Spontaneous Apoptosis of Circulating T Lymphocytes in Patients with Head and Neck Cancer and Its Clinical Importance

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ABSTRACT

Spontaneous apoptosis was observed in a proportion of peripheral blood mononuclear cells obtained from patients with head and neck cancer (HNC) but not from normal healthy donors (T. Saito et al., Clin. Cancer Res., 5: 1263–1273, 1999). To further investigate this phenomenon, peripheral blood mononuclear cells were obtained from patients with HNC or normal controls (NCs) and evaluated for expression of apoptosis markers (annexin V binding and caspase-3 activation), T-cell receptor-associated \( \zeta \) chain, and the death receptor Fas (APO-1, CD95) in CD3\(^{+} \) T cells by multicolor flow cytometry. Soluble Fas ligand (sFasL) in the sera of these individuals was quantitated by ELISA. In patients with HNC, 74 ± 15% (mean ± SD) of CD3\(^{+} \) T cells were Fas\(^{+} \) compared with 52 ± 13% in NCs \( (P < 0.0001) \). Furthermore, 29 ± 16% of the Fas\(^{+} \) CD3\(^{+} \) T cells bound annexin V in patients and only 14% ± 7% of the Fas\(^{+} \) CD3\(^{+} \) T cells bound annexin V in NCs \( (P < 0.0001) \). In patients, Fas\(^{+} \) CD3\(^{+} \) T cells preferentially underwent apoptosis and showed a loss of \( \zeta \) chain expression. Significantly greater proportions of CD8\(^{+} \) T cells than CD4\(^{+} \) T cells were apoptotic \( (P < 0.0002) \), which indicates that CD8\(^{+} \) T cells were especially sensitive to apoptosis. Serum levels of sFasL were lower in HNC patients with active disease than in NCs or in patients with no evident disease \( (P < 0.0183) \). This suggested utilization of sFasL produced in vivo and activation of the Fas/Fas ligand (FasL) pathway in Fas\(^{+} \) T cells. Proportions of apoptotic T cells were higher in HNC patients than in NCs \( (P < 0.0001) \), and a subset of HNC patients with active disease had the highest proportions of circulating Fas\(^{+} \) annexin V\(^{+} \) T lymphocytes. The data indicate that the Fas/FasL pathway is involved in spontaneous apoptosis of circulating Fas\(^{+} \) T lymphocytes in cancer patients. Fas/FasL interactions might lead to excessive turnover of T cells in the circulation and, consequently, to reduced immune competence in patients with HNC.

INTRODUCTION

Evidence has accumulated indicating that immune cells in patients with HNC are functionally impaired (1–3). In comparison with immune cells obtained from the peripheral circulation of normal blood donors, those obtained from HNC patients are found to be poorly responsive to mitogenic or antigenic stimuli, have an altered cytokine profile, are unable to mediate robust cytotoxicity of tumor cell targets, and exhibit impaired signaling via TcR (3, 4). The defects in expression and function of the key signaling molecules associated with TcR in T cells and FcRRIII in natural killer cells as well as cytokine abnormalities were found to be more pronounced in lymphocytes accumulating at the tumor site or in tumor-involved lymph nodes than in the peripheral circulation (2–4). Nevertheless, we and others have reported the presence of frequent, although variable, defects in immune functions of circulating T cells as well as natural killer cells in these patients (5–7). In more recent experiments with tumor-infiltrating as well as circulating T cells of patients with HNC, these variable functional defects in immune cells have been linked to the suppressive influence of the tumor or tumor-derived factors (8, 9). Alternatively, overexpressed tumor-derived antigens could cause persistent polyclonal activation of lymphocytes and, ultimately, their death by apoptosis (10).

The mechanism(s) responsible for apoptosis of T cells present at the tumor site or those in the peripheral circulation of patients with HNC is under intense current investigation. The tumor necrosis factor family of receptors and ligands has been found to mediate immune effector cell interactions with tissue cells (11). For example, the Fas (APO-1, CD95)/FasL pathway represents one of the mechanisms that is involved in inducing the death of T cells interacting with the tumor or tumor-associated antigens. We have recently reported that human HNC cells express both Fas and FasL at the mRNA and protein levels (12). Furthermore, we have obtained evidence that FasL is fully functional on the surface of HNC cells (12). In contrast, its receptor, Fas, may not be functional because various inhibitory

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4 The abbreviations used are: HNC, head and neck cancer; PBMC, peripheral blood mononuclear cell; NC, normal control; TcR, T-cell receptor; NED, no evident disease; FasL, Fas ligand; sFasL, soluble FasL; PE, phycoerythrin; Ab, antibody; FSC, forward angle scatter; SSC, side angle scatter; SCCNH, squamous cell carcinoma of the head and neck.

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proteins regulate signals delivered to the receptor on the tumor cell surface (13). It has been reported that FasL is involved, at least in part, in activation of caspases in Fas+ T lymphocytes (13, 14). Convincing evidence in support of this possibility was recently obtained, confirming that in many but clearly not all instances, tumor-associated FasL mediates the death of Fas+ T cells (12, 15, 16).

In this study, we extend our previous results to demonstrate that Fas+ T lymphocytes constitute the major population of circulating lymphocytes in patients with HNC. These T cells are activated and thus are sensitive to spontaneous or induced apoptosis as shown previously by us (6). Fas+ CD8+ T lymphocytes were observed to preferentially undergo apoptosis. Furthermore, using an ex vivo model of HNC cell lines co-cultivated with Fas+ T cells, we previously demonstrated that the tumor can directly induce T-cell apoptosis, using the Fas/FasL pathway (7, 12). These results are consistent with the hypothesis that Fas+ activated T cells, which are enriched in the peripheral circulation of patients with HNC, are primed to die, leading to a rapid turnover of T lymphocytes and possibly contributing to tumor-related immunosuppression.

MATERIALS AND METHODS

Patients and Normal Donors. PBMCs were obtained from 37 patients with SCCHN seen in the Otolaryngology Clinic over a period of 12 months and from 35 healthy controls (NCs). The patients were not selected for this study; rather, consecutively seen patients were asked to donate blood. The patients and NCs participating in this study all signed an institutional review board-approved informed consent form. The patients and NCs were similar in age, with a mean age of 60 ± 13 (SD) years for the HNC group (17 females and 20 males) and 56 ± 16 years for the control group (17 females and 18 males). At the time of blood draws, 18 patients were tumor free (NED), having undergone surgery at least 12 months previously, and 19 patients had active disease (Table 1). Among the patients with active disease were 10 patients with primary carcinomas and 9 patients with recurrent carcinomas. All 19 were scheduled for surgery and studied before surgery. Eleven of 37 patients were treated with radiotherapy postoperatively, and only 3 of these patients received chemotherapy, which was completed more than 12 months before the blood draw for this study in all cases (Table 1).

Lymphocyte Recovery. Venous blood (20 ml) was obtained from patients and NCs, and PBMCs were separated and processed as described previously (5). Each patient’s cells were processed, stained, and analyzed in parallel with NC cells.

Staining of Cells for Flow Cytometry. Freshly harvested PBMCs were washed in Dulbecco’s PBS (Life Technologies, Inc.), divided into 2 × 10^6 cell aliquots, and individually incubated in the presence of PE-labeled CD95 Ab (clone DX2; PharMingen, San Diego, CA), PerCP-labeled anti-CD3 Ab, or respective isotype controls (all from Becton Dickinson, San Jose, CA) as described previously (5–7). In some experiments, anti-CD8 and anti-CD4 Abs (Becton Dickinson) were used. Anti-FasL Abs and annexin V-FITC conjugate were purchased from PharMingen and used under the conditions described previously (5–7). All Abs were pretitered on normal PBMCs to determine their optimal dilutions.

For Ig chain staining, the cells were washed once with PBS

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Table 1: Clinicopathological parameters of the patients with squamous cell carcinoma of the head and neck included in the study.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients</th>
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<tbody>
<tr>
<td>Sex</td>
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<tr>
<td>Female</td>
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<tr>
<td>Male</td>
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<tr>
<td>Age (yrs)</td>
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<td>3</td>
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<td>Chemotherapy</td>
<td>3</td>
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*All patients had prior therapy more than 12 months before the blood draw for this study.
and once with cold saponin (Sigma, St. Louis, MO) 0.1% (w/v) in PBS + 0.1% (w/v) BSA in solution. The cells were then permeablized for 30 min on ice in 100 μl of the saponin solution. A 10-μl aliquot of TcR-δ-PE (clone 2H2D9; Coulter, Miami, FL) or isotype-IgG1 (Becton Dickinson) was added at the same time. After the incubation period, the cells were washed twice with saponin solution and then washed once with PBS and immediately examined in a flow cytometer.

Flow Cytometry. Three-color flow cytometry analysis was performed on a FACScan (Becton Dickinson) equipped with a single 488 nm argon ion laser. At least 20,000 events were acquired for each sample. The amplification and compensation were set according to the standard procedure, using negative controls and tested cells stained in a single color or combination of colors (FL-1, FITC-annexin V; FL-2, PE-CD95 or PE-δ; and FL-3, CD3-PerCP). Control cells were PBMCs obtained from NC donors. The percentages of apoptotic cells were calculated by scoring annexin V-binding cells after backgating on CD3 cells in the third color. After the incubation period, the cells were washed twice with saponin solution and then washed once with PBS and immediately examined in a flow cytometer.

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The flow cytometry-based caspase-3 activation assay was performed as described previously by us (17).

**Measurement of sFasL in Sera.** sFasL in human sera was measured by a quantitative sandwich enzyme immunoassay (Oncogene Research Products, Boston, MA), using a monoclonal Ab specific for human FasL protein. The lower limit of detection for sFasL was 0.02 ng/ml. Supernatants of a HNC cell line transduced with the human FasL gene and secreting FasL (12) served as a positive control.

**Cell Lines.** The cell lines of human SCCHN were established in our laboratory and maintained as described previously (18). Jurkat cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described previously (7).

**PBMC Activation.** PBMCs obtained from NCs were cultured in the presence of phorbol 12-myristate 13-acetate at 1 ng/ml and 1 μM ionomycin for 18 h at 37°C.

**Statistical Analysis.** All comparisons between patients with HNC and NCs were made using the exact two-tailed Wilcoxon test. The Jonckheere-Tepstra procedure was used to evaluate significance of the data trends observed for different patient groups and NCs. Age effects were checked by fitting separate linear regression models to patients and to NCs. Anal-

![Fig. 2](image_url) Expression of Fas on CD4+ and CD8+ T cells in the peripheral circulation of patients with HNC (n = 17) and NCs (n = 17). The data in A and B are presented as box plots (see the Fig. 1 legend). The percentages of CD4+ Fas+ cells are significantly higher in patients than in controls (P < 0.0001) and are not related to age (NSD) in patients or controls (C). The percentages of CD8+ Fas+ cells are also significantly higher in patients than in controls (P < 0.0009), and they are age-related in NCs (P < 0.0001) and in patients with HNC (P = 0.016; D). The lines show a curvilinear ( "smoothing spline fit") relationship rather than a straight line fit.
ysis of covariance was conducted to determine whether tests for differences between patients and controls needed to be adjusted for differences in age distribution. Linear regression models were also fit to selected pairs of immunological endpoints, including the percentage of Fas⁺, annexin⁺, or Fas⁻ annexin V⁺ T cells and serum levels of sFasL. Estimates of Pearson correlation coefficients and regression slopes were calculated and reported separately for patients and NCs.

RESULTS

Fas Expression on T Lymphocytes in the Peripheral Circulation of Patients with HNC. To determine the mechanisms responsible for high spontaneous apoptosis observed previously in PBMCs obtained from HNC patients, we compared expression of Fas on circulating CD3⁺, CD4⁺, or CD8⁺ T cells obtained from the circulation of HNC patients and NCs. The mean ± SD of CD3⁺ Fas⁺ T cells was 74 ± 15% in patients’ PBMCs as compared with 52 ± 13% in PBMCs of NCs (Fig. 1). Whereas the difference was highly significant at \( P = 0.0001 \), a wide range of values was observed in the circulation of both the patients and NCs. In patients with advanced disease involved in this and other studies in our laboratory, the proportion of Fas⁺ CD3⁺ cells was frequently observed to be close to 100%. The data suggest that patients with HNC have a significantly higher frequency of circulating Fas⁺ T cells than NCs. Importantly, no FasL expression was detected on circulating CD3⁺ T cells of the same patients by flow cytometry. Among CD4⁺ and CD8⁺ T-cell subsets, the proportions of Fas⁺ cells were also significantly increased in patients as compared with NCs (\( P < 0.0001 \) for CD4⁺; \( P < 0.0009 \) for CD8⁺; Fig. 2, A and B, respectively).

Fig. 2, C and D, shows expression of Fas on CD8⁺ and CD4⁺ subsets of circulating T lymphocytes of patients and NCs in relation to age. Clearly, no age-related changes were observed in Fas⁺ CD4⁺ T cells, although the proportion of Fas⁺ CD4⁺ T cells was significantly higher in the peripheral circulation of patients relative to NCs (\( P < 0.0001 \)). In contrast, the proportions of CD8⁺ Fas⁺ T cells increased somewhat with age in patients (slope = 0.43; \( P = 0.016 \)) and more dramatically in NCs (slope = 1.32; \( P < 0.0001 \); Fig. 2D). As a result, the percentage of circulating CD8⁺ Fas⁺ cells in the younger patients with cancer approximated that in the oldest NCs.

Fas⁺ CD3⁺ T Cells Preferentially Bind Annexin V. Cells in early apoptosis have characteristic alterations in their surface membrane. Annexin V binding is used to detect one such alteration, namely, the phosphatidyl serine “flip.” Therefore, annexin V was used to seek evidence for early apoptosis in Fas⁺ CD3⁺ circulating T cells in patients with HNC. To reliably determine annexin V binding to circulating T lymphocytes, the stringent gating strategy described in “Materials and Methods” was used with all control and patient samples. We first determined that the patients had a significantly higher proportion (\( P = 0.0001 \)) of CD3⁺ annexin V⁺ cells in the circulation than did NCs (Fig. 3A). Next, three-color flow cytometry was used to demonstrate that CD3⁺ Fas⁺ annexin V⁺ cells were also significantly more numerous in the circulation of patients than in that of NCs (\( P = 0.0001 \); Fig. 3B). A linear relationship was evident between CD3⁺ annexin V⁺ and CD3⁺ Fas⁺ annexin V⁺ cells in both patients and NCs (with \( r = 0.947 \) and 0.929, respectively, and \( P < 0.0001 \) for both correlations; Fig. 3C). It is apparent in Fig. 3C, however, that only a subset of the patients (12 of 36) had elevated percentages of circulating CD3⁺ annexin V⁺ Fas⁺ cells, relative to controls, with a cutoff of about 30%. Using PBMCs of 1 of these 12 patients and three-color flow cytometry, it was possible to confirm that in this patient, more Fas⁺ T cells than Fas⁻ T cells bound annexin V (Fig. 4A). These results suggested that Fas⁺ T cells were preferentially targeted for apoptosis in the circulation of patients with HNC. In addition, we observed that in the proportion of T cells binding annexin most strongly, Fas expression was decreased (Fig. 4A).

To provide evidence for the loss of Fas expression during
apoptosis, we incubated Jurkat cells or Fas⁺ activated T cells in the presence of Fas cross-linking CH-11 Ab for 18 h and concomitantly monitored them for Fas expression and caspase-3 activity by flow cytometry. We observed that Fas was rapidly internalized upon its cross-linking by CH-11 Ab. Furthermore, caspase-3 activity induced by CH-11 Ab was detectable in both Fas high and Fas low T cells (Fig. 4B). Thus, Fas⁺ CD3⁺ T cells, which are seen to undergo apoptosis in Jurkat cells, and expression of Fas as well as caspase-3 activity was measured by flow cytometry. A representative assay of three assays performed with Jurkat cells is shown. The same experiment was also performed with phorbol 12-myristate 13-acetate/ionomycin-activated T cells obtained from three normal donors.

Caspase-3 Activity in Fas⁺ CD3⁺ T Cells. To determine whether Fas⁺ T lymphocytes also have higher caspase-3 activity than Fas⁻ CD3⁺ T cells, three-color flow cytometry was performed. As shown in Fig. 7, among CD3⁺ T cells of a representative patient with HNC, more than half were Fas⁺. Activity of caspase-3 was detected primarily in CD3⁺ Fas⁺ lymphocytes. This result is consistent with the hypothesis that Fas⁺ T cells are preferentially undergoing spontaneous apoptosis in the circulation of HNC patients.

ζ Chain Expression in Fas⁺ CD3⁺ T Cells. We have reported previously that expression of the TcR-associated ζ chain was found to be decreased in CD3⁺, CD4⁺, or CD8⁺ T cells of patients with HNC compared with that in T cells of NCs.

Fig. 4  A, the CD3⁺ Fas⁺ T lymphocytes in the peripheral circulation of a patient with HNC preferentially bind annexin V. A representative result obtained by three-color flow cytometry using PBMCs obtained from a patient with HNC. The circles show the same cell population either in a FL-1/FL-2 (left panel) or a FSC/FL-1 (right panel) dot plot and indicate that small, apoptotic T cells have the lowest Fas expression. B, Fas expression and caspase-3 activity in apoptotic Jurkat cells. CH-11 agonistic anti-Fas Ab or immunoglobulin control was used to induce apoptosis in Jurkat cells, and expression of Fas as well as caspase-3 activity was measured by flow cytometry. A representative assay of three assays performed with Jurkat cells is shown. The same experiment was also performed with phorbol 12-myristate 13-acetate/ionomycin-activated T cells obtained from three normal donors.
Apoptosis of T Lymphocytes in HNC

To determine whether ζ down-regulation preferentially occurred in circulating Fas⁺ CD3⁺ T cells in patients with HNC, three-color flow cytometry was performed. As shown in Fig. 8, decreased ζ expression was observed in a subset of freshly harvested Fas⁺ CD3⁺ T cells obtained from a representative patient with HNC. Importantly, most Fas⁻ T cells had normal ζ expression, although their proportion was lower in patients than in NCs. The data indicate that down-regulation of ζ occurs preferentially in Fas⁺ CD3⁺ T cells in the circulation of patients with HNC.

sFasL in Sera of Patients and Controls. Because both annexin V binding and caspase-3 activation suggested that Fas⁻ T cells are targeted for apoptosis, we suspected that the Fas/FasL pathway might be involved in this process. The extent of the Fas/FasL pathway contribution to apoptosis of circulating T lymphocytes in patients with HNC was therefore evaluated by examining the presence of sFasL in the patients’ sera. As positive controls, we used cellular supernatants of HNC cell lines transduced with the human FasL gene and secreting FasL into the supernatant, as reported previously (12). The 24 h or 48 h supernatants of the nontransduced SCCHN cell lines tested contained <0.5 ng/ml sFasL. Sera of the patients with HNC or normal donors contained variable but detectable levels of sFasL (0.1–1.2 ng/ml), and there was no significant difference in serum levels of sFasL (P = 0.0978) between these two groups (data not shown). When serum levels of sFasL were determined in a subgroup of 12 patients with elevated percentages of circulating CD3⁺ Fas⁻ annexin V⁻ T cells (see Fig. 3C), no apparent difference from the normal range of sFasL was observed. This suggests increased use of FasL, perhaps via its participation in extensive T lymphocyte apoptosis, in this subgroup of HNC patients. Among the entire cohort of HNC patients, those with NED had sFasL levels comparable with those of NCs (Fig. 9). However, significantly decreased sFasL serum levels were noted in patients with active primary or recurrent disease (P = 0.0183; Fig. 9). The lowest serum levels of sFasL were found in a subgroup of nine patients with recurrent cancer (P = 0.02932). Because HNC patients with active disease also tended to have the highest percentages of annexin V-binding T cells (see below), our observations suggest that the high levels of spontaneous apoptosis were associated with low levels of sFasL in the serum.

Correlation between Apoptosis and Disease. The patients studied included 18 patients with active disease and 19 patients with NED at the time of blood draw. It was therefore possible to compare these two groups of patients with each other and with NCs for expression of the apoptosis markers on T lymphocytes. The data presented in Fig. 10 demonstrate significant differences (P = 0.0002) in the percentage of annexin V-binding CD3⁺ cells between NCs and all HNC patients. There was no significant difference in annexin V-binding to T cells observed between patients with active disease and those with NED. However, the highest levels of T-cell apoptosis were generally observed in the circulation of patients with active disease. Of the 12 patients with elevated percentages (>30%) of CD3⁺ Fas⁻ annexin V⁻ T cells in the circulation (Fig. 3C), 7 had nodal disease (P < 0.018), which is associated with a poor prognosis. Furthermore, a positive correlation between tumor grade and elevated percentage of CD3⁺ Fas⁻ annexin V⁻ T cells in the circulation was statistically significant at P < 0.036. However, neither the tumor stage nor the site correlated with the percentage of apoptotic T cells in the blood of HNC patients.

DISCUSSION

In this study, we investigated the contribution of the Fas/FasL pathway to spontaneous apoptosis of T lymphocytes that occurs in the circulation of patients with HNC. The rationale for the study was based on our previous report (6), which indicated that after a brief incubation in media, significantly more PBMCs underwent spontaneous apoptosis in patients than NCs. Our ex vivo experiments suggested that among PBMCs, Fas⁺ T lymphocytes were especially sensitive to apoptosis (6). To confirm this hypothesis, freshly harvested lymphocytes of patients with HNC or age- and sex-matched NCs were stained for Fas expression and stained simultaneously for markers of early apoptosis, annexin V binding, and caspase-3 activation. We reasoned that using these markers, it might be possible to detect circulating T lymphocytes that are entering the apoptotic pathway but have not yet been removed from the peripheral circulation by the reticuloendothelial system. The expectation was that the proportions of these cells would be higher in the blood of HNC patients than in that of NCs. Furthermore, we expected that the markers of early apoptosis would be found mainly in Fas⁺ (activated) T lymphocytes. Our results confirmed the hypothesis: not only was the percentage of circulating Fas⁺ CD3⁺ lymphocytes significantly higher in patients with HNC than in NCs, but the large majority of Fas⁺ CD3⁺ T cells bound annexin V and had high caspase-3 activity. Not surprisingly, a considerable subset of CD3⁺ T cells was Fas⁻ in NCs, which probably means that these T cells were antigen-primed and susceptible to activation-induced cell death (10).

The focus of our study was circulating CD3⁺ T lympho-
cytes, primarily because they include subsets of effector and helper cells important for tumor-targeted immune responses. By far the most interesting observation made concerns the preferential death of circulating CD8\(^+\) T lymphocytes. CD8\(^+\) T cells preferentially bound annexin V in patients and NCs, but the percentage of annexin V-binding CD8\(^+\) T cells was much higher in patients than in NCs (P = 0.0001). At the same time, <10% of CD4\(^+\) T cells bound annexin V in patients or NCs. This preferential loss of CD8\(^+\) T cells in patients with cancer is in contrast to well-documented apoptosis of CD4\(^+\) T cells in patients with HIV infection (19, 20). Because patients with HNC are often older than 50 years, we were concerned that the origin of sFasL found in the serum becomes an important question. Expression of FasL on the surface of SCCHN has been described by us, and we also have confirmed that this cell surface-associated FasL is functional (12). In addition, tumor cells are known to contain intracellular FasL, which may be secreted. Therefore, the tumor could be, in principle, responsible for inducing apoptosis in circulating T cells of patients with HNC, similar to its ex vivo effects on activated T lymphocytes described previously (12). Serum levels of sFasL in patients with active HNC were found to be significantly lower than those in NCs or in patients with NED. This finding is in contrast to several recent reports, in which increased sFasL serum levels comparable to 75–80-year-old NCs with respect to this parameter.

By multicolor flow cytometry, it was also possible to determine that Fas\(^+\) T cells were significantly more numerous in both CD8\(^+\) and CD4\(^+\) T cell subsets in patients with HNC than in NCs. Fas expression on CD8\(^+\) T cells was age dependent in NCs, but not in patients with HNC. Again, patients were comparable to 80-year-old NCs with regard to Fas expression on CD8\(^+\) T cells. No association of Fas expression with age was evident for CD4\(^+\) T cells.

Our previously published results also showed that expression of TeR-associated \(\zeta\) chain was down-regulated in CD3\(^+\) T cells of patients with HNC (5). Because a high proportion of these T cells expressed Fas, the obvious conclusion was that Fas\(^+\) CD3\(^+\) T cells were also \(\zeta\) low. The current experiments confirmed this conclusion and more strongly linked Fas expression and low \(\zeta\) to early apoptosis of T cells. This finding is in agreement with our data demonstrating that the \(\zeta\) chain, which contains amino acid motifs susceptible to cleavage by caspase-3 and caspase-7, is one of the substrates in the Fas-mediated death pathway (21). Degradation of the \(\zeta\) chain, an important signaling molecule in T cells, in a fraction of Fas\(^+\) CD3\(^+\) T cells during early apoptosis suggests that these circulating lymphocytes are destined to die and that the Fas/FasL pathway is involved in mediating their death.

In considering the possibility that binding of FasL is responsible for the death of Fas\(^+\) T cells in the patients’ circulation, the origin of sFasL found in the serum becomes an important question. Expression of FasL on the surface of SCCHN has been described by us, and we also have confirmed that this cell surface-associated FasL is functional (12).
were found in patients with large granular lymphocytic leukemia or lymphoma (22) and solid cancers (23, 24). It should be noted that all these studies used ELISA based on the use of 4H9 (capture) and 4A5 (detection) Abs (22). In contrast, using an ELISA from Oncogene Research Products, we consistently detect low levels of sFasL in sera of patients with cancer relative to NCs. This ELISA reliably measures levels of sFasL in supernatants of cells transfected with the human FasL gene.5

The subgroup of HNC patients with active advanced disease and the highest proportion of Fas\(^+\) annexin V-binding T cells was not found to have elevated levels of sFasL. We therefore suspected that sFasL in serum of these patients may be consumed by binding to Fas expressed on activated circulating T lymphocytes. Reports in the literature conflict with respect to the role of sFasL in mediating death signals; it apparently can function as an apoptosis-inducing agent or a blocking agent (25–27). These discrepant results could reflect differences in processing of FasL by different cells, leading to the generation of homotrimeric or monomeric sFasL with distinct binding characteristics and distinct functional capacities in mediating apoptosis. We tentatively interpret the low serum sFasL levels in patients with active HNC as indicative of sFasL consumption due to its binding to Fas\(^+\) T lymphocytes and inducing their death.

We have also attempted to correlate apoptosis of CD3\(^+\) T lymphocytes in HNC patients with disease activity as well as the known prognostic factors, such as tumor grade, stage, and nodal involvement. Patients with NED as well as active disease had significantly lower serum levels of sFasL than NCs or NED patients. These observations suggest that spontaneous apoptosis is related to disease activity and that it involves the use of sFasL, particularly in a

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5 T. L. Whiteside, unpublished data.

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Fig. 8 The CD3\(^+\) T lymphocytes in the peripheral circulation of patients with HNC have lower \(\zeta\) chain expression than CD3\(^+\) Fas\(^-\) T cells. A representative result obtained with PBMCs of one patient with HNC and of NC PBMCs tested in the same flow cytometry assay is shown. Similar results were seen with all other patients’ cells.

Fig. 9 Box plots comparing serum levels of sFasL in NCs (n = 35), HNC patients with NED (n = 18), and HNC patients with active disease (n = 19). See the Fig. 1 legend for the description of a box plot.
subgroup of patients with the highest percentages of circulating CD3⁺ Fas⁺ Anx⁺ lymphocytes. Once elevated, however, the proportion of apoptotic T cells does not decrease in patients who are clinically asymptomatic after therapy. This means that either occult disease is present and is recognized by the host immune system or that disease-related changes in lymphocyte turnover are long-lived and do not immediately return to normal in patients with NED. Apoptosis of T cells was significantly associated with the N stage (P < 0.0186) and a high grade of cancer (P < 0.0359) but not with T stage or with disease site. Furthermore, the proportion of annexin V-binding T cells in patients with active recurrent disease was comparable with that in patients with primary active disease. Thus, patients with tumors (primary or recurrent), as a group, did not have a higher mean percentage of CD3⁺ Anx⁺ T cells than patients with NED. Nevertheless, in a subgroup of HNC patients (n = 12), the percentages of apoptotic cells were the highest. This subgroup of HNC patients included seven patients with tumor-involved lymph nodes and thus with poor prognosis.

Our study demonstrates that a significant proportion of circulating T cells, especially CD8⁺ T lymphocytes, in patients with HNC is eliminated by apoptosis. These T cells are Fas⁺ and bind annexin V. Due to extensive apoptosis and concomitant repopulation of the blood compartment with T cells from the immature cell pool, rapid turnover of effector T cells takes place that is reminiscent of that described for patients with HIV (28, 29). This rapid turnover might be responsible for decreased proportions of CD8⁺ antitumor effector cells in the patients’ circulation. The Fas/FasL pathway, including sFasL in the serum, appears to be involved in the regulation of survival and death of circulating T cells in patients with cancer. Whereas this may not be the only responsible mechanism, its contribution to T-cell demise in patients with HNC is significant. A rapid lymphocyte turnover and the loss of CD8⁺ T cells might be critical factors responsible for weakened antitumor defense in these patients.

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Spontaneous Apoptosis of Circulating T Lymphocytes in Patients with Head and Neck Cancer and Its Clinical Importance

Thomas K. Hoffmann, Grzegorz Dworacki, Takashi Tsukihiro, et al.


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