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ORIGINAL ARTICLE

Changes of cytokines in cirrhosis patients with advanced hepatocellular carcinoma treated by intra-arterial chemotherapy

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Abstract
Introduction Tumor necrosis factor (TNF) induces cancer cell-specific apoptosis by binding to a TNF-related apoptosis-inducing ligand. Binding of the Fas ligand on cytotoxic T lymphocytes to the Fas receptor on hepatocytes is also known to induce apoptosis. The aim of this study was to clarify changes of cytokines in patients with liver cirrhosis (LC) and advanced hepatocellular carcinoma (aHCC) receiving intra-arterial combination chemotherapy.
Methods Twenty-one adult Japanese LC patients with aHCC received intra-arterial combination chemotherapy. The serum levels of TNF-alpha, soluble TNF receptor-I (sTNFr-I), soluble Fas ligand (sFas L), and soluble Fas (sFas) were evaluated.
Results Thirteen of the 21 patients (group R) showed an objective response, while the other eight patients (group N) showed no response. The serum level of TNF-alpha was lower after chemotherapy than before chemotherapy in group N, but there was no difference of serum sTNFr-I levels between before and after chemotherapy and there were also no differences between the two groups. The serum sFas levels were higher after chemotherapy than before chemotherapy in group N, while there was no difference among groups.
Conclusions These results indicate that a high serum TNF-alpha level and a low serum sFas level might be important for successful combined arterial chemotherapy in LC patients with aHCC.

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Introduction

Several modalities, such as surgery, PEI, TAE, microwave coagulation therapy, and radiofrequency ablation, are useful in patients with small hepatocellular carcinoma (HCC). However, there are a considerable number of patients with advanced HCC (aHCC), for which intra-arterial combination chemotherapy is one of the few remaining options. The majority of patients with aHCC do not survive for longer than 6 months from the day of diagnosis [1], 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 [2]. The improvement of implanted drug delivery systems has made it 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 QOL [3]. 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 patients with aHCC [3–5]. We have also shown that intra-arterial treatment with a combination of low-dose 5-FU, CDDP, and leucovorin (LV) prolongs the survival of patients with aHCC [6] and that continuous intra-arterial infusion for 24 h was more effective compared with 6-h infusion in patients with HCV mediated liver cirrhosis (LC) and aHCC, although 24-h infusion was associated with stronger hematologic toxicity [7].

HCC is one of the most common types of carcinoma. Unfortunately, therapeutic options are very limited because of the resistance of this tumor to chemotherapy. Both 5-FU

and CDDP have direct antitumor activity [8] In addition, CDDP acts synergistically as a modulator of 5-FU by inhibiting the uptake of neutral amino acids (including L-methionine) into tumor cells, which results in enhancement of the antitumor activity of 5-FU [9]. Thus, use of a combination of CDDP and 5-FU allows administration of lower doses with a consequent reduction in adverse reactions. Hepatic extraction of chemotherapy agents results in low systemic drug concentrations and thus, also minimizes systemic toxicity [10]. It has been reported that staining for tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is observed in 40% of human HCC tissues, with expression of TRAIL being mainly localized to the cytoplasm and membrane [11]. Upregulation of TRAIL receptor expression after treatment with 5-FU has been implicated in sensitising human leukaemia cells and glioma cells [12, 13]. Ganten et al. [14] reported that tumor cell sensitisation by 5-FU leading to TRAIL-induced apoptosis was a receptor-proximal event and that upregulation of the death-inducing TRAIL receptor after treatment with 5-FU was important for the sensitisation of hepatoma cells. Both TRAIL receptor 1 and TRAIL receptor 2 are expressed by the tumor cells of all 5-FU-treated HCC patients regardless of their clinical response, and expression is mainly localized to the cytoplasm and plasma membrane [15]. The TRAIL receptor belongs to the TNF receptor superfamily, which includes TNF receptor I (TNFr-I) and TNF receptor II (TNFr-II), the first members of this family to be discovered and characterized [16, 17]. Nakamura et al. [18] reported that exposure to 5-FU increased Fas expression by HepG2 cells. Therefore, when LC patients with aHCC undergo chemotherapy, we should consider the influence of both tumor factors and host immunity. However, there have been no previous studies using serum cytokines about the evaluation of host immunity in LC patients with aHCC receiving intra-arterial combination chemotherapy. The aim of the present study was to clarify changes of cytokines in LC patients with aHCC receiving intra-arterial combination chemotherapy.

Methods

Patients

Twenty-one adult Japanese patients who had aHCC arising from LC due to HBV, HCV, or non B-non C infection were treated with intra-arterial low-dose 5-FU, CDDP, and LV at our hospital between 2005 and 2006. Blood samples were collected from the patients in the early morning at the start and finish of chemotherapy and were stored at −80°C until measurement within 1 week.

Chemotherapy regimen

All patients were treated by continuous infusion of LV at 12 mg/h, CDDP at 10 mg/h, and 5-FU at 250 mg/m^2/22 h via the proper hepatic artery every 5 days for 4 weeks using a catheter connected to a subcutaneously implanted drug delivery system.

Drug delivery system

In all patients, an intra-arterial catheter was inserted via the femoral artery and was attached to a subcutaneously implanted reservoir [19]. In principle, the gastroduodenal artery and right gastric artery were occluded with steel coils to prevent gastroduodenal injury by the infused anticancer agents. Written informed consent was obtained from all of the patients.

Evaluation of response

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

Assays

Soluble TNF-alpha (sTNF-alpha) was measured in duplicate using a commercially available EIA (Quantikine, R&D Systems Inc., Mineapolis, USA). The assay sensitivity was 2.6 pg/ml, the intra-assay coefficient of variation was ±8.8%, and the inter-assay coefficient of variation was ±16.7%. Serum sTNF-alpha receptor I (sTNFr-I) levels were quantified by a commercially available ELISA (Quantikine, R&D Systems Inc., Mineapolis, USA). Serum soluble Fas (sFas) was measured by a commercially available quantitative sandwich enzyme immunoassay (Quantikine, R&D Systems Inc., Mineapolis, USA). Serum levels of sFas ligand (sFas L) were quantified by using an immunoassay kit for sFas L (MBL, Tokyo, Japan). Concomitant measurement of seven sFas L standards of known concentrations (0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10 ng/ml) was done with the samples. The lowest detectable serum sFas L level was <50 pg/ml. All procedures were performed according to the manufactures recommendations.

Statistical analysis

Tukey's test was used to compare clinical characteristics among the groups. Results are expressed as the mean ± SD. A probability value of less than 0.05 was considered to indicate statistical significance.

Results

The patients were divided into two groups as follows. Thirteen of the 21 patients (group R) showed an objective response (partial response or stable disease), but the other eight patients (group N) showed no response. There were seven men and one women aged 58–70 years (mean ± SD, 61.3 ± 5 years) in group N, while 12 men and 1 women aged 59–73 years (mean ± SD, 65.6 ± 5 years) formed group R. There were three patients with HBV cirrhosis and five patients with HCV cirrhosis in group N, while there were ten patients with HCV cirrhosis and three patients with non B-non C cirrhosis in group R. The Child-Pugh class was A for five patients from group N and seven patients from group R, while for B it was three and five patients, respectively, it was also C for one patient in group R. There were two patients with stage III disease, four patients with stage IVA disease, and two patients with stage IVB disease in group N, while the respective numbers were 4, 5, and 4 in group R. One patients had a Japan integrated staging (JIS) score [20] of 2; three patients had a score of 3, four patients had a score of 4, and no patient had a score of 5 in group N, while the respective numbers were 3, 5, 4, and 1 in group R. In group N, one patient had tumor thrombi in major branches of the portal vein and one patient had a history of thrombosis of the first branch, while there was one patient with tumor invasion into a branch of the right hepatic vein and one patient with invasion of the main right hepatic vein. In group R, one patient had tumor thrombi in major branches of the portal vein and two patients had tumor thrombi in the main portal vein, while there was one patient with invasion of the first branch of the right hepatic vein, one patient with invasion of the right hepatic vein itself, and two patients with invasion into the main hepatic vein (Table 1).

Serum aminotransferases

Figure 1 summarizes the serum aminotransferase levels in group N and group R before and after chemotherapy. There were no significant differences of serum alanine aminotransferase (ALT) or serum aspartate aminotransferase (AST) between group N and group R, and there were also no significant differences between before and after chemotherapy in either of the groups respectively (Fig. 1).

Serum TNF-alpha

In group N, the serum level of TNF-alpha showed a significant decrease after chemotherapy compared with before chemotherapy ($P < 0.05$, Wilcoxon's test), although there were no significant differences between before and after chemotherapy in group R. There were no significant differences between groups N and R either before or after chemotherapy (Fig. 2). These results indicate that host immunity was decreased in group N receiving intra-arterial combination chemotherapy.

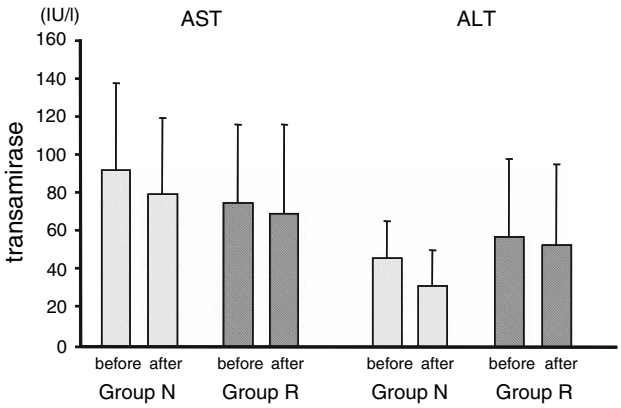

Fig. 1 Comparison of serum aminotransferases between group N and group R. There were no significant differences within either group and also between the groups before or after chemotherapy

Table 1 Clinical characteristics of the 21 patients with liver cirrhosis

	Group N	Group R
No. of patients	8	13
Mean age	61.3 ± 5	65.6 ± 5
Gender (M/F)	7/1	12/1
Type of cirrhosis (HBV/HCV/alcohol)	3/5/0	0/10/3
Child-Pugh classification (A/B/C)	5/3/0	7/5/1
Stage (III/IVA/IVB)	2/4/2 (vv2:1, vv3:1) (vp2:1, vp3:1)	4/5/4 (vv2:1, vv3:1, vv4:2) (vp3:1, vp4:2)
JIS score (2/3/4/5)	1/3/4/0	3/5/4/1

Serum soluble TNF receptor-I

There were no significant differences of serum sTNFr-I levels in groups N and R before and after chemotherapy. There were also no significant differences between groups N and R before or after chemotherapy (Fig. 3). These results indicate that there were no differences in the expression of TNFr-I by hepatocytes related to intra-arterial combination chemotherapy.

Serum soluble Fas and soluble Fas ligand

In group N, the serum sFas level after chemotherapy was significant increased compared with that before chemotherapy ($P < 0.05$, Wilcoxon's test), although there were no significant differences between before and after chemotherapy in group R. There were no significant differences between groups N and R either before or after chemotherapy (Fig. 4). These results might suggest the progression of HCC. Serum levels of sFas L never exceeded 0.15 ng/ml (data not shown).

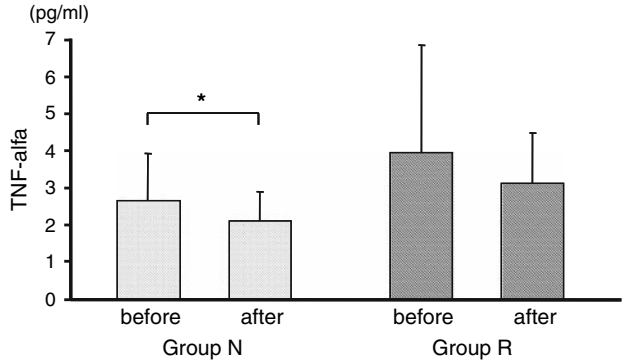

Fig. 2 Comparison of tumor necrosis factor-alpha (TNF-alpha) levels between group N and group R, the serum level of TNF-alpha showed a significant decrease after chemotherapy compared with before chemotherapy ($P < 0.05$, Wilcoxon's test), although there were no significant differences between before and after chemotherapy in group R. There were no significant differences between groups N and R either before or after chemotherapy

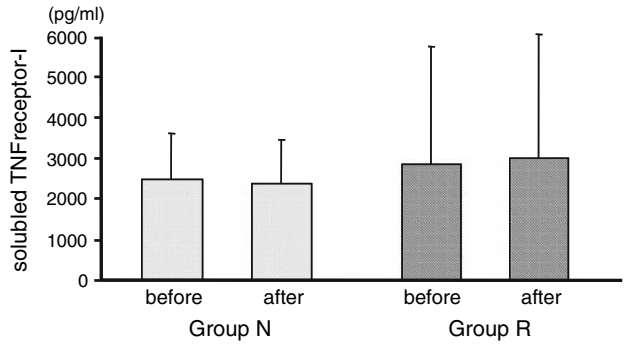

Fig. 3 Comparison of soluble tumor necrosis factor receptor-I (sTNFr-I) levels between group N and group R. There were no significant differences of serum sTNFr-I levels in group N or group R between before and after chemotherapy. There were also no significant differences between groups N and R either before or after chemotherapy

Discussion

TNF-alpha is a mediator of inflammation and cellular immunity that is produced by activated monocytes and Kupffer cells [21, 22]. This cytokine exhibits a wide range of activities, including cytotoxicity against tumors and virus-infected cells, direct antiviral activity, and stimulation of numerous immune effector cells through binding to specific receptors known as TNFr-I and TNFr-II [23, 24]. In the present study, the serum level of TNF-alpha in group N showed a significant decrease after chemotherapy compared with before chemotherapy, although there were no significant differences between before and after chemotherapy in group R. There were no significant differences of serum aminotransferases between groups N and R, and there were also no significant differences between before and after chemotherapy in either group. These results indicated that the decrease TNF-alpha levels in group N after chemotherapy compared with before chemotherapy was not related to changes of the serum AST or ALT. These results might indicate that intra-arterial chemotherapy decreased the cellular immune response mediated by activated monocyte and Kupffer cells in patients with aHCC from group N.

TNFr-I and TNFr-II are surface receptors expressed by different cells, while their soluble forms are released into the blood after cleavage of extra-cytoplasmic domains or alternative splicing. A similar inhibitory effect has been described for soluble Fas [25]. An increase in the circulating levels of these soluble receptors has been reported in HCV infection [26, 27]. Various cytokines, including TNF,

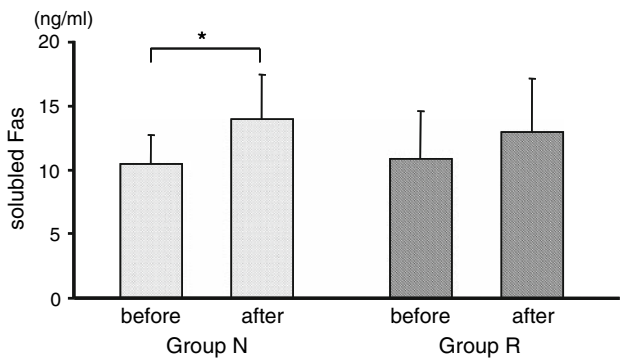

Fig. 4 Comparison of soluble Fas (sFas) levels between group N and group R. Serum sFas levels showed a significant increase in group N after chemotherapy ($P < 0.05$, Wilcoxon's test), although there was no significant change in group R. There were no significant differences between groups N and R either before or after chemotherapy

can induce the shedding of sTNFr, which therefore represents a circulating marker of the inflammatory response that is more stable than TNF-alpha itself. Soluble forms of both TNF receptors can be detected in the serum of healthy subjects and increased circulating levels of sTNFr-I and sTNFr-II have been reported in patients with infections or inflammatory disease including cancer [28]. The TNF/TNF-receptor system has been reported to play a role in hepatitis B infection [29], in which the mechanism of liver injury is related to the immune response directed against hepatitis B antigen expressed by infected hepatocytes. However, circulating sTNFr-II levels were reported to be lower in HCV-infected patients than in HBV-infected patients, although sTNFr-I and TNF-alpha levels were not significantly different [27]. In the present study, our LC patients included those with HCV or HBV as the underlying disease, so we quantified the serum sTNFr-I and observed changes in each patient subgroup before and after chemotherapy. Moreover, there were no significant differences of serum sTNFr-I between before and after chemotherapy in groups N and R. These results indicated that changes of sTNFr-I did not influence the response to chemotherapy in patients with aHCC. In the present study, the serum level of TNF-alpha showed a significant decrease after chemotherapy compared with before chemotherapy in group N, although there was no significant change of serum sTNFr-I after chemotherapy. These results indicate that maintaining a high serum level of TNF-alpha by activating host immunity might be important to obtain a response to intra-arterial combination chemotherapy in LC patients with aHCC.

The serum sFas level of group N showed a significant increase after chemotherapy compared with before chemotherapy, although there were no significant differences between before and after chemotherapy in group R. The average sFas level of patients with chronic hepatitis C was significantly higher compared with that of HIV-infected patients and was also higher than that in healthy controls. Increased sFas levels have been observed in HCV-positive patients with LC [30]. Significantly higher sFas levels have also been found in patients with HCC compared with cirrhosis patients and healthy controls [31]. Furthermore, it was reported that surgical removal of HCC reduced sFas to the undetectable range, suggesting that detection of sFas may be a potential clinical parameter for HCC [32]. In the present study, the increase of serum sFas in group N after chemotherapy compared with before chemotherapy might have indicated progression of HCC. It was reported that immunohistochemical expression of Fas ligand is increased in HCC cells [33]. However, serum sFas L levels were always under 0.15 ng/ml in this study and the sFas L level does not seem to be an index for assessment of LC patients with aHCC receiving arterial chemotherapy. In the present study, the serum level of sFas showed a significant increase after chemotherapy in group N. This finding suggested that sFas might be a useful index for assessment of LC patients with aHCC receiving intra-arterial chemotherapy.

In conclusion, serum cytokines levels might be useful for assessment of LC patients with aHCC during intra-arterial chemotherapy, while maintenance of a high serum TNF-alpha level and a low serum sFas level might be important for successful intra-arterial combination chemotherapy in LC patients with aHCC.

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