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ORIGINAL ARTICLE

Changes of host immunity in relation to efficacy in liver cirrhosis patients with advanced hepatocellular carcinoma treated by intra-arterial chemotherapy

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Abstract
Purpose It is known that tumors develop mechanisms to escape from the immune system and to inhibit antitumor responses. The aim of this study was to retrospectively assess changes of host immunity in relation to efficacy in liver cirrhosis (LC) patients with advanced hepatocellular carcinoma (aHCC) treated by combined intra-arterial chemotherapy.
Methods Thirty-seven adult Japanese LC patients with aHCC were treated by intra-arterial combination chemotherapy. The control group was composed of 19 adult Japanese patients with chronic hepatitis C diagnosed by pathological examination of liver biopsy specimens. All control patients were stage 1 according to the fibrosis score of Desment.
Results Ten of the 37 patients (group PR) showed a partial response and 17 of the 37 patients (group SD) showed stable disease, but 10 of the 37 patients (group PD) showed no response. There were no significant differences in the percentage of Th1 cells between any of the groups either before or after chemotherapy. The percentage of Th2 cells was significantly higher in group PD before and after chemotherapy than in the control group ($P < 0.05$ by Tukey's test). Although there was no significant difference, the percentage of Th2 cells was higher in group SD than in group PR.
Conclusions The percentage of Th2 cells increased in LC patients with aHCC as the efficacy of intra-arterial combination chemotherapy decreased. These results indicated that intra-arterial chemotherapy might be not useful for patients with aHCC, because it induces Th2 dominant host immunity.

Keywords Th1/Th2 balance · Host immunity · Advanced HCC · Liver cirrhosis · Intra-arterial chemotherapy

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Introduction

The majority of primary liver cancers are hepatocellular carcinomas (HCCs) and the incidence of HCC is increasing in many countries [1–5]. Predicting the survival of patients with HCC is difficult, because the prognosis depends on both tumor extent and liver function. Several non-surgical treatment options, including transcatheter arterial emobolization, percutaneous ethanol injection, microwave coagulation therapy, and radiofrequency ablation, have been developed and are widely used in patients with unresectable HCC. However, these modalities are not indicated for patients with multifocal disease, invasion or thrombosis of major blood vessels, and/or poor liver function. The majority of patients with advanced hepatocellular carcinoma (aHCC) do not survive for longer than 6 months from the time of diagnosis [6], while other reports have indicated an average survival period of only 4 months from the onset of symptoms or 2 months from the time of admission [7]. Improvement of catheters and the development of implantable drug delivery systems have enabled us to perform long-term arterial infusion chemotherapy [8]. This means that it is possible to perform repeated hepatic arterial infusion of anticancer agents in patients with aHCC, and hepatic arterial infusion therapy not only improves survival but also improves the quality of life [9]. Intra-arterial combination chemotherapy is one of the few remaining options

for patients with aHCC, and continuous local arterial infusion of 5-fluorouracil (5-FU) and cisplatin (CDDP) via an infuser pump and implanted reservoir has been shown to prolong the survival of these patients [9–11]. We have also reported that combined intra-arterial therapy with low-dose 5-FU, CDDP, and leucovorin (LV) prolongs the survival of aHCC patients [12], with continuous intra-arterial infusion over 24 h being more effective than infusion for 6 h in aHCC patients with liver cirrhosis (LC) due to HCV infection, although 24-h infusion is associated with stronger hematologic toxicity [13].

Th1 and Th2 cells cross-regulate their own development. It has been reported that Th2 cytokines suppress antitumor immunity [14], while activation of the Th1 response promotes antitumor immunity [15, 16]. CD8-positive tumor-infiltrating lymphocytes (TILs) play an important role in host defences against tumor progression. Some studies have indicated that there is a positive correlation between an increase of CD8-positive TILs and tumor cell apoptosis [17, 18]. Ikeguchi et al. reported the significant infiltration of CD8-positive T cells into the fibrous tissue and sinusoidal capillaries of the non-cancerous liver, as well as around the tumor, although the average number of CD8-positive T cells within the tumor was significantly lower than that in the non-cancerous liver tissue and the extent of CD8-positive T cell infiltration into non-cancerous hepatic lobules was not correlated with the severity of liver fibrosis [19]. CD4-positive CD25-positive regulatory T cells (T_{reg}) have an important role in maintaining self-tolerance and regulating the immune response under both physiological conditions and in various diseases [20]. It has been reported that T_{reg} are increased in the peripheral blood and/or tumors of HCC patients and that this increase of T_{reg} suppresses CD4-positive helper T cell responses, and appears to promote the progression of HCC [21–23]. We previously examined the effect of intra-arterial combination chemotherapy on the Th1/Th2 balance in LC patients with aHCC, and found that a combination of low-dose 5-FU, CDDP, and LV might not demonstrate efficacy against aHCC if patients have a high Th2 cell ratio among their CD4-positive T cells [24]. However, we did not examine changes of host immunity in relation to the efficacy of intra-arterial therapy in LC patients with aHCC. Therefore, the present study was performed to retrospectively assess changes of host immunity in relation to the effectiveness of intra-arterial chemotherapy in LC patients with aHCC.

Methods

Patients

Thirty-seven adult Japanese patients who had aHCC and liver cirrhosis due to HBV infection, HCV infection, or excessive alcohol intake were treated intra-arterially with the combination of low-dose 5-FU, CDDP, and LV at our hospital between 2005 and 2008. All of the patients were more than 55 years old. Their tumors were inoperable on the basis of computed tomography findings. Blood samples were collected from the patients in the early morning both before and after chemotherapy. The control group was composed of 20 adult Japanese patients with chronic hepatitis C diagnosed by examination of liver biopsy specimens. All of the control patients were also older than 55 years, and had stage 1 disease according to the fibrosis score of Desment.

Chemotherapy

All patients were treated with 24-h intra-arterial infusion chemotherapy (LV at 12 mg/h, CDDP at 10 mg/h, and 5-FU at 250 mg/m^2 for 22 h). Continuous infusion was performed via the proper hepatic artery every 5 days for 4 weeks using a catheter connected to a subcutaneously implanted drug delivery system [12, 13].

Infusion system

In all patients, an intra-arterial catheter was inserted via the femoral artery and was attached to a subcutaneous reservoir [25]. In principle, the gastroduodenal artery and right gastric artery were occluded with steel coils to prevent gastroduodenal injury by the anticancer agents. Written informed consent was obtained from all of the patients.

Evaluation of therapeutic efficacy

On CT scans obtained after 4 weeks of treatment, the size of the intrahepatic tumors was measured as the product of the two longest perpendicular diameters of the largest tumor. A complete response (CR) was defined as disappearance of the tumor, while a partial response (PR) was defined as more than 50% reduction of the product of the two longest diameters. An increase of the product by more than 25% was defined as progressive disease (PD), and changes between PD and PR were defined as stable disease (SD).

Analysis of CD4-positive T cell subsets

CD4-positive T cell subsets in the peripheral blood were analyzed after nonspecific stimulation with phorbol 12-myristate 13-acetate (PMA), ionomycin, or brefeldin A (Sigma Chemical Co., St. Louis, MO, USA), according to the modified method of Jung et al. [26, 27].

Flow cytometry was used to detect IFN-gamma and IL-4 in the cytoplasm of peripheral blood CD4-positive T cells after culture and staining, as reported previously [26]. Results were expressed as the percentage of cytokine-

producing cells among the CD4-positive T cell population, such as IFN-gamma-positive/IL-4-negative (Th1) cells or IFN-gamma-negative/IL-4-positive (Th2) cells (Fig. 1).

Statistical analysis

Tukey's test was used to compare patient characteristics among the groups. Results are expressed as the mean ± SD. Probability values of less than 0.05 were considered to indicate statistical significance.

Results

The patients were divided into three groups. Ten of the 37 patients (group PR) showed a partial response and 17 of the 37 patients (group SD) showed stable disease, while 10 of the 37 patients (group PD) showed no response. The control group was composed of 12 men and 8 women aged 56–68 years (mean ± SD, 61.6 ± 4 years). There were nine men and one woman aged 58–73 years (mean ± SD, 68.7 ± 7 years) in group PR and 16 men and 1 woman aged 54–74 years (mean ± SD, 67.1 ± 8 years) in group SD, while there were nine men and one woman aged 51–73 years (mean ± SD, 65.2 ± 8 years) in group PD. In group PR, there were seven patients with HCV-related LC (C-LC), one patient with HBV-related LC (B-LC), and two patients with alcoholic LC (ALC). In group SD, there were 11 patients with C-LC, and 1 patient with B-LC, and 5 patients with ALC. Group PD had three patients with C-LC and seven patients with B-LC. The Child–Pugh class was A for 7 patients in group PR, 5 patients in group SD, and 4 patients in group PD, while it was B for 3, 11, and 5 patients, respectively, and C for 1 patient each in groups SD and PD. Three patients had stage III disease, five patients had stage IVA disease, and two patients had stage IVB disease in group PR. There was 1 patient with stage III disease, 12 patients with stage IVA disease, and 4 patients with stage IVB disease in group SD, while the respective numbers were 0, 4, and 6 in group PD. Three patients had a Japan Integrated Staging (JIS) score [28] of 2, four patients had a score of 3, three patients had a score of 4, and no patient had a score of 5 in group PR, while the respective numbers were 1, 4, 11, and 1 in group SD, and 0, 4, 5, and 1 in group PR. One patient from group PR had tumor thrombi in the first branch of the portal vein and one patient with thrombi in the portal trunk, while two patients had tumor invasion into the first branch of the right hepatic vein. In group SD, there were two patients with tumor thrombi in the first branch of the portal vein, one patient with involvement of major branches of the portal vein, and one patient with thrombi in the portal trunk, while three patients had tumor invasion into the first branch of the right hepatic vein, one patient had invasion of the right hepatic vein itself, and one patient had invasion of the main hepatic trunk. In group PD, there were two patients with tumor thrombi in the first branch, two patients with tumor thrombi in major portal vein branches, and two patients with thrombi in the portal trunk, while two patients had tumor invasion into the first branch of the right hepatic vein, two patients had invasion of the right hepatic vein itself, and one patient had invasion of the main hepatic trunk (Table 1).

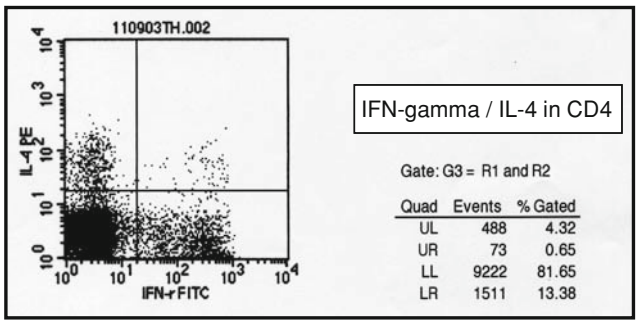

Fig. 1 Flow cytometric detection of interferon (IFN)-γ and interleukin (IL)-4 in CD4-positive T cells. *Upper left* IFN-γ negative and IL-4 positive cells (Th2), *lower right* IFN-γ positive and IL-4 negative cells (Th1), *upper right* IFN-γ positive and IL-4 positive cells (Th0)

Serum aminotransferases

Figure 1 summarizes the comparison of serum aminotransferases between the groups. There were no significant differences among the four groups with respect to serum alanine aminotransferase (ALT) or serum aspartate aminotransferase (AST), and there were also no significant differences of serum aminotransferases between before and after chemotherapy in each group (Fig. 2).

Peripheral blood Th1 and Th2 cells

There were no significant differences in the percentage of Th1 cells between each groups either before chemotherapy (PR, 27.1 ± 9%; SD, 26.7 ± 10%; PD, 25.5 ± 7%; control, 23.5 ± 6%) or after chemotherapy (PR, 26.8 ± 12%; SD, 27.5 ± 13%; PD, 26.1 ± 8%) (Fig. 3). The percentage of Th2 cells in group PD before chemotherapy (4.8 ± 2%) and after chemotherapy (4.4 ± 1%) was significantly higher than in the control group (2.6 ± 0.8%) ($P < 0.05$ by Tukey's test). Although there were no significant differences, the percentage of Th2 cells was also higher in group SD (before, 3.8 ± 2%; after, 4.0 ± 2%) than in group PR (before, 3.2 ± 2%; after, 3.1 ± 1%) (Fig. 4).

Table 1 Clinical characteristics of the 37 patients with liver cirrhosis

	Group PR	Group SD	Group PD
No. of patients	10	17	10
Mean age	68.7 ± 7	67.1 ± 8	65.2 ± 8
Gender (M/F)	9/1	16/1	9/1
Type of cirrhosis (HBV/HCV/alcohol)	1/7/2	1/11/5	7/3/0
Child–Pugh classification (A/B/C)	7/3/0	5/11/1	4/5/1
Stage (III/IVA/IVB)	3/5/2 (vv2:2, vv3:0) (vp2:1, vp4:1)	1/12/4 (vv2:3, vv3:1, vv4:1) (vp2:2, vp3:2, vp4:1)	0/4/6 (vv2:2, vv3:2, vv4:1) (vp2:2, vp3:2, vp4:2)
JIS score (2/3/4/5)	3/4/3/0	1/4/11/1	0/4/5/1

The patients were divided into 3 groups. Ten of 37 patients showed an objective response (group PR, partial response or stable disease), while 10 patients (group PD) showed no response. Seventeen patients (group SD) showed smaller changes between these two limits

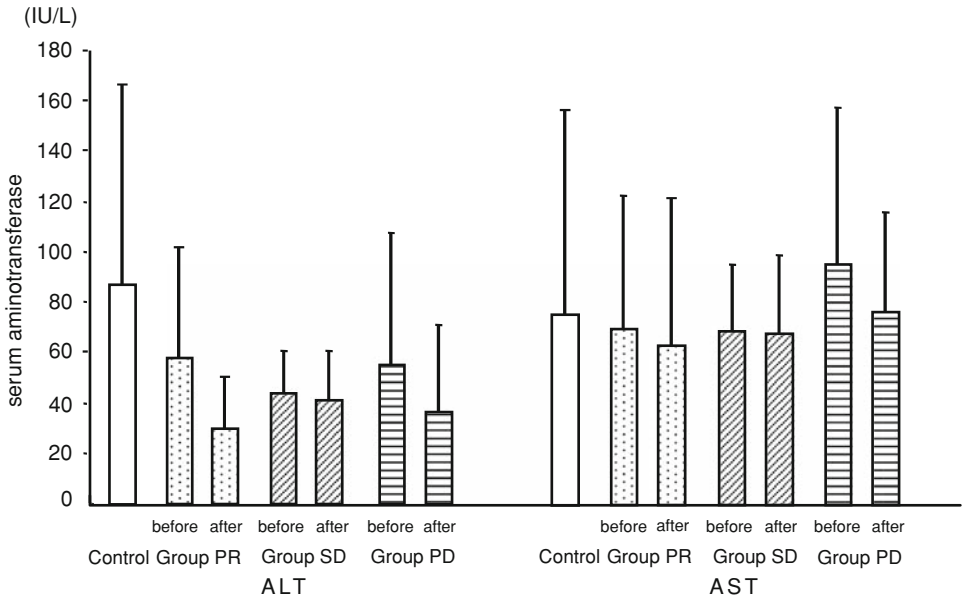

Fig. 2 Comparison of the serum aminotransferases before and after treatment in group PR, group SD, group PD, and the control group. There were no significant differences

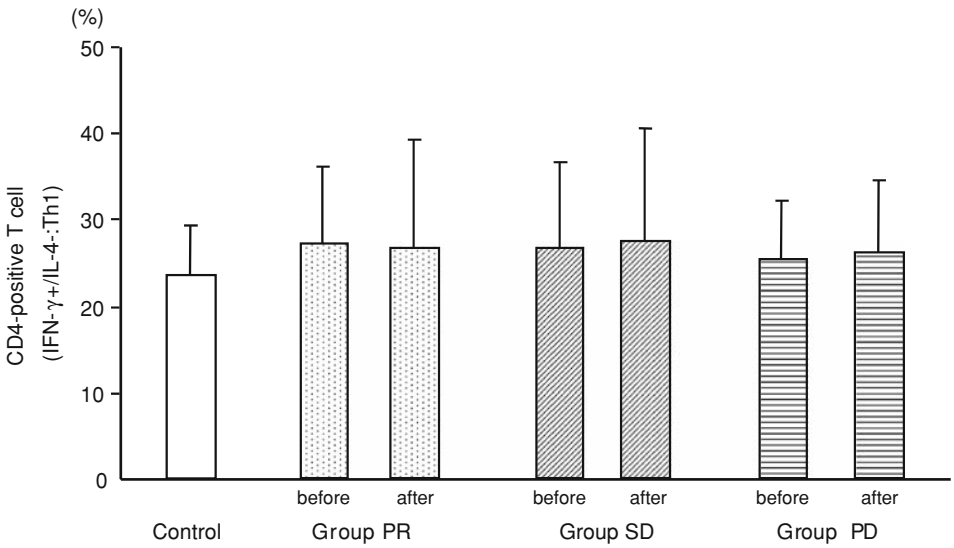

Fig. 3 Comparison of the relative prevalence of CD4-positive T cells (IFN-γ positive and IL-4 negative cells, Th1 cells before and after treatment in group PR, group SD, group PD, and the control group. There were no significant differences

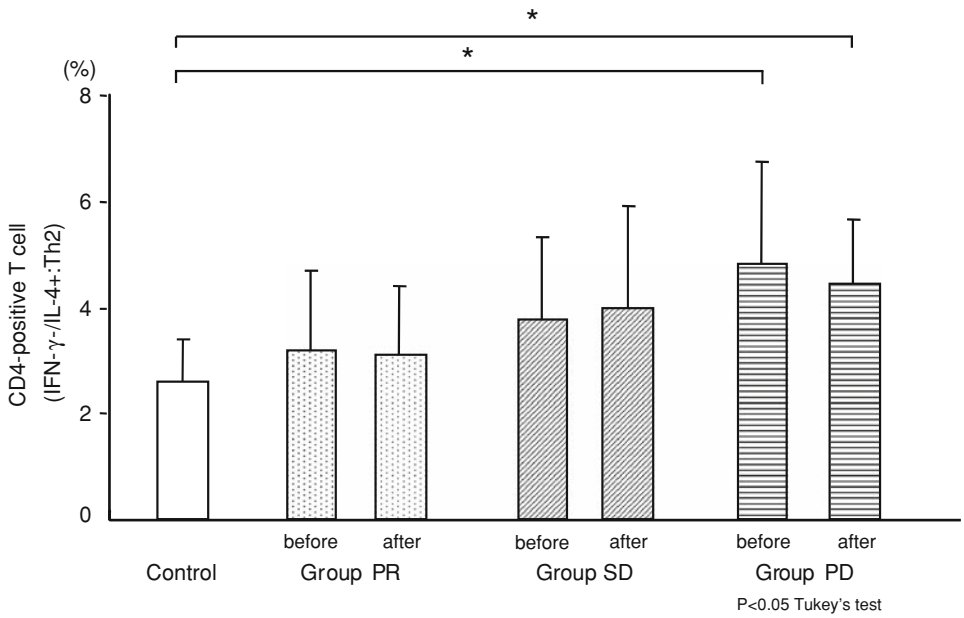

Fig. 4 Comparison of the relative prevalence of CD4-positive T cells (IFN-γ negative and IL-4 positive cells) Th2 cells before and after treatment in group PR, group SD, group PD, and the control group. The percentage of Th2 cells was significantly higher in group PD than in the control group before and after treatment ($P < 0.05$, Tukey's test)

Discussion

It is thought that tumors develop various mechanisms to escape from the immune system and to inhibit antitumor responses. Dendritic cells (DCs) are the most potent antigen-presenting cells with respect to their ability to efficiently prime both CD4-positive and CD8-positive cytotoxic T cells. It has been reported that impaired DC function might be an important factor in tumors escaping from surveillance [29], and that the number of peripheral blood DCs shows significant decrease in cancer patients [30, 31]. It has also been reported that the DCs of cancer patients are mainly immature and cannot stimulate T cells [32–34]. Abnormal differentiation of DCs that results in a decrease of these cells and an immature phenotype is thought to be influenced by tumor-derived factors, such as VEGF [35–37], M-CSF, IL-6 [38–40], and IL-10 [41–43]. Production of immunosuppressive factors, an increase of T_{reg}, and down-regulation of tumor antigens and MHC molecules are all mechanisms by which tumor cells can escape from immune recognition [44, 45]. These escape mechanisms all exist in patients with HCC. Tim et al. have demonstrated that HCC progresses despite the detection of tumor-specific cellular and humoral immune responses in more than 50% of HCC patients [46].

We previously demonstrated reported that the percentage of Th2 cells was significantly higher in group PD before and after chemotherapy compared with the control group, although there were no significant differences of Th2 cells between group PR + SD and the control group either before or after chemotherapy [24]. However, we did not divide group PR + SD into separate PR and SD groups in that study. Therefore, the present study was done to examine host immunity of LC patients with aHCC in relation to efficacy of intra-arterial chemotherapy. The percentage of Th2 cells was significantly higher in group PD before and after chemotherapy than in the control group, although there were no significant differences in the percentage of Th1 cells between the groups either before or after chemotherapy. Moreover, the percentage of Th2 cells was higher in group SD than in group PR and the percentage in group PR was higher than in the control group, although there were no significant differences. Thus, the percentage of Th2 cells increased as the response became worse in LC patients with aHCC treated by intra-arterial combination chemotherapy. We have already reported that Th1 dominance was not lost due to an increase of Th2 cells in HCC patients and that carcinogenesis might occur in patients with chronic HCV infection who show an increase of Th2 cells was a cause or consequence of carcinogenesis [47]. It should also be important that Th1 dominance is not lost due to an increase of Th2 cells in host immunity of patients with aHCC treated by intra-arterial chemotherapy. It has been reported that T_{reg} are increased in the peripheral blood and/or tumors of HCC patients and this increase of T_{reg} suppresses CD4-positive helper T cell responses and appears to promote the progression of HCC [21–23]. In the present study, the percentage of Th2 cells increased as the response becomes worse in LC patients with a HCC receiving intra-arterial combination chemotherapy. These changes might be explained by an increase of T_{reg} with tumor progression, but further study of the changes of T_{reg} in aHCC patients treated by intra-arterial chemotherapy is needed. The present findings suggested that a decrease of DCs, an increase of T_{reg}, or down-regulation of tumor antigens may induce a Th1/Th2 imbalance in LC patients with aHCC receiving

intra-arterial chemotherapy. This imbalance might influence the efficacy of intra-arterial chemotherapy in LC patients with aHCC.

In conclusion, the percentage of Th2 cells increased in LC patients with aHCC as the efficacy of intra-arterial chemotherapy decreased. These results indicated that combined intra-arterial chemotherapy might be not useful for patients with aHCC because it induces Th2 dominant host immunity. It might be important to alter host immunity from Th2 dominance to Th1 dominance when LC patients with aHCC are treated by combined intra-arterial chemotherapy.

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