Ceramide synthases and ceramide levels are increased in breast cancer tissue

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Abbreviations:

- Cer, ceramide
- Sph, sphingosine
- SphK, sphingosine kinases
- LASS, L-serine palmitoyl-CoA synthase
- S1P, sphingosine-1-phosphate
- dhSph, sphinganine
- ER, estrogen receptor
- CerS, dihydroceramide synthase
- dhCer, dihydroceramide
- GAPDH, glyceraldehyde-3-phosphate dehydrogenase
- LASS1–6, L-serine palmitoyl-CoA synthases
- DEGS, dihydroceramide desaturase

Introduction

Sphingolipids are synthesized de novo at the endoplasmic reticulum from serine and palmitoyl-CoA, which condense to form 3-ketosphinganine that enters the sphingolipid metabolic pathway. Sphingosine kinase (SphK) 1 and 2 metabolize ceramides into sphingosine-1-phosphate (S1P), which plays important and general roles in cellular regulation, such as the promotion of tumor cell apoptosis, cell cycle arrest and senescence (4). Furthermore, some sphingolipids have a diverse role in signaling that depends on the synthesizing enzymes (SphK1 and SphK2) as well as on the subcellular location of origin. S1P synthesized by SphK1 is involved in proliferative signaling (8), whereas S1P synthesized by SphK2 is described as an antiproliferative and apoptotic mediator (9).

Several in vitro studies have correlated dysfunction of the sphingolipid-signaling pathway with promotion of tumor cell growth as well as progression and resistance of tumors to chemotherapeutic agents. As ceramides (Cer) constitute the structural backbones of all sphingolipids, we investigated the endogenous ceramide levels in 43 malignant breast tumors and 21 benign breast biopsies and compared them with those of normal tissues using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The total ceramide levels in malignant tumor tissue samples were statistically significantly elevated when compared with normal tissue samples. Upregulation of the total ceramide level averaged 12-fold and 4-fold higher than normal tissue samples, for malignant tumors and benign tissue, respectively. Specifically, the levels of C16:0-Cer, C24:1-Cer and C24:0-Cer were significantly raised in malignant tumors as compared with benign and normal tissue. The augmentation of the various ceramides could be assigned to an increase of the messenger RNA levels of ceramide synthases (SphK) LASS2 (longevity assurance), LASS4 and LASS6. Notably, elevated levels of C16:0-Cer were associated with a positive lymph node status, indicating a metastatic potential for this ceramide. Moreover, the levels of C18:0-Cer and C20:0-Cer were significantly higher in estrogen receptor (ER) positive tumor tissues as compared with ER negative tumor tissues. In conclusion, progression in breast cancer is associated with increased ceramide levels due to an upregulation of specific LASS genes.

Materials and methods

Reagents

The sphingolipid standards were purchased either from Avanti Polar Lipids (Alabaster, AL) or Matreya LLC (Pleasant Gap, PA). The antibodies LASS1 (goat polyclonal), LASS2 (goat polyclonal), LASS3 (goat polyclonal), LASS4 (goat polyclonal), LASS5 (goat polyclonal), SPT (goat polyclonal) and LASS6 (mouse monoclonal) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and from Abnova GmbH (Heidelberg, Germany), whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (mouse monoclonal) was obtained from Applied Biosystems/Ambion (Austin, TX).

Tissue samples

Malignant tissue, benign tissue and normal tissues of breast cancer patients were collected over 7 years with informed consent from patients undergoing surgical resection from the Department of Gynecology (Goethe-University, Frankfurt/Main, Germany). The protocol was approved by the local ethics committee. The samples were taken in the years 2001, 2003, 2005, 2006 and 2007. All tissue samples were stored at –80°C. Normal tissues (not benign, not malignant) were excised at least 1 cm away from the main tumor mass of the patients, but the tumor tissues from these patients were not included in this study. Therefore, the tumor tissues and the normal tissues originate from different patients (unpaired samples). Breast tissue samples were characterized according to standard pathology based on the estrogen receptor (ER) status as determined by ligand binding assays or by immunohistochemistry.

Determination of sphingolipid concentrations in tissue

Tissue samples were minced in phosphate-buffered saline on ice. 10 μl tissue samples were homogenized in 1 ml of ice-cold Tris-HCl buffer (50 mM, pH 7.4, 10 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin) and centrifuged at 16,000 g for 15 min at 4°C. The protein concentration in the supernatant was determined by the bicinchoninic acid (BCA) method. The standard curve was generated using 3-ketosphinganine as the standard. One unit of activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate in 1 min. The activity of the enzyme was determined at a fixed substrate concentration of 10 μM. Enzyme activities were calculated using the Lineweaver-Burk equation.
suspension (0.03 mg/ml) was added to 90 µl of freshly prechilled phosphate-buffered saline. Lipids were extracted in 700 µl of chloroform:methanol (7:1) after the addition of the internal standards (C17:0-Cer, C17:0-Sph, C17:0-SIP). The suspension was vortexed at 25°C for 1 min and centrifuged for 5 min at 23°C and 14,000 rpm. The organic phase was collected and the extraction step was repeated. The combined organic phases were dried under a stream of nitrogen and redissolved in 200 µl of methanol for quantification. After liquid–liquid extraction, concentrations of C16:0-Cer, C18:0-Cer, C20:0-Cer, C24:1-Cer and C24:0-Cer and the internal standards were determined by liquid chromatography coupled with tandem mass spectrometry as described previously (23). Chromatographic separation was accomplished under gradient conditions using a Luna C18 column (150 x 2 mm ID, 5 µm particle size and 10 nm pore size; Phenomenex, Aschaffenburg, Germany). Precursor to product ion transitions of m/z 538 → 264 for C16:0-Cer, of m/z 566 → 264 for C18:0-Cer, of m/z 594 → 264 for C20:0-Cer, of m/z 648 → 264 for C24:1-Cer, of m/z 651 → 264 for C24:0-Cer, of m/z 540 → 284 for C16:0-dhCer, of m/z 568 → 284 for C18:0-dhCer, of m/z 596 → 284 for C20:0-dhCer, of m/z 651 → 284 for C24:1-dhCer, of m/z 653 → 284 for C24:0-dhCer, of m/z 530 → 282 for Sph, of m/z 302 → 284 for dhSph, of m/z 380 → 264 for Sph1P, of m/z 382 → 266 for sphinganine-1-phosphate of m/z 552 → 534 for C17:0-Cer, of m/z 286 → 268 for C17:0-Sph and of m/z 366 → 250 for C17:0-Sph1P were used for the multiple reaction monitoring with a dwell time of 15 ms. The concentrations of Sph, dhSph, S1P and sphinganine-1-phosphate in the selected tumor volume were determined by liquid chromatography coupled with tandem mass spectrometry as described previously (23). Chromatographic separation was accomplished under gradient conditions using a Luna C18 column (150 x 2 mm ID, 5 µm particle size and 10 nm pore size; Phenomenex, Aschaffenburg, Germany). Precursor to product ion transitions of m/z 538 → 264 for C16:0-Cer, of m/z 566 → 264 for C18:0-Cer, of m/z 594 → 264 for C20:0-Cer, of m/z 648 → 264 for C24:1-Cer, of m/z 651 → 264 for C24:0-Cer, of m/z 540 → 284 for C16:0-dhCer, of m/z 568 → 284 for C18:0-dhCer, of m/z 596 → 284 for C20:0-dhCer, of m/z 651 → 284 for C24:1-dhCer, of m/z 653 → 284 for C24:0-dhCer, of m/z 530 → 282 for Sph, of m/z 302 → 284 for dhSph, of m/z 380 → 264 for Sph1P, of m/z 382 → 266 for sphinganine-1-phosphate of m/z 552 → 534 for C17:0-Cer, of m/z 286 → 268 for C17:0-Sph and of m/z 366 → 250 for C17:0-Sph1P were used for the multiple reaction monitoring with a dwell time of 15 ms. The concentrations of Sph, dhSph, S1P and sphinganine-1-phosphate in the selected tumor volume were below the detection limit. The tumor volume could not be scaled up due to the priority for pathological studies that required a greater part of sample. Concentrations of the calibration standards, quality controls and unknowns were evaluated by Analyst software 1.4.2 (Applied Biosystems). A representative chromatogram of C16:0-Cer, C17:0-Cer, C18:0-Cer, C20:0-Cer, C24:1-Cer and C24:0-Cer is displayed in supplement 1 (available at Carcinogenesis Online).

Western blot analysis

Frozen tissue was chopped with a mortar, suspended in lysate buffer [150 mM NaCl, 50 mM Tris, 1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton-X 100, Roche complete (Roche, Mannheim, Germany) pH 7.4], sonicated and centrifuged (14,000 rpm, 10 min, 4°C). The supernatant was stored at -80°C. Forty micrograms of the protein lysate were electrophoresed. The antibodies used were diluted as follows: LASS1, 3, 4, 5, 6, SPT (1:100); LASS2 (1:300) and GAPDH (1:2000). The antibodies used were diluted as follows: LASS1, 3, 4, 5, 6, SPT (1:100); LASS2 (1:300) and GAPDH (1:2000).

Statistical analysis

First, the tissue concentrations of the various ceramides were compared by means of Friedman analysis of variance, with post-hoc Wilcoxon–Wilcox comparisons, a-corrected for multiple testing according to Bonferroni (BIAS software version 8.4, http://www.bias-online.de). Second, to analyze whether or not the ceramide concentrations differed between the tissue type subgroups (malignant, benign and normal), ceramide concentrations were submitted to Kruskal–Wallis analysis (25), with the post-hoc a-corrected Dunn testing, separately for each ceramide. Third, at an exploratory level, ceramide tissue concentrations in the tumor type subgroups (malignant/benign) were compared between samples with respect to tumor size (large or small) by means of Kruskal–Wallis analysis (25), with the post-hoc a-corrected Dunn testing, separately for each ceramide. Furthermore, also at an exploratory level, ceramide tissue concentrations in the malignant tissue subgroup were compared between samples with respect to lymph node (LN) status (positive or negative) and ER status (positive or negative) by means of Mann–Whitney U tests.
separately for each ceramide. Fourth, ceramide tissue concentrations were analyzed for correlations with SPT and LASS mRNA quantities using Spearman’s p correlation analysis.

Results

Ceramide distribution in normal breast tissue

The aim of the study was to compare the endogenous ceramide levels in malignant and benign tissue with normal tissue to gain insight into the importance of ceramide deregulation in cancer development. Ceramide synthases act chain length specific. Therefore, we investigated ceramides of varying chain lengths in malignant, benign and normal tissues by liquid chromatography coupled with tandem mass spectrometry. To present a representative sphingolipid level, three to five different pieces of the tissue samples were employed and the sphingolipid level of each of these pieces was analyzed in duplicate. Figure 2 displays the mean ceramide levels of normal breast tissue normalized to 1 mg tissue (n = 12). The Friedman test showed that the concentration of the various ceramides differ in part significantly from each other. It is obvious that C16:0-Cer [9.40 ± 2.8 (pmol/mg)], C18:0-Cer, C20:0-Cer, C24:1-Cer and C24:0-Cer (Table I).

Fig. 2. Ceramide levels in breast normal tissue. The normal tissue was obtained as described in Materials and Methods. The sphingolipid amounts determined by liquid chromatography coupled with tandem mass spectrometry were related to the weight of the investigated tissue. The values are the mean of at least three values of different parts of the tumor tissue and each experiment was achieved in duplicate. The mean value is shown by a horizontal line. The statistical analysis was done by Wilcoxon–Wilcox test (Bonferroni α-corrected). *P < 0.05 and ***P < 0.001 indicate significant difference among the various ceramides.

Analysis of ceramide levels of malignant, benign and normal breast tissue

The levels of endogenous ceramides in 64 breast tumor tissues [malignant (n = 43); benign (n = 21)] were measured and compared with 12 samples of normal tissues. Malignant tumor tissues comprise the invasive breast cancer types ‘invasive lobular mamma carcinoma’ and ‘invasive ductal mamma carcinoma’ and the pre-stage breast cancer types ‘lobular mamma in situ’ and ‘ductal mamma in situ’. The benign tissues include mastopathies and fibroadenomas. Figure 3A and Table I clearly exhibit that the total ceramide levels were significantly increased in malignant tissue as compared with the benign tissue and the normal tissue. The ceramide levels in the benign tissue were also increased as compared with normal tissue. A significant increase of ceramide levels in malignant tumor tissue versus the benign tissue points to a positive correlation of the ceramide level and disease severity (Table I). An increase in ceramide levels in malignant and benign tissue versus normal tissue was observed for: C16:0-Cer, C18:0-Cer, C20:0-Cer, C24:1-Cer and C24:0-Cer (Table I).

Next, we determined the extent to which ceramides were increased in tumor tissues. For this purpose, we related the mean values (for each specific ceramide) of the malignant and the benign samples to the corresponding mean value of the normal samples. The total ceramide level in malignant (benign) tumor tissue was 12.0-fold (4.0-fold) increased compared to normal tissue. The ceramide specific increase varied: 14.5-fold/4.4-fold (malignant/benign) for C16:0-Cer, 5.2-fold/5.1-fold for C18:0-Cer, 5.6-fold/3.9-fold for C20:0-Cer, 5.9-fold/2.3-fold for C24:1-Cer and 10.7-fold/3.5-fold for C24:0-Cer, respectively (Figure 3B). These data indicate that in malignant tumors the elevation of C16:0-Cer, C24:1-Cer and C24:0-Cer concentrations were more pronounced than that of other ceramides (Table I).

Comparison of sphingolipid level and pathological parameters

To gain insight into the prognostic significance of the sphingolipid level, we investigated whether or not a relationship exists between an elevated sphingolipid level and various pathological parameters such as tumor size, LN status and hormone receptor status. The pathological parameters were not available for all patients, subgroups of patients from which the investigated parameters are known were selected. We missed the information concerning the tumor size of C24:0-Cer [2.80 ± 0.8 (pmol/mg)] and C24:1-Cer [3.49 ± 0.9 (pmol/mg)] are the predominant ceramides in healthy breast tissue. The concentrations of Sph, dSph, S1P and sphinganine-1-phosphate were under the detection limit in the selected tissue sample.
Table I. Ceramide levels of all samples (normal tissue, benign tissue and malignant tumor tissue) and of the subgroups tumor size, LN status, ER status and HER-2/neu status are shown

<table>
<thead>
<tr>
<th>Tumor size</th>
<th>Number of patients</th>
<th>Cer_total (pmol/mg tissue)</th>
<th>C16:0-Cer (pmol/mg tissue)</th>
<th>C18:0-Cer (pmol/mg tissue)</th>
<th>C20:0-Cer (pmol/mg tissue)</th>
<th>C24:0-Cer (pmol/mg tissue)</th>
<th>C24:1-Cer (pmol/mg tissue)</th>
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<td>ALL</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Normal</td>
<td>12</td>
<td>17.47 ± 4.7</td>
<td>9.40 ± 2.8</td>
<td>0.61 ± 0.2</td>
<td>1.17 ± 0.3</td>
<td>2.80 ± 0.8</td>
<td>3.79 ± 0.9</td>
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<tr>
<td>Benign</td>
<td>21</td>
<td>67.43 ± 18.6</td>
<td>41.0 ± 15.5</td>
<td>3.12 ± 0.7</td>
<td>4.56 ± 0.8</td>
<td>6.42 ± 1.7</td>
<td>12.38 ± 3.2</td>
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<td>Malignant</td>
<td>43</td>
<td>200.66 ± 31.7</td>
<td>137.01 ± 26.4</td>
<td>3.16 ± 0.5</td>
<td>6.58 ± 1.0</td>
<td>16.40 ± 3.6</td>
<td>37.50 ± 4.1</td>
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<td>Tumor size</td>
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<tr>
<td>≤2 cm</td>
<td>7</td>
<td>15.70 ± 6.0</td>
<td>6.32 ± 2.9</td>
<td>0.90 ± 0.4</td>
<td>1.57 ± 0.7</td>
<td>2.11 ± 1.0</td>
<td>4.80 ± 2.2</td>
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<td>&gt;2 cm</td>
<td>10</td>
<td>88.00 ± 30.4</td>
<td>46.55 ± 25.9</td>
<td>5.08 ± 1.0</td>
<td>6.60 ± 1.1</td>
<td>9.11 ± 3.0</td>
<td>20.70 ± 5.0</td>
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<td>LN status</td>
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<tr>
<td>negative</td>
<td>24</td>
<td>131.18 ± 24.2</td>
<td>96.80 ± 17.6</td>
<td>2.62 ± 0.5</td>
<td>5.77 ± 0.9</td>
<td>11.9 ± 1.8</td>
<td>38.77 ± 6.9</td>
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<tr>
<td>positive</td>
<td>16</td>
<td>254.90 ± 55.5</td>
<td>141.62 ± 44.5</td>
<td>3.52 ± 0.9</td>
<td>7.05 ± 1.8</td>
<td>18.32 ± 7.3</td>
<td>37.18 ± 3.9</td>
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<td>ER status</td>
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<td>negative</td>
<td>20</td>
<td>207.70 ± 55.4</td>
<td>175.02 ± 49.5</td>
<td>3.62 ± 1.0</td>
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<td>18</td>
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<td>84.75 ± 22.3</td>
<td>2.49 ± 0.9</td>
<td>4.99 ± 0.9</td>
<td>10.60 ± 3.9</td>
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<tr>
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<td>21</td>
<td>116.89 ± 23.8</td>
<td>62.30 ± 17.8</td>
<td>2.60 ± 0.6</td>
<td>5.11 ± 1.0</td>
<td>9.94 ± 2.0</td>
<td>36.94 ± 5.9</td>
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<tr>
<td>positive</td>
<td>10</td>
<td>225.67 ± 85.2</td>
<td>168.31 ± 75.6</td>
<td>3.10 ± 0.9</td>
<td>6.34 ± 1.8</td>
<td>17.66 ± 6.9</td>
<td>30.26 ± 4.7</td>
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</table>

The ceramide levels are shown as mean ± SEM. The pathological parameters were not available for all patients. These patients whose investigated parameters are known were selected. The number of patients that were analyzed is stated in column 2. Data were statistically analyzed using Kruskal–Wallis test with the post-hoc α-corrected Dunn test or the Mann–Whitney U test (*P < 0.05, **P < 0.01, ***P < 0.001). The samples that were compared with each other were marked with parentheses.

six patients, the LN status of three patients, the ER status of five patients and the human epidermal growth factor receptor (HER-2/neu) status of 12 patients. The number of patients that were analyzed is given in parentheses.

Tumor size: To exclude the possibility that the raised ceramide levels were a result of necrosis (mainly occurring in larger tumors), we compared the total ceramide levels of malignant and benign tissue with a diameter >2 cm (n = 20) or ≤2 cm (n = 21) (supplement 3A is available at Carcinogenesis Online). Malignant tumors disclose no significant correlation between tumor size and ceramide level, indicating that necrosis is not responsible for the raised ceramide level. In contrast, malignant tumors independent of tumor size and benign tumors with a diameter >2 cm (n = 10) show a significant increase in ceramide level in comparison with benign tumors with smaller dimensions (n = 7) (Table I, supplement 3A is available at Carcinogenesis Online). These data indicate that the increased ceramide level correlates with disease severity.

LN status: A metastatic potential in colon cancer is discussed in the literature (26) for glycosylated ceramides. Therefore, we investigated whether an elevated ceramide level in tumors could be linked to the LN status. For malignant tumor tissue, the LN status was known from the pathological parameter, the grading status of the malignant tumors, whether an elevated ceramide level in tumors could be linked to the ER or HER-2/neu status. C18:0-Cer and C20:0-Cer are significantly elevated in malignant tumors, whereas C16:0-Cer, C18:0-Cer and C24:1-Cer are independent of the LN status (Figure 4B). However, the observed ceramide increase in malignant tumor tissue is independent of the HER-2/neu status (Table I). There was also no correlation between the various ceramide levels of malignant tumors and a HER-2/neu negative/ER negative status (data not shown).

The mRNA expression levels of SPT and LASS1–6 in breast tumor tissue

In order to obtain insight into the enzymes of the sphingolipid pathway that contribute to the elevated ceramide levels, the mRNA levels of all ceramide synthases (LASS1–6) and SPT (see also Figure 1) were investigated using real-time quantitative polymerase chain reaction. The SPT consists of at least two subunits SPTLC1/SPTLC2. Both subunits are equally expressed in breast tissue (27). Recently, a new subunit of the SPT, SPTLC3, with variable tissue expression was described (28). We therefore investigated the mRNA level of both SPTLC2 and SPTLC3. We examined 10 samples of tumor tissues with elevated (n = 4) and depressed (n = 6) sphingolipid levels. The mRNA expression level of each LASS and SPT were normalized to the average expression level of β-actin and GAPDH (supplement 4 is available at Carcinogenesis Online). The mRNA level of LASS1, LASS3 and SPTLC3 were below the quantification limit. The functionality of the primers, which were used for the quantitative polymerase chain reaction of LASS1 and LASS3 in the breast tumor tissue were tested with brain and prostate tissue as positive controls, where LASS1 and LASS3 are predominantly expressed (29–31). The specific ceramide levels were plotted against the various LASS and
SPTLC2 mRNA levels, respectively. Figure 5A–D shows exemplarily the scatter plots for LASS2 against C_16:0-Cer and C_24:1-Cer and LASS6 against C_18:0-Cer and C_20:0-Cer, respectively. Figure 5E presents the Spearman’s rank correlation coefficient of all data. We observed a significant correlation between LASS2/C_16:0-Cer (r_sp = 0.77, P = 0.009), LASS4/C_18:0-Cer (r_sp = 0.65, P = 0.042), LASS6/C_18:0-Cer (r_sp = 0.74, P = 0.014), LASS4/C_20:0-Cer (r_sp = 0.68, P = 0.030), LASS6/C_20:0-Cer (r_sp = 0.84, P = 0.002), LASS2/C_24:1-Cer (r_sp = 0.72, P = 0.019), LASS6/C_24:1-Cer (r_sp = 0.64, P = 0.047) and LASS2/C_24:0-Cer (r_sp = 0.71, P = 0.022). There was no correlation between ceramide level and SPTLC2 mRNA level. However, SPTLC2 and LASS5 were elevated in patients 1 and 2, which exhibited an outstanding increased ceramide level (supplement 4 is available at Carcinogenesis Online).

The protein expression level of SPT and LASS in breast tumor tissue

Next, we analyzed whether translational modifications also play a role in the altered sphingolipid level in cancerous tissue using western blot analysis. Unfortunately, the currently available antibodies of LASS1, LASS3, LASS4, LASS5, LASS6 and SPT are not specific; therefore, no reproducible western blot or immunohistochemical staining was obtainable. Only LASS2 was reasonably detectable (supplement 5B is available at Carcinogenesis Online). Tissue samples that were previously subjected to the expression analysis of the mRNA level were used for the determination of the protein expression level. Using histochemistry, we showed that the investigated tumor tissues consist predominantly of one cell type (supplement 5A is available at Carcinogenesis Online); therefore, we could exclude cell type specific differences in the expression pattern of the LASS’s. The western blot analysis revealed a correlation between ceramide level and LASS2 expression (supplement 5B is available at Carcinogenesis Online).

Discussion

In this study, we could clearly show that the total endogenous ceramide level was significantly elevated in malignant (12-fold) and benign (4-fold) breast tissue as compared with normal tissue, indicating that elevation of sphingolipid levels correlates with disease status. This elevation in ceramide levels was rather unexpected because in vitro studies predominantly indicated that an increase in ceramides is associated with an induction of apoptosis and therefore primarily linked with anticarcinogenic effects. An elevated concentration of sphingo-myelin, a metabolite of ceramide, was also observed in human cancerous cervical tissues compared with normal cervical tissue (32). In
human head and neck squamous cell carcinomas both an increase of C16:0-Cer, C24:1-Cer and C24:0-Cer and a decrease of C18:0-Cer was detected (33).

Kanto et al. (34) reported that supernatants of tumor cells enriched with ceramides C16:0-Cer and C24:0-Cer induce apoptosis in dendritic cells that helps tumor cells to escape from the immune surveillance system, indicating that an elevated ceramide level in tumor cells could also have tumor protective effects. Moreover, the increase of C16:0-ceramide, synthesized by CerS5 or CerS6, leads to an activation of telomerase activity that is linked to tumor promotion (35). In contrary, C18:0-Cer synthesized by CerS1, which is not expressed in breast tissue, leads to an inhibition of telomerase activity (18,35). In the here investigated breast cancer tissues, we detected elevated levels of C16:0-Cer, C24:0-Cer and C24:1-Cer (Table I, Figure 3). Interestingly, the ratio between C16:0-Cer and C18:0-Cer increases in malignant tumor tissues (C16/C18 = 43) in comparison with normal tissue (C16/C18 = 15). Furthermore, the ratio between C24:0-Cer and C18:0-Cer increased from 5.7 (normal) to 11.8 (malignant). These data indicate that both the relationship between ceramide and phosphorylated sphingolipids [C1P (ceramide-1-phosphate), S1P] (4,6,36) as well as the relationship between ceramides of various chain lengths (especially C16:0-Cer and C18:0-Cer) are indicative for a proliferative or an antiproliferative status.

However, we could not exclude that also S1P and C1P or other metabolites of ceramide are elevated in our tissue samples but due to low expression levels of these substances and the limited tissue amount we could not detect all sphingolipids in a sample; an objective of future studies. Our and literature data raise the question: Are the increased ceramide levels rather an effect or a cause of malignancy? Today, we couldn't make a clear statement about this issue because our data are generated by a snap-reading method. We could not track the ceramide concentrations over the time during the development of tumors from benign to precancerous to cancerous tumors. Therefore, to elucidate whether the increased ceramide levels are a consequence or a cause of malignancy or both and whether this depends on the N-acyl chain length of the ceramides, further experiments are needed.

Using real-time polymerase chain reaction, we could confirm our liquid chromatography coupled with tandem mass spectrometry data, showing that the mRNA levels of LASS2, LASS4, LASS5 and LASS6
are elevated in tumor samples with high ceramide levels. In greater details, we could assign each upregulated ceramide type to one or two specific LASS isoforms (Figure 5). The data suggest that LASS2, which converts lignoceryl-CoA (C24:0-CoA) (30) to C24:0-Cer, also accept nervonyl-CoA (C24:1-CoA) as a substrate. These suggestive data, however, require substantiation from further in vitro experiments. In contrast, we found no correlation between the expression level of SPT, the first and rate-limiting enzyme in sphingolipid de novo synthesis, and upregulated ceramide levels. Recently, Panjanari et al. also demonstrated that an increase in ceramide levels in human leukemia as well as in human colon cancer cells was mediated by induction of sphingolipid de novo synthesis. The effect was independent of SPT expression and activity, but rather due to an increase in CerS activity (37). In fibroblasts, the SPT translocates from the cytosol in quiescent cells to the nucleus in proliferating cells (38). The translocation would allow the enzyme to match up with the substrate and thereby inducing its activity. Therefore, translocation processes of the SPT could contribute to the raised elevated ceramide levels in the tumor tissue. It is also possible that additionally post-translational regulation processes modulate SPT activity. Last but not least, also an enhanced salvage pathway could contribute to an increase in ceramides. This has to be investigated in further studies.

Comparison of ceramide levels with pathological parameters revealed that in patients with LN metastasis C16:0-Cer was significantly elevated. In line with our findings, Kowalska et al. (26) demonstrated an increase of the glycosylated ceramide Gb1 in metastatic colon cancer tissue. Unfortunately, the study failed to specify the chain length of the ceramide backbone. Since the ER status is an important diagnostic marker in breast cancer, it is noteworthy that we observed a significant increase of C18:0-Cer and C20:0-Cer levels in ER positive samples as compared with ER negative samples (Figure 4B). This agrees with our previous findings where we could demonstrate by microarray analysis that the expression level of LASS4 and LASS6 are also ER status dependent regulated (14). Moreover, we checked whether ceramide levels could be related to the clinical outcome of patients. The follow-up data of 20 patients was known. Sixteen patients were without pathological findings and six patients had a recurrence [malignant (n = 5)/benign (n = 1)]. These included the following secondary tumor types: ovarian carcinoma (n = 1); mammary carcinoma (n = 2); osteosarcoma (n = 1) and fibroadenoma (with a history of fibroadenoma) (n = 1). Five of these patients exhibited an elevated total ceramide level in their primary breast tumor sample. Only one patient with osteosarcoma recurrence showed no increase in sphingolipid level in the primary tumor sample. Due to the low patient number, no statistical significance could be achieved, but these data point to a correlation between an increased sphingolipid level and a higher risk for recurrence. In conclusion, in this study, we could demonstrate that ceramides C16:0-Cer, C24:1-Cer and C24:0-Cer were significantly increased in malignant breast tumor tissues as compared with benign and normal tissues indicating that an increase in distinct ceramides correlates with the development of breast cancer. Furthermore, we observed a correlation between elevated ceramide levels and the ER status, whereas a raised C16:0-Cer level was associated with a metastatic LN status.

Supplementary Material
Supplements 1–5 can be found at http://carcin.oxfordjournals.org/

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References


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