RESEARCH ARTICLE

Expression of Fas/FasL in CD8+ T and CD3+ Foxp3+ Treg Cells - Relationship with Apoptosis of Circulating CD8+ T Cells in Hepatocellular Carcinoma Patients

Cun-Li Guo[1], Xiu-Hua Yang[2]*, Wen Cheng[1]*, Yi Xu[1], Jie-Bing Li[1], Yi-Xin Sun[1], Yu-Mei Bi[1], Lei Zhang[1], Qiu-Cheng Wang[1]

Abstract

Aims: Dysfunction of the host immune system in cancer patients can be due to a number of factors, including lymphocyte apoptosis. Several studies showed that Foxp3+T cells take part in inducing this process by expressing FasL in tumor patients. However, the relationship between apoptosis, CD8+T cells and Foxp3+T cells in HCC patients is still unclear. The present study was designed to investigate the correlation between apoptosis levels and Fas/FasL expression in CD8+T lymphocytes and Foxp3+T cells in patients with HCC. Methods: CD8+T cells and CD3+Foxp3+T cells were tested from peripheral blood of HCC patients and normal controls and subjected to multicolor flow cytometry. The expression of an apoptosis marker (annexin V) and the death receptor Fas in CD8+T cells and FasL in CD3+Foxp3+T cells were evaluated. Serum TGF-β1 levels in patients with HCC were measured by enzyme-linked immunosorbent assay. The relationship between apoptosis and Fas expression, as well as FasL expression in CD3+Foxp3+T cells was then evaluated. Results: The frequency of CD8+T cells binding annexin V and Fas expression in CD8+T cells, were all higher in HCC patients than normal controls and the proportion of apoptotic CD8+T cells correlated with their Fas expression. Serum TGF-β1 levels correlated inversely with CD3+Foxp3+T cells. Conclusions: Fas/FasL interactions might lead to excessive turnover of CD8+T cells and reduce anti-tumor immune responses in patients with HCC. Further investigations of apoptosis induction in Fas+CD8+T cells *in vitro* are required.

Keywords: Hepatocellular carcinoma - CD8+T lymphocytes-CD3+Foxp3+T cells - apoptosis -Fas - Fas ligand

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Introduction

Tumor escape from immunological surveillance involve a variety of mechanisms from the down-regulation or loss of surface molecules necessary for tumor recognition to deregulation of immune cell functions and/or the induction of apoptosis in effective T cells (Ferrone et al., 2007; Kerkar et al., 2012). It is likely that these escapes significantly contribute to tumor progression and negatively influence the outcome of patients with cancer. At present, there are numbers researches in studying dysfunction of immune cells in cancer patients (Yoshikawa et al., 2008). One of the mechanisms responsible for dysfunction of immune cells in cancer patients is apoptosis of immune cells (Hoffmann et al., 2002; Reichert et al., 2002; Bauernhofer et al., 2003). It has been reported thus far that tumor cells express molecules such as Fas ligand (FasL) that can induce the apoptosis of T cells (Abusamra et al., 2005). While the Fas/FasL system plays an important role in B and T lymphocyte development and maturation (Rauf et al., 2012), it may also serve as a mechanism for immune evasion by a variety of neoplasms including ovarian carcinoma, colon cancer, melanoma and glioma (Kassouf et al., 2008; Lin et al., 2013). The field of tumor immunology is strongly focused on CD8+T cells in recent years, owing to their ability to directly kill tumor cells and there are strong association between tumor-infiltrating CD8+T cells and patient survival in many cancers (Pagès et al., 2010). However, few reports have reported the apoptosis and Fas expression of CD8+T cells in HCC patients because of the limited availability of appropriate human samples. In the current study, we quantified CD8+T cell ratios by flow cytometry in HCC patients and normal controls. We also assessed the expression of Fas in CD8+T cells and FasL in Foxp+ Treg cells from the same patient and asked what correlation between the apoptosis of CD8+T cell and the expression of Fas and FasL.

*[1]The Third Affiliated Hospital, [2]The First Affiliated Hospital, Harbin Medical University, Harbin, China *For correspondence: docguocunli@163.com*

Materials and Methods

Patients and normal donors

Blood samples were collected from 42 HCC patients and 22 healthy volunteers. HCC was diagnosed based on pathology. The clinical stage of tumor progression was determined according to the International Union against Cancer TNM classification system. We defined I stage as the early stage and II, III, IV stage as the advanced stage. The patients and normal controls (NCs) were similar in age, with a mean age of 52.4±7.8 years for the HCC group and 52.2±11.8 years for the control group. The age, sex and clinical characteristics of the patients are summarized in Table 1. None of the patients had received anticancer therapy or immune therapy before blood sampling, and none had any other autoimmune disease, alcoholic liver disease, human immunodeficiency virus, syphilis infection, or other serious disease. All patients provided written informed consent and study was approved by the ethics committee at our institution.

Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

Venous blood (2 ml) for every exam was obtained from patients and NCs. Peripheral blood mononuclear cells (PBMCs) were isolated from freshly obtained heparinized blood by Ficoll-Paque (TBD science, Tianjin, CHINA) density gradient centrifugation. Blood samples were centrifuged at 2500 rpm/min for 15 min and the cell pellet was separated. The lymphocyte layer was collected, washed and used immediately for further experiments.

Flow cytometry analysis

PBMCs were incubated with the follow conjugated antibodies: ECD-conjugated anti-CD3 and its isotype IgG (Beckman, Los Angeles, CA, USA), PerCP-CY5.5-conjugated anti-CD8, APC-conjugated anti-CD95 and PE-conjugated anti-CD95L and their isotype IgG (eBioscience, San Diego, CA, USA). After the incubation period, the cells were washed twice with PBS and immediately examined in a flow cytometer (BD, CA). Staining for Foxp3 protein was performed using the Foxp3 kit (eBioscience, San Diego, CA, USA).

Apoptosis in peripheral CD8$^+$T Cells

The percentage of apoptotic cells was calculated by scoring Annexin V-binding cells after backgating of CD3$^+$CD8$^+$cells. Control cells were obtained from NC donors. All gated mononuclear cell subpopulations were visualized on forward angle scatter/side angle scatter dot plots. To include all apoptotic cells and avoid debris with a high SCC signal, the gate was set to include a wide boundary of mononuclear cells because apoptotic cells accumulate mainly in the lower forward angle scatter/side angle scatter channels.

Table 1. Clinical Characteristics of Enrolled HCC Patients in this Study

Characteristic		Results
Age(mean±SD, Y)		52.4±7.8
Sex	Mail	34
	Female	8
Hepatitis status	Hepatitis B	24
	Hepatitis C	4
Tumors Number	Single/multiple	28/14
Serum AFP level	+/-	29/13
CA199	+/-	18/24
TNM stage *	I (early stage)	14
	II+III+ IV (advanced stage)	28

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

Enzyme-linked Immunosorbent Assay (ELISA)

TGF-β1 in human sera was measured by enzyme-linked immunosorbent assay in 16 of the patients with HCC and 9 of normal controls according the instructions of manufacturer (Boster, WuHan, CHINA). Some patient do not want to donate their blood second time. Acid activation of the sample was performed before measuring the TGF-β1 level, because the majority of the TGF-β1 is produced in latent form in the body.

Statistical analysis

All data are expressed as means±standard deviation. Statistical significance of differences between two groups was determined using Student's t test or the Mann-Whitney U test if there was evidence of heteroscedasticity. Correlations between parameters were determined by linear regression analysis. Values of $p<0.05$ were considered statistically significant. All data were analyzed using SPSS software version 17.0 for Windows (SPSS Inc., Chicago, IL).

Results

Frequencies of CD8$^+$T cells in peripheral blood of HCC patients

The proportion of CD8$^+$T cells of the total T cell population in peripheral blood was higher in patients with HCC (21.4±1.1%) than in normal controls (17.2±1.3%, $p<0.05$) (Figure 1).

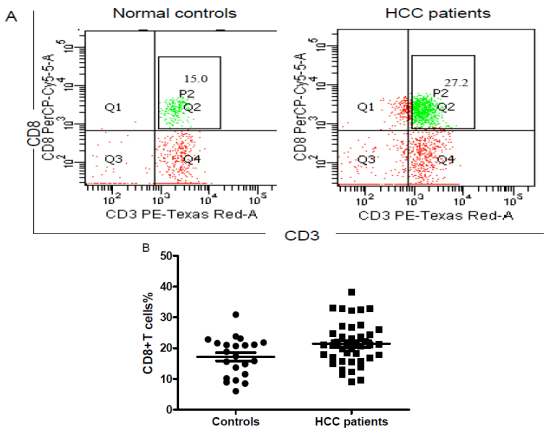

Figure 1. The Proportion of CD8$^+$T Cells of the Total T Cell Population in Peripheral Blood was Higher in Patients with HCC than in Normal Controls. A) a representative result showing the frequency of CD8$^+$T cells in normal controls and HCC patients by FACS. B) statistical chart of the percentage of CD8$^+$ cells in patients with HCC and normal controls

Apoptosis of CD8+T cells in PBS of HCC patients

We then determined the frequency of apoptotic circulating CD8+T lymphocytes in both normal controls and HCC patients. There was significantly higher proportion of AnnexinV+CD8+T cells in the circulation of HCC patients (25.3±6.5%) than in that of normal controls (12.1±6.5%) (p<0.01) (Figure 2). These data indicate that CD8+T cells preferentially bind AnnexinV and are targeted for apoptosis and the AnnexinV expression was significantly correlated with disease progression (Figure 2C). Figure 2D shows expression of AnnexinV on CD8+ subsets of circulating T lymphocytes of normal controls in relation to age. Clearly, no age-related changes were observed in AnnexinV+CD8+T cells between the person who under 60 years old and over 60 years old (p>0.05).

Fas expression of CD8+T cells in PBS of HCC patients

To determine the mechanisms responsible for the increased apoptosis in circulating CD8+T cells from HCC patients, we then compared Fas expression in cells obtained from HCC patients with those from normal controls. The mean number of CD8+Fas+T cells was 62.2±18.5% in HCC patients compared with 42.6±16.5% in NCs and differences were statistically significant (p<0.05). Moreover, Fas expression was significantly correlated with disease progression.

Correlation between increased apoptosis of circulating CD8+T cells and up-regulation of fas expression in CD8+T cell

From our results, we can see that the Fas expression was closely related to the frequency of apoptosis of CD8+T cells (r^2=0.113, p<0.05).

Frequencies of CD3+Foxp3+T Cells in peripheral blood of HCC patients

In recent researches, CD8+Foxp3+ regulatory T cells have recently been detected in tumors; however CD8+Foxp3+ regulatory T cells share phenotype, functional features, and mechanism of action with CD4+ Tregs. So we take the CD3+Foxp3+T cells as the total regulatory T cells in PBMCs of HCC patients. 30 of the HCC patients and 11 of normal controls were examined CD3+Foxp3+T cells. In our results, the frequency of CD3+Foxp3+T Cells in peripheral blood of HCC patients was higher in patients with HCC (9.2±6.8%) than in normal controls (4.7±2.8%, p<0.05, Figure 3: A, B).

FasL expression of CD3+Foxp3+T Cells in PBMCs of HCC patients

Recent studies showed that Treg cells may kill CD8+ and CD4+CD25- lymphocytes through the Fas/FasL or granzyme/perforin way in vitro. Our study has detected the FasL expression of CD3+Foxp3+T cells in peripheral blood of HCC patients and normal controls. We want to evaluate the possibility that the Foxp3+Treg cells take part in the apoptosis of CD8+T cells in HCC patients. Our results showed that 28 of 30 patients had FasL expression and the average percentage is 6.5±0.7%. On the other hand, 10 of 11 normal controls were detected FasL expression and the average percentage is 2.9±0.3%. The difference was statistically significant. (p<0.05, Figure3: C, D).

The relationship between CD3+Foxp3+T Cells and serum TGF-β1

Many types of tumor cells produce high levels of TGF-β, and exogenously added TGF-β can convert CD4+CD25-T cells into Treg cells (Liu et al. 2007).

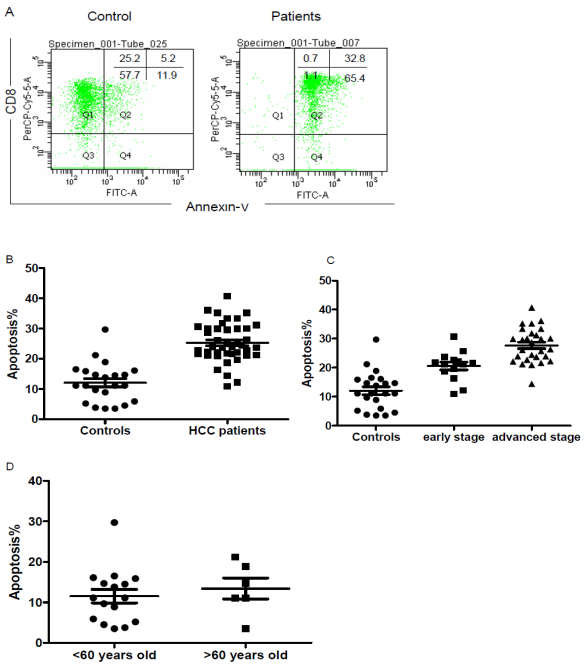

Figure 2. A: A Representative Result Showing the Frequency of Apoptotic CD8+T Cells in Normal Controls and HCC Patients by FACS. B: The frequency of AnnexinV +CD8+T cells in HCC patients was higher than in normal controls. C: AnnexinV expression was significantly higher in advanced stage patients than early stage patients and normal controls. D: no age-related changes were observed in AnnexinV +CD8+ T cells between more or less than 60 years old patients

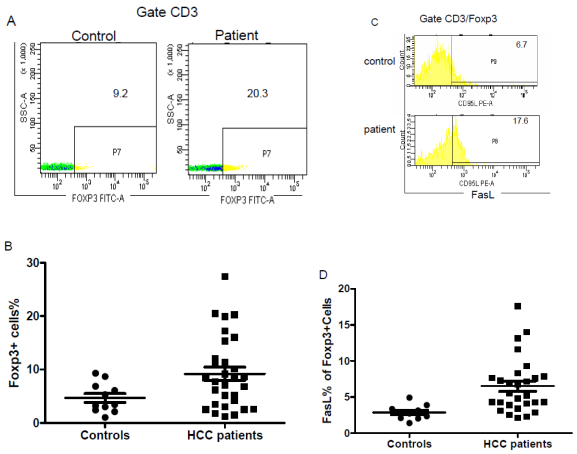

Figure 3. A :A Representative Result Showing the Frequency of Foxp3+T Cells in Normal Controls and HCC Patients by FACS. B: statistical chart of the percentage of Foxp3+T cells in normal controls and HCC patients. (p < 0.05). C: A representative result showing the frequency of FasL+Foxp3+T cells in normal controls and HCC patients by FACS. D: The frequency of FasL+Foxp3+T cells in HCC patients was higher than in normal controls. (p < 0.05)

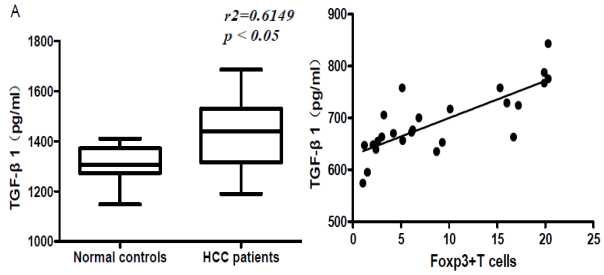

Figure 4. A The Concentration of TGF-β1 in Patients with HCC was Significantly Higher than that in Normal Controls (P <0.05). B: TGF-β1 concentration was significantly related to the frequency of Foxp3+ T lymphocytes (r2=0.6149, p < 0.05)

Figure 6A shows that the concentration of TGF-β1 in patients with HCC (1.43±0.13 ng/mL) was significantly higher than that in normal controls (1.31±0.08 ng/mL) (p<0.05; Figure 4A). Moreover, TGF-β1 concentration was significantly inversely related to the frequency of Foxp3$^+$T cells in PBMCs of HCC patients' and NCs (r^2=0.6149, p<0.05; Figure 4B).

Discussion

Malignant tumor of the generation, development, and the outcome are closely related with the body's immune function. The immune system of the body effectively identify tumor antigen, activate effective lymphocytes, so as to kill the cancer cells. Effective CD8$^+$T cell mediated cytotoxic killing may play a crucial role in the control of cancer development. Adoptive transfer of exvivo stimulated CD8$^+$T cells, for example, can mediate antitumor immunity effectively (Liu et al., 2009). Although it has been reported that the number of lymphocytes are selectively decreased in many cancer patients (Wang et al., 2013), but the exactly mechanism has no coincidence. From our data, we saw the increase in the number of CD8$^+$T cells of the peripheral blood of patients with hepatocellular carcinoma which means that the anti-tumor immune response of the body occurred. However, the disease is still progression indicated that there are functional barriers that causing CD8$^+$T cells which can not effectively kill tumor cells. Previous reports had concluded that the apoptosis of the CD8$^+$T cells increased in Gastric Cancer and head and neck cancer patients (Hoffmann et al., 2002; Reichert et al., 2002). So we suggested that among PBMCs of HCC patients, CD8$^+$ T lymphocytes were especially sensitive to apoptosis and these apoptosis is related the increased expression of Fas. To confirm this hypothesis, freshly harvested lymphocytes of patients with HCC or age- and sex-matched NCs were stained for Fas expression and stained simultaneously for markers of early apoptosis, AnnexinV binding. As a result, we found apoptosis of CD8$^+$T cells was observed more frequently in peripheral blood mononuclear cells obtained from patients with HCC than those from normal donors. And on the other hand, the Fas expression of CD8$^+$T cells was also observed more frequently from patients with HCC than those from normal donors. Fas, a member of the tumor necrosis factor (TNF) receptor family, is a Type 1 membranal protein expressed by a wide variety of cell types. Upon cross-linking by either FasL or an agonistic anti-Fas monoclonal antibody, the Fas-associated death domain and caspase-8 form a death-inducing signaling complex, after which the caspase cascade can be activated, resulting in apoptosis and cell death. Moreover, Fas expression was closely related to the frequency of apoptosis of CD8$^+$T cells, indicating that the function of Fas expressed on CD8$^+$T cells and signal through Fas were maintained and increased apoptosis in HCC patients might be due to elevated Fas expression in CD8$^+$T cells. These results are consistent with the hypothesis that Fas$^+$ activated T cells, which are enriched in the peripheral circulation of patients with HCC, are primed to die, leading to a rapid turnover of T lymphocytes and possibly contributing to tumor-related immuno-suppression. Yoshikawa et al. (Yoshikawa et al., 2008) had the same conclusion in the research of Gastric Cancer patients. Fang et al. (2013) also pointed that Fas and FasL-mediated apoptosis may serve as a proofreading mechanism in human neoplasia and FasL may be involved in the aggressive clinical behavior and invasive potential of the tumors, and thereby play an important role during oral tumor progression.

Recently, the existence of CD4$^+$CD25$^+$T regulatory cells (Tregs) had been described in rodents and humans which suppress the functions of NK and CD8$^+$ lymphocytes (Gogali et al., 2012; Jang 2013). The activity of these cells is known to be closely associated with the expression of forkhead/winged helix transcription factor, Foxp3.

Generally, Foxp3 has been shown to be expressed exclusively in Tregs and plays a very important role in the development and function of these cells. When Foxp3 gene is introduced via retrovirus or enforced transgene expression, naive CD4$^+$CD25$^-$T cells transform to Tregs (Hori et al., 2003). Wolf et al. (2005) demonstrated that high Foxp3 is an independent prognostic factor for overall-survival and progression-free survival in ovarian cancer. Hence, Foxp3 fulfills the criteria of a Treg-specific marker, which at least in differentiated Tregs, is not known to be substantially regulated and represents a suitable surrogate marker for the indirect quantitation of Treg induction in tissues. In other researches, CD8$^+$ Foxp3$^+$ regulatory T cells have recently been detected in tumors; however CD8$^+$ Foxp3$^+$ regulatory T cells share phenotype, functional features, and mechanism of action with CD4$^+$ Tregs (Yang et al., 2010; Horwitz et al., 2013). So we take the CD3$^+$ Foxp3$^+$T cells as the total regulatory T cells in PBMCs of HCC patients. In addition, in our previous experimental studies (Guo et al., 2012) showed that the prevalence of CD4$^+$CD25$^+$ Tregs is associated with fewer CD8$^+$T lymphocyte in the HCC tumor microenvironment. So in the present study, we analyzed the expression of FasL in CD3$^+$Foxp3$^+$Treg cells and to discuss the relationship between CD3$^+$Foxp3$^+$Treg cells and the apoptosis of the CD8$^+$T cell in HCC patients for the first time. As can be seen in the results, our study has detected the FasL expression of CD3$^+$Foxp3$^+$T cells in peripheral blood of HCC patients higher than normal controls.

The difference further confirms that malignant tumors are enriched with CD3$^+$ Foxp3$^+$ Treg cells and might

contribute to the apoptosis of CD8+T lymphocytes. Strauss et al. (2009) reported that Treg cells from all subjects were CD95+, but only Treg cells from cancer patients expressed CD95L (FasL). They and others also pointed out that the "killing" potential of Treg cells is also higher in cancer patients than in NCs (Janssens et al., 2003; Grossman et al., 2004). From all above, we can concluded that in the occurrence of HCC development, apoptosis of CD8+ T lymphocytes may partly be due to the increases of Foxp3+ regulatory T cells, which can weak their immune monitoring and immune killer function, indirectly lead to cancer occur and develop. However the function mechanism of regulatory T lymphocytes and CD8+T cells need to further vitro experiments.

Transforming growth factor beta (TGF-β) is a pleiotropic factor with central functions in the regulation of cell proliferation and differentiation as well as in the maintenance of immune homeostasis (Schramm et al., 2004). In addition, TGF-β seems to be involved in the regulation of Foxp3 in vivo (Khattri et al., 2003; Fontenot et al., 2003). Very recently, TGF-β was described to convert naive mouse CD4+CD25- T cells to suppressive CD4+CD25+ T cells through the induction of Foxp3 (Chen et al., 2003; Nakamura et al., 2014) and the expression of TGF-β in pancreatic islets lead to an increase in CD4+CD25+ T cell numbers with regulatory property. Hu et al. (2013) had concluded, gastric cancer cells may induce Treg cell differentiation through TGF-β, and further promote immunosuppression. In this study, we demonstrated that HCC can up-regulate TGF-β1 expression. Furthermore, inverse correlations between serum TGF-β1 levels and the percentage of CD3+Foxp3+Treg cells were obtained. The results lead to the suggestion that there is high frequency of CD3+Foxp3+Treg cells in PBMCs of HCC patients, which correlated with the level of TGF-β1 in serum, associate with the increased FasL expression and the apoptosis of CD8+T lymphocytes.

In conclusion, a significant proportion of circulating CD8+T cells in patients with HCC is eliminated by apoptosis, thus weakening the anti-tumor defense in these patients. Our results provide new evidence that Fas over expression in CD8+T cells and FasL expression in CD3+Foxp3+Treg cells are related to increased apoptosis of circulating CD8+T cells. On the other hand, detailed mechanisms as to how apoptosis is induced in Fas+CD8+T cells by Foxp3+Treg cells remains unclear in the current study. Further investigations into this matter are urgently required.

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