CD4$^+$ and CD8$^+$ T Cells Cooperate to Improve Prognosis of Patients with Esophageal Squamous Cell Carcinoma

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ABSTRACT

The purpose of this study is to clarify the roles of immune cell types, both individually and synergistically, in esophageal squamous cell carcinoma (ESCC). One hundred and twenty-two patients (105 males and 17 females; mean age, 62.3 years) with primary ESCC underwent surgical tumor resection at the Department of Surgical Oncology, School of Medicine, Hokkaido University and two affiliated hospitals between 1989 and 1999. Immunohistochemical analyses were performed for CD4, CD8, and CD57 (surface markers for natural killer cells). Patient prognosis was found to correlate with the number of CD4$^+$ and CD8$^+$ T cells in the stroma and the number of CD8$^+$ T cells within the cancer cell nest. Furthermore, the number of CD8$^+$ T cells in the stroma and within the cancer cell nest was found to be correlated (correlation coefficient ($r$) = 0.790; $P < 0.0001$). However, no correlation was observed between the number of natural killer cells and patient prognosis. Patients were classified into the following four groups based on CD4$^+$ and CD8$^+$ T-cell count: CD4$^+$/CD8$^+$(+/+), CD4$^+$/CD8$^+$(+/-), CD4$^+$/CD8$^+$(-/+) , and CD4$^+$/CD8$^+$(/-/). For the general patient pool, as well as for selected p-stage III and IV cases ($n$ = 48), the survival rate for CD4$^+$/CD8$^+$(+/+) patients was significantly higher than that for the other three groups (log-rank test, $P = 0.0012$ and 0.0088, respectively). Multivariate analysis identified CD4$^+$/CD8$^+$(+/+) status, T classification, and N classification as independent prognostic factors. In conclusion, cooperation between CD4$^+$ and CD8$^+$ T cells correlates strongly with ESCC patient prognosis.

INTRODUCTION

Although advances in diagnosis and treatment of ESCC have been made in recent years, postsurgery survival rates have not improved in the last decade (1, 2). Therefore, new clinical parameters for prognosis and new approaches for adjuvant treatment are needed.

TILs are considered to be a manifestation of the host immune response against cancer cells (3). TILs have been studied in various types of carcinoma and reported to be prognostic factors (4, 5). Inclusion of CD8$^+$ T cells among the TIL population appears to be important in the anticancer immune response. Infiltration of CD8$^+$ T cells within cancer cell nests is a reliable prognostic indicator in human colorectal cancer (6). Additional studies revealed that i.t. CD8$^+$ T-cell infiltration is more strongly associated with a favorable outcome than peritumor infiltration in both ESCCs and adenocarcinomas. In fact, it was shown that i.t. CD8$^+$ T-cell infiltration is itself an independent positive prognostic factor (7).

It was recently reported that CD4$^+$ T cells, which had received little attention with regard to tumor immunity, play a central role in initiating and maintaining anticancer immune responses (8–10). The potential and feasibility of cancer immunotherapy using tumor antigens recognized by CD8$^+$ T cells were demonstrated; however, the overall immune responses were weak and transient without CD4$^+$ T cells (11). Immunohistochemical analysis of CD4$^+$ T cells is important; however, few studies have been conducted.

Clinical immune responses to autologous cancer cells may be mediated not only by tumor-specific TILs such as CD8$^+$ T cells but also by nonspecific TILs such as NK cells. Recently, in patients with colorectal (12), gastric (13), and lung cancer (14), an immunohistochemical analysis of NK cells was performed and reported that extensive i.t. infiltration of NK cells was found to be associated with a favorable tumor outcome. However, it is not clear whether NK cells play a role in the antitumor resistance in patients with ESCC.

The purpose of this study is to clarify the individual and cooperative roles of CD4$^+$ T cells, CD8$^+$ T cells, and NK cells in ESCC.

MATERIALS AND METHODS

Patients and Esophageal Specimens. One hundred and twenty-two patients (105 males and 17 females; mean age, 62.3 years) with primary ESCC underwent radical esophagectomy between September 1989 and May 1999 at the Department of Surgical Oncology, School of Medicine, Hokkaido University or at an affiliated hospital (Department of Surgery, Teinekeijinkai Hospital and Department of Surgery, Hokkaido Gastroenterology Hospital). No distant metastasis was detected in any patient upon preoperative examination. No patients had received prior anticancer treatment. Cases of in-hospital death were excluded from the current study. The clinical typing of tumors was determined according to the tumor-node-metastasis (TNM) classification system of the International Union Against Cancer (15).

All specimens were fixed in 10% formalin and embedded in paraffin wax. One of the deepest sections from each tumor was selected for evaluation, and serial 4-μm-thick sections were examined by immunohistochemistry.

Immunohistochemistry. Immunohistochemical reactions were carried out using the streptavidin-biotin-peroxidase method. The mouse monoclonal primary antibodies used were anti-human CD4 and anti-human CD8 (Histofine CD8 mouse IgG1κ monoclonal antibody and Histofine CD4 mouse IgG1 monoclonal antibody; Nichirei Corp., Tokyo, Japan) at a 1:500 dilution in PBS and anti-human CD57 at a dilution of 1:5 (Leu 7; Becton Dickinson Immunocytometry System, San Jose, CA) to identify cells originating from the neural crest, including NK cells (12–14). As positive controls, normal adenoid tissue was used for CD4 and CD8, and prostate gland was used for CD57. Sections were deparaffinized in xylene, washed in PBS (pH 7.4), and rehydrated in a graded series of ethanol solutions. Endogenous peroxidase activity was blocked by a 10-min incubation with 3% hydrogen peroxide in methanol. After washing in PBS, specimens were saturated with 10% normal goat serum (Histofine SAB-PO kit; Nichirei Corp.) for 5 min and then incubated at room temperature for 30 min with primary antibody. After washing in PBS, a biotinylated goat antimouse immunoglobulin antibody (Histofine SAB-PO kit; Nichirei Corp.) was applied for 60 min at room temperature. Immunohistochemical reactions were visualized with freshly prepared 3,3'-diaminobenzidinium tetrahydrochloride chromogen.

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2 The abbreviations used are: ESCC, esophageal squamous cell carcinoma; NK, natural killer; TIL, tumor-infiltrating lymphocyte; i.t., intratumoral.
Table 1: Classification by the number of CD4+ T cells, CD8+ T cells, and NK cells in the stroma.

One hundred and twenty-two patients with primary ESCC underwent radical esophagectomy. One of the deepest sections from each tumor was selected for evaluation, and immunohistochemical reactions were carried out. The number of CD4+ T cells, CD8+ T cells, and NK cells was counted in the stroma. We classified patients into four groups (the most abundant group, abundant group, moderate group, and scanty group) containing almost equal number of patients. Mean ± SD, range and number of patients are shown.

<table>
<thead>
<tr>
<th></th>
<th>No. of immune reactive cells/microscopic field, mean ± SD (range, no. of patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4+ T cells</strong></td>
<td>Most abundant</td>
</tr>
<tr>
<td>121.4 ± 26.1</td>
<td>68.7 ± 11.1</td>
</tr>
<tr>
<td>(89.0–183.8, 31)</td>
<td>(54.6–87.8, 30)</td>
</tr>
<tr>
<td><strong>CD8+ T cells</strong></td>
<td>65.3 ± 30.6</td>
</tr>
<tr>
<td>(35.8–160.3, 31)</td>
<td>(19.2–35.4, 30)</td>
</tr>
<tr>
<td><strong>NK cells</strong></td>
<td>8.3 ± 0.2</td>
</tr>
<tr>
<td>(2.1–41.3, 31)</td>
<td>(0.9–1.9, 30)</td>
</tr>
</tbody>
</table>

Evaluation and Classification of CD4+ T Cells, CD8+ T Cells, and NK Cells. The immunostained sections were evaluated under a microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Immunohistochemistry and evaluation of immune cells were done according to the previous reports of Naito et al. (6) and Schumacher et al. (7), with some modifications. The degree of immune cell infiltration was observed in more than 10 independent high-power (×200) microscopic fields for each tissue sample. The five areas with the most abundant distribution were selected. The number of CD4+ T cells, CD8+ T cells, and NK cells was counted both in the mesenchymal stroma and within the cancer cell nest.

In the present study, the threshold values used to demarcate group boundaries were selected such that each group contained equal numbers of patients. Initially, we classified patients into four groups (the most abundant group, abundant group, moderate group, and scanty group) containing an almost equal number of patients as described in Table 1. Patients with various CD4+ T cell, CD8+ T cell, and NK cell counts were classified in the same manner. Subsequently, we classified patients into two groups: (a) CD4+ (++) group (the most abundant group and abundant group) and (b) CD4+ (--) group (moderate group and scanty group). Patients were also classified by CD8+ T-cell count in the same manner. Finally, we classified all patients into the following four groups for analysis: patients classified as both CD4+ (+++) and CD8+ (+++) were described as CD4+/8+ (+++). By the same manner, other three groups were designated as CD4+/8+ (+–), CD4+/8+ (–+), and CD4+/8+ (––). The current study was performed in a retrospective manner, but all specimens were evaluated by two investigators blinded to the patients’ clinical information. In case of interobserver variability, the classification was decided using the average of counted positively stained cells by two observers.

Statistical Analysis. The χ² test and Fisher’s exact test were used as appropriate. The Kaplan-Meier method was used to estimate overall survival, and survival differences were analyzed by the log-rank test based on the number of immune cells. Correlation coefficient described r, and r ≥ 0.7 is defined as strong correlation. Univariate and multivariate analyses of immune cells and clinicopathological features were performed using the Cox proportional hazard regression model. P values < 0.05 were regarded as significant in all of the analyses. All analyses were performed with statistical software (StatView 3 version 5.0; SAS Institute Inc., Cary, NC).

RESULTS

Immunohistochemistry for Immune Cells. CD4+ T cells, CD8+ T cells, and NK cells were detected within cancer cell nests or the stroma in contact with cancer cells. Fig. 1 shows representative photomicrographs of immunohistochemical staining of CD4, CD8, and CD57.

Overall Survival Rates for CD4+ T Cells, CD8+ T Cells, and NK Cells in the Stroma. Four groups classified by the number of immune cells in the stroma included 30 or 31 patients/group (Table 1). Better prognosis was found to correlate with increasing numbers of CD4+ or CD8+ T cells in the stroma (Fig. 2, A and B) and within the cancer cell nest (data not shown). In contrast, prognosis did not correlate with the number of NK cells in the stroma (Fig. 2C) or within the cancer cell nest (data not shown). In cases containing more than 10 NK cells/high power field in the stroma (n = 7), prognosis was not improved (data not shown).

Correlation between the Number of CD8+ T Cells in the Stroma and CD8+ T Cells within the Cancer Nest. CD8+ T cells in the stroma and within the cancer cell nest of individual cases are shown in Fig. 3. There was strong correlation (r = 0.790; P < 0.0001) between the number of CD8+ T cells in the stroma and the number of CD8+ T cells within cancer cell nest. The numbers of CD4+ T cells (r = 0.417; P < 0.0001) and NK cells (r = 0.405; P < 0.0001), however, did not exhibit similar correlation between the two sample regions (data not shown).

Kaplan-Meier Survival Analysis of CD4/8(+/+), CD4/8(–/+), CD4/8(–/–), and CD4/8(+/–) Patient Populations. Survival curves constructed according to the Kaplan-Meier method are shown in Fig. 4, A and B. Among 122 patients with ESCC, those in the CD4/8(+/+), CD4/8(–/+), and CD4/8(–/–) groups numbered 44, 17, 17, and 44, respectively. Only the CD4/8(+/+) group showed a significantly higher survival rate compared with the other three groups, which all exhibited similar survival (log-rank test, P = 0.0012; Fig. 4A). Among stage III and IV cancer patients (n = 48), those in the CD4/8(+/+), CD4/8(–/+), and CD4/8(–/–), and...
Correlation between CD4/8 Status and Clinicopathological Features. Correlations between CD4/8 status and various clinicopathological features are summarized in Table 2. CD4/8 status was found to correlate with gender ($P = 0.0352$). However, no significant correlation was found between CD4/8 status and age, pathological data on TNM classification, p-stage grouping, histopathological grading, tumor size, surgical margin, or postoperative adjuvant therapy.

Univariate and Multivariate Analyses. Univariate analysis for overall survival using a Cox regression model identified T classification, N classification, M classification, tumor size, surgical margin, and CD4/8 status as significant prognostic predictors. Multivariate analysis of the same set of patients was performed for pathological predictors and CD4/8 for survival time using the Cox regression model. The results indicated that CD4/8 status is an independent favorable prognostic factor. T classification and N classification also had independent prognostic value, with hazard ratios of 2.390 ($P = 0.0213$) and 4.585 ($P = 0.0002$), respectively (Table 3).

DISCUSSION

CD8$^+$ T cells can lyse tumor cells directly and destroy large tumor masses in vivo. Previous immunohistochemical data (6, 7) demon-
CD4/8 status and various clinicopathological features are shown. The correlation between CD4 and CD8 were designated as CD4/8. Patients were classified into the following four groups for analysis: patients classified as both CD4(+) and CD8(+) group (moderate group and scanty group), patients classified as either CD4(+) or CD8(+) group (medium group), and patients classified as either CD4(−) or CD8(−) group (abundant group). Patients were classified as both CD4(−) and CD8(−) group (moderate group and scanty group). Patients were classified as both CD4(+) and CD8(+) group (moderate group and scanty group).

On the other hand, cytokines such as IFN-γ secreted by CD4+ T cells can have systemic effects. These immune cells may prevent postsurgical systematic micrometastasis rather than actually suppressing stage progression.

CD4/8(+/+) status may be a valuable prognostic marker and index of postsurgical host immune reactivity. However, patient prognosis may be difficult to predict before surgical resection because biopsy specimens obtained by endoscopy may not be useful for determining CD4/CD8 status.

NK cells are components of the innate immune system capable of lysing target cells without prior sensitization. However, contrary to previous reports (12–14), the number of NK cells did not improve prognosis in our study. Although NK cells may work as effectors of nonspecific antitumor immunity, they comprise only 15% of all circulating lymphocytes (23) and a small fraction of the TILs (24). We think that the scarcity of NK cells explains their lack of prognostic effect. In fact, NK cells are present in the stroma in smaller numbers than CD4+ and CD8+ T cells, as shown in Table 1. Thus, CD4+ T cells and CD8+ T cells, rather than NK cells, appear to participate in antitumor immunity in patients with ESCC.

In conclusion, cooperation of CD4+ and CD8+ T cells appears to drastically improve the prognosis of patients with ESCC. Thus, combination immunotherapy including both activated CD4+ and CD8+ T cells may be effective in patients with ESCC.

Table 2 Clinicopathological features of 122 patients with ESCC according to combination of CD4+ T cells and CD8+ T cells

<table>
<thead>
<tr>
<th>Variables</th>
<th>CD4/8(+/+) (n = 44)</th>
<th>Others (n = 78)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Female</td>
<td>0.0352</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>≥60</td>
<td>&lt;60</td>
<td>0.4140</td>
</tr>
<tr>
<td>p stage</td>
<td>I</td>
<td>II</td>
<td>0.5445</td>
</tr>
<tr>
<td>Grade</td>
<td>G1</td>
<td>G2</td>
<td>0.2945</td>
</tr>
<tr>
<td>pT classification</td>
<td>T1</td>
<td>T2</td>
<td>0.3548</td>
</tr>
<tr>
<td>pN classification</td>
<td>N0</td>
<td>N1</td>
<td>0.1001</td>
</tr>
<tr>
<td>pM classification</td>
<td>M0</td>
<td>M1</td>
<td>0.2235</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>≥4.5</td>
<td>&lt;4.5</td>
<td>0.4319</td>
</tr>
<tr>
<td>Surgical margin</td>
<td>Positive</td>
<td>Negative</td>
<td>0.4861</td>
</tr>
<tr>
<td>Adjuvant therapy</td>
<td>Yes</td>
<td>No</td>
<td>0.9690</td>
</tr>
<tr>
<td>Performance status</td>
<td>0</td>
<td>1</td>
<td>0.3404</td>
</tr>
</tbody>
</table>

*p was calculated by χ² test.

Table 3 Univariate and multivariate analyses

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>2.812 (0.872–9.070)</td>
<td>0.0835</td>
</tr>
<tr>
<td>Age (≥60 yrs/≤60 yrs)</td>
<td>1.024 (0.563–1.861)</td>
<td>0.9388</td>
</tr>
<tr>
<td>p stage (I, III, II)</td>
<td>7.828 (3.969–15.437)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Grade (2, 3/1)</td>
<td>1.745 (0.781–3.898)</td>
<td>0.1745</td>
</tr>
<tr>
<td>pT classification (2, 3, 4/1)</td>
<td>3.767 (1.913–7.418)</td>
<td>0.0001</td>
</tr>
<tr>
<td>pN classification (1/0)</td>
<td>5.880 (2.885–11.985)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pM classification (1/0)</td>
<td>3.056 (1.615–5.780)</td>
<td>0.0006</td>
</tr>
<tr>
<td>Tumor size (≥4.5 cm/≤4.5 cm)</td>
<td>1.847 (1.013–3.366)</td>
<td>0.0452</td>
</tr>
<tr>
<td>Surgical margin (positive/negative)</td>
<td>5.126 (2.255–11.655)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Adjuvant therapy (no/yes)</td>
<td>1.078 (0.601–1.934)</td>
<td>0.8016</td>
</tr>
<tr>
<td>Performance status (1, 2/0)</td>
<td>1.222 (0.547–2.731)</td>
<td>0.6251</td>
</tr>
<tr>
<td>CD4/8 (CD4/8(+/+)/others)</td>
<td>0.208 (0.088–0.492)</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

*CI, confidence interval.

Univariate and multivariate analyses of immune cells and clinicopathological features were performed using the Cox proportional hazard regression model. T classification, N classification, and CD4/8(+/+) status are independent favorable prognostic factors.
ACKNOWLEDGMENTS

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