Increased Soluble CD95 (sFas/CD95) Serum Level Correlates with Poor Prognosis in Melanoma Patients

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ABSTRACT
Functional impairment of the Fas/CD95 receptor-ligand system is associated with the development and progression of malignancies. One possible cause might be the inhibition of the formation of a functional Fas/CD95-FasL complex by soluble Fas/CD95 molecules (sFas/CD95). In the present study we determined sFas/CD95 serum concentration in 125 melanoma patients of different clinical stages of disease compared with 30 healthy controls using an ELISA. sFas/CD95 serum level was significantly elevated (P < 0.0005) in melanoma patients (mean ± SE = 8.60 ± 0.26 ng/ml) compared with healthy controls (mean ± SE = 6.27 ± 0.25 ng/ml). Univariate analysis revealed a correlation of sFas/CD95 serum concentration with advanced stages of disease (P = 0.009). Only a slight increase in sFas/CD95 serum level (P = 0.057) could be observed in regard to the tumor burden. Patients undergoing current treatment with cytostatics (n = 18) revealed a strong increase in sFas/CD95 serum level (P < 0.0005), whereas treatment with IFN-α alone or combined with cytostatics (n = 19) showed no change in serum sFas/CD95 concentration. According to univariate analysis, elevated sFas/CD95 serum levels were associated with a poor overall (P < 0.005) and a progression-free (P < 0.0005) survival. Multivariate analysis revealed sFas/CD95 serum concentration as an independent predictive factor for progression-free (P = 0.011), but not overall (P = 0.078), survival. Our results show a prognostic relevance of serum sFas/CD95 in melanoma patients, indicating that the evaluation of sFas/CD95 serum level may be important for the selection of therapeutic strategies.

INTRODUCTION
The membrane-bound type I protein Fas/CD95 and its ligand, FasL, play a key role in maintaining tissue homeostasis via induction of apoptosis (1). Besides regulation of lymphatic cells and tissues providing immunotolerance, limiting clonal expansion, and maintaining immunoprivileged sites of the organism (2–3), the Fas/CD95-FasL system has recently been described to exert important functions in the control of malignant proliferation (4). It could be shown in various studies, that Fas/CD95-mediated apoptosis can be impaired or abrogated in neoplastic cells of different origins because of the down-regulation, total loss, or dysfunctional signaling of Fas/CD95 (5, 6). Furthermore, the Fas/CD95-FasL system has recently been shown to suppress metastatic progression in mice (7). As known from other cell-surface receptors, soluble isoforms of Fas/CD95 (sFas/CD95) lacking parts of the transmembrane domain, attributable to alternative splicing, could be detected (8–10). These soluble molecules were shown to specifically inhibit the Fas receptor-ligand binding in the extracellular space (8), which enables them to impair the homeostatic regulation of immune responses. Indeed, sFas/CD95 was found to be elevated in serum from patients suffering from autoimmune disorders or hematopoietic malignancies compared with healthy controls (11–13). Additional investigations revealed increased sFas/CD95 serum levels in patients with nonhematopoietic malignancies, which was associated with poor prognosis (14–19). These observations, however, could not be confirmed by other groups (20–22), leaving the function and clinical significance of serum sFas/CD95 in patients with malignancies still a matter of controversial debate.

In the present work, we investigated sFas/CD95 serum concentration in malignant melanoma patients regarding its correlation with clinical parameters, such as stage of disease and tumor burden, and its prognostic significance for progression-free and overall survival of patients.

MATERIALS AND METHODS
Patients. After informed consent was obtained, blood was drawn from 125 unselected patients with histologically confirmed malignant melanoma of different stages of disease, presenting at the Department of Dermatology, The Saarland University Hospital, Homburg/Saar, Germany, and from 30 healthy controls matched in age and gender. Patients were enrolled in this study between September 1997 and November 1998. Clinical staging of patients was performed according to the criteria of the American Joint Committee on Cancer (23). Follow-up was performed in at least 3-month intervals, including physical examination, X-ray of the chest, ultrasound of the abdomen and lymph nodes, and blood chemistry. Patients in advanced stages of disease also underwent computed tomography of the brain and scintigraphy of the skeleton. Patients enrolled in the study before surgical removal of the primary tumor and/or with positive detection of metastatic tumor mass by the diagnostic methods mentioned above were scored as patients with detectable tumor mass; all other patients were scored as tumor-free. Patients were treated according to therapy
protocols of the Dermatological Cooperative Oncology Group, including cytostatic (dacarbazine, cisplatinum, temozolomide, and vincristine) and immunomodulatory (IFN-α) agents in different combinations and schedules. Patients who were enrolled in the study 3 days to 4 weeks after application of therapeutic agents were scored as patients under ongoing therapy. Patients who received no systemic therapy until inclusion in the study or whose last systemic treatment was applied more than 4 weeks previously were scored as untreated patients. Detailed characteristics of the patients enrolled in this study are presented in Table 1.

**Sera**. Sera of healthy controls were kindly provided by the Department of Hematology and Blood Transfusion of the Saarland University Hospital. All controls were blood donors undergoing regular physical and laboratory examinations.

**Serum Analysis.** Sera were harvested by centrifugation at 1500 [time] g and thereafter stored at −70°C until further processing. A commercially available ELISA kit (Quantikine human sFas; R&D Systems, Minneapolis, MN) recognizing recombinant and natural sFas/CD95 was used for quantification of sFas/CD95 according to the manufacturer’s instructions. Briefly, serum samples were diluted 10-fold and thereafter subjected to a polystyrene multiplate precoated with a murine monoclonal antibody directed against Fas. After 2 h of incubation, plates were washed and thereafter incubated with a polyclonal anti-Fas antibody conjugated to horseradish peroxidase. A color reaction by hydrogen peroxide and tetramethylbenzidine was performed for detection of bound antibody and subsequently quantified using a microtiter plate reader (Multiskan MCC/340; Labsystems, Helsinki, Finland). Standard curves were constructed using serial dilutions of recombinant sFas/CD95. The minimum detectable concentration was determined as 20 pg/ml. The intra- and interassay variation was determined as <10%. Each serum sample was tested in duplicate.

**Statistical Methods.** Student’s t test (gender and tumor burden), Bonferroni post hoc test (therapy versus no therapy), ANOVA analysis (patients versus controls), and ANOVA regression analysis (stage of disease) were used for statistical comparisons. The Kolmogorov-Smirnov test revealed sFas/CD95 serum concentration as normally distributed data. A cutoff point was determined for serum sFas/CD95 according to the best discrimination between patients and controls regarding optimal values of sensitivity and specificity using the ROC analysis. The calculated cutoff value was used for all comparative analyses if not otherwise indicated. Probabilities of survival and progression-free survival were analyzed using the Kaplan-Meier method in combination with the log-rank test; end points were death from melanoma and any detectable progress or relapse of melanoma, respectively. Multivariate analysis was performed using Cox’s proportional hazard model and ANOVA analysis. Differences with a P < 0.05 were considered statistically significant. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

### RESULTS

**sFas/CD95 Is Increased in Melanoma Patients.** Sera were obtained from 125 patients diagnosed with malignant melanoma. They included 14 males with a mean age of 52.8 ± 2.8 years and 17 females with a mean age of 51.3 ± 3.3 years in stage I/II (primary melanoma), 19 males with a mean age of 55.7 ± 2.3 years and 27 females with a mean age of 56.1 ± 3.0 years in stage III (regional lymph node and/or in-transit metastases), and 30 males with a mean age of 60.2 ± 1.7 years and 18 females with a mean age of 56.3 ± 2.5 years in stage IV (distant metastases). The healthy controls consisted of 15 males with a mean age of 54 ± 5.3 years and 15 females with a mean age of 52 ± 7.5 years. As shown in Table 1, the mean sFas/CD95 serum concentration was significantly increased in melanoma patients (P < 0.0005) compared with the healthy donors tested. No relationship was observed between serum level of sFas/CD95 and gender (Table 1) or age (data not shown) of the patients tested.

**sFas/CD95 Serum Concentration Correlates with Disease Progression.** Regression analysis using the ANOVA method revealed a positive correlation (P = 0.009) between the sFas/CD95 serum level and the patients’ stage of disease (Table 1). Regarding the tumor burden, univariate analysis revealed a slight increase (P = 0.057) of sFas/CD95 serum concentrations in patients with a detectable tumor load compared with tumor-free patients (Table 1).

**Differential Effects of Therapy Modalities on sFas/CD95 Serum Level.** To study the impact of different treatment modalities on sFas/CD95 serum concentration, the melanoma patients tested were subdivided into three groups: (a) patients currently receiving cytostatic therapy only; (b) patients currently treated with IFN-α alone or in any combination with cytostatics; and (c) patients without any current therapy (Table 1). Patients treated with cytostatics alone showed a strong increase in sFas/CD95 serum concentration (P < 0.0005) compared with untreated patients. In contrast, patients currently

### Table 1 Patient characteristics

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>No. of cases</th>
<th>sFas/CD95 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>30</td>
<td>6.27 ± 0.25</td>
</tr>
<tr>
<td>Patients</td>
<td>125</td>
<td>8.60 ± 0.26</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>63</td>
<td>9.10 ± 0.35</td>
</tr>
<tr>
<td>Female</td>
<td>62</td>
<td>8.09 ± 0.38</td>
</tr>
<tr>
<td>Stage (AJCC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>31</td>
<td>7.61 ± 0.38</td>
</tr>
<tr>
<td>III</td>
<td>46</td>
<td>8.51 ± 0.46</td>
</tr>
<tr>
<td>IV</td>
<td>48</td>
<td>9.32 ± 0.43</td>
</tr>
<tr>
<td>Tumor load</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detectable</td>
<td>47</td>
<td>9.24 ± 0.43</td>
</tr>
<tr>
<td>Not detectable</td>
<td>78</td>
<td>8.21 ± 0.32</td>
</tr>
<tr>
<td>Therapy</td>
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<td></td>
</tr>
<tr>
<td>Cytostatics</td>
<td>18</td>
<td>11.17 ± 0.84</td>
</tr>
<tr>
<td>IFN-α</td>
<td>19</td>
<td>9.04 ± 0.68</td>
</tr>
<tr>
<td>No therapy</td>
<td>88</td>
<td>7.98 ± 0.26</td>
</tr>
</tbody>
</table>

*Data represent mean ± SE.

*P < 0.0005; ANOVA analysis (patients versus controls), Student’s t test (gender, tumor load), Bonferroni post hoc test (therapy versus no therapy), ANOVA regression analysis (stage).

*AJCC, American Joint Committee on Cancer.

*P < 0.05, **P < 0.005.
treated with IFN-\(\alpha\) revealed no significant changes in sFas/CD95 serum level.

**Predictive Value of Serum sFas/CD95 for Progression-free and Overall Survival.** To analyze the clinical consequences of elevated sFas/CD95 serum levels, melanoma patients tested were divided into two categories in regard to their sFas/CD95 serum concentration according to a cutoff value of 7.92 ng/ml obtained by ROC analysis. The stage-dependent percentage of patients showing sFas/CD95 serum levels above the calculated threshold is shown in Fig. 1. Elevated sFas/CD95 serum concentrations were strongly associated with a poor overall (\(P = 0.0047\)) and progression-free (\(P = 0.0002\)) survival of melanoma patients, as revealed by Kaplan-Meier analysis using the log-rank test using the date of serum sampling as the starting point of the calculation (Fig. 2). The same analysis considering the time point of diagnosis as the starting point revealed similar results (data not shown). Multivariate analysis performed using the proportional hazards model of Cox including the sFas/CD95 serum concentration in combination with the prognostic factors stage of disease and tumor burden revealed the stage of disease (\(P = 0.0275\)) and the tumor burden (\(P = 0.0323\)), but not the sFas/CD95 serum level (\(P = 0.0780\)), as independent predictive factors for overall survival. Regarding the progression-free survival, tumor burden (\(P = 0.0073\)) and sFas/CD95 serum concentration (\(P = 0.0112\)) proved to be independent predictive factors by multivariate analysis.

**DISCUSSION**

Malignant melanoma cells and tissues have been shown to express Fas/CD95 to variable extents (24, 25). Recent studies revealed Fas/CD95 expression on melanoma cells to be partly (26, 27) or completely (25, 28, 29) dysfunctional in regard to its ability to mediate apoptotic cell death. On the other hand, Owen-Schaub et al. (7) demonstrated that a functional Fas/CD95-FasL system can suppress metastatic spread in comparing the progression of Fas/CD95-sensitive murine melanoma cells in wild-type and FasL-deficient mice. One possible mechanism causing impairment of a functional Fas/CD95-FasL signal transduction might be quantitative abnormalities in sFas/CD95 molecules, inhibiting appropriate receptor-ligand binding (8). This mechanism has been proposed to be used by cancer cells to escape from immunosurveillance (9); this was strengthened by recent investigations showing elevated sFas/CD95 serum levels in a variety of different malignancies (14–19). However, the clinical relevance of abundant sFas/CD95 molecules in serum from cancer patients remains unclear.

Several studies have been performed in recent years showing increased sFas/CD95 serum concentration associated with poor prognosis in patients suffering from hepatocellular cancer, bladder cancer, renal cell cancer, non-Hodgkin’s lymphoma, and breast cancer (15–17, 30, 31). Nevertheless, some studies found elevated sFas/CD95 serum levels lacking a prognostic relevance in cancer patients (21, 32). Preliminary data obtained from eight patients indicated that sFas/CD95 also can be elevated in the serum of melanoma patients (14). Because of the limited number of patients investigated, no correlations regarding stage of disease, tumor burden, and prognosis could be performed in this study, but the authors indicate a trend toward higher sFas/CD95 serum amounts in melanoma patients with advanced disease.

In the present study we demonstrate elevated sFas/CD95 serum levels in a larger panel of 125 melanoma patients of all the different clinical stages of disease. Statistical analyses clearly showed that enhanced sFas/CD95 serum concentrations were correlated significantly with the stage of disease but not with the tumor burden. Furthermore, cytostatic treatment was associated with a strong increase of sFas/CD95 serum concentration. A possible explanation for this observation might be the up-regulation of Fas/CD95 expression in tumor cells induced by cytotoxic agents, as described previously for colon carcinoma cells (33). IFN-\(\alpha\) treatment did not increase sFas/CD95 serum levels, confirming our previous observations that IFN-\(\gamma\), but not IFN-\(\alpha\) up-regulates Fas/CD95 expression on melanoma cell lines (26).

Regarding the cellular origin of the increased sFas/CD95 serum levels observed in this study, the correlation of sFas/CD95 serum concentration with the patients’ stage of disease suggests that tumor tissue itself might be one possible source of sFas/CD95 production. On the other hand, it seems dubious to propose melanoma cells as the only site of origin of elevated serum sFas/CD95 because of the lack of an association between sFas/CD95 serum concentration and the patients’ tumor burden. We suggest activated peripheral blood lymphocytes as another potential source of elevated sFas/CD95 serum concentration, as recently indicated by others (8). Furthermore, serum sFas/CD95 in melanoma patients potentially might originate by shedding from plasma membrane-derived extracellular vesicles instead of alternative splicing (34).

The present study demonstrates for the first time a highly significant correlation of elevated sFas/CD95 serum level with poor prognosis in melanoma patients, presenting sFas/CD95 serum concentration as an independent predictive factor for progression-
free survival. This observation may be causally related to the inhibition of a functional Fas/CD95-FasL interaction by sFas/CD95, enabling tumor cells to evade from immunosurveillance by tumor-infiltrating lymphocytes (8) or impairing Fas/CD95-mediated apoptosis induced by cytostatic drugs (35). Additional studies are necessary to specify the site of origin of the increased serum sFas/CD95 and, furthermore, to clarify the mechanisms of function of sFas/CD95 in melanoma patients.

In conclusion, our study shows significantly increased sFas/CD95 serum concentration in melanoma patients that was associated with a poor prognosis, indicating serum sFas/CD95 as an independent predictive factor for progression-free survival of melanoma patients.

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