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Galectin-3 is strongly up-regulated in nonapoptosing mammary epithelial cells during rat mammary gland involution

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Galectin-3 is an endogenous mammalian lectin that binds to ABH carbohydrate antigens. Here we show that galectin-3 is strongly up-regulated during mammary gland involution and that it is expressed virtually exclusively on nonapoptotic cells. We demonstrate that dexamethasone, an inhibitor of the second phase of mammary gland involution, potently suppresses up-regulation of galectin-3 as judged immunohistochemically and on western blots, suggesting that systemic hormone levels regulate galectin-3 expression during involution. However, at the RNA level galectin-3 expression is rapidly up-regulated on the onset of involution but remains consistantly high during the first and second phase of involution regardless of dexamethasone treatment. These data suggest that the up-regulation of galectin-3 in the involuting mammary gland is not only controlled transcriptionally but also regulated posttranscriptionally under the control of systemic glucocorticoid hormones involved in coordinating the involution process.

Key words: ABH histo-blood group antigen/apoptosis/galectin-3/mammary gland/tissue remodeling

Introduction

Using subtractive immunization to identify cell surface epitopes expressed in a metastasis-specific fashion, we generated a monoclonal antibody called M-N1 that recognizes carbohydrate antigens (Sleeman *et al.*, 1999). Specifically, the M-N1 antibody binds to blood group antigen B subtypes 2, 3, and 4 with slight cross-reactivity with blood group antigen A subtype 2. The expression of these carbohydrate epitopes on tumor cells is functionally important because the M-N1 antibody efficiently inhibits tumor growth in spontaneous metastasis assays. These results suggest that expression of the subtypes of blood group antigen B recognized by the M-N1 antibody do not directly participate in the metastatic cascade but rather confer a growth or survival advantage on the tumour cells.

The M-N1 antigen is virtually nonexpressed in normal mammary tissue but is strongly up-regulated during involution of these glands after the cessation of lactation (Mengwasser and Sleeman, 2001). However, the incidence of apoptotic bodies during tissue involution inversely correlates with M-N1 staining. Thus M-N1 expression may confer a growth or survival property onto those epithelial cells required for tissue remodeling during mammary gland involution. This finding is consistent with the notion that the expression of the M-N1 antigen promotes tumor cell growth or survival *in vivo*.

A possible explanation for the blocking of tumor growth by the M-N1 antibody is that it inhibits the interaction of tumor cells with ligands or extracellular matrix components vital for their growth or survival *in vivo*. A candidate for such a ligand would be galectin-3. Galectin-3 is an endogenous mammalian lectin that binds to ABH antigens (Sato and Hughes, 1992), and can be located in the cytoplasm, located in the nucleus, or secreted extracellularly (reviewed in Hughes, 1999). A number of important biological functions have been ascribed to galectin-3, including modulation of cell adhesive properties (Inohara and Raz, 1995; Kuwabara and Liu, 1996; Warfield *et al.*, 1997; Ochieng *et al.*, 1998) and regulation of cell motility (Sano *et al.*, 2000). Additionally it has anti-apoptotic and growth-enhancing properties (Yang *et al.*, 1996; Akahani *et al.*, 1997) and can promote cell invasiveness (Le Marer and Hughes, 1996). These abilities of galectin-3 are obviously closely tied with aspects of tumorigenesis and metastasis. Indeed, in several cases expression of galectin-3 has been shown to correlate with histological grade and to be prognostically significant (Nakamura *et al.*, 1999; Honjo *et al.*, 2000). More significantly, ectopic expression or inhibition of galectin-3 has been shown to increase or decrease tumorigenicity and metastatic proclivity of tumor cells, respectively (Bresalier *et al.*, 1998; Yoshii *et al.*, 2001; Honjo *et al.*, 2001).

Our aim in the study presented in this article was to examine the expression pattern of galectin-3 in the mammary gland to determine whether galectin-3 expression correlates with M-N1 antigen expression. Prior to pregnancy and lactation, mammary glands contain branching networks of ducts formed by mammary epithelial cells (reviewed in Vonderhaar, 1985). During pregnancy and lactation, lateral buds form along the ducts and subsequently develop into alveoli. These alveoli are made up of differentiated mammary epithelial cells that secrete milk during lactation. After weaning, the mammary glands regress in a process called involution. During involution the mammary gland is restructured through the coordinated processes of apoptosis of luminal epithelial, myoeptithelial, and endothelial cells, and lobular-alveolar remodeling (Pitelka, 1988; Lund *et al.*, 1996; Strange *et al.*, 1992). Loss of suckling

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leads to accumulation of milk in the alvoeli and a fall in the levels of systemic lactogenic hormones. These events trigger the involution process (Feng *et al.*, 1995; Marti *et al.*, 1997).

There are two distinct phases to mammary gland involution. The first phase is reversible and controlled by local factors. Alveolar cells apoptose but no remodeling of the lobular-alveolar structure occurs (Li *et al.*, 1997). In the second phase, proteases degrade extracellular matrix and basement membrane components and the lobular-alveolar structures collapse (Lund *et al.*, 1996). Continued apoptosis, replacement of most of the epithelial component with adipose tissue and reestablishment of the resting mammary gland ductal structures, leads to remodeling of the gland. The involution process is completed within 10–15 days (Lascelles and Lee, 1978).

Here we show that galectin-3 is specifically and strongly up-regulated during mammary gland involution, virtually exclusively on nonapoptosing mammary epithelial cells. There is considerable colocalization of galectin-3 and M-N1 expression. The increased expression of galectin-3 during mammary gland involution is regulated at the transcriptional and posttranscriptional levels and is sensitive to glucocorticoid hormones.

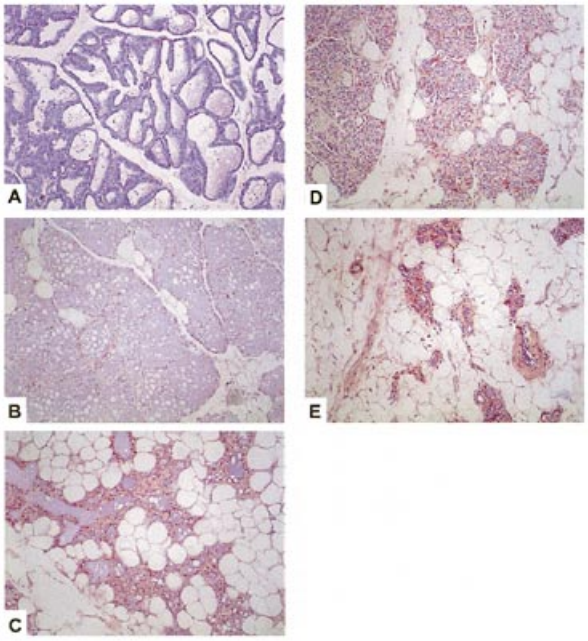

Fig. 1. Immunohistochemical staining of paraffin wax–embedded sections of female rat mammary tissue with galectin-3 antibodies (red/brown stain). The hematoxylin counterstain is blue. Sections are from lactating animals (**A**) and from animals 2 days (**B**), 4 days (**C**), 6 days (**D**), and 8 days (**E**) postweaning. Pups were withdrawn from the rats at day 0. Bar: 50 μm

Results

The essentially blood group B antigen M-N1 is strongly up-regulated in involuting mammary glands (Mengwasser and Sleeman, 2001). We reasoned that as a lectin with the ability to bind to this antigen, galectin-3 might be coordinately expressed with the M-N1 antigen. We therefore investigated galectin-3 expression in lactating and postlactating rat mammary tissue. No staining was observed in mammary glands during lactation. However, within 2 days of cessation of lactation and the onset of mammary gland regression, galectin-3 was dramatically up-regulated and remained highly expressed on mammary epithelial cells during the involution process (Figure 1).

The expression pattern of galectin-3 in the involuting mammary glands is highly reminiscent of that of the M-N1 antigen. We have previously demonstrated that the M-N1 antigen is not expressed on apoptosing cells in this context (Mengwasser and Sleeman, 2001). Using double-stain immunohistochemistry, we investigated whether galectin-3 expression colocalizes with apoptotic cells or M-N1 antigen expression (Figure 2). Apoptotic cells were labelled using Apoptag®, a system that allows detection of the DNA fragmentation that occurs during apoptosis. We focused particularly on mammary glands 4 days postlactation, because at this time point galectin-3 and M-N1 expression and apoptosis are at their highest levels. As shown in Figure 2 and Table I, virtually no galectin-3-positive cells were apoptotic. However, a significant proportion of galectin-3-positive cells were also positive for M-N1 antigen expression (Table I). The fact that galectin-3 staining can be clearly seen in occasional apoptotic cells (Figure 2F) and that β-actin can be readily detected in apoptotic cells (Figure 2G) demonstrates that antigens can still be detected mammary epithelial cells undergoing apoptosis. Thus galectin-3 expression is strongly up-regulated during mammary gland involution and is inversely correlated with apoptosis.

Mammary involution occurs in two stages, a first reversible stage controlled by local factors in which alveolar structure remains intact, and a second stage dependent on systemic hormone levels, characterized by alveolar collapse and tissue remodeling and that begins around 4 days postweaning (Lund *et al.*, 1996; Li *et al.*, 1997). Glucocorticoid hormones have been reported to be able to inhibit the second stage of involution in postlactating animals (Lund *et al.*, 1996; Li *et al.*, 1997; Mengwasser and Sleeman, 2001). Blockade of the onset of the second phase of involution by dexamethasone does not inhibit up-regulation of M-N1 antigen expression (Mengwasser and Sleeman, 2001). To determine the hormone sensitivity of galectin-3 expression in this context, we used galectin-3 antibodies to stain sections of involuting mammary glands from animals that had been treated with dexamethasone. As can be seen in Figure 3, dexamethasone treatment dramatically inhibited the onset of alveolar collapse and tissue remodeling and also potently inhibited the expression of galectin-3. Quantification of the results showed that galectin-3 expression is strongly suppressed by dexamethasone until 6–8 days postweaning, when its expression slowly begins to increase (Table II). Thus galectin-3 expression is regulated by systemic hormone levels in the involuting mammary gland

The immunohistochemistry results in Figures 1–3 suggest that galectin-3 is up-regulated during mammary gland involution in a hormone sensitive manner. To confirm this we also analyzed galectin-3 expression during involution using northern blot and western blot analyses. In the northern blots, as expected, no significant levels of galectin-3 were detected prior to involution (Figure 4). Note that the lactating mammary gland sample is overloaded to permit a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal to be seen because we have previously shown that GAPDH expression is up-regulated

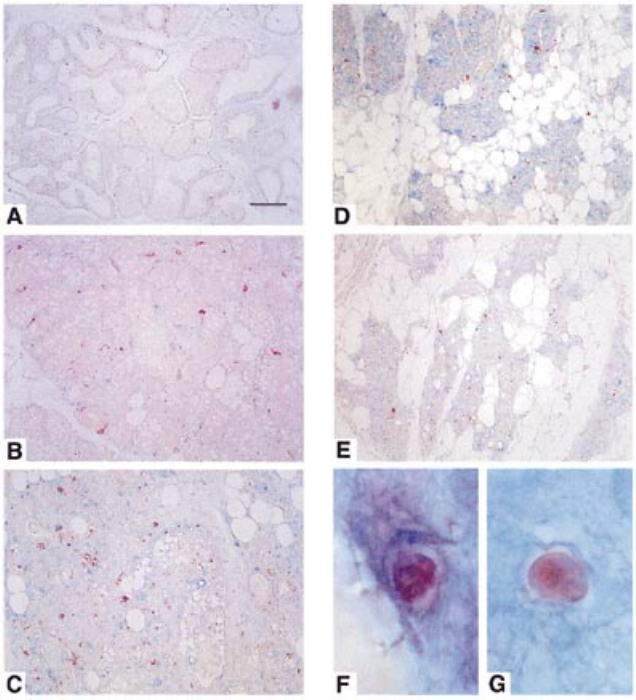

Fig. 2. Apoptotic cells are not galectin-3-positive. Sections are from lactating animals (**A**) and from animals 2 days (**B**), 4 days (**C**, **F**, **G**), 6 days (**D**), and 8 days (**E**) postweaning. Pups were withdrawn from the rats at day 0. (**A–F**) Sections were double-stained with galectin-3 antibodies (blue staining) and Apoptag® to show apoptotic bodies (red stain). (**G**) The section was double-stained with β-actin antibodies (blue staining) and Apoptag® to show apoptotic bodies (red stain). Counterstaining was omitted for clarity. Bar: **A–E**: 50 µm; F, **G**: 5 µm

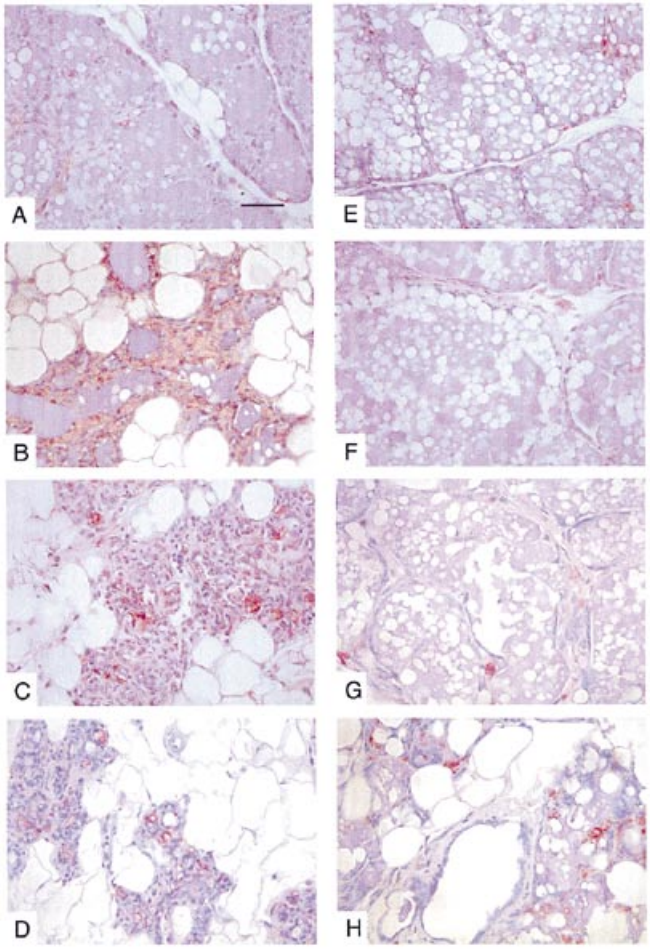

Fig. 3. Sections of mammary glands taken from animals 2 days (**A**, **E**), 4 days (**B**, **F**), 6 days (**C**, **G**), and 8 days (**D**, **H**) postweaning. The animals were either treated (**E–H**) or not treated (**A–D**) with dexamethasone. Sections were immunostained with galectin-3 antibody. Positive staining is red/brown and the hematoxylin counterstain is blue. Bar: 50 µm.

during mammary gland involution (Mengwasser and Sleeman, 2001). Galectin-3 was strongly upregulated at the northern blot level after the onset of involution; surprisingly we observed that it remained consistently high for 8 days postlactation, even if the mammary glands came from animals that had been treated with dexamethasone. However, in western blots galectin-3 protein levels rose strongly during involution, peaking at 4–6 days postlactation (Figure 5), consistent with the immunohistochemistry data. Furthermore, in dexamethasone-treated animals, galectin-3 protein levels remained low and only slightly increased later in the involution process, as observed in the immunohistochemistry. We conclude that in addition to transcriptional regulation, galectin-3 is also subject to posttranscriptional regulation in the involuting mammary gland.

Discussion

Here we show that galectin-3 is strongly up-regulated during mammary gland involution postweaning. Although this increase in expression begins during the first stage of involution, the major increase in expression occurs during the second stage. This is underscored by the fact that dexamethasone, a glucocorticoid hormone that inhibits the onset of the second stage of involution,

Table I. Coincidence of M-N1 expression, galectin-3 expression, and apoptosis in epithelial cells of involuting mammary glands

Stain	M-N1 pos. cells/mm^2	Gal-3 pos. cells/mm^2	Apoptotic cells/mm^2	Double pos. cells/mm^2
M-N1 + Apoptag®	283 ± 18	–	156 ± 14	1 ± 0.4
Galectin-3 + Apoptag®	–	364 ± 22	146 ± 3	4 ± 0.4
M-N1 + Galectin-3	329 ± 20	398 ± 28	–	87 ± 9

Sections of rat mammary glands 4 days postweaning were double stained with combinations of M-N1 antibody, galectin-3 antibody, or with Apoptag® stain to highlight apoptotic cells. The mean and standard error of the number of positively stained (pos) cells in five independent 1-mm^2 fields of view are indicated. Representative data are shown for mammary glands from one individual rat. Mammary glands from three rats were analyzed to exclude possible animal to animal variations.

Table II. Influence of dexamethasone treatment on the number of galectin-3-positive cells and apoptotic cells in involuting rat mammary glands

	α-galectin-3 pos. cells/mm^2		Apoptotic cells/mm^2	
Days postweaning	–Dex	+ Dex	–Dex	+ Dex
0	8 ± 1.4	8 ± 1.4	1 ± 0.6	1 ± 0.6
2	74 ± 11	29 ± 4	81 ± 6	184 ± 17
4	307 ± 14	16 ± 2	164 ± 16	140 ± 21
6	174 ± 10	99 ± 2	43 ± 8	70 ± 4
8	132 ± 3	122 ± 3	18 ± 1	49 ± 8

Sections of rat mammary glands at various stages of involution with (+Dex) and without (– Dex) dexamethasone treatment were stained with galectin-3 antibodies or with Apoptag® stain to highlight apoptotic cells. The mean and standard error of the number of stained cells in five independent 1-mm^2 fields of view are indicated. Representative data are shown for mammary glands from one individual rat. Mammary glands from at least two rats were analyzed for each timepoint/condition to exclude possible animal to animal variations.

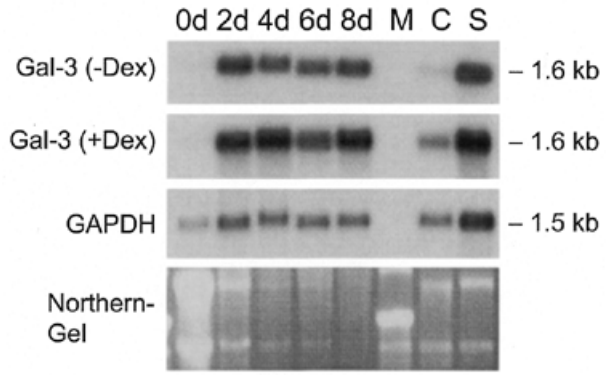

Fig. 4. Northern blot analysis of galectin-3 expression during mammary gland involution. The tissue used for making the RNA came from animals that were either treated (+Dex) or nontreated (–Dex) with dexamethasone. Poly (A+) RNA from mammary glands taken 0, 2, 4, 6, and 8 days postweaning was hybridized with probes for galectin-3 (Gal-3) or GAPDH. As positive controls for these probes, poly (A+) RNA from rat colon (C) or stomach (S) was used. The sizes of the transcripts by reference to the New England Biolabs RNA markers (M) loaded on the gels are indicated. All lanes were loaded with 5 µg RNA, except for the 0 days postweaning lanes, where the amount of RNA loaded was increased to obtain a hybridization signal with the loading control GAPDH. This was necessary because GAPDH is regulated during mammary gland involution (Mengwasser and Sleeman, 2001). The original ethidium-stained gel is therefore also shown to demonstrate the relative amounts of RNA loaded per lane (Northern-Gel). The GAPDH and gel shown correspond to the Gal-3 (–Dex) northern blot. Equivalent GAPDH and gel results were obtinaned for the Gal-3 (+Dex) northern blot.

strongly suppresses the expression of galectin-3. These data point to a possible function for galectin-3 during the second stage of involution, which in contrast to the first stage is typified by tissue remodeling (Li *et al.*, 1997).

Galectin-3 has a number of properties that could contribute to tissue remodeling, incuding the regulation of cell adhesiveness and motility, the suppression of apoptosis, and the promotion of cell growth and invasiveness. Furthermore, galectin-3 has been shown to modulate branching morphogenesis of the uretic bud/collecting duct in the embryonic kidney (Bullock *et al.*, 2001), and we note that this branching morphogenesis has many similarities to the tissue remodeling that occurs in the

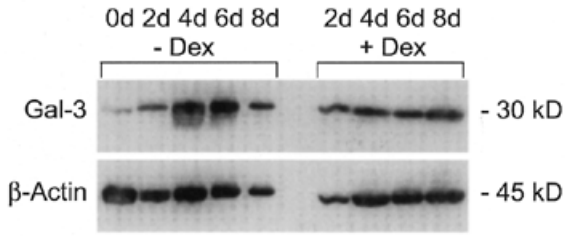

Fig. 5. Western blot analysis of galectin-3 expression during mammary gland involution. The tissue used for making the protein samples came from animals that were either treated (+Dex) or nontreated (–Dex) with dexamethasone. Protein samples from mammary glands taken 0, 2, 4, 6, and 8 days postweaning were subjected to SDS–PAGE and western blotted. The blots were first probed with galectin-3 antibodies (Gal-3), then stripped and probed with anti-β-actin antibodies. The size of the proteins calculated by reference to protein molecular weight markers run on the gel is indicated.

postlactation mammary gland. Which of the properties of galectin-3 might contribute to tissue remodeling? We observed virtually exclusive expression of galectin-3 on nonapoptotic cells. Thus in the context of tissue remodeling, expression of galectin-3 might suppress the induction of apoptosis. Additionally, dexamethasone treatment inhibits tissue remodeling but not apoptosis in involuting mammary glands (Lund *et al.*, 1996; Li *et al.*, 1997; Mengwasser and Sleeman, 2001; data presented herein) and also suppresses galectin-3 expression (Figure 3, Figure 5, Table II). If galectin-3 were to suppress apoptosis in the involuting mammary gland, one prediction would be that dexamethasone treatment might result in an increased rate of apoptosis. We note that dexamethasone treatment results in a modest increase in the number of apoptotic cells in involuting mammary glands (Table II). However, it is equally possible that properties of galectin-3 other than or in addition to the suppression of apoptosis might contribute to tissue remodeling.

If galectin-3 plays a role in postlactational tissue remodeling, one might expect to see disturbed tissue remodeling in mice bearing a targeted deletion of the galectin-3 gene. We examined this histologically by comparing postlactational mammary glands from wild-type mice with those from galectin-3 knockout animals (Hsu *et al.*, 2000), but could observe no obvious morphological or kinetic differences in the involution process (data not shown). However it is important to remember that there are many members of the galectin family, and it is highly likely that other members may compensate for the lack of galectin-3 in the knockout animals. Therefore it will be important to use conditional knockout techniques to examine the role of galectin-3 in mammary gland involution.

An unexpected observation in this study was the fact that galectin-3 protein levels are regulated differently to galectin-3 RNA levels, suggesting that galectin-3 is both transcriptionally and posttranscriptionally regulated. The glucocorticoid hormone dexamethasone appears to act posttranscriptionally because no difference in galectin-3 RNA levels was observed when involuting mammary glands from dexamethasone-treated and nontreated animals were compared. This would suggest that dexamethasone acts indirectly, perhaps by regulating galectin-3 translation or by affecting galectin-3 protein stability.

Such a scenario is not without precedent. For example, dexamethasone blocks the IL-4-induced increase in protein levels of IL-4Ralpha on T and B cells without altering IL-4Ralpha

mRNA levels (Mozo *et al.*, 1998). Glucocorticoids are known to negatively modulate protein synthesis, in part through inhibiting the assembly of the functional eIF4F holocomplex required for translation initiation (Shah *et al.*, 2000a,b), and through attenuation of the activation of ribosomal protein S6 kinase (Shah *et al.*, 2000b,c) whose role is to preferentially select those mRNA species bearing a cis-acting terminal oligopyrimidine (5′-TOP) motif for translation. Furthermore, glucocorticoids can also stimulate proteasome- and calcium-dependent proteolysis (Wang *et al.*, 1998; Thompson *et al.*, 1999). If our suggestion that galectin-3 plays a role in tissue remodeling is correct, then the suppression of galectin-3 protein expression by systemic glucocorticoid hormones during the first stage of involution (despite the high galectin-3 RNA levels present at this time) might be important for ensuring that tissue remodeling does not occur during this first, reversible stage.

Our initial motivation for analysing expression of galectin-3 in involuting mammary glands was to compare its expression with that of the M-N1 antigen, to determine the likelihood that this carbohydrate antigen might be a ligand for galectin-3 during involution. Like M-N1, galectin-3 is strongly up-regulated during involution and is also virtually never expressed in apoptotic cells. M-N1 and galectin-3 also colocalize (Table I). Thus galectin-3 and M-N1 are spatially and temporarily expressed such that M-N1 could act as a ligand for galectin-3. However, the colocalization of M-N1 and galectin-3 is by no means complete. Furthermore, M-N1 is not regulated by glucocorticoid hormones (Mengwasser and Sleeman, 2001). Future work will therefore focus on determining whether M-N1 and galectin-3 have a functional relationship.

Materials and methods

Mammary gland preparation

Female BDX rats were maintained and treated according to German regulations. For virgin mammary glands, 8-week-old rats were used. Pregnancies were timed by plug analysis. Lactating rats were separated from their pups after 22 days of lactation. Dexamethasone-treated animals were injected subcutaneously daily with 2 mg/kg dexamethasone dissolved in a volume of 200 µl benzyl benzoate:castor oil (1:2.5). At different times after weaning, rats were sacrificed and the no. 4 mammary glands removed for histology or protein or RNA analysis. All experiments were repeated independently at least twice.

Immunohistochemistry

Mammary glands were fixed in 4% paraformaldehyde and then embedded in paraffin wax and sectioned. The sections were subsequently immunostained as described (Dall *et al.*, 1995), using 3,3-amino-9-ethyl carbazole for color development. The monoclonal antibodies used in this study were M-N1 (Sleeman *et al.*, 1999) A1D6 anti-galectin-3 (Liu *et al.*, 1996), and AC-15 anti-β-actin (Sigma). For double-staining experiments, sections were stained first with galectin-3 or β-actin antibodies using alkaline phosphatase standard (Vector Laboratories) and the alkaline phosphatase Substrate Kit III (blue color, Vector Laboratories) for color development. Subsequent staining of apoptotic cells was performed using an Apoptag® kit (Oncor) according to the manufacturer's instructions, except that alkaline phosphate Substrate Kit I (red color, Vector Laboratories) was used for color development. Quantification of staining was performed by taking photographs of stained sections and counting the number of stained cells in five independent 1-mm square fields of view.

Cloning of rat galectin-3

Full-length rat galectin-3 was amplified by reverse-transcription polymerase chain reaction from rat spleen cDNA and cloned into the BamHI/EcoRI sites of pcDNA3.1. The primers used were 5′-GCGGA TCCAT GGCAG ACGGC TTCTC ACTTA ATGAT G-3′ and 5′-GCGAA TTCTT AGATC ATGAT GGCGT GGGAA GCGCT GG-3′. The amplified sequence corresponds to bases 41–830 of the published rat galectin-3 sequence (GenBank accession number NM031832). The amplified cDNA was multiply sequenced on both strands to ensure the sequence was correct. The galectin-3 cDNA was cut out of the pcDNA3-1 vector and used as a probe for northern blots.

Northern blots

RNA was prepared from snap-frozen mammary gland, colon, and stomach tissue using peqGOLD RNA Pure (Peqlab) according to the manufacturer's instructions. Poly (A)+ RNA was subsequently purified from the total RNA using standard protocols, and 5-µg aliquots were size-fractionated on 1.0% formaldehyde-agarose gels and blotted onto Hybond N^+ membrane (Amersham). The membranes were then cross-linked (UV Stratalinker 2400, Stratagene) and hydridized at 65°C in QuickHyb® (Stratagene). Probes were generated by ^{32}P-labeling of cDNA fragments (ReadyPrime, Amersham). Unincorporated label was removed prior to hybridization using an Elutip (Schleicher & Schüll) according to the manufacturer's specifications. After hybridization with the labeled probes, membranes were washed twice in 2× SSC, 0.1% sodium dodecyl sulfate (SDS), and twice in 1× SSC, 0.1% SDS at 64°C, after which they were exposed to film (20× SSC contains 175.3 g/l NaCl and 88.2 g/l sodium citrate, pH 7.0).

SDS–PAGE and western blotting

Protein lysates from mammary glands were prepared as previously described (Hebbard *et al.*, 2000). Briefly, freshly dissected mammary glands were snap-frozen in liquid nitrogen, then ground to a powder in liquid nitrogen using a pestle and mortar. Frozen powdered mammary gland was mixed with a preweighed vial of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer (2% SDS, 125 mM Tris–HCl, pH 6.8, 100mM dithiothretol, 10% glycerol). The vial was then weighed again to determine to amount of frozen powdered mammary that had been added to the vial. The concentration was then adjusted to 25 mg frozen powdered mammary gland/ml sample buffer. The resulting protein lysate was sonified and then boiled for 5 min. Proteins were resolved by size by SDS–PAGE (1 mg frozen powdered mammary gland per cm loading slot) using a resolving gel containing 10% polyacrylamide. For western blotting, proteins separated by SDS–PAGE were electrically transferred to Immobilon™-P (Millipore) by the method of Towbin *et al.* (1979). Blots were probed first with A1D6 anti-galectin-3 (Liu *et al.*, 1996) antibody and subsequently with anti-β-actin antibody clone AC-15 (Sigma) using

the electrochemiluminescence detection system (Amersham, Braunschweig) as previously described (Sleeman, 1993).

Abbreviations

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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