Fas Ligand Expression in Metastatic Renal Cell Carcinoma During Interleukin-2 Based Immunotherapy: No In vivo Effect of Fas Ligand Tumor Counterattack

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ABSTRACT

Purpose: It has been hypothesized that tumor cells expressing Fas ligand (FasL) might be able to counterattack and neutralize tumor-infiltrating lymphocytes. We assessed the effect of FasL tumor counterattack on the clinical outcome of interleukin-2 (IL-2)–based immunotherapy in metastatic renal cell carcinoma.

Experimental Design: Tumor core needle biopsies were obtained before IL-2–based immunotherapy in 86 patients and repeated within the first cycle in 57 patients. Tumor cells expressing FasL and intratumoral lymphocyte subsets expressing CD4, CD8, CD56, and CD57 were analyzed by immunohistochemistry.

Results: At baseline, negative FasL staining in tumor cells was seen in 10 of 86 (12%) biopsies, whereas intense FasL staining was seen (a) in fewer than 10% of tumor cells in 26 (30%) biopsies; (b) in 11 to 50% of tumor cells in 25 (29%) biopsies; (c) in 51 to 90% of tumor cells in 18 (21%) biopsies; and (d) in >90% of tumor cells in 7 (8%) biopsies. On treatment, tumor FasL expression did not change from baseline levels. Moreover, tumor FasL expression was not correlated with objective response or survival whereas the absolute number of CD4+, CD8+, CD56+, and CD57+ cells per mm² tumor tissue at baseline was significantly higher in responding patients compared with nonresponding patients (P = 0.01, P = 0.008, P = 0.015, and P < 0.001, respectively). During the first course of immunotherapy, the absolute number of CD4+, CD8+, and CD57+ cells per mm² tumor tissue was significantly higher in responding patients compared with nonresponding patients (P = 0.034, P < 0.001, and P < 0.001, respectively). However, no correlation was observed between the number of intratumoral lymphocytes and tumor FasL expression level.

Conclusion: These observations do not support the hypothesis that FasL tumor “counterattack” has an effect on the clinical outcome in metastatic renal cell carcinoma during IL-2–based immunotherapy.

INTRODUCTION

The explanation for the low levels of responses seen after interleukin-2 (IL-2)– and interferon-α (IFN-α)–based immunotherapy is probably extremely complex. However, the hypothesis that tumors might create a zone of immune “privilege” by expressing Fas ligand (FasL, CD95L) and counterattack tumor-infiltrating lymphocytes by delivering apoptotic death signals may represent one of several factors of importance for treatment failure.

FasL, cloned in 1993, was initially thought to be expressed in immune cells only, including activated T-lymphocytes and natural killer cells, and to play a key role in cytotoxicity and immune homeostasis. However, FasL expression in the eye and placenta was noted, and thus the concept of immune privilege was established (1).

Furthermore, studies subsequently reported the expression of FasL by human malignancies, including melanoma (2, 3), astrocytomas (4) and colorectal (5), esophageal (6), lung (7), ovarian (8), head and neck (9), and pancreatic carcinoma (10). Although controversial (11–15), these studies led to the hypothesis that tumor tissues could represent sites of immune privilege that enable cancers to “counterattack” the immune system (16, 17).

In 1999, Uzzo et al. (18) provided the first evidence that FasL was expressed in renal cell carcinoma cell lines and renal tumor tissue, and they also suggested that FasL expression was functional. The observation that FasL is expressed in renal cell carcinoma has subsequently been confirmed by others (19–25).

Metastatic renal cell carcinoma (mRCC) is a treatment-resistant disease, that, however, is responsive to IL-2– and IFN-α–based immunotherapy. The antineoplastic actions of IL-2 and IFN-α are associated with infiltration of immune effector cells within the tumor tissue (26, 27). However, functional interaction occurring in vivo between tumor cells expressing FasL and tumor-infiltrating lymphocytes during IL-2–based immunotherapy has not been investigated previously. Thus, in the present...
study, we have assessed tumor cells expressing FasL and tumor-infiltrating lymphocyte subsets in situ in repeated tumor tissue core needle biopsies obtained at baseline and within the first cycle of IL-2–based immunotherapy in patients with mRCC, and correlated the findings with the clinical outcome.

MATERIALS AND METHODS

Patients and Response Evaluation. A total of 120 consecutive single-institution patients with inoperable histologically confirmed mRCC were treated on an outpatient basis with IL-2–based immunotherapy from February 1999 to August 2002. Twenty-six of these patients were enrolled in a multicenter prospective phase II trial of IL-2, IFN-α, and histamine dihydrochloride (28). A further 23 patients received standard treatment with the same schedule but without histamine (26). Sixty-three patients were enrolled in a randomized phase II trial of IL-2 ± histamine dihydrochloride, and the final eight patients were treated with IL-2 alone. The local ethical committee and the Danish Medical Agency approved the studies.

For the interferon-containing regimens, the treatment plan consisted of 1 priming-week of daily IFN-α and up to 9 treatment cycles of 4 weeks with IFN-α (human leukocyte IFN-α, Interferon Alfanative, BioNative, Umeå, Sweden, for the phase II trial, otherwise Introna, Schering-Plough, Farum, Denmark) 3.0 million IU as a fixed dose subcutaneously once daily, 7 days per week; IL-2 (Aldesleukin, rIL-2, Proleukin, Chiron, Amsterdam-Zuidoost, the Netherlands) 2.4 million IU/m² subcutaneously twice daily, 5 days per week twice, weeks 1 and 2 every cycle; and histamine dihydrochloride (Ceplene, Maxim Pharmaceuticals Inc, San Diego, CA) 1.0 mg in 1.0 mL by a 20-minute slow subcutaneous injection, twice daily, 5 days per week throughout the study. Patients were evaluated for objective response every 3 cycles.

For the noninterferon-containing regimens, the treatment plan consisted of up to 4 treatment cycles of 5 weeks with IL-2, 18 million IU as a fixed dose subcutaneously once daily, 5 days per week for 3 weeks followed by 2 weeks rest. In case of randomization, 1.0 mg of histamine dihydrochloride was added twice daily, concomitantly with IL-2. Patients were evaluated for objective response every 2 cycles.

Objective response was defined according to standard World Health Organization criteria: (a) complete response (CR), defined as total disappearance of all clinical disease; (b) partial response (PR), defined as a reduction of >50% in the bidimensional product diameter; (c) stable disease (SD), defined as a reduction of <50% or an increase in size of <25%; and (d) progressive disease (PD), defined as an increase in size of >25% in the bidimensional product diameter.

Collection of Samples. Core needle biopsies (18G cutting needle) were collected by standard ultrasound-guided procedures at baseline and before the start of week 3 (non-IFN-containing schedules) or week 5 (IFN-containing schedules). These time points for repeated biopsies were selected to coincide with routine outpatient clinical visits, according to the immunotherapy schedule. For the present study analysis, week 3 and week 5 biopsies were classified as on-treatment biopsies. The local ethical committee had approved the biopsy study. Of the 120 consecutive patients, written informed consent and baseline biopsies were obtained from 101 patients. Four patients did not complete one course of therapy because of toxicity and were not evaluable for objective response. Two patients had only fine needle biopsies done. These six patients were excluded from all further analyses. Thus, 95 patients were included in the study (Table 1). Three patients had biopsies containing necrotic tissue only and six patients had biopsies with insufficient tumor tissue. Thus at baseline, 86 patients had evaluable biopsies. On-treatment biopsies were only evaluable in 57 of the 95 patients for the following reasons: (a) 21 patients refused repeated biopsies; (b) 8 had biopsies with insufficient tumor tissue; (c) 5 had biopsies showing necrosis only and (d) 4 had no biopsies done for safety reasons. On the basis of well-known prognostic factors of Memorial Sloan Kettering Cancer Center (29), there were no significant differences between the 95-patient group and the 86-patient group or the 57-patient group, \( P = 0.24 \) and \( P = 0.27 \), respectively, Fisher’s exact test.

Biopsies were done from accessible tumor locations: kidney, \( n = 68 \); abdominal soft tissue, \( n = 16 \); liver, \( n = 17 \); pleura/nerve wall, \( n = 10 \); lymph node, \( n = 9 \); muscle, \( n = 5 \); kidney bed, \( n = 4 \); subcutis, \( n = 5 \); and lung, \( n = 3 \) (some patients had biopsies from more than one location). No complete responding patients had accessible tumors for core needle biopsies. On-treatment biopsy was obtained from the same tumor as the baseline biopsy.

**Immunohistochemistry.** Sections (2 μm) of formalin-fixed, paraffin-embedded biopsies were mounted on ChemMate slides (S2024, DakoCytomation, Glostrup, Denmark), dried for 1 hour at 60°C, deparaffinized, and rehydrated. After endogenous peroxidase blocking (0.5% hydrogen peroxidase in water for 30 minutes), antigens were retrieved by microwave oven

<table>
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<th>Table 1 Patient characteristics (N = 95)</th>
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<td>Median age, years (range)</td>
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<td>Male</td>
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<td>Karnofsky performance status</td>
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<td>100</td>
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<td>90</td>
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* Memorial Sloan-Kettering Cancer Center (29)
heating [3 × 5 minutes at 850 W in Tris/EGTA retrieval buffer (pH 9.0)]. The tissue sections were incubated for 1 hour with the following antibodies: anti-FasL (F 37720, clone 33 (Transduction Laboratories, Newington, NH), diluted 1:40; and G247-4 (PharMingen San Diego, CA), diluted 1:10, 1:25, and 1:50 with and without the copper signal enhancement; anti-CD4 (NCL-CD4–1F6, 1:50, NovoCastra); anti-CD8 (M7103, 1:100, DakoCytomation); anti-CD56 (NCL-CD56–B16, 1:40, NovoCastra) and anti-CD57 (33251A, 1:500, PharMingen). Sections were then incubated for 30 minutes with peroxidase-conjugated EnVision (K4000, DakoCytomation). Staining was visualized with 3,3'-diaminobenzidine solution. Sections were counterstained in Mayer’s hematoxylin and mounted with Aquetex (64912–50, Struers KEBO Lab, Albertslund, Denmark). All staining was done in a TechMate automatic immunohistochemistry staining machine (DakoCytomation). Sections from normal tonsil, normal kidney, and from various kidney tumors were used as positive controls. Negative controls consisted of either relevant isotype controls from PharMingen and DakoCytomation or stains in which the primary antibody was omitted from the reaction.

Immunohistochemical Evaluation. Tumor cell FasL staining was assessed in a semiquantitative fashion, incorporating both the intensity and distribution of specific staining: (0) no intensely stained cells; (1+) 1 to 10% intensely stained cells, almost all tumor cells positive; (2+) 11 to 50% intensely stained cells, almost all tumor cells positive; (3+) 51 to 90% intensely stained cells, almost all tumor cells positive; (4+) > 90% intensely stained cells, almost all tumor cells positive. Tumor cells were identified based on morphology. Semiquantitative scoring was assessed blinded by two observers (F. D. and N. M.). Intratumoral immune cells were enumerated by a stereological method (30). In short, this was done with a morphometric system consisting of an Olympus AH-3 microscope with a motorized stage that was controlled by a computer for manual interactive counting on the computer screen. The software used was CAST-grid version 2.0, developed by Olympus (Albertslund, Denmark). Each microscopic field of vision was projected onto the computer screen with a video camera, and the computer generated an unbiased counting frame in which the measurements were done. On the projected image of the section, the tumor area was encircled. Necrosis, artifacts and fibrous areas were omitted. The first field of vision was chosen at random, after which the computer sampled systematically the following fields of vision within the entire encircled area. Using a ×40 objective, we counted a total number of 40 fields (4,951 μm² each), if the size of the tumor allowed for it. The entire core needle biopsy was assessed. Only a cell with staining restricted to the plasma membrane, a visible nucleus, and located within the counting frame was counted as positive. The mean number of cells per mm² tumor tissue was assessed for each patient. Staining was analyzed blinded by one observer. Selected sections were counted blinded by a senior histopathologist (N. M.) and a high level of reproducibility was found, as reported previously (26).

Statistics. Overall survival time was measured from first day of treatment until death or last follow-up evaluation. We evaluated the relationship between assessed variables and objective response using the nonparametric Mann-Whitney U test. The Spearman correlation test was used to evaluate the relationship between tumor FasL expression and intratumoral lymphocyte subsets. The Wilcoxon signed rank test for paired samples was used to assess the significance of changes from baseline to week 5. We evaluated the relationship between assessed variables and survival using the Kaplan-Meier method and the log-rank test. Dichotomy of the patients was done at the median value for each evaluated variable. The median follow-up period was 30.2 months (range 18–61 months). No patients were lost to follow-up. Data were updated March 11, 2004. SPSS v11.0 (SPSS, Chicago, IL) was used to do statistical analyses.

RESULTS

Clinical Treatment Results. A total of 95 patients treated with IL-2–based immunotherapy were evaluable for consecutive tumor biopsies. Table 1 lists patient characteristics. Of these, seven patients achieved PR, thirty-five patients achieved SD and 53 patients had PD. Median survival was 13.3 months (range 0.5–60.9 + months). Four patients (3 with PR and 1 with SD) had no evidence of disease (NED) and were alive at 44+, 53+, 59+, and 61 + months, respectively, after immunotherapy followed by subsequent resection of residual tumor.

Correlation between Tumor FasL Staining and Objective Response. Baseline and on-treatment tumor FasL expression was evaluated and correlated with objective response. We could not find detectable levels of FasL using the monoclonal G247-4 antibody. However, with the clone 33 antibody, tumor cells stained positive for FasL with a cytoplasmatic, often granular, perinuclear reaction. At baseline, FasL tumor staining was negative (0+) in 10 of 86 (12%) biopsies; with <10% intensely stained tumor cells (1+) in 26 of 86 (30%) biopsies; with 11 to 50% intensely stained cells (2+) in 25 of 86 (29%) biopsies; with 51 to 90% intensely stained cells (3+) in 18 of 86 (21%) biopsies; and with >90% intensely stained cells (4+) in 7 of 86 (8%) biopsies (Fig. 1). On-treatment, tumor FasL expression did not change from baseline (P = 0.31). When FasL staining of responding (PR) and nonresponding (SD+PD) patients at baseline or on-treatment were compared, no significant differences were noted (P = 0.46 and P = 0.16, respectively). Patients with NED did not show any significant difference in FasL staining compared with non-NED patients at baseline or on-treatment (P = 0.9 and P = 0.4, respectively). In this group of four patients, we also analyzed FasL expression in the resected “residual” tumors. However, there were no significant differences in FasL staining in the residual tumors compared with baseline staining.

Correlation between Tumor FasL Staining and Survival. Baseline and on-treatment tumor FasL expression were examined and compared with survival. However, no statistically significant correlations with survival were shown (log-rank P = 0.89 and 0.64, respectively).

Correlation between Tumor-Infiltrating Lymphocytes and Objective Response. Baseline and on-treatment intratumoral lymphocyte subsets defined by immunohistochemistry were evaluated and correlated with objective response. At baseline, the absolute number of CD4, CD8, CD56, and CD57 cells/ mm² tumor tissue was significantly higher in responding patients (PR) compared with nonresponding patients (SD+PD);
During the first course of immunotherapy, the absolute number of CD4, CD8, and CD57 cells per mm² tumor tissue was significantly higher in responding patients compared with nonresponding patients ($P = 0.01$, $P = 0.008$, $P = 0.015$, and $P < 0.001$, respectively; Fig. 1). During the first course of immunotherapy, the absolute number of lymphocyte subsets per mm² tumor tissue in individual patients for A, CD4⁺; B, CD8⁺; C, CD56⁺; and D, CD57⁺. E, tumor cell FasL staining with no correlation to response, assessed in a semiquantitative fashion: 0, no intensely stained cells; 1+, 1 to 10% intensely stained cells; 2+, 11 to 50% intensely stained cells; 3+, 51 to 90% intensely stained cells; 4+, > 90% intensely stained cells. Only patients with a complete set of two data points are displayed in Fig. 1.

Correlation between Tumor-Infiltrating Lymphocytes and Tumor Cells Expressing FasL. Correlation between baseline or on-treatment tumor-infiltrating CD4, CD8, CD56, or CD57 lymphocyte subsets and FasL expressing tumor cells were evaluated by use of the Spearman's correlation test. However, no statistically significant correlations were found (data not shown).
**DISCUSSION**

This is to our knowledge the first *in vivo* serial assessment of tumor tissue FasL expression during IL-2–based immunotherapy. Although the explanation for the low level of responses seen in IL-2– and IFN-α–based immunotherapy is probably extremely complex, the hypothesis that tumor cells expressing FasL may escape the immune response and counterattack tumor-infiltrating lymphocytes by delivering apoptotic death signals has intuitive appeal. However, this hypothesis is not supported by our data. We showed that tumor FasL expression was not correlated with objective response or survival, whereas high numbers of intratumoral lymphocyte subsets at both baseline and during treatment were significantly positively correlated with objective response. Moreover, no correlation, neither negative nor positive, was observed between the number of intratumoral lymphocytes and tumor FasL expression. Thus, in the tumor milieu during IL-2–based immunotherapy, FasL-mediated counterattack seems to be less important than would be predicted from *in vitro* assays, suggesting that the FasL counterattack hypothesis is of no significance *in vivo*.

After the initial publications in 1996 (5), many types of tumor have been reported to express FasL (2, 4–10). Moreover, FasL expression has been reported to be negatively correlated with prognosis in melanoma (31) and in breast (32), ovarian (33), liver (34), and renal cell carcinoma (RCC; ref. 25). However, the counterattack hypothesis is controversial and has been questioned recently, primarily based on major concerns about laboratory methods and reagents (13, 35) including the use of possible nonspecific antibodies in the analyses (36, 37). A revised hypothesis has been proposed, suggesting that FasL is expressed by T lymphocytes upon activation after tumor cell recognition, causing them to kill themselves (“suicide”) and also each other (“fracticide”; ref. 13). It has also been suggested that melanoma cells express FasL intracellularly confined to lysosomal-like microvesicles and that these FasL-bearing microvesicles can be released extracellularly mediating FasL cell death that does not necessarily imply cell-to-cell contact (3). Nevertheless, in all these models, it is the presence of the tumor that initiates the events culminating in immune cell death (17).

In 1999, Uzzo et al. (18) provided the first evidence that FasL is expressed in RCC cell lines and renal tumor tissues, and they also suggested that FasL was functional. Because of the controversy over the specificity of certain commercially available anti-FasL antibodies (36), Uzzo et al. (18) documented FasL in RCC by multiple techniques. For the immunohistochemical analyses, they used the clone 33 antibody. Therefore, we also used this antibody in the present study. For comparison, we also used the G247-4 antibody (37). However, we were not able to detect levels of FasL using G247-4. Gerharz et al. (21) were also unable to detect FasL using the G247-4 antibody in RCC cell lines that had been shown to be positive for FasL expression by RT-PCR. Only after immunoprecipitation before Western blotting, G247-4 antibodies revealed expression of FasL in RCC cells (21). Therefore, they concluded that tumor FasL was present, but only weakly in RCC, and that its detection depended on the sensitivity of the analytical methods used (21).

Although the ultimate test of a molecules role in disease pathology is whether its expression correlates with the patients outcome, we assessed the role of tumor FasL expression *in vivo* during “extreme conditions,” (i.e., during manipulation of the immune system by IL-2 and IFN-α to induce tumor regression). Thus, objective response was selected as end point whereas tumor shrinkage seems a more valid end point than numbers of apoptotic tumor-infiltrating lymphocytes. Moreover, this end point better reflects the “attack-counterattack” model used. However, independently of tumor FasL expression levels, we observed *in situ* both at baseline and during the first course of IL-2–based immunotherapy, a significantly higher number of tumor-infiltrating lymphocytes in patients who subsequently achieved response compared with nonresponding patients. This observation challenges the FasL tumor “counterattack” hypothesis during IL-2–based immunotherapy.

Indeed, immune privilege cannot be associated with a single protective FasL-dependent mechanism but rather involves an intricate orchestration of different processes (38). Moreover, it has been shown that tumor specific CD4+ and CD8+ T cells that are isolated from melanoma lesions seem to be resistant to tumor FasL expression, and thus they have developed strategies for overcoming FasL escape mechanisms (39). This indicates that the role of FasL in the homeostatic regulation of immune responses seems to be much more complex than initially thought. A direct impact on tumor FasL expression for IL-2, IFN-α, or histamine has, to the best of our knowledge, not been reported.

Repeated attempts have been made to identify variables important for objective response and survival in patients with mRCC undergoing IL-2–based immunotherapy to select those patients most likely to benefit from treatment. However, our data suggests that the fate of a patient with mRCC before IL-2– and IFN-α–based immunotherapy cannot be determined by measuring tumor FasL expression. Either mRCC tumor FasL expression *in vivo* is not functional or tumor-infiltrating lymphocytes have developed strategies for overcoming this escape mechanism, thus supporting the development of immunotherapeutic strategies for the treatment of mRCC.

In summary, we provide the first quantitative analysis of the clinical relevance of tumor FasL expression in mRCC at baseline and during IL-2–based immunotherapy. Our observations do not support the hypothesis that FasL tumor “counterattack” has an effect on the clinical outcome in mRCC during IL-2–based immunotherapy.

**ACKNOWLEDGMENTS**

Thanks go to Karin Vestergaard for cutting sections and to Tom Nordfeld for help with immunohistochemistry. Staff members at the Department of Oncology are acknowledged for careful management of the patients.

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