Elevated Soluble Fas (sFas) Levels in Nonhematopoietic Human Malignancy

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Abstract

Fas is a widely expressed membrane-anchored protein that induces apoptosis. Soluble Fas (sFas), generated by alternative mRNA splicing, can antagonize cell-surface Fas function. We have investigated sFas in 104 cancer patients with nonhematopoietic malignancies using a Fas-specific ELISA and immunoprecipitation. Our studies demonstrate an elevated 40—42-kDa sFas species in both patient serum and tumor explants. These observations provide the first evidence that sFas is increased in patients with solid tumors in a manner reflective of disease stage and tumor burden and argue that sFas can be synthesized and released both systematically and locally within the tumor microenvironment.

Introduction

The human Fas protein (CD95) was independently identified by two laboratories on the basis of specific monoclonal antibody reactivity (reviewed in Ref. 1). Subsequent CDNA cloning revealed that Fas is a 48-kDa Type I membrane protein and a prototypic member of the nerve growth factor/TNF-R superfamily (1). Fas can occur as both a cell-surface and a soluble protein. Cell-surface Fas is anchored by a single membrane-spanning domain and is widely expressed on normal and malignant cells (1—4). sFas is generated by alternative mRNA splicing events (5—8). To date, four isoforms of sFas have been described (7, 9). The most predominant sFas isoform results from the deletion of exon 6 encoding the last 5 amino acid residues of the extracellular domain and 16 of 17 amino acids in the transmembrane domain (5—8). This isoform has been identified in the supernatants of activated human lymphocytes (5—8) and several tumor cell lines, including hepatoma (8), glioma (10), osteosarcoma (6), and T- and B-cell leukemias and lymphomas (7, 11). Moreover, elevated levels of sFas have been observed in the serum of patients with hematopoietic malignancies (11). The interaction of FasL with cell-surface Fas is known to induce apoptosis in sensitive cells (1). Expression of cell-surface Fas, although requisite for the induction of apoptosis, does not predict the biological function of this protein. The molecular mechanisms mediating Fas resistance are complex and involve both postreceptorial and prereceptorial events. Postreceptorial resistance mechanisms are known to include expression of the intracellular Fas-associated tyrosine phosphatase FAP-1 (12) and the apoptosis-protective proteins bcl-2 and bcl-xL (1, 13). Prereceptorial resistance can be mediated by the sFas protein, which antagonizes both anti-Fas and Fasl killing in a dose-dependent manner (5—8). Because the loss of Fas function has been causally implicated in tumor progression (14), sFas production may contribute to the biology of malignant disease. In the present study, we have investigated sFas levels in the serum of a variety of cancer patients using a Fas-specific ELISA, immunoprecipitation, and Western blotting. We now report, for the first time, that patients with nonhematopoietic malignancies exhibit elevated sFas levels compared to normal controls and that sFas can be synthesized and released in the culture supernatants of human solid tumor explants. Significantly, our results suggest that the relative elevation of sFas levels in nonhematopoietic cancer patients may be reflective of both disease stage and tumor burden. The known involvement of Fas/Fasl function in immune effector function (1) and tumor progression (14) underscores the potential biological significance of our findings.

Materials and Methods

Human Serum Samples. Residual human serum samples were obtained under an Institutional Review Board-approved protocol from the Clinical Chemistry Laboratory, University of Texas M. D. Anderson Cancer Center. All samples were maintained 3—7 days after initial phlebotomy at 4°C, centrifuged at 10,000 rpm for 15 min, and then immediately used for assay or frozen at −70°C until use.

Human Tumor Explants. Resected human tumor specimens were provided by the Department of Pathology, University of Texas M. D. Anderson Cancer Center, under an Institutional Review Board-approved protocol. Freshly isolated human tumor specimens were procured within 15 min of devascularization and maintained on ice for 1—3 h in PBS until processing. Tumor specimens were finely minced under sterile conditions and incubated in 3—5 ml of complete medium containing penicillin/streptomycin at 37°C at 10% CO2 for 48 h. Cell-free medium was recovered by centrifugation at 10,000 rpm and used for immunoprecipitation/Western blot analyses or stored at −20°C until use.

Fas-specific ELISA. A double antibody sandwich ELISA was constructed to detect sFas in patient serum and supernatants from tumor explants. For this assay, Nunc Immuno-module Maxisorb plates (Nunc, Inc., Naperville, IL) were coated with 150 ng of monoclonal anti-Fas (Kaiyma Biomedical Co., Thousand Oaks, CA) in a total volume of 50 µl PBS/well and incubated overnight at RT. The next day, wells were washed three times with PBS and blocked with PBS containing 5% BSA in a volume of 200 µl for 1 h at RT. After being blocked, plates were washed three times in PBS, and 50 µl undiluted human serum or human Fas:Fc standard were added to each well for 2 h at RT. Human Fas:Fc produced in baculovirus (15) was the kind gift of Dr. Carl F. Ware (University of California-Riverside, Riverside, CA). After incubation with serum or standards, plates were washed three times with PBS, and 250 ng/well polyclonal rabbit anti-Fas serum (the generous gift of Dr. Thomas Gesner, Chiron Corporation, Emeryville, CA) was added in 50 µl PBS containing 5% BSA. Plates were then incubated for 30 min at RT and washed with PBS three times, and 15 ng/well goat antirabbit horseradish peroxidase-conjugated IgG (BioSource International, Camarillo, CA) was added in 50 µl PBS containing 1% goat serum for 30 min at RT. After a final wash in PBS, 75 µl/well TMB peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added for 1 h, and the absorbance was read at 630 nm with a Dynatech MR5000 spectrophotometer.

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4 The abbreviations used are: TNF-R, tumor necrosis factor receptor; FasL, Fas ligand; sFas, soluble Fas; RT, room temperature.

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Immunoprecipitation and Western Blot Analyses. Protease inhibitors (15 μM aqueous phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin) were added to human serum or cell-free supernatants from human tumor explants, and the samples were precleared with goat IgG bound to protein G-agarose followed by protein G-agarose, or with protein A-agarose (human serum only). Immunoprecipitation was carried out as described previously (6) using normal goat serum or anti-Fas-specific goat serum bound to protein G at 4°C overnight. Protein G beads were collected by centrifugation, washed four times in PBS, and resuspended in 2× SDS-PAGE loading buffer. Samples were boiled for 5 min and run on a 7.5% SDS-polyacrylamide gel under nonreducing conditions, and the proteins were blotted to nitrocellulose. sFas was detected using anti-Fas-specific rabbit serum and horseradish peroxidase-conjugated donkey antirabbit antibody (Tago Immunologicales, Carpinterio, CA) as described previously (6), and proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) according to the manufacturer's recommendations.

Statistical Analyses. Differences between sFas levels among the cancer patient group and normal controls were determined using the nonparametric Kruskal-Wallis test. The distribution of sFas across the different cancer groups are presented by box plots (Figs. 1 and 2). Box plots portray median values, quartiles, and outliers of the empirical distribution of the data. Statistical significance was defined as the P-value of a two-sided test (P < 0.050).

Results

Detection of sFas by ELISA. This study was undertaken to expand our knowledge of sFas production in cancer patients with nonhematopoietic malignancies. To this end, we have examined serum sFas levels using a Fas-specific double sandwich ELISA developed in our laboratory. Sensitivity and reproducibility of the ELISA assay was initially verified with a baculovirus-produced human Fas:Fc chimeric protein (data not shown). In repetitive assays, detection of Fas:Fc was shown to be linear from 6.25 to 100 ng/ml, with an interassay variation of <7%. Specificity and sensitivity of the ELISA assay for sFas was documented by the addition of baculovirus-produced sFas to healthy donor serum. Using this approach, detection of sFas was shown to be linear, with 60–80% recovery of the initially added protein detectable. Furthermore, baculovirus-produced sFas protein was shown to be stable in normal human control serum for up to 7 days at 4°C (equivalent to the maximum storage times for collected patient samples) and after several freeze-thaw cycles when stored at −70°C.

To determine circulating sFas levels in a variety of malignant disease states, residual serum samples were collected from patients undergoing cancer treatment at the University of Texas M. D. Anderson Cancer Center. For the sake of comparison, healthy donor serum was also evaluated. As shown in Fig. 1, moderate to abundant sFas levels were detectable in the 67 patient serum samples from patients with bladder adenocarcinoma, ovarian carcinoma, lung carcinoma, head and neck tumors, melanoma, sarcoma, lymphoma, and leukemia. The majority of the cancer patients examined (58 of 67) demonstrated statistically significant elevations in sFas levels relative to normal controls (see below). These results confirm those of Knipping et al. (11), who found elevated levels of sFas in patients with leukemias, and demonstrate for the first time that sFas levels can be elevated in patients with nonhematopoietic malignancies. Interestingly, however, not all cancer patients exhibited increased sFas relative to normal controls.

Limited sample size and accurate staging of the nonhematopoietic tumors shown in Fig. 1 precluded our ability to address the relationship between sFas levels and stage of malignant disease. For these reasons, we have examined 29 breast cancer patients and 23 colon carcinoma patients presenting with primary disease, localized metastases, or widely disseminated metastases. Fig. 1A shows sFas levels for colon and breast cancer patients compared to normal controls and patients with malignancies at other sites. Among the three cancer groups (colon, breast, and all other sites), sFas levels were, overall, significantly elevated compared to normal controls (P < 0.001 using the two-sided Kruskal-Wallis test). With the adjustment of type I error in multiple pairwise comparisons by multiplying each P-value by the number of comparisons being made, sFas levels in each cancer group were still significantly higher than the normal group (breast versus control, P < 0.001; colon versus control, P = 0.001; all other sites versus control, P < 0.001). Furthermore, when patients with breast or colon carcinoma were considered by disease stage, elevated sFas levels were shown to correlate with both metastatic phenotype (breast and colon) and tumor burden (colon). Fig. 1B shows the distribution of sFas levels in breast carcinoma patients with primary and metastatic disease. Patients with metastatic disease (stage IV) demonstrated significantly higher sFas levels than patients with primary disease (stage I, II, or III) in the overall Kruskal-Wallis test (P = 0.037). Interestingly, patients who had undergone surgical resection of their primary disease (N. E. D., no evidence of disease) showed a strong trend toward lower sFas levels, suggesting a causative role of tumor burden in the elevation of sFas. Additional sample collection will be necessary to confirm whether such differences are statistically significant.

When sFas levels were compared among colon cancer subgroups, significant differences were also observed among those patients with local and metastatic disease. As shown in Fig. 2C, patients with local colon cancer (stage I or II) had significantly lower sFas levels than those with widely metastatic disease (stage III; P = 0.007). To assess whether elevated sFas levels more accurately mirrored metastatic disease or tumor burden (measurable disease volume), two metastatic subgroups were created to include those patients with widely metastatic disease (liver metastases with at least one other organ involved) and those patients with isolated liver metastases amenable to local therapy. Patients with widely metastatic disease demonstrated significantly higher sFas levels than did those with isolated liver metastases (P = 0.002, for overall Kruskal-Wallis test). No differences were noted between patients with primary colon cancer and those with isolated liver metastases (P = 0.462). Collectively, our findings suggest that similar to breast carcinoma, sFas is more highly elevated in metastatic disease. Furthermore, circulating sFas levels in colon carcinoma may be more reflective of tumor burden than metastatic disease per se.

Fig. 1. sFas levels in cancer patient serum were examined by ELISA as described in "Materials and Methods." The distribution of patients' sFas levels compared to controls are presented by box plots. Median sFas levels are represented by a horizontal line across individual box plots. Sample size is shown in parentheses above each box plot.
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Fig. 2. A, sFas levels in cancer patients with breast or colon carcinoma was examined by ELISA as described in "Materials and Methods." sFas levels are presented by box plots, and the numbers of individual samples analyzed are indicated. sFas levels from colon and breast patients are shown compared to those patients with tumors at all other sites (compiled from Fig. 1). B, sFas levels from breast cancer patients by disease stage. Serum samples were obtained from patients with primary or metastatic breast carcinoma. In addition, serum samples were obtained from patients who had previously undergone surgical resection for primary disease during routine follow-up. These patients showed no evidence of disease (N.E.D.) at the time of evaluation. The number of individual samples analyzed are indicated for each subgroup. C, sFas levels from colon cancer patients by disease stage. Serum samples were obtained from patients with local, widely metastatic (METS.) or isolated liver metastases (LIVER METS.). The numbers of individual samples analyzed are indicated for each subgroup.

Detection of sFas in Human Serum by Immunoprecipitation/ Western Blotting. Although elevated sFas levels were detectable by ELISA in the serum of cancer patients, the nature of the reactive moiety remained unresolved. Therefore, serum from patients with a variety of solid tumors, including breast, colon, and melanoma, was subjected to immunoprecipitation and Western blotting. Fig. 3A shows the results of two such experiments using melanoma patient serum. Consistent with our ELISA results, patient serum demonstrated an immunoreactive sFas protein with an apparent molecular mass of 40—42 kDa when immunoprecipitated with anti-Fas but not control antibody (Fig. 3A, Lanes 4 and 8 compared to Lanes 3 and 7). In control experiments, sFas was not at detectable levels in pooled normal control serum containing 0—19 ng/ml sFas (Lanes 1, 2, 5, and 6). To verify that the sFas protein detectable by immunoprecipitation was the ELISA-reactive moiety, serum samples were reanalyzed by ELISA after immunoprecipitation. These experiments showed a 45—67% decrease in sFas levels following a single round of immunoprecipitation (data not shown), confirming specificity for the 40—42-kDa sFas protein in our ELISA.

Detection of sFas in Culture Supernatants from Human Tumor Explants. Previous reports have demonstrated sFas production by human lymphocytes (5—8) and nonhematopoietic tumor cell lines (6—8, 10). To determine whether sFas was also present in freshly isolated human tumor explants, regional nodes from patients with metastatic melanoma were obtained. After immunoprecipitation and Western blotting of cell-free supernatants from explants cultured in vitro for 48 h, a 40—42-kDa sFas protein was detectable in four of the five melanoma lesions examined. An experiment representative of the four positive explants is shown in Fig. 3B. These studies demonstrate that sFas can be synthesized and released locally within the tumor microenvironment. However, because these metastatic lesions contain an admixture of resident lymphocytes, tumor associated lymphocytes, and tumor cells, the precise cellular source of sFas remains enigmatic.

Discussion

Recently, we (6) and others (5, 7, 8) have documented the existence of sFas lacking the transmembrane domain of full-length Fas as a result of an alternate mRNA splicing event. To date, sFas production has been observed in vivo in normal individuals and patients with rheumatoid arthritis, systemic lupus erythematosus, and B- and T-cell leukemias (5, 11). In the current study, we examined serum from 104 patients with primary and metastatic nonhematopoietic malignancies at various anatomical sites and show that a majority of the samples contain elevated sFas levels compared to controls. Furthermore, the magnitude of sFas production, at least for breast and colon carcinoma, correlates with both metastatic phenotype and tumor burden. Our findings are further supported by the detection of a 40—42-kDa sFas protein in both patient serum and fresh tumor explants by immunoprecipitation and Western blotting. This is the first study to document the presence of elevated sFas levels in vivo from patients with solid tumors.

sFas production is unique among TNF receptor superfamily members in two critical aspects. First, sFas is generated by alternative mRNA splicing events (5—9) rather than proteolytic cleavage, and second, at least one sFas isoform retains an intact intracytoplasmic domain. The sFas protein lacking the transmembrane domain is the most predominant soluble isoform (5—9). In addition, three other, less abundant isoforms of sFas have been shown to exist (7, 9). Each of these isoforms is encoded by a unique, prematurely truncated transcript resulting from alternative mRNA splicing and shares only 49 NH₂-terminal amino acids in common with full-length Fas as a result of a frameshift mutation. To date, these prematurely truncated sFas transcripts have been identified only in human lymphocytes (7, 9). Although multiple sFas isoforms exist, it is important to note that commercially available monoclonal antibodies (such as the CH-11...
used in our assay) have not been shown to cross-react with the truncated sFas splice variants (7). Thus, our ELISA most likely detects only the predominant sFas isoform lacking the transmembrane domain, a premise further strengthened by detection of a single 40–42-kDa Fas-reactive protein after immunoprecipitation of cancer lime 9 was immunoprecipitated with nonspecific control antiserum and Lane 10 with specific anti-Fas antiserum. Molecular mass markers are indicated (Wa); arrow, sFas-specific band. B, sFas is detectable in cell-free supematants from involved regional lymph nodes in metastatic melanoma.

Our studies clearly demonstrate elevated sFas levels in patients with solid tumors, although the absolute sFas levels differ from those previously reported for hematopoietic tumors (11). In the study by Knipping et al. (11), patients with B- and T-cell leukemias showed median sFas levels of approximately 3.3 ng/ml, whereas in the present study, nonhematopoietic patients with colon carcinoma, breast carcinoma, and tumors at a variety of other sites showed median sFas levels of approximately 20, 30, and 35 ng/ml, respectively (Fig. 2A). The absolute sFas values reported in our study are lower than those from patients with autoimmune disease (5), who demonstrated approximate median values ranging from 60 to 125 ng/ml. Such intrastudy variation may reflect innate differences in the patient populations examined in the three studies, the nature of the sFas standards or secondary anti-Fas antibodies employed for detection, or the existence of sFas isoforms preferentially recognized by particular anti-Fas antibodies. Our study demonstrated a bimodal distribution of normal sFas levels, precluding direct statistical comparison of controls with other cancer groups by parametric statistical methods and suggesting potential differences within the normal control population. Importantly, however, our control values (0–19 ng/ml sFas; median, 0.5 ng/ml) are in close agreement with both Knipping et al. (11) and Cheng et al. (5), who reported median values of 1.75–2.0 ng/ml and a range of 15–35 ng/ml, respectively.

It was not particularly surprising that cancer patients demonstrated a considerable range of sFas levels, even when a particular tumor site was considered (Figs. 1 and 2A). For example, melanoma patients demonstrated a range of sFas levels from 7 to 80 ng/ml. In patients with elevated levels of sFas, the magnitude of sFas production was widely variable, even among those patients with similar cancer types (e.g., 7–80 ng/ml in melanoma cancer patients). The nature of this variation is currently unknown but may be related to inherent differences in therapeutic regimen (chemotherapy, radiotherapy, or immunotherapy), the duration of patient treatment, the extent of tumor burden, or the degree of disease progression (primary versus metastatic disease). In this regard, one-half of the melanoma patients accrued for this study had stage II disease (previous resection with no measurable tumor burden), whereas the other half had stage III disease (measurable tumor burden). Our findings in patients with breast and colon cancer (Fig. 2, B and C) suggest that sFas levels may correlate with metastatic phenotype. Whether elevated patient sFas levels more adequately reflect disease stage or tumor burden is difficult to assess in these studies; however, colon cancer patients with isolated liver metastases (stage IV) show sFas levels similar to those of patients with localized colon disease (stage I–III), thereby suggesting that the magnitude of sFas production may be more closely associated with tumor burden. Although additional studies will be required to definitively correlate sFas levels with disease stage or tumor burden in a particular disease group, it should be noted that the levels of other soluble TNF-R superfamily members (such as CD30 and TNF) have been shown to correlate more closely with tumor burden (16).

The precise cellular origin of sFas in our study is unresolved. We speculate that sFas may be derived from the tumor itself, corroborating previous observations in tumor cell lines (6–8, 10). However, because sFas can be produced by activated peripheral blood lymphocytes (5, 11), it is possible that sFas is derived from the lymphocytes themselves. In support of the latter possibility, we have been unable to detect sFas in other solid tumor explants, including those derived from breast and colon. It is interesting to note, however, that sFas levels were significantly reduced in breast cancer patients following removal of their primary tumor (Fig. 2B). Tumor versus lymphoid origin of sFas may not be exclusive possibilities and may be dependent upon the tumor type and/or the degree of lymphoid infiltration. Alternatively, it is possible that surrounding stromal tissues produce sFas in response to tumor or as a secondary response to immune activation. Clearly, additional studies will be necessary to resolve the origin of the sFas protein detected.

To date, production of sFas has been observed only in those cells expressing the membrane-anchored Fas protein (5–10). Importantly, however, the introduction of sFas expression constructs as well exposure to cell-free supernatants containing sFas have been shown to confer Fas resistance (5, 7, 10). These results imply a potential autocrine, paracrine, and juxtacrine role for sFas. It has been previously suggested that soluble cytokine receptors may regulate immune function by binding to free ligand and preventing target cell activation through the membrane-anchored receptor. Human FasL is a 40-kDa type II membrane protein and a member of the TNF ligand family occurring as a cell-surface or soluble protein, most likely as a result of cell-surface proteolysis (1). FasL is highly expressed, albeit transiently, on NK and T lymphocytes and is a critical component of cytotoxic effector cell function (1). Thus, the biological role of sFas may be envisioned to involve binding and neutralization of either soluble of cell-surface FasL before interaction and membrane-anchored Fas protein. Alternately, given the known requirement for...
receptor oligomerization in TNF-R superfamily signal transduction pathways (1, 17), it is also possible that sFas forms complexes with cell-surface Fas to prevent or alter signal transduction. Whatever the mechanism, the functional antagonism of sFas in FasL-dependent apoptosis has been well documented (7, 8).

The biological ramifications of our findings for malignancy are compelling, especially considering the accumulating evidence for participation of Fas/FasL in T-cell-mediated cytotoxicity (1) and for the critical role of apoptosis in tumorigenesis (18, 19). With this in mind, we hypothesize that the loss of Fas function may be an important event in the evolution and progression of malignant disease. Indeed, Möller et al. (20) demonstrated that Fas expression is markedly reduced in colon carcinomas compared to normal adjacent tissue and, further, that the loss of Fas expression in the primary tumor correlates with disease progression (metastasis). The first experimental evidence that the loss of Fas can enhance tumor development has been recently reported (14). In this system, lymphomagenesis driven by the Eμ-myc transgene was shown to be markedly accelerated in lpr/lpr (Fas-deficient) mice compared to wild-type littermates, confirming a causal, rather than correlative, role for Fas loss in tumor progression. Because sFas can functionally antagonize FasL to effectively inactivate cell-surface Fas function, it is conceivable that elevated sFas production may serve as a novel mechanism to promote tumorigenesis and progression.

Clearly, additional work will be required to determine whether sFas can serve as a marker of malignant disease or host reaction to disease or as a potential target for antineoplastic therapy. With respect to the latter, it is possible that overexpression of sFas can protect malignant cells from host destruction and that strategies aimed at inhibiting sFas production could increase tumor cell susceptibility to such killing. Before such manipulation, however, it is imperative to understand the molecular controls favoring the generation of sFas and membrane-anchored Fas.

References

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