FasL:Fas Ratio–A Prognostic Factor in Breast Carcinomas

Toralf Reimer, Christina Herrnring, Dirk Koczan, Dagmar Richter, Bernd Gerber, Dieter Kabelitz, Klaus Friese, and Hans-Jürgen Thiesen

Department of Obstetrics and Gynecology [T. R., C. H., D. R., B. G., K. F.], Institute of Immunology [D. Ko., H-J. T.], University of Rostock, 18055 Rostock, and Department of Immunology, Paul-Ehrlich-Institute, 63225 Langen [D. Ka.], Germany

Abstract

Programmed cell death (apoptosis) is primarily mediated by Fas ligand (FasL; CD95L) and the Fas receptor (Fas; CD95). In this study, FasL was detected by immunohistochemical analysis in 85% of breast carcinomas and 14% of fibroadenomas randomly chosen, indicating that high expression of FasL might play a role in tumor pathology. FasL and Fas levels as well as FasL:Fas ratios were further ascertained in 215 human breast tumors, including 199 invasive ductal carcinomas, by real-time quantitative reverse transcription-PCR and compared with expression levels and ratios found in 25 normal human tissues, in 37 fibroadenomas, and in 5 normal breast tissues. Among breast carcinomas, high FasL mRNA expression seems to be positively correlated with histological grading (n = 212; P < 0.0001). A ratio of FasL:Fas mRNA transcripts > 1 is found to be significantly associated with decreased patient’s disease-free survival (n = 211; P < 0.03) and increased mortality (n = 211; P = 0.19). A FasL:Fas ratio > 1 is related to tumor progression scored by histological grading (n = 212; P < 0.02). The selection process leading to highly aggressive breast tumor variants might be enhanced by FasL-mediated tumor fratricide, eventually a possible target for novel therapeutic strategies.

Introduction

Breast cancer is the most common cancer of women in many parts of the world. Despite intensive research over the past decades, mortality from this disease has hardly decreased (1). Although TILs are capable of infiltrating the tumor, their functional cytotoxic activities seem to be hampered (2, 3). Different mechanisms, for example alterations in the expression of the MHC of the tumor (4), lack of costimulatory molecules like B7.1 (CD 80; Ref. 5), or secretion of soluble tumor-derived inhibitory factors (6), have been considered to play a role in this immune escape of the tumor.

Recently, it has been shown that an additional mechanism might play an important role in the immune escape of tumors. A variety of apoptosis-inducing ligands like the tumor necrosis factor, FasL, and the tumor necrosis factor-related apoptosis-inducing ligand have been found to induce apoptosis in target cells that express the corresponding receptors. Fas, the receptor of FasL, triggers upon activation by FasL and binds to an intracellular death domain containing protein called Fas-associated death domain protein, thereby activating a cascade of caspases perpetuating the apoptotic process of cell killing (7). Expression of Fas has been detected in numerous different solid tissues (8) and in the hematopoietic system.

Elevated FasL expressions have been found in tumor cells of colon (9), esophageal (10), stomach (11, 12), lung (13), and ovarian cancer (14). In esophageal cancer, areas of the tumor that expressed high FasL levels showed a reduction in the number of TILs and an increased apoptosis of TILs (10). FasL expressed by tumor cells seemed to induce apoptosis in Fas-sensitive TILs. In another study, FasL-positive hepatoma cells displayed reduced Fas expression, in accordance with loss of sensitivity to Fas-mediated apoptosis (15).

To assess FasL and Fas expressions in malignant human tissues in comparison with normal human tissues, FasL and Fas levels as well as FasL:Fas ratios were determined in mRNA pools derived from 25 normal human tissues and correlated with results obtained from the analysis of 257 human breast specimens. Absolute copy numbers of FasL and Fas mRNAs were determined by quantitative RT-PCR, and their distribution within the tumor was visualized by immunohistochemical analysis. Expression and distribution of FasL and Fas were analyzed in breast carcinoma (n = 215), fibroadenoma (n = 37), and normal breast tissue (n = 5) at the mRNA and partly at the protein level. The present study was designed to address the role of the apoptosis-inducing ligand FasL and its receptor Fas in breast carcinoma as a possible mechanism for the immune escape of the tumor.

Materials and Methods

Human body screening of Fas and FasL was done by using human total RNAs commercially available from Clontech ( Palo Alto, CA). Total RNA samples from 25 different human tissues were analyzed by Fas- and FasL-real-time quantitative RT-PCR (TaqMan analysis; see below). The RNA samples were pooled from tissues of the following origins (see the data sheet from Clontech).

In brief, RNA of heart came from 2 female Caucasians, ages 16 and 36; pancreas RNA from 18 male/female Caucasians, ages 17–69; liver RNA from 2 male/female Caucasians, ages 15 and 35; and RNA from cerebellum from a 64-year old male Caucasian (death by acute heart failure). Small intestine RNA was pooled from 3 male/female Caucasians, ages 15–60; stomach RNA from 15 male/female Caucasians, ages 23–61; spleen RNA from 5 male Caucasians, ages 22–48; RNA of bone marrow from 3 male/female Caucasians, ages 24–59; and fetal brain RNA from 8 male/female spontaneously aborted Caucasian fetuses, ages 20–34 weeks. Fetal liver RNA came from spontaneously aborted Caucasian fetuses, ages 15–24 weeks; adrenal gland RNA from 6 male Caucasians, ages 32–50; kidney RNA from 8 male/female Caucasians, ages 24–55; brain RNA from 2 female Caucasians, ages 16 and 36, and salivary gland RNA from 43 male/female Caucasians, ages 13–78. Lung RNA was pooled from 5 male/female Caucasians, ages 14–40; thyroid RNA from 4 male/female Caucasians, ages 10–46; trachea RNA from 84 male/female Caucasians, ages 17–70; mammary gland RNA from 8 female Caucasians, ages 23–47; colon RNA from 2 male Caucasians, ages 35 and 50; prostate RNA from 23 male Caucasians, ages 26–64; and spinal cord RNA from 31 male/female Caucasians, ages 17–72. Skeletal muscle RNA came from 10 male/female Caucasians, ages 23–56; thymus RNA from 13 male/female Caucasians, ages 17–37; testis RNA from 25 male Caucasians, ages 28–64; and placenta RNA from normal afterbirth of 15 female Caucasians, ages 22–41.

Patients and Tissue Collection. Tissue samples of 215 unselected primary breast carcinomas, 37 fibroadenomas, and 5 normal breast tissues were collected during surgery at the Department of Obstetrics and Gynecology of the

Received 9/14/99; revised 12/1/99; accepted 1/4/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.


2 T. Reimer and C. Herrnring contributed equally to this work.

3 To whom requests for reprints should be addressed, at Institute of Immunology, Post Office Box 10 08 88, D-18055 Rostock, Germany.

4 The abbreviations used are: TIL, tumor-infiltrating lymphocyte; FasL, Fas ligand; Fas, Fas receptor; RT-PCR, reverse transcription-PCR; CI, confidence interval.
University of Rostock between 1994 and 1998. None of the patients had received chemo-, radio-, or immunotherapy prior to surgery. The mean age for breast cancer patients was 58 years (range, 29–90 years); for patients with a fibroadenoma, 39.7 years (range, 15–67 years); and for patients with normal breast tissue, 30.2 years (range, 20–42). Sixty-three (29.3%) patients with breast cancer were premenopausal, and 152 (70.7%) were postmenopausal. The majority of breast tumors (n = 199; 92.5%) were invasive ductal carcinomas; the others were invasive lobular carcinomas (n = 7, 3.3%), mixed mucinous carcinomas (n = 1, 0.5%), and medullary (n = 5, 2.3%) and tubular carcinomas (n = 3, 1.4%). All carcinoma patients of this study underwent either total or partial axillary lymph node dissection (mean number of nodes examined, 18). The main classifying prognostic factors are shown in Table 1. One hundred and five women (48.8%) were treated with simple mastectomy and lymph node dissection, and 110 (51.2%) with lumpectomy plus axillary clearance. Breast-conserving surgery was followed by postoperative radiotherapy (n = 98). Two hundred patients (93%) received adjuvant therapy, consisting of chemotherapy (n = 52), hormone therapy (n = 96), or both (n = 52).

The malignancy of invasive breast carcinomas was scored according to the histopathologic grading of Bloom and Richardson (16). Tumor grading (n = 212) included G1 (n = 107, 50.5%), G2 (n = 81, 38.2%), and G3 (n = 24, 11.3%). Women with bilateral breast carcinomas or multiple fibroadenomas were excluded. Within the fibroadenoma subgroup, the mean tumor size was 21 mm (range, 12–45 mm). The median follow-up time for malignant cases was 32 months (range, 2–53 months). The cutoff date for this analysis was June 1999.

Of these patients, paraffin-embedded sections of 40 unselected breast carcinomas, 7 fibroadenomas, and 5 normal breast tissues were analyzed immunohistochemically. Upon submission of the investigation to the ethic committee of the Rostock Medical Association, it was ruled that no ethical approval was necessary.

Real-Time RT-PCR. Total RNA of breast tissue was prepared using the acid guanidinium thiocyanate-phenol-chloroform protocol (17). All specimens were tested by analysis of housekeeping gene expression using conventional RT-PCR. First-trimester placenta (FasL) from women undergoing legal abortions and liver mRNA (Fas) served as positive control.

The primer pairs and probes were designed using the Primer Express 1.0 program (PE Applied Biosystems, Foster City, CA). Oligonucleotide hybridization probes (18) and primer pairs with the following sequences were synthesized as follows: FasL: TaqMan probe 5'-TGG. Primers and probes were obtained from Applied Biosystems GmbH (Weiterstadt, Germany). The primers yielded RT-PCR products of 82 (FasL) and 105 (Fas) nucleotides. Direct sequencing of the PCR product was performed with the automatic DNA sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) using ABI dye-terminator chemistry according to the manufacturer’s protocol. The sequencing reactions were repeated at least twice in both directions from independent template preparations to avoid the possibility of PCR artifacts.

Preparation of RNA Standard. For calibration of the FasL and Fas TaqMan assays, two RNA standards were generated by using an in vitro T7-polymerase transcription system (RibomAX Large Scale RNA Production System; Promega Corp., Madison, WI). Using the TaqMan 5' and 3' primers, a preparative standard PCR reaction was performed to produce a FasL- or Fas-specific DNA fragment. The cloning procedure was carried out by ligating the fragments into a Smal linearized pBluescript KS vector (Stratagene, La Jolla, CA). The correct plasmid clone was purified by ionic exchange column DNA preparation (Qiagen, Valencia, CA) and linearized by Xhol cleavage to generate a useful template for producing a cutoff in vitro transcript. A starting mixture was made by diluting the specific RNA molecules with a competitor yeast tRNA (Life Technologies, Grand Island, NY) to a final concentration of 10^10 in vitro transcripts in 2 μg of yeast tRNA per 10 μl (in water). This starting mixture was used to prepare stock dilution series over eight logs from 10^5 to 10^2 specific RNA molecules.

RT-PCR Procedure. The TaqMan EZ RT-PCR kit (PE Applied Biosystems) was used for reverse transcription and amplification of both targets and standards (19). Production of cDNA and PCR amplification was carried out in a single-tube, single-enzyme system without the addition of subsequent enzymes or buffers. All RT-PCR reactions were performed in duplicates with a final volume of 25 μl. The reaction conditions for 100 ng of total RNA were 2 min at 50°C, 30 min at 60°C, 5 min at 95°C, 35 cycles with 20 s at 94°C, and 1 min at 60°C. The quantification of FasL and Fas RNA standards was linear over eight logs, and the assay measured as little as 100 copies of FasL or Fas mRNA copies per tube. The threshold cycle values decreased linearly with increasing target quantity. In all experiments, the correlation coefficient was between 0.985 and 0.995.

Immunohistochemical Detection of FasL and Fas. Paraffin-embedded sections of breast carcinomas, fibroadenomas, and normal breast tissues were deparaffinized in xylene and rehydrated before analysis. Slides, treated with a pepsin solution and endogenous peroxidase, quenched with 0.5% hydrogen peroxide, were washed in PBS, blocked with 5% normal goat serum, and incubated overnight at 4°C with a rabbit polyclonal antihuman Fas-specific IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at 0.4 μg/ml. A Vectastain ABC detection kit (Vector Laboratories, Alexis Corp., San Diego, CA) containing a biotinylated secondary antibody and avidin-conjugated horseradish peroxidase was used to identify antibody binding. Diaminobenzidine was used as substrate for the horseradish peroxidase. Slides were counterstained with

---

Table 1: Clinicopathological characteristics of the 215 breast cancer patients and relationship to FasL:Fas ratio mRNA copy number

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients (%)</th>
<th>Median</th>
<th>Range</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menopausal status</td>
<td>209</td>
<td>0.64</td>
<td>0.01–4.26</td>
<td>0.65–0.98</td>
<td>0.103</td>
</tr>
<tr>
<td>Premenopausal</td>
<td>62 (29.7)</td>
<td>0.77</td>
<td>0.07–14.4</td>
<td>0.98–1.48</td>
<td>0.019</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>147 (70.3)</td>
<td>1.14</td>
<td>0.18–4.5</td>
<td>1.14–2.14</td>
<td>0.335</td>
</tr>
<tr>
<td>Histological grade</td>
<td>206</td>
<td>0.66</td>
<td>0.07–7.74</td>
<td>0.79–1.21</td>
<td>0.148</td>
</tr>
<tr>
<td>I</td>
<td>101 (49.0)</td>
<td>0.76</td>
<td>0.01–14.4</td>
<td>0.73–1.48</td>
<td>0.755</td>
</tr>
<tr>
<td>II</td>
<td>81 (39.3)</td>
<td>1.14</td>
<td>0.18–4.5</td>
<td>1.14–2.14</td>
<td>0.018</td>
</tr>
<tr>
<td>III</td>
<td>24 (11.7)</td>
<td>0.69</td>
<td>0.04–4.23</td>
<td>0.81–1.11</td>
<td>0.529</td>
</tr>
<tr>
<td>Lymph node status</td>
<td>203</td>
<td>0.82</td>
<td>0.1–14.4</td>
<td>0.95–1.76</td>
<td>0.032</td>
</tr>
<tr>
<td>Negative</td>
<td>116 (57.1)</td>
<td>0.64</td>
<td>0.01–4.23</td>
<td>0.81–1.11</td>
<td>0.148</td>
</tr>
<tr>
<td>Positive</td>
<td>88 (42.9)</td>
<td>0.82</td>
<td>0.1–14.4</td>
<td>0.95–1.76</td>
<td>0.353</td>
</tr>
<tr>
<td>Tumor size</td>
<td>205</td>
<td>0.64</td>
<td>0.16–0.43</td>
<td>0.74–1.05</td>
<td>0.755</td>
</tr>
<tr>
<td>pT1</td>
<td>93 (45.4)</td>
<td>0.88</td>
<td>0.01–14.4</td>
<td>0.98–1.69</td>
<td>0.720</td>
</tr>
<tr>
<td>pT2</td>
<td>96 (46.8)</td>
<td>0.78</td>
<td>0.11–4.5</td>
<td>0.48–1.8</td>
<td>0.529</td>
</tr>
<tr>
<td>pT3 and pT4</td>
<td>16 (7.8)</td>
<td>0.75</td>
<td>0.01–4.26</td>
<td>0.79–1.21</td>
<td>0.229</td>
</tr>
<tr>
<td>EEC status</td>
<td>208</td>
<td>0.74</td>
<td>0.07–14.4</td>
<td>0.89–1.43</td>
<td>0.353</td>
</tr>
<tr>
<td>≤10 fmol/mg</td>
<td>74 (35.6)</td>
<td>0.74</td>
<td>0.01–4.26</td>
<td>0.79–1.21</td>
<td>0.720</td>
</tr>
<tr>
<td>&gt;10 fmol/mg</td>
<td>134 (64.4)</td>
<td>0.74</td>
<td>0.01–4.26</td>
<td>0.79–1.21</td>
<td>0.720</td>
</tr>
<tr>
<td>≤10 fmol/mg</td>
<td>118 (57.6)</td>
<td>0.74</td>
<td>0.01–4.26</td>
<td>0.79–1.21</td>
<td>0.720</td>
</tr>
<tr>
<td>&gt;10 fmol/mg</td>
<td>87 (42.4)</td>
<td>0.74</td>
<td>0.01–4.26</td>
<td>0.79–1.21</td>
<td>0.720</td>
</tr>
<tr>
<td>Overall</td>
<td>209</td>
<td>0.74</td>
<td>0.01–4.26</td>
<td>0.79–1.21</td>
<td>0.720</td>
</tr>
</tbody>
</table>

*ER, estrogen receptor; PgR, progesterone receptor.*
For control staining, the immunizing peptide (Fas, amino acids 316–335; Santa Cruz Biotechnology) was coincubated at 4 μg/ml at primary antibody incubation. In all cases, staining was inhibited by the immunizing peptide. Fas, peptide controls, and negative control of one tissue sample were detected at the same time. A mouse monoclonal antihuman FasL-specific IgG1 (NOK-1; PharMingen, San Diego, CA) at 40 μg/ml was used for FasL detection following the same protocol as described above. The stained slides were evaluated by the department’s pathologist. Immunohistochemical results were analyzed by light microscopy. Localization and intensity of staining reaction of different cellular components were compared with negative con-
trols. Immunohistochemical staining was considered positive when at least 10% of breast cells were positive. **Statistical Analysis.** Clinical, histological, and biological parameters were compared using the most appropriate among the Spearman correlation coefficient, $\chi^2$ test, Mann-Whitney $U$ test, and Kruskal-Wallis $H$ test. Disease-free and overall survival were analyzed using Kaplan-Meier method, and comparison of study groups was performed with the log-rank test. The Cox regression model was applied over both univariate and multivariate analyses, with the associated likelihood ratio test used for tests of trend differences. In all tests, the significance level was set at $P < 0.05$, and all were two-tailed tests.

**Results**

**Human Body Screening of FasL:Fas RNA.** Absolute copy numbers of FasL and Fas mRNAs were determined in a large panel of total RNAs extracted from pools of 25 different normal tissues (Fig. 1). For FasL, low copy numbers of <10,000 molecules/µg total RNA were detected in heart, pancreas, cerebellum, fetal brain, and skeletal muscle. High copy numbers of >100,000 FasL/µg RNA molecules are found in spleen, lung, and trachea (Fig. 1A). For Fas, low copy numbers in the range of 30,000 to 60,000 molecules/µg RNA were found in skeletal muscle, thyroid, salivary gland, fetal liver, and cerebellum. High copy numbers of >300,000 molecules/µg RNA were found in liver, spleen, adrenal gland, lung, trachea, prostate, thymus, and testis (Fig. 1B). In mammary glands, mRNAs levels of 34,000 FasL molecules and 268,000 Fas molecules/µg total RNA were found, resulting in a FasL:Fas ratio of 0.14. A FasL:Fas ratio <1 were determined in all normal tissues tested except a FasL:Fas-ratio >1 for the spleen with known antigen-induced turnover of lymphocytes (Fig. 1C).

**Immunohistochemical Expression of FasL and Fas in Breast Carcinoma, Fibroadenoma, and Normal Breast Tissue.** Tissue samples were randomly chosen for immunohistochemical analysis for 40 of the 215 breast carcinomas, for 7 of the 37 fibroadenomas, and for 5 normal breast tissues. In breast carcinoma tissue, Fas was detected in 88% of the tissue samples (34 of 40), of which 10 showed a weak reaction only. Staining was distributed nonhomogeneously throughout the tumor. Weak reaction for FasL could be detected in 14% of fibroadenomas (one of seven) and 20% of normal breast tissue samples (one of five). A positive reaction was seen along the cell membrane and in the cytoplasm of the tumor cells. Fas expression in breast carcinoma specimens was detected in 88% of the analyzed tissue samples (35 of 40). Staining was distributed mainly inhomogeneously along cell membranes of the tumor cells. Infiltrating lymphocytes stained strongly positive for Fas but only weakly or not at all for FasL. The number of infiltrating lymphocytes did not significantly vary from specimen to specimen, not exceeding 10% of total amount of cells. Fas expression was detected homogeneously in all fibroadenomas and normal breast tissue samples. All immunohistochemical results and corresponding mRNA copy numbers (n = 52) are summarized in Table 2.

**Expression of FasL and Fas mRNA in Subgroups.** FasL and Fas mRNA copy numbers per 1 µg of total RNA were determined in 257 human breast specimens (215 breast carcinomas, 37 fibroadenomas, and 5 normal breast tissues). Of 215 probes investigated, positive TaqMan results were obtained for FasL mRNA (n = 212; 98.6%) or Fas (n = 211; 98.1%). The absolute RNA values of Fas and FasL determined in 215 breast carcinomas were grouped depending on their grading (G1–G3), of which 199 tumors had been classified as invasive ductal carcinomas.

Taking the absolute number of FasL and Fas molecules, elevated FasL copy numbers of >100,000 molecules/µg total RNA was found in 8 of 107 tumors of G1 (7.5%), in 14 of 81 tumors of G2 (17.3%), and in 8 of 24 tumors of G3 (33.3%). Of 37 fibroadenomas, only 2 had FasL values slightly >100,000 molecules. Normal breast tissue displayed 51,000 molecules as highest value. Compared with nonmalignant breast tissue (n = 42), invasive breast cancers of grade 1 (n = 105; P = 0.461) and of grade 2 (n = 81; P = 0.881) did not show significant differences regarding FasL mRNA expression. However, FasL expression was significantly higher in breast cancers of grade 3 (n = 24; P < 0.0001) compared with nonmalignant breast tissue.

In case of Fas expressions, high copy numbers of >300,000 molecules/µg RNA were found in 1 of 107 tumors of G1 (0.9%), in 1 of 81 tumors of G2 (1.1%), and in 2 of 24 tumors of G3 (8.3%). Two of 37 (5.4%) were detected in the group of fibroadenomas, and of one of five in normal tissues (20%). Comparison of Fas mRNA copy number per 1 µg total RNA revealed a significant difference, being highest in tissues of fibroadenoma, followed by breast carcinoma and normal breast tissue (P = 0.001/Kruskal-Wallis H test).

Within each group, the tumors were ranked according to their FasL:Fas ratio. Of 107 G1 tumors, 30 had a ratio >1 (28%), of which 11 had ratios of >2 (10.3%). Of 81 G2 tumors, 25 had a ratio of >1 (30.9%), of which 9 displayed higher ratios than 2 (11.3%). Of 24 G3 tumors, 16 had a ratio >1 (66.7%), of which 6 had a ratio >2 (25%). Of 37 fibroadenomas tested, none had a FasL:Fas ratio >1, all falling within a range of 0.11–0.97. Normal tissues had ratios within the range 0.11 to 0.73. Breast cancer tissue expressed the highest ratio of FasL:Fas mRNA transcripts, followed by fibroadenoma and normal breast tissue (P < 0.0001). The FasL:Fas ratios of 199 invasive ductal breast carcinomas are specified according their histological grades in Fig. 2. The median for the FasL:Fas ratio in breast cancer was 0.74 (95% CI for mean, 0.92–1.29); in fibroadenoma, 0.31 (95% CI, 0.31–0.48); and in normal breast tissue, 0.28 (95% CI, 0–0.64). All fibroadenomas and normal breast tissues showed a FasL:Fas ratio <1, whereas 34% (72 of 211) of breast cancers showed a ratio >1.

Table 2 Immunohistochemical and corresponding RT-PCR results (copies) of Fas and FasL expression in different breast tissue (n = 52)

<table>
<thead>
<tr>
<th>Histology (n = 52)</th>
<th>Breast cancer (n = 12)</th>
<th>Breast cancer (n = 11)</th>
<th>Breast cancer (n = 17)</th>
<th>Fibroadenoma (n = 7)</th>
<th>Normal breast tissue (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FasL HIC* positive/tested</td>
<td>8/12</td>
<td>10/11</td>
<td>16/17</td>
<td>1/7</td>
<td>1/5</td>
</tr>
<tr>
<td>FasL copies median (95% CI)</td>
<td>(6 weak pos.)</td>
<td>(4 weak pos.)</td>
<td>(10907)</td>
<td>(weak pos.)</td>
<td>(weak pos.)</td>
</tr>
<tr>
<td>Fas IHC positive/tested</td>
<td>11/12</td>
<td>10/11</td>
<td>14/17</td>
<td>7/7</td>
<td>5/5</td>
</tr>
<tr>
<td>Fas copies median (95% CI)</td>
<td>7023</td>
<td>6073</td>
<td>4377</td>
<td>13786</td>
<td>2924</td>
</tr>
<tr>
<td>FasL:Fas mRNA ratio</td>
<td>0.17–0.48</td>
<td>0.57–0.98</td>
<td>1.2–7.74</td>
<td>0.11–0.78</td>
<td>0.18–0.73</td>
</tr>
</tbody>
</table>

* IHC-immunohistochemistry, pos.-positive.
Relationship between FasL or Fas mRNA Copy Numbers and Other Variables in Breast Carcinoma.

Among 215 breast cancers, a positive correlation was found between FasL mRNA copy numbers and histopathological grading \((n = 210; P < 0.0001)\). Tumor biopsies of patients with positive lymph nodes status \((n = 86)\) showed a tendency toward higher FasL mRNA copy numbers \((P = 0.08)\). Until now, no association has been detected between FasL mRNA copy numbers and tumor size, menopausal status, age, histology, estrogen or progesterone receptor status, body mass index, lymphatic or vascular invasion, age of menarche, and partus. For Fas expression, a positive correlation was determined between Fas mRNA copy numbers and tumor grade \((n = 210; P < 0.03)\). All other tested variables showed no association with Fas mRNA copy numbers. Using the cutoff value of 1, a FasL:Fas ratio >1 significantly decreased the patient’s disease-free survival \((P < 0.03; \text{Fig. 3A})\) and showed a tendency toward increased mortality \((P = 0.19; \text{Fig. 3B})\).

To compare independent prognostic impact, FasL:Fas ratio was combined with the established prognostic factors in a Cox regression analysis for disease-free and overall survival. Histological grade \((n = 212; P < 0.05)\), FasL:Fas ratio \((n = 211; P < 0.03)\), and tumor size \((n = 214; P < 0.005)\) were significantly related to reduced disease-free survival in the univariate analysis. Among these factors, only tumor size \((P < 0.01)\) was of independent significance in the multivariate model \((n = 208)\). For overall survival analysis, tumor size \((P < 0.0002)\) and histological grade \((P < 0.03)\) were significant prognostic factors in the univariate test. However, only tumor size \((P < 0.002)\) was an independent prognostic parameter for overall survival in the multivariate model \((n = 208)\). In this study, axillary lymph node involvement did not seem to be a prognostic factor for risk of recurrence and death.

Relationship between FasL and Fas mRNA Copy Numbers and Other Variables in Fibroadenoma.

Among 37 fibroadenomas, no association was seen between FasL or Fas mRNA copy numbers and tumor size, age, body mass index, partus, or day of menstrual cycle.

Discussion

Initially, the FasL:Fas system was identified to eliminate unwanted cells by apoptosis, such as activation-induced cell death in immune...
between breast cancers and fibroadenomas, the ratio of FasL:Fas level was determined in breast carcinoma, fibroadenoma, and normal variants by tumor fratricide, leading to tumor cells that become highly aggressive tumor clones. In particular, tumor cells expressing FasL might essentially escape the attack by immune cells (counter-attack model). However, in addition, FasL-expressing tumor cells deliver qualitative data on protein expression. Thus, the RT-PCR analysis confers higher sensitivity and accuracy compared with immunohistochemical staining. Furthermore, the sensitivity of the immunohistochemical findings increases with the binding affinities of antibodies being used. Fibroadenomas expressed the highest copy numbers of Fas mRNA, which was consistent with the immunohistochemical finding of strong, homogeneous expression of Fas in these benign tumors. Surprisingly, we also found a high amount of immunohistochemical staining. Furthermore, the sensitivity of the immunohistochemical data might vary depending on the median of mRNA copy numbers. However, the comparison of the two techniques used is limited because the TaqMan RT-PCR analysis quantitates mRNA copy numbers, whereas immunohistochemical studies deliver qualitative data on protein expression.

Finally, the ratio of FasL:Fas mRNA transcripts has been found to be the most reliable parameter to determine the prognostic status of the disease. A subgroup of one-third of breast cancer patients with a FasL:Fas ratio > 1 showed a significant shorter disease-free survival (Fig. 3A). From 31 patients having breast tumor of grade 1 and a FasL:Fas ratio > 1, one patient died and three had a relapse; from 24 patients having tumors of grade 2, four died and one relapsed; and from 16 patients having tumors of grade 3, three died and three relapsed (Fig. 2). The importance of the FasL:Fas ratio as a prognostic factor needs to be assessed over a longer period with extended numbers of patients. However, ratios >1 identify a group of highly malignant breast cancers and therefore might be considered as criteria for the determination of therapeutic regimens.

Ideally, a new prognostic factor should be evaluated only in the setting of individuals who have not received any systemic therapy. According to the Sixth International Conference on Adjuvant Therapy of Primary Breast Cancer held in St. Gallen, Switzerland (February 1998), all patients with axillary lymph node-positive tumors should have adjuvant systemic treatment, and >95% of node-negative patients are candidates for adjuvant therapies. Ninety-three % of all patients in our study received adjuvant therapy, of which 57% were lymph node negative.

The possibility that there is an interaction between the FasL:Fas ratio and response to various therapies must be taken into account. However, until now there has not been any evidence that apoptotic parameters like FasL or Fas are predictive factors in treatment of breast carcinomas. The reverse distribution of G1 (50.5% G1) versus
G2 (38.2%) tumor grades in our study might be explained by the limited sample number of n = 215 and by low intra- and interobserver reliabilities known to occur by semiquantitative grading analysis.

Several mechanisms of escaping Fas-mediated apoptosis have been described. Down-regulation of Fas has been shown in some carcinomas, such as hepatoma, intestinal-type stomach carcinoma, or breast cancer cell lines (12, 15, 23). However, other carcinomas, such as esophageal carcinoma (10) and the breast carcinomas in this study, still express Fas on the cell surface. Therefore, other mechanisms for evasion of Fas-mediated apoptosis downstream of the receptor are required. The Fas-resistant cell line MCF-7 became sensitive to Fas-mediated apoptosis upon transfection with the interleukin-1-β-converting enzyme, an important protein acting downstream of Fas (23). Recently, 39 of 40 breast carcinoma tissues were found to express only weak or no bax-α mRNA. Transfection of the MCF-7 cell line with bax-α cDNA increased strongly the sensitivity of these cells to Fas-mediated apoptosis (24).

Initial studies (25) undertaken on a small number of ductal invasive breast carcinoma (n = 18) confirm our own observation that the presence of FasL mRNA in the whole breast tissue is not a product of leukocytic contamination. In their breast cancer sections, FasL was localized to the cytoplasm and membrane of tumor cells. In our study, FasL:Fas ratios of >1 strongly correlate with the grade of invasive ductal carcinomas (Fig. 2). The proportion of invasive ductal carcinomas with FasL:Fas ratios <1 seems to decrease with grades 1–3. These data are highly suggestive of selection processes involving tumor fratricide. Up-regulation of FasL expression probably induces killing of Fas-bearing tumor cells by promoting the selection of malignant tumor variants, the Fas pathway of which has become insensitive to FasL binding. Initially, induced Fas expression might have been expected to favor the elimination of cellular tumor mutants, but on the contrary, the contemporary expression of FasL promotes the selection of cells also defective in the Fas pathway. Furthermore, reducing the tumor size by eliminating fewer malignant cells enables limited nutrients and oxygen to be used predominantly for propagating more aggressive tumor variants.

Acknowledgments

We thank Dr. J. Makovitzky for immunohistochemical evaluation and H. Krentz for statistical advice.

References

FasL:Fas Ratio–A Prognostic Factor in Breast Carcinomas

Toralf Reimer, Christina Herrmring, Dirk Koczan, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/4/822

Cited articles
This article cites 24 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/4/822.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/4/822.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/60/4/822. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.