Expression of Pro- and Antiapoptotic Proteins in Circulating CD8+ T Cells of Patients with Squamous Cell Carcinoma of the Head and Neck

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ABSTRACT

Objective: Apoptosis of T lymphocytes in the circulation of patients with squamous cell carcinoma of the head and neck (SCCHN) was shown to target effector CD8+ rather than CD4+ T cells. This study evaluates the contribution of pro- and antiapoptotic components of the mitochondria-dependent pathway to apoptosis of circulating CD8+ T cells in these patients.

Experimental Design: Blood samples were obtained from 77 patients with SCCHN and 51 normal control(s) (NC). Percentages of CD8+ Annexin V+ (ANX+) and CD8+CD95+ cells, changes in mitochondrial membrane potential and levels of expression of Bcl-2, Bcl-XL, and Bax in CD8+ T lymphocytes were measured by quantitative flow cytometry.

Results: Elevated percentages (P < 0.001) of early apoptotic (CD8+ANX+CD95+) T cells in the circulation distinguish SCCHN patients from NCs but not patients with no evidence of disease (NED) from those with active disease (AD). Circulating CD8+ but not CD4+ T cells in patients were found to contain higher levels of proapoptotic Bax and antiapoptotic Bcl-XL (P < 0.01) than NC cells. The Bax/Bcl-2 ratio was elevated in CD8+ T cells of patients relative to NCs (P < 0.01), and it correlated with the percentage of ANX+CD8+ T cells (P = 0.007). The Bax/Bcl-XL ratio discriminated AD from NED patients.

Conclusion: Apoptosis of circulating CD8+ T cells is found in SCCHN patients with AD or NED. Up-regulated Bax and Bcl-XL expression, the elevated Bax/Bcl-2 ratio and its association with ANX binding implicate the mitochondrial pathway in death of CD8+ T cells of patients with SCCHN. Understanding of molecular mechanisms of T-cell death and survival is essential for the development of more effective biotherapies for SCCHN.

INTRODUCTION

In patients with cancer, including squamous cell carcinoma of the head and neck (SCCHN), antitumor functions of T lymphocytes are often compromised (1, 2). One of the responsible mechanisms is extensive T-cell apoptosis, which has been demonstrated to involve T lymphocytes at the tumor site as well as those in the peripheral circulation of patients with SCCHN (3, 4) and with other cancers (5, 6). CD8+ T lymphocytes appear to be particularly sensitive to apoptosis in patients with SCCHN (3, 4), and the proportion of circulating proapoptotic CD8+ T cells is significantly higher in patients than in age-matched normal controls (NC; Ref. 3, 4, 7). Among CD8+ T lymphocytes, the effector cell subsets (i.e., CD8+CD45RO−CD27− or CD8+CD28−) appear to be preferentially targeted for apoptosis (8–10). Further, we recently have shown that tumor antigen-specific tetramer+CD8+ T cells are more sensitive to apoptosis than nontumor-specific CD8+ T cells in patients with SCCHN or with melanoma. Overall, it appears that T lymphocytes responsible for tumor epitope-specific effector activity are readily eliminated in patients with cancer, contributing to tumor escape.

It has been shown previously that most circulating CD3+CD8+ T lymphocytes targeted for apoptosis express Fas (CD95), and for this reason, the Fas/FasL pathway is considered, at least in part, responsible for the T-cell demise in patients with cancer (3, 11). On the other hand, in vitro experiments, involving coinoculation of activated T lymphocytes with tumor cells, indicate that both receptor-mediated and mitochondrial pathways mediate tumor-induced apoptosis of T cells (12). The role of the mitochondria-dependent pathway and its pro- or antiapoptotic components (13) in the death of circulating T cells in patients with SCCHN has not been investigated. Therefore, in this study, we evaluate expression of antiapoptotic proteins, such as Bcl-2 and Bcl-XL, as well as the proapoptotic member of the Bcl-2 family, Bax, in circulating CD8+ T cells of patients with SCCHN. We report that in Annexin-V binding (ANX+) CD8+ T cells, which are destined for apoptosis, expression of Bax and of Bcl-XL is significantly increased, whereas that of...
Bcl-2 remains unaltered relative to NC. This finding implicates the mitochondrial pathway in the control of survival and death of T cells in the peripheral circulation of patients with SCCHN.

**MATERIALS AND METHODS**

**Patients and Normal Controls.** Seventy-seven patients with SCCHN seen over a period of 4 years (2000–2003) in the Outpatient Otolaryngology Clinic at the University of Pittsburgh Cancer Institute were included in this study. Two patient cohorts were evaluated: the first included 41 patients seen from 2000 to 2002, and the second group of 36 patients was studied more recently (April–November, 2003). Among 77 patients, there were 42 with active disease (AD) whose blood was studied before surgery and 35 patients with no evidence of disease (NED) at the time of blood draws. All patients and NC (n = 51) signed the informed consent approved by the Institutional Review Board. Attempts were made to age match the patients and NC, but the mean age of patients was 59.4 years and that of NC was 54.9. The cohort of 36 patients whose specimens were acquired in 2003, included 21 with AD (14 males and 7 females) and 15 with NED (10 males and 5 females). Characteristics of the patients are presented in Table 1.

**Antibodies.** The FITC-conjugated anti-human CD95 monoclonal antibody (mAb) was obtained from PharMingen (San Diego, CA). Extra cellular domain-conjugated anti-human CD3 and PECy5-conjugated antihuman CD8 mAbs and 7-amino-actinomycin D were purchased from Immunotech (Mar- saille, France). FITC-conjugated ANX was also purchased from Immunotech. FITC-conjugated anti-human Bcl-2 mAb (IgG1) was obtained from DAKO Corp. (Carpenteria, CA), and FITC-conjugated anti-human Bcl-XL (IgG1) and Bax (IgG2b) mAbs were purchased from Santa Cruz Biotechnology Biotechnol- ogy (Santa Cruz, CA). Isotype control Abs (IgG1 and IgG2b) were obtained from Immunotech.

**Isolation of Peripheral Blood Mononuclear Cells(s) (PBMC).** Venous blood (30 ml) was collected from patients or NC into heparinized tubes and immediately delivered to the laboratory. PBMC were separated on Ficoll-Hypaque gradients (Amersham Biosciences, Uppsala, Sweden), and cells recovered from the gradient interface were washed in PBS, counted in a trypan blue dye and immediately used for experiments. The specimens from patients and NC were processed and tested simultaneously.

**CD95 Expression on T Cells.** To study surface expression of CD95 (Fas receptor) in CD8+ T cells, PBMC (1 × 10^6 cells/tube) were stained with FITC-conjugated anti-human CD95, extra cellular domain-conjugated anti-human CD3, and PECy5-conjugated anti-human CD8 for 30 min at 4°C. Isotype controls were included in all experiments. Cells were washed in cold PBS-sodium azide buffer, and the percentage of CD95+ cells was determined by multicolor flow cytometry.

**Analysis of Apoptosis.** To document apoptosis in CD8+ T cells, staining with ANX was performed. After surface staining with the labeled Abs, as described above, the cells were washed in ANX-binding buffer and incubated with FITC-conjugated ANX for 15 min at 4°C. Immediately after staining, the cells were analyzed by flow cytometry. An aliquot of PBMC was treated with UV-B light (213.6 mJ cm⁻²) to induce apoptosis and used as a positive control for ANX binding.

**Expression of the Bcl-2 Family Members in Lymphocytes.** Expression of antiapoptotic proteins, Bcl-2 and Bcl-XL, and the proapoptotic protein, Bax, was investigated in CD8+ or CD4+ T cells of SCCHN patients and NC using multicolor flow cytometry. PBMCs were first stained for surface expression of the CD3, CD4, or CD8 antigens, as described above. Staining for intracytoplasmic proteins was then performed as follows. PBMC were fixed with 1% (v/v) paraformaldehyde in PBS for 10 min at room temperature and then permeabilized with 0.1% (v/v) saponin (Sigma, St. Louis, MO) in PBS for 5 min at 4°C. Cells were next stained with FITC-conjugated anti-human Bcl-2, anti-Bcl-XL, or anti-Bax mAbs for 30 min at 4°C. FITC-conjugated mouse IgG1, and IgG2b were used as isotype controls. After several washes with 0.1% saponin solution, cells were analyzed by multicolor flow cytometry.

**Mitochondrial Membrane Potential (MMP).** Flow cytometry-based assay was used to measure loss of MMP in freshly isolated PBMC, gating on CD8+ T cells or in PBMC incubated in medium at 37°C for 18 h. An aliquot of PBMC was incubated for 15 min at 37°C in AIM-V medium containing 200 nM tetramethyl rhodamine methylester (Molecular Probes, Eugene, OR). As a positive control for a loss of MMP, another aliquot of PBMC was treated with 5 mM mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxy phenylhydrazone (Sigma) during the staining period. The red tetramethyl rhodamine methylester fluorescence was analyzed using FL2 in a flow cytometer immediately after staining.

**Flow Cytometry.** Four-color flow cytometry was performed on a Coulter EPICS XL instrument (Beckman Coulter,
the expression levels of Bcl-2, Bcl-XL, or Bax in CD4 T cells of patients with SCCHN. Linear regression analysis was
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The mean fluorescence intensity was calculated for Bcl-2, Bcl-XL, and Bax in each subset of CD8+ T cells separately using

results show that both CD95+ expression on and ANX binding to CD3+CD8+ T cells are significantly higher (P < 0.0001) in patients than NC (Fig. 1). Although ANX binding and expression of CD95 discriminated between patients with SCCHN and NC, neither characteristic was able to distinguish AD from NED groups (Fig. 1).

Our previous data indicate that in patients with SCCHN, ANX binds mainly to CD95+CD8+ T cells, as previously demonstrated by double staining for ANX binding and CD95 expression (3, 5, 6). The more detailed analysis of the data shown in Fig. 2 suggests that in the circulation of NC, relatively few CD95+ cells bind ANX, as the percent of CD95+ cells is much higher than that of ANX+ cells (Fig. 2). In patients with SCCHN, whereas the percentages of both CD95+ and ANX+ cells are significantly elevated, proportions of CD95+ cells still exceed the percent of ANX+ cells, and no evident correlation exists between the two markers. Thus, expression of CD95 on CD8+ T cells may or may not be followed or accompanied by ANX binding. This suggests that the molecular mechanisms responsible for spontaneous apoptosis of circulating CD8+ T cells in patients with SCCHN involve not only the external or Fas receptor-mediated signals but are also regulated by other signals. The most likely possibility is that intrinsic or mitochondrial pathway also contributes to T-cell apoptosis or survival. To evaluate this possibility, we examined CD8+ T cells obtained from a smaller cohort of 36 SCCHN patients and 11 NC for expression of the Bcl-2 family members, which are known to responsible for spontaneous apoptosis of circulating CD8+ T cells.

RESULTS

Apoptosis of CD8+ T Cells and CD95 Expression.

was considered to be statistically significant.

discrimination between live and early apoptotic cells exists between the two markers. Thus, expression of CD95 on CD8+ T lymphocytes involves not only the external or Fas receptor-mediated signals but are also regulated by other signals. The most likely possibility is that intrinsic or mitochondrial pathway also contributes to T-cell apoptosis or survival. To evaluate this possibility, we examined CD8+ T cells obtained from a smaller cohort of 36 SCCHN patients and 11 NC for expression of the Bcl-2 family members, which are known to play a central role in regulation of cellular death or survival (13).
We used a quantitative flow cytometry-based method allowing for precise measurements of expression levels of the Bcl-2 family members in a defined T-cell subset.

**Bcl-2, Bcl-XL, and Bax Expression in CD8+ T Cells.** The Bcl-2 family members are expressed in circulating CD3+CD8+ T cells, but the expression levels for different members of this large family (11) were found to differ in cells of SCCHN patients relative to those of NC. As shown in Fig. 3, Bcl-2 expression in CD8+ T lymphocytes of a representative SCCHN patient was comparable with that in a NC. In contrast, both Bax and Bcl-XL levels were significantly elevated in CD8+ T cells of this patient relative to NC. Significantly elevated levels of Bax ($P < 0.01$) and Bcl-XL ($P < 0.03$), but not of Bcl-2, were consistently seen in CD8+ T cells of patients compared with NC (Figs. 4, A and B). Consequently, the Bax/Bcl-2 ratio was significantly higher in CD8+ T cells of SCCHN patients than in NC ($P = 0.008$). Importantly, expression levels of Bcl-2, Bcl-XL, or Bax and the Bax/Bcl-2 ratio were not significantly different in CD4+ T cells of NC and SCCHN patients (data not shown).

The Bax/Bcl-XL ratio was also determined for CD8+ T cells and found to be no different in SCCHN patients and NC (Fig. 4B), presumably because the expression levels of both Bax and Bcl-XL were increased in CD8+ T cells of SCCHN patients. These observations suggested that the intracellular levels of the two antiapoptotic proteins, Bcl-2 and Bcl-XL, might be regulated differently relative to Bax expression in CD8+ T cells of patients with SCCHN. Next, levels of Bax in CD8+ T cells of individual patients with SCCHN and of NC were compared with those of Bcl-XL or Bcl-2. As shown in Fig. 5, the intracellular expression levels of Bcl-XL positively correlated with increased Bax expression in the CD8+ T lymphocytes of patients. In contrast to Bcl-XL, the levels of Bcl-2 were not increased in the CD8+ T cells of patients. Also, a significantly higher number of patients than NC had circulating CD8+ T cells with elevated Bax and Bcl-XL levels (Fig. 5). In aggregate, these data suggest that Bcl-XL plays a more significant biological role than Bcl-2 in CD8+ T cells of patients with SCCHN.

We next compared expression levels of Bcl-2, Bcl-XL, and Bax in CD4+ and CD8+ T cells of patients with SCCHN. Fig. 6 shows that in the patients, up-regulated expression of Bax and Bcl-XL is confined to CD8+ T cells and that Bcl-2 expression is similar or decreased in CD8+ versus CD4+ T cells. These data support the notion that elevated expression of Bax and Bcl-XL might be related to apoptosis of CD8+ T cells in the circulation of patients with SCCHN.

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**Fig. 2** Percentages of circulating CD8+ CD95+ T cells are higher than those of CD8+Annexin V+ (ANX V) cells in patients with squamous cell carcinoma of the head and neck (SCCHN) and in NC. The stippled lines indicate the normal means. The circle shows that both markers are significantly higher in the patients than in NC ($P < 0.0001$).

**Fig. 3** Expression levels of Bcl-XL and Bax, but not Bcl-2, are up-regulated in circulating CD3+CD8+ T cells obtained from a representative patient with SCCHN relative to those in a NC. Isotype controls were used and the backgate was set on CD8+ T cells. Molecules of equivalent soluble fluorochrome (MESF) units for each component of the Bcl-2 family are indicated. FITC, fluorescein isothiocyanate.
ANX Binding and Expression of the Bcl-2 Family Members in CD8+ T Cells. To evaluate the involvement of Bax, Bcl-2, and Bcl-XL in apoptosis of CD8+ T cells of patients with SCCHN, intracytoplasmic levels of proapoptotic Bax were related to those of the antiapoptotic proteins and then correlated to ANX binding. We observed a significant positive correlation ($P < 0.007$) between the Bax/Bcl-2 ratio and the percentage of ANX+ cells in CD8+ T cells of patients with SCCHN (Fig. 7A). This finding indicates that the mitochondrial pathway is involved in apoptosis of circulating CD8+ T cells in these patients. On the other hand, no significant correlation could be established between the Bax/Bcl-XL ratio and ANX binding to CD8+ T cells (Fig. 7B). In fact, ANX+CD8+ T cells tended to have higher levels of Bcl-XL than Bax ($i.e.$, a lower Bax/Bcl-XL ratio), indicating perhaps that a compensatory increase in the Bcl-XL protein level allows these cells to maintain viability and remain in the circulation. No significant correlations between CD95 expression on CD8+ T cells and Bax or Bcl-XL levels were evident in patients with SCCHN (data not shown).

Mitochondrial Membrane Potential Assessments. A flow cytometry-based assay (tetramethyl rhodamine methyl-ester) was established to measure changes in MMP in CD8+ T cells obtained from the peripheral circulation of patients with SCCHN (14). The assay was standardized using Jurkat cells treated with CH-11 mAb to induce apoptosis via the receptor-pathway. Although the assay precisely measured a loss of MMP in Jurkat cells (data not shown), it was not informative when applied to testing of MMP changes in CD8+ T cells freshly obtained from patients and NC because a loss of MMP in circulating CD8+ T cells obtained from patients with SCCHN and NC was not significantly different. To explain this potentially confounding result, freshly isolated PBMC of SCCHN patients were tested for ANX binding and MMP loss, then incubated in medium at 37°C for 24 h (15) and retested in both assays. As shown in Fig. 8, ANX+CD8+ T cells were at 37% versus 7% cells with MMP loss immediately after phlebotomy. As expected, ANX binds to early apoptotic cells without detectable mitochondrial changes. After incubation of PBMC, as CD8+ T cells progress to later stages of apoptosis, ANX binding goes up and concomitantly, a loss of MMP becomes detectable. Thus, only cells in very early apoptosis are detectable in the peripheral circulation of patients by ANX binding. CD8+ T cells with a loss of MMP ($i.e.$, those in later stages of apoptosis)}
are efficiently cleared and thus not detectable in the circulation of patients.

Expression of the Apoptosis-Related Markers and Disease. Up-regulation in expression CD95, Bax, Bcl-XL, and in ANX binding were consistently and significantly greater in patients with cancer than in NC (Figs. 1, 2, 4). However, as indicated above, neither expression of CD95 nor ANX binding discriminated patients with AD from those with NED. This result suggests that elevated ANX binding and CD95 positivity are stable features of CD3+/CD8+ T cells in patients with SCCHN and do not significantly change after curative therapies. When the Bax/Bcl-2 ratios were compared, no difference was observed between patients with AD and NED. In contrast, Bcl-XL expression was found to be significantly decreased ($P < 0.05$) in CD8+ T cells in patients with NED, resulting in the significantly higher Bax/Bcl-XL ratio in NED patients relative to the patients with AD (Fig. 9). These data suggest that Bax and not Bcl-XL determine the sensitivity to apoptosis of circulating CD8+ T cells in patients with both AD and NED.

Studies of expression of apoptosis-related markers in CD8+ T cells of patients with AD at the time of blood draws showed that none convincingly discriminated early from advanced-stage (I/II versus III/IV) disease (Table 2). Only small numbers of patients were studied, however, and trends toward higher values for Bax or Bcl-XL expression in cells of patients with more advanced disease are evident in Table 2. Importantly, the evidence of nodal disease was associated with a significant up-regulation in Bax and Bcl-XL expression as well as in the Bax/Bcl-2 ratio, indicating that in SCCHN patients with a poor prognosis, these apoptosis-related markers may have special significance.

**Fig. 6** Comparisons of the expression levels of Bcl-2, Bcl-XL, and Bax in circulating CD4+ and CD8+ T cells of patients with squamous cell carcinoma of the head and neck (SCCHN). Paired analysis showed a significantly higher expression for Bcl-XL and Bax in CD8+ than CD4+ T cells. MESF, molecules of equivalent soluble fluorochrome.
DISCUSSION

Apoptosis of T cells in the circulation of cancer patients has consistently been shown to involve more CD8+ than CD4+ T cells in our studies (3, 5–7, 15). This apoptosis is viewed as a manifestation of the tumor escape in patients with AD and as a long-lived consequence of malignancy and perhaps also anti-tumor therapy in patients with NED. Our studies have shown that apoptosis of circulating CD8+ T cells preferentially targets various mature effector T cell subsets (9, 10) and that clonally restricted as well as tumor epitope-specific (tetramer+) T cells are also preferentially destined to apoptose. In AD patients studied before therapy, apoptosis of tumor-infiltrating as well as circulating CD8+ T cells has been linked to their chronic activation state, expression of CD95 on the T-cell surface and defective signaling via T-cell receptor (2, 4, 8). In patients with NED, persistent apoptosis of circulating CD8+ T cells is more difficult to explain. It is the fact, however, that ANX binding to CD8+ T cells remains elevated for months even years after therapy, and that absolute numbers of T cells do not normalize in SCCHN patients presumably cured of their cancer (16). We hypothesize that once cancer induces death functional abnormalities in effector T cells and alters their homeostasis, restoration of a normal lymphocyte turnover and immune balance becomes very difficult. Furthermore, persistent apoptosis and altered lymphocyte homeostasis might explain the inability of these “cured” patients to mount effective antitumor responses and may be the reason for frequent recurrences of cancer after therapy.

The current study was undertaken to explore the contribution of mitochondrial pathway to apoptosis of circulating CD8+

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**Fig. 8** Annexin-V (ANX V) binding and mitochondrial membrane potential (MMP) loss in CD8+ T cells in fresh isolation of peripheral blood mononuclear cells (PBMC; A) and after the same PBMCs were incubated in medium at 37°C for 24 h. Note a significant increase in both ANX V+CD8+ T cells and CD8+ T cells with a lower MMP. *Ex vivo* incubation was performed as described previously (15).

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**Fig. 9** Expression of Bcl-XL, but not Bax, is significantly decreased (p < 0.05) in circulating CD8+ T cells of patients with no evidence of disease (NED). The Bax/Bcl-XL ratio distinguishes patients with active disease (AD) from those with NED.
Bcl-2 and Bcl-XL in Apoptosis of CD8+ T Cells in Cancer

In support of the above hypothesis, this study shows that the mitochondria-dependent pathway is, in part, involved in spontaneous apoptosis of circulating T cells in patients with SCCHN. Mitochondrial response to apoptotic stimuli is determined by the balance between the pro- and antiapoptotic Bcl-2 family members. Antiapoptotic Bcl-2 family members, including Bcl-2 and Bcl-XL, protect against mitochondrial apoptotic events, whereas proapoptotic members such as Bax promote the release of apoptogenic proteins from the mitochondria. It is now known that the release of apoptogenic factors, such as cytochrome c, Smac/Diablo, apoptosis-inducing factor, and others from the intermembrane space in mitochondria requires translocation of Bax from the cytosol into the outer membrane of mitochondria and formation of Bax homodimers (21). Because Bcl-2 and Bcl-XL, which are mainly located in the outer membrane of mitochondria, can bind to Bax and prevent it from forming homodimers, relative changes of Bcl-2, Bcl-XL, and Bax vary in disease, including malignancy (20, 22-27). We found that in SCCHN patients, Bcl-2 expression in T cells was not altered compared with that in T cells of NC. In contrast, Bcl-XL and Bax expression levels were up-regulated in the T cells of patients, and quantitative expression data obtained by flow cytometry in this study are more robust than any of the methods used previously. The increased Bax/Bcl-2 ratio was associated with enhanced apoptosis of CD8+ T cells in the patients. Therefore, circulating ANX+CD8+ T cells in patients with SCCHN clearly are susceptible to apoptosis mediated through the mitochondrial pathway. However, elevated levels of an antiapoptotic mitochondrial protein, Bcl-XL, seen in these cells suggest that it may be compensating for an increase in proapoptotic Bax. If so, then the role of mitochondria in spontaneous apoptosis of effector CD8+ T cells might be largely confined to protective effects. It is clear that compensatory mechanisms are involved in protection of at least some ANX+CD8+ T cells, because the addition of cytokines is able to rescue CD8+ T cells of patients from apoptosis, as indicated by our ex vivo experiments.6 Nevertheless, concomitant elevations of Bax and Bcl-XL expression seen in CD8+ T cells of patients with advanced nodal disease seem to suggest a role for Bcl-XL in the proapoptotic rather than protective mechanisms.

The main objective of our studies was to evaluate the phenomenon of spontaneous CD8+ T cell apoptosis in the context of the disease and its activity. The focus on apoptosis of CD8+ T cells is particularly important in patients with cancer because of the crucial role this subset of immune cells is thought to play in antitumor responses (1, 11). Interestingly, the markers of apoptosis, specifically CD95 or Bax expression, the Bax/Bcl-2 ratio, or ANX binding to CD8+ T cells, consistently discriminated among patients with SCCHN and NC but did not

T cells in patients with SCCHN. Both Fas receptor-mediated and mitochondria-dependent apoptotic pathways could be responsible for the higher susceptibility to spontaneous apoptosis of CD8+ T cells in patients with cancer. Our studies have initially focused on the former mechanism, because of the finding that Fas is expressed on the surface of a high percentage of circulating lymphocytes in patients with malignancies (4, 5, 17-20) and that these Fas+ (CD95+) T cells have higher caspase activity and lower T-cell receptor chain expression than the counterparts in NC (3, 5). Although we showed earlier that ANX binds to CD95+CD8+ T cells (3, 6), the percentages of CD95+CD8+ T cells clearly exceed those of ANX-binding cells (Fig. 2). This implies that expression of CD95 is not always accompanied by ANX binding and suggests that mechanisms not involving the Fas/Fasl pathway may be responsible for regulating apoptosis of CD8+ T cells in the peripheral circulation of patients with cancer.

In support of the above hypothesis, this study shows that the mitochondria-dependent pathway is, in part, involved in spontaneous apoptosis of circulating T cells in patients with SCCHN. Mitochondrial response to apoptotic stimuli is determined by the balance between the pro- and antiapoptotic Bcl-2 family members. Antiapoptotic Bcl-2 family members, including Bcl-2 and Bcl-XL, protect against mitochondrial apoptotic events, whereas proapoptotic members such as Bax promote the release of apoptogenic proteins from the mitochondria. It is now known that the release of apoptogenic factors, such as cytochrome c, Smac/Diablo, apoptosis-inducing factor, and others from the intermembrane space in mitochondria requires translocation of Bax from the cytosol into the outer membrane of mitochondria and formation of Bax homodimers (21). Because Bcl-2 and Bcl-XL, which are mainly located in the outer membrane of mitochondria, can bind to Bax and prevent it from forming homodimers, relative changes of Bcl-2, Bcl-XL, and Bax expression may be more significant than absolute changes of individual Bcl-2 family members. Indeed, it has been reported that levels of Bcl-2,

### Table 2

Expression of apoptosis-related markers in CD3+CD8+ T cells of patients with active disease stratified according to the disease status *

<table>
<thead>
<tr>
<th>Units</th>
<th>%</th>
<th>Bax/Bcl-2</th>
<th>MESF × 10³</th>
</tr>
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<tbody>
<tr>
<td>ANX+</td>
<td>CD95+</td>
<td>Bcl-XL</td>
<td>Bax</td>
</tr>
<tr>
<td>Disease stage</td>
<td></td>
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<td></td>
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<tr>
<td>I or II</td>
<td>32 ± 12</td>
<td>70 ± 19</td>
<td>0.32 ± 0.05</td>
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<td>(n = 17)</td>
<td>(n = 17)</td>
<td>(n = 13)</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>III or IV</td>
<td>47 ± 20</td>
<td>76 ± 14</td>
<td>0.42 ± 0.13</td>
</tr>
<tr>
<td>(n = 25)</td>
<td>(n = 25)</td>
<td>(n = 13)</td>
<td>(n = 13)</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
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</tr>
<tr>
<td>T₁ or T₂</td>
<td>33 ± 13</td>
<td>72 ± 16</td>
<td>0.35 ± 0.05</td>
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<tr>
<td>(n = 26)</td>
<td>(n = 26)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
</tr>
<tr>
<td>T₁ or T₄</td>
<td>37 ± 23</td>
<td>76 ± 17</td>
<td>0.46 ± 0.16</td>
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<td>(n = 16)</td>
<td>(n = 16)</td>
<td>(n = 6)</td>
<td>(n = 6)</td>
</tr>
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<td>N stage</td>
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<td></td>
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</tr>
<tr>
<td>N₀</td>
<td>31 ± 13</td>
<td>72 ± 18</td>
<td>0.31 ± 0.05</td>
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<td>(n = 24)</td>
<td>(n = 24)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>N₁, N₂, N₃</td>
<td>40 ± 21</td>
<td>75 ± 14</td>
<td>0.43 ± 0.013 †</td>
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<td>(n = 18)</td>
<td>(n = 18)</td>
<td>(n = 11)</td>
<td>(n = 11)</td>
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* The data are means ± SD. The numbers of patients studied are in parentheses.
† Indicates a significant difference (P < 0.05) between patients with or without nodal involvement.

* Unpublished data.
distinguish AD from NED patients. Thus, it would appear that CD8+ T cells of patients with cancer, regardless of the disease activity or stage, are highly susceptible to apoptosis. This observation suggests that immune dysregulation induced by the disease does not readily recover after curative therapies but persists for weeks or months afterward. Evidence for persistently decreased total lymphocyte counts in patients with NED reported previously by us (16) is consistent with the hypothesis that dysregulated lymphocyte homeostasis may be long-lived and not readily corrected even in patients who are cured by anticancer therapies.

Clinical significance of immunological “markers” for SCCHN was emphasized in a recent study by Shibuya et. al (28). These investigators found that a proliferative response of lymphocytes to CD3 antibodies was the single greatest predictor of reduced disease-free interval (28). Both the presence at the tumor site of T cells with functioning T-cell receptor, as measured by expression of the ζ chain, and of dendritic cell infiltrates were found to be highly significant and robust predictors of a better prognosis and a better 5-year survival in patients with oral carcinoma (29, 30). These studies strongly suggest that functional T cells at the tumor site are important for an effective control of tumor progression by the immune system. In SCCHN, tumor-associated dysfunction of T cells often extends to include T cells in the peripheral circulation (3–8). The present study, focused on apoptosis of circulating CD8+ T cells, indicates that in active disease, pro- and antiapoptotic proteins, Bcl-2, Bcl-XL, or Bax, might be potentially useful markers of immune competence. Thus, the Bax/Bcl-2 ratio or Bax and Bcl-XL expression in CD8+ T cells are significantly different in patients with nodal disease versus those without nodal involvement. There is also a trend of increased values for these markers in more advanced (T3 or T4) disease, despite a small number of patients in the study groups (Table 2). Additional experiments are needed to confirm these data. The demise or survival of CD8+ T cells in patients with SCCHN is a complex process regulated in part by mechanisms related to the external and intracytoplasmic pathways of apoptosis (13). In this context, it might be first necessary to acquire understanding of interactions between individual components of these pathways in disease to be able to discriminate between immunologically competent patients with a better prognosis from those who are immunologically compromised and thus have a worse prognosis.

In conclusion, our data documenting a simultaneous up-regulation in the Bax/Bcl-2 ratio and Bcl-XL expression levels in circulating CD8+ T cells and a strong association of Bax expression with spontaneous apoptosis of CD8+ T cells in patients with SCCHN are compelling. The mitochondria, in addition to the Fas-mediated pathway, seem to be involved in the regulation of apoptosis of circulating CD8+ T cells. Mitochondrial antiapoptotic protein, Bcl-XL, seems to play an especially important compensatory role in protecting these T cells from demise, but it also may contribute to regulation of proapoptotic Bax by a distinct mechanism. Clearly, additional studies correlating expression of apoptotic markers with prognostic factors and survival data are necessary to determine the impact of CD8+ T cell apoptosis on disease progression.

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Expression of Pro- and Antiapoptotic Proteins in Circulating CD8+ T Cells of Patients with Squamous Cell Carcinoma of the Head and Neck

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