Abstract. Background: Interleukin-12 (IL-12) is a powerful cytokine that plays an important role in cell-mediated immunity. Although IL-12 is produced by antigen-presenting cells (APCs), the relationship between IL-12 expression and APCs in colorectal cancer tissue remains unknown. Patients and Methods: Immunohistochemical detection of APCs and IL-12 was performed in 22 colorectal cancer specimens. CD83 and CD68 were used for the markers of mature dendritic cells (DCs) and macrophages, respectively. Double staining with CD83 or CD68 and IL-12 was also performed to detect IL-12-secreting cells. CD83-, CD68- and IL-12-positive-cell densities, clinicopathological factors and survival were analyzed. Results: CD83-, CD68- or IL-12-positive-cells were stained in the tumor stroma. Double-stained CD83/IL-12- or CD68/IL-12-positive-cells were also detected in the same area. The CD83-positive-cell density was significantly higher in patients with a high IL-12-positive-cell density than those with a low IL-12-positive-cell density. The CD83-positive-cell density was significantly lower in patients with T3-T4 depth of invasion, lymph node metastasis or tumors more advanced than stage II. The IL-12-positive-cell density tended to be lower in patients with T3-T4 depth of invasion or venous invasion. Patients with high CD83- or IL-12-positive-cell density in their cancer specimens showed significantly better prognosis. Conclusion: This study provides new information on the significance of mature DCs, macrophages and IL-12-secreting cells in the local environment of colorectal cancer. Survival in patients with colorectal cancer was reflected by mature DCs and/or IL-12-positive-cell density.

Key Words: IL-12, CD83, CD68, dendritic cell, macrophage, antigen-presenting cell, colorectal cancer.
secrete IL-12 at the local site of colorectal cancer. Furthermore, the relationship between these cell densities and clinicopathological factors or prognosis remains unknown. Therefore, we attempted to clarify these questions.

**Patients and Methods**

**Patients and specimens.** Twenty-two specimens were obtained from primary colorectal cancer patients who underwent surgery at the Department of Surgery I, University Hospital of Occupational and Environmental Health, Japan, between February 1998 and September 1999. The patient and tumor characteristics are shown in Table I. Informed consent was obtained from all patients prior to the study. The patients had not received chemotherapy or radiotherapy prior to surgery. Moreover, no patient was under treatment with drugs that influence cytokine secretion or immunity, such as steroid or interferon.

**Immunohistochemistry.** Immunohistochemistry of endogenous IL-12 was performed using the labelled polymer method. For each tumor, mouse anti-human monoclonal antibody to IL-12 p40 (1-1A4) (Cosmobio, Tokyo, Japan) was used as the primary antibody to identify IL-12-positive cells. At the time of operation, primary colorectal cancer specimens were collected and fixed in 10% formalin. Two-μm-thick sections from paraffin-embedded blocks were deparaffinized in xylene and rehydrated. The sections were pretreated with Proteinase K Enzyme Digestion (DAKO, Carpinteria, CA, USA). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 10 min. After washing with phosphate-buffered saline (PBS), the sections were pre-incubated with 10% goat serum albumin for 10 min and then incubated with IL-12 antibody at a 1:100 dilution for 60 min. After washing with PBS, each slide was treated with Envision+® (DAKO) for 30 min. Diaminobenzidine (DAB) -hydrogen peroxide was used as the chromogen in color development, and nuclear counterstaining with Mayer’s hematoxylin solution was performed.

Immunohistochemical detection of CD83 and CD68 was performed by the labelled streptavidin-biotin method. Mouse anti-human monoclonal antibodies to CD83 (1H4b, Novocastra Laboratories, Newcastle-upon-Tyne, UK) and CD68 (KP-1, DAKO) were used to identify mature DCs and macrophages, respectively. The deparaffinized 2-μm-thick sections were pretreated twice for 5 min with citrate buffer (0.01 mol/L; pH 6.0) at 100°C in a microwave oven. The slides were preincubated with 10% normal rabbit serum for 10 min and then incubated with antibodies to CD83 and CD68 at dilutions of 1:20 and 1:200, respectively. After washing with PBS, the slides were treated with anti-mouse IgG for 10 min and then incubated with streptavidin-biotinylated horseradish peroxidase complex (Nichirei, Tokyo, Japan) for 5 min. Colorization and nuclear counterstaining were subsequently carried out.

For double staining, sections were incubated in 1% hydrochloric acid for 120 min after IL-12 immunohistochemistry as the first-step. The second-step for CD83 or CD68 immunohistochemistry was performed using the alkaline phosphatase-conjugated streptavidin (Nichirei) staining method, and sections then colorized using the FUCHSIN+ substrate-chromogen system (DAKO).

Staining for negative controls followed the methods, except for incubation with PBS instead of the primary antibody, resulting in no detectable staining.

**Measurement of CD83-, CD68- and IL-12-positive-cell densities.** The mean values of CD83-, CD68- and IL-12-positive-cell densities were calculated as follows. Three areas with the highest density of positive cells at the tumor stroma were selected at low-power (x 100). The positive cells were then counted at 200x magnification, using an ocular grid. The sections were examined by two independent investigators without prior knowledge of the clinicopathological data or prognosis.

**Clinicalopathological assessment.** The tumors were staged by two pathologists, who had no prior knowledge of the results of the assays, according to the International Union Against Cancer (UICC) TNM Classification of Malignant Tumors (20). Clinicopathological factors such as age, gender, tumor size, nodal involvement, depth of invasion, distant metastasis, vessel invasion, histological type and staging were analyzed for association with CD83-, CD68- and IL-12-positive-cell densities.

**Statistical analysis.** Data are expressed as mean±SD. The statistical analyses were performed using Student’s t-test and regression theory, as appropriate. Survival analysis was performed using the Kaplan-Meier method, and statistical significance was calculated using the log-rank test. P-values less than 0.05 were considered statistically significant. Statistical calculations were performed using the StatView-J statistical package (version 5.0, SAS Institute, Inc., Cary, NC, USA).

**Results**

**Patient characteristics.** The profiles of the patients diagnosed with colorectal cancer, recruited in the present study, are provided in Table I. Three patients showed disease recurrence, including 1 pulmonary and brain metastasis, 1 bone metastasis and 1 lymph node metastasis in the hepatoduodenal ligament. Four other patients...
showed a worsening of their disease with residual hepatic or lymph nodes metastasis after surgery. Five of these 7 patients died from cancer during follow-up period.

**Immunohistochemistry.** Tumor cells of the colorectal carcinomas were not stained by CD83, CD68 and IL-12 monoclonal antibodies, although positively-stained cells were identified in the tumor stroma of the colorectal carcinoma. Representative photomicrographs of double-stained CD83/IL-12- and CD68/IL-12-positive cells are shown in Figure 1. Not all, but some of these cells were stained with both CD83 or CD68 (in pink) and IL-12 (in brown) at the same cells. The rate of double-stained cells was about 22.8% of CD83- and 12.5% of CD68-positive cells in the specimen of Figure 1, respectively.

**Relationship between CD83- or CD68-positive-cell density and IL-12-positive-cell density.** The mean CD83-, CD68- and IL-12-positive-cell densities were 75.5±45.4 cells/mm² (22.7-213.3), 190.6±67.7 cells/mm² (106.8-304.0) and 139.6±131.3 cells/mm² (21.3-562.7), respectively. The CD83-positive-cell density was significantly higher in patients with high IL-12-positive-cell density (≥70 cells/mm²) than those with low IL-12-positive-cell density (<70 cells/mm², p=0.0436, Figure 2). CD68-positive-cell density was not statistically associated with IL-12-positive-cell density in the colorectal cancer patients (data not shown).

**Correlation between clinicopathological factors and CD83-, CD68- or IL-12-positive-cell density.** The relationships between each positive-cell density and the clinicopathological factors in patients with colorectal cancer are given in Table II. CD83-positive-cell density was significantly lower in patients with T3-T4 depth of invasion (p=0.003), lymph node metastasis (p=0.004) and tumors more advanced than stage II (p=0.004), and tended to be lower in patients without well-differentiated adenocarcinoma (p=0.052). IL-12-positive-cell density tended to be lower in patients with T3-T4 depth of invasion (p=0.131) and venous invasion (p=0.097). CD68-positive-cell density was not significantly correlated with any clinicopathological factors (data not shown).

**Correlation between overall survival and CD83-, CD68- or IL-12-positive-cell density.** Kaplan-Meier survival curves for all patients, according to each positive-cell density, are provided in Figure 3A-C. The 5-year survival rates of low CD83- (<60 cells/mm²), low CD68- (<185 cells/mm²) and low IL-12-positive-cell density groups (<70 cells/mm²) were 50.0%, 69.2% and 50.0%, respectively. In contrast, the 5-year survival rates of high CD83- (≥60 cells/mm²), high CD68- (≥185 cells/mm²) and high IL-12-positive-cell density groups (≥70 cells/mm²) were 92.9%, 98.8% and 92.9%, respectively. The overall survival rates of the high CD83- or high IL-12-positive-cell density groups were significantly higher than that of the low CD83- or low IL-12-positive-cell density groups, respectively (p=0.0265 and p=0.0312, log-rank test, Figures 3A and 3C). On the other hand, the overall survival rate of the high CD68-positive-cell density group tended to be higher than that of the low CD68-positive-cell density group, however this difference was not statistically significant (p=0.2988, log-rank test, Figure 3B).

**Discussion**

Mature DCs and macrophages are thought to be major producers of IL-12. However, it is not known which cells actually secrete IL-12 at the tumor site. In this study, double
staining with CD83 or CD68 and IL-12 were detected on the same cells in tumor stroma (Figure 1). The rate of double-stained cells was about 22.8% of CD83- and 12.5% of CD68-positive cells in the specimen in Figure 1. These results suggest that IL-12 may be produced by both mature DCs and macrophages at the tumor site of colorectal cancer.

Another purpose of the present study was to evaluate the relationship among mature DCs, macrophages and IL-12-positive cells at local environments of colorectal cancer. CD83-positive cell density was significantly increased in patients with high IL-12-positive cell density (Figure 2), although CD68-positive cell density was not statistically correlated with IL-12-positive cell density (data not shown). One of the reasons for this difference might be that the mean CD68-positive cell density was over twice the CD83-positive cell density. Our data also suggest that CD83-positive cells, as well as CD68-positive cells, might be important for production of IL-12.

We also speculated that mature DCs, macrophages and IL-12-secreting cells might reflect the local immunity of colorectal cancer specimens. IL-12 has been demonstrated to have antitumor and antimetastatic effects in vivo (21, 22) and has been previously measured systemically and evaluated in several malignancies, including colorectal cancer. The pre-operative serum level of IL-12 was significantly lower in patients presenting with a more advanced stage in colorectal or esophageal cancer (23-25). Moreover, the production of IL-12 by peripheral blood mononuclear cells in patients with gastric or colorectal cancer decreased significantly with advancing disease, and was lowest in patients with distant metastases or cachexia (26). On the other hand, IL-12-secreting cells have been evaluated immunohistochemically at the inflammatory site, but not at the local site of colorectal cancer (27-32). In the present study, IL-12-positive cell density tended to be associated with depth of tumor invasion and venous invasion in the patients with primary colorectal cancer (Table II). Furthermore, overall survival was significantly higher in patients with high IL-12-positive cell density (Figure 3C). These results suggest that high local IL-12 secretion is associated with good prognosis and limited spreading of colorectal cancer. These findings may be consistent with previous studies noting the antitumor immunity and antimetastatic effect of IL-12.

CD83 has been identified as a highly specific marker for DCs, and is associated with the development of a mature DC phenotype. A previous study demonstrated a trend toward improved survival of patients with greater CD83- and CD86-double-positive cell density in colorectal cancer (33). In breast cancer, the number of CD83-positive tumor-infiltrating DCs was inversely correlated with positive lymph node metastasis, and an increased number of CD83-positive tumor-infiltrating DCs contributed greatly to both the relapse-free and overall survival rates (34). In the present study, CD83-positive cell density was also significantly

![Figure 2. Relationship between CD83-positive-cell density (cells/mm²) and high (≥70 cells/mm²) or low (<70 cells/mm²) IL-12-positive-cell density group, respectively. Closed box; mean value, Error bar; standard deviation, *; p=0.0436.](image)
inversely correlated with depth of tumor invasion, lymph node metastasis and tumor stage (Table II). Moreover, the number of patients recruited in this study was small. A more detailed and larger study will be needed to clarify these problems.

In conclusion, this study provided new information on the significance of mature DCs, macrophages and IL-12 in colorectal cancer. The results of the present study suggest that survival in patients with colorectal cancer may be reflected not only by CD83-, but also by IL-12-positive-cell density. Thus, colorectal cancer patients with low CD83- or IL-12-positive-cell density may require additional immunochemotherapy following surgery.

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