Mammaglobin Expression in Primary, Metastatic, and Occult Breast Cancer

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Abstract

The mammaglobin gene encodes a novel, breast cancer-associated glycoprotein. In this study, we have evaluated the frequency with which mammaglobin expression can be detected in primary and metastatic breast tumors and in breast tumor cells present in the peripheral circulation. Of 100 primary human breast tumors examined, 81 were strongly immunopositive for mammaglobin protein. Staining was independent of tumor grade and histological type. Ten of 11 lymph nodes from patients with metastatic breast cancer contained detectable mammaglobin mRNA, whereas mammaglobin expression in uninvolved lymph nodes was undetectable. Using a nested reverse transcription-PCR assay, mammaglobin mRNA was also detected in 9 of 15 products (60%) used for autologous stem cell transplant. These results suggest that larger clinical studies are warranted to investigate the full clinical utility of mammaglobin as a tool for breast cancer patient management.

Introduction

The mammaglobin gene was first identified using a differential screening approach directed at the isolation of novel, human breast cancer-associated genes (1, 2). Mammaglobin encodes a Mr 10,000 glycoprotein and is distantly related to a family of epithelial secretory proteins that includes rat estramustine-binding protein/prostatein (3, 4) and human Clara cell Mr 10,000 protein (CC10)/uteroglobin (5). Although the function of the mammaglobin protein is unknown, mammaglobin displays two characteristics to suggest that its expression is particularly relevant to breast cancer biology: (a) bothNorthern blot analysis and a more sensitive RT-PCR analysis of several adult human tissues have demonstrated that mammaglobin expression is restricted to the mammary gland (2); and (b) mammaglobin mRNA is present at high levels in human breast tumor cell lines and primary breast tumors as compared to nonmalignant breast tissue. In a preliminary survey, 5 of 10 breast carcinoma cell lines and 8 of 35 primary human breast tumors exhibited high levels of mammaglobin mRNA (2). Based on its breast cancer-associated and somewhat unique breast-specific pattern of expression, we believe that mammaglobin is an excellent candidate for a novel and clinically useful breast tumor marker. As a first step to determine the clinical utility of mammaglobin, we have further evaluated the frequency with which mammaglobin expression can be detected in primary breast tumors, metastatic breast tumors, and occult breast tumor cells present in the peripheral circulation.

Materials and Methods

Immunohistochemical Staining for Mammaglobin. The COOH-terminal sequence of the mammaglobin protein was synthesized as a 16-residue peptide (EVFMQLYDSSLCDLF), conjugated to carrier, and injected into rabbits for antiserum production (Research Genetics, Huntsville, AL). Archived breast tumor specimens were chosen at random from the Vanderbilt University Department of Pathology and the Washington University Cancer Center Tumor Repository. Formalin-fixed, paraffin-embedded tissues were cut at 5 μm, mounted on charged slides, and dried.

For immunohistochemical analysis, slides were deparaffinized and rehydrated in graded solutions of ethanol and distilled water. Tissue sections were preincubated with normal goat serum (Vector Laboratories, Burlingame, CA) at a 1:1000 dilution in 3% BSA/PBS and then with anti-mammaglobin rabbit antiserum at a 1:5000 dilution for 1 h at room temperature. After several rinses in PBS, sections were incubated in a solution of normal goat serum (1:1000), 5% BSA, and 6 μg/ml biotinylated goat anti-rabbit IgG (Vector Laboratories) in PBS for 1 h. The secondary antibody solution was rinsed four times in PBS, and tissues were then incubated with a 1:1000 dilution of streptavidin peroxidase (Boehringer Mannheim, Indianapolis, IN) in a solution of 3% BSA/PBS. After a 30-min incubation, slides were again rinsed four times in PBS and exposed to chromagen solution containing 1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAKO, Carpinteria, CA) and 0.02% hydrogen peroxide for 3 min. Slides were rinsed briefly in deionized water, counterstained with Harris’ hematoxylin, and mounted under coverslips. For negative controls, tissue sections were processed identically, except that a 1:500 dilution of preimmune rabbit serum was substituted for the anti-mammaglobin antiserum. Alternatively, for peptide competition experiments, mammaglobin antiserum was first incubated with the 16-residue mammaglobin peptide at a concentration of 100 μg/ml in 3% BSA/PBS for 1 h at room temperature and then applied to tissue sections. Immunopositivity was scored as follows: 0, no staining; 1, weak and sporadic staining in less than 50% of tumor cells; 2, weak staining in greater than 50% of tumor cells; 3, strong, diffuse cytoplasmic staining in less than 50% of tumor cells; and 4, strong, diffuse cytoplasmic staining in greater than 50% of tumor cells. Only sections scoring 3 or 4 were considered to be mammaglobin positive.

Specimen Collection and RNA Preparation. All human tissue specimens were obtained and analyzed with approval from the Washington University Human Studies Review Board. Anonymous lymph node specimens containing metastatic lesions were obtained from the Cooperative Human Tissue Network (6) and the Washington University Cancer Center Tumor Repository. Tissue specimens were cryosectioned at 50 μm, and three to four sections were dissolved in 500 μl of Trizol reagent (Life Technologies, Inc., Rockville, MD). An adjacent, H&E-stained section confirmed the presence of neoplastic cells in involved nodes. Aliquots of ~1 × 106 peripheral leukocytes were obtained from leukopheresis products of breast cancer patients undergoing peripheral stem cell collection for autologous stem cell transplant. For positive controls, 105 mammaglobin-expressing MDA-MB175 human breast tumor cells were mixed with 106 human OM431 melanoma cells to yield a 1:105 breast cancer cell dilution. A pure population of OM431 cells was used as a negative control. Frozen cell pellets were immediately lysed in 1 ml of Trizol reagent (Life Technologies, Inc.).

All RNA isolations were performed exactly as recommended in the manufacturer’s protocol, and resulting RNAs were resuspended at a concentration of 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: RT-PCR, reverse transcription-PCR; dNTP, deoxynucleotide triphosphate; DCIS, ductal carcinoma in situ; PBSC, peripheral blood stem cell, K19, Keratin 19; CEA, Carcinoembryonic Antigen.
2 μg/μl in RNase-free water. RNA integrity and concentration were also assessed by agarose gel electrophoresis.

**RT-PCR Assays.** The mammaglobin RT-PCR assays were performed in the Washington University Cancer Center Molecular Diagnostics Core Facility. Approximately 1 μg of total RNA was converted to first-strand cDNA using a T12–18 primer and the Superscript II preamplification system (Life Technologies, Inc.) as per the supplier’s protocol. After treatment with and inactivation of RNase H, samples were stored at –20°C. To assess the integrity of synthesized cDNA, 10% of the cDNA was subjected to a 25-μl PCR reaction containing a final concentration of 1× Taq DNA polymerase buffer, 2.0 mM MgCl₂, 200 μM dNTPs, 0.6 μM glyceraldehyde-3-phosphate dehydrogenase forward amplification primer (5’-CCACCCATGGCAAATTCCATGGCA-3’), 0.6 μM glyceraldehyde-3-phosphate dehydrogenase reverse amplification primer (5’-TCTAGACGGCAGGTCAGGTCCACC-3’), and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.). Reactions were heated to 94°C for 1 min and then subjected to 40 cycles of 94°C for 45 s, 57°C for 60 s, 72°C for 45 s, followed by a final extension step at 72°C for 5 min. A preliminary study was performed to determine the optimal number of PCR cycles. After amplification, 2-μl aliquots were run on a 2% agarose gel to determine the presence of a product of the expected size. The remaining cDNA was stored at –20°C until use.

### Table 1 Mammaglobin immunoreactive staining in human breast cancer

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Staining score 4</th>
<th>Staining score 3</th>
<th>Staining score 2</th>
<th>Staining score 1</th>
<th>Staining score 0</th>
<th>Total</th>
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<tr>
<td>Lobular</td>
<td>0</td>
<td>1 (33%)</td>
<td>1 (33%)</td>
<td>0</td>
<td>1 (33%)</td>
<td>3</td>
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<tr>
<td>Well differentiated</td>
<td>11 (78%)</td>
<td>0</td>
<td>1 (7%)</td>
<td>0</td>
<td>2 (14%)</td>
<td>14</td>
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<tr>
<td>Moderately differentiated</td>
<td>28 (67%)</td>
<td>5 (12%)</td>
<td>3 (7%)</td>
<td>2 (5%)</td>
<td>4 (10%)</td>
<td>42</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>24 (63%)</td>
<td>9 (24%)</td>
<td>2 (5%)</td>
<td>0</td>
<td>3 (8%)</td>
<td>38</td>
</tr>
<tr>
<td>DCIS</td>
<td>1 (33%)</td>
<td>2 (67%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td>17</td>
<td>7</td>
<td>2</td>
<td>10</td>
<td>100</td>
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</tbody>
</table>

* a Except for lobular and DCIS, all specimens are invasive ductal cancers. b The degree of immunopositive staining of breast tumor tissue sections with anti-mammaglobin antibody was rated as follows: 0, no staining; 1, weak staining in <50% of tumor cells; 2, weak staining in >50% of tumor cells; 3, strong, cytoplasmic staining in <50% of tumor cells; 4, strong, cytoplasmic staining in >50% of tumor cells. For purposes of discussion, mammaglobin-positive tumors are those that scored 3 or 4.
and 72°C for 45 s. PCR products were analyzed on a 2% agarose gel, and a single, uniformly intense fragment of 599 nucleotides indicated that each cDNA synthesis reaction had been successful.

For expression studies in lymph node tissue, 10% of each lymph node cDNA reaction was subjected to a 25-μl PCR reaction containing a final concentration of 1× Taq DNA polymerase buffer, 1.5 mM MgCl₂, 200 μM dNTPs, 0.6 μM mammaglobin forward amplification primer (5'-AGCAGTCACCAGGTGGTGG-3'), 0.6 μM mammaglobin reverse amplification primer (5'-ATAAGAAGAAGGTGGTGG-3'), and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.). Reactions were heated to 94°C for 1 min and then subjected to 40 cycles of 94°C for 45 s, 55°C for 60 s, and 72°C for 45 s. An additional 10% of each cDNA was subjected to an identical 25-μl PCR reaction containing 2.0 mM MgCl₂, 0.6 μM K19 forward amplification primer (5'-GAGGTTGATTCCGCTCGGGA-3'), and 0.6 μM K19 reverse amplification primer (5'-ATCTTTCCGTTCCTCAGGCCAG-3') with reaction conditions of 94°C for 45 s, 58°C for 60 s, and 72°C for 45 s for 40 amplification cycles. All amplification products were delivered to an alternate laboratory site and electrophoresed on a 2% agarose gel.

For nested RT-PCR analysis of stem cell products, 10% of each stem cell cDNA reaction was subjected to a 25-μl PCR reaction containing a final concentration of 1× Taq DNA polymerase buffer, 2.0 mM MgCl₂, 200 μM dNTPs, 0.6 μM mammaglobin outer forward amplification primer (5'-CAGCGGTCTCCTGATCTGTG-3'), 0.6 μM mammaglobin outer reverse amplification primer (5'-TACGGTTTCAACAATGTGC-3'), and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.). Reactions were heated to 94°C for 1 min and then subjected to 25 cycles of 94°C for 45 s, 58°C for 60 s, and 72°C for 30 s. A 5-μl aliquot (10%) of the primary PCR product was added to an identical, secondary 25-μl PCR reaction containing a final concentration of 1× Taq DNA polymerase buffer, 1.5 mM MgCl₂, 200 μM dNTPs, 0.6 μM mammaglobin inner forward amplification primer (5'-AGCAGTGCTACGGGTGCCTC-3'), 0.6 μM mammaglobin inner reverse amplification primer (5'-ATAAGAAGAAGGTGGTGG-3'), and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.). Reactions were heated to 94°C for 1 min and then subjected to 40 cycles of 94°C for 45 s, 55°C for 60 s, and 72°C for 45 s. Secondary PCR products were electrophoresed on a 2% agarose gel and subjected to Southern blot analysis using a 0.2 μm Nitran Plus membrane and the supplier’s protocol for the neutral transfer method (Schleicher & Schuell, Keene, NH). The resulting filter was hybridized with 1 × 10⁶ cpm/ml mammaglobin cDNA probe and washed as described previously (2).

**Results and Discussion**

High levels of mammaglobin mRNA have been observed previously in a small set of primary human breast tumors (2). To study mammaglobin protein expression, control for cellular heterogeneity between tumor specimens, and develop a reagent that could be implemented for routine immunohistochemical analysis, we synthesized a peptide corresponding to the COOH-terminal mammaglobin protein sequence and generated rabbit polyclonal anti-mammaglobin antibodies. The specificity of the generated antibody was confirmed by Western blot analysis of several human breast tumor cell lines and primary human breast tumors that had been examined previously for mammaglobin mRNA expression (2). A single, immunoreactive protein band was observed only in protein extracts from a subset of samples known to express mammaglobin mRNA. This immunoreactivity was lost when the antibody was preincubated with the peptide used for immunization. Furthermore, when the human A293 cell line was transfected with a mammaglobin cDNA expression vector, an identical immunoreactive band was detected by Western blot analysis of the transfected cell protein extract. Nontransfected A293 cells yielded no immunoreactive species with the mammaglobin antibody. Having demonstrated the specificity of the antibody, this reagent was used in a larger survey of primary breast tumors of varying grades and histological types. A summary of mammaglobin protein expression in these specimens is presented in Table 1. Overall, 80% of ductal carcinomas examined demonstrated strong cytoplasmic staining for mammaglobin protein. Interestingly, staining was equally frequent among well-differentiated (78%), moderately differentiated (67%), and poorly differentiated (63%) tumors (Fig. 1). Strong staining was also seen in three of three cases of pure DCIS. The predominant mammaglobin staining pattern in tumor cells was diffuse and cytoplasmic, although some cells demonstrated intense staining localized adjacent to the nucleus. In nonneoplastic breast tissue, rare and scattered positive epithelial cells were seen within the acini of type I (Fig. 1c) and type II (Fig. 1d) lobules and within the columnar cells of terminal ducts (Fig. 1e). However, the overall frequency of mammaglobin immunoreactivity in nonneoplastic ducts and acini was less than 10%. This suggests that although mammaglobin expression is not truly tumor specific, there is a quantitative and/or qualitative difference in the level of expression between malignant and nonmalignant breast epithelium. As has been observed with other secretory proteins, such as the M₁ 15,000 gross cystic disease fluid protein (GCDP15) (7–9), increased expression of mammaglobin coincided with features of apocrine change. However, unlike GCDP15, breast tumor cells with both apocrine and nonapocrine features express mammaglobin.

MAMMAGLOBIN EXPRESSION IN BREAST CANCER

![Mammaglobin and keratin 19 expression in lymph nodes containing metastatic breast cancer. RT-PCR analysis using keratin 19-specific primers (top panel) or mammaglobin-specific primers (bottom panel) in parallel assays. Lanes 2–12 are lymph node specimens containing histologically documented metastases from primary breast tumors. Lanes 13–15 are lymph node biopsies from patients without any known malignancy. Lane 1 is a primary breast tumor previously shown to express mammaglobin and serves as a positive control for the assay. Lane −, a negative (no reverse transcriptase enzyme) control for the assay; Lane M is a 100-bp molecular ladder.](image1)

![Detection of mammaglobin in circulating breast tumor cells. RNA from patients with metastatic breast cancer (CA) or normal donors (NL) was obtained from peripheral stem cell collection products and subjected to nested RT-PCR and Southern hybridization analysis to determine mammaglobin expression. Negative control RNA (−) is derived from the OM431 melanoma cell line. Positive control RNA (+) is derived from a mix of 1:10² MDA-MB175 breast tumor cells in a background of OM431 cells.](image2)

4 M. A. W. and T. P. F., unpublished data.
5 M. A. W., unpublished data.
with roughly equal frequency and intensity. The specificity of all positive staining patterns was documented by the lack of signal from identical specimens incubated with either preimmune rabbit serum or anti-mammaglobin antiserum preincubated with competing COOH-terminal peptide.

We also examined whether mammaglobin expression could be detected in metastatic breast tumor cells within lymph nodes. Several epithelial-specific genes have been used as surrogate markers to detect occult epithelial malignancies. A nested RT-PCR assay for CEA has been used to identify metastases in histologically negative lymph nodes from patients with breast and gastrointestinal cancer (10). Many similar assays based on cytokeratin 20 (11) and keratin 19 (12–14) have been used to detect occult breast tumor cells in lymph nodes and the peripheral circulation. These molecular assays demonstrate enhanced sensitivity over serial sectioning and immunohistochemical approaches. However, their utility is often limited by a low level of keratin gene expression normally present in lymphoid and other nonepithelial cells (14, 15). Recently, a study comparing seven different gene expression markers found that both mammaglobin and CEA were frequently expressed among human breast tumor cell lines but were not expressed in a sample of 20 normal lymph nodes. The study concluded that both mammaglobin and CEA were excellent candidate markers for detecting breast cancer metastases in sentinel lymph nodes (16). Using a similar RT-PCR approach, we directly compared mammaglobin expression to that of keratin 19 in a group of lymph node specimens from patients with metastatic breast cancer. Fig. 2 shows the results of this study. Among 11 patients with lymph nodes containing histologically documented metastatic breast cancer, mammaglobin expression could be detected in 10 cases (91%), whereas keratin 19 expression was detected in all 11 cases (100%). However, in three cases of lymph nodes from patients without malignant disease, keratin 19 was detected in all cases, whereas mammaglobin was not detectable in any cases.

Because metastatic breast tumor cells frequently express mammaglobin and because mammaglobin cannot be detected in lymphoid populations, we investigated whether a more sensitive RT-PCR assay for mammaglobin mRNA could detect circulating breast tumor cells. Samples of PBSC collections from 15 patients undergoing high-dose chemotherapy and autologous stem cell transplant for metastatic breast cancer were subjected to a nested RT-PCR assay to detect mammaglobin mRNA. The results of this assay are shown in Fig. 3. Based on the robust hybridization signal obtained from duplicate positive controls containing 1:10⁵ breast tumor cells, the limit of detection for the assay is probably well below 1:10⁶ cells. Mammaglobin mRNA was not detected in any PBSC samples from healthy donors at this level of amplification. Among 15 cases of metastatic breast cancer, 9 patients (60%) yielded detectable mammaglobin mRNA from their PBSCs, suggesting that the collected products contained contaminating breast tumor cells. In the six negative cases, we were unable to determine whether a lack of signal represented a lack of tumor cells that express mammaglobin, a true absence of tumor cells, or assay insensitivity. Limiting future studies to patients whose primary tumors definitively express mammaglobin (either by immunohistochemical or molecular assays of the primary tumor) will simplify interpretation of a negative result.

The clinical significance of occult tumor cells in lymph nodes, bone marrow, and the circulation remains controversial. Nevertheless, mammaglobin expression may have sufficient sensitivity and specificity to be a useful tool to further explore this issue in larger clinical trials. For example, mammaglobin expression may be useful to evaluate tumor contamination of peripheral stem cell products after cytoreductive therapy and tumor cell purging protocols (18). In summary, the current study demonstrates that mammaglobin expression is a promising marker for neoplastic breast epithelial cells and provides sufficient evidence to warrant larger clinical studies using mammaglobin as a molecular marker for early detection, staging, prognosis, and/or relapse monitoring of breast cancer.

References
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