Elevated Caspase-3 Activity in Peripheral Blood T Cells Coexists with Increased Degree of T-Cell Apoptosis and Down-Regulation of TCR Zeta Molecules in Patients with Gastric Cancer

Akihiro Takahashi, Koji Kono, Hideki Amemiya, Hidehiko Iizuka, Hideki Fujii, and Yoshiro Matsumoto
First Department of Surgery, Yamanashi Medical University, Yamanashi 409-3898, Japan

ABSTRACT

To evaluate the mechanisms of T-cell dysfunction in patients with gastric cancer, we investigated the caspase activity of T cells, the induction of spontaneous T-cell apoptosis, the expression of T-cell receptor (TCR) ζ molecules, and the ability of T cells to produce cytokines in peripheral blood lymphocytes from patients (n = 22) and healthy controls (n = 14). The caspase-3 activity of T cells was studied as the protease activity of caspase-3 using the cell-permeable substrate of PhiPhiLux G1D2. Flow cytometric analysis was performed with triple staining by annexin V-FITC, propidium iodide, and CD3-R-phycoerythrin-Cy5 for the detection of T-cell apoptosis and with intracellular staining using permeabilized cells for the expression of TCR-ζ molecules. IFN-γ and tumor necrosis factor α production from T cells was evaluated in response to anti-CD3 stimulation. Caspase-3 activity of peripheral blood T cells from patients with advanced disease was significantly increased compared with that from controls [15.5 ± 3.6 mean fluorescence intensity (MFI) versus 11.5 ± 3.3 MFI; P = 0.0068]. Parallel to this, the apoptosis of peripheral blood T cells from patients with advanced disease was significantly higher than for those from controls (16.5 ± 15.5% versus 4.8 ± 2.7%; P = 0.010). Furthermore, the expression of TCR-ζ molecules in patients with advanced disease was significantly decreased in comparison with that of the controls (41.0 ± 13.9 MFI versus 56.7 ± 16.3 MFI; P = 0.014), and this decreased expression coexisted with impaired IFN-γ (42.4 ± 43.2 pg/ml versus 1757.4 ± 2449.0 pg/ml; P = 0.031) and tumor necrosis factor α (682.6 ± 519.3 pg/ml versus 1686.0 ± 1533.7 pg/ml; P = 0.041) production of T cells. Thus, peripheral blood T cells from gastric cancer patients simultaneously exhibit an elevated caspase-3 activity, an increased degree of T-cell apoptosis, a down-regulation of TCR-ζ molecules, and impaired cytokine production. These observations suggest that induction of T-cell apoptosis coexisting with a down-regulation of TCR-ζ molecules may be responsible for T-cell dysfunction in patients with gastric cancer.

INTRODUCTION

Tumor-infiltrating and, to a lesser extent, peripheral lymphocytes from patients with advanced-stage cancer are known to have a poor immune response (1), and tumor cells have developed mechanisms to evade the immune system. A multitude of different effector mechanisms could account for this, such as deficient antigen presentation by the down-regulation of MHC class I expression on tumor cells (2, 3), decreased or lost expression of T-cell epitopes on tumor cells (4, 5), immunosuppressive factors derived from tumor cells (6, 7), or T-cell dysfunction in the cancer-bearing host (8–13).

With regard to T-cell dysfunction, we and others have shown that alteration in the signal-transducing molecules associated with the TCR3 was responsible for impaired T-cell response in patients with various types of cancer (8–13). The cytoplasmic domain of the TCR-ζ subunit on the TCR complex is involved in signal transduction and subsequent activation of T cells (14). A decrease in TCR-ζ levels on T cells from patients with several types of malignancies has been observed (8–13). In addition, decreased expression of TCR-ζ has been correlated with reduced proliferative responses after antigenic challenge (8) and with reduced cytokine production (9, 10, 12). Moreover, Zea et al. (9) demonstrated that the overall survival rate of melanoma patients with low TCR-ζ levels was significantly lower than that of patients with normal TCR-ζ levels. Thus, abnormalities in the expression of signal-transducing ζ molecules are frequently observed in the cancer-bearing host.

Recently, we have shown that one possible mechanism behind these structural changes in the TCR-complex is related to oxygen metabolites, such as hydrogen peroxide secreted from tumor-associated macrophages (15). This observation was subsequently confirmed in a tumor-bearing animal model (16). Furthermore, Rabinowich et al. (17) reported another possible...
mechanism: that a loss in the expression of TCR-ζ molecules is associated with T-cell apoptosis mediated by Fas L-expressing tumor cells. In fact, Saito et al. (18) reported that PBMCs undergo spontaneous apoptosis more frequently in patients with head and neck cancers than in healthy controls. Moreover, it has been shown recently that activated caspase inside T cells induced by apoptotic signals directly degraded TCR-ζ molecules (19). These observations suggest that activated caspase induced by apoptotic stimuli, including Fas-Fas L interaction or oxygen metabolites, is involved in the reduced expression of TCR-ζ molecules.

These findings prompt us to investigate the caspase activity of T cells in the cancer-bearing host. This is the first study demonstrating that an elevated activity of caspase in T cells coexists with an increased degree of spontaneous T-cell apoptosis and decreased expression of TCR-ζ molecules in patients with gastric cancer.

MATERIALS AND METHODS
Patients and Controls. Twenty-two patients, treated at Yamanashi Medical University Hospital and Yamanashi Prefec-

Table 1  Patient characteristics

<table>
<thead>
<tr>
<th>Gender (M/F)</th>
<th>Age (years)</th>
<th>TNM* classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 14)</td>
<td>8/6</td>
<td>54.36 ± 7.90</td>
</tr>
<tr>
<td>Gastric cancer patients (n = 22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early disease (n = 10)</td>
<td>6/4</td>
<td>56.55 ± 7.08</td>
</tr>
<tr>
<td>Advanced disease (n = 12)</td>
<td>8/4</td>
<td>60.82 ± 8.10</td>
</tr>
</tbody>
</table>

*TNM, Tumor-Node-Metastasis.

**Fig. 1** Representative flow cytometric data of caspase-3 activity in a patient and in a control subject. After incubation for 24 h as described in “Materials and Methods,” the caspase-3 activity of peripheral T cells was evaluated with PhiPhiLux G1D2 with gating for CD3-PE by flow cytometry. The caspase-3 activity of T cells was increased in the patient compared with that in the healthy control.

**Fig. 2** Caspase-3 activity of peripheral T cells. After incubation for 24 h under the above-described conditions, the caspase-3 activity of peripheral T cells was evaluated with PhiPhiLux G1D2 with gating for CD3-PE by flow cytometry. The caspase-3 activity of T cells from the patients with advanced disease was significantly increased compared with that of healthy controls (15.5 ± 3.6 MFI versus 11.5 ± 3.3 MFI; P = 0.0068). A significant difference was also observed between T cells from the patients with advanced disease and those with early disease (15.5 ± 3.6 MFI versus 12.3 ± 3.0 MFI; P = 0.041).

Clinical Hospital and pathologically diagnosed with gastric cancer, were enrolled in this study. None of the patients received surgery, radiotherapy, chemotherapy, or other medical interventions during this study. Informed consent for blood donations was obtained for all individuals. The patients were divided into two groups: those with early disease corresponding to stages I and II according to the Tumor-Node-Metastasis classification for gastric cancer (n = 10); and those with advanced disease corresponding to stages III and IV (n = 12). Patient characteristics are shown in Table 1. Healthy controls (n = 14) were
age-matched (54.36 ± 7.90 years for the controls versus 58.77 ± 7.72 years for the patients; \( P = 0.108 \)), and each experiment was performed in parallel for the gastric cancer patients and the healthy controls.

**Preparation of PBMCs.** Twenty ml of peripheral blood were drawn from each of the patients and the controls and centrifuged by a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient. Aliquots of PBMCs (1.0 \( \times \) 10\(^7\)cells) were used directly for the evaluation of TCR-\(\zeta\)-molecule expression and the IFN-\(\gamma\)- and TNF-\(\alpha\)-releasing assay. Aliquots of PBMCs (1.0 \( \times \) 10\(^7\)cells) were incubated in a 12-well plate with AIM-V medium (Life Technologies, Inc., Gaithersburg, MD) at 37°C in 5.0% CO\(_2\) for 24 h to evaluate apoptosis and caspase activity in T cells.

**Caspase-3 Activity in Peripheral T Cells.** The caspase-3 activity of T cells was evaluated as the protease activity of caspase-3 by using the PhiPhiLux G1D2 kit (MBL, Nagoya, Japan; Ref. 20), after a 24-h *in vitro* incubation. A substrate of PhiPhiLux G1D2, which can penetrate into the cell nucleus, is converted to the fluorescent form when it is cleaved by the protease activity of caspase-3. The assessment was performed according to the manufacturer’s recommendations with some modifications. Briefly, PBMCs were incubated with 75 \( \mu\)l of PhiPhiLux G1D2 for 1 h at 37°C in 5% CO\(_2\), and then double staining was performed with PE-conjugated CD3 mAbs (Becton Dickinson, Mountain View, CA). The caspase-3 activity in T cells, which were gated for the PE+ cells in PBMCs, was analyzed by fluorescence-activated cell sorting caliber flow cytometry.

**Apoptosis in Peripheral T Cells.** T-cell apoptosis was measured by triple staining with FITC-conjugated annexin V, PI, and CD3-RPE-Cy5 (DAKO, Glostrup, Denmark) using a

---

**Fig. 3** Increased caspase-3 activity of peripheral T cells shown with epifluorescence microscopy. After incubation for 24 h as described in “Materials and Methods,” peripheral T cells were treated with PhiPhiLux G1D2 and subjected to epifluorescence microscopy. The fluorescent cells were more frequently observed in patients with advanced disease (A) than in healthy controls (B).
MEBCYTO Apoptosis Kit (MBL, Nagoya, Japan) according to the manufacturer’s recommendations with flow cytometric analysis. The proportion of apoptosis was measured by flow cytometry using annexin V-FITC and PI, with gating of T cells by RPE-Cy5-CD3 staining. Most of the T cells from the healthy control were FITC-negative and PI− (A), whereas some proportion of T cells from the patient was FITC+ and PI−, corresponding to apoptotic cells (B).

**Expression of TCR-ζ Molecules.** The expression of TCR-ζ molecules was examined by techniques described previously using flow cytometric analysis (10, 11). In brief, the isolated PBMCs were fixed with 0.5% formaldehyde in PBS for 20 min on ice after permeabilization by digitonin (10 µg/ml) for 15 min on ice. The intracellular component of ζ molecules in the CD3 complex was stained by anti-ζ mAbs (TIA-2, IgG1; Coulter) or by IgG1 isotype control mAbs in a saturating concentration. The mAbs, which were conjugated with ζ molecules, were stained by using rabbit antimouse FITC antibodies (DAKO). Then, double staining was performed by PE-conjugated CD3 mAbs (Becton Dickinson). The double-stained cells were assessed by flow cytometric analysis. The MFI of ζ molecules, which were gated as CD3+ cells, was measured.

**Production of IFN-γ and TNF-α.** PBMCs (5.0 × 10⁵ cells) were incubated with 500 µl of AIM-V medium for 30 h in a 48-well plate (Coaster, Cambridge, MA) precoated with mAbs to CD3 (10 µl/ml, final concentration; UCHT 1) to induce IFN-γ and TNF-α production from T cells. The obtained supernatants were stored at −70°C. Then the IFN-γ and the TNF-α contents were determined using the sandwich ELISA technique with the BIOTRAK IFN-γ ELISA system (Amersham Pharmacia Biotech, Buckinghamshire, England) and the MEDGENIX TNF-α EASIA kit (BioSource Europe S.A., Nivelles, Belgium), respectively, according to the manufacturers’ recommendations.

**Caspase-3 Activity by Epifluorescence Microscopy.** Activated caspase in T cells was detected using the PhiPhiLux G1D2 kit as described above. Then the obtained cells were centrifuged onto silane-coated glass slides and observed in an epifluorescence microscope (Olympus, Tokyo, Japan) with a filter for FITC.

**Statistical Analysis.** To determine statistical differences between two groups, Student’s t test was used. Statistical significance was determined when Ps were <0.05.

---

**Fig. 4** Representative flow cytometric data for apoptosis in peripheral T cells in a patient and a control. The proportion of apoptosis was measured by flow cytometry using annexin V-FITC and PI, with gating of T cells by RPE-Cy5-CD3 staining. Most of the T cells from the healthy control were FITC-negative and PI− (A), whereas some proportion of T cells from the patient was FITC+ and PI−, corresponding to apoptotic cells (B).

**Fig. 5** Apoptosis in peripheral T lymphocytes. T-cell apoptosis was detected by flow cytometry using annexin V-FITC and PI, with gating of T cells by RPE-Cy5-CD3 staining. The proportion of FITC+ and PI− cells was evaluated in all T cells. Peripheral T cells from patients with advanced-stage cancer had a significantly increased apoptosis compared with those from the controls (16.5 ± 15.5% versus 4.8 ± 2.7%; P = 0.010). No significant differences was seen in the proportion of T-cell apoptosis between early disease and control (6.7 ± 6.1% versus 4.8 ± 2.7%; P = 0.317).
versus advanced disease and those with early disease (15.5 ± 0.0068), as indicated in Fig. 2. Furthermore, a significant difference in the proportions of T cells that underwent apoptosis in a comparison of cells from the patients with early disease and the healthy controls (6.7 ± 6.1% versus 4.8 ± 2.7%; P = 0.317). These results indicate that T-cell apoptosis frequently occurs in patients with advanced-stage cancer in comparison with healthy controls.

Expression of TCR-ζ Molecules. To evaluate the expression of TCR-ζ molecules in peripheral T cells, flow cytometric analysis was performed using intracellular staining for TCR-ζ molecules in peripheral T cells. The expression of TCR-ζ molecules in the T cells of the patients with advanced disease was significantly decreased in comparison with that in the controls (41.0 ± 13.9 MFI versus 56.7 ± 16.3 MFI; P = 0.014). There is no significant difference of TCR-ζ expression in the patients with early disease and the controls (53.1 ± 12.4 MFI versus 56.7 ± 16.3 MFI; P = 0.560).

RESULTS

Caspase-3 Activity of Peripheral T cells from Cancer Patients. To investigate the caspase activity of T cells, flow cytometric analysis was performed using PhiPhiLux G1D2 for the detection of protease activity of caspase-3. Representative flow cytometric data are shown in Fig. 1, which indicates that the caspase-3 activity of T cells is increased in cancer patients compared with healthy donors. The MFI corresponding to caspase-3 activity in T cells from the patients with advanced disease was significantly increased compared with that of the healthy controls (15.5 ± 3.6 MFI versus 11.5 ± 3.3 MFI; P = 0.0068), as indicated in Fig. 2. Furthermore, a significant difference in MFI is also observed in T cells from the patients with advanced disease and those with early disease (15.5 ± 3.6 MFI versus 12.3 ± 3.0 MFI; P = 0.041). To further confirm the status of caspase activity, an epifluorescence-microscope examination was performed. The epifluorescence microscope showed that yellow fluorescent cells were more often observed in the patient than in the healthy control (Fig. 3). These results indicate that caspase-3 activity in T cells from patients in the advanced stage was increased in comparison with that in healthy controls.

Apoptosis in Peripheral T Cells from Cancer Patients. To investigate whether peripheral T cells from the patients undergo spontaneous apoptosis, the proportion of cells that underwent apoptosis was measured by flow cytometry using annexin V-FITC and PI, with gating of T cells by staining with RPE-Cy5-CD3 mAbs after a 24-h in vitro incubation. Representative flow cytometric data (Fig. 4) revealed that most of the T cells from the healthy control were FITC− and PI−, whereas some proportion of T cells from the patient was FITC+ and PI−, corresponding to apoptotic cells. As shown in Fig. 5, a significant increase in apoptosis of peripheral T cells from the patients in the advanced stage of cancer was found in comparison with those from the healthy donors (16.5 ± 15.5% versus 4.8 ± 2.7%; P = 0.010). No significant difference was seen in the proportions of T cells that underwent apoptosis in a comparison of cells from the patients with early disease and the healthy controls (6.7 ± 6.1% versus 4.8 ± 2.7%; P = 0.317). These results indicate that T-cell apoptosis frequently occurs in patients with advanced-stage cancer in comparison with healthy controls.

DISCUSSION

The present report contains several findings of relevance to T-cell dysfunction in the cancer-bearing host. Caspase-3 activity of peripheral blood T cells from the patients with advanced-stage gastric cancers was significantly increased when compared with that from the healthy controls, and this observation coexists with the increased degree of spontaneous apoptosis and the down-regulation of TCR-ζ molecules.
Recently, it was demonstrated that TCR-ζ molecules are cleaved by a caspase protease activity in cells undergoing apoptosis (19). Caspase-3 and caspase-7 were identified as the specific enzymes involved in the degradation of TCR-ζ molecules (19). Our new finding that caspase-3 activity of T cells was increased in gastric cancer patients supports the possibility that activated caspase directly degrades TCR-ζ molecules in T cells in cancer-bearing hosts.

Furthermore, in the present study, the spontaneous apoptosis of peripheral T cells from gastric cancer patients was more frequently observed, in line with a previous report of patients with head and neck cancers (18). This phenomenon coexists with the fact that the caspase-3 activity of peripheral blood T cells was increased in the patients in comparison with that in the healthy donors. In general, several stimuli such as the Fas-Fas L-mediated system (21), the perfolin-granzyme-mediated system (22), and the hydrogen peroxides (23) induce the apoptotic signal, which converts procaspase to activated caspase and consequently causes DNA fragmentation. In fact, it has been reported that several types of tumor cells express Fas L and, concomitantly, T-cell apoptosis was detected inside the tumor microenvironment (24). Furthermore, it was also shown that tumor cells expressing functional Fas L trigger the apoptosis of Fas-sensitive T cells in vitro (17, 25). Thus, Fas and Fas L interaction might be one possible mechanism for inducing T-cell apoptosis in the cancer-bearing host. In addition, another possibility can be extracted from our previous work, i.e., that hydrogen peroxide derived from tumor-associated macrophages induces a decreased expression of TCR-ζ molecules (15).

Thus, it is suggested that peripheral T cells from cancer patients, but not from healthy donors, are preprogrammed to undergo apoptosis in vivo, regardless of the nature of the apoptotic stimuli. In the present study, the patients did not receive chemotherapy or radiation therapy. Furthermore, the increased caspase activity of T cells was observed in the patients with advanced-stage cancers and not in those with early-stage cancer, indicating that the extent of the tumor burden affects the induction of T-cell apoptotic events with increased caspase activity.

Taken together, the mechanisms responsible for T-cell dysfunction in the cancer-bearing host, that is, both abnormalities of signal-transducing molecules and T-cell apoptosis, may originate from the same tumor-induced events. We have shown that the coexisting phenomena that elevated caspase activity and increased the degree of apoptosis occurred in peripheral blood T cells from gastric cancer patients, which parallels the down-regulation of TCR-ζ and reduced cytokine production.

ACKNOWLEDGMENTS

We thank Drs. Itsuki Ashizawa, Junich Okuda, and Hidenori Akaike for providing us with precious blood samples from gastric cancer patients, and Tamiyasu Shimamiya for excellent technical assistance.

REFERENCES

Increased Caspase Activity of T Cells in Gastric Cancer


Elevated Caspase-3 Activity in Peripheral Blood T Cells Coexists with Increased Degree of T-Cell Apoptosis and Down-Registration of TCR Zeta Molecules in Patients with Gastric Cancer

Akihiro Takahashi, Koji Kono, Hideki Amemiya, et al.