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 serum levels were associated with a poor overall (P < 0.005) survival. Multivariate analysis revealed sFas/CD95 serum concentration as an independent predictive factor for progression-free (P = 0.011), not but over (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

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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 before were scored as untreated patients. Detailed characteristics of the patients enrolled in this study are presented in Table 1. 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 multplate 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 microtitre 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 cut-off 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 treated with IFN-α alone or in combination with cytostatics showed a slight increase of serum concentration (P < 0.01) compared with untreated patients. The group of patients treated with cytostatics and IFN-α (Table 1) showed a slight decrease in serum concentration (P < 0.05) compared with untreated patients. The correlation between sFas/CD95 serum level and the patients’ stage of disease (P < 0.05) was not observed in patients currently treated with IFN-α alone or in combination with cytostatics.

### Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of cases</th>
<th>sFas/CD95 (μg/ml) ± SE</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>
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<tr>
<td>Therapy</td>
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<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.

**b 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.
Soluble Fas/CD95 in Malignant Melanoma Patients

P. serum concentrations were strongly associated with a poor over-
calculated threshold is shown in Fig. 1. Elevated sFas/CD95
age of patients showing sFas/CD95 serum levels above the
ng/ml obtained by ROC analysis. The stage-dependent percent-
CD95 serum concentration according to a cutoff value of 7.92
quences of elevated sFas/CD95 serum levels, melanoma patients
free and Overall Survival.
To analyze the clinical conse-
CD95 serum level.
ROC analysis, providing the best discrimination between patients and
controls regarding optimal values of sensitivity and specificity. Stage
III, primary melanoma; Stage III, regional lymph node and/or in-transit metastases; Stage IV, distant metastases.

Fig. 1 Stage-dependent increase of sFas/CD95 serum level in 125
melanoma patients as measured by ELISA. Bars, percentage of patients
tested with elevated sFas/CD95 serum concentration. The cutoff value
for sFas/CD95 serum concentration (7.92 ng/ml) was calculated using
Tested with elevated sFas/CD95 serum concentration. The cutoff value
was calculated using

Predictive Value of Serum sFas/CD95 for Progression-
free and Overall Survival. To analyze the clinical conse-
quences 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 percent-
age 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 over-
all (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 sur-
vival, tumor burden (P = 0.0073) and sFas/CD95 serum concen-
tration (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
cauing impairment of a functional Fas/CD95-FasL signal trans-
duction might be quantitative abnormalities in sFas/CD95 mol-
ecules, 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 show-
ing 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 ele-
uted in the serum of melanoma patients (14). Because of the
limited number of patients investigated, no correlations regard-
ing 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 concen-
tration. 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-α treatment did not increase sFas/CD95 serum
levels, confirming our previous observations that IFN-γ, but not
IFN-α 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-
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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REFERENCES


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