FOXP3$^+$ Regulatory T Cells Affect the Development and Progression of Hepatocarcinogenesis

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Abstract

Purpose: Tumor-infiltrating lymphocytes represent the host immune response to cancer. CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells (Tregs) suppress the immune reaction. The aim of the present study was to investigate the clinicopathologic significance and roles of Tregs and CD8$^+$ T cells during hepatocarcinogenesis.

Experimental Design: We examined the infiltration of FOXP3$^+$ Tregs and CD8$^+$ T cells in the tumor stroma and nontumorous liver parenchyma using 323 hepatic nodules including precursor lesions, early hepatocellular carcinoma (HCC), and advanced HCC, along with 39 intrahepatic cholangiocarcinomas and 59 metastatic liver adenocarcinomas. We did immunohistochemical comparative studies.

Results: The prevalence of Tregs was significantly higher in HCC than in the nontumorous liver ($P < 0.001$). The patient group with a high prevalence of Tregs infiltrating HCC showed a significantly lower survival rate ($P = 0.007$). Multivariate analysis revealed that the prevalence of Tregs infiltrating HCC was an independent prognostic factor. The prevalence of Tregs increased in a stepwise manner ($P < 0.001$) and that of CD8$^+$ T cells decreased during the progression of hepatocarcinogenesis ($P < 0.001$). Regardless of the presence of hepatitis virus infection or histopathologic evidence of hepatitis, the prevalence of Tregs was significantly increased in nontumorous liver bearing primary hepatic tumors.

Conclusions: Tregs play a role in controlling the immune response to HCC during the progression of hepatocarcinogenesis. It has been suggested that primary hepatic cancers develop in liver that is immunosuppressed by a marked infiltration of Tregs. A high prevalence of Tregs infiltrating HCC is thought to be an unfavorable prognostic indicator.

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, representing the third most common cause of mortality among deaths from cancer (1). Even with remarkable advances in diagnostic and therapeutic techniques, the incidence of HCC is still on the increase. Hepatitis virus B (HBV) and hepatitis virus C (HCV) are known to be major risk factors, and chronic infection with these viruses is responsible for ~80% of HCCs in humans (2). Most of the HCCs occur in damaged liver (chronic hepatitis or liver cirrhosis), even if the liver is not infected with HBV or HCV (3). HCC is also characterized by an obvious multistage process of tumor progression (4–7), from a regenerative nodule to adenomatous hyperplasia (AH), and thereafter to atypical adenomatous hyperplasia (AAH), early HCC (defined as *in situ* or microinvasive cancer), and advanced HCC. It is important to detect cancers at an early stage, including their precursor lesions, and to assess their risk in order to provide appropriate treatment and reduce cancer-related mortality.

Previous studies have investigated the changes in morphology, genetics, and molecular biology of epithelial cells during tumorigenesis. Recently, many studies have suggested that the tumor microenvironment also plays an important role in the establishment and progression of tumors. Lymphocytes contribute to the tumor microenvironment through immunity and inflammation. CD8$^+$ CTLs can directly kill target cells by releasing granules including membrane-lytic materials such as perforin and granzymes in acquired immune responses, thereby playing a central role in antitumor immunity. Indeed, a high frequency of CD8$^+$ T cells infiltrating cancer tissue can be a favorable prognostic indicator in ovarian cancer (8) and colorectal cancer (9). In HCC, extremely marked infiltration of T cells including predominant CD8$^+$ T cells has been shown to be closely associated with a low recurrence rate and good prognosis (10). On the other hand, another study using a mouse model has

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shown that marked infiltration of $CD8^+$ T cells exacerbates liver damage, thus accelerating the development of HCC (11).

In contrast to $CD8^+$ CTL, which generally exert a suppressive influence on tumor growth, regulatory T cells (Tregs) are thought to have a positive effect on tumor growth through suppression of antitumor immune cells. $CD4^+CD25^+$ Tregs are a minor but functionally unique population of T cells, which maintain immune homeostasis in immune tolerance and the control of autoimmunity. Tregs can inhibit immune responses mediated by $CD4^+CD25^-$ and $CD8^+$ T cells *in vitro* by a contact-dependent and cytokine-independent mechanism (12–14), although more recent reports suggest that the immune suppression mechanisms of Tregs *in vivo* are more complex (15, 16). Forkhead or winged helix family of transcription factor P3 (FOXP3) is critical for the development and function of Tregs in mice and humans (16, 17), and is still the only marker for evaluating real Tregs that have a suppressive function. In murine models, it has been described that Tregs inhibit the antitumor immune response (15, 18–20). Involvement of $CD4^+CD25^+$ Tregs in human cancer has been observed in peripheral blood and tumor tissues from patients with several types of cancer (21–25). A few groups have reported that Tregs are increased in peripheral blood and among tumor-infiltrating lymphocytes of patients with HCC (26–28), although these were not large-scale studies and did not estimate the clinicopathologic significance of Tregs infiltrating HCC, including their prognostic value. Early studies detected Tregs not as $FOXP3^+$ T cells but as $CD4^+CD25^+$ T cells, although recent studies have revealed that $CD4^+CD25^+$ T cells consist of Tregs and activated effector T cells, the latter being increased in inflammatory lesions (29). Furthermore, no previous study has investigated host immune responses in multistage hepatocarcinogenesis.

In the present study, we first investigated the clinicopathologic values of both $FOXP3^+$ Tregs and $CD8^+$ T cells infiltrating the tumor stroma of HCC, and then examined the prevalence of $FOXP3^+$ Tregs and $CD8^+$ T cells during multistage hepatocarcinogenesis. Precursor lesions of HCC are small nodular lesions that can be detected and evaluated only by microscopic analysis, making it difficult to extract living immune cells from them and to analyze their immunophenotypes and immune functions. Therefore, we selected an immunohistochemical comparative approach for evaluating host immune responses in these HCC precursor lesions. This approach was used in the other experiments as well. We also investigated whether Tregs are involved in the development of HCC, and compared the host immune responses by measuring and comparing the infiltration of Tregs and $CD8^+$ T cells between HCC and primary hepatic adenocarcinoma, intrahepatic cholangiocarcinoma (ICC) as well as between primary and metastatic liver tumors. We compared the prevalence of Tregs in nontumorous liver parenchyma among patients with and without primary hepatic tumors, and those with and without hepatitis viral infection. The results showed that the prevalence of Tregs increases during the progression of established cancers as well as that of their precursor lesions. Furthermore, the prevalence of Tregs was significantly correlated with patient survival, independent of other prognostic factors.

Materials and Methods

Patients and samples. This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan. Clinical and pathologic data and the specimens used for immunohistochemical analysis were obtained through a detailed retrospective review of the medical records of 218 patients with 323 hepatic nodules of HCC or its precursor lesions who had undergone initial surgical resection between 1992 and 2000 at the National Cancer Center Hospital, Tokyo, Japan. None of the nodules had been treated previously with techniques such as radiofrequency ablation, percutaneous ethanol injection therapy, or transcatheter arterial embolization or injection, and none of the patients with nodules had received systemic chemotherapy. Sixty-five patients had hepatic cancers that had been treated by surgical resection, radiofrequency ablation, percutaneous ethanol injection therapy, or transcatheter arterial embolization or injection; the current nodules were also located in different lobes, as well as distant from, the previous cancers. In another six patients, curative resection was not done. The remaining 147 patients were studied in order to evaluate the clinicopathologic correlation of the prevalence of $FOXP3^+$ Tregs and $CD8^+$ T cells with specific variables. Tumors were classified according to the WHO classification (30) and the International Union against Cancer tumor-node-metastasis (TNM) classification (31). If patients had multiple nodules in the liver, we selected the nodule showing the most advanced histologic grade for our study. If a tumor had different grades of histology, the grade of the tumor was regarded as the most advanced one among them. Nontumorous liver was classified histopathologically into four categories: non–chronic hepatitis (NCH), chronic hepatitis (CH), chronic hepatitis with cirrhotic change (pre-cirrhotic stage; PC), and liver cirrhosis (LC), which corresponded to 0, 1-3, 4-5, and 6 of the fibrosis stages of the modified histological activity index system (32). There were 5 patients with HBV infection and 15 patients without HBV or HCV infection in NCH, which included liver with fatty changes and/or slight inflammatory infiltrates in the portal area. All patients had complete medical records and had been followed by the tumor registries for survival and outcome. Follow-up was available in all cases and ranged from 0.5 to 169.1 months (mean, 52.8 months). The latest survival data were collected on April 30, 2006. The overall survival rate at 5 years and the disease-free survival rate were 39.5% and 18.4%, respectively. The clinicopathologic features of the patients are summarized in Table 1.

We also investigated 39 patients with ICC and 59 patients with metastatic liver tumors from primary colorectal cancer who had undergone initial surgical resection between 1991 and 2005 at the National Cancer Center Hospital. The patients with ICC or metastatic liver cancer without hepatitis viral infection were randomly selected and those with hepatitis viral infection were all the patients we had. The patients with ICC comprised 22 males and 17 females, and their median age at surgery was 63 years (range, 44-85 years). HBV and HCV infection were detected in four and five patients, respectively. Their livers were diagnosed histopathologically as CH in eight patients and as PC in one patient. NCH were found in the liver of 30 patients without any HBV or HCV infection. Tumor diameters ranged from 15 to 140 mm (mean, 64.6 $\pm$ 30.6 mm). There were 8 patients at stage I, 9 patients at stage II, 3 patients at stage IIIa, 7 patients at stage IIIb, and 12 patients at stage IIIc according to the International Union against Cancer staging classification (31). ICCs were classified histopathologically as well-differentiated adenocarcinoma in 7 cases, moderately differentiated adenocarcinoma in 27, and poorly differentiated adenocarcinoma in 5 according to the WHO classification (30). The patients with liver metastasis from colorectal cancer comprised 37 males and 22 females, and their median age at surgery was 62 years (range, 34-81 years). HBV and HCV virus infection were detected in 8 and 21 patients, respectively, and their livers were diagnosed histopathologically as CH in 18 and as NCH in 11. The other 30 patients had not been infected with HBV or HCV and their nontumorous liver showed no inflammatory or fatty changes. Therefore, the nontumorous liver tissue from these patients was defined as "healthy liver." Thirty-three patients had a solitary tumor and 26 had multiple tumors. Tumor diameters ranged from 12 to 150 mm

Table 1. Clinicopathologic features of the patients

Variables	Results
Characteristics of the patients with HCC (218 cases)	
Age, y (median, range)	62, 17-84
Gender (male/female)	170/48
Virus infection [HBV/HCV/HBV+HCV/(−)]	57/117/10/34
Nontumor liver (NCH/CH/PC/LC)	20/101/35/62
Tumor nodules (AH/AAH/early HCC/WD HCC/MD HCC/PD HCC)	11/9/68/58/123/54
Clinicopathologic findings of the patients with HCC (147 cases)	
Age, y (median, range)	62, 17-83
Gender (male/female)	113/34
Virus infection [HBV/HCV/HBV+HCV/(−)]	47/79/9/12
Nontumor liver (NCH/CH/PC/LC)	17/71/23/36
Child-Pugh classification (A/B/C)	136/11/0
TNM stage (I/II/III/IV)	57/53/37/0
Histologic grade (early HCC/WD HCC/MD HCC/PD HCC)	17/15/77/38
AFP, ng/mL (median, range)	27.1, 1-27,170
VP (presence/absence)	57/90
IM (presence/absence)	33/114
Tumor size, mm (median, range)	35, 6-185

Abbreviations: MD, moderately differentiated; PD, poorly differentiated; WD, well differentiated.

(mean, 42.3 ± 28.2 mm). Histopathologically, the tumors were well-differentiated adenocarcinoma in 5 cases, moderately differentiated adenocarcinoma in 53 cases, and poorly differentiated adenocarcinoma in 1 case.

Immunohistochemical analysis. Immunohistochemistry was done on the formalin-fixed, paraffin-embedded tissue sections as described previously (33). We reacted 4-μm-thick sections of representative blocks with monoclonal antibodies against the following: CD4 (1F6; 1:50), CD8 (4B11; 1:50), and perforin (5B11; 1:50) from Novocastra Laboratories, Ltd. (Newcastle upon Tyne, United Kingdom), and FOXP3 (clone 42; ref. 25). Briefly, the sections were deparaffinized and rehydrated. After blocking of endogenous peroxidase with methanol containing 0.3% H_2O_2, the sections were autoclaved at 121°C for 10 min in citrate buffer (10 mmol/L sodium citrate; pH 6.0) for antigen retrieval. After blocking with normal goat serum, the sections were reacted overnight with appropriately diluted primary antibodies. The sections were then reacted sequentially with biotin-conjugated anti-mouse IgG antibodies (Vector Laboratories, Burlingame, CA) and Vectastain Elite ABC reagent (Vector Laboratories). For staining CD4 and CD8, a CSA system (DAKO, Glöstrup, Denmark) and EnVision$^+$ Polymer system (DAKO) were used, respectively, instead of the avidin-biotin complex system. Diaminobenzidine was used as the chromogen, and the nuclei were counterstained with hematoxylin.

Serial sections were prepared from each paraffin block. The first section was stained with H&E and the second, third, and fourth sections were subjected to immunohistochemistry to detect the CD8, CD4, and FOXP3 antigens. CD8$^+$, CD4$^+$, or FOXP3$^+$ lymphocytes were counted in the corresponding visual fields. Quantitative evaluation of lymphocytes was done by analyzing at least three different high-power fields (×40 objective and ×10 eyepiece). The proportion of FOXP3$^+$ lymphocytes among CD4$^+$ lymphocytes and that of CD8$^+$ lymphocytes among total T cells, together with the sum of CD4$^+$ and CD8$^+$ lymphocytes, were calculated for each field and the averages were compared.

Statistical analysis. Values were expressed as mean ± SD. Statistical analyses were done with StatView-J 5.0 software (Abacus Concepts, Berkeley, CA). Associations among the variables were assessed by the χ^2 test, Student's t test, Mann-Whitney U test, and Kruskal-Wallis test. If there was evidence of non-normality, the Mann-Whitney U test or the Kruskal-Wallis test was used to test the difference in medians among the groups. Survival rates were calculated by the Kaplan-Meier method. Differences between survival curves were analyzed by the log-rank test. To assess the correlation between survival time and multiple clinicopathologic variables, multivariate analyses were done by the Cox proportional hazards regression model. Differences were considered significant at $P < 0.05$.

Results

Increased populations of FOXP3$^+$ Tregs among CD4$^+$ T cells in tumor stroma of HCC. In order to assess the infiltration of Tregs in the stroma of HCC (n = 235) and nontumorous liver (n = 248), we evaluated both the absolute numbers of FOXP3$^+$ Tregs and the prevalence of FOXP3$^+$ Tregs among CD4$^+$ T cells. The absolute number of FOXP3$^+$ Tregs that had infiltrated HCC was significantly higher than that of Tregs in nontumorous liver from patients with HCC or healthy liver tissue (versus healthy controls, $P < 0.001$; versus NCH, $P < 0.001$; versus CH, $P = 0.002$; versus PC, $P = 0.023$; versus LC, $P < 0.001$; Fig. 1A). The prevalence of tumor-infiltrating FOXP3$^+$ Tregs among CD4$^+$ T cells in HCC was also significantly higher (versus healthy controls, $P < 0.001$; versus NCH, $P < 0.001$; versus CH, $P < 0.001$; versus PC, $P < 0.001$; versus LC, $P < 0.001$; Fig. 1B). Among advanced HCCs, the prevalence of FOXP3$^+$ Tregs was significantly higher in less differentiated HCCs (Kruskal-Wallis test, $P < 0.001$; Fig. 1B). No significant difference in the infiltration of Tregs was found among CH, PC, and LC. The prevalence of Tregs in NCH was lower than that in CH ($P = 0.021$), PC, and LC, but was significantly higher than that in healthy controls ($P < 0.001$; Fig. 1B).

The absolute number of CD8$^+$ T cells was increased in CH, PC, and LC, and was significantly higher than that in HCC ($P < 0.001$; Fig. 1C). The prevalence of CD8$^+$ T cells in HCC was significantly lower than that in any type of damaged and nontumorous liver from patients with HCC (versus NCH, $P = 0.025$; versus CH, $P < 0.001$; versus PC, $P = 0.015$; versus LC, $P < 0.001$; Fig. 1D). In advanced HCCs, the prevalence of CD8$^+$ T cells was significantly lower in less differentiated HCC (Kruskal-Wallis test, $P = 0.034$; Fig. 1D). CD8$^+$ T cells

were increased slightly in NCH and viral hepatitis including CH, PC, and LC compared with healthy controls. These results suggested that an immunoreaction had also occurred in nontumorous liver bearing HCC without viral hepatitis.

Clinicopathologic features of HCC and the prevalence of tumor-infiltrating Tregs and CD8⁺ T cells. We analyzed the correlation between clinicopathologic features of HCC and the prevalence of tumor-infiltrating Tregs or that of $CD8^+$ T cells in HCC (Table 2A and B). Patients with HCC were divided into two groups either by the median value for the prevalence of tumor-infiltrating Tregs (29.0%) or by $CD8^+$ T cells (51.5%). The high Treg group (n = 73) showed a significant correlation with high histologic grade (P = 0.021) and tended to show a lower number of infiltrating $CD8^+$ T cells in HCC (P = 0.064) among the various clinicopathologic characteristics (Table 2A).

Prognostic significance of the prevalence of Tregs and CD8⁺ T cells in HCC. Overall and disease-free survival were analyzed in these patients. Of the 147 patients with HCC who underwent hepatic resection, 88 (59.9%) died. The overall 5-year survival and disease-free survival rates were 39.5% and 18.4%, respectively. The low-Treg group showed significantly better overall survival than the high-Treg group (log-rank test, P = 0.007; Fig. 1E). Mean overall survival was 60.3 (±33.8) months for the low-Treg group and 45.1 (±38.7) months for the high-Treg group. The low-Treg group also showed significantly better disease-free survival than the high-Treg group (log-rank test, P = 0.015; Fig. 1F). Mean disease-free survival was 36.2 (±31.7) months for the low-Treg group and 27.3 (±32.9) months for the high-Treg group. The 15 clinicopathologic factors listed in Table 3A and B were examined for their association with overall and disease-free survival after initial resection of the tumor. Univariate analysis of overall survival revealed that the following variables had a negative influence: Child-Pugh classification (B), TNM stage (III and IV), high serum α-fetoprotein (AFP; >27.1 IU/mL), presence of portal vein invasion (VP), presence of histologic intrahepatic metastatic foci (IM), and high prevalence of tumor-infiltrating Tregs (Table 3A). In multivariate Cox proportional hazard analysis for clinicopathologic variables and prevalence of tumor-infiltrating Tregs, the

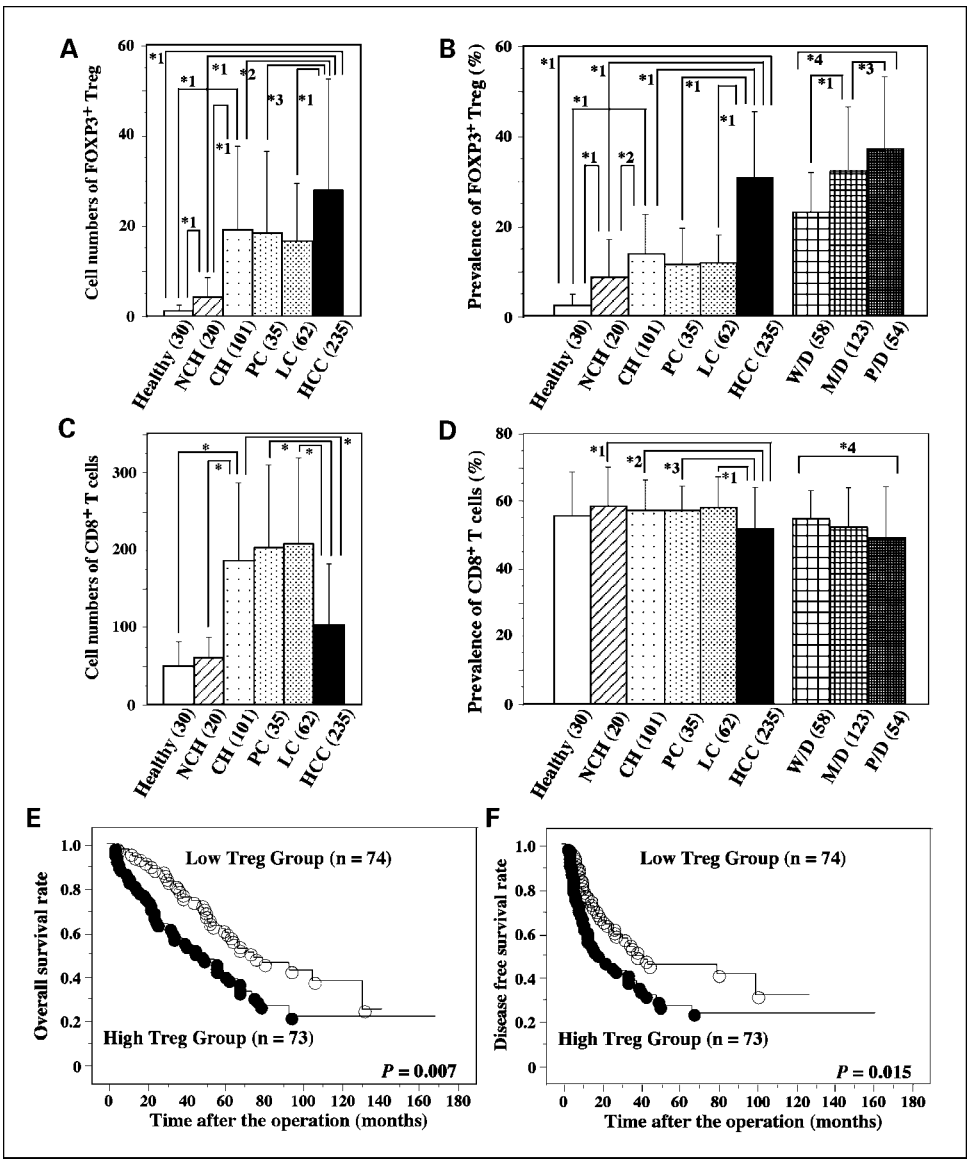

Fig. 1. Increased population of $FOXP3^+$ Tregs and decreased population of $CD8^+$ T cells in tumor stroma of HCC. *A* and *B,* absolute number of Tregs (*A*) and prevalence of Tregs (*B*) in HCC and nontumorous liver. Right column, the contents of HCC according to histologic grade (*B*). Number of cases tested in parentheses: *A,* *1, $P < 0.001$; *2, $P = 0.002$; *3, $P = 0.023$. *B,* *1, $P < 0.001$; *2, $P = 0.021$; *3: $P = 0.044$; *4, $P < 0.001$ (Kruskal-Wallis test); thin bars, SD. *C* and *D,* absolute number of $CD8^+$ T cells (*C*) and prevalence of $CD8^+$ T cells (*D*) in HCC and nontumorous liver. Right column, the contents of HCC according to histologic grade (*D*). Number of cases tested in parentheses: thin bars, SD. *C,* *, $P < 0.001$. *D,* *1, $P = 0.025$; *2, $P < 0.001$; *3, $P = 0.015$; *4, $P < 0.001$ (Kruskal-Wallis test). *E* and *F,* Kaplan-Meier survival curves of 147 patients with HCC. Overall survival curve (*E*) and disease-free survival curve (*F*) are shown. The prognosis was significantly worse in the high Treg prevalence group (*solid dots, n* = 73) than in the low Treg prevalence group [*white dots, n* = 74; log-rank test, $P = 0.007$ (*E*) and $P = 0.015$ (*F*)].

hazard ratio for poor prognosis was 1.640 for patients in the high-Treg group compared with patients in the low-Treg group ($P = 0.040$; Table 3A). Worse Child-Pugh classification and the presence of VP were also independent factors for overall patient survival. Univariate analysis for disease-free survival revealed that six variables negatively affected the survival rate and all of them were the same with the six variables of overall survival (Table 3B). In multivariate analysis for disease-free survival, two variables—the presence of IM and the high prevalence of Tregs infiltrating HCC—were significant factors. The hazard ratio for poor prognosis was 1.706 for patients in the high-Treg group compared with patients in the low-Treg group ($P = 0.024$; Table 3B). There was no significant difference in the overall survival rate or disease-free survival rate between the low and high $CD8^+$ T cell groups. These results indicated that the prevalence of tumor-infiltrating Tregs was an independent prognostic factor in patients with HCC, whereas the prevalence of tumor-infiltrating $CD8^+$ T cells was not.

Increased populations of Tregs among $CD4^+$ T cells in tumor stroma correspond to progression during multistage hepatocarcinogenesis. It was suggested that Tregs play important roles in the progression of HCC. Therefore, the prevalence of Tregs among $CD4^+$ T cells in the precursor lesions, AH ($n = 11$; Fig. 2E-H) and AAH ($n = 9$), and early HCC ($n = 68$; Fig. 2I-L), was analyzed during tumorigenesis of HCC. As shown in Fig. 3A, the prevalence of Tregs increased significantly in a stepwise manner during the progression of hepatocarcinogenesis (Kruskal-Wallis test, $P < 0.001$; viral hepatitis containing CH, PC, and LC versus precursor lesions containing AH and AAH, $P = 0.038$; precursor lesions versus early HCC, $P = 0.121$; early HCC versus advanced HCC, $P < 0.001$). These findings suggest that the prevalence of Tregs is closely correlated with the progression of multistage hepatocarcinogenesis. In contrast, the prevalence of $CD8^+$ T cells showed a clear, but not drastic, decrease during the progression of hepatocarcinogenesis (Kruskal-Wallis test, $P < 0.001$; Fig. 3B).

Table 2. Correlation between clinicopathologic findings and the prevalence of Tregs and $CD8^+$ T cells infiltrating HCC

(A) Correlation between clinicopathologic findings and the prevalence of Tregs infiltrating HCC

Variables	Prevalence of Tregs among $CD4^+$ T cells		
	High Treg	Low Treg	*P*
Age, y (mean ± SD)	62.6 ± 8.92	61.4 ± 10.4	0.444*
Gender (male/female)	59/14	54/20	0.259†
Viral infection			
HBV and/or HCV/(−)	66/7	69/5	0.531†
HBV(+)/(−)	27/7	29/5	0.525†
HCV(+)/(−)	45/7	43/5	0.640†
Nontumor liver (NCH/CH/PC/LC)	7/41/9/16	10/30/14/20	0.289†
Child-Pugh score (A/B/C)	68/5/0	68/6/0	0.772†
TNM stage (I/II/III/IV)	28/22/23/0	29/31/14/0	0.155†
Tumor size, mm (median, range)	40, 9-185	30, 6-150	0.113‡
Histologic grade (early HCC/WD HCC/MD HCC/PD HCC)	7/3/38/25	10/12/39/13	**0.021**†
AFP, ng/mL (median, range)	24.1 (1.8-27,170)	28.3 (1.0-25,000)	0.681‡
VP (presence/absence)	33/40	24/50	0.112†
IM (presence/absence)	20/53	13/61	0.152†
Number of $CD8^+$ T cells infiltrating tumor (median, range)	75 (12-405)	91 (9-435)	0.064‡

(B) Correlation between clinicopathologic findings and the prevalence of $CD8^+$ T cells infiltrating HCC

Variables	Prevalence of $CD8^+$ T cells in total T cells		
	High $CD8^+$ T cells	Low $CD8^+$ T cells	*P*
Age, y (mean ± SD)	63.6 ± 9.08	60.5 ± 10.0	0.051*
Gender (male/female)	51/23	62/12	**0.045**†
Viral infection			
HBV and/or HCV/(−)	69/4	66/8	0.238†
HBV(+)/(−)	30/4	26/8	0.203†
HCV(+)/(−)	42/4	46/8	0.348†
Nontumor liver (NCH/CH/PC/LC)	11/35/12/15	6/36/11/21	0.471†
Child-Pugh score (A/B/C)	67/6/0	69/5/0	0.736†
TNM stage (I/II/III/IV)	28/29/16/0	29/24/21/0	0.560†
Tumor size, mm (median, range)	40, 6-185	31, 10-150	0.075‡
Histologic grade (early HCC/WD HCC/MD HCC/PD HCC)	9/8/39/17	8/7/38/21	0.907†
AFP, ng/mL (median, range)	21.5 (1.0-27,170)	36.3 (1.8-17,430)	0.105‡
VP (presence/absence)	31/42	26/48	0.362†
IM (presence/absence)	18/55	15/59	0.524†

Abbreviations: MD, moderately differentiated; PD, poorly differentiated; WD, well differentiated.
*Student's *t* test.
†χ^2 test or Fisher exact test.
‡Mann-Whitney *U* test.

Table 3. Univariate and multivariate analyses of prognosis factors associated with overall and disease-free survival in patients with HCC

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% confidence interval)	*P*	Hazard ratio (95% confidence interval)	*P*
(A) Univariate and multivariate analyses of prognosis factors associated with overall survival in patients with HCC				
Prevalence of tumor-infiltrating FOXP3$^+$ Tregs among CD4$^+$ T cells in HCC (high/low)	1.791 (1.163-2.760)	**0.008**	1.640 (1.023-2.628)	**0.040**
Prevalence of tumor-infiltrating CD8$^+$ T cells in total T cells in HCC (high/low)	1.055 (0.687-1.620)	0.806	1.109 (0.681-1.805)	0.678
Age* (≥63 y/<63 y)	1.003 (0.653-1.540)	0.989	0.909 (0.573-1.440)	0.684
Gender (male/female)	1.052 (0.636-1.740)	0.844	0.957 (0.554-1.655)	0.876
Viral hepatitis (presence/absence)	1.140 (0.496-2.620)	0.757	0.972 (0.332-2.842)	0.958
Nontumor liver (NCH/CH, PC, LC)	0.766 (0.369-1.592)	0.475	0.503 (0.207-1.225)	0.130
Child-Pugh score (A/B, C)	0.462 (0.222-0.962)	**0.039**	0.395 (0.171-0.913)	**0.030**
TNM stage (I, II/III, IV)	0.412 (0.256-0.663)	<**0.001**	1.079 (0.456-2.548)	0.863
Tumor size* (≥37 mm/<37 mm)	1.398 (0.909-2.149)	0.127	1.018 (0.553-1.875)	0.954
AFP* (≥27.1 ng/mL/<27.1 ng/mL)	1.673 (1.084-2.581)	**0.020**	1.454 (0.901-2.347)	0.125
Histologic grade (WD HCC/MD HCC, PD HCC)	0.637 (0.376-1.078)	0.093	0.931 (0.489-1.772)	0.828
VP (presence/absence)	2.843 (1.825-4.429)	<**0.001**	2.546 (1.323-4.900)	**0.005**
IM (presence/absence)	2.880 (1.786-4.641)	<**0.001**	2.081 (0.916-4.730)	0.080
Prevalence CD8$^+$ T cells in total T cells in nontumor liver (high/low)	0.754 (0.490-1.159)	0.198	0.688 (0.419-1.131)	0.140
Prevalence of FOXP3$^+$ Tregs among CD4$^+$ T cells in nontumor liver (high/low)	0.756 (0.491-1.165)	0.205	0.737 (0.442-1.229)	0.241
(B) Univariate and multivariate analyses of prognosis factors associated with disease-free survival in patients with HCC				
Prevalence of tumor-infiltrating FOXP3$^+$ Tregs among CD4$^+$ T cells in HCC (high/low)	1.701 (1.105-2.619)	**0.016**	1.706 (1.073-2.713)	**0.024**
Prevalence of tumor-infiltrating CD8$^+$ T cells in total T cells in HCC (high/low)	1.150 (0.750-1.765)	0.522	1.330 (0.817-2.165)	0.251
Age* (≥63 y/<63 y)	0.917 (0.597-1.407)	0.691	0.803 (0.508-1.271)	0.350
Gender (male/female)	0.992 (0.600-1.641)	0.976	0.941 (0.546-1.619)	0.825
Viral hepatitis (presence/absence)	0.931 (0.405-2.140)	0.866	0.754 (0.249-2.287)	0.619
Nontumor liver (NCH/CH, PC, LC)	0.902 (0.435-1.871)	0.782	0.537 (0.215-1.342)	0.184
Child-Pugh score (A/B, C)	0.458 (0.220-0.955)	**0.037**	0.463 (0.206-1.039)	0.062
TNM stage (I, II/III, IV)	0.357 (0.220-0.577)	<**0.001**	0.808 (0.320-2.038)	0.651
Tumor size* (≥37 mm/<37 mm)	1.455 (0.947-2.235)	0.087	1.171 (0.635-2.159)	0.614
AFP* (≥27.1 ng/mL/<27.1 ng/mL)	1.556 (1.010-2.397)	**0.045**	1.503 (0.932-2.421)	0.944
Histologic grade (WD HCC/MD HCC, PD HCC)	0.810 (0.481-1.365)	0.429	1.354 (0.722-2.538)	0.345
VP (presence/absence)	2.284 (1.476-3.535)	<**0.001**	1.692 (0.870-3.294)	0.121
IM (presence/absence)	3.512 (2.163-5.704)	<**0.001**	2.487 (1.020-6.064)	**0.045**
Prevalence CD8$^+$ T cells in total T cells in nontumor liver (high/low)	0.727 (0.473-1.118)	0.146	0.644 (0.393-1.054)	0.080
Prevalence of FOXP3$^+$ Tregs among CD4$^+$ T cells in nontumor liver (high/low)	0.800 (0.520-1.230)	0.309	0.788 (0.482-1.290)	0.344

Abbreviations: MD, moderately differentiated; PD, poorly differentiated; WD, well differentiated.
*Two groups were divided by the median.

Infiltration of Tregs shows no difference among different histologic types of tumor, but differs between primary and metastatic hepatic tumors. Although metastatic liver tumors are common, the most frequent type of tumor developing primarily in the liver is HCC, and the second major type is ICC. In order to examine whether antitumor immune response was affected by tumor histology, we compared the prevalence of Tregs and CD8$^+$ T cells between HCC and primary hepatic adenocarcinoma, ICC (n = 39). The prevalence of Tregs in the tumor stroma was comparable between HCC and ICC (Fig. 4A), whereas the prevalence of CD8$^+$ T cells in ICC was significantly lower than that in HCC (P = 0.004; Fig. 4A). The prevalence of Tregs in nontumorous liver was also comparable between patients with HCC and patients with ICC (Fig. 4B), although their prevalence was significantly higher than that in healthy liver (versus HCC, $P < 0.001$; versus ICC, $P < 0.001$). The prevalence of CD8$^+$ T cells in nontumorous liver was comparable among patients with HCC, ICC, and healthy liver. These findings suggest that the Treg response is almost the same in both histologic types of primary hepatic tumor, HCC, and ICC, whereas the CD8$^+$ T cell response is reduced to a greater degree in ICC than in HCC.

We then analyzed the prevalence of tumor-infiltrating Tregs and CD8$^+$ T cells in the liver of patients with primary HCC, its IM (n = 27), ICC (n = 39), and metastatic liver adenocarcinoma originating from colorectal cancer (n = 59), to examine whether the antitumor immune response differs between primary and metastatic tumors of the liver. The prevalence of Tregs

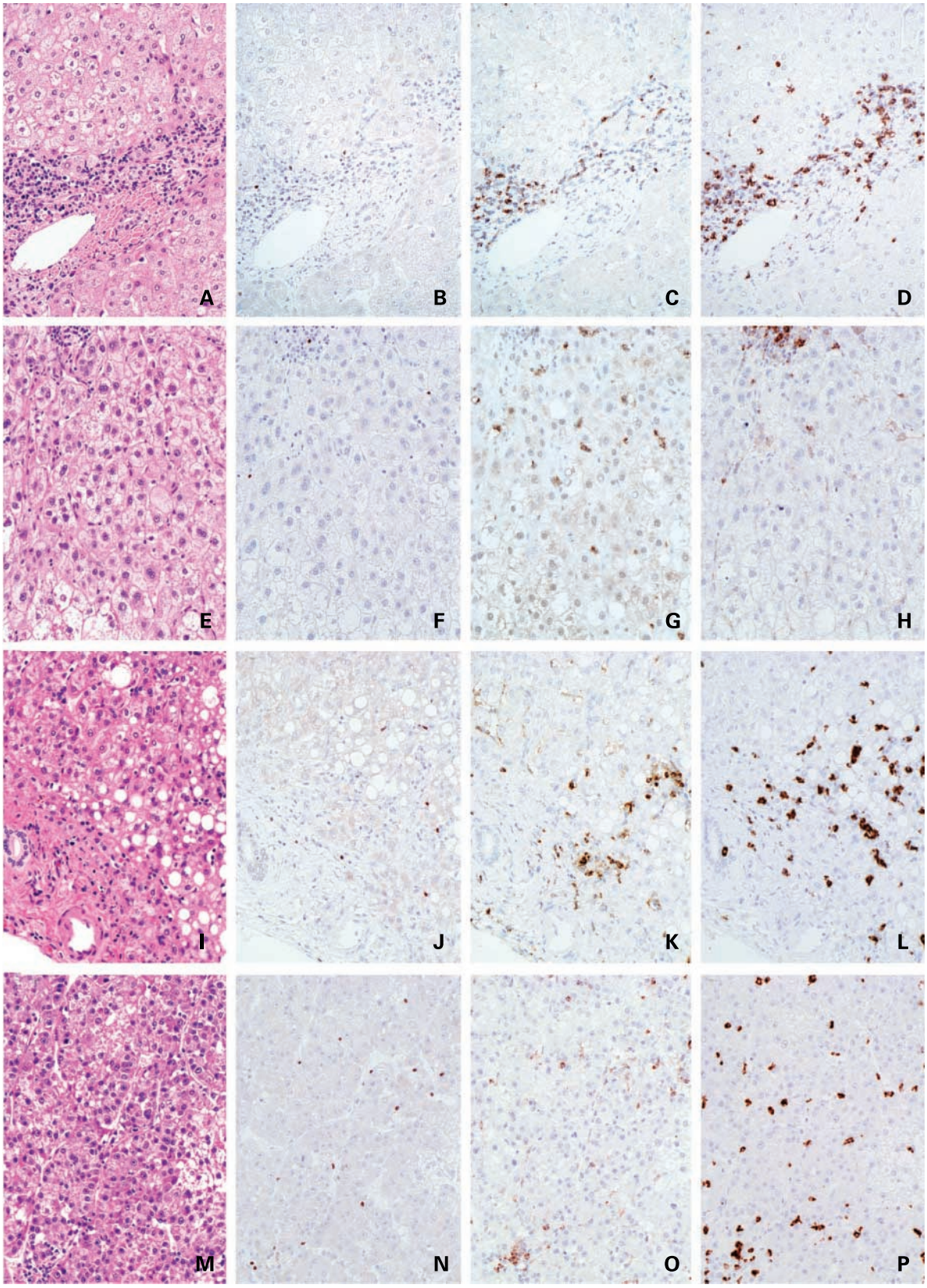

Fig. 2. Representative features of tissue-infiltrating FOXP3$^+$, CD4$^+$, or CD8$^+$ T lymphocytes. CH (*A-D*), AH (*E-H*), early HCC (*I-L*), and MD HCC (*M-P*) by HE staining (*A, E, I*, and *M*) and immunostaining for FOXP3 (*B, F, J,* and *N*), CD4 (*C, G, K,* and *O*), and CD8 (*D, H, L*, and *P*).

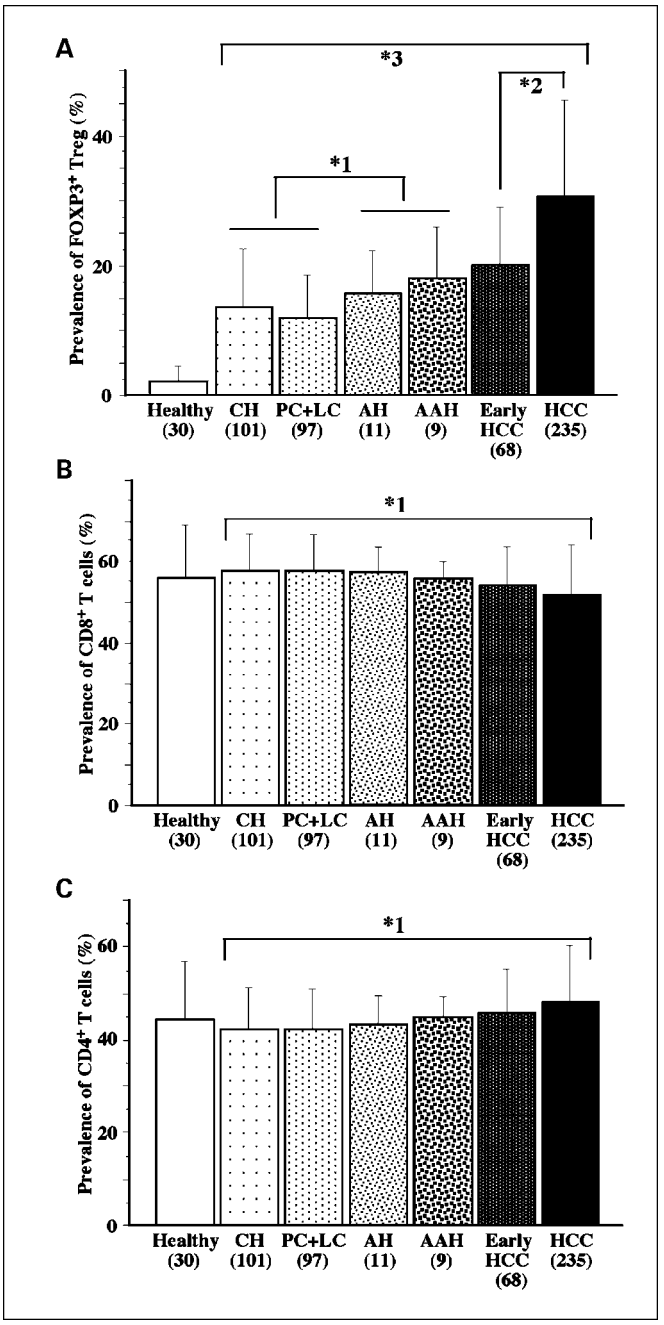

Fig. 3. Increased population of Tregs and decreased population of $CD8^+$ T cells in tumor stroma corresponding to the progression of multistage hepatocarcinogenesis. Prevalence of Tregs among $CD4^+$ Tcells (*A*), prevalence of $CD8^+$ Tcells among total Tcells (*B*), and prevalence of $CD4^+$ Tcells among total T cells (*C*) in HCC, its precursor lesions, and nontumorous liver. Number of cases tested in parentheses; Thin bars, SD. *A,* *1, $P = 0.038$; *2, $P < 0.001$; *3, $P < 0.001$ (Kruskal-Wallis test). *B* and *C,* *1, $P < 0.001$ (Kruskal-Wallis test).

was significantly higher in primary HCC than in IM ($P = 0.003$; Fig. 4A). Also, the prevalence of Tregs in primary hepatic adenocarcinoma was higher than that in metastatic hepatic adenocarcinoma ($P = 0.020$; Fig. 4A). The prevalence of $CD8^+$ T cells was comparable between primary and metastatic tumors.

Hepatitis viral infection and antitumor host immune response. HBV or HCV infection is a risk factor for the development of HCC (2), and it is also reported that these chronic viral infections suppress the host immune response (2). Some investigators have suggested that HCV infection increases ICC development, although this remains to be proven (34). The prevalence of Tregs in nontumorous liver of patients infected with HBV or HCV was significantly higher than in healthy liver (Fig. 4B), even in patients who were in the so-called "carrier" stage, with infection but no detectable manifestations or histologic changes. To investigate whether Tregs affected the development of primary liver tumors, we compared the prevalence of Tregs in nontumorous areas of liver bearing HCC, ICC, or metastatic liver adenocarcinoma among patients with and without hepatitis viral infection. The prevalence of Tregs in nontumorous liver bearing HCC or ICC without any HBV or HCV infection was apparently higher than that in healthy liver (versus HCC, $P < 0.001$; versus ICC, $P < 0.001$; Fig. 4B). In contrast, the prevalence of Tregs in nontumorous liver bearing HCC or ICC with HBV or HCV infection was slightly, but not significantly, higher than that in liver bearing no primary liver tumor with hepatitis virus infection (Fig. 4B). These findings suggest that a further increase of Treg infiltration in nontumorous liver with hepatitis virus infection is not closely correlated with the development of primary liver tumors. An interesting observation was that the prevalence of Tregs in nontumorous liver bearing HCC without HBV or HCV infection was higher than that in healthy liver, and was slightly lower than that in HBV-infected liver (Fig. 4B and C). The prevalence of Tregs in liver infected with HCV was higher than that in liver with HBV infection ($P = 0.016$), and

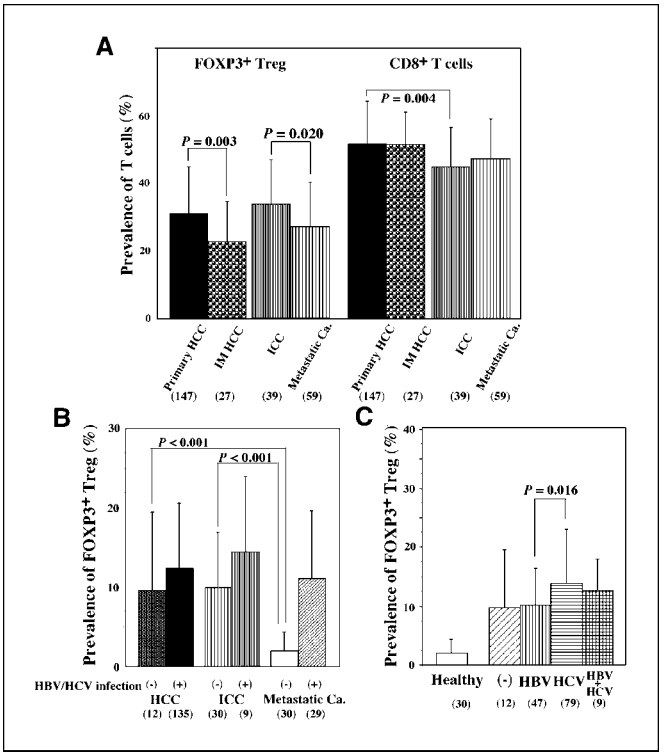

Fig. 4. *A,* prevalence of Tcells in primary liver cancer (HCC and ICC) and metastatic HCC (IM) and adenocarcinoma from colon cancer (metastatic ca.). Left and right columns, the prevalence of Tregs and $CD8^+$ Tcells, respectively. Number of cases tested are in parentheses. *B,* prevalence of Tregs in nontumorous liver of patients bearing HCC, ICC, or metastatic liver cancer, with or without HBV or HBC infection. Number of cases tested in parentheses. *C,* prevalence of Tregs in nontumorous liver of patients bearing HCC. Prevalence of Tregs in nontumorous liver with or without (−) hepatitis B and/or C viral infection were significantly higher than that in healthy controls. Number of cases tested in parentheses.

that in both HBV- and HCV-infected liver was intermediate between that in HBV- and HCV-infected liver. These observations were also recognized in patients with ICC (data not shown).

Discussion

Tumor-infiltrating lymphocytes represent the host immune response to a tumor, and include $CD8^+$ cytotoxic T cells and natural killer cells as positive responders and Tregs as immunosuppressors. There has been no large-scale or clinicopathologic study of Tregs in HCC and tumor-infiltrating lymphocytes in hepatocarcinogenesis. In the present study, we investigated the relationship between host immune response and hepatocarcinogenesis, focusing especially on Treg infiltration. First, we showed the clinicopathologic significance of Tregs among $CD4^+$ T cells infiltrating advanced HCC based on the following findings: (*a*) the prevalence of Tregs in HCC ($n = 235$) was significantly higher ($P < 0.001$) than that in nontumorous liver ($n = 248$), which included healthy liver, NCH, CH, PC, and LC. (*b*) Patients with HCC in the high-Treg group showed a significantly lower survival ratio. Both overall survival (log-rank test, $P = 0.007$) and disease-free survival (log-rank test, $P = 0.015$) were lower than for patients with HCC belonging to the low-Treg group. (*c*) Multivariate analysis revealed that the prevalence of tumor-infiltrating Tregs was an independent prognostic factor, along with Child-Pugh classification and presence of VP, for overall survival and that the prevalence of tumor-infiltrating Tregs and that of IM were independent prognostic factors for disease-free survival. (*d*) The prevalence of tumor-infiltrating Tregs was increased in poorly differentiated HCC (Kruskal-Wallis test; $P < 0.001$). In addition, we found that the prevalence of tumor-infiltrating Tregs increased in a stepwise manner (Kruskal-Wallis test, $P < 0.001$), whereas the prevalence of $CD8^+$ T cells decreased (Kruskal-Wallis test, $P < 0.001$) during the progression of hepatocarcinogenesis. These findings suggest that Treg infiltration was closely correlated with the progression of neoplastic cells in hepatocarcinogenesis. Furthermore, we showed that the prevalence of Tregs was increased in nontumorous liver tissue from patients with primary hepatic tumors, regardless of the presence of hepatitis virus infection or histopathologically evident hepatitis or liver cirrhosis. This indicates that primary hepatic tumors develop in liver, in which Tregs show marked infiltration and immune reactivity is suppressed. This is the first report to show that infiltration of Tregs is closely correlated with the development and progression of hepatocarcinogenesis, and that the prevalence of Tregs is a useful prognostic factor in patients with HCC.

It was reported previously that $CD8^+$ T cells infiltrating tumors are associated with good prognosis (8, 9), and that tumor-infiltrating Treg is increased in a variety of tumors (21–28). A few studies have also investigated the clinicopathologic significance of Treg infiltration (23–25), but conclusions about its correlation with prognosis were contradictory. Marked infiltration of Tregs in cancer stroma was reported to be an unfavorable prognostic factor in ovarian (23) and pancreatic (25) cancers, and was associated with control of tumor progression in head and neck cancers (24). No prognostic influence of Tregs was found in anal squamous cell carcinomas (35). In the present study, using multivariate analyses, we showed that the prevalence of Tregs in HCC was significantly correlated with both overall survival and disease-free survival. A high prevalence of Tregs was closely correlated only with histologic grade among a number of clinicopathologic variables. These findings indicate that the prevalence of tumor-infiltrating $FOXP3^+$ Tregs can be an independent prognostic factor for patients with HCC. In addition to the prevalence of Tregs, our multivariate analysis revealed that among 15 prognostic factors, Child-Pugh classification and the presence of VP and IM were independent indicators of unfavorable overall and disease-free survival, respectively, consistent with previous studies (36, 37). In contrast, infiltration of $CD8^+$ T cells as well as perforin-positive cells (data not shown) in HCC was found to have no prognostic significance. A positive prognostic effect of infiltrating $CD8^+$ T cells has been reported in various solid cancers such as colorectal (8) and ovarian (9) cancer. Only patients bearing HCC with exceptionally marked infiltration of $CD8^+$ T cells were reported to have a good prognosis (10). It is interesting that a negative prognostic effect of $CD8^+$ T cell infiltration has been observed in virus-related tumors, including EB virus–associated nasopharyngeal carcinomas (38) and human papilloma virus–associated anal carcinomas (35). This effect observed in other tumors was not observed in HCC, even though HCC is closely associated with hepatitis virus infection, and might be attributable to an organ-specific immune response.

In established HCC, Treg infiltration might play an important role in tumor progression and clinical behavior by modifying the host immune response. Furthermore, our data showed that the prevalence of Tregs increased in a stepwise manner from viral hepatitis containing CH, PC, and LC, to precursor lesions of AH and AAH, early HCC, and advanced HCC, indicating that Treg infiltration was closely involved in the progression of hepatocarcinogenesis ($P < 0.001$; Fig. 3A). It has been suggested that Tregs suppress the immune response through cell contact–dependent (12–14) or cell contact–independent mechanisms (15, 16), and that immune suppression occurs in several steps (12, 14, 16). Various immune cells could be the targets of Treg suppression, such as $CD8^+$ T cells, $CD4^+CD25^-$ T cells, B cells, natural killer cells, natural killer T-cells, and dendritic cells (12, 14, 16, 39, 40). In this study, the prevalence of $CD8^+$ tumor-infiltrating lymphocytes was found to decrease significantly during hepatocarcinogenesis ($P < 0.001$; Fig. 3B), and this was inversely correlated with Treg infiltration. The group of patients with advanced HCC showing marked Treg infiltration showed a tendency to have a lower prevalence of $CD8^+$ tumor-infiltrating lymphocytes (Table 2A). Thus, it is possible that Tregs contribute to reducing the infiltration of $CD8^+$ T cells during hepatocarcinogenesis. Unitt et al. reported that Tregs isolated from advanced HCC suppressed the proliferation and perforin expression of autologous circulating $CD8^+$ T cells (27).

Persistent viral infection requires host immune suppression. $CD4^+CD25^+$ Tregs have been reported to be linked to the chronicity and progression of viral hepatitis in patients with HBV or HCV infection by down-regulating the hepatitis virus-specific T cell response (41–43). Our present observations confirm marked infiltration of Tregs in the liver of patients infected with HBV or HCV. Regardless of the presence of hepatitis virus infection and histopathologic changes indicative of hepatitis, the prevalence of Tregs in nontumorous liver tissue

of patients bearing HCC was significantly higher than that in healthy liver, but was slightly lower than that in liver with viral hepatitis. It has been suggested that even in patients with HCC of unknown etiology, immunosuppression might have started in the liver before tumor development. In patients with primary hepatic adenocarcinoma, ICC, the prevalence of FOXP3$^+$ Tregs in nontumorous liver without viral infection was also higher than that in healthy liver. These findings suggest that primary hepatic tumors can develop in the liver with a certain degree of Treg infiltration. A subsequent increase of Treg infiltration seemed to accelerate the development of hepatic tumors in patients infected with hepatitis virus, but not to a significant degree. Further studies will be necessary to clarify the threshold of Treg prevalence at which the risk of hepatic tumor development becomes high. The prevalence of Tregs in primary hepatic tumors, both HCC and adenocarcinoma, was significantly higher than that in metastatic hepatic tumors with the corresponding histology. These findings support the hypothesis that the development and progression of primary hepatic tumors involves high accumulation of Tregs.

In conclusion, our data suggest that Tregs play a role in controlling the immune response to HCC from the precursor stage to established cancer, and also that primary hepatic cancers might develop in liver that is immunosuppressed by marked infiltration of Tregs, regardless of the presence of hepatitis viral infection. A high prevalence of Tregs seems to be an indicator of poor prognosis.

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