Infiltrating regulatory T cell numbers is not a factor to predict patient's survival in oesophageal squamous cell carcinoma.
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In previous studies, we found that cooperation between CD4+ and CD8+ T cells appears to drastically improve the prognosis of patients with oesophageal squamous cell carcinoma (OSCC) (Cho et al., 2003). Thus, the host immune response against cancer cells appears to play a critical role in the inhibition of recurrence and determines the postsurgical prognosis in OSCC. Recent reports suggested that thymic-derived CD4+ CD25+ regulatory T cells (Treg; Foxp3+ lymphocytes) participate in the control of tumour immunity, but whether Treg control tumour immunity in OSCC has not been established.

Treg represent a minor fraction (5–10%) of the CD4+ T cells, and they maintain immune homeostasis in immunotolerance and control autoreactive T cells. In addition to their role in autoimmunity, Treg participate in transplantation tolerance and tumour immunity. Although the mechanisms of suppression by Treg remain to be determined in vivo, many investigators have reported that Treg can inhibit immune responses mediated by CD4+ CD25+ T cells and CD8+ T cells in vitro via cell–cell contact (Sakaguchi et al., 1995; Sakaguchi, 2000; Shevach, 2002; Camara et al., 2003; Anatomy et al., 2005). Treg express CD25 (interleukin-2 receptor α), glucocorticoid-induced tumour necrosis factor receptor, and cytolytic T lymphocyte-associated antigen 4 on their surface. The nuclei of these cells also contain Foxp3, which is a member of the forkhead or winged helix family of transcription factors. Foxp3 is reported to be a key regulatory gene for the development and function of Treg and the most specific marker of Treg (Chatila et al., 2000; Brunkow et al., 2001; Mchugh et al., 2002; Fontenot et al., 2003; Horii et al., 2003; Khattri et al., 2003; Fontenot and Rudensky, 2005).

Several studies in mice have shown that Treg inhibit the anti-tumour immune response (Onizuka et al., 1999; Shimizu et al., 1999; Nishikawa et al., 2005) and that depletion of Treg can enhance effector T cell anti-tumour responses (Sutmuller et al., 2001; Tanaka et al., 2002). Additional studies have reported that the Treg population increases in peripheral blood and tumour tissues from patients with several types of human cancer (Woo et al., 2001; Liyanage et al., 2002; Ichihara et al., 2003; Curiel et al., 2004; Kawaida et al., 2005; Ormandy et al., 2005), but the relationship between the Treg population and the prognosis has not been clarified in OSCC. The purpose of this study, therefore, was to determine the effect of Treg on CD4+ and CD8+ T cells in OSCC. The present study was performed with same cohort as in Cho et al. (2003).

MATERIALS AND METHODS

Patients and specimens

One hundred and twenty-two patients (105 male and 17 female; mean age, 62.3 years) with primary OSCC underwent radical
oesophagectomy 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, Teine keijinkai Hospital and Department of Surgery, Hokkaido Gastroenterology Hospital). Preoperative examination did not find distant metastasis in any of the patients, and none of the patients had received prior anticancer treatments. Cases of in-hospital death were excluded from the current study. The clinical typing of tumours was determined according to the tumour-node-metastasis (TNM) classification system of the International Union Against Cancer (Sobin and Wittekind, 2002). All tumour specimens were fixed in 10% formalin and embedded in paraffin wax. One of the deepest sections from each tumour was selected for evaluation, and serial 4-μm thick sections were examined by immunohistochemistry. All the informed consent process for immunohistochemical staining were conducted in accordance with the guidelines of the Hokkaido University Institutional Review Board Authorization for this study.

Immunohistochemistry

For immunohistochemical analysis, formalin-fixed and paraffin-embedded specimens were deparaffinised in xylene and dehydrated through a graded series from ethanol to water. For antigen retrieval, sections were floated on 1 mM EDTA buffer (pH 9.0) in a plastic container and then heated in a domestic pressure cooker for 3 min after it reached the maximum pressure. Once cooled, the heat-treated sections were washed three times for 5 min each with PBS (pH 7.4). Before staining the sections, endogenous peroxidase activity was eliminated by a 20-min incubation in 0.3% hydrogen peroxide in methanol. After washing in PBS, specimens were blocked with 10% normal goat serum (Nichirei Corporation, Tokyo, Japan) for 30 min and then incubated at room temperature for 60 min with 1:40 mouse anti-human Foxp3 antibody (clone 246A/E7; Abcam, Cambridge, UK) in antibody diluent (Dako-Cytomation, Glostrup, Denmark). Normal adenoid tissue was used as a positive control for Foxp3. After washing with PBS, the sections were incubated for 60 min at room temperature with a biotinylated goat antibody to mouse immunoglobulin (Histofine Simple Stain MAX PO [MALTII]; Nichirei Corporation, Tokyo, Japan). After washing in PBS, immunohistochemical staining was developed by incubating the sections in freshly prepared 3,3′-diaminobenzidine tetrahydrochloride (Histofine Simple Stain DAB Solution; Nichirei Corporation) for approximately 10 min. The sections were washed in distilled water, counterstained with haematoxylin for 15 s, and mounted in Permount (Muto-Glass, Tokyo, Japan). Mouse IgG1 (DakoCytomation, Glostrup, Denmark) was used in place of the Foxp3 antibody for negative controls.

Quantification of Treg

Immunohistochemically stained sections were evaluated under a microscope (Olympus Optical Co. Ltd, Tokyo, Japan). The current study was performed in a retrospective manner, but all specimens were evaluated by two investigators blinded to the patients’ clinical information. Treg were quantified by analyzing five different high power fields (×400). Between 0 and 848 Treg were detected in the five fields, and the median (109) was used as a cutoff to define the subgroups.

Statistical analysis

Statistical analysis was performed using the χ² test. The Kaplan–Meier method was used to estimate overall survival and survival differences were analysed by the log-rank test based on the number of immune cells. Univariate and multivariate analyses of immune cells and clinicopathological features were performed using the Cox proportional hazard regression model. The F-test was used to analyse the variance in the CD8/CD4 ratio. The Mann–Whitney U-test was used to analyse the number of Treg. In all cases, P-values less than 0.05 were regarded as indicating statistical significance.

**Figure 1** Immunohistochemical staining. (A) Positive control tonsil stained with anti-human Foxp3 antibody. (B) Negative control tonsil stained with isotype-matched IgG. (C, D) OSCC stained with anti-human Foxp3 antibody at (C) × 200 and (D) × 400.
RESULTS

Immunohistochemical staining of Treg

Figure 1 shows representative photomicrographs of immunohistochemical staining for Treg using an antibody to human Foxp3. Treg were detected in cancer cell nests or in the stroma in contact with cancer cells.

Correlation between Foxp3 status and clinicopathological features

Correlations between Foxp3 status and various clinicopathological features are summarised in Table 1. Foxp3 status was found to correlate with CD4 status (P = 0.0186), CD8 status (P = 0.0021), and CD4/8 status (P = 0.0002). No significant correlation was found between Foxp3 status and age, gender, pathological data according to TNM classification, or p-stage grouping.

Table 1  Correlation between clinicopathological features of the 122 patients with OSCC and the number of Foxp3 positive cells

| Variable | No. of cases | Low n = 61 | High n = 61 | P-value |
|----------|--------------|------------|-------------|---------|
| Age (Years) |              |            |             |         |
| <62      | 58           | 28         | 30          | 0.7169 |
| ≥62      | 64           | 33         | 31          |         |
| Gender   |              |            |             |         |
| Male     | 105          | 53         | 52          | 0.7938 |
| Female   | 17           | 8          | 9           |         |
| pT classification |          |            |             |         |
| T1/T2    | 67           | 32         | 35          | 0.5852 |
| T3/T4    | 55           | 29         | 26          |         |
| pN classification |          |            |             |         |
| Negative | 60           | 28         | 32          | 0.4688 |
| Positive | 62           | 33         | 29          |         |
| pM classification |          |            |             |         |
| M0       | 101          | 50         | 51          | 0.8105 |
| M1       | 21           | 11         | 10          |         |
| pStage   |              |            |             |         |
| III      | 76           | 38         | 38          | >0.9999 |
| III/IVA  | 46           | 23         | 23          |         |
| CD8 status |            |            |             |         |
| Abundant | 61           | 24         | 37          | 0.0186 |
| Scanty   | 61           | 37         | 24          |         |
| CD4 status |            |            |             |         |
| Abundant | 61           | 22         | 39          | 0.0021 |
| Scanty   | 61           | 39         | 22          |         |
| CD4/8 status |         |            |             |         |
| CD4/8(+/) | 44           | 12         | 32          | 0.0002 |
| Others   | 78           | 49         | 29          |         |

Kaplan–Meier survival analysis of low Foxp3 and high Foxp3 patients

Survival curves were constructed according to the Kaplan–Meier method (Figure 2). In the 122 patients with OSCC (Figure 2A), the survival rates for patients with low Foxp3 were significantly lower than for patients with high Foxp3 (P = 0.0028 by log-rank test). In CD4/8 (+/+) patients (n = 44; Figure 2B) or CD4/8 (+/-) or (-/-) patients (n = 34; Figure 2C), Foxp3 status was not significantly related to the prognosis (P = 0.5185 and 0.8479, respectively, by log-rank test). In CD4/8(-/-) patients (n = 44; Figure 2D), the survival rates for patients with low Foxp3 were significantly lower than for patients with high Foxp3 (P = 0.0050 by log-rank test). Similar results were found in patients divided into subgroups of p-stages I/II (P = 0.0018; Figure 2E) and III/IV (P = 0.0352; Figure 2F).

Univariate and multivariate analyses

Univariate analysis for overall survival using a Cox regression model identified T classification, N classification, M classification, CD8 status, CD4 status, and Foxp3 status as significant predictors of the prognosis. Multivariate analysis of the same set of patients was performed for pathological predictors, CD4 status, CD8 status, and Foxp3 status for survival time using the Cox regression model. T classification, N classification, CD8 status, and Foxp3 status were of independent prognostic value (Table 2). Although Foxp3 status was not independent when multivariate analysis was performed using CD4/8 status and Foxp3 as factors, Foxp3 status had independent prognostic value in selected cases of CD4/8(-/-) patients (Hazard ratio = 3.382; P = 0.0207; data not shown).

Correlation between CD4/8 status and the number of Treg

A significant correlation was found between CD4/8 status and the number of Treg by the Mann–Whitney U-test (for CD4/8(+/+)) vs (-/-), P = 0.0021; for CD4/8 (+/+)/ vs CD4/8 (-/-), P = 0.0005; Figure 3). The median value of the number of Treg in CD4/8(+/+) and CD4/8(+/-), CD4/8(-/-), and CD4/8(-/-) patients was 203, 93, 68 and 67 respectively.

Correlation between Foxp3 status and the variance on CD8/CD4 ratio

In the 122 patients with OSCC (Figure 4A), the heterogeneity of the variance in the CD8/CD4 ratio was significantly different between high Foxp3 patients and low Foxp3 patients (P<0.0001 by F-test). The variance on CD8/CD4 ratio of low Foxp3 patients (variance: 30.046) was larger than high Foxp3 patients (variance: 0.279) in the same set of patients. Multivariate analysis of the same set of patients identified T classification, N classification, M classification, CD4 status, CD8 status, and Foxp3 status as significant predictors of survival time using the Cox regression model. Univariate and multivariate analyses showed that CD8/CD4 ratio was not independent when multivariate analysis was performed using CD4/8 status and Foxp3 as factors, Foxp3 status had independent prognostic value in selected cases of CD4/8(-/-) patients (Hazard ratio = 3.382; P = 0.0207; data not shown).

Kaplan–Meier survival analysis according to the CD8/CD4 ratio

The patients were divided into four groups according to their CD8/CD4 ratios (Figure 4C). The survival rates for patients with the highest and lowest CD8/CD4 ratios were significantly lower than for the other two groups (P = 0.0026 by log-rank test). In the CD8/8(-/-) patients (n = 44; Figure 4D), the CD8/CD4 ratio was not significantly related to the prognosis (P = 0.3566 by log-rank test).

DISCUSSION

In recent years, factors that regulate immune responses to malignant tumour cells have received a great deal of attention. Several of these studies have shown that Treg are recruited to
human carcinomas and they may influence the prognosis of cancer patients (Woo et al., 2001; Liyanage et al., 2002; Ichihara et al., 2003; Curiel et al., 2004; Kawaida et al., 2005; Ormandy et al., 2005). To adequately evaluate antitumour immune function, however, multiple factors should be evaluated simultaneously. Here, we found that the CD8$^{+}$ T cell effect was significant only when CD4$^{+}$ T cells were also present. This is the first report that has clarified the correlation of Treg with CD4$^{+}$ and CD8$^{+}$ T cells in OSCC and examined the effect of Treg on these T cells. In addition, we did not find a correlation between Foxp3 status and TNM classification, suggesting that Treg do not influence the progression of cancer.

Based on evidence from murine models, the prognosis of high Foxp3 patients is expected to be poor (Onizuka et al., 1999; Shimizu et al., 1999; Sutmuller et al., 2001; Tanaka et al., 2002; Nishikawa et al., 2005); however, the current results do not support this hypothesis because the high Foxp3 patients had a dramatically better prognosis than the low Foxp3 patients. Thus, the increase in Treg seemed to be due to an increase in the total number of

Table 2 Univariate and multivariate analyses of immune cells and clinicopathological features using the Cox proportional hazard regression model

| Variable                      | Univariate Hazard ratio (95% CI) | P-value | Multivariate Hazard ratio (95% CI) | P-value |
|-------------------------------|----------------------------------|---------|-----------------------------------|---------|
| Gender (male/female)          | 2.812 (0.872–9.070)              | 0.0835  |                                   |         |
| Age (≥62 vs <62) (years)      | 1.404 (0.783–2.519)              | 0.2543  |                                   |         |
| pT classification (3/4 vs 1/2) | 4.164 (2.238–7.747)              | <0.0001 |                                   |         |
| pN classification (1 vs 0)     | 5.880 (2.885–11.985)             | <0.0001 |                                   |         |
| pM classification (1 vs 0)     | 3.056 (1.615–5.780)              | 0.0006  |                                   |         |
| pStage (III/IV vs I/I)        | 7.828 (3.969–15.437)             | <0.0001 |                                   |         |
| CD4 status (abundant vs scanty)| 0.350 (0.184–0.664)              | 0.0013  |                                   |         |
| CD8 status (abundant vs scanty)| 0.451 (0.248–0.819)              | 0.0089  |                                   |         |
| Foxp3 status (low vs high)    | 2.474 (1.338–4.577)              | 0.0039  |                                   |         |
| CD4/8 (CD4/8(+/-) vs others)  | 0.208 (0.088–0.492)              | 0.0003  |                                   |         |
| Foxp3 status (low vs high)    | 2.474 (1.338–4.577)              | 0.0039  |                                   |         |

CI, confidence interval.
T lymphocytes, and Treg do not appear to suppress the anti-tumour immune response. In fact, the number of Treg in CD4/8(+/+) patients was significantly higher than in CD4/8(−/−) patients and CD4/8(+/−) patients, and survival curves divided on the basis of Foxp3 status in CD4/8(+/+) patients were similar. Interestingly, in CD4/8(−/−) patients, the survival rates for low Foxp3 patients were significantly lower than for high Foxp3 patients. Given these results, it appears that the presence of Treg suggested a normal antitumour immune response, and Treg do not appear to inhibit the proliferation of tumour-specific T lymphocytes. Similar results were shown in patients divided on the basis of p-stage. Curiel et al (2004) reported that tumour cells produce the chemokine CCL22, which mediates trafficking of Treg to the tumour and that the percentage of Treg in CD4+CD3− T cells is higher in the later than the earlier stage of disease; however, we did not find a correlation between p-stage and Foxp3 status, and high Foxp3 patients had a better prognosis in both earlier stage and advanced stage patients. Although the intratumor balance of Treg and CD8+ T cell has been shown to correlate with prognosis of several cancer types such as ovarian cancer and hepatocellular carcinoma (Sato et al 2005; Gao et al 2007), we did not find a significant correlation between CD8+ T cell/Treg ratio and prognosis. These data also support the idea that Treg do not suppress antitumour immunity in OSCC.

Various immunological factors must be considered to understand the state of antitumour immunity. We previously reported that the prognosis of the CD4/8(+/+) group is remarkably better than that of the other groups (Cho et al, 2003). Around the same time, Diederichsen Axel et al (2003) reported that a high CD8/CD4 ratio is associated with a better prognosis in colorectal cancer. Furthermore, Piersma et al (2007) also reported that a higher CD8/CD4 ratio is associated with the lack of tumour metastases in the draining lymph nodes of cervical cancer patients. Therefore, it is obvious that the balance of CD4+ T cell and CD8+ T cell is critical for prognosis of patients with cancer. In the present study, the favourable prognosis of high Foxp3 patients in the CD4/8(−/−) group appears to be related to the CD8/CD4 ratio. When the patients were divided into four groups on the basis of the CD8/CD4 ratio, the prognoses of the groups with the highest and lowest ratios were poorer than those of the other two groups. In addition, the CD8/CD4 ratio was disrupted in low Foxp3 patients with poorer prognoses. Similar results were observed in the CD4/8(+/−) patients. These findings show that a suitable CD8/CD4 ratio corresponds with a favourable prognosis and that it reflects the immune response against OSCC.

In conclusion, the increase of infiltrating Treg in OSCC patients is due to an increase in the total number of T lymphocytes, and the
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