Mammaglobin and Lipophilin B Expression in Breast Tumors and their Lack of Effect on Breast Cancer Cell Proliferation

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Abstract. Background: Mammaglobin (SCGB2A2) and lipophilin B (SCGB1D2) are members of the secretoglobin polypeptide family. Mammaglobin has been shown to be overexpressed in breast tumor tissue, indicating that mammaglobin might confer a growth advantage to mammaglobin-expressing tumor cells. Materials and Methods: The mammaglobin and lipophilin B mRNA expression levels were investigated in seven breast tumors and matched non-neoplastic tissues from the same patients using quantitative real-time RT-PCR. The effect of mammaglobin and lipophilin B expression on breast cancer cell proliferation rates was investigated by analyzing retrovirally transduced Hs578T cell clones. Cell proliferation rates were determined during the exponential growth phase by analyzing the change in lactate dehydrogenase activity over time. Results: All analyzed breast cancer tumors had lower expression levels of mammaglobin and lipophilin B than the respective mean level of the non-neoplastic breast tissues; no prominent overexpression was evident. There was high variability in the expression of mammaglobin and lipophilin B among the non-neoplastic samples, showing that caution should be taken when evaluating their over- and underexpression in tumors. The expression levels of mammaglobin and lipophilin B correlated with each other in the analyzed samples (p=0.001). Ectopic overexpression of mammaglobin and lipophilin B did not affect the cell proliferation rate of Hs578T breast carcinoma cells in vitro. Conclusion: Our findings suggest that the overexpression of mammaglobin observed in certain breast tumors is an epiphenomenon not causally involved in breast carcinogenesis.

Mammaglobin (SCGB2A2) and lipophilin B (SCGB1D2), also known as BU101, belong to the secretoglobin polypeptide family, which consists of small secreted polypeptides (1). Human mammaglobin was identified in a search for genes with altered expression in primary human breast tumors compared to normal breast tissue (2). In breast tissue, mammaglobin forms a heterodimer with lipophilin B, and its expression is confined to breast epithelial cells (3-5). Since mammaglobin transcripts are not normally expressed by cells in blood and lymph nodes, it is considered a promising molecular marker for disseminated and circulating breast cancer cells (reviewed in (6)). Lipophilin B transcripts have not been detected in blood, however, antibodies against lipophilin B are found in serum from patients with late-stage breast cancer (7).

The biological function of the mammaglobin-lipophilin B protein complex is not known, but mammaglobin has been suggested to be involved in breast cell proliferation (8). Whether the expression of mammaglobin and lipophilin B changes during breast cancer tumorigenesis is controversial. Several studies have shown overexpression of mammaglobin and lipophilin B in breast tumors (2, 5, 9-13). However, a recent study showed downregulation of both mammaglobin and lipophilin B in a large proportion of breast tumors (14). We have shown down-regulation of mammaglobin in sweat gland-derived cylindromas (15) and down-regulation of lipophilin B in pituitary adenomas (16). Other secretoglobins, such as uteroglobin and high in normal-1 (HIN-1), are generally down-regulated in tumors and have been suggested to function as tumor suppressors (17-24).

The aim of this study was to clarify the clinical and biological role of mammaglobin and lipophilin B in human breast cancer. Seven breast tumors and matched non-neoplastic tissues were analyzed for mammaglobin and lipophilin B expression. In addition, the effect of ectopic mammaglobin and lipophilin B overexpression on breast cancer cell proliferation was studied.

Materials and Methods

Patients. Tumor samples and matched non-neoplastic breast tissues were collected from six patients with breast carcinoma and one patient with fibroadenoma (Table I). None of the patients had...
tissues by using RNAqueous kit (Ambion Inc.) and from cells using Quantitative real-time RT-PCR.

Infection efficiency was stock was replaced by fresh medium and the cells were cultivated to a 24-well plate. The plate was centrifuged for 2 hours at 1350 x g, gives an indirect measurement of the cell proliferation rates. Western blotting. Cells were kept on ice and lysed for 30 min in 1% Triton X-100 solution. Thereafter the lysates were transferred to Eppendorf tubes and stored at –20°C until use. Protein concentrations of the cell lysates were determined using the BCA method ( Pierce Chemical Co., Rockford, IL, USA). Proteins were separated under reducing conditions on 4-12% bis-Tris Nu-PAGE gradient gels (Invitrogen AB) using MES-SDS running buffer. The separated proteins were incubated with 1 µg/ml of affinity purified anti-mammaglobin antibodies (15) for 60 min at room temperature, followed by peroxidase-conjugated anti-rabbit IgG antibody (Amersham Biosciences). The blocked membranes were incubated with 0.1% Tween 20. Thereafter, the blocked membranes were incubated with 1 µg/ml of affinity purified anti-mammaglobin antibodies (15) for 60 min at room temperature, followed by peroxidase-conjugated anti-rabbit IgG antibody (Amersham Biosciences). The blocked membranes were incubated with 1 µg/ml of affinity purified anti-mammaglobin antibodies (15) for 60 min at room temperature, followed by peroxidase-conjugated anti-rabbit IgG antibody (Amersham Biosciences).

Production of retroviral stocks and infection of Hs578T cells. Mammaglobin, lipophilin B and green fluorescent protein (GFP) cDNAs were cloned into pMX vector (27), which was kindly provided by J. Bergh (Uppsala University Hospital, Sweden). Phoenix A packaging cells were obtained from the American-Type Culture Collection (Manassas, VA, USA). The cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 50 µg/ml gentamicin. Cell culture media and reagents were from Invitrogen AB (Täby, Sweden).

Cell lines and cell culture. The breast cancer cell line Hs578T was kindly provided by J. Bergh (Uppsala University Hospital, Sweden) and has been described elsewhere (26); the breast cancer cell line MDA-MB-415 was kindly provided by T. Fleming (Washington University, St. Louis, MO, USA). Phoenix A packaging cells were provided by T. Kitamura (University of Tokyo, Japan). Phoenix A packaging cells were kindly provided by J. Bergh (Uppsala University Hospital, Sweden).

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Cell proliferation assay. Cell proliferation rates were determined during the exponential growth phase by analyzing the change in lactate dehydrogenase (LDH) activity over time (30). The reduction of pyruvate by NADH to form lactate is catalyzed by LDH and produces a change in optical density at 340 nm (31). Since every viable cell contains the same amount of LDH enzyme, the LDH-activity correlates with the number of cells and its change gives an indirect measurement of the cell proliferation rates.

Cells were plated in 96-well flat-bottom plates at 4,000 cells/well and maintained in 90 µl medium. LDH activity was measured 24, 48, 72 and 96 hours after the plating. For each cell clone, eight wells were analyzed at respective time points. To induce complete cell lysis, 10 µl of 1% Triton X-100 were added to each well, followed by one hour of incubation at room temperature. After cell lysis, 20 µl supernatant was transferred to a new plate and mixed with 180 µl substrate buffer containing 8x10^-4 M sodium pyruvate (Sigma-Aldrich) and 2x10^-4 M β-NADH (Sigma-Aldrich) in 0.1 M phosphate buffer, pH 7.0. Absorbance at 340 nm was recorded immediately after the addition of substrate buffer and thereafter received any treatment prior to specimen collection. Samples of the tumor and non-neoplastic breast tissues were fixed in RNAlater (Ambion Inc., Austin, TX, USA) immediately after excision. Adjacent parts of the tissue samples were fixed in formalin and paraffin embedded, followed by routine morphological examination and tumour grading according to Page et al. (25).

Table I. Clinical and pathological features of the breast tumors included in the study.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>TNM</th>
<th>Grade</th>
<th>Receptor status</th>
<th>Tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Estrogen Progesterone</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>58</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Fibroadenoma</td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>T1N0M0</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>T1N0M0</td>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>T1N1M0</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>74</td>
<td>T2N0M0</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
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<td>T1N0M0</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>7</td>
<td>72</td>
<td>T1N0M0</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

ND, not determined.
recorded for up to 10 minutes. Background control absorbance values, measured for the culture medium without cells, were subtracted from these of cell cultures and the mean of the absorbance change (ΔA/min) was calculated.

Statistical analysis. Spearman’s rho test was used to test the statistical significance for correlation of mammaglobin and lipophilin B expression in breast tumor tissues and matched non-neoplastic breast tissues. Linear regression was used to estimate the coefficients of the linear equation, involving cell doubling time and secretoglobin expression. All calculations were carried out with the Statistical Package for Social Scientists (SPSS) for Windows version 11.5 (SPSS Inc., Chicago, IL, USA).

Results

In order to examine the mammaglobin and lipophilin B mRNA expression in breast tumors and matched non-neoplastic breast tissues, RNA samples from tumors and matched non-neoplastic breast tissues were analyzed by quantitative real-time RT-PCR. In the six matched breast cancer samples, the mammaglobin/18S rRNA-ratio was higher in three, and lower in the remaining three breast cancer tumors, compared to their matched non-neoplastic breast tissues (Figure 1A, patients 2-7). The lipophilin B/18S rRNA-ratios exhibited a similar pattern as mammaglobin; the lipophilin B/18S rRNA-ratio was higher in two, and lower in four of the breast cancer tumors, compared to their matched non-neoplastic breast tissue (Figure 1B, patients 2-7). Analysis of a fibroadenoma (Figure 1A and 1B, patient 1) showed an approximately two-fold difference in expression of both mammaglobin and lipophilin B between the tumor and the matched non-neoplastic tissue. The mRNA expression of mammaglobin and lipophilin B positively correlated with each other (Spearman’s rho=0.796, p=0.001, 2-tailed test). Notably, all the breast cancer tumor samples exhibited levels of both mammaglobin and lipophilin B lower than the respective mean level of the seven non-neoplastic samples.

To evaluate the effect of mammaglobin and lipophilin B overexpression on the cell proliferation rate, overexpression of their respective mRNAs in the breast cancer cell line Hs578T, which did not express any endogenous secretoglobins (data not shown), was generated by retrovirally transducing cells with expression constructs for mammaglobin or lipophilin B, which were thereafter cloned. The infection efficiency of the Hs578T cell line was approximately 20% when measured as a proportion of fluorescent cells after a parallel infection with a green fluorescent protein control construct.

Quantitative real-time RT-PCR analysis of nine isolated mammaglobin-infected Hs578T cell clones showed mammaglobin mRNA expression ranging from none mammaglobin expression to 15 times the mean of that of the non-neoplastic breast samples (Figure 2A). Similarly, analysis of ten isolated lipophilin B-infected Hs578T cell clones showed lipophilin B mRNA expression ranging from no lipophilin B mRNA to 72 times the mean of that of the non-neoplastic breast samples (Figure 2B). For comparison, the breast cancer cell line MDA-MB-415, which expresses endogenous mammaglobin and lipophilin B, was analyzed.
The level of mammaglobin and lipophilin B overexpression in MDA-MB-415 was 34- and 11-fold, respectively, that of the mean of the non-neoplastic samples (data not shown). The nine Hs578T clones expressing no or various levels of mammaglobin mRNA and the ten clones expressing no or various levels of lipophilin B mRNA were evaluated for their cell proliferation rates under standard cell culture conditions. Cell doubling times were calculated for cells in the exponential growth phase. Hs578T cells expressing no mammaglobin or lipophilin B showed cell doubling times between 15.1 and 24.2 hours (Figure 2A, B). Mammaglobin-expressing Hs578T clones had cell doubling times between 14.6 and 19.2 hours (Figure 2A), and lipophilin B-expressing Hs578T clones had cell doubling times between 14.0 and 19.4 hours (Figure 2B). Overexpression of mammaglobin showed no association with cell doubling time \((p=0.33)\), while lipophilin B over-expression showed a weak association with a shorter cell doubling time \((p=0.043)\). To confirm mammaglobin protein expression in the ectopically mRNA-expressing clones, mammaglobin protein was analyzed by Western blot analysis. Cell lysates from an Hs578T cell clone expressing 15 times the mean mammaglobin level of the non-neoplastic cells and the breast cancer cell line MDA-MB-415, were separated by SDS polyacrylamide gel electrophoresis and immunoblotted. The immunoblot showed that the mammaglobin mRNA-expressing Hs578T cell line expressed mammaglobin protein, albeit at a lower level than the MDA-MB-415 cell line (Figure 3).

To investigate whether co-expression of mammaglobin and lipophilin B had any effect on cell proliferation rates, lipophilin B was retrovirally transduced into the mammaglobin-transduced Hs578T clone exhibiting the highest mammaglobin expression level. The fraction of cells with chromosomally integrated retrovirally transduced lipophilin B cDNA was analyzed by quantitative real-time PCR 36 and 64 days after the retroviral infection. The fraction of cells with transduced lipophilin B genes, in a population of cells in which all cells stably expressed mammaglobin, did not change over time (Figure 4), showing that co-expression of mammaglobin and lipophilin B did not affect the cell proliferation rate of Hs578T breast carcinoma cells.

**Discussion**

The rationale for conducting the present study was based on previous studies showing that mammaglobin and lipophilin B are over expressed in certain breast tumors, indicating that expression of these two secretoglobins might confer a growth advantage to tumor cells (2, 5, 9-13). In the present study we found that all of the six breast cancer tumors analyzed expressed mammaglobin and lipophilin B at levels lower than the mean levels of the non-neoplastic tissues; no
overexpression was apparent. In addition, we found that forced overexpression of mammaglobin and/or lipophilin B did not appreciably affect the proliferation rate of Hs578T breast carcinoma cells.

Although the low number of patients included (n=7) precluded performing any thorough statistical analysis, we were, nevertheless, able to confirm that the expression of mammaglobin and lipophilin B correlated with each other in breast tissue (14). Notably, all of the six breast cancer tumors analyzed expressed mammaglobin and lipophilin B at levels lower than the mean levels of the non-neoplastic tissues. This is in line with a recent study by Zafrakas et al. (14); in their breast tumor material, down-regulation of mammaglobin and lipophilin B was more common than up-regulation. The high variability in the expression among the non-neoplastic samples suggests that caution should be taken when evaluating over- and underexpression of mammaglobin and lipophilin B in breast tumors.

Ectopic overexpression of mammaglobin and lipophilin B did not appreciably affect the cell proliferation rate of Hs578T breast carcinoma cells. Importantly, the levels of over-expression in carcinoma tissue compared to the non-neoplastic tissues was up to 14-fold for mammaglobin and up to 72-fold for lipophilin B, thus being in the range of what has previously been described in over expressing clinical breast tumors (9, 10, 13). We also assessed the effects of co-expression of mammaglobin and lipophilin B, but neither did it have any appreciable effect on the cell proliferation rate of Hs578T cells.

From this we conclude that overexpression of mammaglobin and/or lipophilin B does not confer a growth advantage, or disadvantage, for breast carcinoma cells under standard in vitro cell culture conditions. This does not exclude the possibility that mammaglobin and/or lipophilin B could be of some advantage to the tumor cells in vivo. However, together with previous reports showing an association between elevated mammaglobin and less aggressive breast tumors (32, 33), it does suggest that the overexpression of mammaglobin observed in certain breast tumors is an epiphenomenon not causally involved in breast carcinogenesis.

Acknowledgements

This work was supported by grants from the Cancer Research Foundation in Northern Sweden and the Swedish Cancer Society. We thank Anders Johansson, Annika Holmberg, Robert Johansson and Yvonne Jonsson for technical help.

References


Figure 4. Quantitative real-time PCR analysis of gene copy numbers in retrovirally transduced Hs578T cells. Lipophilin B and APP gene copy numbers were analyzed 36 and 64 days after retroviral infection of Hs578T cells with lipophilin B expression vector. Error bars indicate standard deviations of quadruplicate samples.


