Fas Ligand Expression by Neoplastic T Lymphocytes Mediates Elimination of CD8+ Cytotoxic T Lymphocytes in Mycosis Fungoides: A Potential Mechanism of Tumor Immune Escape?1

Xiao Ni, Parul Hazarika, Chunlei Zhang, Rakhashandra Talpur, and Madeleine Duvic2
Department of Dermatology, University of Texas, M. D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Mycosis fungoides (MF) is the most common form of cutaneous T-cell lymphoma (CTCL) and arises from the accumulation and clonal proliferation of epidermotropic, CD4+/CD45RO+ (helper/memory) T lymphocytes. Loss of CD8+ CTLs within MF lesions is associated with poor prognosis and disease progression. Because T-lymphocyte apoptosis is controlled mainly through the Fas/Fas ligand (FasL) pathway and tumor cells may escape immune surveillance by expressing FasL, triggering apoptosis of tumor-infiltrating T lymphocytes, we studied the role of this system in MF. T-cell subsets, Fas/FasL expression, and apoptosis were evaluated in normal and lesional skin biopsy specimens from 21 patients with all stages of MF and in cultured CTCL cell lines (MJ, HUT78, and HH) using immunohistochemistry, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), and Western blotting. MF lesions and paired, clinically “normal,” uninvolved skin showed increased numbers of both TUNEL-positive epidermal keratinocytes (n = 13; F = 31.146; P < 0.01, ANOVA) and dermal lymphocyte infiltrates (n = 13; F = 15.825, P < 0.01, ANOVA) compared with the normal control skin. FasL expression was highest in lesional epidermal keratinocytes, in CTCL tumor cell lines, and in dermal tumor lymphocytes in MF lesions compared with uninvolved skin. FasL colocalized with CD45RO+ cells. CD8+ cells in or adjacent to CD45RO+ cells were positively labeled by TUNEL for apoptosis. In addition, CD8+ cell numbers were decreased in areas in which FasL+ tumor cells were abundant (2.01 ± 0.86%) compared with non-FasL expressing areas (13.53 ± 3.54%; P < 0.02). These results suggest that a potential mechanism of tumor immune escape in MF is FasL-mediated apoptosis of infiltrating CD8+ CTLs.

INTRODUCTION

CTCLs3 are a heterogeneous group of peripheral, extranodal, non-Hodgkin’s lymphomas that arise in skin-homing lymphocytes and may progress by disseminating to lymph nodes and viscera. MF, the most indolent form of CTCL, and its leukemic variant, the SS, are the most common CTCLs, representing a disease spectrum. MF and SS originate from a clonal expansion of epidermotropic, CD4+/CD45RO+ helper/memory T cells (1, 2). Between 1974 and 1984, the incidence of MF increased by 3.2-fold, and the proportion of MF cases among all lymphomas increased from 1.6 to 2.8% (2, 3).

The stage and prognosis of MF has been defined by the skin or T-stage as reported by Bunn and Lamberg in 1979 (4). Early-stage patients (T1–2 ) with patches or plaques on less than 10% of their skin have an excellent overall survival and prognosis, when they respond to skin-directed therapy (5, 6). With the exception of electron beam used in early patients, no curative therapies exist, and progressive disease can be associated with morbidity, disfigurement, and death from sepsis (5–7).

One hypothesis to explain the indolent nature of MF suggests that clonal T cells arise and accumulate in response to chronic antigen stimulation (8). It has been suggested that antigens may differ from one patient to another and could be provided by persistent infectious agents (9–11). Also in support of chronic antigen-driven T-lymphocyte proliferation is the finding of significant HLA Class II associations, including HLA-DRB1*11 and DQB*03 with MF and DQB*02 with SS (12). Regardless of the initial cause, with disease progression, accumulation of malignant T cells is followed by loss of normal T-cell immunity leading to an AIDS often exacerbated by treatment and to death from opportunistic infections (13).

Hoppe et al. (14) reported that T1,2 MF patients tend to

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2 To whom requests for reprints should addressed, at Department of Dermatology, M. D. Anderson Cancer Center, Box 434, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 745-1113; Fax: (713) 745-3597; E-mail address: mdavic@mdanderson.org.

3 The abbreviations used are: CTCL, cutaneous T-cell lymphoma; FasL, Fas ligand; L0, MF skin lesion at baseline; MF, mycosis fungoides; N0, clinically uninvolved “normal” skin from MF patient at baseline; NS, normal skin from normal control subjects; TIL, tumor-infiltrating lymphocyte; Tdt, terminal deoxynucleotidyl transferase; TUNEL, Tdt-mediated dUTP nick end labeling; SS, Sézary syndrome; IL, interleukin; DAB, dianinobenzidine; AI, apoptosis index; TBS, Tris-buffered saline; HRP, horseradish peroxidase.
have a higher proportion of CD8+ cells in their skin biopsy specimens, compared with patients with more advanced disease. Heald et al. (15, 16) also found that the loss of CD8+ cells, tumor-infiltrating CTLs in MF lesions and in the peripheral blood of SS patients is associated with poor prognosis and an inability to respond to photopheresis therapy. Within each T stage, patients with a larger proportion of CD8+ cells had a better survival rate than did patients with fewer numbers of CD8+ cells. Defects in cell-mediated immunity are reported to arise during disease progression (17, 18). The excess production of T-helper type 2 cytokines (IL-4, 5, 6, and 10) by malignant T cells can antagonize production of T-helper type 1 cytokines (IFN-γ and IL-2) and interfere with cell-mediated immunity (19). However, the mechanism by which CD8+ CTLs are lost with the progression of MF has not been fully elucidated at the molecular level.

Classical mechanisms to account for tumor evasion of the immune system include defective antigen presentation, interference with tumor/C-cell interaction, and production of immunosuppressive factors (20). New evidence points to expression of FasL as a possible mediator of tumor immune privilege (20). FasL (also called CD95L/APO-1 L) is a type-II integral membrane protein member of the tumor necrosis factor family that transduces an apoptotic death signal by binding to its receptor Fas (CD95). Fas is a type I transmembrane protein of the tumor necrosis factor receptor family. The Fas/FasL system is implicated in several aspects of immune regulation and development. It modulates T-cell selection and clonal deletion of activated T cells by the thymus, regulation of activated B-cell-specific killing of virally infected or transformed cells, and establishment of “immune-privileged” sites such as testis, brain, and eyes (20, 21).

Recent data suggest that de novo expression of FasL in tumors might result in the elimination of Fas+ antitumor lymphocytes, referred to as Fas counterattack, by which tumors may foil the immune system and become immune privileged sites. Both hematological malignancies (22) and solid tumors, such as melanomas, have been found to induce apoptotic cell death of TILs through Fas and FasL interactions (23). In human colorectal carcinomas, apoptosis in TILs was more frequent in tumors that expressed FasL than in FasL-negative tumors (24). FasL expression in esophageal cancer was associated with apoptosis and depletion of TILs in vivo (25). These findings suggest that the Fas/FasL system is an important mechanism by which tumors avoid attack by CTLs.

FasL-expressing tumor cells have been reported in T-cell-derived malignancies including natural killer cell lymphoma, large granular lymphocytic (LGL) lymphoma (26) and T-cell acute lymphocytic leukemia (T-ALL; Ref. 27). FasL expression has been studied in MF, but the results were not conclusive (28–30). This study shows that the depletion of specific CD8+ CTLs by apoptosis within MF lesions is associated with FasL expression in tumor cells. FasL expression by MF tumor cells might lead to the loss of CD8+ CTLs, which clinically is associated with poor prognosis. These results suggest strategies for monitoring therapy in the future.

**MATERIALS AND METHODS**

**Tissue Specimens.** Twenty-one patients with either newly diagnosed MF or with established MF, who were off therapy because they were being enrolled in clinical research trials, signed informed consent for the use of tissue for research. All clinical research was approved by the M. D. Anderson Cancer Center Institutional Review Board and was conducted according to good clinical practice guidelines. Biopsy specimens were taken under local anesthesia from clinically uninvolved skin (N0) and from representative untreated skin lesion (L0) using 6-mm punch biopsies. Skin tumor (T) staging of all of the patients at baseline was conducted according to the recommendations of the European Organization for Research and Treatment of Cancer classification for primary cutaneous lymphoma (31). Normal control skins (NS; n = 8) were obtained from adult breast surgeries.

**Cell Lines.** CTCL cell lines HH, MJ (MF), HUT78 (SS; Ref. 32) and Jurkat cell line were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in HEPES-buffered RPMI 1640 with 2 mmol/liter glutamine, supplemented with 10% fetal bovine serum, 0.25 mg/ml amphotericin B, 100 units penicillin G, 100 units streptomycin, and 1 mmol/liter sodium pyruvate. Cytosin preparations of naïve CTCL cells lines were air-dried and fixed for 10 min with cold acetone prior to immunocytochemistry staining.

**TUNEL.** After proteinase K treatment at 20 μg/ml for 30 min, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 60 min. Sections were treated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. The labeling reaction was performed in buffer with TdT and fluorescein-dUTP according to the instructions (In situ cell death detection kit-POD; Roche, Mannheim, Germany). Slides were coverslipped and incubated at 37°C for 60 min in a humidity chamber before incubation with sheep antifluorescin antibody Fab fragment conjugated with HRP at 37°C for 30 min. TUNEL-positive color development (brown) was obtained by incubating the sections with DAB substrate solution for 15 min. DNase I-treated specimens (1.0 μg/ml for 10 min at room temperature) served as positive controls, and TdT was omitted from negative control slides.

**Immunohistochemical Detection of FasL in Skin Lesions and Cytospin Preparations.** Six to eight 5-μm consecutive sections from each specimen were stained using immunohistochemistry methods described by Müllauer et al. (33) with slight modifications. After deparaffinization and rehydration, sections were microwaved in 0.01 m citrate buffer for 10 min at high power for antigen retrieval. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol for 30 min. After blocking with 1% normal horse serum-0.1 m PBS (pH 7.2) for 30 min, sections were incubated at 37°C for 60 min, and then overnight at 4°C with 1:100 dilution of mouse monoclonal antibody (clone 33). The antibody was raised against a peptide comprising the COOH-terminal amino acid 116–277 of human FasL. (Transduction Laboratories, Lexington, KY) and used in 1% normal horse serum-0.1 m PBS (pH 7.2). Sections were rinsed in 0.1 m PBS (pH 7.2), and bound antibody was detected with a three-step biotin-avidin method (ABC-Elite kit; Vector Laboratories, Burlingame, CA). DAB was used as a
Correlation between FasL+ and CD8+ T-Cell Loss in MF

chromogen substrate. Tissues were counterstained with hematoxylin. The lymph nodes from a patient with B-cell lymphoma was included as a positive control. Omission of the primary antibody was the negative control. A rabbit polyclonal antihuman FasL IgG (NH2-terminal amino acid, 2–19; Santa Cruz Biotechnology, Santa Cruz, CA) and immune peptide were used to confirm the results. Staining of cytopsins was by the same protocol without antigen retrieval, using 0.1% Saponin (w/v %)-0.1 M PBS (pH 7.2).

Colocalization of FasL and CD45RO or CD8/FasL by Doublestaining. CD45RO or CD8 staining was performed first and was followed by FasL doublestaining to identify FasL+ T-cell subsets within MF lesions. Staining was performed at room temperature according to the manufacturer’s instructions (Dako Envision Doublestain System; Dako, Carpinteria, CA) with minor modifications. Briefly, after microwave antigen retrieval and endogenous peroxidase blocking, mouse antihuman CD45RO monoclonal IgG (1:20; UCHL1; Dako A/S, Denmark) or mouse antihuman CD8 monoclonal IgG (1:20; clone C8/144; Dako, A/S) or negative control reagent was added to the slides and incubated for 30 min. The slides were then next incubated with labeled polymer–HRP for 30 min and then with DAB substrate for 10 min. Sections were blocked for 3 min and then incubated with mouse monoclonal antihuman FasL-specific IgG1 at 1:100 in blocking buffer for 30 min and with polymer-alkaline phosphatase for 30 min. FasL-positive color was detected after application of alkaline phosphatase substrate solution (Vector Blue; Vector Laboratories) for 10 min. When viewed under light microscopy, FasL single-positive cells stained blue. CD45RO or CD8/FasL dual-positive cells appeared with blue cytoplasmic/cell surface and brown cell-surface staining.

Detection of TUNEL Staining in CD45RO or CD8+ T Cells by Doublestaining. CD45RO or CD8 staining was performed first on MF sections followed by TUNEL detection to identify cells undergoing apoptosis. Briefly, doublestain-blocking solution (Dako Envision Doublestain System) was applied for 3 min, followed by primary antibody (anti-CD45RO or anti-CD8) as above for 30 min, and labeled polymer–AP for 30 min (Dako Envision Doublestain system). Sections were then incubated for 10 min with Vector Blue substrate solution. TUNEL was performed in the same sections as above, but with 3-amino-9-ethylcarbazole (AEC) color development. When viewed under light microscopy, FasL CD45RO or CD8/FasL dual-positive cells showed red nucleus staining and blue cytoplasmic/cell surface staining.

Semi-quantitative Analysis of Immunohistochemistry Staining and Cell Number. To quantify TUNEL-positive cells, FasL staining, and CD45RO+ or CD8+ cells, stained sections were observed under light microscopy at ×400. The number of TUNEL-positive cells per 500 epidermal keratinocytes and 500 dermal infiltrating cells within each of five areas per section were counted. AIs for keratinocytes and infiltrating cells was determined by the number of positive staining cells per 500 epidermal keratinocytes and 500 dermal infiltrating cells within each of five areas per section.

RESULTS

Patient Demographic Data. The demographics and stages of the 21 patients (10 male and 11 female) are shown in Table 1. The median age of the subjects was 67 years old (range, 40–84). Clinical staging of lymphoma was done on all patients.
FasL-positive cells were studied in 21 MF patients. MF patients had higher AIs than did MF skin lesions, shown in Fig. 1. Surprisingly, clinically uninvolved skin from MF patients was similar to that seen in NS from control subjects (Fig. 3, A and B). Eighty-one % (17 of 21) MF patients’ lesions showed weak to strong (+ to ++++) staining of Fasl in epidermal keratinocytes, and the number of positive lesions was similar across all four stages (Table 2). Fasl+ infiltrating dermal lymphocytes were detected in only one-half of the Stage I (four of eight) or II (three of five) lesions but were seen in five of six Stage IV lesions (Table 2), but the differences were not statistically significant as Fisher’s exact test ($P > 0.05$). We also analyzed Fasl staining in patients classified by T stage as shown in Table 3 with similar results. In T3 and T4 lesions, Fasl-positive lymphocytes were detected in four of five and three of five patients, respectively. Staining of Fasl was more intense in dermal lymphocytic infiltrates of all stages of MF lesions than it was in dermal lymphocytes found in paired, clinically uninvolved skin (10 of 15; sign test, $\chi^2 = 8.1; P < 0.01$) and in control NS.

**Table 1** Fasl expression in MF lesion epidermal keratinocytes and dermal infiltrates

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/Sex</th>
<th>Stage</th>
<th>Keratinocyte</th>
<th>Infiltrating cell</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>43/F</td>
<td>IA, T1</td>
<td>N0' + + +</td>
<td>N0' - 0</td>
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<tr>
<td>2(^{a})</td>
<td>67/M</td>
<td>IA, T1</td>
<td>+ + + +</td>
<td>+ + + 2</td>
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<td>3(^{a})</td>
<td>84/F</td>
<td>IA, T1</td>
<td>+ + + + +</td>
<td>N0' - 0</td>
</tr>
<tr>
<td>4</td>
<td>72/M</td>
<td>IA, T1</td>
<td>+ + + +</td>
<td>N0' - 0</td>
</tr>
<tr>
<td>5</td>
<td>53/F</td>
<td>IB, T2</td>
<td>N0' + +</td>
<td>N0' - 0</td>
</tr>
<tr>
<td>6</td>
<td>71/M</td>
<td>IB, T2</td>
<td>N0' + +</td>
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</tr>
<tr>
<td>7</td>
<td>40/F</td>
<td>IB, T2</td>
<td>+ + + +</td>
<td>N0' - 0</td>
</tr>
<tr>
<td>8(^{a})</td>
<td>70/F</td>
<td>IB, T2</td>
<td>+ + + +</td>
<td>N0' - 0</td>
</tr>
<tr>
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<td>65/M</td>
<td>IA, T1</td>
<td>+ + + + +</td>
<td>N0' + + + +</td>
</tr>
<tr>
<td>10(^{a})</td>
<td>66/M</td>
<td>IB, T1</td>
<td>N0' + +</td>
<td>N0' - 0</td>
</tr>
<tr>
<td>11</td>
<td>78/M</td>
<td>IB, T1</td>
<td>N0' + +</td>
<td>N0' - 0</td>
</tr>
<tr>
<td>12</td>
<td>69/M</td>
<td>IB, T1</td>
<td>+ + + + +</td>
<td>N0' - 0</td>
</tr>
<tr>
<td>13(^{a})</td>
<td>54/F</td>
<td>IB, T1</td>
<td>+ + + +</td>
<td>N0' - 0</td>
</tr>
<tr>
<td>14</td>
<td>76/F</td>
<td>III, T4</td>
<td>N0' + +</td>
<td>N0' - 0</td>
</tr>
<tr>
<td>15(^{a})</td>
<td>68/F</td>
<td>III, T4</td>
<td>+ + + +</td>
<td>N0' - 0</td>
</tr>
<tr>
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<td>63/F</td>
<td>IVA, T1</td>
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<td>- + + 1</td>
</tr>
<tr>
<td>17(^{a})</td>
<td>67/F</td>
<td>IVA, T1</td>
<td>+ + + + +</td>
<td>N0' + + + +</td>
</tr>
<tr>
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<td>- 0 - 0</td>
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<td>IVA, T4</td>
<td>+ + + + +</td>
<td>N0' + + + +</td>
</tr>
<tr>
<td>20</td>
<td>66/M</td>
<td>IVB, T4</td>
<td>+ + + + +</td>
<td>N0' + + + +</td>
</tr>
<tr>
<td>21(^{a})</td>
<td>57/M</td>
<td>IVB, T3</td>
<td>+ + + + +</td>
<td>N0' + + + +</td>
</tr>
</tbody>
</table>

\(^{a}\) T stage: T1, patches and plaques <10% body surface area; T2, patches and plaques >10% body surface area; T3, tumors; T4, generalized erythroderma.

\(^{b}\) Intensity (-, +, ++, ++++) and frequency (0, 1, 2, 3) of immunohistochemical staining.

\(^{c}\) Selected for study of Fasl versus CD8+ and CD45RO double-staining.

At baseline, and representative patients from each stage were included as follows: Stage IA (n = 4), Stage IB (n = 4), Stage IIA (n = 1), Stage IIB (n = 4), Stage III (n = 2), Stage IVA (n = 4), and Stage IVB (n = 2). Representative skin lesions of each patient’s skin T stage (Table 1) were selected for study as follows: T1, patch or plaque covering <10%; T2, patch or plaque covering >10%; T3, tumor stage; and T4, erythroderma.

Increased AI Is Present in Baseline Untreated MF Lesions and in Clinically Uninvolved Skin. It has been suggested that Fasl, expressed by several neoplastic cell lines and some tumors in vivo, is able to trigger the apoptotic process in activated T lymphocytes (23, 25). This system may constitute a key element of the immunological escape mechanism used by many types of neoplasms. To see whether this mechanism was important in MF, we first examined the amount of baseline apoptosis by TUNEL in situ in 13 untreated MF lesions paired with the same patient’s uninvolved skin. MF lesions and paired, clinically “normal” uninvolved skin both contained increased numbers of TUNEL-positive epidermal keratinocytes (n = 13; $F = 31.146; P < 0.01$, ANOVA) and dermal lymphocyte infiltrates (n = 13; $F = 15.825; P < 0.01$, ANOVA) compared with normal control skins (n = 8). Representative sections are shown in Fig. 1. Surprisingly, clinically uninvolved skin from MF patients had higher AIs than did MF skin lesions, which supports the systemic nature of this disease (Fig. 2).

MF Lesions Contain Higher Fasl Expression Than NS. Fasl-positive cells were studied in 21 MF patients’ skin lesions and clinically normal skin at all stages of the disease (Tables 1, 2, and 3). Granular staining was observed in both the cytoplasm and cell membrane of positive cells. Representative sections demonstrating the pattern and degree of Fasl staining in the normal skin, the uninvolved skin, and paired lesional skin are shown in Fig. 3, A, B, and C–D, respectively. The strongest immunoreactivity for Fasl was found in lymphocytes with cerebriform nuclei within lesions (Fig. 3D). Low Fasl staining within the epidermal keratinocytes of normal uninvolved skin from MF patients was similar to that seen in NS from control subjects (Fig. 3, A and B). Eighty-one % (17 of 21) MF patients’ lesions showed weak to strong (+ to ++++) staining of Fasl in epidermal keratinocytes, and the number of positive lesions was similar across all four stages (Table 2). Fasl+ infiltrating dermal lymphocytes were detected in only one-half of the Stage I (four of eight) or II (three of five) lesions but were seen in five of six Stage IV lesions (Table 2), but the differences were not statistically significant as Fisher’s exact test ($P > 0.05$). We also analyzed Fasl staining in patients classified by T stage as shown in Table 3 with similar results. In T3 and T4 lesions, Fasl-positive lymphocytes were detected in four of five and three of five patients, respectively. Staining of Fasl was more intense in dermal lymphocytic infiltrates of all stages of MF lesions than it was in dermal lymphocytes found in paired, clinically uninvolved skin (10 of 15; sign test, $\chi^2 = 8.1; P < 0.01$) and in control NS.

**Fasl Expression Is Higher in CD45RO+ T Cells Than in CD8+ Lymphocytes in MF Lesions.** To determine which cells in MF lesions were expressing Fasl, we studied 10 representative MF lesions (patients 2, 3, 8, 10, 13, 15, 17, 19, 20, and 21). Double-labeling methods were used to first detect either CD45RO or CD8 followed by Fasl. These patients were selected because their lesions contained Fasl+ dermal lymphocytes (shown in Table 1). All of the 10 lesions contained infiltrates of lymphocytes staining for both CD8 and CD45RO. CD45RO+ infiltrating cells were located in groups or clusters around dermal vessels and in the epidermis. Most CD45RO+ -infiltrating cells stained blue for Fasl, with intensity varying from + to ++. The highest immunoreactivity for Fasl was observed in CD45RO+ atypical dermal and epidermal lymphocytes with cerebriform nuclei as shown in Fig. 4, A and B. CD8+-infiltrating cells also showed weak (+) to moderate (+++) blue counter-staining for Fasl, but it was less intense than that seen in the CD45RO+ tumor cells (Fig. 4, C and D).

**Fasl Is Expressed by CTCL Tumor Cell Lines in Vitro.** To confirm that MF tumor cells express Fasl, we also examined Fasl expression in three well-established CTCL cell lines (HH, MJ, and Hut78). Cytosin preps from all three of the cell lines showed homogeneous, strong immunoreactivity to Fasl antibody (Fig. 5, A–C). The Fasl bands by Western blot analysis were stronger in three CTCL cell lines than in human endothelial cells as a positive control, shown in Fig. 5D. We used ELISA to determine further the relative amount of cellular (membrane-bound) versus soluble Fasl in CTCL lines and in Jurkat control T cells. The level of expression of cellular Fasl was higher than soluble Fasl in all three of the CTCL cell lines ($n = 3; t = 1.976; P > 0.05$, paired t test) as shown in Fig. 6. HUT78 had the highest expression of both forms of Fasl, especially the membrane-bound form of Fasl. Cellular Fasl
was higher in all three of the CTCL lines than in the control Jurkat T cells.

**High FasL Expression by MF Lesion Tumor Cells Is Associated with Low Numbers of CD8+ Infiltrating Cells.** To evaluate whether FasL expression by MF tumor cells influenced or was associated with CD8+ CTLs in MF lesions, we counted the number of CD8+ infiltrating cells in predetermined FasL-expressing versus non-FasL-expressing areas in lesions from seven MF patients. Three of 10 patient samples used above were excluded because they had either strong staining (patients 3 and 13) or weak staining (patient 8) of FasL over the entire infiltrate. As shown in Fig. 4, C and D, we observed that the CD8+ infiltrating lymphocytes were most commonly distributed at the periphery of FasL+-expressing tumor cell aggregates, and only a few single CD8+ lymphocytes were found inside areas of CD45RO+ cell infiltrates. CD8+ infiltrating cell numbers were lowest in areas in which FasL-expressing lymphocytes were most abundant compared with areas that did not contain CD45RO+ cells in the same MF lesion sections. As shown in Table 4, mean numbers of CD8+ lymphocytes were significantly less (a 7.41-fold decrease) in areas of high expression of FasL on CD45RO+ cells as compared with areas in which the expression of FasL was low (P < 0.02; n = 7; range,
In fact, the absolute numbers of CD8+ /H11001/ H11001/ cells within non-FasL-expressing areas of the MF lesions (Fig. 7, TUNEL, regardless of whether they were in FasL-expressing or TUNEL (Fig. 7, blestaining also showed some of CD45RO quantitative analysis or comparison. CD45RO/TUNEL dou-

Areas of dermal infiltrates that were high-FasL-expressing areas contained significantly fewer CD8+ T cells than did areas lacking high FasL-expression. In addition, CD8+ T cells in these areas of high FasL were undergoing apoptosis, as determined by a TUNEL assay. The significance of these data are that MF CD45RO+ tumor cells may escape immune recognition and elimination by FasL-induced apoptosis of CD8+ TILs.

Seven of 10 patients that were selected for high-Fas-L-expressing lesions, had late-stage disease, suggesting that this mechanism may play important roles in advanced MF patients. They support the previous correlation between loss of CD8+ TILs and disease progression, stage, and response to immunotherapy, i.e., photopheresis (15, 16).

CTCL represents a unique tumor model because both the neoplastic and the reactive cells are T lymphocytes. In the case of MF studied here, the tumor cells are clonal, expressing one type of variable regions of the β chain (Vb) of the T-cell receptor, CD4 and CD45RO (memory) protein. These cells are epidermotropic, based on the expression of various molecules including chemokines and their receptors, especially IFN-inducible protein-10 (IP-10) and T cell chemokine receptor CXCR3 (38), and CTL-associated antigen-4 (CTLA-4; Ref. 30). Although in several human cancer models (22–26) tumor cells expressing FasL can destroy TILs by the Fas/FasL pathway, this possibility has not been previously investigated in CTCLs. We have shown for the first time that tumor cells expressing CD45RO coexpress FasL in MF lesions, and that three CTCL cell lines express membrane FasL in vitro. We hypothesize that FasL-bearing neoplastic T cells may be capable of killing CD8+ cytotoxic T cells via Fas/FasL-mediated apoptosis in MF lesions, based on our finding of high apoptotic labeling within lesional CD8+ cells.

Colocalization of TUNEL and CD8+ in MF Dermal Lymphocytes. To further investigate the status of CD8+ T-cell infiltrates observed in the areas of high versus low FasL-expression in MF lesions, we performed CD8/TUNEL doublestaining. The majority of CD8+ T cells were labeled by the TUNEL, regardless of whether they were in FasL-expressing or non-FasL-expressing areas of the MF lesions (Fig. 7, C and D). In fact, the absolute numbers of CD8+ TUNEL+ cells within areas of high FasL expression in MF lesions were too low for quantitative analysis or comparison. CD45RO/TUNEL doublestaining also showed some of CD45RO+ cells labeled by the TUNEL (Fig. 7, A and B).

**DISCUSSION**

We demonstrate that MF tumor cells in skin lesions from all stages of the disease express FasL, and confirm tumor expression of membrane-bound FasL in CTCL lines. Both lymphocytes and keratinocytes appear to have a high-apoptotic rate in normal and lesional MF skin biopsy specimens, based on TUNEL staining. The high rate of keratinocyte apoptosis in MF normal and lesional skin compared with control NS could explain why epidermal atrophy is common in MF patients’ skin. The high rate of apoptosis in normal uninvolved skin appears to be unique for MF, because normal apoptosis was found in the uninvolved skin of patients with hyperproliferative skin diseases including Darier’s disease, pityriasis rubra pilaris, and psoriasis (37). The most important finding was an inverse correlation between the distribution of FasL+ and CD8+ cells within MF lesions. Areas of dermal infiltrates that were high-FasL-expressing areas contained significantly fewer CD8+ T cells than did areas lacking high FasL-expression. In addition, CD8+ T cells in these areas of high FasL were undergoing apoptosis, as determined by a TUNEL assay. The significance of these data are that MF CD45RO+ tumor cells may escape immune recognition and elimination by FasL-induced apoptosis of CD8+ TILs. Seven of 10 patients that were selected for high-Fas-L-expressing lesions, had late-stage disease, suggesting that this mechanism may play important roles in advanced MF patients. They support the previous correlation between loss of CD8+ TILs and disease progression, stage, and response to immunotherapy, i.e., photopheresis (15, 16).

CTCL represents a unique tumor model because both the neoplastic and the reactive cells are T lymphocytes. In the case of MF studied here, the tumor cells are clonal, expressing one type of variable regions of the β chain (Vb) of the T-cell receptor, CD4 and CD45RO (memory) protein. These cells are epidermotropic, based on the expression of various molecules including chemokines and their receptors, especially IFN-inducible protein-10 (IP-10) and T cell chemokine receptor CXCR3 (38), and CTL-associated antigen-4 (CTLA-4; Ref. 30). Although in several human cancer models (22–26) tumor cells expressing FasL can destroy TILs by the Fas/FasL pathway, this possibility has not been previously investigated in CTCLs. We have shown for the first time that tumor cells expressing CD45RO coexpress FasL in MF lesions, and that three CTCL cell lines express membrane FasL in vitro. We hypothesize that FasL-bearing neoplastic T cells may be capable of killing CD8+ cytotoxic T cells via Fas/FasL-mediated apoptosis in MF lesions, based on our finding of high apoptotic labeling within lesional CD8+ cells.

Colocalization of TUNEL and CD8+ in MF Dermal Lymphocytes. To further investigate the status of CD8+ T-cell infiltrates observed in the areas of high versus low FasL-expression in MF lesions, we performed CD8/TUNEL doublestaining. The majority of CD8+ T cells were labeled by the TUNEL, regardless of whether they were in FasL-expressing or non-FasL-expressing areas of the MF lesions (Fig. 7, C and D). In fact, the absolute numbers of CD8+ TUNEL+ cells within areas of high FasL expression in MF lesions were too low for quantitative analysis or comparison. CD45RO/TUNEL doublestaining also showed some of CD45RO+ cells labeled by the TUNEL (Fig. 7, A and B).

**DISCUSSION**

We demonstrate that MF tumor cells in skin lesions from all stages of the disease express FasL, and confirm tumor expression of membrane-bound FasL in CTCL lines. Both
CTCLs are reported to have reduced apoptosis rates (43). The recent study by Zoi-Toli et al. (28) demonstrated the loss of Fas expression in aggressive types of CTCLs but not in indolent types of CTCLs.

Tateyama et al. (44) reported that CD4+ T lymphocytes are primed to express FasL by CD4 cross-linking and to contribute to CD8+ T-cell apoptosis via Fas/FasL death-signaling pathway. Piazza also verified that activated CD4+ helper T-
MF patients with earlier-skin-stage (T1,2) disease tended to have sion characterize disease progression in CTCL patients (17, 18). CD8 membrane-bound form. These findings suggest that MF tumor cells that FasL expressed by CTCL cell lines is mainly the mem-
brane-bound form of FasL. Cellular FasL was higher in all three of the CTCL lines than in control Jurkat T cells.

cells were able to mediate apoptotic events in Fas-positive CD8+ T cells (45). In the present study, we studied the possibility that expression of FasL by MF tumor cells could decrease CD8+ T cells by quantitating CD8+ T-cell number in FasL-expressing versus non-FasL-expressing areas of MF lesions. There was a 7-fold reduction in CD8-positive CTLs in areas in which FasL+ tumor cells were abundant compared with non-FasL-expressing areas in identical sections. Direction interactions between CD4+ and CD8+ T cells are necessary to mediate apoptotic signal in CD8+ T cells (45). We have shown that FasL expressed by CTCL cell lines is mainly the mem-
brane-bound form. These findings suggest that MF tumor cells in vivo express FasL, which in turn enhances apoptosis of CD8+ T cells, resulting in their depletion over time.

Loss of cellular immunity and increased immunosuppression characterize disease progression in CTCL patients (17, 18). MF patients with earlier-skin-stage (T1,2) disease tended to have a higher proportion of CD8+ cells in their skin biopsy specimens, compared with patients with more advanced disease stages (14). We also found that a higher percentage of MF infiltrates expressed FasL in later stages of MF than in earlier stages of the disease. Hence, loss of CD8+ CTLs may occur via FasL mechanisms, leading to disease progression. Nevertheless, CD8+ cells undergoing apoptosis were also found in areas that lacked high FasL expression, which suggested that there may be other mechanisms by which TILs are lost in CTCLs. Of interest, a longitudinal study by Terheyden et al. (46), analyzing primary melanomas and subsequent metastases, showed that higher numbers were positive for FasL among metastatic melanoma than among primary melanomas.

On the other hand, it has been demonstrated that most TILs are not functional. This has been attributed to inhibition by tumor-derived immunosuppressive factors, such as transforming growth factor β, gangliosides, nitric oxide, IL-10, and FasL (47). transforming growth factor β inhibits the expression of pore-forming protein gene in human peripheral blood CD8+ T lymphocytes (48). The development of immunodeficiency in MF has been explained by a shift toward Th2 cytokine production observed in the peripheral blood and skin lesions of patients with CTCL (17, 19, 49). Functional and molecular assays have demonstrated a cytokine profile in CTCL patterns similar to that produced by the Th2-type helper T cells (50). We also suggest that FasL may serve as yet another immunosuppressive factor, a potent mediator of immunological tolerance and privilege in the context of MF. Additional studies are now under way to confirm and to elucidate this mechanism.

FasL also plays a role in mediating immune privileged sites, in contributing to immunological tolerance in the periphery, in down-sizing immune responses, and in supporting allograft survival (50). All of these involve FasL-mediated apoptotic depletion of leukocytes. A recent study reports that FasL gene transfer prolongs rat renal allograft survival and down-regulates antiapoptotic Bcl-2-associated athanogene (Bag-1) in parallel with enhanced Th2-type cytokine expression (51). This role is contradicted by the finding that, in certain cases, murine allografts, genetically manipulated to express FasL, caused neu-
trphil infiltration and allograft destruction (52). Neutrophilic infiltrates are never a feature of MF lesions. Of interest, adenovirus-mediated overexpression of FasL in mouse ankle joints ameliorated collagen-induced arthritis, thus providing powerful evidence for an anti-inflammatory role for FasL in vivo (53). Why FasL transfection could have such contradictory effects in experimental models is not yet clear (54).

It has been reported that cyclosporin A (CsA) treatment induces an inhibition of FasL mRNA expression in T cells (55) as well as inhibiting the ability of CD4+ T cells to kill CD8+ target cells (45). Cyclosporin A administration has been associated both with progression to more aggressive lymphomas (56) and with remission of MF (57). Thus, understanding the significance of the Fas counterattack as a mechanism of immune evasion in CTCL should facilitate development of more effective therapy for CTCL that does not further contribute to the destruction of the remaining tumor cytotoxicity. Fas sensitization of tumor cells or prote-
ction of CD8+ T cells from FasL could represent promising targets for future therapeutic strategies.

Table 4  Reduction of CD8+ TILs in FasL-expressing versus non-FasL-expressing areas of MF lesions

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>FasL-expressing areaa (CD8+ TILs %)</th>
<th>Non-FasL-expressing areaa (CD8+ TILs %)</th>
<th>(3) = (2)/(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.20</td>
<td>10.5</td>
<td>4.77</td>
</tr>
<tr>
<td>10</td>
<td>1.28</td>
<td>15.7</td>
<td>12.33</td>
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<tr>
<td>15</td>
<td>1.54</td>
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</tr>
<tr>
<td>19</td>
<td>1.43</td>
<td>10.0</td>
<td>6.99</td>
</tr>
<tr>
<td>20</td>
<td>1.41</td>
<td>12.5</td>
<td>8.90</td>
</tr>
<tr>
<td>21</td>
<td>3.66</td>
<td>20.33</td>
<td>5.55</td>
</tr>
</tbody>
</table>

Mean ± SD 2.01 ± 0.86 13.53 ± 3.54 7.41 ± 2.67

a Areas stained with strong and moderate intensity of FasL (+++++) were defined as FasL-expressing.

b Areas stained with negative or weak intensity (+ + +) were defined as non-FasL-expressing areas.

![Fig. 6](image-url) Cellular FasL protein is highly expressed in CTCL cell lines. Soluble and cellular (membrane-bound) FasL in supernatants and lysates of CTCL cell lines (HH, MJ, and HUT78) and Jurkat T cell lines were measured by a sandwich ELISA. The level of expression of cellular FasL was higher than of soluble FasL in all three of the CTCL cell lines (n = 3; t, 1.976; P > 0.05 paired t test). HUT78 had the highest expression of both forms of FasL, especially the membrane bound-form of FasL. Cellular FasL, was higher in all three of the CTCL lines than in control Jurkat T cells.
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REFERENCES


Fig. 7 Colocalization of TUNEL and CD8+ in MF lesions. CD45RO or CD8 antigen was first labeled (blue) by immuno-alkaline phosphatase on paraffin-embedded MF lesional sections followed by in situ TUNEL (brown-red). In A, CD45RO/TUNEL doublestaining showed that keratinocytes in MF epidermis from patient 20 have single TUNEL-positive nuclear staining (brown-red, inset). Blue, in MF dermis, CD45RO single-positive cells; brown-red, nuclear staining, and blue cytoplasmic/cell surface staining (×100). CD45RO/TUNEL dual-positive cells. In B, higher magnification showed that the large atypical single CD45RO+ cells were more common than CD45RO+/TUNEL+ cells in the upper dermal infiltrates (×400). In C, in a sequential section of same lesion, a high proportion of keratinocytes with single TUNEL-positive nuclear staining are present in epidermis. The majority of dermal CD8+ cells were positive by TUNEL (×100). D, arrow, at a high magnification, condensation of TUNEL-positive nuclei are seen against the blue, CD8+ cytoplasmic/cell surface background. ×400.


Fas Ligand Expression by Neoplastic T Lymphocytes Mediates Elimination of CD8+ Cytotoxic T Lymphocytes in Mycosis Fungoides: A Potential Mechanism of Tumor Immune Escape?

Xiao Ni, Parul Hazarika, Chunlei Zhang, et al.


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