Expression of Fas (CD95/APO-1) Ligand by Human Breast Cancers: Significance for Tumor Immune Privilege

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Breast cancers have been shown to elicit tumor-specific immune responses. As in other types of cancer, the antitumor immune response fails to contain breast tumor growth, and a reduction in both the quantity and cytotoxic effectiveness of tumor-infiltrating lymphocytes (TILs) is associated with a poorer prognosis. Fas ligand (FasL) induces apoptotic death of activated lymphocytes that express its cell surface receptor, FasR (CD95/APO-1). FasL-mediated apoptosis of activated lymphocytes contributes to normal immune downregulation through its roles in tolerance acquisition, immune response termination, and maintenance of immune privilege in the eye, testis, and fetus. In this report, we demonstrate that breast carcinomas express FasL. Using in situ hybridization and immunohistochemistry, we show that breast tumors constitutively express FasL at both the mRNA and protein levels, respectively. FasL expression is prevalent in breast cancer: 100% of breast tumors (17 of 17) were found to express FasL, and expression occurred over more than 50% of the tumor area in all cases. By immunohistochemistry, FasR was found to be coexpressed with FasL throughout large areas of all the breast tumors. This suggests that the tumor cells had acquired intracellular defects in FasL-mediated apoptotic signaling. FasL and FasR expression were independent of tumor type or infiltrative capacity. FasL expressed by tumor cells has previously been shown to kill Fas-sensitive lymphoid cells in vitro and has been associated with apoptosis of TILs in vivo. We conclude that mammary carcinomas express FasL in vivo as a potential inhibitor of the antitumor immune response.

Despite expression of tumor-associated antigens such as MAGE 1-3, HER-2/neu (9), and DF3/MUC-1 (11) and the presence of tumor-specific cytotoxic T lymphocytes (12), the immune system fails to contain breast carcinoma. Evidence suggests that a poor local immune response contributes to a poor prognosis in patients with breast cancer. As with other cancers (30), a reduction in the level of tumor-infiltrating lymphocytes (TILs) correlates with a poorer prognosis in patients with breast cancer (22). Also in common with other cancers (24), TILs residing in breast cancers exhibit decreased cytotoxic effectiveness relative to that of peripheral blood lymphocytes (32). The mechanisms by which breast cancers inhibit and evade antitumor immune responses are poorly understood.

Fas ligand (FasL) induces apoptotic death of sensitive lymphoid cells expressing its cell surface receptor, FasR (CD95/APO-1) (25). FasL-mediated apoptosis of activated lymphocytes contributes to immune downregulation through its roles in tolerance acquisition (23), T-cell activation-induced cell death (1), and immune response termination (8). FasL is expressed as a mediator of immune privilege in the eye (13), the testis (6), and the placenta (15). By inducing apoptosis of infiltrating proinflammatory immunocytes, the FasL expressed in these organs may help to prevent potential inflammatory damage to vision and reproduction. In rodent transplantation experiments, prolonged allograft survival has been obtained for FasL-expressing tissues (6, 36) or for FasL-negative pancreatic islets coengrafted with FasL-expressing cells (18, 20). Transplantation of murine tumor cell allografts stably transfected with the Fasl gene showed that FasL can cause local suppression of both humoral and cellular allograft-specific immune responses (4).

Recent evidence has shown that tumors can also express FasL as a possible mediator of tumor immune privilege (29). Cancer cell lines that express FasL have been shown to kill lymphoid cells by Fas-mediated apoptosis in vitro (28). This suggests a Fas counterattack mechanism of tumor immune escape, by which a cancer cell, by expressing FasL, can delete Fas-sensitive antitumor immune effector cells by apoptosis. Melanoma (14), hepatocellular carcinoma (35), lung cancer (27), astrocytoma (31), and liver metastases of colon adenocarcinomas (34) have been shown to express FasL in vivo. FasL expression by esophageal carcinoma cells was found to be associated with apoptotic depletion of tumor-infiltrating lymphocytes in vivo (7).

The aim of this study was to establish if mammary carcinomas expressed FasL as a possible mediator of tumor immune privilege in breast cancer. Immunohistochemistry and in situ hybridization were used to localize both FasL protein and mRNA within neoplastic breast tissue in vivo.

MATERIALS AND METHODS

Specimens. Human mammary carcinomas (n = 17) were collected following surgical resections performed at the Mercy Hospital, Cork, Ireland, by a protocol approved by the University Teaching Hospitals Ethics Committee. Specimens were from patients with newly diagnosed breast carcinoma, and the clinicopathological characteristics of the tumors are shown in Table 1. Sections of normal breast tissue, distal to the tumors, were used as controls (n = 10). None of the patients had received chemo-, radio-, or immunotherapy prior to resection.

Immunohistochemical detection of FasL and FasR protein. Formalin-fixed, paraffin-embedded, surgically resected tumor sections were deparaffinized in xylene followed by rehydration in a graded series of alcohol. Sections were postfixed in 4% paraformaldehyde for 1 h and were washed twice for 5 min each time in a wash buffer containing 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 0.001% saponin. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide in methanol for 5 min. Sections were then washed as described above except that the wash buffer for this and all subsequent steps

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TABLE 1. Clinicopathological characteristics and extent of expression of FasL and FasR in breast tumors

| Patient | Tumor type                        | Extent of expression | FasL | FasR |
|---------|-----------------------------------|----------------------|------|------|
|         |                                   |                      |      |      |
| 1       | Intraduct and infiltrating carcinoma | ++++                | +++  | +++  |
| 2       | Intraduct and infiltrating carcinoma | ++++                | +++  | +++  |
| 3       | Intraduct and infiltrating carcinoma | ++++                | +++  | +++  |
| 4       | Intraduct and infiltrating carcinoma | ++++                | +++  | +++  |
| 5       | Intraduct and infiltrating carcinoma | ++++                | +++  | +++  |
| 6       | Intraduct and infiltrating carcinoma | ++++                | +++  | +++  |
| 7       | Intraduct and infiltrating carcinoma | ++++                | +++  | +++  |
| 8       | Infiltrative ductal carcinoma      | +++                  | +++  | +++  |
| 9       | Infiltrative ductal carcinoma      | +++                  | +++  | +++  |
| 10      | Infiltrative ductal carcinoma      | +++                  | +++  | +++  |
| 11      | Ductal carcinoma in situ           | NA                   | NA   | NA   |
| 12      | Ductal carcinoma in situ           | NA                   | NA   | NA   |
| 13      | Infiltrative mucinous carcinoma    | Low<sup>a</sup>     | +++  | +++  |
| 14      | Mucinous adenocarcinoma            | ND<sup>b</sup>      | +++  | +++  |
| 15      | Infiltrative lobular carcinoma     | +++                  | +++  | +++  |
| 16      | Infiltrative lobular carcinoma     | +++                  | +++  | +++  |
| 17      | Tubular carcinoma                  | 1                    | +++  | +++  |

<sup>a</sup> Graded by the Nottingham modification of Scarff-Bloom-Richardson.

<sup>b</sup> Extent of expression was graded on the basis of the percentage of positive cells, as follows: +, 0 to 25%; +, 25 to 50%; +++, 50 to 75%; +++++, 75 to 100%.

<sup>c</sup> NA, not applicable.

<sup>d</sup> Graded by conventional histopathological classification.

<sup>e</sup> ND, not determined.

In situ hybridization was performed with paraffin-embedded human breast tumor sections (4-μm thick) mounted on amino-propylsilane-treated slides. In situ hybridization was performed with the GenPoint in situ hybridization kit (DakoKOP Corp., Glostrup, Denmark), according to the manufacturer’s instructions. Briefly, this involved microwave treatment followed by limited protease K digestion to enable probe access to tissue mRNA. Hybridization was performed at 42°C for 16 h with the biotinylated FasL-specific riboprobe at a final concentration of 0.5 ng/μl. Following posthybridization washes at 42°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the sections were incubated with streptavidin-conjugated horseradish peroxidase at room temperature for 15 min. Following washes in TBST (50 mM Tris-HCl [pH 7.6], 300 mM NaCl, 0.1% Tween 20), a signal amplification step was performed by incubating the sections with biotinyl-tyramide at room temperature for 5 min. Horseradish peroxidase catalyzes oxidation of biotinyl-tyramide, which rapidly forms covalent bonds with adjacent aromatic groups in the tissue. This resulted in additional biotin deposition at sites of riboprobe binding. Sections were washed in TBST, and a second incubation with streptavidin-conjugated horseradish peroxidase was performed. Following final TBST washes, color development was performed with the diaminobenzidine chromogenic substrate, which generates a brown color. A control hybridization was performed with a consecutive section from each specimen by using conditions identical to those described above, except that a biotinylated Fasl. sense riboprobe was used.

RESULTS

Mammary carcinomas express FasL protein. FasL expression by tumor cells was immunohistochemically detected in all (n = 17) surgically resected breast carcinomas examined (Fig. 1). Immunohistochemistry was performed with a FasL-specific polyclonal IgG (Santa Cruz Biotechnology) raised against a synthetic FasL peptide. FasL specificity was confirmed in consecutive control sections by using the FasL peptide immunogen (FasL amino acids 260 to 279) as an internal competitive control. Inclusion of the soluble peptide immunogen during primary antibody incubation resulted in direct, competitive displacement of positive staining (Fig. 1). Inclusion of an irrelevant peptide had no effect on FasL staining. Detection of the FasL protein in the breast tumors was confirmed by immunohistochemistry with a FasL-specific monoclonal antibody (FasL clone G427-4; Pharmingen). With consecutive tumor sections, the Pharmingen monoclonal antibody resulted in a pattern of staining identical to that obtained with the Santa Cruz Biotechnology polyclonal antibody. An isotype-matched monoclonal antibody did not stain control sections.

The magnitude and extent of FasL protein expression detected immunohistochemically were variable both within individual tumors and between tumors. FasL staining varied from weakly positive neoplastic areas to intensely staining regions of tumors, where the intensity of staining was stronger than that observed in local FasL-positive TILs. However, FasL staining was of locally uniform intensity within nests of tumor cells. FasL-positive and -negative tumor islands were frequently found to occur within the same tumor, although all tumors expressed FasL throughout more than 50% of the tumor area (Table 1). Expression of FasL in mammary tumors occurred in ductal carcinomas (12 of 12), lobular carcinomas (2 of 2), mucinous carcinomas (2 of 2), and a tubular carcinoma (1 of 1). There was no apparent difference in FasL expression between infiltrative and in situ carcinomas.

Localization of FasL mRNA expression by in situ hybridization. A biotinylated, FasL-specific RNA hybridization probe (riboprobe) was generated as follows. A 344-bp fragment of the human FasL cDNA sequence corresponding to codons 96 to 210 was amplified by PCR with a proofreading thermostable polymerase (ULTma DNA polymerase; Perkin-Elmer, Norwalk, Conn.). The fragment was cloned into the EcoRV site of pBluescript (Strategene, La Jolla, Calif.), which is flanked by the T3 and T7 RNA promoters in opposite orientations. The orientation of the cloned insert relative to those of these promoters was ascertained by restriction mapping. By using the recombinant plasmid as a template, the FasL-specific antisense riboprobe was synthesized by in vitro transcription of the cloned insert with biotin-16-UTP and T3 RNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany). A sense control riboprobe was synthesized from the same template in the opposite direction by using T7 RNA polymerase (Boehringer Mannheim GmbH). The nucleotide sequence of the FasL-specific riboprobe showed no significant homology to any other sequence in the EMBL DNA sequence database.

Localization of FasL mRNA to breast carcinoma cells. Non-tumor cells, including lymphocytes and neurons, are known to express FasL. Detection of FasL mRNA in whole tumor tissue by Northern blotting or reverse transcription-PCR would not necessarily confirm that the mRNA detected was expressed by tumor cells. In order to confirm that the FasL protein detected in the breast tumors was expressed by tumor cells, we performed in situ hybridization to detect and localize FasL mRNA within the tumor tissue. A biotinylated, FasL-specific RNA probe (riboprobe) was synthesized by in vitro transcription of a 344-bp fragment of
FasL cDNA (codons 96 to 210) cloned into pBluescript. The nucleotide sequence of the FasL riboprobe showed no significant homology to any other sequence within the EMBL DNA sequence database. Using in situ hybridization with this probe, FasL mRNA expression was detected in tumor cells in the resected mammary carcinomas. Positive hybridization occurred within neoplastic cells throughout extensive areas of the tumors (Fig. 1). FasL mRNA detection colocalized with the
FasL protein detected immunohistochemically with serial tumor sections. Colocalization of FasL mRNA and protein confirmed that breast carcinoma cells expressed FasL. Cells of lymphoid morphology were also positive by hybridization with the FasL-specific riboprobe, possibly representing activated, FasL-expressing cytotoxic T lymphocytes and natural killer (NK) cells. The specificity of hybridization was confirmed with a biotinylated control riboprobe with a sequence complementary to that of the FasL-specific probe (sense control probe). This sense control probe failed to generate positive signals in control hybridizations with consecutive tumor sections, thereby confirming the specificity of FasL mRNA detection (Fig. 1).

**Coexpression of FasR and FasL in breast cancers.** FasR expression was immunohistochemically detected in all (n = 17) surgically resected breast carcinomas examined (Fig. 2). Immunohistochemistry was performed with a FasR-specific polyclonal IgG (Santa Cruz Biotechnology) raised against a synthetic FasR peptide. FasR specificity was confirmed in consecutive control sections with the FasR peptide immunogen (FasR amino acids 316 to 335) as an internal competitive control. Inclusion of the soluble peptide immunogen during primary antibody incubation resulted in direct, competitive displacement of positive staining. Inclusion of an irrelevant peptide had no effect on FasR staining.

The magnitude and extent of FasR expression were variable both within individual tumors and between tumors (Table 1). FasR was expressed over more than 50% of the tumor area in all specimens. By using consecutive immunohistochemically stained tumor sections, FasL and FasR were found to be coexpressed by tumor cells throughout large areas of all tumors (Fig. 2). This suggests that the tumor cells had acquired intracellular defects in FasL-mediated apoptotic signaling.

**DISCUSSION**

In this report, we demonstrate that breast cancers express FasL, an inducer of immunocyte cell death, via the FasR-mediated pathway of apoptosis. Because activated leukocytes express abundant cell-surface FasR, expression of FasL potently enables breast tumors to counterattack and kill Fas-sensitive, antitumor immunocyte effector cells. We and others have previously demonstrated that FasL expressed by diverse tumor cells in vitro is biologically active: FasL-expressing tumor cells previously demonstrated that FasL expressed by diverse tumor cells in vitro is biologically active: FasL-expressing tumor cells precluded from the possibility that the magnitude and extent of FasL expression were variable, extensive detection of FasL protein was derived from TILs. While the magnitude and extent of FasL expression were variable, extensive expression (>50% of the tumor area) occurred in all tumors. Although we noted that myoepithelial cells were immunohistochemically positive, FasL expression was otherwise absent in all FasL-expressing areas of the mammary tumors. In the present study, significant neutrophil infiltration was absent from all FasL-expressing areas of the mammary tumors. All available evidence indicates that in its native context of expression, FasL mediates immunological downregulation, tolerance, and privilege, and its absence, through mutation, leads to autoimmune disease in mice (26).

Evidence which directly implicates FasL as an inhibitor of immunological responses to tumors in vivo has accumulated. When a murine FasL-expressing melanoma cell line was injected into syngeneic host mice, this cell line quickly developed tumors. In syngeneic hosts that express a defective, mutant FasR (lpr [lymphoproliferation]), tumor formation was impaired (14). The greater efficiency of tumor containment by these syngeneic lpr mice may have been due to their lymphocytes’ insensitivity to tumor-expressed FasL. Although other mechanisms of immune evasion enabled the eventual establishment of tumors in FasL-insensitive lpr mice, these experiments showed that FasL contributed to the immune privilege of the tumor, expediting tumor formation in wild-type mice. A recent experiment involving allograft transplantation of murine tumor cells stably transfected with the FasL gene showed that FasL caused profound local suppression of both humoral and cellular allograft-specific immune responses (4). FasL expression by human esophageal carcinoma cells was found to be associated with apoptotic depletion of tumor-infiltrating lymphocytes in vivo (7).

In order to express FasL, tumor cells must be insusceptible to FasL-mediated apoptosis. In the present study, FasR and FasL were found to be coexpressed throughout large areas of all the breast tumors (n = 17). This suggests that complete loss of FasR cannot account for the resistance of breast cancer cells to FasL-mediated apoptosis. Breast cancer cells have been shown to have defective Fas signal transduction in vitro (17). Resistance to Fas-mediated apoptosis is a common feature of cancers, irrespective of cell surface expression of FasR (17, 28, 29). Fas resistance of breast cancer cells has been overcome in vitro by transfection of DNAs encoding the intracellular pro-
FIG. 2. Human breast carcinomas coexpress FasR and FasL. Immunoperoxidase staining with a FasR-specific rabbit polyclonal IgG antibody (FasR Ab) was performed with paraffin-embedded breast carcinoma sections. A consecutive section from each tumor was used for immunohistochemical detection of FasL with a FasL-specific rabbit polyclonal IgG antibody (FasL Ab). Slides were counterstained with hematoxylin. FasR-positive immunohistochemical staining (brown) is shown in a representative breast carcinoma (magnification, ×100). The same tumor region is also positive for FasL expression, indicating coexpression of FasR and FasL by breast tumor cells in vivo. As a control for specificity of antibody detection, the appropriate immunizing peptide (FasR or FasL) was included during primary antibody incubation. Competitive displacement of staining by the soluble peptide immunogen confirmed the specificity of FasL detection (Fig. 1) and FasR detection (data not shown). These results are representative of 17 breast carcinomas.
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