Prognostic Significance of Mature Dendritic Cells and Factors Associated With Their Accumulation in Metastatic Liver Tumors From Colorectal Cancer

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Although dendritic cells (DCs) play an important role in tumor immunity, their prognostic significance and factors related to mature DCs have not been addressed in metastatic liver tumors. In surgically resected, paraffin-embedded tissue sections from 70 patients with colorectal liver metastasis, CD83 (a marker of mature DCs) positive cells and cancer cells positive for the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assay were counted. Expression of gp96, which is considered to participate in the maturation of DCs, was also evaluated. CD83-positive cells were observed predominantly in the cancer invasive margin. Patients with CD83-positive cell counts of <2 per field had a significantly poorer prognosis (5-year survival rate 47.5% vs 23.1%; $P = 0.0184$). Patients with >0.83% apoptotic cancer cells had significantly higher numbers of CD83-positive cells (7.3 ± 7.3 vs 4.0 ± 5.1; $P = 0.039$). Patients with immunohistochemically positive gp96 expression in tumors had significantly higher numbers of CD83-positive cells than those with negative gp96 expression (6.0 ± 6.5 vs 1.4 ± 2.3; $P = 0.0108$). Patients with metachronous occurrence of liver metastasis had significantly higher numbers of CD83 positive cells than those with synchronous detection (6.3 ± 6.5 vs 3.9 ± 5.9; $P = 0.0313$). Although the number of apoptotic cancer cells, degree of tumor gp96 expression, and synchronous or metachronous occurrence of liver metastasis did not directly influence patient outcome, they did influence the number of CD83-positive cells in the cancer invasive margin, which was a significant prognostic factor in patients with colorectal liver metastasis. HUM PATHOL 35:1392-1396. © 2004 Elsevier Inc. All rights reserved.

Key words: CD83, dendritic cell, apoptotic cancer cell, heat shock protein, gp96.

Abbreviations: DC, dendritic cell; HSP, heat-shock protein; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing 0.1% (v/v) Tween-20; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

Several studies have shown that high numbers of dendritic cells (DCs) in tumor tissue, including lung cancer, breast cancer, and head and neck cancer, correlate with good prognosis.[1-3] However, no study to date has investigated the prognostic significance of mature DCs in metastatic liver tumors.

The most potent antigen-presenting cells, DCs are central to the regulation, maturation, and maintenance of the cellular immune response against cancer. After taking up antigens, immature DCs differentiate into mature DCs that prime naive T cells and initiate antigen-specific T-cell responses to the cancer.[4] Killed tumor cells have been shown to be a source of tumor-associated antigens for processing and presentation by DCs. However, there remains some disagreement as to the optimal form of killed tumor cells for DC uptake, maturation, and capacity to stimulate antigen-reactive T cells via the separate major histocompatibility class I and II pathways.[5] Sauter et al[6] reported in an in vitro study that although immature DCs phagocytosed both apoptotic and necrotic tumor cells, only necrotic cells induced DC maturation. This study also found that the range of dying cell to DC ratio was limited, such that some mixture ratios failed to induce DC maturation and that large amounts of apoptotic cells induced DC death. Because human cancers involve both apoptotic and necrotic tumor cells, the ratio of apoptotic to necrotic tumor cells can vary widely, making the quantitative assessment of tumor necrosis difficult in human carcinoma tissue. There have not yet been any reports on the relationship between the degree of apoptotic tumor cells and mature DCs in in vivo cancer tissue.

Among several factors that induce DC maturation,[7-15] heat-shock proteins (HSPs) are the most likely candidates for this effect.[16] Surface expression of gp96 on tumor cells stimulates DC maturation in vitro and induces efficient T-cell priming and tumor rejection in vivo.[17] It also has been reported that HSPs are released from tumor lysate but not from apoptotic cells. On the other hand, Feng et al[18] reported that once stressed, HSPs were identified on apoptotic cells.

The aims of the present study were to evaluate the prognostic significance of CD83 (a marker of mature DCs) positive cell infiltration,[19-22] and clinicopathologic factors associated with their accumulation in patients with metastatic liver tumor.

PATIENTS AND METHODS

Between January 1990 and January 2004, 113 patients underwent macroscopically curative hepatic resection due to colorectal liver metastasis at the First Department of Surgery, Shinshu University Hospital. Informed consent was obtained preoperatively from each patient to use part of the resected

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cancer lesion for research. Of the 113 patients, more than 5 years had passed after initial hepatectomy in 70. The patient group comprised 44 men and 26 women with a mean age of 61.1 years (range, 33 to 82 years). None of the patients received adjuvant chemotherapy. After discharge, all patients were followed up at our outpatient clinic on a monthly or bimonthly basis.

Clinicopathologic data on these patients were also reviewed retrospectively. As prognostic factors, age, sex, presence or absence of other distant metastasis (lung) at initial hepatectomy, number and maximum diameter of metastatic liver tumors, histological differentiation, presence or absence of cancerous exposure at the transected margin, and synchronous or metachronous detection and resection of liver metastasis were examined.

Immunohistochemistry for CD83 and gp96

Formalin-fixed, paraffin-embedded tissues were cut into 4-μm-thick sections and mounted on glass slides coated with poly-L-lysine. Deparaffinized rehydrated sections were incubated in 0.01 N HCl and 0.5% (v/v) pepsin for 20 minutes at 37°C to allow antigen retreval of CD83. Endogenous peroxidase activity was blocked by incubation for 10 minutes with methanol containing 3% (v/v) hydrogen peroxide. Washing, blocking, and immunoreactivity signal amplification were performed using a tyramide signal amplification kit (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions. Subsequently, primary antibodies were applied and left overnight at 4°C for CD83 and 1 hour at room temperature for gp96. The following primary antibodies were used: anti-CD83 (HB15A17.11, 1:1000 dilution; Serotec, Oxford, UK) and anti-gp96 (1:2000 dilution; NeoMarkers, Fremont, CA). Antibody binding was visualized using 3,3'-diaminobenzidine. After rinsing in water, cell nuclei were counterstained with hematoxylin, and the sections were dehydrated and coverslipped. As a negative control, normal mouse IgG (mouse primary antibody control; Zymed, San Francisco, CA) was used as the primary antibody.

CD83-positive cells were distributed predominantly in the invasive margin. Screening was performed at low magnification to first identify the 10 areas with the greatest numbers of CD83-positive cells in the invasive margin, then count the number of CD83-positive cells in each of these areas at high magnification (× 400).

Immunohistochemical staining for gp96 was evaluated by 2 independent observers who were blinded to the source of the specimens, and the entire area of each section was observed. Immunoreactivity of cancer or noncancer cells for gp96 was classified as negative if ≤10% of the total number of cancer or noncancer cells were positive, and positive if >10% of the total number of cancer or noncancer cells were positive.

Western Blot Analysis of gp96

Immediately after hepatectomy, samples obtained from both cancerous and noncancerous areas were snap-frozen in an acetone bath cooled in liquid nitrogen, and the specimens were then stored at −80°C. Liver tissue was homogenized in a buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol NaCl, 1% (v/v) Nonidet P-40, 0.1% (v/v) sodium dodecyl sulfate, 1% (w/v) sodium deoxycholate, 2 mmol EDTA, 1 mmol phenylmethyl sulfonyl fluoride, 2 mg/L aprotinin, 10 mg/L leupeptin, and 5 g/L pepstatin. The homogenates were centrifuged at 12,000 × g for 10 minutes at 4°C, and the supernatants were collected. The protein concentration was measured by bicinchoninic acid protein assay (Pierce, Rockford, IL). The same amounts of protein from liver homogenates were dissolved in sample buffer consisting of 25 mmol Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (v/v) sodium dodecyl sulfate, 0.02% (w/v) bromophenol blue, and 3% (v/v) 2-mercaptoethanol, loaded on 8% (w/v) polyacrylamide gels, and electrophoresed. The proteins were transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA) by electroblotting. The membranes were blocked for 1 hour at room temperature with 5% (w/v) nonfat dried milk and 0.1% (w/v) bovine serum albumin in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBS-T), then incubated for 1 hour with an anti-grp94 antibody diluted in TBS-T containing 5% (v/v) fetal bovine serum. After 3 washings in TBS-T, the membranes were incubated for 1 hour with peroxidase-conjugated rabbit anti-rat antibody (Dako, Glostrup, Denmark) diluted in TBS-T containing 5% (v/v) fetal bovine serum. After washing in TBS-T, blots were developed by enhanced chemiluminescence (Amersham) and exposed to X-ray film (RX-U; Fuji, Kawasaki, Japan).

Apoptotic Cancer Cell Count

To estimate apoptotic cancer cells, deparaffinized rehydrated sections were stained by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay[23] with some modifications. Briefly, formalin-fixed, paraffin-embedded sections were dewaxed, rehydrated, and digested with 20 μg/mL proteinase K (Sigma) for 15 minutes. Endogenous peroxidase was blocked by treatment with 0.3% (v/v) hydrogen peroxide. Sections were then rinsed in water and incubated with 50 μL terminal deoxynucleotidyl transferase buffer (30 mmol/L Tris-HCl [pH 7.2], 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride) containing 8.3 U terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, Germany) and 0.83 nmol biotinylated 16-deoxyuridine triphosphate (Boehringer Mannheim) in a moist chamber at 37°C for 60 minutes. Sections were then rinsed and incubated with horseradish peroxidase–conjugated streptavidin (Dako) diluted 1:500 in 0.01 mol/L Tris-HCl (pH 7.5) and 150 mmol/L NaCl (TBS) containing 1% (v/v) bovine serum albumin (Sigma, St. Louis, MO) for 30 minutes at room temperature, then rinsed in TBS and stained with diaminobenzidine. The percentage of apoptotic cancer cells was calculated by TUNEL positivity in 5 randomly chosen high-power (× 400) fields on each section.

Statistical Analysis

Data are shown as mean ± standard deviation. The Mann-Whitney *U*-test was used to compare the 2 groups. Survival curves were calculated by the Kaplan-Meier method, and statistical significance was tested by the log-rank test. Multiple regression analysis was performed using the Cox proportional hazards model. Variables to be entered into the multivariate analysis were chosen on the basis of the results of univariate analysis. A stepwise procedure and a likelihood ratio test were used to select the variables for the final model. All analyses were performed using the Statview 5.0 statistical software package (Abacus Concepts, Berkley, CA).

RESULTS

CD83-positive cells were observed predominantly in sinusoid-like vessels very close to cancer cells and among infiltrating cells in the cancer invasive margin (Fig 1). When patients were divided into 2 groups based on the median value of CD83-positive cell count

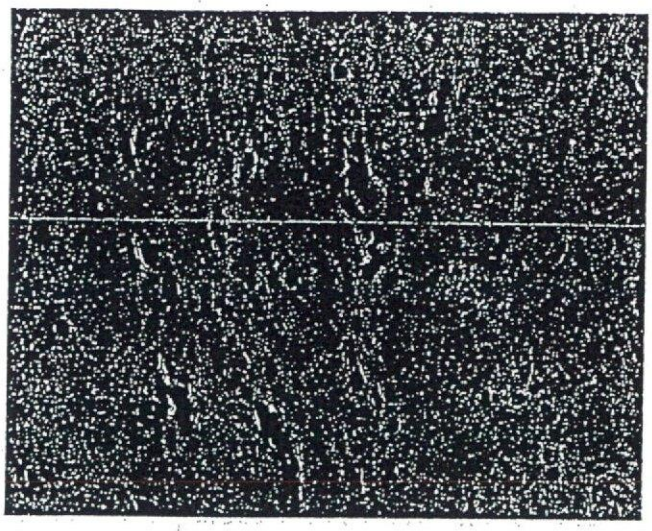

FIGURE 1. CD83-positive cells distributed predominantly in an invasive margin. (Original magnification × 400.)

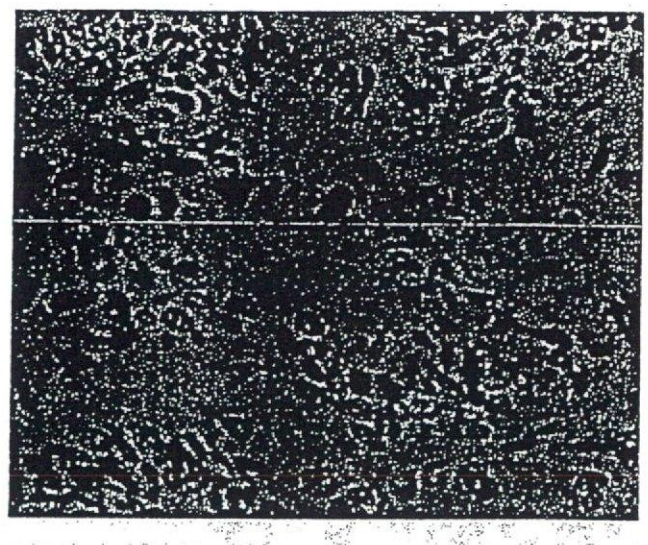

FIGURE 3. TUNEL-positive apoptotic tumor cells in colorectal liver metastasis. (Original magnification × 400.)

over a total of 700 areas (ie, 10 areas from 70 patients), patients with CD83-positive cell counts of <2 per field (n = 26) had a significantly poorer prognosis than patients with ≥2 CD83-positive cells per field (n = 44) (5-year survival rate, 47.5% vs 23.1%; $P = 0.0184$) (Fig 2).

The mean percentage of apoptotic cancer cells per slide was 0.83%, with a range of 0 to 3.9% (Fig 3). Patients with >0.83% apoptotic cancer cells (n = 32) had a significantly higher number of CD83-positive cells than patients with ≤0.84% apoptotic cancer cells (n = 38) (7.3 ± 7.3 vs 4.0 ± 5.1; $P = 0.039$). However, the percentage of apoptotic cancer cells itself had no influence on patient survival.

Western blot analysis demonstrated gp96 expression in both cancer and noncancer regions (Fig 4); gp96 immunostaining also demonstrated positivity in both cancer and noncancer regions. Immunohistochemically, 61 patients exhibited positive gp96 expression in tumor tissue (Fig 5), and these patients had a significantly higher number of CD83-positive cells than the remaining 9 patients who exhibited negative gp96 expression (6.1 ± 6.6 vs 1.4 ± 2.3; $P = 0.0107$). Noncancerous gp96 expression had no influence on the number of CD83-positive cells. Also, gp96 itself was not a significant prognostic factor.

Univariate analysis demonstrated that patients with mucinous tumor ($P < 0.0001$), a positive surgical margin ($P = 0.0121$), and lung metastasis ($P = 0.001$) had significantly poorer prognosis. However, age, sex, number of liver tumors, maximum tumor diameter, and synchronous or metachronous detection did not significantly correlate with patient survival after initial hepatectomy (Table 1). Patients with metachronous resection had a significantly higher number of CD83-positive cells than those with synchronous resection (6.3 ± 6.5 vs 3.9 ± 5.9; $P = 0.0313$), although the remaining factors were not related to CD83-positive cell count. Cox multivariate regression analysis demonstrated that lung metastasis, histological differentiation, and number

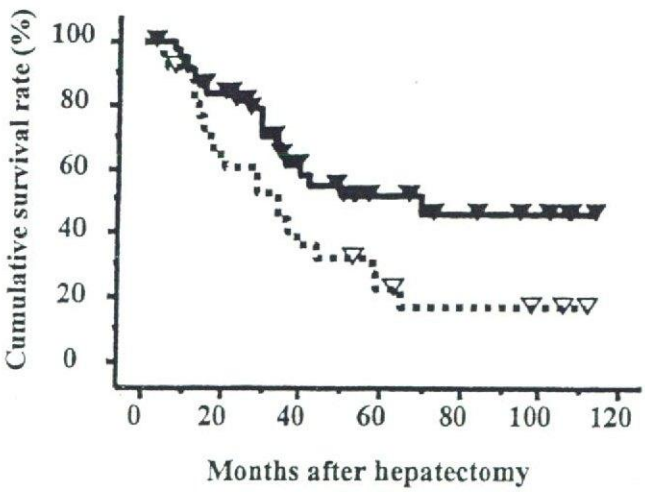

FIGURE 2. Comparison of the cumulative survival rate between patients with CD83-positive cell counts of <2 per field (dotted line, n = 26) and patients with ≥2 CD83-postive cells per field (solid line, n = 44).

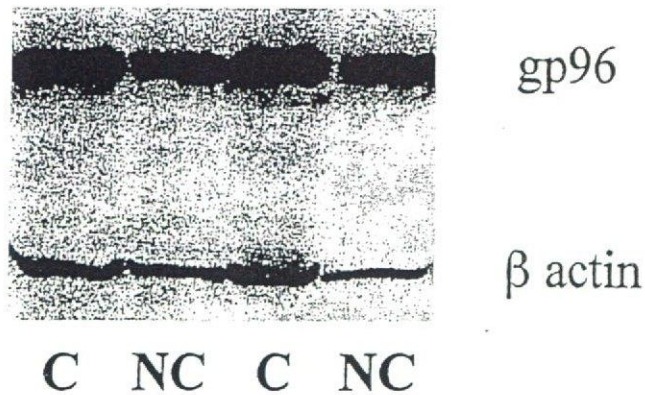

FIGURE 4. Western blot of gp96 and β-actin in cancer (C) and noncancer tissues (NC).

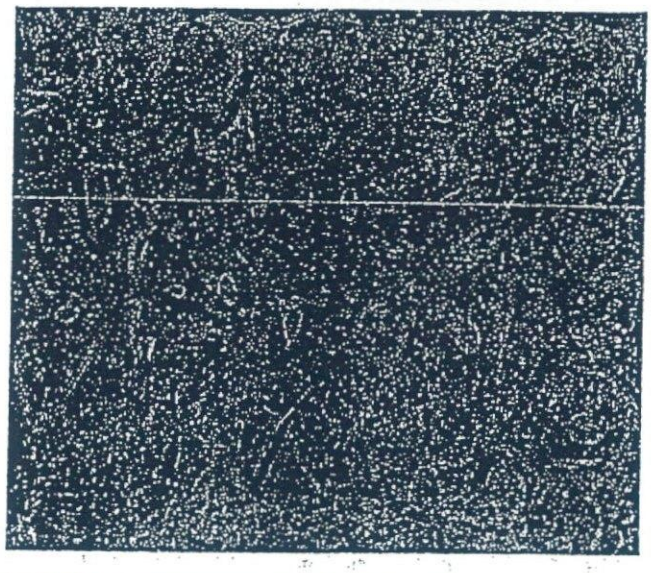

FIGURE 5. Immunostaining of gp96-positive cancer tissue. (Original magnification × 100.)

of CD83-positive cells in the cancer invasive margin were significant independent prognostic factors (Table 2).

DISCUSSION

An abundance of intratumor DCs is associated with better patient outcomes for a variety of carcinomas, including cervical, colorectal, gastric, lung, and nasopharyngeal cancers.[1-3] CD83 is widely used in in vitro studies as a marker for mature DCs.[19-22] Bell et al[24] reported that immature DCs reside within the tumor, whereas mature DCs tend to be located in the peritumoral areas in breast carcinoma. Suzuki et al[25] reported that in the invasive margins of colorectal cancer stroma, mature CD83-positive DCs form clusters with T cells to promote T-cell activation and the development of tumor-specific immunity. However, no study has investigated the prognostic significance of mature DCs in metastatic liver tumors. The present study demonstrated that patients with mature DC counts of <2 per field had a significantly poorer prognosis. Sauter et al[6] reported that although immature DCs phagocytosed both apoptotic and necrotic cells, only necrotic cells induced DC maturation. Their study also described that the range of dying cells to DC ratio was limited, such that some mixture ratios failed to induce DC maturation and that large amounts of apoptotic cells induced DC death.[6] The uptake of apoptotic cells may be involved in the production of anti-inflammatory signals that establish peripheral self-tolerance.[26-28] Pietra et al[29] reported that tumor cells in early phases of apoptosis inhibited DC maturation, whereas cells in late apoptosis or even primary necrosis delivered a partial maturation signal. In vitro studies have yielded controversial results. Also, human cancers involve both apoptotic and necrotic tumor cells, and the ratio of apoptotic to necrotic tumor cells can vary widely. To date, there have been no reports on the relationship between apoptotic tumor cells and mature DCs in in vivo cancer tissue. Our study found that the mean percentage of apoptotic cancer cells was only 0.84%. This did appear to influence the number of CD83-positive cells, although it was not a prognostic factor. This result suggests that mature DCs are positively associated with apoptotic cancer cells in in vivo human cancer tissue.

Several factors induce DC maturation. These include microorganisms,[7-9] presence of the CD40 ligand on activated T cells,[10-12] cytokines (eg, TNF-α, IL-1β), bacterial and viral products,[13-15] and nucleotides, but HSPs are the most likely candidates for this effect.[16] Surface expression of gp96 on tumor cells stimulates DC maturation in vitro and induces efficient T-cell priming and tumor rejection in vivo.[17] In the present study, gp96 itself was not found to be a prognostic factor. However, in cancers immunohistochemically positive for gp96, a significantly higher number of

TABLE 1. Univariate Analysis of Predictive Factors

Factor	Number of patients	5-year survival rate (%)	*P* value
Age (years)			0.6625
<62	35	42.7	
≥62	35	33.6	
Sex			0.3594
Male	44	38.3	
Female	26	38.5	
Histological types			<0.0001
Well differentiated	30	49.6	
Moderately differentiated	35	34.3	
Mucinous	5	0	
Number of metastatic liver tumors			0.3266
1	31	41.2	
≥2	39	35.7	
Maximum tumor diameter (mm)			0.3254
<47	42	32.5	
≥47	28	46.4	
Lung metastasis			0.001
Presence	16	12.5	
Absence	54	45.7	
Occurrence of metastasis			0.4822
Synchronous	24	29.2	
Metachronous	46	42.9	
Surgical margin			0.0121
Positive	20	15.0	
Negative	50	47.3	

TABLE 2. Cox's Multivariate Regression Analysis

Variables	χ^2	*P* value	Hazard ratio	95% confidence interval
Histological type	20.362	<0.0001		
Moderately differentiated	8.345	0.0039	2.53	1.348–4.751
Mucinous	18.5	<0.0001	12.223	3.906–38.252
CD83-positive cell count < 2	8.227	0.0041	2.385	1.317–4.319
Absence of lung metastasis	9.773	0.0018	0.33	0.171–0.638

CD83-positive DCs was observed at the invasive margin than in gp96-negative cancers. This finding suggests that cancer gp96 expression is related to CD83-positive DCs in patients with metastatic liver tumors.

The effect of the time interval between the primary procedure and liver resection on the patient's prognosis remains controversial. The plausible causes of better prognosis in patients with metachronous occurrence of liver metastasis have not been defined, even though the prognostic significance of metachronous occurrence of liver metastasis has been described.[30-33] The present study has revealed that although synchronous or metachronous resection of liver metastasis did not influence prognosis after initial hepatectomy, patients who underwent metachronous resection and had a disease-free interval from primary to metastasis had a significantly higher number of mature DCs. This finding suggests a difference in host immunity between patients with synchronous and metachronous occurrence of liver metastasis. This difference in mature DC count might be related to the prognostic difference between these patient groups, as demonstrated by some previous studies.[30-33]

Although the number of apoptotic cancer cells, tumor gp96 expression, and synchronous or metachronous occurrence of liver metastasis had not influence on patient's outcome, they did appear to influence the number of CD83-positive cells in the cancer invasive margin. This was a significant prognostic factor in patients with colorectal liver metastasis.

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Dendritic Cells, T-Cell Infiltration, and Grp94 Expression in Cholangiocellular Carcinoma

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Although dendritic cells (DCs) play an important role in tumor immunity, there have been no reports on their role in cholangiocellular carcinoma (CCC). In 26 formalin-fixed, paraffin-embedded tissue sections from patients with CCC, cells positive for CD83 (a marker of mature DCs), CD1a (a marker of immature DCs), and CD8 and CD4 (T cell markers) were counted, and expression of glucose-regulated protein (grp) 94, which is considered to participate in the maturation of DCs, was evaluated by immunohistochemistry and Western blot analysis to study the relationship between their expression and patients' disease outcome. The number of CD83-positive DCs at the invasive margin of CCCs correlated significantly with the number of CD8-positive or CD4-positive T cells in the cancerous region and was significantly higher in grp94-positive cancer than in grp94-negative cancer ($P = 0.0006$). CD83-positive patients (positive cells in invasive margin > 12.4/field) had both a significantly lower incidence of lymph node metastasis (23.1% vs 69.2%; $P = 0.0206$) and a better outcome than CD83-negative patients ($P < 0.001$). We conclude that mature DCs are distributed predominantly at the invasive margin of cancers, and a significantly higher number of mature DCs at the invasive margin are observed in patients with grp94-positive cancer cells. Mature DCs may enhance CD8- and CD4-positive cell infiltration into cancers and improve prognosis in patients with CCC, due in part to abatement of lymph node metastasis. HUM PATHOL 35:881-886.

Key words: cholangiocellular carcinoma, dendritic cells, CD83, grp94.

Abbreviations: CCC, cholangiocellular carcinoma; CR, cancerous region; DC, dendritic cell; grp94, glucose-regulated protein 94; HSP, heat-shock protein; IM, invasive margin; NC, noncancerous region; TBS-T, Tris-buffered saline containing 0.1% Tween-20; TIL, tumor-infiltrating lymphocyte.

Dendritic cells (DCs) are the most potent antigen-presenting cells and are central to the regulation, maturation, and maintenance of a cellular immune response to cancer. After taking up antigens, immature DCs differentiate into mature DCs that prime naive T cells and initiate antigen-specific T-cell responses to the cancer.[1] The T-cell antigen receptors recognize fragments of antigens bound to molecules of major histocompatibility complex classes I and II on the surface of DCs, which stimulate CD8-positive cytotoxic T lymphocytes and CD4-positive helper T cells.[2]

Several studies have shown that the presence of a high number of DCs in tumor tissue (eg, lung cancer, breast cancer, head and neck cancer) correlates with a good prognosis.[3-5] Among several DC-specific and maturation-associated markers, CD83 is a marker for mature DCs[6] and CD1a is a marker for immature DCs.[7,8]

The ability to present exogenous antigens through a process called "cross-presentation" is a key feature of DCs. Peptides chaperoned by heat-shock protein (HSP), like glucose-regulated protein 94 (grp94), which is resident in the endoplasmic reticulum, can be presented to cytotoxic T lymphocytes by DCs. Such presentation requires the uptake of grp94 via a cell surface receptor, CD91, which is expressed by DCs.[9-11]

Until now, the emergence and prognostic significance of DCs and grp94, which stimulate T lymphocytes and may play a central role in the antitumor immune response, have not yet been examined in cholangiocellular carcinoma (CCC). The aims of this study were, therefore, to evaluate the infiltration of mature DCs, CD8-positive T cells, CD4-positive T cells, and tumor grp94 expression in patients with CCC.

MATERIALS AND METHODS

Patients

Between 1992 and 2002, 26 patients underwent hepatectomy for CCC at the First Department of Surgery, Shinshu University Hospital, leaving no macroscopic evidence of residual cancer. This group comprised 15 men and 11 women with a mean age of 67 years (range, 52 to 81 years). After discharge, all of the patients were followed at our outpatient clinic on a monthly or bimonthly basis. The median follow-up time was 17 months (range, 2 to 47 months). Permission was obtained preoperatively from each patient to use part of the resected tumor lesion for research.

Immunohistochemistry of CD83, CD1a, grp94, CD4, and CD8

Formalin-fixed, paraffin-embedded tissues were cut into 4-μm-thick sections and mounted on glass slides coated with poly-L-lysine. The endogenous peroxidase activity was blocked for 30 minutes with 3% (v/v) hydrogen peroxidase. We used the tyramide signal amplification system (NEN Life Science

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Products, Boston, MA) according to the manufacturer's instructions. The sections were preincubated with TNB blocking buffer containing 0.1 mol Tris-HCl (pH 7.5), 0.15 mol NaCl, and 0.5% (w/v) blocking reagent (supplied in a kit). Subsequently, primary antibodies were applied and left overnight at 4°C. The primary antibodies used were anti-CD83 (Serotec, Oxford, UK), anti-CD1a (Serotec), anti-grp94 (Neo-Markers, Fremont, CA), anti-CD4 (Novocastra, Newcastle Upon Tyne, UK), and anti-CD8 (Dako, Glostrup, Denmark). The sections were washed in TNT wash buffer containing 0.1 mol Tris-HCl (pH 7.5), 0.15 mol NaCl, and 0.05% (v/v) Tween 20. Antibody binding was visualized using 3,3'-diaminobenzidine. After rinsing in water, cell nuclei were counterstained with hematoxylin, and the sections were dehydrated and coverslipped.

For double immunostaining, sections were first incubated in 0.01 mol (pH 6.0) or 1 mmol (pH 8.0) ciytric acid buffer for 10 minutes after staining with alkaline phosphatase–conjugated streptavidin (Dako). The second step for the immunohistochemistry was done as described earlier using diaminobenzidene as the stain. As a negative control, normal mouse IgG (mouse primary antibody control; Zymed, San Francisco, CA) was used as the primary antibody.

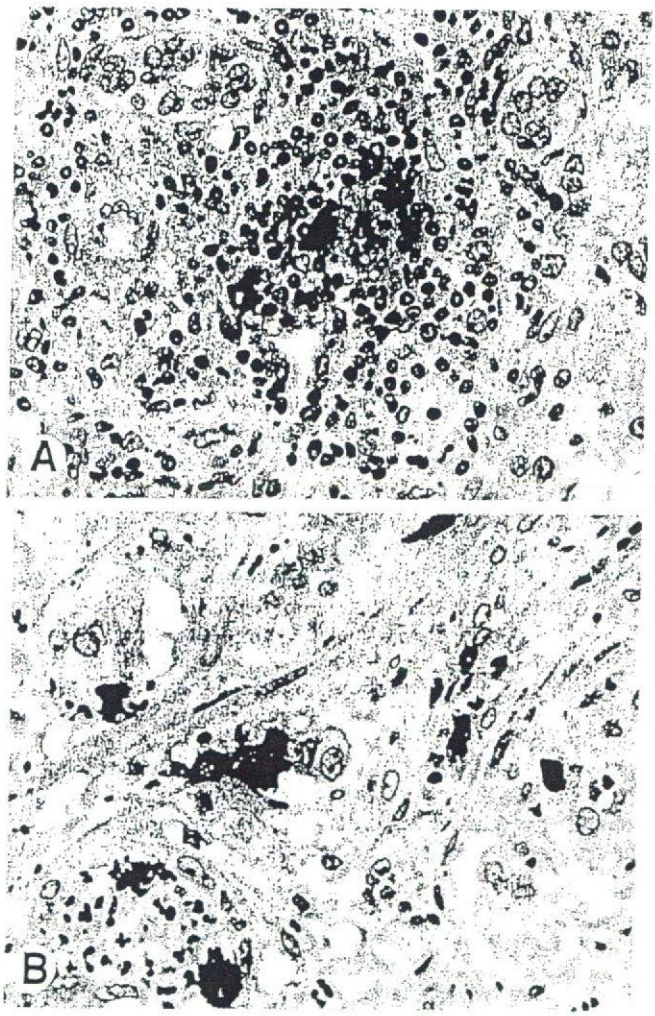

FIGURE 1. Immunostaining for CD83 in cancer tissues of CCC. (A) CD83-positive DCs at the IM. (B) CD1a-positive DCs in the CR. (Original magnification × 400.)

In 5 randomly chosen high-power fields (× 400) from 3 areas (CR, cancerous region; IM, invasive margin; and NC, noncancerous region), the numbers of cells positive for CD83, CD1a, CD8, and CD4 were counted using an Olympus BX50 microscope (Olympus, Lake Success, NY) and the same microscope objective. The immunohistochemical staining for grp94 was evaluated by 2 independent observers who were blinded to the source of the specimens, and the entire area of each section was observed. Immunoreactivity of cancer and noncancer cells for grp94 was classified as negative (−) if ≤5% of the total number of cancer or noncancer cells were positive, and as positive (+) if >5% of the total number of cancer or noncancer cells were positive.

Western Blot Analysis of grp94

Immediately after hepatectomy, samples obtained from both cancerous and noncancerous areas were snap-frozen in an acetone bath cooled in liquid nitrogen, and the specimens were then stored at −80°C. Liver tissue was homogenized in a buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol NaCl, 1% (v/v) Nonidet P-40, 0.1% (v/v) sodium dodecyl sulfate, 1% (w/v) sodium deoxycholate, 2 mmol EDTA, 1 mmol phenylmethyl sulfonyl fluoride, 2 mg/L aprotinin, 10 mg/L leupeptin, and 5 g/L pepstatin. The homogenates were centrifuged at 12,000 × *g* for 10 minutes at 4°C, and the supernatants were collected. The protein concentration was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The same amounts of protein from liver homogenates were dissolved in sample buffer (25 mmol Tris-HCl [pH 6.8], 10% [v/v] glycerol, 2% [v/v] sodium dodecyl sulfate, 0.02% [w/v] bromophenol blue, and 3% [v/v] 2-mercaptoethanol), loaded on 8% (w/v) polyacrylamide gels, and electrophoresed. The proteins were transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA) by electroblotting. The membranes were blocked for 1 hour at room temperature with 5% (w/v) nonfat dried milk and 0.1% (w/v) bovine serum albumin in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBS-T), and then incubated for 1 hour with an anti-grp94 antibody diluted in TBS-T containing 5% (v/v) fetal bovine serum. After 3 washings in TBS-T, the membranes were incubated for 1 hour with peroxidase-conjugated rabbit anti-rat antibody (Dako) diluted in TBS-T containing 5% (v/v) fetal bovine serum. After washing in TBS-T, blots were developed by enhanced chemiluminescence (Amersham) and exposed to X-ray film (RX-U; Fuji, Kawasaki, Japan).

Statistical Analysis

The Mann-Whitney U-test and Spearman's rank correlation were used for statistical analyses. The χ^2 method with Yates's correction or Fisher's exact test was used for qualitative variables. The survival curves were calculated by the Kaplan-Meier method and compared using the log-rank test. Differences with a P value <0.05 were considered statistically significant.

RESULTS

CD83-positive cells (Fig 1A) were distributed predominantly in the IM (Fig 2), but CD1a-positive cells (Fig 1B) were observed only in the CR. Double immunostaining of CD83 and CD8 or of CD83 and CD4 (Fig 3A and B) showed each CD83-positive cell surrounded

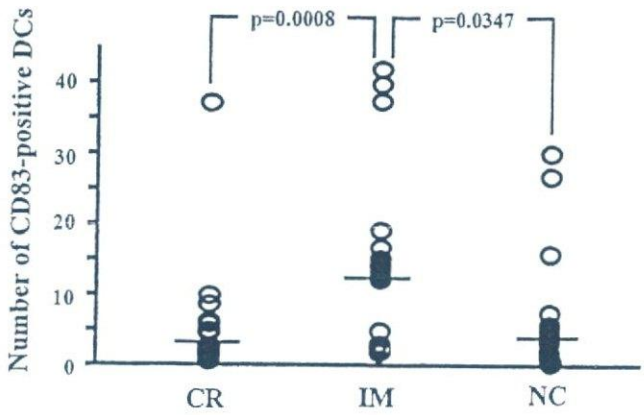

FIGURE 2. Predominant distribution of CD83-positive cells in the IM.

by CD8- or CD4-positive cells. The number of CD83-positive cells in the IM correlated significantly with the number of CD8-positive (Spearman's rank correlation coefficient = 0.507; $P = 0.0113$) or CD4-positive cells in the CR (Spearman's rank correlation coefficient = 0.598; $P = 0.0028$). CD8-positive patients (positive cells in CR >16/field) or CD4-positive patients (positive cells in CR >26/field) had significantly better prognoses than CD8-negative ($P = 0.0407$) or CD4-negative patients ($P = 0.0024$).

Western blot analysis (Fig 4) and immunostaining for grp94 (Fig 5) revealed grp94 expression in both CR and NC. However, the number of CD83-positive DCs in IM did not differ between patients with positive grp94 expression in NC and those with negative grp94 expression in NC ($P = 0.109$). Patients with grp94-positive cancer cells had a significantly high number of CD83-positive DCs in IM than those with grp94-negative cancer cells ($P = 0.0006$) (Fig 6), although grp94 itself was not a significant prognostic factor.

When patients were divided by the TNM classification,[12] the stage II patients had significantly longer survival than the stage IIIC patients ($P < 0.01$) (Fig 7).

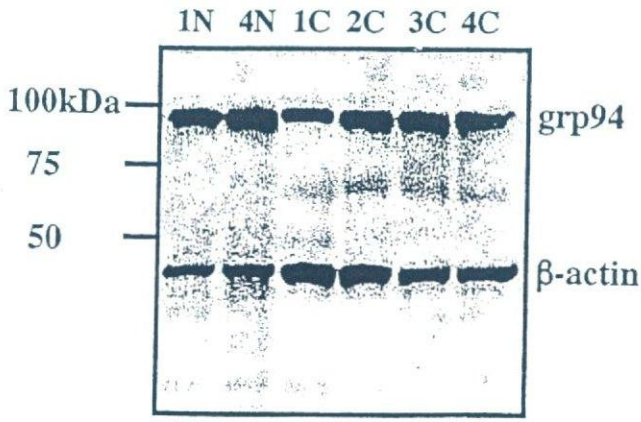

FIGURE 4. Western blot of grp94 and β-actin in cancerous (lanes 1C to 4C) and noncancerous tissues (lanes 1N and 4N). All samples show a strong band at 94 kDa, indicating grp94.

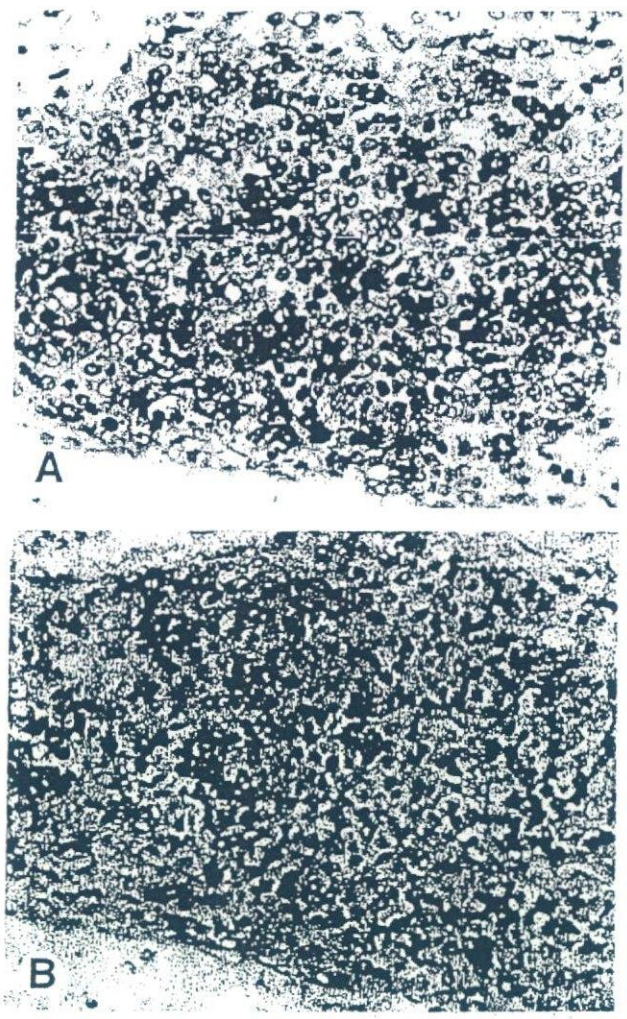

FIGURE 3. Double immunostaining of CD83 and CD8, and CD83 and CD4. (A) Double immunostaining of CD83 (pink) and CD8 (brown). (B) Double immunostaining of CD83 (pink) and CD4 (brown). (Original magnification × 400.)

Also, the stage II patients had significantly more CD83-positive DCs at the invasive margin than did the stage IIIC patients ($P = 0.0296$) (Fig 8).

When patients were divided into 2 groups (positive or negative) based on the median value of each factor, CD83-positive patients (positive cells in IM >12.4/field), whose CD83-positive cell count was 20.3 ± 11.1 (mean ± standard deviation), had both a significantly lower incidence of lymph node metastases (23.1% vs 69.2%; $P = 0.0206$) and better prognosis than CD83-negative patients (CD83-positive cell count, 2.7 ± 3.3) ($P < 0.001$) (Fig 9). CD1a-positive patients (positive cells in CR > 5.3/field) had also significantly better prognoses than CD1a-negative patients ($P = 0.0119$). The number of CD1a-positive cells in CR was significantly correlated with that of CD83-positive cells in IM (Spearman's rank correlation coefficient = 0.671; $P < 0.001$). Other clinicopathologic factors, including age, sex, preoperative serum carcinoembryonic antigen and carbohydrate antigenic determinant 19-9 levels,

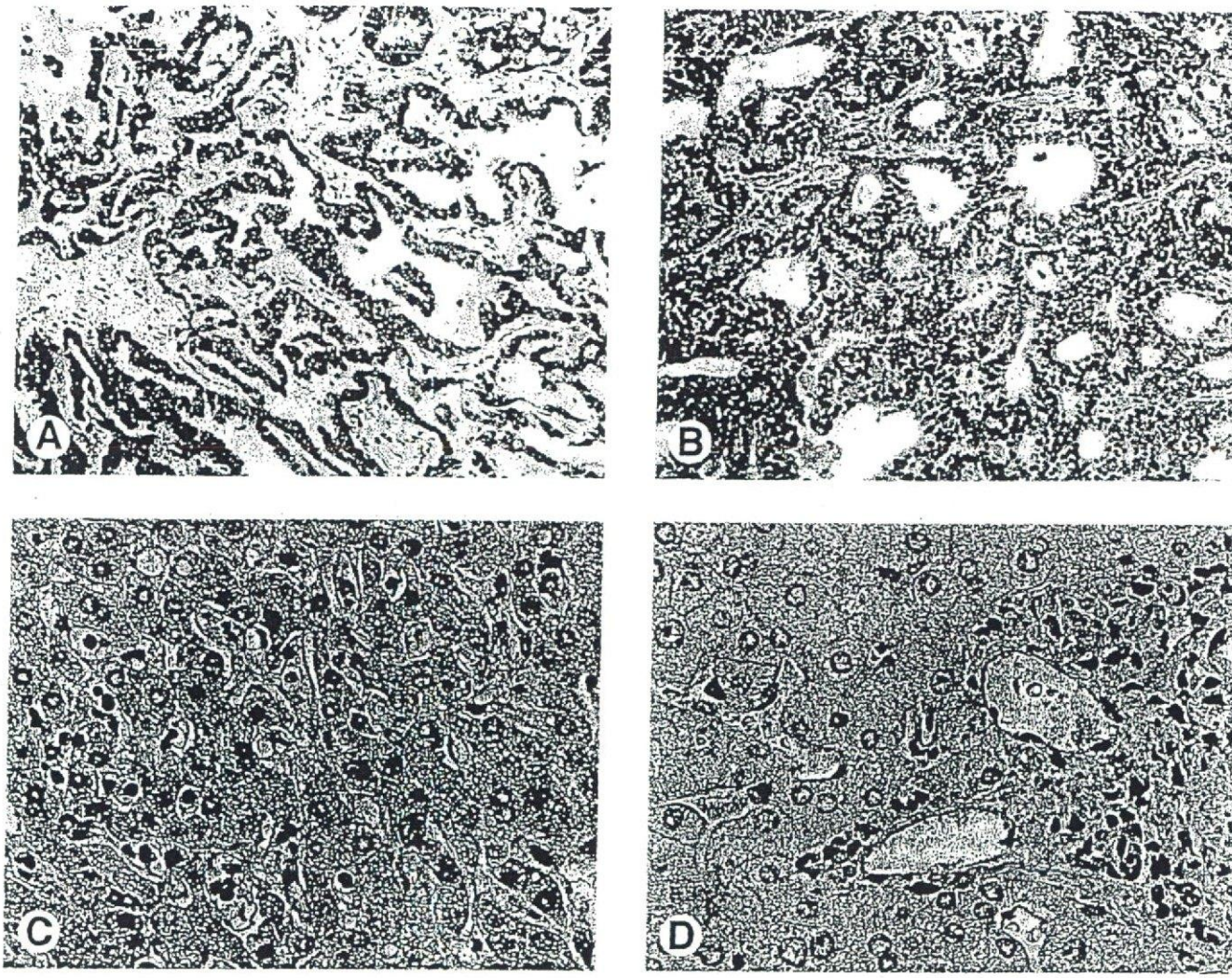

FIGURE 5. Immunostaining of grp94 of the tumor. (A) Immunostaining of grp94-positive CR. (B) Immunostaining of grp94-negative CR. (C) Immunostaining of grp94-positive NC. (D) Immunostaining of grp94-negative NC. (Original magnification × 400.)

tumor diameter, histological differentiation, vascular invasion, microscopic intrahepatic metastasis, and residual cancer at the transection margin showed no significant difference between CD83 or CD1a-positive and -negative patients.

DISCUSSION

CCC is the second most common primary malignancy of the liver. Its resectability rate is extremely low, but surgical resection is the only definitive treatment,[13,14] because the nonsurgical methods available to date have failed to change the outcomes. Therefore, there is a need to investigate new treatment modalities for CCC. An abundance of intratumor DCs is associated with a better outcome in patients with various of carcinomas, including cervical, colorectal, gastric, lung and nasopharyngeal cancers.[2-4] However, there has been no report on the relationship between the number of CD83-positive DCs or CD1a-positive DCs and survival in patients with CCC.

CD83 has been used widely for in vitro studies as a marker of mature DCs.[15-18] Bell et al[19] reported that in breast carcinoma immature DCs reside within the tumor, whereas mature DCs are located in peritumoral areas. Suzuki et al[20] reported that in the invasive margin of colorectal cancer stroma, mature CD83-positive DCs form clusters with T cells to promote T-cell activation for the generation of tumor-specific immunity. The present study also showed that in patients with CCC, the distribution of CD83-positive DCs differs from that of CD1a-positive DCs. CD83-positive DCs were located predominantly in the invasive margin, whereas CD1a-positive DCs were located only in the cancerous region. Immature DCs take up tumor-specific antigen, migrate to lymphoid tissue, and undergo maturation there. However, so far, no report has described the prognostic significance of immature DCs in the cancer-

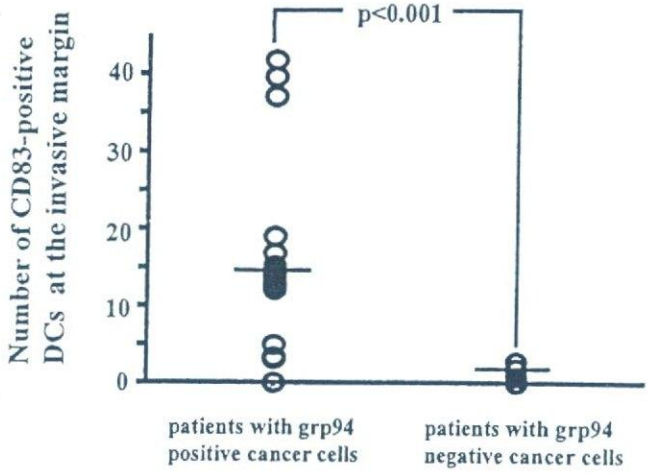

FIGURE 6. Numbers of CD83-positive DCs at the IM in patients with grp94-positive and grp-negative cancer cells. (14.7 ± 12.0 vs. 0.8 ± 1.3 cells/high power field; $P < 0.001$.)

ous region and mature DCs in the peritumoral area. The present study showed that the number of immature DCs in the cancerous region is correlated with that of mature DCs at the invasive margin and that both are significant prognostic factors, suggesting a potential relationship between DCs in the cancerous region and those at the invasive margin. However, this morphological analysis alone did not directly prove that immature DCs mature in the cancerous region and migrate to the invasive margin. The present study also showed that more advanced staged patients had fewer CD83-positive cell count, suggesting that a host immune response depends negatively on cancer progression.

Although previous studies showed that a greater abundance of tumor-infiltrating lymphocytes (TILs) and a stronger proliferative ability of TILs are associated with a better survival of patients with cancer,[21-22] including colorectal and renal cell carcinoma, there have been no reports on the relationship between TILs and DCs in patients with CCC. The present results obtained by double-immunostaining for CD83 and CD8 or CD83 and CD4 revealed that CD83-positive cells

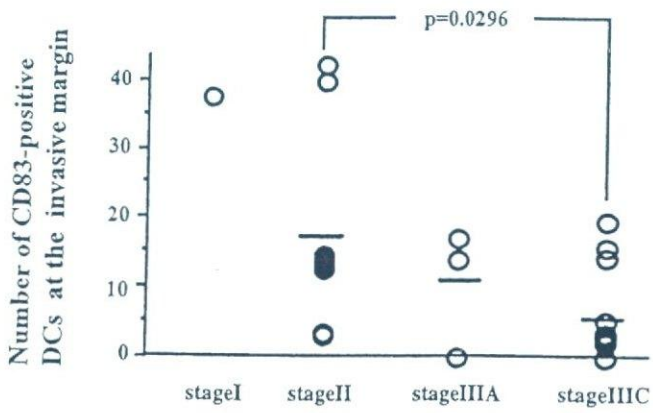

FIGURE 8. Numbers of CD83-positive DCs at the IM in stage I, II, IIIA and IIIC patients divided by TNM classification.

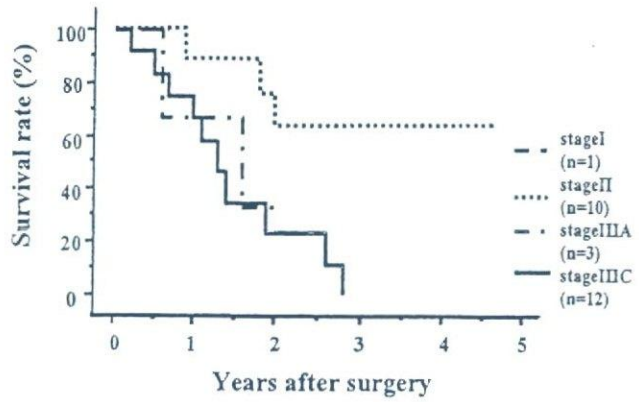

FIGURE 7. Five-year survival curves in patients with stages I, II, IIIA, and IIIC TNM classifications. (Stage II vs. stage IIIC, $P < 0.01$.)

were surrounded by CD8- or CD4-positive cells. The number of mature DCs at the invasive margin was correlated significantly with that of CD8-positive T cells or that of CD4-positive T cells in the cancerous region. Patients with high numbers of CD8-positive or CD4-positive T cells in the cancerous region had significantly better prognoses than those without. The present study also showed that CD83-positive patients had a significantly lower incidence of lymph node metastases, suggesting a relationship between the number of mature DCs at the invasive margin and the likelihood of lymph node metastasis.

Taken together, the prognostic significance of CD83-positive DCs at the invasive margin may be explained as follows. When more abundant mature DCs are located at the invasive margin, there is a greater chance of T cells encountering mature DCs, and an increase in the number of activated T cells is expected, which might result in prevention of the spread of the cancer and a reduction in the incidence of lymph node metastases and, consequently, a better prognosis.

Various factors induce DC maturation. These include microorganisms,[23-25] presence of the CD40 li-

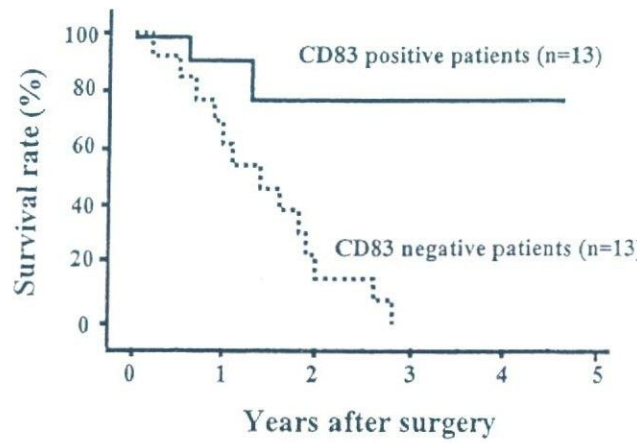

FIGURE 9. Five-year survival curves of CD83-positive (solid line) and CD83-negative (dotted line) patients (5-year survival rate, 78.6% vs 0%, respectively; $P < 0.001$).

gand on activated T cells,[26-28] cytokines (eg, tumor necrosis factor α, interleukin-1β), bacterial and viral products, [29-31] and nucleotides, but HSPs are the most likely candidates to explain this effect.[32] Surface expression of grp94 on tumor cells stimulates DC maturation in vitro and induces efficient T-cell priming and tumor rejection in vivo.[33] After maturation, DCs are no longer able to bind grp94 molecules; hence the grp94 receptor, CD91, is down-regulated.[34] Regression of some tumors, including melanoma and breast cancer, after injection with DCs has been observed, and associated with up-regulation of tumor HSP expression and infiltration of lymphocytes in response to HSP derived from autologous tumors.[35] In the present study, grp94 itself was not found to be a prognostic factor. In cancers immunohistochemically positive for grp94, however, a significantly higher number of CD83-positive DCs was observed at the invasive margin than those in grp94-negative cancers. This finding suggests that cancer grp94 expression is related to CD83-positive DCs in patients with CCC, but there is no relationship with CD1a-positive immature DCs.

In conclusion, mature DCs may influence the infiltration of CD8- and CD4-positive cells into CCC, with a consequent improvement in prognoses, due in part to abatement of lymph node metastasis.

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Significance of Macrophage Chemoattractant Protein-1 Expression and Macrophage Infiltration in Squamous Cell Carcinoma of the Esophagus

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OBJECTIVES: Macrophage chemoattractant protein-1 (MCP-1) is a chemokine-inducing infiltration of macrophages, which can play several roles in tumor growth and metastasis. We have attempted to clarify the relationship between MCP-1 expression and macrophage infiltration in esophageal squamous cell carcinoma (SCC).

METHODS: Paraffin-embedded sections of tissue samples taken from 56 patients with esophageal SCC after curative surgery were immunohistochemically stained for MCP-1, CC chemokine receptor 2 (CCR-2), and thymidine phosphorylase (TP). Macrophage recruitment in SCC was evaluated by monocytic count based on CD68 immunostaining. Microvessels immunostained for Factor VIII-related antigen were counted in SCC, and microvessel density (MVD) was determined. Ki-67 labeling index was calculated based on Ki-67 immunostaining, and an apoptotic index was calculated based on the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end labeling.

RESULTS: MCP-1 was expressed in cancer cells of 31 SCC (55.4%) and in stromal cells mainly identified as macrophages of 16 SCC (28.6%). CCR-2 was expressed in stromal cells of all SCC and in vascular endothelial cells of 15 SCC (26.8%). There was a significant correlation between the expression of MCP-1 in cancer cells and of CCR-2 in stromal cells. TP was expressed in stromal cells in 76.7% of the SCC. Monocytic count, MVD, and Ki-67 LI in SCC with MCP-1 expression in cancer cells were higher than that without, and apoptotic index in SCC with MCP-1 expression in cancer cells were lower than that without. Furthermore, the monocytic count was positively correlated with MVD, while it was inversely correlated with apoptotic index. Clinicopathologically, MCP-1 expression in cancer cells was correlated with venous invasion, distant metastasis, and lymph node metastasis. Monocytic count in SCC with venous invasion, distant metastasis, or lymph node metastasis was higher than that without them. Five-year survival rate in the patients with high monocytic count or MCP-1 expression was worse than that with a low monocytic count or without MCP-1 expression.

CONCLUSIONS: These results suggest that MCP-1 expression and macrophage infiltration is associated with angiogenic promotion in esophageal SCC. MCP-1 expression may be interactively associated with macrophage infiltration in esophageal SCC; MCP-1 may play an important role in tumor angiogenesis through production of angiogenic factors, such as TP, by recruited macrophages in esophageal SCC. Furthermore, CCR-2 expression in vascular endothelial cells may participate partially in angiogenesis. Clinicopathologically, esophageal SCC patients with MCP-1 expression have no favorable prognosis.

(Am J Gastroenterol 2004;99:1667-1674)

INTRODUCTION

Angiogenesis in tumor stroma plays a key role in tumor growth, invasion, and metastasis, and is stimulated by several angiogenic factors and cytokines releasing from tumor cells and/or stromal cells (1, 2). Furthermore, interaction between tumor and stromal cells is essential for angiogenesis and tumor growth. Some of these stromal cells have been identified as tumor-associated macrophages, and they play an important role in tumor growth, invasion, and metastasis (3). Infiltrating macrophages in tumor stroma are regulated by several kinds of cytokines and growth factors (4, 5), including macrophage

chemoattractant protein-1 (MCP-1) (6). MCP-1 is a member of C-C chemokines (6), and possesses chemotaxic activity for macrophages (7). MCP-1 has been reported to be upregulated in several tumors (8, 9). Furthermore, MCP-1 may be an indicator of angiogenesis because MCP-1 gene transfection in tumor cells promotes angiogenesis, and MCP-1 transfection induces angiogenesis (10). In addition, MCP-1 may also have a direct effect on tumor angiogenesis by inducing chemotaxis of endothelial cells, as evidenced by the detection of CC chemokine receptor-2 (CCR-2), which is a receptor for MCP-1 in these cells. Thymidine phosphorylase (TP) is an angiogenic factor that is identified as platelet-derived endothelial cell growth factor (11, 12). In several tumors, such as breast cancer (13), TP was expressed in tumor-associated macrophages, and TP expression participates in tumor angiogenesis and metastasis. On the other hand, angiogenesis influences proliferation and apoptosis in tumors. However, it is not yet well known how MCP-1, which may be an angiogenic promoter, influences apoptosis and cell proliferation in tumors.

In squamous cell carcinoma (SCC) of the esophagus, it has already been reported that vascular endothelial cell growth factor (VEGF) (14) and TP (15) are major components of angiogenic factors, and their expression is associated with angiogenesis, a high incidence of distant metastasis, and a poor progonosis of patients. In the present study, MCP-1 expression in esophageal SCC was immunohistochemically examined, and the significance of MCP-1 expression and macrophage infiltration as well as the relationship between them were investigated in esophageal SCC. Furthermore, the relationship between TP and MCP-1 expression, and the relationship between MCP-1 expression and apoptosis/cell proliferation were investigated in esophageal SCC.

MATERIALS AND METHODS

Patients and Tissue Preparation

Tissue samples of esophageal SCC were obtained from 56 patients who underwent esophagectomy with curative intent, without having received preoperative chemotherapy and radiotherapy, between 1989 and 1998 in the Department of Surgery, Shinshu University Hospital. These tissues were fixed in 10% formalin buffered with phosphate at pH 7.4 and embedded in paraffin. Serial sections were made and mounted on poly L-lysine-coated glass slides. Routine histopathological examination was performed to determine histological differentiation, depth of invasion, presence of lymph node metastasis, and lymphatic and venous invasion according to the TNM classification (16).

Immunostaining for MCP-1, CCR-2, and TP

The avidin-biotin complex (ABC) method was used for MCP-1 immunostaining. The immunostaining was performed using anti-human MCP-1 monoclonal antibody (5D3-F7, Pharmingen, San Diego, CA) diluted 50-fold. Sections immersed in citrate buffer were treated in a microwave oven before the staining procedure. CCR-2 immunostaining was performed using anti-human CCR-2 monoclonal antibody (48607.121; Genzyme/Techne, MN) diluted 100-fold. TP immunostaining was performed using a mouse anti-TP IgG antibody (clone 654-1; Nippon Roche Research Center, Kamakura, Japan) (17) diluted 500-fold according to the ABC method. Visualization of the immunoreaction for MCP-1, CCR-2, and TP was performed with the staining medium for peroxidase containing 0.05% 3,3'-diaminobenzidine tetrachloride. For negative controls, non-immunized mouse immunoglobulin-G was substituted for primary antibody at the same concentration as the test antibody in every run.

Expression of MCP-1 and TP in cancer or stromal cells was characterized as negative or positive, according to the extent of the immunostaining. If immunoreactivity of MCP-1 and TP was randomly expressed, it was judged to be positive when more than 10% of cancer cells were stained in each section. In well χ^2 and moderately differentiated SCC, the reaction was judged by excluding the keratinized portion localized at the central part of the cancer nest, called cancer pearl. MCP-1 and TP expression of stromal cells in SCC was judged to be positive if more than 10% of stromal cells were stained in each section.

Immunostaining and Count of Microvessels

Microvessels in SCC tissue were immunostained by the ABC method using rabbit antihuman factor VIII-related antigen polyclonal antibody (Dako, Glostrup, Denmark) diluted 300-fold. Microvessel count was performed according to the method of Maeda *et al.* (18). Briefly, the factor VIII-stained sections were screened at five-fold magnification under a microscope to identify the areas of highest vascular density within SCC tissue. Microvessels in cancer stroma were counted in the five areas with the highest density at 200-fold magnification. Microvessel density (MVD) was expressed as the average of the microvessel count in these areas.

Immunostaining for CD68 Antigen and Monocytic Count

Macrophages were identified by positive immunostaining for CD68 antigen. CD68 immunostaining was performed by the ABC method using monoclonal antibody KP1 (Dako Patts, Copenhagen, Denmark) diluted 100-fold. For counting macrophages in esophageal SCC, a method similar to that used to determine MVD was performed. After screening at low magnification under a microscope, five areas with a high density of CD68-positive stromal monocytes were selected, and CD68-positive stromal monocytes were counted at 200-fold magnification, and taken as the monocytic count.

Ki-67 Immunostaining and TUNEL

Ki-67 immunostaining was performed by the ABC method using anti-Ki-67 monoclonal antibody (MIB-1; Immunotech. S.A., Marseille, France) diluted 100-fold. Sections were treated in a microwave oven before the staining procedure. Visualization of the immunoreaction was performed with DAB.

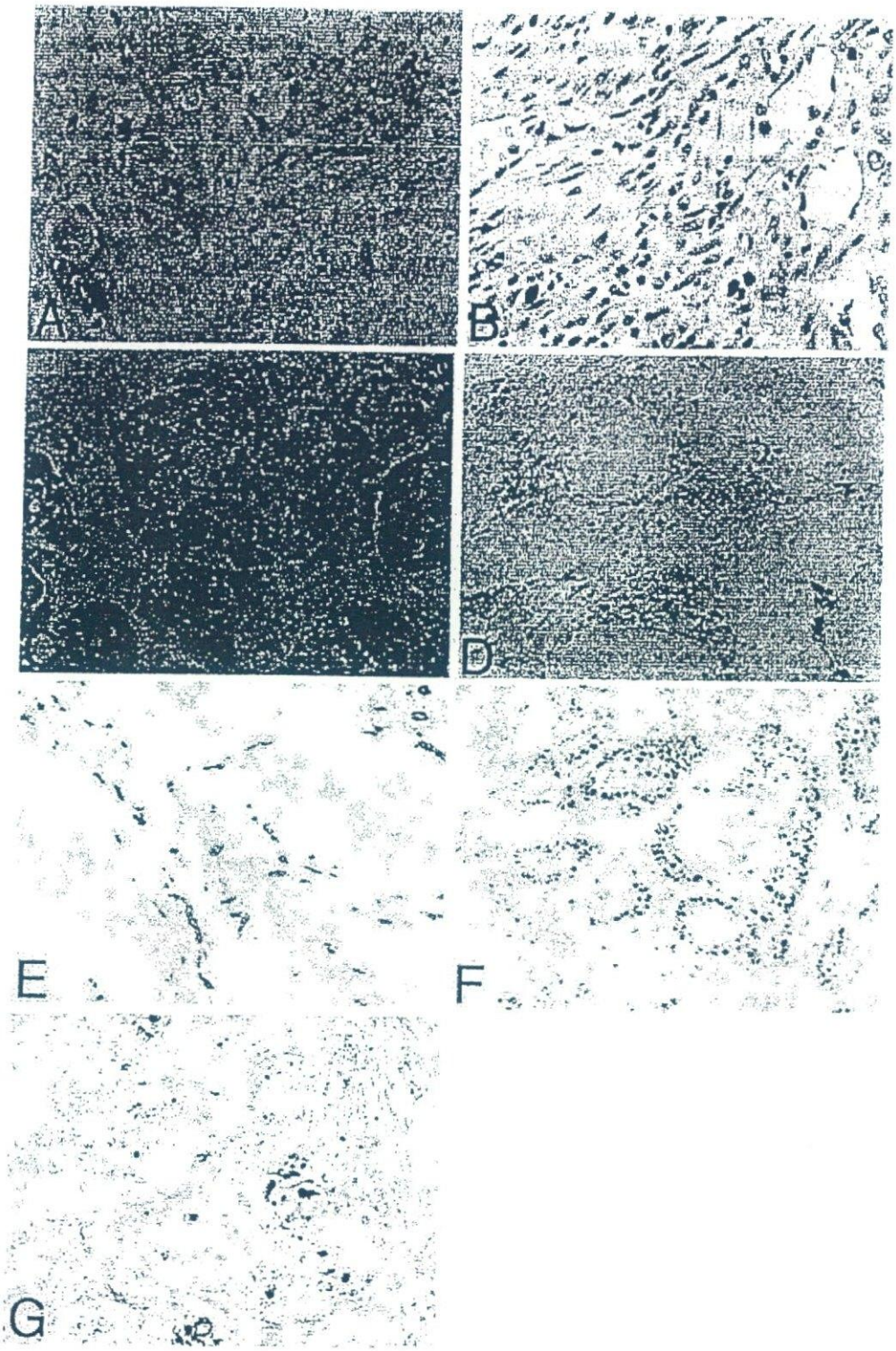

Figure 1. Immunohistochemical findings. (*A*) Macrophage chemoattractant protein-1 (MCP-1) immunostaining. Not only squamous cell carcinoma (SCC) cells but infiltrating stromal cells revealed a positive reaction for MCP-1. (*B*) CC Chemokine receptor-2 (CCR-2) immunostaining. Stromal cells revealed a positive reaction for CCR-2, as did vascular endothelial cells occasionally. (*C*) Tymidine phosphorylase (TP) immunostaining. TP was expressed in cancer and stromal cells. (*D*) CD 68 immunostaining. Stromal cells infiltrated in SCC mainly showed a positive reaction for CD 68. Most of these cells showed CD 68-positive reaction. (*E*) Factor VIII-related antigen immunostaining. A positive reaction for Factor VIII-related antigen is observed in the microvessels in the tumor stroma. (*F*) Ki-67 immunostaining. Ki-67-positive reaction is observed in the nuclei of the cancer cells. (*G*) TUNEL. A TUNEL-positive reaction is observed in the condensed nuclei of the cancer cells.

Table 1. Relationship Between MCP-1 and TP Expression of Cancer Cells and/or Stromal Cells

TP Expression in Stromal Cells	MCP-1 Expression					
	Cancer Cells			Stromal Cells		
	Positive	Negative	*p* Value	Positive	Negative	*p* Value
Positive	28	15	0.009	15	28	0.054
Negative	3	10		1	12	

MCP-1, macrophage chemoattractant protein-1; TP, thymidine phosphorylase.

A dark accumulation of DAB in the nuclei was judged to indicate a positive reaction for Ki-67. The percentage of cancer cells with nuclei stained for Ki-67, the Ki-67-labeling index, was calculated for each section on the basis of staining of about 2,000 cancer cell nuclei.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate–biotin nick end labeling (TUNEL) was performed according to the method of Gavrieli *et al.* (19), and briefly used subsequent reactions with proteinase K (20 mg/ml, Dako), and terminal deoxynucleotidyl transferase (6.75 × 10−2 unit/ml, Life Sciences, St. Petersburg, FL) and biotinylated deoxyuridine triphosphate (3.75 pM/ml, Enzo Diagnosis, New York). The TUNEL reaction was visualized with DAB. Dark accumulation of DAB in the cells (nuclei and apoptotic bodies) was judged to indicate a positive reaction to TUNEL. When a few apoptotic bodies were clustered in a portion of a section, this aggregate was judged to have originated from an apoptotic cell and was counted as one apoptotic cell. The rate of TUNEL-positive cells (apoptotic index) was calculated for each section by examining about 2,000 cancer cells, excluding the keratinized portion (cancer pearl) in the central part of the carcinoma nests in the well χ^2 and moderately differentiated SCC.

Statistical Analysis

The clinicopathological features and the histochemical results were analyzed by the χ^2 test or the Mann-Whitney test. The significance of correlation among the monocytic count, MVD, apoptotic index, and Ki-67 labeling index was evaluated by Pearson's analysis. Survival rates were analyzed by the Cox-Mantel test. Statistical significance was defined as $p < 0.05$.

RESULTS

Expression of MCP-1, CCR-2, and TP

In esophageal SCC, a positive reaction for MCP-1 was revealed in the cytoplasm of cancer cells and/or infiltrating stromal cells (Fig. 1A). Thirty-one SCC (55.4%) and 16 SCC (28.6%) were positive for MCP-1 in cancer and stromal cells, respectively. There was a significant correlation between MCP-1 expression in cancer and stromal cells. CCR-2 was expressed in infiltrating stromal cells in all SCC, and occasionally, in vascular endothelial cells in 15 SCC (26.8%).

TP was expressed in both cancer cells and stromal cells, which are considered to be mainly macrophages (Fig. 1B) because most of them showed CD68-positive staining. TP expression in the stromal cells was observed in 43 of 56 SCC (76.8%). There was a significant correlation between MCP-1 expression in cancer cells and TP expression in stromal cells (Table 1).

MCP-1 Expression and Monocytic Count/Microvessel Count/Ki-67 Labeling Index/Apoptotic Index

Monocytic count and MVD were calculated in the immunostained sections (Figs. 1C and D). Monocytic count in SCC with MCP-1 expression in cancer or stromal cells was higher than that without (Table 2). MVD in SCC with MCP-1 expression in cancer or stromal cells was higher than that without it.

Ki-67 labeling index was calculated in the sections that were immunostained (Fig. 1E). Ki-67 labeling index in SCC with MCP-1 expression in cancer cells was higher than that without, while there was no difference between SCC with and without MCP-1 expression in stromal cells (Table 2). The apoptotic index was calculated in the sections stained by TUNEL (Fig. 1F). The apoptotic index in SCC with MCP-1 expression in cancer cells was lower than that without, while there was no difference between SCC with and without MCP-1 expression in stromal cells (Table 2).

Correlations between monocytic count and MVD, Ki-67 labeling index, and apoptotic index are shown in Figure 2. There was a significant correlation between monocytic count and MVD, and there was an inverse correlation between monocytic count and apoptotic index.

Table 2. MCP-1 Expression and Histochemical Results in Esophageal SCC

Variable	MCP-1 Expression					
	Cancer Cells			Stromal Cells		
	Positive (n = 31)	Negative (n = 25)	*p* Value	Positive (n = 16)	Negative (n = 40)	*p* Value
Monocytic count	254.1 ± 84.3c	126.7 ± 73.5c	<0.0001	258.9 ± 92.9c	172.6 ± 95.1c	0.004
Microvessel density	33.0 ± 10.0e	19.5 ± 8.90	<0.0001	32.7 ± 10.1e	24.7 ± 11.5e	0.0175
Ki-67 labeling index	53.5 ± 7.5b	48.6 ± 10.0e	0.04	49.8 ± 7.20	51.8 ± 9.60	0.38
Apoptotic index	1.40 ± 0.6c	1.90 ± 0.63	0.0013	1.50 ± 0.65	1.70 ± 0.69	0.368

MCP-1, macrophage chemoattractant protein-1.

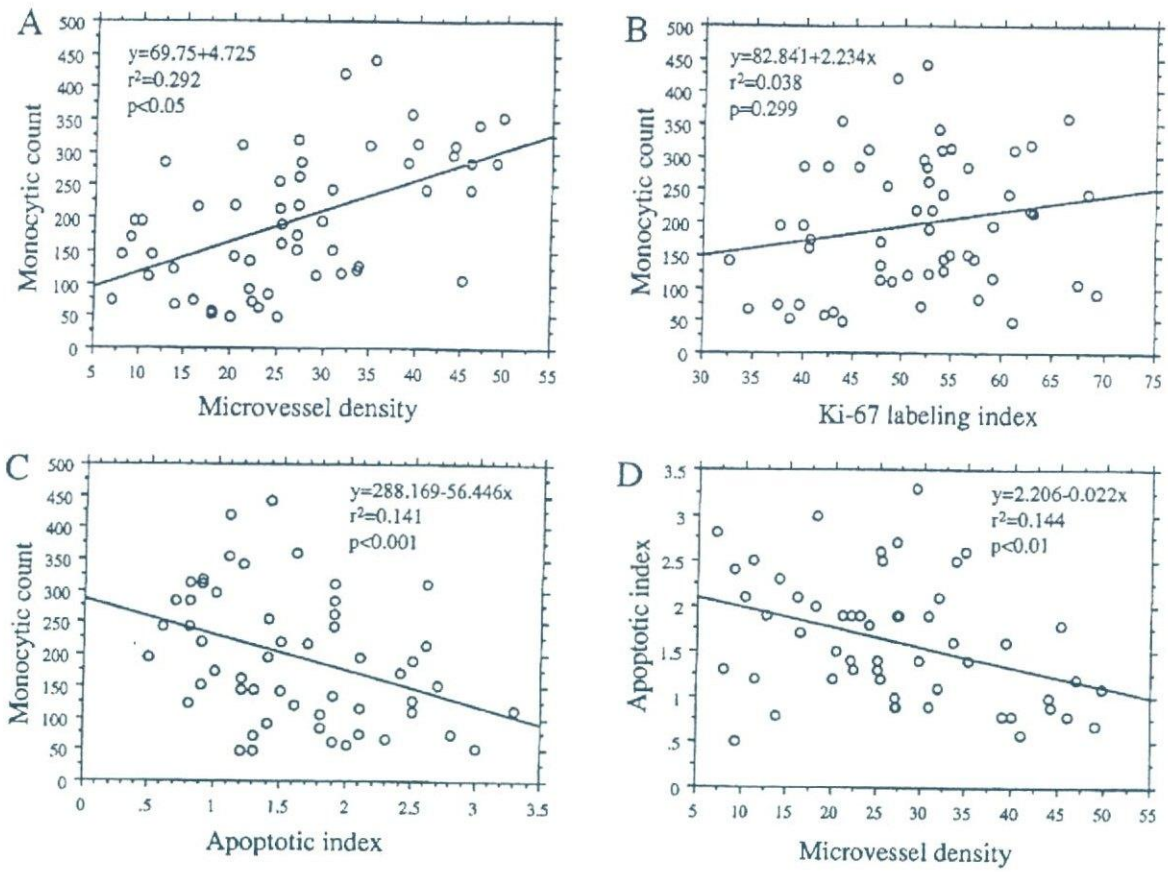

Figure 2. Relationships among monocytic count, microvessel density, Ki-67 labeling index, and apoptotic index. (*A*) Monocytic count and microvessel density. (*B*) Monocytic count and Ki-67 labeling index. (*C*) Monocytic count and apoptotic index. (*D*) Apoptotic index and microvessel density. There was a significant correlation between the monocytic count and the microvessel density, and there was an inverse correlation between the apoptotic index and the monocytic count, or the microvessel count.

Correlation with Clinicopathological Findings and Survival Rates

Regarding the relationship between MCP-1 expression and the clinicopathological features of the patients with esophageal cancer, MCP-1 expression in cancer cells was correlated with the depth of invasion, histological venous invasion, lymph node metastasis, and occurrence of clinical distant metastasis after surgery. However, there was no correlation between MCP-1 expression in stromal cells and the clinicopathological features (Table 3). The monocytic count in SCC with venous invasion, lymph node metastasis, and distant metastasis after surgery was higher than that without them, respectively (Table 4).

Five-year survival rate in esophageal SCC patients with MCP-1 expression was lower than that without (Fig. 3A). When the patients were divided into two groups based on the mean value of the monocytic count, the 5-yr survival rate in esophageal patients with a high monocytic count showed a worse outcome than that with a low monocytic count (Fig. 3B).

DISCUSSION

MCP-1 is a chemokine, which is thought to be a potent angiogenic factor. Ueno *et al.* (20) reported that the concentration of MCP-1 in breast cancer tissue was closely correlated with the concentration of TP and VEGF, which are major components of angiogenic factors. Furthermore, Saji *et al.* (21) reported a significant correlation between MCP-1 expression and neovascularization by histochemical evaluation in breast cancer. In the present study, MVD in the esophageal SCC with MCP-1 expression of cancer cells or stromal cells is higher than that without. Therefore, MCP-1 expression may play an important role in angiogenic promotion in esophageal SCC.

Infiltration of macrophages in tumor stroma is an important role for angiogenesis in several malignancies, including breast cancer (22) and oral SCC (23). In the present study, the monocytic count in esophageal SCC with MCP-1 expression was higher than that without it, and the monocytic count showed a significant correlation with MVD in esophageal

Table 3. MCP-1 Expression and the Clinicopathological Features in Esophageal SCC

Variable	No. of Patients	MCP-1 Expression: Cancer Cells Positive (n = 31)	Cancer Cells Negative (n = 25)	Cancer Cells *p* Value	Stromal Cells Positive (n = 16)	Stromal Cells Negative (n = 40)	Stromal Cells *p* Value
Sex							
Man	42	23	19	0.88	13	39	0.78
Woman	14	8	6		3	11	
Histological type of SCC							
Well- and moderately	47	25	22	0.36	15	32	0.2
Poorly	9	6	3		1	8	
Depth of invasion							
Submucosa	13	4	9	0.042	4	9	0.54
Muscularis propria or deeper	43	27	16		12	31	
Lymphatic invasion							
Positive	44	27	17	0.83	14	30	0.25
Negative	12	4	8		2	10	
Venous invasion							
Positive	31	23	8	0.0016	12	19	0.56
Negative	25	8	17		4	21	
Lymph node metastasis							
Positive	30	22	8	0.0037	8	22	0.73
Negative	26	9	17		8	18	
Distant metastasis after surgery							
Positive	16	14	2	0.002	7	9	0.11
Negative	40	17	23		9	31	

SCC. This suggests that macrophage recruitment might play an important role in tumor angiogenesis of esophageal SCC. Furthermore, these macrophages infiltrating in esophageal SCC may be identified as tumor-associated macrophages. On the other hand, TP, which acts as an angiogenic factor for tumors, is considered to be a protumor marker of infiltrating macrophages in several tumors (13). In the present study, TP was expressed in the infiltrating stromal cells that were identified mainly as macrophages, in esophageal SCC, and 76.7% of esophageal SCC had TP-expressed macrophages. MCP-1 expression was closely associated with TP expression in esophageal SCC, which suggests that MCP-1 produced by SCC cells stimulates infiltration of macrophages, which have CCR-2, in cancer stroma. Consequently, MCP-1 promotes tumor angiogenesis by angiogenic mediators, such as TP released from these infiltrating macrophages. Moreover, these macrophages may also play an important role in recruitment of macrophages because MCP-1 was expressed in infiltrating macrophages themselves, which indicates that there may be an autocrine function of MCP-1 on macrophages via the CCR-2. Interaction between cancer and stromal cells may be important for the tumor environment, including angiogenesis in esophageal SCC.

Table 4. Monocytic Count and the Clinicopathological Features

Variable	Monocytic Count	*p* Value
Histological type of SCC		
Well- and moderately	193.4 ± 101.4d	0.6
Poorly	217.2 ± 106.5d	
Depth of invasion		
Submucosa	1654.4 ± 96.8a	0.17
Muscularis propria or deeper	206.9 ± 102.1i	
Lymphatic invasion		
Positive	205.7 ± 102.2n	0.24
Negative	166.3 ± 97.0e	
Venous invasion		
Positive	233.0 ± 96.3e	0.003
Negative	152.9 ± 91.3e	
Lymph node metastasis		
Positive	224.1 ± 108.6m	0.035
Negative	166.2 ± 84.6e	
Distant metastasis after surgery		
Positive	290.1 ± 79.5e	<0.0001
Negative	160.1 ± 84.7e	

In addition, Salcedo *et al.* (24) reported that MCP-1 may also have a direct effect in tumor angiogenesis inducing chemotaxis of endothelial cells of blood vessels. Ohta *et al.* (25) reported that CCR-2 was immunohistochemically detected in monocytic cells but not in vascular endothelial cells. In the present study, CCR-2 was expressed in the endothelial cells of blood vessels in esophageal SCC. It was suggested that MCP-1 may be a potent angiogenic promoter by migration of the endothelial cells, although it was not a major promotion.

In the esophageal SCC patients with distant metastases after surgery, high monocytic counts were shown in the present study. These infiltrating monocytes may act as the tumor-associated macrophages, mentioned above. It suggested that these macrophages may play important roles for not only angiogenesis to maintain and control tumor environment but distant metastases of tumor cells in esophageal SCC, because

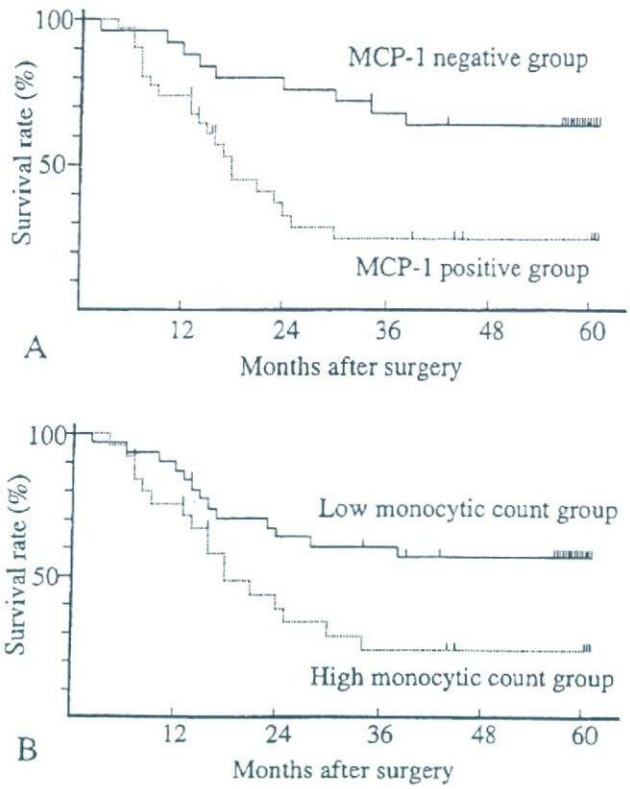

Figure 3. Survival curves in esophageal cancer patients. (*A*) Five-year survival rate in patients with MCP-1 expression was worse than that without it (24.5% *vs* 63.8%; $p < 0.01$). (*B*) Regarding monocytic count, the patients were divided into two groups: a high and a low monocytic count group. Five-year survival rate in patients with a high monocytic count was worse than those with a low monocytic count (24.2% *vs* 56.9%; $p<0.05$).

these macrophages may supply tumors many growth and angiogenic factors, including TP and cytokines. Previously, in esophageal SCC patients with TP expression (14) and the other growth factors (15), distant metastases has been frequently shown.

Regarding proliferative activity of esophageal SCC, we investigated the findings of Ki-67 labeling index and apoptotic index. Ki-67 labeling index of esophageal SCC with MCP-1 expression in cancer cells was higher than that without, while there was no difference in the Ki-67 labeling index between esophageal SCC with and without MCP-1 expression in stromal cells. Furthermore, the apoptotic index of esophageal SCC with MCP-1 expression in cancer cells was lower than that without, while there was no difference in the apoptotic index between esophageal SCC with and without MCP-1 expression in stromal cells. Tumor angiogenesis may be closely associated with apoptosis of tumor cells (26). In the present study, MVD is inversely correlated with the apoptotic index, and MVD was correlated with the monocytic count in esophageal SCC. Therefore, MCP-1 expression of cancer cells may contribute to macrophage infiltration and angiogenesis, probably as protumor macrophages, and thus MCP-1 expression may be interactively associated with proliferative activity and apoptosis in esophageal SCC.

Regarding the clinicopathological features of patients with esophageal SCC, MCP-1 expression of cancer cells and macrophage infiltration were associated with histologic venous invasion and distant metastasis after surgery. As a result, 5-yr survival rate was worse in patients with MCP-1 expression or with a high monocytic count. Leek *et al.* (11) reported that macrophage infiltration was associated with angiogenesis and a poor prognosis in patients with invasive breast cancer. Therefore, in esophageal SCC, MCP-1 expression and macrophage infiltration also play important roles in not only angiogenic promotion but clinicopathological aggressive behavior as well, and they may be useful for their ability to predict the clinical outcome of esophagectomized patients.

In conclusion, MCP-1 expression and macrophage infiltration may be associated with angiogenic promotion. MCP-1 expression may be interactively associated with macrophage infiltration; MCP-1 may play an important role in tumor angiogenesis through production of angiogenic factors, such as TP, by recruited macrophages in esophageal SCC. Furthermore, CCR-2 expression in vascular endothelial cells may participate partially in angiogenesis. Clinicopathologically, patients of esophageal SCC with MCP-1 expression have no favorable prognosis.

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DISEASES OF THE ESOPHAGUS

Case report

Thoracoscopic enucleation of esophageal stromal tumor

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SUMMARY. **Gastrointestinal stromal tumor is a rare entity, especially in the esophagus. We report a patient with a stromal tumor of the esophagus who underwent a thoracoscopic enucleation of the tumor. The patient was a 61-year-old man complaining of slight dysphagia. A submucosal tumor of the middle thoracic esophagus was found endoscopically. The tumor was approximately 4.0 cm in diameter measured by endoscopic ultrasonography. On 17 May 2001, thoracoscopic enucleation of the esophageal tumor was performed using a Kodama Di-suction. The Kodama Di-suction was useful for the thoracoscopic enucleation of the submucosal tumor of the esophagus, acting as both a dissector and a sucker. The patient's course was uneventful after surgery. Histopathologically the esophageal tumor revealed a high cellularity, consisting of spindle cells, and the tumor cells were immunohistochemically positive for CD34 and c-kit protein, but not for a-smooth muscle actin or S-100 protein. From these findings, the esophageal submucosal tumor was diagnosed as gastrointestinal stromal tumor, distinguished from leiomyoma.**

KEY WORDS: **esophagus, GIST, Kodama Di-suction, thoracoscopy.**

INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the stomach and the intestine, however, in the esophagus, leiomyoma is most common.[1] Herein, we report a patient with a GIST of the esophagus which was enucleated thoracoscopically, using a thoracoscopic instrument functioning as a sucker as well as a dissector.

CASE REPORT

A 61-year-old man, complaining of slight dysphagia, was monitored endoscopically for an esophageal submucosal tumor from July 2000, and admitted to Shinshu University Hospital to remove this tumor surgically on 7 May 2001. A barium meal study showed a tumor with a smooth surface in the right wall of the middle thoracic esophagus, and approximately 4 cm in longitudinal diameter (Fig. 1). Endoscopy showed a protruded lesion covered with a normal mucosa in the middle thoracic esophagus (Fig. 2A), and no morphological change of the tumor was detected in endoscopic findings between July 2000 and March 2001. Biopsy specimens taken from the tumor in July 2000 histologically revealed a stromal tumor. An endoscopic ultrasonogram (EUS) in July 2000 showed that the esophageal tumor was 3.5 × 2.0 cm in diameter, and originated from the proper muscle layer of the esophagus (Fig. 2B). However, in EUS in May 2001, the diameter of the tumor was 4 × 2.5 cm in diameter. A computed tomogram showed the esophageal tumor with heterogeneous density, and no metastasis in the lung and the liver.

On 17 May 2001, thoracoscopic enucleation of the submucosal tumor of the esophagus was performed under general anesthesia administered using a double-lumen endothoracheal tube. A 10-mm-video-trocar was inserted into the 7th intracostal space on the mid-axillary line of the right lateral chest. Four 10 mm-trocars were inserted into the intracostal spaces on the anterior or posterior axillary lines for operative and assisting instruments. As the lung was collapsed, the tumor was found in the middle thoracic esophagus covered with the

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