Mammary-Derived Growth Inhibitor Protein and Messenger Ribonucleic Acid Concentrations in Different Physiological States of the Gland

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ABSTRACT

Expression of mammary-derived growth inhibitor in tissue from lactating and involuting bovine mammary glands was investigated. Seventeen lactating, pregnant (220 to 272 d in gestation) cows were divided into two groups of 8 and 9 cows each. Cows of the first group were slaughtered while in lactation. Cows of the second group (9 involuting cows) were slaughtered at 13 to 52 d following sudden cessation of milking. High concentrations of mammary-derived growth inhibitor (63% of the total protein) were detected in mammary tissue of lactating cows. Mammary-derived growth inhibitor (<10% of the total protein) was dramatically reduced during most of the involution period (13 to 45 d following cessation of milking). Mammary-derived growth inhibitor was again detected (28% of the total protein) during the last stage of involution (46 to 53 d after cessation of milking), which coincided with colostrum formation. When steady state concentrations of mammary-derived growth inhibitor mRNA were examined, the results obtained mirrored those obtained at the protein concentration. These data suggest that regulation of mammary-derived growth inhibitor occurs via modulation of the steady state concentration of its mRNA. Furthermore, there is a strong correlation between mammary-derived growth inhibitor expression and lactation in dairy cows.

(Key words: mammary-derived growth inhibitor, lactation, involution)

Abbreviation key: MDGI = mammary-derived growth inhibitor.

INTRODUCTION

Mammary-derived growth inhibitor (MDGI) is a 13-kDa protein purified from the lactating bovine mammary gland (2, 9, 10). In situ hybridization and immunohistochemical analysis showed that MDGI is actually produced by mammary epithelial cells (9). Mammary-derived growth inhibitor inhibits proliferation of several normal and transformed mammary epithelial cell lines in a dose-dependent and reversible manner (6). The MDGI protein and mRNA are higher in fully differentiated lactating glands than in rapidly developing glands of pregnant animals (9, 10). Mammary-derived growth inhibitor may fulfill a role in the onset of differentiation coupled with inhibition of mammary epithelial cell proliferation (6).

Dairy cows require a nonlactating period of approximately 60 d prior to lactation to achieve maximal milk production in the subsequent lactation (8, 11, 15). The nonlactating period of the dairy cow is divided into three distinct stages: 1) an initial period of active involution, which begins just after the cessation of milking and is completed by approximately 30 d in the dry period; 2) a period of steady state involution (31 to 45 d after cessation of milking); and 3) a period of colostrum formation prior to the initiation of lactation (46...
to 60 d following cessation of milking) (8, 14, 15). Politis et al. (12, 13) detected very low concentrations of MDGI in the mammary glands of dairy cows during the first two stages of mammary involution. It is not known whether MDGI is expressed in the bovine mammary gland during the last stage of involution, which coincides with colostrum formation. Furthermore, there is no published information on changes of MDGI mRNA between lactating and involuting animals.

The objective of the present study was to investigate changes in MDGI mRNA and protein concentrations between lactating and involuting animals. Emphasis was placed on the last stage of involution, which coincided with colostrum formation. Our work provides more basic knowledge toward understanding the molecular events affecting MDGI expression during mammary growth and development.

MATERIALS AND METHODS

Animals

Seventeen lactating, pregnant (220 to 272 d in gestation) Holstein cows were used throughout this investigation. Eight cows were slaughtered while in lactation. The remaining 9 cows were slaughtered during the dry period according to the following schedule: 3 cows at 12 to 30 d, another set of 3 cows at 31 to 45 d, and the remaining 3 cows at 46 to 53 d following sudden cessation of milking. These days were chosen to correspond with the three distinct stages of the involution process: active involution, steady state involution, and colostrum formation (8, 14, 15). Cessation of milking occurred at approximately 300 DIM. At slaughter, the udder was removed quickly, and approximately 15 to 20 g of mammary tissue parenchyma were dissected, frozen in liquid nitrogen, and transported immediately to our laboratory.

Tissue Preparation for MDGI

Protein Determination

Mammary tissue (10 g), obtained at the local slaughter house, was minced with scissors and homogenized using a Brinkman homogenizer (Kinematica GmbH, Steinhofhalde, Switzerland) in 25 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl2) supplemented with .25 M sucrose and protease inhibitors: pepstatin A (.5 μg/ml) and phenylmethylsulfonylsulfate (.5 mM). The homogenate was centrifuged at 1000 × g for 10 min. The supernatant was recovered and centrifuged at 100,000 × g for 30 min to obtain a pellet containing microsomes and mitochondria and a supernatant denoted as the cytoplasmic fraction.

Detection of MDGI Antigen

Western blot analysis using anti-MDGI rabbit serum was employed to examine the presence or absence of MDGI in the cytoplasmic fraction of bovine mammary tissue (12, 13). The cytoplasmic fraction contains the majority of MDGI present in the mammary cells (10, 12, 13). Anti-MDGI rabbit IgG was produced by R. Grosse in the Central Institute of Molecular Biology, Academy of Sciences, Berlin-Buch, Germany. All details regarding preparation and the specificity of this antibody have been described earlier (2, 10, 12, 13).

The SDS-PAGE was carried out using 15% acrylamide-resolving gels and 4% acrylamide-stacking gels as described by Bohmer et al. (2). Samples were electrophoresed in .02 M Tris, .15 M glycine buffer (pH 8.3) at 30 mA for 5 h along with standards from 2.5 to 116 kDa (Sigma Chemical Co., St. Louis, MO) in adjacent lanes. Gel portions containing standards were cut, stained for 30 min in 1% (wt/vol) Coomassie brilliant blue dye and 50% (wt/vol) trichloroacetic acid, and destained in 20% (vol/vol) acetic acid. Portions of the gels containing fractionated proteins present in the cytoplasmic fraction of mammary tissue were further processed for Western blots.

Western blots were prepared using immobilon membranes (Millipore, Bedford, MA). Electrostatic transfer of samples previously fractionated by SDS-PAGE (30 V, 12 h, 4°C; Bio-Rad Transblot apparatus, Bio-Rad, Richmond, CA) was carried out in .02 M Tris, .15 M glycine (pH 8.3) containing 20% methanol. Initially, membranes were incubated at room temperature for 30 min in Tris-buffered saline (50 mM Tris, .9% NaCl, pH 7.5) containing 3% gelatin. Subsequently, all washes and incubations were carried out with Tris-buffered saline containing 1% gelatin. After washing, the membranes were incubated for 12 h with
anti-MDGI rabbit IgG (2 μg/ml); they were then washed and incubated for 2 h with the secondary antibody, peroxidase-conjugated anti-rabbit IgG (1:1000 dilution; Sigma Chemical Co.). To ensure antibody specificity, a control lane was loaded with 1 μg of purified MDGI. The MDGI antigen was purified by R. Grosse in the Central Institute of Molecular Biology. All details of this purification procedure have been described earlier (6). All membranes were washed and stained with 0.003% hydrogen peroxide and 4-chloro-1-naphthol (0.6 mg/ml; Bio-Rad).

Determination of MDGI Protein Content

An ELISA methodology was developed to measure MDGI concentration in the cytoplasmic fraction of bovine mammary cells obtained from lactating and involuting glands. Briefly, test samples were initially incubated in 0.1% SDS at 37°C for 30 min. This step is necessary because MDGI has the tendency to precipitate. Samples were then diluted in 0.1 M sodium bicarbonate buffer (pH 9.6), incubated overnight at 4°C in a 96-well polystyrene microtiter plate, and then equilibrated for 1 h at 37°C with 100 μl of anti-MDGI rabbit IgG (0.8 μg/ml). Wells were washed six times with PBS (.14 M NaCl, .01 M NaH₂PO₄, pH 7.2) containing 0.05% Tween 80, incubated for 1 h at 37°C with 100 μl of anti-rabbit IgG-peroxidase conjugate (1:2000 dilution), and washed again six times with PBS. One hundred microliters of 0.182 mM 2′,2′-azineo-diethylbenzothiazoline sulphonate (Sigma Chemical Co.) in 1.2% SDS at 37°C with 100 μl of anti-rabbit IgG peroxidase conjugate (1:2000 dilution), and washed again six times with PBS. One hundred microliters of .182 mM 2′,2′-azineo-diethylbenzothiazoline sulphonate (Sigma Chemical Co.) in 1 M citrate buffer (pH 5.2) containing 0.003% hydrogen peroxide were then added to each well. Absorbance was measured at 405 nm after 10 min. Controls included in the assays were 1) wells coated with bovine serum albumin without sample and 2) wells with the first antibody layer substituted by nonimmune serum. The sensitivity of this assay system was 1 ng/ml, and absorbance was linear with MDGI concentration up to 10 ng/ml. Recovery of an added mass of MDGI was 91.8%, and intraassay and interassay coefficients of variation were less than 10%.

The MDGI protein concentration was expressed as micrograms of MDGI per milligram of total protein. Total protein in cytoplasmic and microsomal fractions of mammary tissue was determined by the method of Bradford (3) using bovine serum albumin as a standard.

Isolation of Total RNA from Mammary Tissue

Total cellular RNA from mammary tissue was isolated by the method of Chomczynski and Sacchi (4). Mammary tissue (1 g) was cut into small pieces, while frozen, with a sterile razor blade in a petri dish. Mammary gland pieces were lysed by the addition of 10 ml of RNAzol (CINNA/BIOTECH, Frienswood, TX). The larger pieces of the tissue were homogenized by drawing the lysis buffer through a syringe fitted with an 18-gauge needle and subsequently were transferred to a fresh polypropylene tube. One milliliter of chloroform was then added, and the final suspension was mixed vigorously and then placed on ice for 15 min. Samples were centrifuged at 10,000 × g for 20 min at 4°C. After centrifugation, the upper aqueous phase containing RNA was transferred to a new tube, mixed with an equal volume of isopropanol, and then placed at −70°C for 1 h to precipitate RNA. Sedimentation at 10,000 × g for 20 min was then performed, the supernatant was discarded, and the RNA pellet was washed twice with 95% ethanol, sedimented, vacuum dried (15 min), and dissolved in .2 ml of H₂O.

Northern Blot Analysis

Hybridization of immobilized RNA to radiolabeled cDNA probes was performed as described previously (1). Briefly, 8 μg of total RNA were separated by electrophoresis on a 1.2% agarose gel containing 6% formaldehyde. The gel was rinsed twice in 10 × SSC (0.15 M NaCl, .015 M sodium citrate, pH 7.0) for 10 min at room temperature, and the RNA was transferred to zeta probe membranes (Bio-Rad) by capillary blotting with 20 × SSC for 20 h. Following transfer, the membrane was placed in a plastic bag containing 8 ml of prehybridization buffer [50% formamide, 4 × (.15 M NaCl, .01 M NaH₂PO₄, 1 mM EDTA, pH 7.4), 1% SDS, 200 μg/ml of salmon sperm DNA, and 5 mg/ml of skim milk powder] for 20 h at 42°C. Membranes were hybridized in the same solution containing 4 × 10⁵ cpm/ml of ³²P-labeled MDGI cDNA, labeled using nick-translation (1). The membranes were washed at 42°C for 20 min in approximately 300 ml of 2
Western blot analysis of mammary tissue mammary-derived growth inhibitor (MDGI). Proteins from cytoplasmic fraction of mammary cells were separated by SDS-PAGE, blotted onto nitrocellulose, and detected with anti-MDGI rabbit serum and anti-rabbit IgG-peroxidase conjugate. Lane a, 15 μg of cytoplasmic mammary cell extract obtained from involuting cows (4 to 53 d following cessation of milking); lane b, 5 μg of cytoplasmic mammary cell extract obtained from lactating cows; lane c, .35 μg of purified MDGI.

Statistical Analysis

All data were expressed as means and standard deviations. Differences between means were evaluated using the Student’s t test (P < .05).

RESULTS

Western blot analysis was performed to establish the presence of MDGI antigen in mammary tissue obtained from glands at different physiological stages. Figure 1 shows the presence of a 13-kDa immunoreactive MDGI antigen in mammary tissue obtained from involuting cows at 46 to 53 d after cessation of milking (lane a) and from lactating cows (lane b), which comigrated with purified MDGI (lane c).

To gain more insight into the pattern of MDGI production, ELISA methodology was employed to measure MDGI protein concentrations in mammary tissue. Table 1 shows the presence of high concentrations of MDGI (6.3 μg/mg of total protein) in mammary tissue obtained from lactating cows. However, MDGI was present at very low concentrations (<.8 μg/mg of total protein) in mammary tissue obtained from mammary glands at the first stage (less than 30 d after cessation of milking) or second stage (31 to 45 d after cessation of milking) of involution. The MDGI was again detected (2.8 μg/mg of total protein) during the last stage of involution (46 to 53 d after cessation of milking), which coincided with colostrum formation.

To investigate the level at which the expression of MDGI is regulated, the steady state concentrations of MDGI mRNA concentra-
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Figure 2. Mammary-derived growth inhibitor (MDGI) mRNA isolated from mammary tissue obtained from lactating and involuting glands. Total RNA (8 µg) was electrophoresed on agarose gel and transferred to membrane, and the filter was probed with a nick-translated MDGI cDNA probe. Lanes a and b, total RNA isolated from lactating mammary glands; Lanes c and d, total RNA isolated from involuting mammary glands (46 to 53 d following cessation of milking).

DISCUSSION

The first novel finding emerging from this study is the presence of MDGI in mammary tissue obtained from glands during the last stage of involution, which coincided with colostrum formation. Western blot analysis, however, reflects the presence or absence of MDGI antigen. It does not address the possibility of quantitative differences in concentrations were explored. Total RNA from bovine mammary tissue was hybridized with a bovine MDGI cDNA, and the results are presented in Figure 2. As shown, bovine MDGI was encoded by a single RNA species of approximately 800 nucleotides long, a size in agreement with that previously reported (6). Relative amounts of MDGI mRNA were initially determined by scanning the autoradiogram. Then, the blot was stripped, hybridized with a 18-S rRNA probe, and scanned for a second time to estimate the amount of total RNA in each lane. Values obtained for MDGI mRNA from the first scanning were then divided by the corresponding 18-S rRNA values from the second scanning, therefore accounting for variability in the amount of RNA loaded to each lane and transfer efficiency. The normalized values were then expressed as percentage of the maximal stimulation; the maximally stimulated sample was arbitrarily assigned a value of 100%. The results are presented in Table 2. As shown, maximal stimulation of MDGI mRNA occurred in mammary tissue obtained from lactating animals. Lower (threefold) MDGI mRNA were detected in mammary tissue obtained from involuting animals at the last stage of involution (46 to 53 d following cessation of milking). This coincided with colostrum formation. We were unable to detect appreciable mRNA in mammary glands of involuting cows (first and second stages).

TABLE 1. Mammary-derived growth inhibitor (MDGI)1 antigen in mammary tissue obtained from lactating and involuting Holstein cows.

<table>
<thead>
<tr>
<th>Source</th>
<th>MDGI Antigen (µg/mg of proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Lactating</td>
<td>6.3 1.2</td>
</tr>
<tr>
<td>Involution stage2</td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>.08 .06</td>
</tr>
<tr>
<td>Second</td>
<td>.04 .03</td>
</tr>
<tr>
<td>Third</td>
<td>.28 .12</td>
</tr>
</tbody>
</table>

1The MDGI expression was determined by ELISA.
2First stage corresponds to 12 to 30 d, second stage to 31 to 45 d, and third stage to 46 to 53 d following sudden cessation of milking.

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TABLE 2. Mammary-derived growth inhibitor (MDGI) mRNA in mammary tissue obtained from lactating and involuting Holstein cows.

<table>
<thead>
<tr>
<th>Source</th>
<th>Relative amounts of MDGI mRNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactating</td>
<td>100</td>
</tr>
<tr>
<td>Involution stage 2</td>
<td>ND 2</td>
</tr>
<tr>
<td>First</td>
<td>ND</td>
</tr>
<tr>
<td>Second</td>
<td>ND</td>
</tr>
<tr>
<td>Third</td>
<td>36</td>
</tr>
</tbody>
</table>

1First stage corresponds to 12 to 30 d, second stage to 31 to 45 d, and third stage to 46 to 53 d following sudden cessation of milking.

2Not detected.

The presence of high concentrations of MDGI during the prepartum period likely is consistent with a role for MDGI in differentiation rather than proliferation of mammary epithelial cells. The question of whether MDGI production in the third stage of involution is a consequence of initiation of colostrum production, or involvement of MDGI in the regulation of these phenomena, or both cannot be answered at the moment.

The second major finding of the present study was that the regulatory mechanism for control of MDGI expression in mammary cells in vivo is via modulation of the steady state concentration of its mRNA. This is because changes in MDGI mRNA concentrations were almost identical to those observed at the protein level. The MDGI mRNA was highest in lactating glands; MDGI mRNA in involuting glands (third stage) was still approximately 36% of the fully induced concentrations observed in lactating glands. The MDGI mRNA was not detected in involuting glands obtained during the first and second periods.

Two mechanisms for MDGI mRNA regulation are consistent with these observations: 1) modulation of MDGI mRNA through changes in transcriptional activity of the MDGI gene and 2) modulation through stabilization of MDGI mRNA. Further experiments involving measurements of transcription rates will more clearly define how the message levels are being modulated.

Based on the results obtained at the protein level, one should expect some, even though limited, MDGI mRNA in involuting cows (first and second stages). These are two possible explanations for this inconsistency. First, the MDGI protein may have been actually produced at an earlier stage of lactation and remained within the mammary gland in association with milk fat. This explanation is consistent with earlier findings (6), suggesting that MDGI is found in milk in strong association with milk fat globule membrane. Second, MDGI mRNA was present at concentrations lower than the detection limit of our system. It is generally more difficult to detect mRNA species in tissues from cells in culture, especially when the particular mRNA species is scarce.
Considering the limited MDGI expression in mammary glands of involuting cows (first and second stages), two major possibilities can be envisaged: limited secretion from substantial loss of mammary epithelial cells or reduced synthetic activity per cell, alone or in combination. It is unlikely that the lack of MDGI mRNA is due to cell loss, because loss of mammary epithelial cells does not occur to a significant extent during involution in the bovine species (8, 15). We favor the second hypothesis, that the limited secretion is due to reduced synthetic ability per cell, based on several studies showing that inactive mammary epithelial cells prevail during the first and second stages of mammary involution (8, 11, 13). The question of whether lack of MDGI production is a consequence of these cellular events, or whether it is involved in the regulation of these events, or both cannot be answered at the moment.

The presence of MDGI in lactating and involuting glands 2 wk prior to parturition, at the stage of colostrum formation, suggests that a relationship exists between the presence of MDGI and the functional state of the gland. These results and recent results of others (6) that MDGI can substitute for prolactin in inducing β-casein expression led us to hypothesize a role for MDGI in differentiation of epithelial cells along with inhibition of cell proliferation.

In summary, the present observations document the presence of high concentrations of MDGI in lactating and involuting mammary glands at the time of colostrum formation. Our initial investigation of the molecular events that occur in mammary glands has led to the realization that regulation of MDGI production occurs at the level of the steady-state concentration of MDGI mRNA. Additional experiments should lead to a further clarification of the mechanism or mechanisms affecting MDGI expression and ultimately to a better understanding of the physiological significance of these events.

ACKNOWLEDGMENTS

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REFERENCES