manuscript to cancer research (in the appendix) where we demonstrate that galectin-3 producing cells require fetuin, a serum glycoprotein, to be able to rapidly secrete galectin-3. The secreted galectin-3 then binds to the cell surface and is then used to ligate cells to elastin .

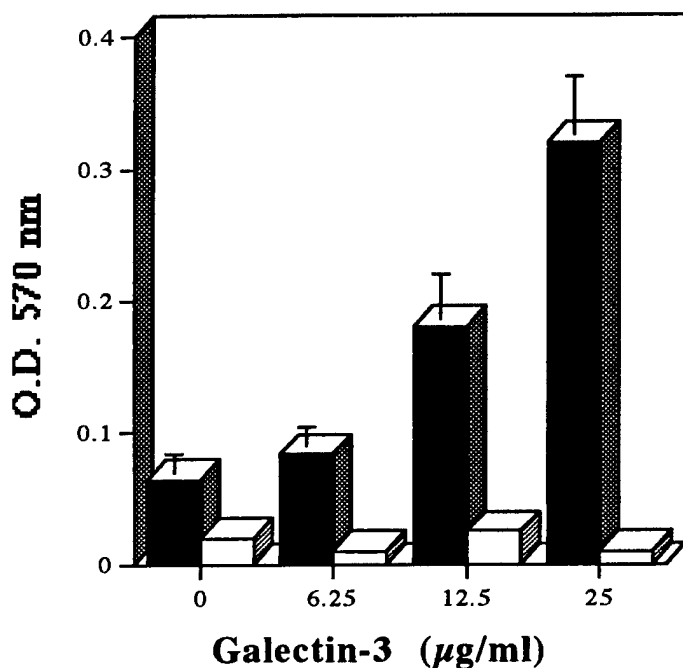
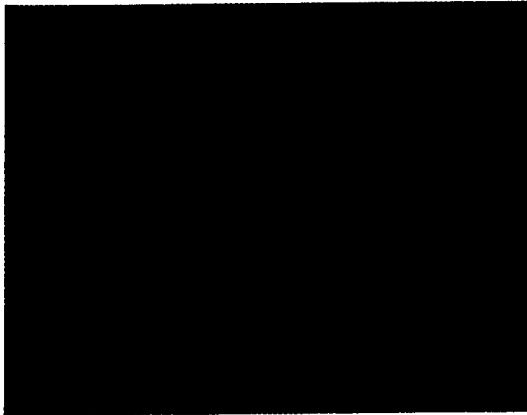


Figure 2. The ligation of breast carcinoma cells (clone 11-9-1-4) to elastin by galectin-3. The cells in calcium free, serumless DMEM/F12 medium were plated at 5×10^4 cells/well in elastin coated microtiter wells (solid bars) in the presence of recombinant galectin-3 (0-25 µg/well). The same number of cells/well were also plated in wells coated with bovine serum albumin (open bars) in the presence of galectin-3 (0-25 µg/well). The adherent cells were determined after 1 hr.

Task 2b. In order to establish that galectin-3 is the lectin which is used when breast epithelial cells interact with elastin, we proposed to use galectin-3 antibodies to abrogate this interaction. We have only been able to block the binding interaction partially with the polyclonal antibodies we have. Interestingly, the polyclonal antibodies effectively retarded the growth of breast carcinoma cells on the elastin fibers. We are currently repeating these experiments for publication. We also hope to obtain function blocking monoclonal antibodies to galectin-3 from Dr. Avraham's laboratory. He has kindly availed all these reagents for us as per letter of agreement (see appendix).

Task 2c. *To evaluate the adhesion of galectin-3 expressing and null expressing cells on elastin rich human lung tissue specimens.* This has turned out to be a very promising study which we hope to develop much further. Briefly, we obtained cryosections of human lung on slides. The slides were immediately flooded with 3% BSA for blocking. Galectin-3 expressing breast epithelial cells (clone 11-9-1-4) and null expressing cells (BT-549) were then detached from the culture flasks by 2 mM EDTA, washed twice in Ca/Mg free PBS and labeled with fluorescein isothiocyanate for 10 min in Ca/Mg free PBS. The labeled cells were washed extensively in PBS and finally suspended in the same buffer containing 0.5% fetuin. The cells (500,000 cells/slide) were then allowed to incubate on the lung sections for approximately 30 min. The slides were washed twice in PBS and the attached cells examined under an epifluorescence microscope. As seen in Figure 3B, the galectin-3 expressing cells interacted very well with the lung tissue (whose extracellular matrix is mainly composed of elastin) compared to galectin-3 null expressing cell line (Figure 3 A). The adhesion of cells to the elastin tissue is drastically reduced in the absence of fetuin which triggers the release of galectin-3 from the cells. Another important observation was that the breast epithelial cells tend to interact with human lung elastin far much better when compared with their interactions with bovine elastin. Many cells were seen under the light microscope adhered to elastin fibers. This experiments has been repeated numerous times with highly reproducible results. We therefore hope to further improve the techniques and use this as a simple assay to characterize different breast epithelial cells with differing metastatic abilities.

A



B



Figure 3. The adhesion of Breast Carcinoma cells to cryosectioned human lung tissue. The parental BT-549 which do not express galectin-3 did not adhere to the tissue within 1 hr (panel A) while the galectin-3 expressing cells, clone 11-9-1-4 did adhere well (panel B, arrow).

Discussion.

The overall goal of this proposal was to establish the significance of the involvement of galectin-3 in cell to elastin interaction in breast cancer tumorigenesis. The interaction of breast carcinoma cells with elastin can either be of no consequence or can be of great significance in the growth of breast carcinoma cells in the organs colonized by them during the metastatic process. One of the sites that breast cancer normally metastasizes to is the lung. We are currently dissecting the significance of elastin especially in elastin rich tissues such as the lungs in the proliferation of breast carcinoma cells. In these studies we incubate breast carcinoma cells on cryosectioned lung tissue in PBS which is devoid of divalent ions to rule out the participation of integrins in the adhesive interactions. The central question here is whether or not the interaction of cells with lung elastin stimulate the growth of the breast carcinoma cells with the implication that this growth stimulation could be taking part in vivo. Interestingly, our observation that breast carcinoma cells

clone 11-9-4 interact far much better with human lung elastin as opposed to bovine elastin poses newer thought provoking questions. One of our objectives is to now explore these questions in more details.

In the next grant period we will concentrate more in addressing specific aim # 2 and Tasks 2 and 3. We will use antisense oligos as well as function blocking antibodies. We will then proceed to use the dominant negative galectin-3 constructs in an attempt to block the adhesion of breast carcinoma cells to elastin rich tissues.

6. KEY RESEARCH ACCOMPLISHMENTS:

- We have firmly established that galectin-3 does indeed interact with elastin in vivo.
- We have shown that when breast epithelial cells interact with elastin, they are able to proliferate.
- The rapid interaction of human breast carcinoma cells with human lung extracellular matrix implies a simple assay to examine the propensity of breast epithelial cells to colonize the lung tissue.

7. REPORTABLE OUTCOMES.

- A manuscript which was submitted before the award notice was received was accepted and published in December 1999 and is included in appendix
- A manuscript which was submitted this month to Cancer Research in which the support by USAMRMC was acknowledged is included in the appendix.
- A graduate student, Jacqueline Akech is supported entirely by this grant.

8. CONCLUSIONS:

In conclusion, we have demonstrated that the interactions of breast carcinoma cells with elastin has significant implications in cellular growth in vivo. It is therefore our intention to pursue this line of research by using the preliminary data obtained thus far and applying for major grants to support the work. I would like to take this opportunity to thank USAMRMC for the support. We, in minority institutions find it very difficult to attract research dollars because of inadequate critical mass of manpower and this grant has significantly pushed my research, enabling me to submit a publication to Cancer Research. We are in the process of developing a simple assay to evaluate the ability of tumor cells to interact with lung extracellular matrix. I think this assay will be able to address a number of critical questions regarding the biological properties of metastatic cells. We have also for the first time demonstrated a link between fetuin expression and galectin-3 secretion in breast carcinoma cells. These are indeed 'medical products' obtained from this research effort so far.

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APPENDICES

Rapid Release of Intracellular Galectin-3 from Breast Carcinoma Cells by Fetuin ¹.

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Running Title: Release of Galectin-3 by Fetuin.

Key words: Fetuin, Galectin-3, Carcinoma, Secretion, Breast.

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ABSTRACT.

Galectin-3, a beta galactoside binding protein, plays a significant role in cell to extracellular matrix interactions. Despite its extracellular expression, the precise physiological mechanisms which 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 which is abundant in the fetal serum, is capable of inducing the rapid release (~ 1 minute) of intracellular galectin-3 from the cells. The mechanism by which galectin-3 is released rapidly appears to be novel and does not depend on changes in intracellular calcium levels. We also report that galectin-3 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 beta galactosides and significant sequence homology in their carbohydrate binding domains (1-3). Galectin-3 is expressed in the nucleus, 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 which may be responsible for tumor emboli (5,14). It has also been shown to be responsible for rapid adhesion of breast carcinoma cells to extracellular matrix proteins such as collagen IV, laminin and elastin (6,7).

Galectin-3 like many other cytosolic proteins such as thioredoxin (8), interleukin-1 β (9), acidic and basic fibroblast growth factor (10) can traverse the plasma membrane and yet they lack signal peptides necessary for secretion via the classical secretory pathway. The mechanisms by which galectin-3 and other proteins which lack signal peptides are secreted via the non-classical pathway have yet to be elucidated. Galectin-3 may be concentrated in secretory vesicles which are concentrated in the membrane domains (11). It has been shown that N-terminal domain of galectin-3 is critical for its secretion and is the driving force which localizes it in the secretory vesicles (12,13,22). How galectin-3 moves from these vesicles into the ECM or the mechanism which 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 containing serum, it is dramatically reduced in serumless medium (4). In the present study, we have exposed breast carcinoma cells to different extracellular matrix 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 induces cell aggregation by binding to lectin-like molecules (14). More recently, it was demonstrated that insect cells which 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 which 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. All the cell lines were cultured in DMEM/F12 (Sigma; Cat. # D-9785) supplemented with 100 μ g/ml penicillin-streptomycin, 2.5 μ g/ml Fungizone, 20 ng/ml epidermal growth factor, 98 ng/ml cholera toxin and 10% heat inactivated fetal bovine serum, 2 mM glutamine and non-essential amino acids. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Human extracellular matrix was purchased from Collaborative Research and all 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 μ l of serumless medium added to siliconized eppendorf tubes containing 100 μ l 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 extracellular matrix (hECM) 2 mg/ml d) Thiodigalactoside (TDG) 100 mM e) Lactose 100 mM f) 10% serum and g) 0.25% fetuin. The samples were incubated for 10 min. at room temperature, centrifuged to pellet the cells and the supernatant from each tube (20 μ l) assayed for galectin-3 by western blot as previously described (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. In order to demonstrate that the secreted fetuin is from intracellular domains and not the cell surface, cell surface proteins were labeled with biotin and then the labeled proteins chased after treatment of the cells with fetuin. Briefly, cells in 75 cm² culture dishes were washed with serumless medium (X5) and then with PBS (X2). 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 flasks washed thoroughly with PBS, and then trypsinized. Trypsin was inactivated by addition of complete medium containing serum and centrifuged. The cells were washed twice with serumless medium and then divided into two eppendorf tubes (500,000 cells/tube) in

100 μ l of serumless medium without (control) or with 0.25% fetuin. The cells were then incubated for 30 min. at 37°C, centrifuged and 20 μ l of the supernatant 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 subjected to SDS-PAGE. The gels were blotted onto nitrocellulose, incubated with avidin-peroxidase followed by chemiluminescence reagents as described (6).

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 μ M 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 made using a SPEX dual wavelength (AR-CM) fluorometer. After about 100 sec. of stabilization, fetuin was added to a final concentration of 0.25% and changes in intracellular calcium ion concentrations monitored.

Effects of Calcium ionophore A23187 and Thapsigargin in the Rapid release of galectin-3 from Breast Carcinoma Cells. To determine whether agents which increase intracellular calcium could induce the rapid release of galectin-3, the assay was done in the absence or presence of 5 μ M of A23187 in serumless DMEM/F12 containing 1 mM CaCl_2 . The assay was also done in the presence of 5 μ M of A23187 and 0.25% fetuin. Thapsigargin (1 μ g/ml), a known inhibitor of the endoplasmic reticulum Ca^{2+} 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 to galectin-3 null expressing cells (6,16). In all 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 Ca^{2+} ions. The adhesion assay was also done in the presence of 0.25% fetuin and 100 mM thiodigalactoside (TDG). As a negative control (lack of rapid adhesion and spreading), BT-549 cells which 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 photographed by a digital camera and images analyzed by adobe photoshop.

Adhesion of Breast Carcinoma Cells to Elastin in the Absence and Presence of Fetuin. In order to further implicate galectin-3 in cell to extracellular matrix 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 hours at 80°C until all the elastin was dissolved. This was

then diluted with PBS to a final concentration of 2mg/ml. The wells of a microtiter plate were then coated with the solubilized elastin for 1 hr at 37°C. Non-specific sites were blocked with 2% BSA and cells 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 hours and the non-adherent cells washed off in the serumless medium. The adhered cells were photographed and the number of cells adhered 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 ten minutes as we routinely did in this report. As shown in Figure 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, alpha 2HS glycoprotein (2 mg/ml) (data not shown). It is evident from Figure 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 one minute of exposure of cells to fetuin (Figure 1C). Prechilling the cells at 4°C prior to adding fetuin did not affect the release of galectin-3 (data not shown) demonstrating 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), implying that 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 which is rapidly secreted into the medium upon fetuin stimulation is cell surface bound and 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 Figure 2, all the label was retained in the cell membrane (Figure 2, lanes 1 and 2). The conditioned medium in both the control (Figure 2, lane 3) and fetuin treated (Figure 2, lane 4) tubes did not reveal 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 both the control and fetuin treated conditioned medium. Moreover all these bands were above 40 kDa (data not shown).

Intracellular Calcium Ion Concentration in Fetuin Induced Galectin-3 Release. In order 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 addition of fetuin. Increases in intracellular calcium ion concentration have been shown to stimulate galectin-3 secretion (4,13). As can be seen in Figure 3A, fetuin raised intracellular calcium in 11-9-1-4 transiently by about 30%. However in the MDA-MB-435 cell line, addition of fetuin actually reduced intracellular calcium levels (Figure 3A). We also challenged the cells with agents which are known to increase intracellular Ca^{2+} ion concentration in the absence and presence 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 (Figure 3B, lanes 1 and 6 respectively). The inclusion of A23187 in serumless medium (Figure 3B, lanes 2 and 7) failed to release galectin-3. The inclusion of A23187/fetuin (Figure 3B, lanes 3 and 8) in the serumless medium triggered the release of galectin-3 in levels comparable to 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 (Figure 4A) compared to adhesion in the absence of fetuin (Figure 4B). The rapid adhesion and spreading in the presence of fetuin was slowed down considerably in the presence of 100 mM TDG (Figure 4C). The parental BT-549 which do not express galectin-3 failed to spread and adhere in the presence of fetuin (Figure 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 suggest that galectin-3 and not galectin-1 is the relevant lectin. The parental BT-549 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. In fact galectin-3 binds specifically to elastin and is associated with tropoelastin in breast carcinoma cells, suggesting that this interaction is physiologically relevant (7). The BT-549 (galectin-3 null expressing cells) adhered poorly to elastin in the absence (control) or presence of 0.25% fetuin as was expected (Figure 5A and B). The 11-9-1-4 cells (galectin-3 expressing) on the other hand adhered very well to elastin in the presence of fetuin. The interaction was significantly better when compared to adhesion in the absence of fetuin (Figure 5A and B). 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 non-classical pathway of secretion of galectin-3 is critical in order to understand its precise physiological role in cell to extracellular matrix interactions. Galectins, particularly galectin-1 and -3 have long been suspected of regulating the adhesion of a wide variety of cell types to extracellular matrix (19). We have previously demonstrated that galectin-3 plays a crucial role in the plating and cloning efficiencies of breast carcinoma cells. The cells which express galectin-3 interact more efficiently with substrata such as laminin, collagen IV, elastin, and soft agar compared to galectin-3 null expressing cells (6,7,20). It is well documented that the galectin-3 gene is involved in tumorigenesis (14,15,21) 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 to 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 upon 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 carbohydrate recognition domain (CRD) of the lectin. Normally galectin-3 is expressed on the cell surface presumably bound via its CRD to glycans containing polylectosamine residues such as lysosomal associated membrane

proteins (25). Galectin-3 molecules can also interact with each other via their N-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 that have polylectosamine residues may trigger the release of more galectin-3 from intracellular stores. The present data demonstrate that this is not the case for all 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 at cytoplasmic side of plasma membranes (11-13). This step of accumulation is rate limiting and can be up-regulated by heat shock and calcium ionophores (4,12,26,27). The next step in galectin-3 secretion appears to be evagination of plasma membrane, a process that requires N-terminal domains of the protein (22). Finally, the process consists of 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 (22). It is this pathway that appears to be supported by our data, because we are defining a process which 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 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.

Based on 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 since this interaction is abrogated by TDG. Cells which lack galectin-3 but express galectin-1 such as BT-549 breast carcinoma lack this rapid cellular adhesion and spreading as previously observed (6). Interestingly, fetuin has been implicated in cell spreading, stretching and adhesion in other cell systems (18). The rapid cellular adhesion and spreading which is catalyzed by fetuin and galectin-3 may well explain cell growth promoting activities of fetuin (18). The cells which have the capacity to adhere and spread quickly to substrata obviously will have a growth advantage over those which 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 (7). We now show that fetuin mediated release of galectin-3 in serumless DMEM/F12 is sufficient to ligate galectin-3 expressing cells to elastin while galectin-3 null expressing cells are not ligated. The ligation occurred over 12 hour 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 vivo* 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, which

may trigger the rapid release of galectin-3. Alternatively, rapid release of galectin-3 may be relevant only in tumor cells where 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 one minute 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 galectin-3 is most likely released from vesicles close to the plasma membrane. The release it is not influenced by factors which normally affect the exocytosis or endocytosis pathways. The present data suggest the mechanism (s) by which fetuin may modulate the cellular adhesion and growth of breast epithelial cells in vitro and in vivo.

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Figure Legends.

Figure 1. *Fetuin mediated release of Galectin-3 from MDA-MB-435 breast carcinoma cells into the medium.* In panel A, the cells (500,000 cells/tube) were exposed to the extracellular matrix proteins, beta galactoside sugars, serum and fetuin in serumless DMEM/F12 for 10 min. and released galectin-3, assayed by western blot. In panel B, the cells were exposed to various concentrations of fetuin for 10 min. and galectin-3 assayed. In panel C, the cells were exposed to 0.25% fetuin for the stated time points and galectin-3 assayed.

Figure 2. *Cell surface is not the source of released galectin-3.* The cells (11-9-1-4) were biotinylated, washed and the cells incubated without or with fetuin in serumless medium for 30 min. Aliquots of the cell pellet fraction and conditioned medium were run through SDS-PAGE, transferred to immobilon and the membrane incubated with avidin-peroxidase, and chemiluminescent reagents and exposed to X-ray film as described in Materials and Methods. All the label was retained in the cell pellet fraction.

Figure 3. *Intracellular calcium ion concentration changes and release of galectin-3.* In panel A, intracellular calcium ion changes were determined after the addition of 0.25% fetuin (arrow) to fura 2 AM loaded breast carcinoma cells. The experiment was repeated 3 times with similar results. In panel B, 11-9-1-4 and MDA-MB-435 respectively, were treated with the following reagents in serumless DMEM/F12 containing 1 mM Ca^{2+} and galectin-3 assayed as described: 0.25% fetuin (lanes 1 and 6); calcium ionophore A23187 (5 μM)(lanes 2 and 7); a combination of A23187 and fetuin (lanes 3 and 8); thapsigargin (1 $\mu\text{g/ml}$)(lanes 4 and 9); and a combination of thapsigargin and fetuin (lanes 5 and 10).

Figure 4. *Control of rapid cell spreading and adhesion by fetuin.* The 11-9-1-4 cells (galectin-3 expressing) were plated in the wells of a microtiter plate in the presence (panel A) or absence (panel B) of 0.25% fetuin in serumless DMEM/F12 containing 1 mM Ca^{2+} . The cells were also plated in the presence of both fetuin and 100 mM TDG (panel C). As a

control BT-549 (no galectin-3) were plated in the presence of fetuin (panel D). The cells were allowed to adhere for 30 min. and then photographed by a digital camera (phase contrast)

Figure 5. *Ligation of Breast carcinoma cells to elastin.* In panel A, the wells of a microtiter plate were coated with hydrazine solubilized elastin. After blocking non-specific sites with 2% BSA, the cells (5×10^4 cells/well) were plated in quadruplicates and allowed to adhere to the wells in serumless DMEM/F12 medium without divalent ions and without (control) or with 0.25% fetuin and allowed to adhere for at least 12 hours at 37°C. The non-adherent cells were washed twice and the adherent cells photographed. In panel B, after photographing the cells, they were fixed in methanol and cell number determined. The bars represent average number of cells/well .

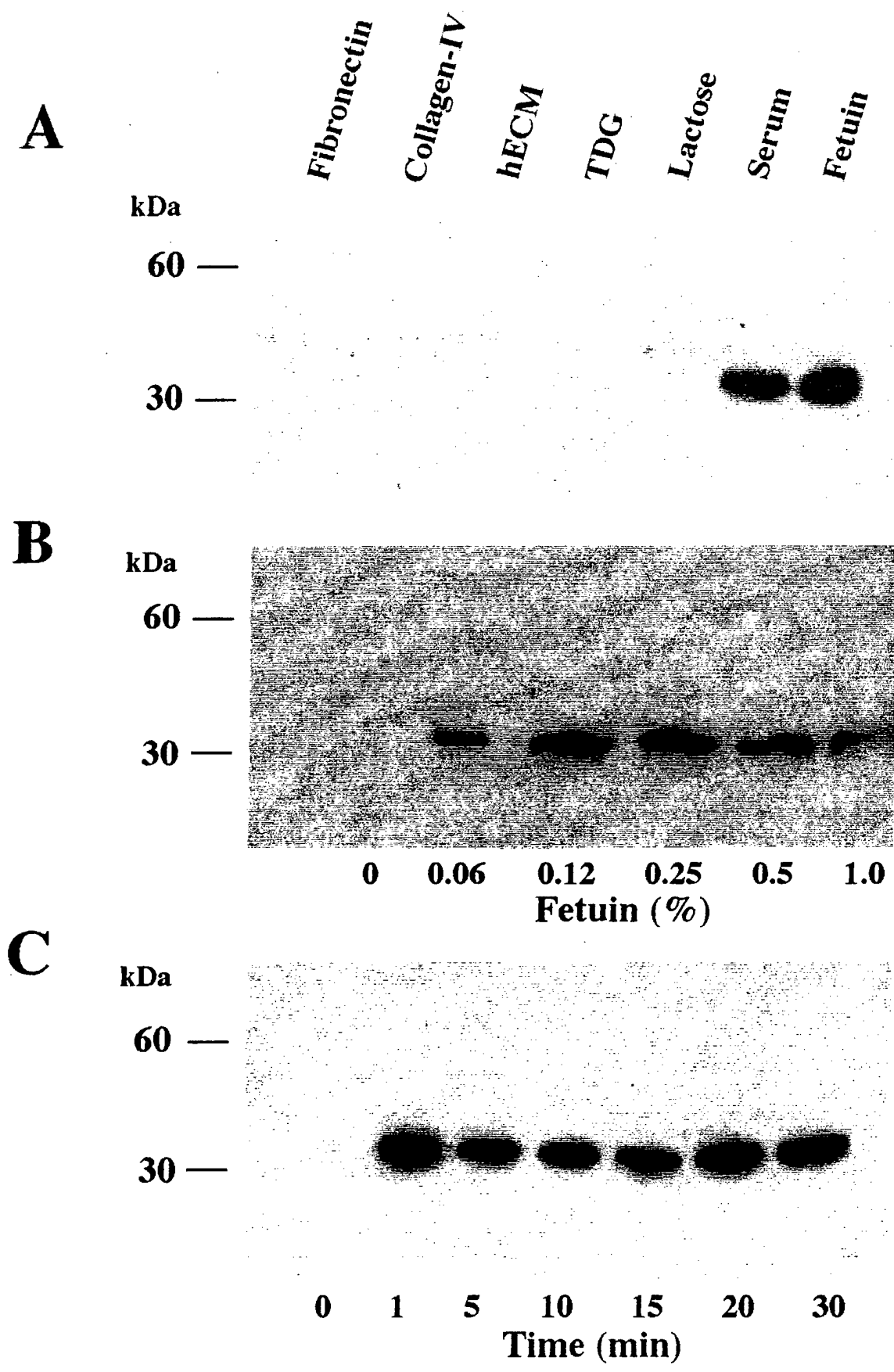


Figure 1

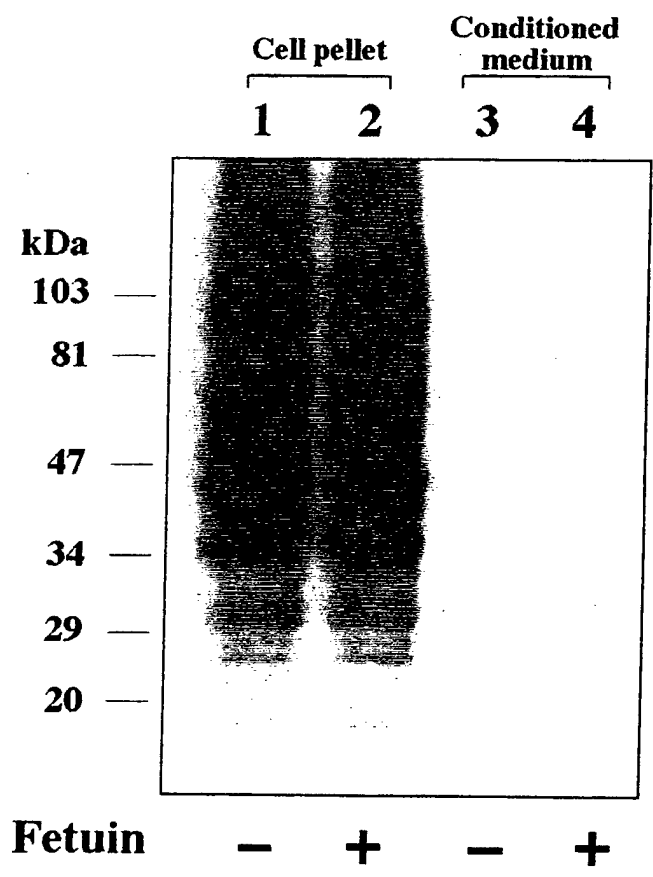
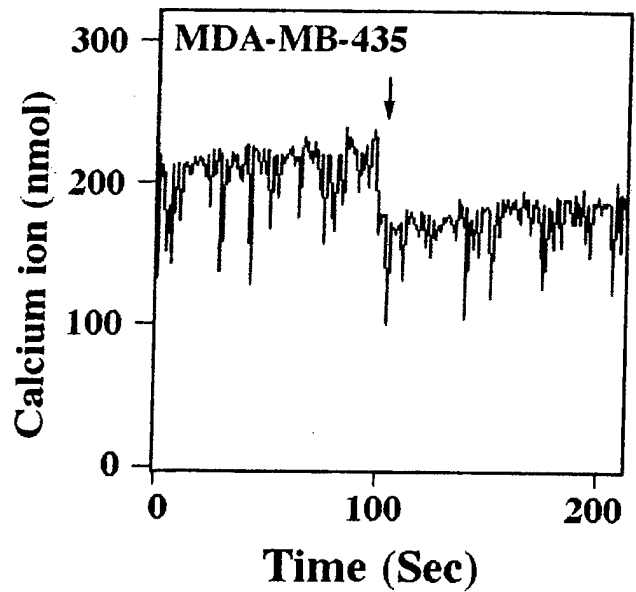
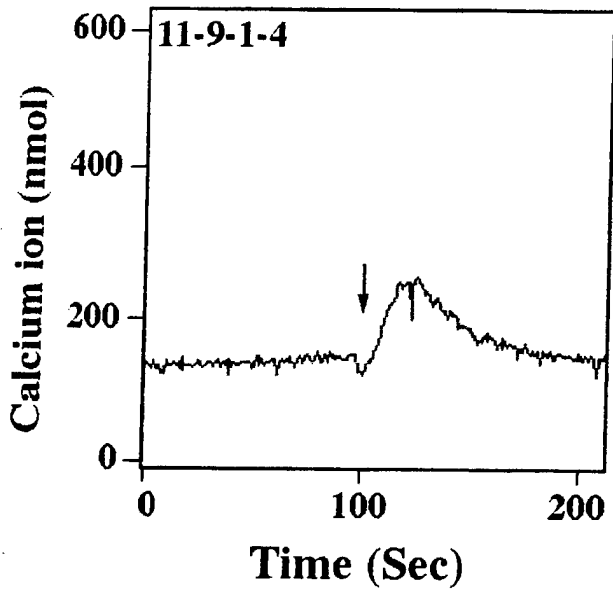


Figure 2

A



B

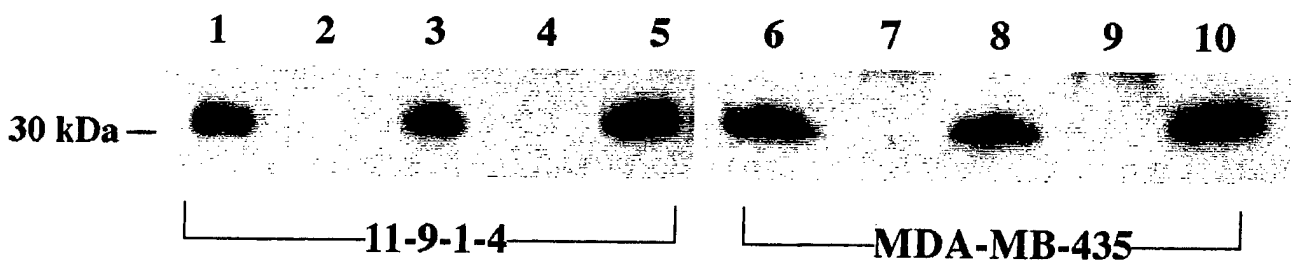


Figure 3

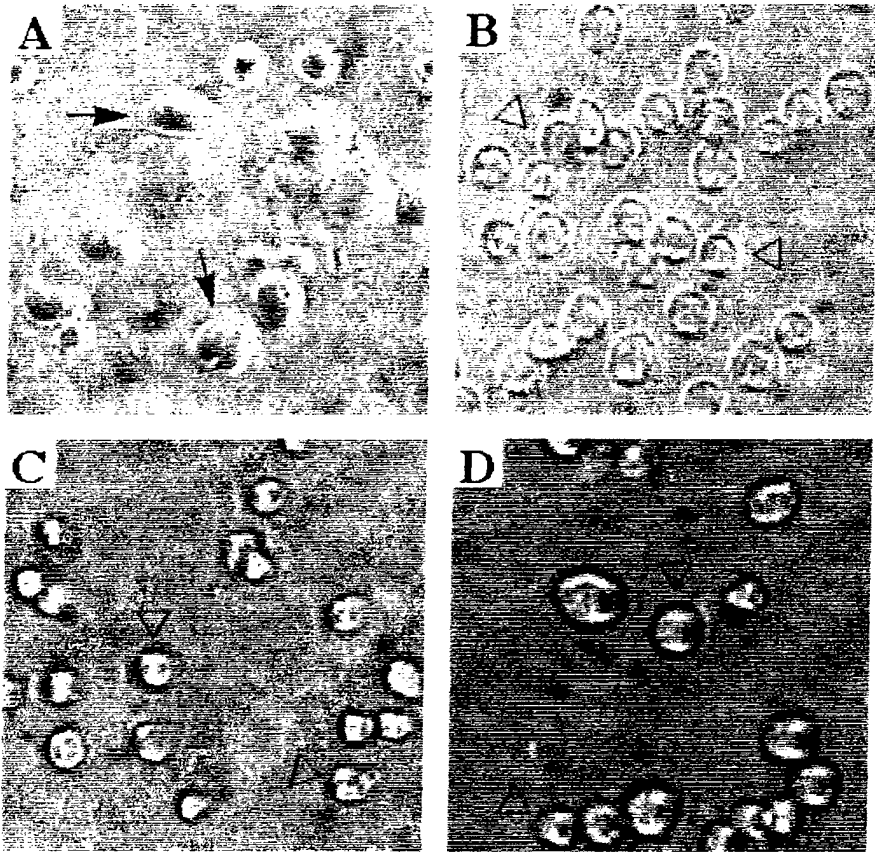


Figure 4

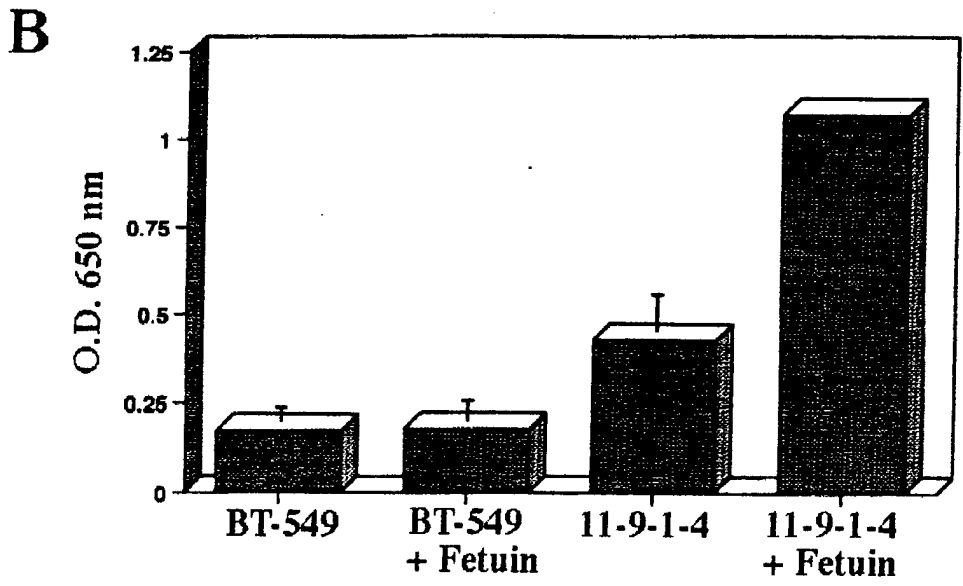
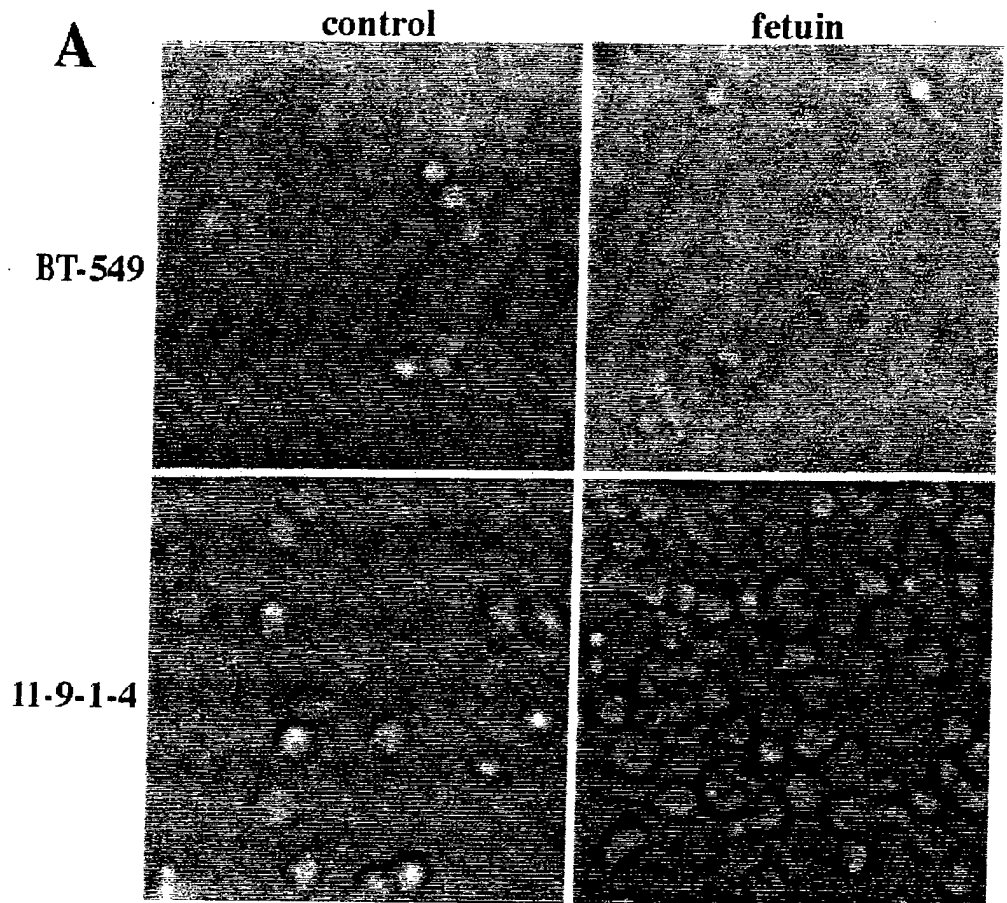


Figure 5

Galectin-3 Regulates the Adhesive Interaction Between Breast Carcinoma Cells and Elastin

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Abstract Galectin-3 is a beta-galactoside binding lectin whose precise physiological role is not yet defined. In the present studies, we questioned whether galectin-3 plays a role in the adhesion of breast carcinoma cells to elastin. The impetus for this analysis was the initial observation that the cellular receptor for elastin, the 67 kDa elastin/laminin protein may have galectin-like properties (Mecham et al. [1989] *J. Biol. Chem.* 264:16652–16657). We therefore analyzed the adhesion of breast carcinoma cells to microtiter wells coated with elastin under conditions which eliminate integrin participation in adhesion. The adhesion assay was done in the absence and presence of purified recombinant galectin-3. We hereby demonstrate that high concentrations of galectin-3 ligate breast carcinoma cells to microtiter wells coated with elastin. Galectin-3 also demonstrated a specific binding interaction with purified elastin in a dose and lactose dependent manner. Furthermore we demonstrated by immunoprecipitation that endogenous galectin-3 in breast carcinoma cells is associated with tropoelastin. Lastly, the breast carcinoma cells which expressed galectin-3 on their surface, demonstrated enhanced cellular proliferation on elastin compared to galectin-3 null expressing cells. These studies suggest that galectin-3 is capable of regulating the interactions between cells and elastin. *J. Cell. Biochem.* 75:505–514, 1999. © 1999 Wiley-Liss, Inc.

Key words: galectin-3; elastin; ligation; breast; carcinoma

Whereas the precise physiological role of galectin-3 is still elusive, mounting evidence suggests its involvement in the regulation of cell-extracellular matrix interactions [Sato and Hughes, 1992; ochieng et al., 1992; Warfield et al., 1997; Kuwabara and Liu, 1996]. In breast epithelial cells, low expression of galectin-3 has been linked to low cloning and plating efficiencies while high expression improves the interaction of cells with different substrata [Makker et al., 1995; Warfield et al., 1997]. The mechanisms by which galectin-3 modulates the interactions of cells with extracellular matrices is not well defined, and may vary in different cell types and at different stages of differentiation or transformation. For example, it has been demonstrated that galectin-3 expression is crucial for transformed cells to acquire the anchorage independent growth in soft agar [Raz et al.,

1990; Makker et al., 1995]. Recent studies suggest that galectins modulate cell to extracellular matrix interactions in a novel fashion by interaction with integrins such as $\alpha 7\beta 1$ or $\alpha 1\beta 1$ [Gu et al., 1994; ochieng et al., 1998].

It is presumed that cells interact with elastin via the non-integrin 67 kDa elastin/laminin receptor [Lesot et al., 1983; Malinoff and Wicha, 1983; Rao et al., 1983]. Whereas the mature receptor on the cell surface is 67 kDa, its full-length gene encodes only a 37 kDa precursor protein [Yow et al., 1988; Rao et al., 1989]. It is believed the precursor is bonded to a ~30 kDa protein to form the mature chimeric receptor. Since the discovery of this receptor, it has been known that it has galectin-like properties because lactose modifies its biological activities. More recently, the work of Buto et al. [1998], suggested that the 67 kDa receptor is a heterodimer stabilized by strong intramolecular hydrophobic interactions, carried by fatty acids bound to the 37 kDa precursor and to a galectin-3 cross-reacting molecule. We have therefore hypothesized that galectin-3 is part of the 67 kDa elastin/laminin receptor complex

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and contributes to the cell-elastin or cell-laminin interactions.

On the surface of mesenchymal cells, there are high affinity receptors for elastin-peptides. These peptides are capable of triggering intracellular signaling which may be responsible for synthesis or recruitment of cell surface ~120 kDa receptors for insoluble elastin [Groult et al., 1991]. It is possible that similar signaling responses may be triggered in non-mesenchymal cells. Breast carcinoma cells interact well with insoluble elastin [Parsons et al., 1991], and the elastin receptors in these cells may be activated in a fashion similar to the mesenchymal cells. Despite recent progress in the characterization of the 67 kDa elastin receptor, our knowledge of cell-elastin interactions still lag behind the well characterized cell-laminin or cell-collagen interactions. It is likely that in regards to tumor growth either in situ or in micrometastases, cell-elastin interactions are as important as the interactions of cells with either laminin or collagen IV.

We hereby demonstrate that galectin-3 at high extracellular concentrations, can ligate breast carcinoma cells to elastin. We further demonstrate that galectin-3 is tightly associated with insoluble elastin, an association which can be downregulated by lactose. Our data also suggest that galectin-3 is associated with soluble tropoelastin intracellularly and is an integral part of the cell to elastin receptor complex.

MATERIALS AND METHODS

Human breast carcinoma cell lines BT-549; Sk-Br-3; MDA-MB-435; and 11-9-1-4 (derived from BT-549) [Makker et al., 1995] were obtained from Dr. Avraham Raz, Karmanos Cancer Institute. All the cell lines were cultured in DMEM (Sigma) supplemented with 100 µg/ml penicillin-streptomycin, 2.5 µg/ml Fungizone, 20 ng/ml epidermal growth factor, 98 ng/ml cholera toxin, 10% heat inactivated fetal bovine serum, 2 mM glutamine, and non-essential amino acids. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Recombinant galectin-3 was isolated and purified as described [Ochieng et al., 1993].

Ligation of Breast Carcinoma Cells to Elastin by Recombinant Galectin-3

Insoluble elastin was washed extensively with PBS containing 1 M NaCl to dissociate any non-specifically bound protein. The elastin fi-

bers were then resuspended in PBS at 4 mg/ml and the suspension (100 µl/well) added to the wells of an ELISA micro-titer plate (Immulon 1B, Dynex Technologies) and incubated overnight at 37°C. The wells were then washed once with PBS and 200 µl of 3% heat inactivated fatty acid free bovine serum albumin in PBS added to each well to block non-specific sites for 1 h at 37°C. The wells were once more washed with PBS and serumless, calcium-free DMEM/F12 medium containing increasing doses (0-25 µg/well) of galectin-3 added to each well and allowed to incubate for 15 min at 37°C. The breast carcinoma cells, BT-549 (subclone 11-9-1-4) cells were then added to the wells at 5×10^4 cells/well in the serumless medium and allowed to incubate for 1 h at 37°C. The same number of cells/well were also plated in wells coated with 3% bovine serum albumin (open bars) in the presence of galectin-3 (0-25 µg/well) and incubated as above. The wells were then washed (3×) with serumless medium and the number of cells ligated to elastin per well determined by the Alamar blue method as previously described [Warfield et al., 1997].

To test whether the cells adhere to glycoproteins which may be associated with elastin fibers such as fibrillin, the elastin (5 mg/ml) was treated with anhydrous hydrazine and 1% hydrazine sulfate overnight at 80°C. The solubilized elastin was then used to coat the wells of a microtiter plate and cellular adhesion performed as described above.

Growth of Breast Carcinoma Cells on Elastin

Insoluble elastin was washed and added to ELISA microtiter plates as described above. Breast carcinoma cells (galectin-3 expressing and null expressing cell lines, 2×10^4 cells/well) were then added to wells containing elastin after blocking with BSA, in DMEM/F12 medium containing 10% serum and allowed to grow for up to 10 days in a humidified CO₂ incubator. The cells were then photographed using a digital camera (Kodak) and the images analyzed by Adobe Photoshop.

Binding of Galectin-3 to Elastin

Insoluble elastin was washed with high salt and added to ELISA microtiter plate as described above. After blocking the non-specific sites with 3% bovine serum albumin, serially diluted aliquots of recombinant galectin-3 (0-16 µg/well) were added to the wells in duplicates

in the absence and presence of 200 mM lactose. To demonstrate elastin binding specificity, galectin-3 in the same concentration range was also added to wells coated with BSA alone. After 1 h of incubation at 37°C, the wells were washed twice with PBS containing 0.05% tween and 100 µl of rabbit polyclonal antibodies to galectin-3 (1:500) added to each well and incubated at 37°C for 30 min. The wells were then rinsed 3× with PBS and 50 µl of biotinylated goat anti-rabbit IgG (Vectastain-ABC kit) added to each well and incubated for 5 min at 37°C. After washing the wells (3×) with PBS, 50 µl of Vectastain ABC reagent was added to each well and incubated for a further 5 min at 37°C. The wells were then washed extensively (6×) with PBS and then alkaline phosphate substrate (Blue Phos™) added and absorbance (650 nm) determined by a microplate reader (Dynex Tech.).

Immunoprecipitation of Elastin-Galectin-3 Complex

In order to establish the physiological relevance of elastin-galectin-3 interactions, we questioned whether the two proteins are associated either intracellularly or on the cell surface of breast carcinoma cells which express both galectin-3 and elastin. The cells were lysed in lysis buffer (calcium/magnesium free PBS containing, 0.5% NP 40, 1 mM EDTA, 2 mM PMSF, pH 7.5). The lysate was pre-cleared by incubating for 2 h with protein A/G agarose and aliquots (200 µg of protein each) of the lysate incubated with 0, 5, 10, and 20 µg of recombinant galectin-3 for 2 h at 4°C followed by either rabbit pre-immune serum or rabbit polyclonal anti-galectin-3 antibodies (10 µg/tube) for 10 h at 4°C in eppendorf tubes. Protein A/G agarose was then added to each tube and allowed to incubate for 2 h at 4°C. The agarose was then washed 5× with PBS containing 0.05 % tween and boiled for 5 min with 1X SDS-sample buffer. The samples were then analyzed by 10% SDS-PAGE, transferred to immobulin-P membrane and probed with mouse anti-human elastin which recognizes bands of tropo-elastin in Western blots. The membranes were washed and probed with peroxidase labeled sheep anti-mouse IgG, followed by incubation with Amersham chemiluminescence reagents and exposed to X-ray film. In another set of experiments, the lysates (200 µg of protein each) were incubated with either non-immune mouse

antibodies or mouse monoclonal anti-elastin (Sigma) after pre-clearance. The immunoprecipitation was done as described above, and the membranes probed with rat monoclonal anti-galectin-3 (TIB 166) [Warfield et al., 1997]. The membranes were washed and then probed with peroxidase labeled goat anti-rat IgG and the bands visualized as described above.

RESULTS

Interactions of Breast Carcinoma Cells With Elastin

Strong adhesion of cells to extracellular matrix proteins depend on a number of factors, such as the activation state of the cell surface receptors and optimal biochemical conditions such as pH, divalent cations, and incubation temperatures. In the case of cell to elastin adhesion, despite the lack of involvement of integrins, divalent cations, and serum factors are needed for optimal interaction with insoluble elastin [Ochieng et al., unpublished data]. The divalent ions and serum factors may be necessary for the activation or recruitment of the 67 kDa elastin receptor. In analyzing the involvement of galectin-3 in cell to elastin interaction, we employed conditions which were unfavorable for integrins (lack of divalent ions). Biological activity of galectin-3 is independent of divalent cations and serum, especially when using exogenously supplied protein. Serum was excluded in the medium because serum proteins such as fibronectin and fetuin could potentially interact with elastin and promote cellular adhesion via other receptors.

In the adhesion assays, the insoluble elastin after washing in buffer containing high salt, was immobilized very well to the floor of the wells and could not be removed by gentle washing. We have consistently shown that galectin-3 expressing breast carcinoma cells such as 11-9-1-4 and MDA-MB-435 interact much better with elastin compared to low galectin-3 expressing cells such as SK-Br-3. In our hands, we have repeatedly observed that approximately 10–20% of the galectin-3 expressing cells when added to micro-titer wells coated with elastin will adhere within 1 h of incubation at 37°C. In the case of low galectin-3 expressing cells, less than 5% of the added cells adhere after 1 h of incubation (data not shown). This phenomenon is true for other extracellular matrix proteins, laminin, and collagen IV [Warfield et al. 1997].

We therefore wanted to determine whether exogenously added galectin-3 could improve the interaction of breast carcinoma cells with elastin. Our data (Fig. 1) demonstrate that galectin-3 by itself can significantly promote the adhesion of cells to immobilized elastin. The galectin-3 mediated ligation of cells to elastin was concentration dependent. It is likely that for cells to interact optimally with elastin, secreted galectin-3 is concentrated on the cell surface to ligate or improve their adhesion to elastin. If this is so, then galectin-3 mediated adhesion of cells to elastin could be a physiologically relevant mechanism of cell-elastin interaction. The experiment was repeated three times with BT-549 clone 11-9-1-4, and once with MDA-MB-435. Both are tumorigenic cell lines of breast cancer. In all cases, high concentrations of galectin-3 were capable of ligating cells to elastin. We estimated that approximately 40–50% of the cells added to the wells were ligated to elastin at 25 $\mu\text{g/ml}$ of galectin-3. Similar concentrations of galectin-3 were unable to ligate the cells to wells coated with BSA alone (Fig. 1).

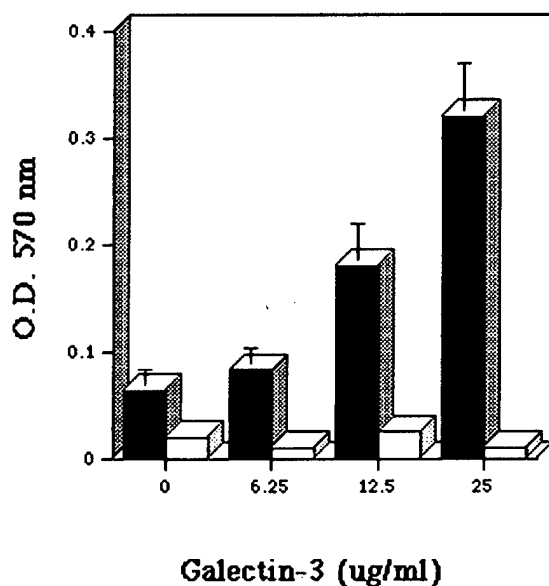


Fig. 1. The ligation of breast carcinoma, BT-549, subclone 11-9-1-4 cells to elastin by galectin-3. The cells in calcium free, serumless DMEM/F12 medium were plated at 5×10^4 cells/well in elastin coated microtiter wells (solid bars) in the presence of recombinant galectin-3 (0–25 $\mu\text{g/well}$). The same number of cells/well were also plated in wells coated with bovine serum albumin (open bars) in the presence of galectin-3 (0–25 $\mu\text{g/well}$). The adherent cells were determined after 1 h. The experiment was repeated three times with 11-9-1-4 cells.

To further explore the interaction of breast carcinoma cells with elastin, the cells were added to elastin coated wells and allowed to incubate for up to 10 days. In this experiment, we were interested in testing the hypothesis that galectin-3 expressing cells have a higher propensity to adhere and proliferate on elastin compared to low galectin-3 expressing cells. By day 3 of incubation of the various cell lines with elastin, we consistently observed that the cells (11-9-1-4) which express high levels of galectin-3 [Makker et al., 1995; Warfield et al., 1997], interacted very well with insoluble elastin (Fig. 2A). There were numerous cells (arrow heads) associated with individual elastin fibers (arrows). This was in contrast to the cells (SK-Br-3) with little or no expression of galectin-3 (Fig. 2B). In this case, there were fewer cells associated with elastin fibers and were easily detached by washing. Interestingly, the cells which had better interactions with elastin (high galectin-3 expression) also had a tendency to proliferate while on the elastin fibers or in close proximity to the fibers as shown in Figure 2A. After 7 days of growth on elastin fibers, the galectin-3 expressing cells (11-9-1-4) literally covered the elastin fibers, resulting in expanding colonies of proliferating cells (Fig. 2C). The galectin-3 null expressing cells (SK-Br-3), on the other hand, showed little or no proliferation on elastin after 7 days of growth (Fig. 2D). The experiment was repeated four times with similar results each time. The experiment was also repeated with MDA-MB-435 (high galectin-3 expression) and BT-549 (lack galectin-3 expression) cell lines. The MDA-MB-435 cells as expected proliferated rapidly on elastin fibers, while BT-549 only interacted marginally with elastin (data not shown).

Interaction of Galectin-3 With Elastin

To explain the ability of galectin-3 to ligate cells to elastin, we questioned whether the lectin interacts specifically with insoluble elastin. Indeed in five separate experiments, we demonstrated by ELISA that galectin-3 interacts very strongly with insoluble elastin. The binding curve was saturable and lactose dependent (Fig. 3). Similar concentration of galectin-3 did not bind to bovine serum albumin coated wells (non-specific binding). To eliminate the possibility that galectin-3 interacts via the sugar moieties which may be present in elastin, the elastin

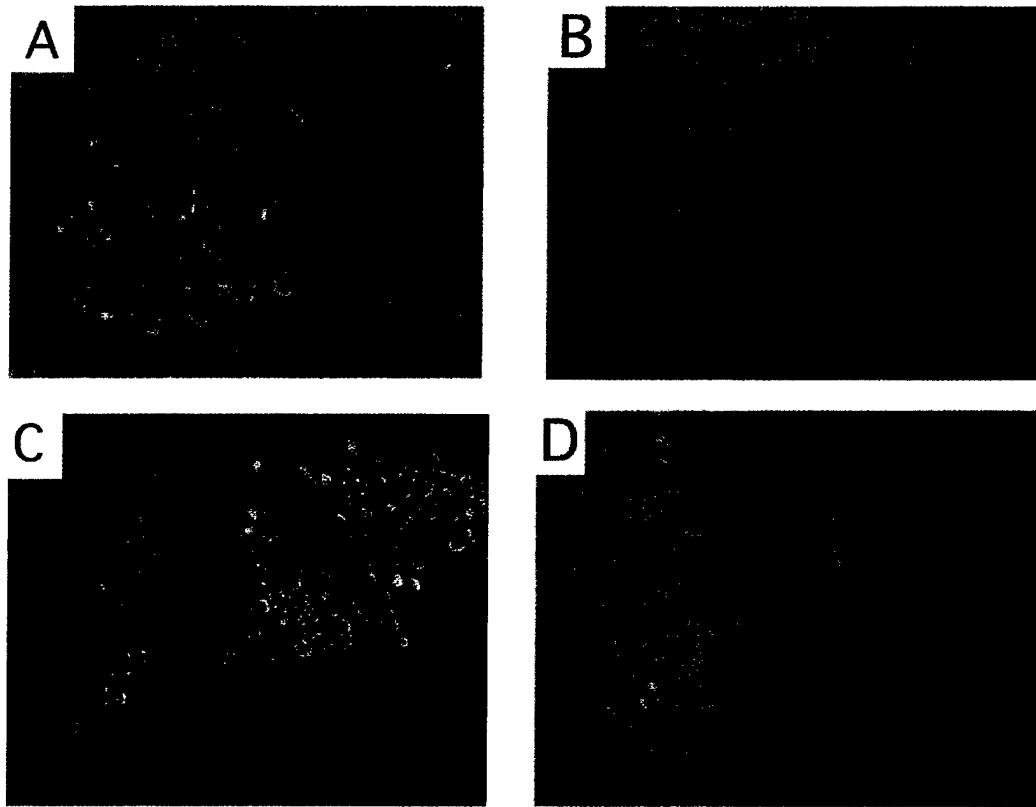


Fig. 2. Growth of galectin-3 expressing and null expressing cell lines on elastin. The wells of a microtiter plate were coated with elastin as described in Materials and Methods. The cells (2×10^4 cells/well) were then added to the elastin coated wells in DMEM/F12 medium containing 10% bovine serum and allowed to grow for 3 days (A,B) or 7 days (C,D). Galectin-3 expressing 11-9-1-4 cells (A,C) and SK-Br-3 (B,D). The cells are depicted by arrow-heads and elastin-fibers by arrows.

fibers were treated with anhydrous hydrazine which cleaves glycosidic bonds. The anhydrous hydrazine completely solubilized the elastin after about 5 h of the treatment. The addition of galectin-3 to wells coated with hydrazine solubilized elastin yielded saturable binding curves similar to Figure 3 (data not shown), suggesting that galectin-3 was directly interacting with peptide domains of elastin in a novel fashion.

To further explore the interaction of galectin-3 with elastin, we questioned whether intracellularly expressed elastin (tropoelastin) is associated with galectin-3 in cells which express both proteins. It has been demonstrated that whereas tropoelastin is mainly synthesized by fibroblasts in the mammary gland, breast epithelial cells can also express the protein [Krishnan and Cleary, 1990]. Our data clearly demonstrate that polyclonal antibodies to galectin-3 can bring down a complex of proteins containing tropoelastin (with bands in the range

of ~55–70 kDa) from lysed breast carcinoma cells (Fig. 4A). Non-immune rabbit serum (control) failed to specifically immunoprecipitate the complex (Fig. 4A, lane 1). Addition of excess recombinant galectin-3 to the lysates (lanes 3 to 5) did not increase the amount of immunoprecipitable complex, implying that the complex probably exists intracellularly in a saturated state with all the galectin-3 binding sites on tropoelastin occupied. The complex was also brought down with mouse monoclonal antibodies against elastin. When the membrane was probed with rat anti-galectin-3, a strong band at 30 kDa (galectin-3) and a smaller band at ~62 kDa, were observed (Fig. 4B). The data strongly suggest that the association between galectin-3 and elastin is a physiologically relevant phenomenon. Studies are currently ongoing to evaluate further the nature of this complex, including the ~62 kDa protein which cross-reacts with anti-galectin-3 antibodies.

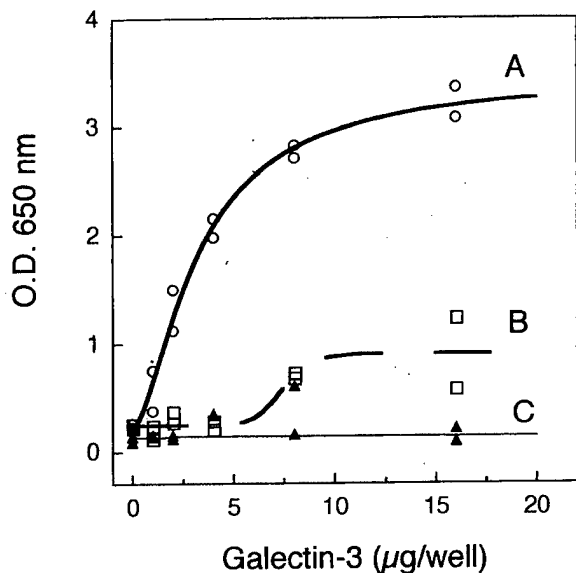


Fig. 3. Binding of galectin-3 to elastin. The wells of a microtiter ELISA plate were coated with elastin as described in Materials and Methods. Recombinant galectin-3 (0–16 µg/well) was then added to the wells in duplicates in the absence of lactose (circles; line A) or presence of 200 mM of lactose (squares; line B). Galectin-3 in the same concentration range was also added in duplicates to wells coated with bovine serum albumin alone (triangles; line C). After 1 h of incubation at 37°C, galectin-3 bound to elastin was determined by ELISA as described in Materials and Methods. The absorbance of the alkaline phosphate substrate was determined at 650 nm.

DISCUSSION

In the present study, we have demonstrated that galectin-3 associates in a novel fashion with both the soluble and insoluble elastin. This specific association could be important in the regulation of cell-elastin interactions. The adhesive interactions of cells with elastin is not well defined compared to the other extracellular matrix proteins, because the main physiological function of elastin is to impart elasticity to vertebrate elastic tissues. Nevertheless, it has been demonstrated that insoluble elastin as well as elastin peptides are capable of promoting not only cellular adhesion, but also proliferation [Parsons et al., 1991; Jung et al., 1998]. It is probable that in the case of certain primary as well as metastatic tumors growing in elastin rich tissues such as the lungs, their interaction with elastin is pivotal to their proliferative potential and formation of micrometastases in those tissues [Timar et al., 1991; Svitkina and Parsons, 1993]. For example, in the case of breast elastosis, interaction of the tumor cells with excess elastin in the breast may exacer-

bate tumor growth [Kao et al., 1986; Khatun et al., 1992].

It is established that the 67 kDa elastin/laminin receptor has galectin-like properties because lactose and anti-galectin-1 antibodies are capable of modifying its biological activities [Mecham et al., 1989]. This, taken together with the recent studies suggesting galectin-3 may be a novel component of the 120 kDa elastin/laminin receptor complex [Buto et al., 1998], prompted us to question whether galectin-3 has a direct role in cell-elastin interactions. Presently it is not clear which part of the chimeric 67 kDa receptor protein is responsible for strong cellular adhesion to elastin. Sequences in the 37 kDa precursor protein have been shown to interact with elastin peptides [Castronovo et al., 1991]. However the contribution of the ~30 kDa component in elastin binding is virtually unknown.

The ability of galectin-3 to interact with elastin in a lactose dependent manner suggests that this is a novel interaction. The interaction may involve both the R- and carbohydrate recognition domains which have been shown to participate in non-covalent homodimerization of the molecule leading to positive cooperativity [Hsu et al., 1992; ochieng et al., 1993; Kuklinski and Probstheimer, 1998]. To explain the ability of galectin-3 to ligate the cells to elastin, we postulate that the exogenously supplied recombinant galectin-3 forms a covalent complex with the 37 kDa precursor of the 67 kDa elastin/laminin receptor on the cell surface resulting in the mature protein which in turn promotes the cellular adhesion to elastin. Alternatively high concentrations of galectin-3 may form higher order oligomers by positive cooperativity [Hsu et al., 1992; ochieng et al., 1993], with some molecules binding specifically to elastin and others to its cell surface expressed ligands such as lysosomal associated membrane proteins [Do et al., 1990], thereby acting as a bridge which links the cells to elastin coated wells (Fig. 5). Other cellular mechanisms may also be involved. For example, cell-elastin interactions may take place in two or more phases. Galectin-3 on the cell surface, may form a temporary bridge between cells and insoluble elastin, followed by the full participation of the 67 kDa in the adhesion as a late response.

We compared growth potentials of various cell lines on elastin, to further implicate galectin-3 in cell-elastin interactions. All the galec-

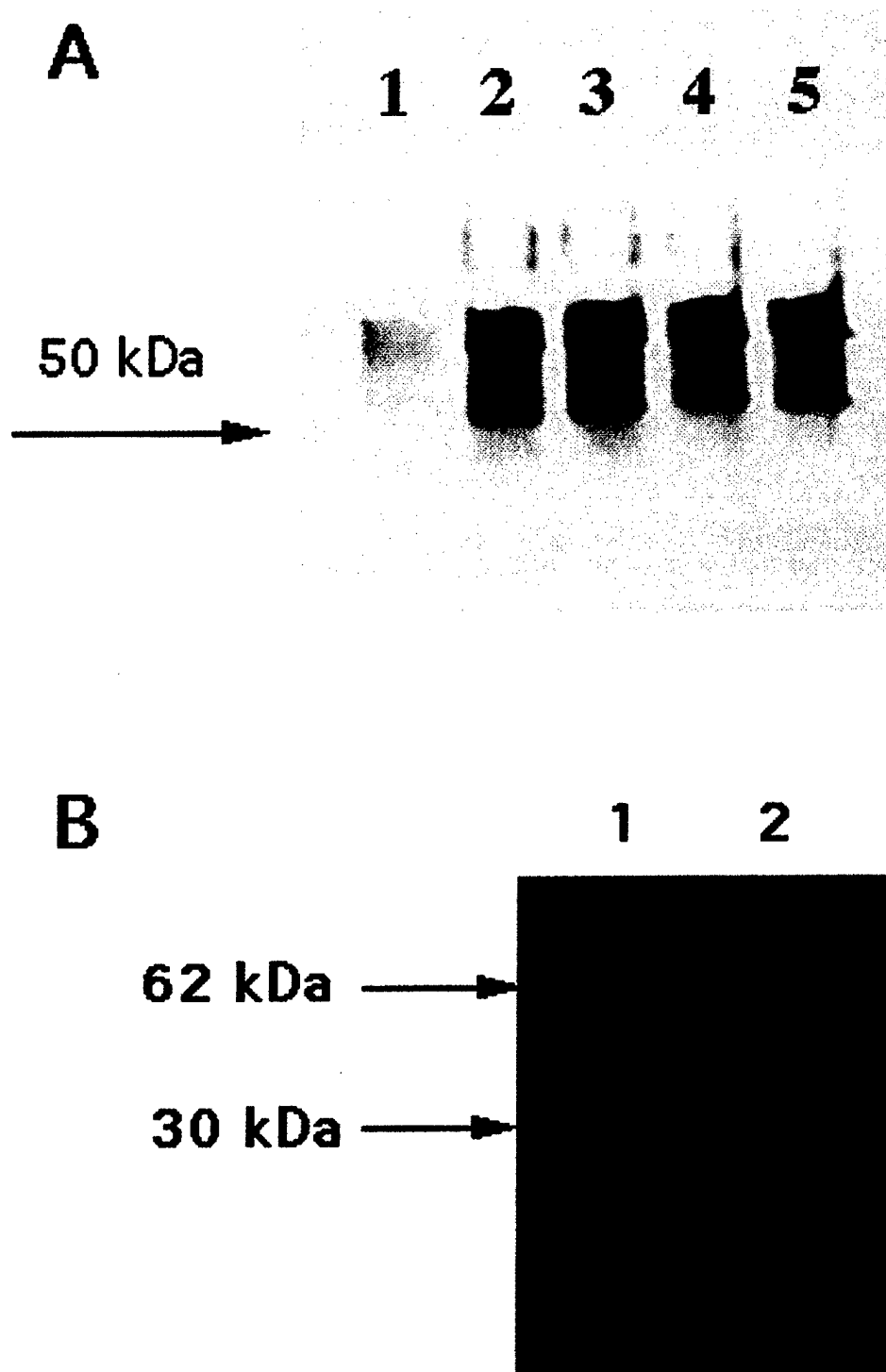


Fig. 4. Association of Elastin and galectin-3. Galectin-3 expressing breast carcinoma cells were lysed and the lysate immunoprecipitated with either rabbit pre-immune serum (**A, lane 1**) or rabbit polyclonal antibodies to recombinant human galectin-3 (**A, lanes 2–5**). In lanes 2–5, the lysates were incubated with 0, 5, 10, and 20 μ g of recombinant galectin-3 for 2 h at 4°C prior to the addition of galectin-3 antibodies. The samples then resolved by 10% reducing SDS-PAGE, blotted, and the membrane probed

with mouse monoclonal antibodies to elastin. The bands represent tropoelastin ~62–67 kDa. The immunoprecipitation was also done with either non-immune mouse serum (**B, lane 1**) or mouse monoclonal anti-elastin (**B, lane 2**). In this case the membrane was probed with rat monoclonal anti-galectin-3. The galectin-3 band (30 kDa) as well as a band which most likely represents a galectin-3 dimer (~62 kDa) are represented in **B, lane 2**.

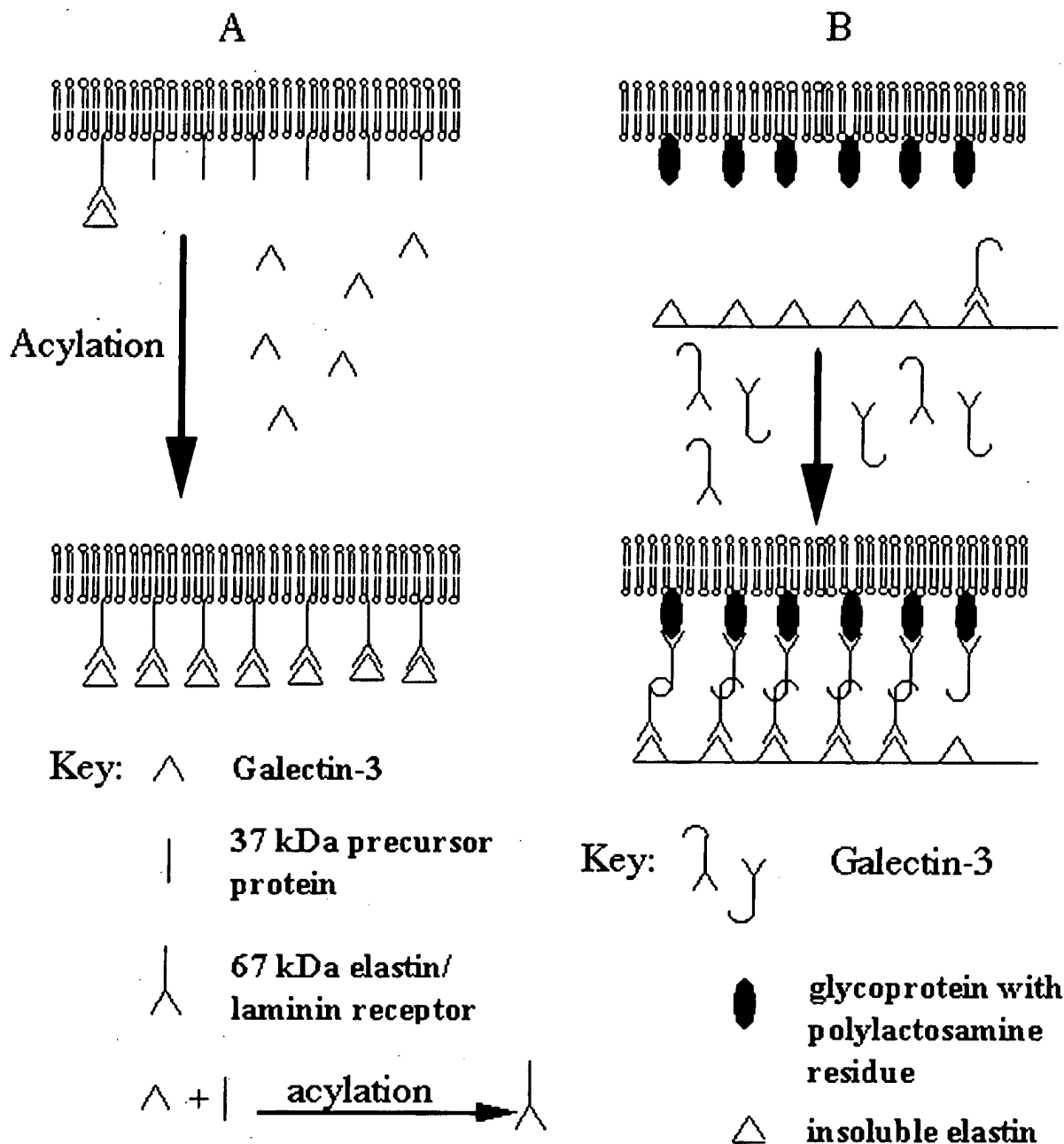


Fig. 5. A model depicting two possible ways by which galectin-3 ligates cells to elastin. In **A**, galectin-3 and the precursor of 67 kDa laminin receptor are joined by acylation to form the mature 67 kDa laminin receptor which is then used for adhesion to elastin. In **B**, some of the galectin-3 molecules interact with elastin while others with polylactosamine containing glycans on the cell surface via their carbohydrate recognition domains. The galectin-3 molecules then interact with each other via their R-domains, forming bridges which ligate the cells to the elastin.

tin-3 expressing cell lines proliferated rapidly on elastin while the galectin-3 null expressing cell lines only interacted marginally with elastin. Presently we do not know whether galectin-3 expression is the rate limiting step in such interactions. One approach to address this quandary is to stably transfect the galectin-3 express-

ing cell lines with anti-sense galectin-3 gene and show whether the transfected cells have reduced proliferative capacity on elastin. Galectin-3 expression has already been demonstrated to play a significant modulatory role in the interaction between cells and two extracellular matrix proteins, laminin and collagen IV [Warf-

ield et al., 1997]. Interestingly, the 67 kDa elastin/laminin receptor can also mediate the interaction of cells with collagen [Minafra et al., 1992].

The ability of anti-galectin-3 polyclonal antibodies to immunoprecipitate a complex consisting of tropoelastin, suggests that galectin-3 is tightly associated with the intracellular soluble elastin. Presently, we do not know the significance of this association. It could be a novel pathway by which galectin-3 is secreted to the extracellular space since this protein lacks the signal peptide [Barondes et al., 1994]. We cannot however, rule out the possibility that the complex also comprises the 67 kDa receptor which has been shown to be associated with tropoelastin [Hinek and Rabinovitch, 1994]. Therefore the association of galectin-3 and intracellularly expressed tropoelastin and possibly the insoluble extracellular elastin implies a modulatory role in cell to elastin interactions. Moreover this modulatory role of galectin-3 may extend to the interaction of cells with other extracellular matrix proteins such as laminin [ochieng and Warfield, 1995].

In summary, we have demonstrated that galectin-3 on the cell surface has the propensity to ligate or increase the adhesion of breast carcinoma cells to insoluble elastin. We have also demonstrated that galectin-3 interacts specifically with both soluble and insoluble elastin. It appears that galectin-3 uses its carbohydrate recognition domain in its interaction with elastin. The enhanced interaction of breast carcinoma cells (which express galectin-3) with elastin, further implicates galectin-3 in cell to elastin interactions. We also postulate based on our data, that galectin-3 may cooperate with the 67 kDa elastin/laminin receptor protein or its precursor to regulate cell-elastin interactions. Furthermore, it is tempting to speculate that the expression of galectin-3 and the 67 kDa elastin-laminin receptor confer on breast carcinomas, the propensity to form micrometastasis on elastin-rich tissues such as the lungs.

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