Advances in Brief

Spontaneous ex Vivo Apoptosis of Peripheral Blood Mononuclear Cells in Patients with Head and Neck Cancer

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Abstract

Proportions of apoptotic (TUNEL+) peripheral blood mononuclear cells (PBMCs) were measured by flow cytometry in patients with head and neck cancer and normal controls at the time of blood draws (0 time) and after 24-h incubation. PBMCs were incubated at 37°C in medium (spontaneous apoptosis) and in the presence of CH-11 anti-Fas antibody (anti-Fas) or tumor necrosis factor (TNF)-α, both capable of inducing DNA fragmentation in activated T cells expressing the TNF family of receptors. PBMCs obtained from the patients had significantly higher (P < 0.0001) proportion of apoptotic cells than PBMCs of controls at 0 time as well as after 24-h incubation. Ex vivo apoptosis included all subsets of PBMCs: CD3+ T cells, CD16+CD56+ natural killer cells, CD19+ B cells, and CD14+ monocytes, as determined by two-color flow cytometry. However, T cells represented the largest PBMC subset undergoing apoptosis, and lymphocytes rather than monocytes were the major TUNEL+ PBMC population. Among T cells, the level of spontaneous ex vivo apoptosis was nearly as high as that of CH-11 antibody-induced or TNF-α-induced apoptosis, indicating that activated Fas+ and TNFR1+ T cells were pre-programmed in vivo to die. Also, elevated levels of spontaneous apoptosis at time 0 in patients with head and neck cancer (P < 0.0001) indicated that a higher fraction of PBMCs was undergoing apoptosis in vivo in patients than controls. Together, the data suggest that an increased rate of turnover of lymphocytes is associated with cancer and may be responsible for functional lymphocyte imbalance, even in treated patients who have no evident disease.

Introduction

The tumor microenvironment may influence the functional potential of immune cells that accumulate at the site of tumor growth or metastasis. Evidence has accumulated that TILs isolated from human tumors and tested for phenotypic and functional characteristics express activation markers (1–3) and yet are functionally impaired (1–6). In addition to impairments in cell proliferation in response to mitogens as well as tumor-associated antigens, fresh TILs or tumor-associated lymphocytes are poorly cytotoxic against tumor targets, have an altered cytokine profile, including deficiencies in interleukin 2 and IFN-α and do not signal normally upon T-cell receptor engagement (1, 2, 7–12). More recent data from our and other laboratories indicate that lymphocytes undergo apoptosis at the tumor site (5, 13–16). The ability of human tumors to induce apoptosis in activated normal lymphocytes has been confirmed by in vitro experiments in which fresh or cultured tumor cells were shown to induce caspase activation and DNA fragmentation in lymphocytes coincubated with fresh or cultured tumor cells for 24 h or less in the absence of any other apoptosis-inducing agent (13).

Immunoeffector cells obtained from the peripheral blood of patients with cancer, including HNC, have also been reported to have a variety of functional abnormalities, which may vary in magnitude from patient to patient and which may be related to the extent of disease (1, 5, 9, 17). Thus, it appears that immunoinhibitory influence of the tumor might extend far beyond the tumor microenvironment and might become a systemic effect, especially in patients with advanced metastatic disease. The possibility that immune cells may be actively dying in patients with cancer and that a rapid turnover of these cells occurs as a consequence of this process has profound implications for the development of adequate antitumor immune responses and for immunotherapy of cancer. Clearly, the ability to assess the level of this turnover might be viewed as an important component of or indicator for therapeutic interventions aimed at the protection of immune cells from apoptosis.

We have observed recently that mononuclear cells obtained from the peripheral blood of patients with HNC spontaneously undergo DNA cleavage upon their incubation in culture medium for several hours. In contrast, PBMCs of normal individuals have very low levels of ex vivo apoptosis under the same...
experimental conditions. This observation has prompted us to investigate the nature of this \textit{ex vivo} phenomenon and to provide a rationale and evidence for the hypothesis that a substantial number of mononuclear cells in the peripheral circulation of patients with HNC are preprogrammed \textit{in vivo} to undergo apoptosis.

Materials and Methods

\textbf{Patients and Controls.} Twenty-two patients with histologically proven SCCHN, who were consecutively seen at the University of Pittsburgh Medical Center (Pittsburgh, PA) between January 1998 and June 1998, were included in this study. All patients signed the informed consent. The patients underwent surgery and, when indicated, radio- or chemotherapy for their tumors at various time periods (1 month to 17 years; median, 28 months) prior to the visit during which they donated peripheral blood for the study. At the time of blood draws, all of the patients had NED. There were eight patients with stage I, four with stage II, four with stage III, and six with stage IV disease. Comorbidity was retrospectively graded according to the method of Kaplin-Finestein. No patients had grade 2 or 3 comorbidity, and no patient was on \textit{beta} blockers or calcium channel inhibitors. One patient was taking oral prednisone.

\begin{table}
\centering
\caption{Characteristics of patients with SCCHN included in the study.} 
\begin{tabular}{cccccccc}
\hline
Patient no. & Site & Age & Sex & Stage & Differ. & Treatment & Rec. & Disease course & Time from last surgery \\
\hline
1 & Oral & 58 & F & I & Mod & Surg & NED & 8 months \\
2 & Larynx & 46 & F & I & Mod & Surg & NED & 1 year 7 months \\
3 & Larynx & 65 & M & I & Mod & Surg & NED & 4 years \\
4 & Larynx & 66 & M & I & Mod & Surg & NED & 17 years 4 months \\
5 & Oral & 70 & F & I & Well & Surg & NED & 2 months \\
6 & Larynx & 74 & M & I & Well & Surg & NED & 5 years 5 months \\
7 & Skin & 57 & F & I & Well & Surg & NED & 3 years 5 months \\
8 & Man & 76 & F & IV & Well & Surg & NED & 5 months \\
9 & Oral & 83 & M & IV & Mod & Surg & NED & 4 years 7 months \\
10 & Larynx & 60 & M & IV & Mod & Surg & NED & 1 year 3 months \\
11 & Pharynx & 56 & M & III & Well & Surg & NED & 2 years 5 months \\
12 & Larynx & 68 & M & IV & Mod & Surg & NED & 6 months \\
13 & Larynx & 78 & M & III & Mod & Surg & NED & 4 months \\
14 & Larynx & 76 & M & I & Mod & Surg & NED & 3 years 11 months \\
15 & Pharynx & 66 & M & IV & Mod & Surg & NED & 4 years 6 months \\
16 & Oral & 41 & F & I & Mod & Surg & NED & 6 years 1 month \\
17 & Oral & 51 & M & IV & Mod & Surg & NED & 1 month \\
18 & Oral & 55 & M & II & Poor & Surg & T & 2 months \\
19 & Oral & 71 & M & II & Well & Surg & NED & 2 years 2 months \\
20 & Larynx & 78 & M & III & Mod & Surg & NED & 6 months \\
21 & Larynx & 58 & M & III & Mod & Surg & NED & 3 years 11 months \\
22 & Oral & 33 & M & II & Mod & Surg & NED & 2 years 11 months \\
\hline
\end{tabular}
\end{table}

\begin{itemize}
\item[a] Tumor differentiation determined by histopathology.
\item[b] Surg., surgery; Rad, radiation; Chem, chemotherapy.
\item[c] Rec., recurrence; N, involving cervical lymph nodes; T, local recurrence.
\end{itemize}

Table 1 lists patient characteristics, including sex (16 males and 6 females) and age (median, 65 years), tumor site and stage, the presence and number of recurrent or second primary tumors, and clinical status of at the time of a blood donation for this study.

Normal volunteers (\textit{n} = 16) were recruited from among the laboratory personnel and included 6 males and 10 females. The controls were younger than patients, and their median age was 33 years. All normal donors were in excellent health at the time of this study, and none had a history of smoking or drinking. In addition, a smaller group of seven normal controls who were age matched with the patients (median age, 66 years; 6 males and 1 female) were also included. In all experiments, each patient specimen was always tested in parallel with a normal control. Patient and control specimens were processed and handled in the same way, and patient samples were always tested together with at least one laboratory control.

\textbf{PBMCs.} Venous blood was obtained from patients and controls (20 mls) in the morning and collected into heparinized tubes. Blood samples were hand carried to the laboratory and processed by Ficoll-Hypaque gradient centrifugation. PBMCs were recovered from the gradient interface, washed in DPBS, counted in trypan blue, and immediately used for experiments.

The experimental design for \textit{ex vivo} apoptosis of PBMCs is outlined in Fig. 1. To determine the proportion of PBMCs with evidence of DNA fragmentation, a TUNEL assay (see below) was performed on an aliquot (3.5 \times 10^6 cells) of patients' or control cells immediately after their isolation (\textit{i.e.}, at time 0). The remaining PBMCs were divided into aliquots (3.5 \times 10^6 cells), and each aliquot was incubated for 24 h at 37°C under the following conditions: (a) medium alone; (b) medium plus CH-11 Ab (agonistic anti-Fas Ab; UBI Biotechnology, Lake Placid, NY) at the concentration of 400 ng/ml; and (c) medium plus recombinant TNF-\alpha (Knoll Pharmaceuticals, Whippany, NJ) used at the concentration of 50 ng/ml. After incubation, cells were harvested and immediately tested in TUNEL assays for evidence of apoptosis. The proportions of PBMCs that underwent spontaneous apoptosis (medium alone) or induced apoptosis (medium plus CH-11 Ab or medium plus TNF-\alpha) during the 24-h incubation period were determined by flow cytometry. The medium used for all experiments was RPMI 1640; it was supplemented with 1 mm l-glutamine, 10\% (\textit{v/v}) of
PBMCs were washed twice in DPBS containing 0.1% BSA. PBMCs were washed in DPBS (Life Technologies, Inc.), divided into 5

PBMCs were washed twice in DPBS containing 0.1% BSA and 0.1% NaN₃ and fixed with 2% (w/v) paraformaldehyde in DPBS for 30 min at room temperature prior to flow cytometry.

The TUNEL assay was performed to study DNA fragmentation in freshly harvested or ex vivo incubated cells. PBMCs were washed after fixation with paraformaldehyde and permeabilized with 0.1% (w/v) sodium citrate in phosphate saline buffer containing 0.1% (v/v) Triton X-100 for 3 min on ice. After washing, cells were incubated with FITC-conjugated dUTP in the presence of terminal deoxynucleotidyl transferase enzyme solution for 1 h at 37°C, using reagents purchased from Boehringer Mannheim Corp. (Indianapolis, IN). After incubation, the cells were washed, and 10,000 events were acquired and analyzed by two-color analysis, using FACScan. Controls included cells incubated without the enzyme in labeling buffer. To determine the assay reliability, intra- and inter-assay correlations were established based on blood samples collected from six normal individuals on different days and tests after splitting each sample into three aliquots. The analysis of this data set indicated that the assay was highly reliable, with the Interassay correlation of 0.909.

Flow Cytometry. Two-color flow cytometry was performed on a FACScan (Becton Dickinson) equipped with a single 488-nm argon ion laser. To reliably detect small subpopulations of cells, at least 10,000 events were acquired for each sample. The following settings were used: 640 and 615 V on photomultiplier tubes for FL1 (FITC-TUNEL) and FL2 (PE), respectively. FITC and PE fluorescence were measured through 530/30 and 585/42 bandpass filters. Compensation for FL1-%FL2 and FL2-%FL1 was 0.7% and 29%, respectively. Threshold was set on 30 for forward scatter.

The percentages of apoptotic cells were calculated by scoring TUNEL⁺ cells in two-color gated subpopulations: CD3⁺/TUNEL⁺, CD3⁻/TUNEL⁺ in comparison to CD3⁻/TUNEL⁻, and CD3⁺/TUNEL⁻. All of the gated mononuclear cell sub-populations were visualized on FCS/SSC dot-plots. To include all apoptotic cells and avoid debris with a high SSC signal, the gate was set to include a wide boundary of mononuclear cells ("open gate"), because apoptotic cells accumulated mainly in the lower FSC/SSC channels (18). The strategy of double gating eliminated a majority of debris, which usually overlaps with apoptotic cells. The level of debris within the gated populations did not exceed 10%, and it was not significantly higher in samples with high than low (control) apoptotic values. MFI was determined for all gated cell populations.

The percentages or MFI of Fas⁺ or TNFR1⁺ PBMCs were determined after staining of the cells with anti-Fas Ab (ZB4; Upstate Technology, Lake Placid, NY) or with Ab to TNFR1 (R&D Systems, Minneapolis, MN). For these determinations, we individually gated on lymphocytes and monocytes, based on the FCS/SSC dot-plots.

Cytospin Preparation. PBMCs obtained from patients and normal control samples were doubly stained for TUNEL and CD3 surface marker, as described above. Stained cells were centrifuged onto glass slides, using a cytocentrifuge (Cytospin 2; Shandon) and directly mounted with fluorescent mounting medium (Dako). The slides were examined independently by two observers, using a fluorescent microscope (Microphot-FX; Nikon), and photographs of representative fields were taken, using appropriate filters.

Data Analysis and Statistics. The probability values for differences among the TUNEL⁺ cell population between the normal volunteers with no known cancer and SCCHN patients were calculated by Mann-Whitney U test using StatView software version 4.54 (Abacus Concepts, Inc. Berkeley, CA). Twenty-four-h assays were compared by subtracting least squares means derived from a mixed linear model for percentage of TUNEL⁺ cells. The model independent variables were group (patient or control), assay time 0, 24-h spontaneous, 24-h CH-11 Ab, 24-h TNF-α, and group by time interactions. Differences in least squares means were tested for significance, using a pooled within-person estimate of SE.

Results

Spontaneous and Induced Apoptosis in Patients’ PBMCs. When PBMCs obtained from patients and normal controls were examined at the time of blood draws (time 0) for the presence of cells with the evidence of DNA fragmentation (i.e., TUNEL⁺ cells), only a very low proportion of PBMCs was found to be TUNEL⁺ (Table 2). Nevertheless, even at time 0, a small but significantly higher proportion of PBMCs were TUNEL⁺ in patients (mean ± SD, 4 ± 2%) than in controls (mean ± SD, 1 ± 0.5%). After 24-h incubation of patients’ PBMCs in medium alone at 37°C, a significantly higher proportion (P < 0.0001) of these cells became TUNEL⁺. As shown in Table 2, the proportion of total TUNEL⁺ PBMCs with spontaneous apoptosis after 24-h incubation ranged from 9 to 36% in patients with SCCHN, and it was significantly higher (P < 0.0001) than that in PBMCs obtained from normal controls and tested in parallel with patients’ cells in the same assays. It
was also significantly higher than spontaneous apoptosis measured among PBMCs of age-matched controls (data not shown). In these and all other experiments, the data obtained for age-matched controls were not significantly different from those obtained for laboratory controls.

When the percentage of CD3⁺ TUNEL⁺ cells among PBMCs was determined by flow cytometry (Table 2), it became clear that T lymphocytes represented only one subset of total PBMCs undergoing ex vivo apoptosis. As shown in Table 2, CD3⁺ TUNEL⁺ cells accounted for less than one-half of total PBMCs with evidence of DNA fragmentation in patients with SCCHN.

The data shown in Fig. 2 indicate that after 24 h of incubation in medium or medium supplemented with anti-Fas CH-11 Ab or TNF-α, the patients’ PBMCs contained a significantly higher proportion (P < 0.0001) of TUNEL⁺ cells than control PBMCs. The PBMCs susceptible to agonistic CH-11 Ab or to TNF-α (presumably, cells expressing Fas or TNFR1, respectively) were significantly more numerous among PBMCs of patients with SCCHN than of controls (Fig. 2). The mean interassay differences among percentages of apoptotic PBMCs are listed in Table 3. Despite closeness of the means for results of 24-h assays among controls as well as patient cells and a high degree of correlation found for these assays in individual subjects, statistical analysis indicated significant differences (Table 3).

Thus, in both controls and patients, incubation of PBMCs with CH-11 Ab induced significantly more apoptosis than incubation with medium alone. In patients, incubation of PBMCs with TNF-α also induced more apoptosis than medium alone (Table 3). This observation suggested not only that the mean proportion of Fas⁺ cells was significantly lower (10%) in control PBMC than in the patients’ PBMCs (23%) but also that most of these cells (i.e., 7% in control and 19% in patients) were

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**Table 2** Percentages of total TUNEL⁺ PBMCs or CD3⁺/TUNEL⁺ T cells in normal controls and patients with SCCHN

<table>
<thead>
<tr>
<th>No.</th>
<th>Controls</th>
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<td></td>
<td>Time 0</td>
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<tr>
<td>1</td>
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<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
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</tbody>
</table>

* The data were obtained by flow cytometry for TUNEL⁺ cells or TUNEL⁺CD3⁺ cells among PBMCs obtained from normal volunteers or patients with SCCHN. Apoptosis was measured at the time of blood draws (time 0) and 24 h later after incubation of cells in medium at 37°C. ND, not done.
undergoing spontaneous apoptosis ex vivo. We determined the actual percentages and/or MFI of Fas+ and TNFR1+ cells in PBMCs of a subset of the patients and controls. In patients, 66 ± 7% of PBMCs were found to be Fas+ compared with 44 ± 15% Fas+ PBMC in controls (P < 0.05). TNFR1 was expressed mainly on monocytes (20% in patients versus 9% in controls), and MFI for TNFR1 on monocytes was considerably higher in the patients than controls (data not shown). As illustrated in Fig. 2, the magnitude of spontaneous or induced apoptosis observed with different patients’ PBMCs was highly variable, compared with a relatively narrow range of apoptotic cells seen within PBMCs of normal donors.

**Spontaneous or Induced Apoptosis of CD3+ T Cells.** To determine the fate of T cells after 24-h incubation of PBMCs with medium, CH-11 Ab, or TNF-α, we also measured the percentage of TUNEL+ cells among CD3+ lymphocytes in patients and normal controls (Fig. 3). Among CD3+ lymphocytes, a significantly higher proportion of cells was TUNEL+ in patients than in controls, and this was observed for spontaneous apoptosis as well as apoptosis induced by incubation of these cells with CH-11 Ab or TNF-α for 24 h (Fig. 3). A high degree of correlation was observed between the three assays for apoptosis in the individual control or patients’ cells incubated with medium, CH-11 Ab, or TNF-α (Fig. 4). In aggregate, however, CH-11 Ab or TNF-α induced a somewhat higher proportion (borderline significance) of T cells to undergo apoptosis in control PBMCs than that observed after lymphocyte incubation with medium alone. In the patients’ PBMCs, both CH-11 Ab and TNF-α induced significantly higher (P < 0.0001 and 0.0004, respectively) apoptosis in CD3+ T cells than medium alone (Table 4). These observations suggest that the proportion of all T cells expressing Fas or TNFR1 and undergoing ex vivo apoptosis is significantly higher in patients with SCCHN than in controls.

The data in Table 4 suggest that two distinct subsets of patients could be identified based on the percentage of spontaneous apoptosis found among T cells. Thus, if the mean ± 2 SD proportion of CD3+/TUNEL+ lymphocytes for controls is 9%, then the patients with the percentage of CD3+/TUNEL+ cells above the mean could be placed in one subset. Those patients with CD3+/TUNEL+ cells <9% could be placed in the second subset. As indicated in Table 4, 13 of 20 patients with measurements of spontaneous CD3+/TUNEL+ cells were in the first subset. No such variability was seen with T cells of normal individuals (Fig. 3B; Table 4). This considerable variability among patients with regard to the percentage of apoptotic T cells suggested that susceptibility of T cells to ex vivo apoptosis could be related to the disease process, despite the fact that all patients were NED at the time of this study. However, no associations were detected between T-cell apoptosis ex vivo and any of the following clinical-pathological criteria: tumor site, stage, or recurrence. There was a significant association (P < 0.02) observed for a lower percentage of apoptotic CD3+ cells present in PBMCs of patients with well-differentiated tumors, which have a more favorable prognosis in SCCHN (19).

**Flow Cytometry Analysis of CD3+/TUNEL+ Lymphocytes.** To quantitate the proportions of CD3+/TUNEL+ T lymphocytes among patients’ and normal PBMCs, two-color flow cytometry was performed (Fig. 5). An open gate was used, and FSC versus SSC indicated the presence of a heterogeneous population of TUNEL+ cells (Fig. 5, green).

The two-color flow cytometry analysis revealed the presence of CD3+ TUNEL+ and CD3+ TUNEL+ fractions in PBMCs of both patients and normal controls (Fig. 5, B and D, respectively). However, the percentages of TUNEL+ cells were always considerably higher in patients than in controls. It is interesting to note that the proportion of CD3+ TUNEL+ cells (Fig. 5, red) is considerably smaller in patients’ than in control PBMCs, whereas that of CD3+ TUNEL+ cells is larger (Fig. 5, B and D, gray). This observation suggests that a loss of the CD3ε chain from the surface of T cells occurs during 24-h ex vivo incubation, and that it may actually precede DNA fragmentation. Furthermore, an average proportion of CD3+ cells was generally lower in patients than in normal controls.

As shown in Fig. 6, a considerable proportion of CD3+ cells in the patients’ PBMCs were TUNEL+ (Fig. 6A, blue). This fraction contained cells with lowest FSC (red) and higher

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**Table 3** Differences in spontaneous and induced ex vivo apoptosis in PBMCs obtained from controls or patients with HNC

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients</th>
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<tbody>
<tr>
<td>Absolute value</td>
<td></td>
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<tr>
<td>Spontaneous vs. CH-11 Ab</td>
<td>2.3 ± 0.8</td>
<td>3.7 ± 0.7</td>
</tr>
<tr>
<td>CH-11 Ab vs. TNF-α</td>
<td>2.3 ± 1.1</td>
<td>5.5 ± 0.9</td>
</tr>
<tr>
<td>TNF-α vs. spontaneous</td>
<td>0.1 ± 1.0</td>
<td>1.7 ± 0.8</td>
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</table>

*The data are differences ±SE among the percentages of TUNEL+ PBMCs incubated in medium (spontaneous apoptosis) or in the presence of CH-11 Ab or TNF-α for 24 h at 37°C. TUNEL+ PBMCs were quantified by flow cytometry as described in “Materials and Methods.”

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**Fig. 3** Percentages of TUNEL+ cells among CD3+ T lymphocytes for all patients and controls were determined by two-color flow cytometry at the time of blood draw (time 0) or after 24-h incubation in the presence of medium, CH-11 Ab, or TNF-α. Horizontal bars, means. *, Ps for differences between patients and controls.
FSC (Fig. 6B, green). The red cells were strongly TUNEL\(^+\) and had the weakest expression of CD3, as indicated by their fluorescence intensity (Fig. 6C). The CD3\(^-\) fraction also contained strongly TUNEL\(^+\) cells (Fig. 6C). In addition, when we examined PBMCs undergoing ex vivo spontaneous apoptosis in medium at 2, 6, and 24 h of incubation by two-color (CD3\(^+\)/TUNEL\(^+\)) flow cytometry, it became apparent that the fraction of weakly CD3\(^+\) but strongly TUNEL\(^+\) T cells was increasing with the incubation time (data not shown). This experiment indicated that T cells gradually lose CD3 expression in the course of ex vivo incubation. Surface density of CD3 expression decreases (hence, the appearance of cells that are weakly CD3\(^+\) or CD3\(^-\) and still TUNEL\(^+\)). By FSC, it was possible to identify a subset of small cells, which were most intensely TUNEL\(^+\) (Fig. 6, B and C, red) and which did not express CD3 or were weakly CD3\(^+\). These cells most likely represent a fraction of T cells at the fairly advanced stage of apoptosis that have lost surface markers, resulting in the increased proportion of CD3\(^-\) TUNEL\(^+\) cells at 24 h of incubation (Fig. 6C).

By preparing cytocentrifuge smears of PBMCs undergoing spontaneous ex vivo apoptosis (Fig. 6, D and E), it was possible to identify individual cells at various stages of the apoptosis process, from nonapoptotic CD3\(^+\) TUNEL\(^-\) (red) to CD3\(^+\) TUNEL\(^+\) (green with red rim) to CD3\(^-\) TUNEL\(^+\) (green only) cells. It is important to note that although this progressive apoptosis (spontaneous or induced) was readily detectable in PBMCs obtained from patients, only a few PBMCs obtained from normal controls showed evidence of DNA fragmentation under the same incubation conditions. As shown in Fig. 6, only a few normal T cells underwent DNA fragmentation ex vivo, in comparison to numerous apoptotic T cells seen in patients’ PBMCs (Fig. 6, compare D and E).

Flow Cytometry Analysis of CD3\(^-\) TUNEL\(^+\) Cells.

Because we observed that DNA fragmentation occurred not only in CD3\(^+\) T cells but also in PBMCs that were CD3\(^-\), two-color flow cytometry was used to discriminate between these two populations and to identify the CD3\(^-\) mononuclear cell subsets, which become TUNEL\(^+\) during 24-h incubation of PBMCs in medium alone. The data shown in Table 5 indicated that TUNEL\(^+\) cells were detected in the CD16\(^+\)CD56\(^+\) (NK) cell, CD19\(^+\) (B) cell, and CD14\(^+\) (monocyte) cell populations. Thus, TUNEL\(^+\) cells were present among each of the subsets of PBMCs. The proportions of TUNEL\(^+\) among all of the PBMC subset cells were higher in patients than the accompanying controls. Together, the CD3\(^-\) cell subpopulations accounted for ~60%, and CD3\(^+\) T cells accounted for the other 40% of TUNEL\(^+\) cells. No preferential spontaneous or induced apoptosis was observed in any of the PBMC subsets in patients or controls. However, lymphocytes were the major PBMC subset undergoing apoptosis ex vivo.

Discussion

In this study, we observed the presence of significantly higher proportions of TUNEL\(^+\) PBMCs in patients with HNC than in normal controls at the time of blood draw (time 0), as well as 24 h after incubation. These observations suggest that in patients with HNC, apoptosis of PBMCs initiated in vivo continues after these cells are placed in medium and incubated. Apparently, in patients but not in normal controls, many PBMCs are already preprogrammed in vivo to die, and their incubation...
at 37°C allows the process of apoptosis to proceed, leading to cell death in a considerable proportion of the cells. No comparable cell death was observed with PBMCs obtained from normal controls. Hence, this phenomenon appears to be related to the disease and may have biological significance. In this context, it is important to note that the cancer patients who participated in this study had NED at the time of blood donations, having undergone surgery or surgery plus chemo- and/or radiotherapy in the past. No significant differences could be found in the level of *ex vivo* apoptosis between a group of 9 patients treated with surgery plus chemo- and/or radiotherapy and a group of 12 patients treated with surgery alone. In a parallel study to be reported separately, we examined PBMCs obtained from a group of patients with metastatic malignant melanoma and observed the same *ex vivo* phenomenon of apoptosis in patients’, but not control, PBMCs. Thus, this appears to be a generalized phenomenon, not associated with any particular type of cancer and evident even at the time where patients have no clinically detectable disease after therapy for their cancers.

A characteristic feature of *ex vivo* apoptosis we observed was that it included all subsets of PBMCs: T, NK, and B cells as well as monocytes. Lymphocytes accounted for a great majority of apoptotic cells. We initially focused on CD3⁺ T cells as a target for apoptosis, because of the previous data indicating that: (a) a substantial proportion of T lymphocytes present at the tumor site showed evidence of apoptosis (5, 13); (b) functional impairments were demonstrable in TILs as well as circulating T cells in patients with HNC and other cancers (1–20); (c) decreased expression of the ε and ζ chains associated with TcR was linked to functional defects and perhaps apoptosis in CD3⁺ TILs or PBMCs of patients with cancer (1, 5, 7–12, 20); and (d) coincubation of activated T cells with tumor cells induced the apoptotic pathway in these T cells, culminating in their death (13). Cumulatively, these data suggested that T lymphocytes, especially those accumulating at the tumor site, were activated in patients with cancer (1–3) and were thus susceptible to AICD or TICD or both (3, 13). However, our present data indicate that in addition to CD3⁺ T cells, other subsets of PBMCs were undergoing apoptosis at a significantly higher level than that observed in PBMCs of normal controls. Thus, apoptosis was observed within all of the mononuclear cell subsets examined in this study.

The second interesting characteristic of the process of *ex vivo* apoptosis was that the level of spontaneous apoptosis at 24 h was nearly as high as that induced by agonistic anti-Fas Abs (CH-11) or by exogenous TNF-α. This finding suggested that most of PBMCs expressing Fas or TNFR1 were undergoing spontaneous apoptosis. Furthermore, the level of CH-11 Ab-induced apoptosis was higher than that of spontaneous apoptosis, indicating that not all Fas⁺ T cells had died upon incubation with medium alone. In all cases, the proportion of PBMCs sensitive to Fas or TNFR1 engagement was significantly higher in patients with HNC than in normal controls. Also, the actual percentages and/or MFI of Fas⁺ or TNFR1⁺ cells were found

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*ᵃ The data were obtained by two-color flow cytometry for TUNEL⁺ cells in CD3⁺ T cells. The Ps indicate differences between time 0 apoptosis and 24-h spontaneous or induced apoptosis. ND, not done.

ᵇ Patients with the percentage of spontaneously apoptotic cells that was >9% (the mean + 2 SD for controls).
to be significantly higher in PBMCs of the patients than of controls. However, no attempts were made in this study to correlate expression of these receptors with the level of apoptosis. Nevertheless, these data suggest that more PBMCs in patients than in controls were activated and thus presumably sensitive to AICD. However, AICD by definition involves TcR-mediated signaling (21, 22), whereas in our study, non-T cells constituted a considerable proportion of PBMCs undergoing apoptosis (Table 5). Therefore, a possibility of FasL-mediated or TNF-α-mediated cell death not involving the TcR pathway has to be considered as a mechanism responsible in vivo for apoptosis of PBMCs in patients, and to a much smaller extent, in normal individuals. The question remains as to why PBMCs expressing Fas or TNFR1 spontaneously undergo apoptosis ex vivo. We suggest that these PBMCs are preprogrammed in vivo to die, and that the proportion of such preprogrammed PBMCs is significantly higher in patients than in controls.

The third aspect of our studies that, in part, helps to explain spontaneous ex vivo apoptosis concerns the proportion of TUNEL+ PBMCs at time 0, i.e., at the time of blood draws. Spontaneous apoptosis was observed at that time in a small but significantly higher proportion of PBMCs in patients than in controls (4% versus 1% in Fig. 2). Because in vivo apoptotic PBMCs are cleared rapidly from the circulation by the reticuloendothelial system, this observation suggests that: (a) a higher fraction of PBMCs is undergoing apoptosis in vivo in patients than in controls; and (b) a more rapid turnover of PBMCs must be occurring in patients than in controls. This means that PBMCs are dying and are replaced at a much higher rate in patients than in controls. If this speculation were correct, it could explain why lymphocytes are often found to be functionally impaired in the circulation of patients with cancer (7–12). In particular, our earlier results, indicating that the proportion of lymphocytes with low or absent expression of the ζ chain is significantly increased in the circulation of patients with HNC, could be explained by the rapid rate of apoptosis of such cells (1). Our in vitro and in situ data have indicated that a link might exist between the ζ chain degradation and apoptosis in cancer (23, 24), and more recent results indicate that ζ protein contains caspase-sensitive amino acid motifs.4 Taken together, the findings of decreased expression of ζ in T and NK cells in patients with HNC (5, 23, 24) and other cancers (6, 8, 11, 9, 17, 25–28), functional defects observed in lymphocytes (1, 20), and the increased rate of PBMC turnover might all represent events related to tumor-induced and/or activation-induced apoptosis.

The distinction between the two mechanisms of apoptosis (AICD versus TICD) operative in patients with cancer is not clear at present. It is highly likely that the presence of tumor or even of occult metastases induces activation not only of TILs but also of a fraction of PBMCs and thus predisposes these cells to AICD. On the other hand, we and others have provided

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4 H. Rabinowich, unpublished data.
evidence that human tumors, including HNC, can directly induce apoptosis in activated lymphocytes via the Fas-FasL, TRAILR-TRAIL, or other tumor-derived signals (13, 29). These mechanisms of TICD are under extensive examination in our laboratory.5

The biological and clinical significance of the findings reported here is of considerable interest. Certainly, not all patients with HNC show elevated spontaneous apoptosis ex vivo. Thus, as illustrated in Fig. 2, a broad range of values for spontaneous or induced ex vivo apoptosis can be observed in patients relative to a narrow range in controls. On the basis of the proportions of TUNEL+ cells among CD3+ T lymphocytes, the patients with HNC can be divided into two groups, one which is similar to normal controls (i.e., with TUNEL+/CD3+ cells <9% which is less than the normal mean + 2SD) and second, which is significantly higher than normal controls (i.e., with TUNEL+/CD3+ cells >9%), as shown in Table 4. However, we could not discern any apparent clinical differences between the two groups, except that patients with a lower percentage of apoptotic T cells in PBMCs tended to have well-differentiated tumors.

We could not detect an association between signaling defects present in T cells at time 0 (e.g., decreased $\zeta$ chain expression) and levels of spontaneous or induced apoptosis of these cells after 24-h incubation (data not shown). We expected to find such association based on the new data in our laboratory, suggesting a link between $\zeta$ abnormalities in T lymphocytes and

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apoptosis (13). Finally, the extent of *ex vivo* spontaneous apoptosis in SCCHN patients with NED is surprising. It suggests that tumor-induced effects on the hematopoietic system are long lived. It is possible that in SCCHN patients with large tumor burden, *i.e.*, in patients studied before surgery, *ex vivo* apoptosis might be even more pronounced. When confirmed and extended, this finding could provide a basis for estimating the degree of immunoincompetence in patients with SCCHN and other cancers. The presence of extensive *ex vivo* apoptosis of T lymphocytes in HIV has been well documented in the literature (30–32), suggesting that in infections as well as cancer, circulating activated lymphocytes are preprogrammed to die. The mechanisms responsible for this phenomenon remain to be elucidated.

### References


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6 H. Rabinowich, personal communication.
Spontaneous ex Vivo Apoptosis of Peripheral Blood Mononuclear Cells in Patients with Head and Neck Cancer

Takao Saito, Iris Kuss, Grzegorz Dworacki, et al.


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