RESEARCH ARTICLE

Associations Between Infiltrating Lymphocyte Subsets and Hepatocellular Carcinoma

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Abstract

Aims: We aimed to analyze the phenotype of tumor-infiltrating lymphocytes (TILs) and non-tumor infiltrating lymphocytes (NILs) in HCC and non-tumor tissues, and evaluate relationships between changes in these cells and the prognosis of HCC. Methods: Lymphocytes were isolated from HCC and corresponding non-tumor tissues and tested by flow cytometry. For comparison, clinical parameters were analyzed. Results: Compared with the non-tumor tissue, tumor tissue had a lower intensity of NK, NKT andCD8+T cell infiltration. TILs had higher intensity of CD4+CD25+Foxp3+regulatory T cell (Treg cells) infiltration compared with that in NILs. The prevalence of Treg cells was associated with fewer CD8 + T lymphocytes in the HCC immune microenvironment. The frequencies of NK cells and CD8+T cells in TILs of HCC patients with metastasis less than 12 months were lower than those without metastasis. However, the frequency of Treg cells was higher than those without metastasis. Conclusion: These results suggest that the frequencies of CD8+T, NK and NKT cells as well as Treg cells in the tumor tissue of HCC are significantly associated with patient survival, and could be applied as predictive indicators for HCC prognosis.

Keywords: Hepatocellular carcinoma - tumor microenvironment - tumor-infiltrating lymphocytes - regulatory T cells

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, it is the fifth most common cancer in men (523 000 cases, 7.9% of the total) and the seventh in women (226 000 cases, 6.5% of the total) and most of the burden is in developing countries (IARC 2008). Although treatment options such as hepatic resection, liver transplantation, chemotherapy and tyrosine kinase inhibitors are widely used for HCC, it is still frequent recurrence (Shirabe et al., 1991).

Immunotherapy seems to one of new therapies for HCC, and previous studies reported that patients undergoing hepatic resection for HCC with prominent lymphocyte infiltration show reduced recurrence and better prognosis as compared with those without prominent lymphocyte infiltration (Shirabe et al., 1995). Previous studies indicated recurrence after liver transplantation for HCC is related to immunosuppression, and subpopulations of tumor-infiltrating lymphocytes (TILs) could predict recurrence (Unitt et al., 2006).

There are a variety of tumor-associated immune cells such as macrophages, lymphocytes, B cells in the tumor tissue (Li et al., 2009; Pan et al., 2009; Shen et al., 2010). The numbers of TILs and the changes of their function are seen to reflect the anti-tumor response of the body. Previous studies reported that TILs contribute greatly to the interaction between the host immune system and neoplastic growth in terms of several cancers, such as melanoma, colorectal and ovarian cancer (Matkowski et al., 2009).

A detailed understanding of the functional peculiarities of individual T lymphocyte subtypes may explain the paradox that the presence of TILs does not always correlate with improved prognosis and may also allow the development of targeted approaches that specifically augment antitumor immune responses (Schumacher et al., 2001; Nakano et al., 2001). Previously, there were several studies showed the specific types of immune cells regulate the host defense against HCC, which were based on the subpopulations of TILs with the development of immunohistochemistry and flow cytometry (Chen et al., 2007; Gao et al., 2007; Kasper et al., 2009; Chew et al., 2010). However, the results are inconsistence and inconclusive. No definitive conclusion has been reached regarding the efficacy of T cell dependent immune mechanism or the correlation between the extent, type of lymphocyte infiltration and the tumor progression in HCC.

In this study, we aim to investigate the frequency of many lymphocytes subsets including CD3, CD4, CD8, CD56, CD57, CD20 cells in TILs and NILs of HCC patients, and the role of CD25+, CD8+, CD56+, CD57+

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Table 1. Clinical Characteristics of Enrolled HCC Patients

Variable	Results
Age (mean±SD, Y)	63±10.8
Sex, male/female	29/21
HBsAg, +/-	27/23
HCsAg, +/-	12/38
Background Chronic hepatitis/ Cirrhosis/fat liver/alcohol liver	24/15/7/4
Tumor factors	
Number of tumors (Single/double/multiple)	22/18/10
α-fetoprotein level[ng/ml] >400/400-200/≤200	26/13/11
TNM stage I/II/III/IV*	15/23/8/4
GOT/GPT ≤1.0/>1.0	19/31

*HCC patient disease stage was evaluated according to TNM classification system of the International Union against Cancer, 2002

cell distribution in determining the location of infiltrating immune cells in HCC tumor microenvironment by immunohistochemistry methods. We aimed to clarify the relationship between their distribution, other clinic pathological variables and patient prognosis.

Materials and Methods

Study subjects

Specimens of liver tissues were obtained by biopsy or surgery from 50 HCC patients. Surgically resected or biopsy specimens were diagnosed by routine pathology. The clinical stage of tumor progression was determined according to the International Union against the Cancer TNM classification system. The age, sex and clinical characteristics were collected from medical record and summarized in Table 1. All patients were asked to provide written informed consent, and our study was approved by the ethics committee in our institution.

Isolation of tumor infiltrating T cells

The freshly excised tissue samples were separated into two parts, including tumor region and non-tumor region tissue that were >3 cm away from the tumor margin. The specimens from the tumorous region contained some tumor and normal tissues. TILs were collected from the tumor region, and the NILs were collected from the non-tumor region. The tissues were cut into small pieces and pressed through a 200 stainless steel mesh and suspended in Eagle's minimum essential medium (Life Technologies, Grand Island, NY) supplemented with 5 mM HEPES and 5% heat-inactivated fetal calf serum. Then the cells' suspensions were loaded onto 2 superimposed layers of 100% and 75% ficoll-paque and centrifuging at 2500 rpm for 20 minutes at 20°C. The lymphocytes were washed twice in phosphate-buffered saline (PBS).

Anti-body and Flow Cytometry Analysis

NILs and TILs were incubated with the follow conjugated antibodies: CD3-ECD, CD4-PE-Cy7, CD8-FITC, CD56-FITC, CD57- FITC, CD20- FITC (Beckman, Los Angeles, CA, USA). CD25-Percp-cy5.5, Foxp3-FITC, (eBioscience, San Diego, CA, USA). After washing, the labeled cells were analyzed on FACSAria (BD, San Jose, CA, USA) and FACS FC500 (Beckman, Los Angeles, CA, USA) with CellQuest software.

Immunohistochemical Staining

Cryostat sections 5-μm thick were cut and fixed in cold acetone for 5 min. After immersion in blocking serum, sections were incubated with mouse anti-human CD25 , CD4, CD56, CD8 Ab (DAKO) at a 1:50 or 1:100 dilution, respectively, in PBS supplemented with 3% BSA at 4ºC overnight. After successive washing in PBS, sections were incubated with biotinylated anti-mouse immunoglobulin at a 1:100 dilution in PBS supplemented 5% BSA.

Immunohistochemical detection was performed according to the avidin–biotin-peroxidase complex method using the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA). Sections were finally developed with diaminobenzidine (DAB) substrate (Muto Pure Chemicals, Tokyo, Japan) or Vector SG (VectorLaboratories). Specimens then were counterstained with Methyl Green solution and mounted.

Following-up

The disease recurrence after surgery were followed up, including items of physical examinations, (AFP), chest radiography, and spiral abdominal computed tomography scans every 6 monthsin the first years. Other examinations, such as abdominal ultrasound, were selected and performed every 6 to 12 months, depending on the patient's status.

Statistical Analysis

The association between between two groups was analyzed statistically by Student's t test. If there was heteroscedasticity, Mann-Whitney U test would be used. Correlations between parameters were analyzed by linear regression analysis. SPSS software version 17.0 for Windows (SPSS Inc., Chicago, IL) was used for analysis, and values of $p < 0.05$ were considered statistically significant.

Results

Frequencies of lymphocytes in tumor tissue and non-tumor tissue of HCC patients

The prevalence of lymphocytes of TILs and NILs in 50 HCC patients was showed in Figure 1. In population with CD3+, the percentage of CD4+ cells was be significantly higher in TILs than that in NILs (27.4±15.4% vs. 18.7±8.0%, $p < 0.05$). Concurrently, the percentage of CD8+ cells was lower in TILs than that in NILs (32.6±12.3% vs. 39.9±12.5%, $p < 0.05$). For other lymphoid subsets, we found reduced in CD3-CD56+ NK cells (20.6±10.4%% vs. 27.9±13.5% %, $p < 0.05$), CD3+CD56+ NKT cells (8.5±3.7% vs. 12.7±8.4%, $p = 0.06$) and CD3-CD57+ NK cells (6.9±3.7% vs 9.6±5.1%, $p < 0.05$) as well as CD3+CD57+ NKT cells (10.8±3.9% vs. 14.8±7.9%, $p = 0.08$) (Figure 1). The frequency of CD4+CD25+Foxp3+regulatory T cell's (Treg cells) in TILs of hepatocellular carcinoma was significantly higher than NILs (7.1±4.0% in TILs vs. 5.1±3.3% in NILs, $p < 0.05$).

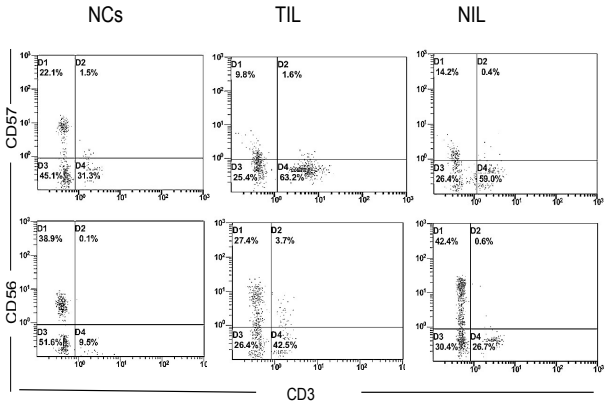

Figure 1. Frequencies of CD3-CD56+NK Cells, CD3-CD57+NK Cells, CD3+CD8+T Cells in NCs, TILs and NILs

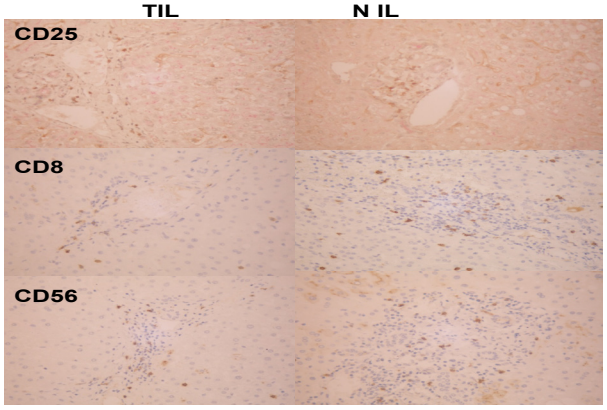

Figure 2. The Distribution of CD4+CD25+ T Cells in the Liver with Immunohistochemistry Using Serial Sections

Immunohistochemical detection of CD4+CD25+T cells, CD8+T cells and CD3-CD56+NK cells in TIL and NIL

The distribution of immune cells in the liver was detected by immunohistochemistry of serial section. Hematoxylin-eosin staining of marginal region in HCC shows lymphocytes infiltrated around the well-differentiated HCC. Serial sections revealed CD25+ cells abundant around the tumor than that of non-tumor tissue, but only a small number of CD8+T cell and NK cells were found in the tumor (Figure 2).

Relationship between CD8+T cells, CD3-CD56+NK cells and Treg cells in the liver with HCC

In tumor regions, the increased number of Treg cells was associated with the decreased number of CD8+T cells. Our study showed the proportion of CD8+T cells were inversely proportional to Treg cells in tumor regions ($r = -0.62$, $p < 0.05$). But CD3-CD56+NK cells did not show relationship with Treg cells.

The changes of tumor-infiltrating lymphocytes for the survival of HCC patients

All patients were divided into two groups according to the results of the CT scan, including patients who has intrahepatic recurrence and metastatic within a year and patients without recurrence and metastasis. In TILs, the frequency of CD3-CD56+, CD3-CD57+, and CD3+CD8+ were lower in patients with metastasis less than 12 months than those without metastasis (P<0.05) (Figure 3).

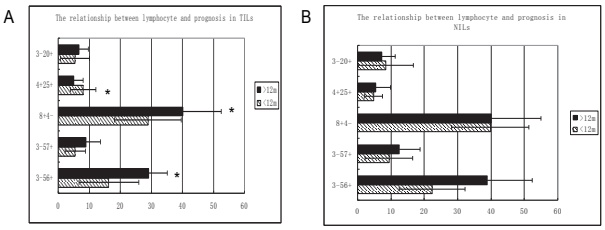

Figure 3. Frequency of CD3-CD56+, CD3-CD57+ and CD3+CD8+ in Patients with and Without Metastasis

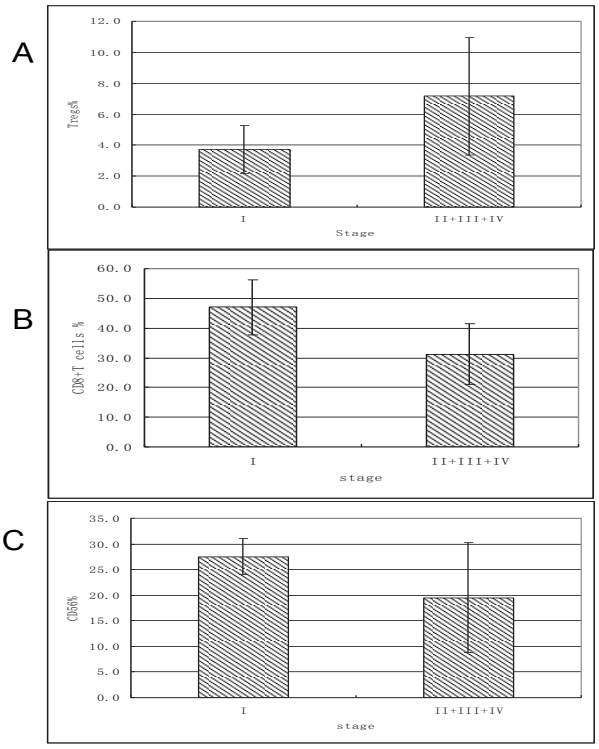

Figure 4. Proportion of Tregs, CD8+T Cells and NK Cells in Different Stages of HCC Patients

However, frequency of Treg cells was higher in patients with metastasis less than 12 months than those without metastasis (P<0.05).

Relationship between clinical parameters and frequency of TILs

All lymphocytes were not correlated with the other clinical indicators such as TNM, AFP, HBV, HCV, GOT and GPT (data not show). The proportion of Treg cells was significantly lower in the early stage (i.e., stage I) than that in the advanced stage (i.e., II+III+ IV) ($p < 0.01$). The frequencies of CD8+Tcells and CD3-CD56+ NK cell were higher in the early stage than those in advanced stages ($p < 0.05$) (Figure 4).

Discussion

It has been demonstrated that a lower intensity of NK, NKT and CD8+T cell's infiltration was found in tumor tissue, and they were negatively associated with the prognosis of HCC patients. Although the responses of tumor-specific cytotoxic T-lymphocyte had been reported in HCC patients, tumor regression is rarely observed (Gehring et al., 2009). Two major mechanisms are suggested to be responsible for the compromised tumor-

specific killing: the dysfunction of cytolytic machineries and the increase of the inhibitory elements (Wu et al., 2009). NK cells, NKT cells and CD8+T cells represent the major cytolytic components of the TILs in the liver. These cells are able to rapidly release immunomodulatory cytokines, which activate leukocytes of both the innate and adaptive immune system. Therefore, reduction of these cells may cause the tumor progression. In our study, CD8+ T cells, NK cells and NKT cells were found decreased in TILs when comapred with those in NILs, especially for CD3+CD8+T cells and CD3-CD56+ NK cells. Previous studies showed that CD8+ T cells, NK cells and NKT cells were reduced in both peripheral blood and liver of HCC patients (Smyth et al., 2005; Fu et al., 2007), which was in line with our study. The main reason is that tumor cells could suppress the infiltration of lymphocytes and may escape from the host immune attack. Some researches had reported that tumor cells can express Fas ligand (FasL) to induce apoptosis of Fas expressing in TILs (Okada et al., 2000). Another study indicated that PD-1/PD-L1 plays a pivotal role in HCC evasion, which could promote apoptosis of CD8+ T cells (Shi et al., 2011).

Treg cells are the main regulatory in the body and inhibit the proliferation and the function of many different types of cells in the immune system such as conventional CD4+ and CD8+ T lymphocytes, NK cells, NKT cells, B cells, dendritic cells and monocytes/macrophages (Smyth et al., 2006). Recent studies have reported that the increased number of Treg cells in the peripheral blood and TILs in patients with ovarian, gastric or esophageal cancer could impair cell-mediated immunity and promote disease progression (Morse et al., 2008). Previous studies have demonstrated that removing or suppressing Treg cells can strengthen antitumor immunity (Ohmura et al., 2008; Setiady et al., 2010), and these studies indicate that Treg cells play a negative regulatory role in human antitumor immunity. In our study, we showed an increased numbers of CD4+CD25+Foxp3+ T cells in the TILs of HCC, which are inconsistence with previous results.

The mechanisms that Treg cells suppress these cells are not fully understood. Previous studies demonstrated that human circulating Treg cells can kill autologous responder cells and monocytes via Fas/FasL-mediated apoptosis (Streuss et al., 2009). Strauss et al. (2009) reported that Treg cells from all subjects were CD95+, but only Treg cells from cancer patients expressed CD95L (FasL). These Treg cells, when activated via T cell receptors (TCR) and IL-2, up regulated CD95 and CD95L expression and suppressed CD8+RC proliferation by inducing Fas-mediated apoptosis. The same conclusion was reached by Venet et al. (2006). In our study, we found the reduced number of the CD8+T lymphocytes may partly be due to the increases of Treg cells which can weaken their immune surveillance and immune killer function, and this reduced number could indirectly lead to cancer occurrence or development. Nevertheless, the pathological significance of Treg cells in tumor development and progression need more researches.

The frequency of Treg cells was lowered in TILs from early stage patients as compared with the advanced stages, and the frequency of CD8+T cells and CD3-CD56+ NK cells were higher in the early stage than in the advanced stage. Previous experimental study reported a similar finding (Kobayashi et al., 2007). Moreover, we also found the frequency of CD3-CD56+, CD3-CD57+ and CD3+CD8+ cells of the patients with the metastasis less than 12 months were lower than those without metastasis. The frequency of Treg cells was higher in the patients with metastasis than those without metastasis. These phenomena showed us that CD3-CD57+NK cells, CD3-CD56+NK cells and CD3+CD8+T cells may kill the tumor cells and have the function of inhibiting cancer cell migration. The decrease of these cells may be a risk factor of metastasis.

Our results showed that CD3-CD56+ NK cells, CD3-CD57+ NK cells, and CD3 +CD8+ T cells have a strong correlation with the prognosis of HCC patients and can be a good indicator for judging patients' prognosis. The mechanism of these lymphocytes' changes in HCC remains unclear in the current study. Further investigations are still warranted.

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