RESEARCH ARTICLE

Increased Frequency of Foxp3+ Regulatory T Cells in Mice with Hepatocellular Carcinoma

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Abstract

The CD4+CD25+ regulatory T cell (Treg) is a special kind of T cell subset. Studies have showed that Treg cells are involved in a number of physiological processes and pathologic conditions such as autoimmune diseases, transplantation tolerance and cancer. Tregs with unique capacity for immune inhibition can impair anti-tumour immunity and help tumor cells to escape from immune surveillance. The aim of our study was to investigate whether Tregs are involved in hepatocellular carcinoma (HCC). A BABL/C mouse with HCC in situ model was established to evaluate the Treg existence in carcinoma tissues and the changes of Tregs in spleen using flow cytometry and immunohistochemistry methods. Granzyme B expression in carcinoma tissues was analyzed by immunohistochemistry to investigate the tumor local immune status.The proportion of CD4+CD25+/CD4+ spleen lymphocytes of tumor bearing mice (18.8%±1.26%) was found to be significantly higher than that in normal mice (9.99%±1.90%) (P<0.01). Immunohistochemistry of spleen tissue also confirmed that there was an increase in Treg in tumor-bearing mice, while in carcinomas it showed Treg cells to be present in tumor infiltrating lymphocyte areas while Granzyme B was rarely observed. Anti-tumour immunity was suppressed, and this might be associated with the increase of Tregs. Our observations suggest that the CD4+CD25+Treg/ CD4+ proportion in spleen lymphocytes can be a sensitive index to evaluate the change of Tregs in hepatocellular carcinoma mice and the Treg may be a promising therapeutic target for cancer.

Keywords: Hepatocellular carcinoma - regulatory T cell - CD4+CD25+ T cell - Foxp3 - Granzyme B

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Introduction

Hepatocellular carcinoma (HCC)is one of the leading causes of cancer related death in the world and about 60-70 ten thousands have died in this disease (Kudo et al., 2010). Its early manifestations is not typical and often easy to ignore . When there is a clear in clinical symptoms , it often goes to middle-late progress and looses opportunity to surgery , so that the patients have limited treatment options and even with the current treatments of transarterial chemoembolization and sorafenib,the prognosis is not particularly ideal (Llovet et al., 2008). The pathogenesis is still not clear but the recognized risk factors include hepatitis B/C infection, aflatoxins and excess alcohol consumption (Luke, et al., 2010; Gao, et al., 2012). Modern oncology think , the occurrence and development of cancer is closely related to the body's immune state and cellular immune is the main force of antineoplastic immune (Kalos et al., 2003) .

Sakaguchi et al. for the first time described that Tregs had potent immunoregulatory functions and could control self- tolerance (Sakaguchi et al., 1995). It has been shown that Tregs play a critical role in anti-tumour immune responses. Tregs with their unique immune inhibition can impair the anti-tumour immune and help tumor cells to escape from immune surveillance. Many researchs have found an increased number of Treg in various cancers, such as gastric carcinoma, pancreatic cancer, prostate cancer and breast carcinoma, etc (Miller et al., 2006; Nummer et al., 2007; Gupta et al., 2007; Ghebeh et al., 2008; Mizukami et al., 2008) and deletion of CD25+ cells may cause tumor regression (Onizuka et al., 1999; Shimizu et al., 1999). The expansion of Treg correlates with a poor prognosis. Although studies have also reported an increase in Treg population in both the peripheral blood and tumor microenvironment in HCC patients (Thakur et al., 2011), there are almost no researches that describe the change of Treg in spleens and the tumor local immune status.

The principle objective of our study was to evaluate the existence of Treg in tumor microenvironment and the changes of Treg in spleens in HCC mice using flowcytometry and immunohistochemistry. To investigate the tumor local immune status, immunohistochemical staining of Granzyme B was performed.

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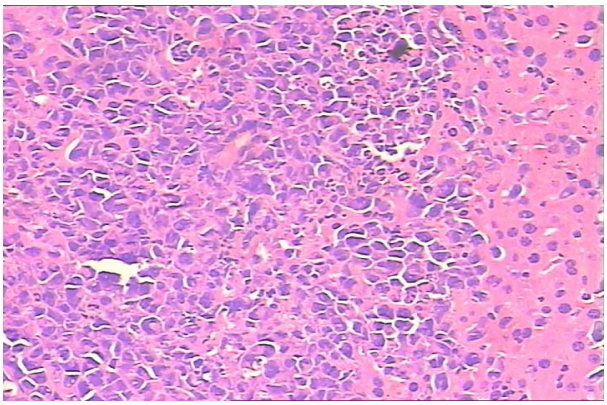

Figure 1. Histology of Mice with Hepatocellular Carcinoma (magnification×200). Cells were pleomorphism obviously, arranging disorderly, and the liver normal structure was lost. Necrosis was obvious

Materials and Methods

Reagents and Materials

Fluorescentlylabeled antibody: CD4-FITC and its homotype antibody(the United States BD company), CD25-PE and its homotype antibody (eBioscience). Immunohistochemical antibody: anti- mouse Foxp3 and granzyme B antibodies (Abcam, Cambrideg, MA, USA); Flow cytometry (the United States BD company). SPF BALB/C mice (male, 6-8 weeks old, mean weight,18±2 g ,purchased from the SLACCAS company, China), mouse H22 hepatocellular carcinoma cell lines (China Center for Type Culture Collection, wuhan, CN).

Mice and tumor model

Young male BALB/C mice were bred under specific pathogen-free (SPF) conditions in 60 square inch plastic cages. Rooms were maintained at 23.3±2.2 °C with a 12 h light/dark cycle. Animal protocols were approved by the Experimental Animal Management Committee Of Wenzhou Medical College. All surgery was performed under chloralic hydras anesthesia, and all efforts were made to minimize suffering.

Tumor model: The H22 cells were cultured in RPMI 1640 medium (Gibco Invitorogen Corporation) suplemented with 10% fetal calf serum (Gibco Invitorogen Corporation), 100 U/ml of penicillin G and 100 μg/ml of streptomycin. The medium was renewed every 2 days. After growing to confluency, the cells were collected and inoculated to abdomen with 1 ×10^6 cells per mouse. After 8-9 days, cancerous ascites were extracted aseptically and washed with culture mediumn for three times. Cell counts were performed with a hemocytometer using trypan blue exclusion and the suspension was resuspended to a concentration of 1 ×10^6 /ml for making model. The mice were randomly divided into the experimental group and control group. The mouse was celiac anesthesia with 4% hydration chlorine aldehyde (0.1ml per 10g weight). It was fixed to the operating table in supine position and disinfected with alcohol. Then we did a longitudinal incision about 0.8cm in the ventral line below the xiphoid , put a piece of wet gauze below the incision, pressed bilateral rib bow gently to expose the liver, injected 0.01 ml cancer cells suspension (10^4 cells)to the liver with 1 ml syringe, stopped bleeding and last closed enterocoelia. After operation, the mice were free to eat and drink.

Histopathological examination

About 25 days later, the models were formed. We could observe that the mental state of the mice became poor, the action became sluggish and a lot of ascites developed. When anatomy, we could see gray and nubbly carcinoma tissues at different sizes from 0.5 to 1.0 cm in diameters in livers. 4-μm thick sections were prepared and stained with haematoxylin and eosin by standard histological procedures. Slices were evaluated using light microscopes (Nikon, Japan). Through the histopathological examination,we could confirm the model construction successful (Figure 1).

Flow Cytometry

The spleen was grinded with a plunger of a disposable syringe, and then passed through a nylon mesh. Collected the cell suspension and washed it once. Single- cell suspension was acquired and cells were surface stained with CD4 FITC and CD25 PE at 4 °C for 30 minutes. Background fluorescence was assessed by the appropriate isotype and fluorochrome-matched control antibodies. Then, erythrocytes were lysed by red blood cell lysis buffer. After washed with PBS, the samples were fixed with fixation solution. Cells were detected using a FACScalibur flow cytometer. Ten thousand-gated events were acquired and the data was analyzed using CellQuest software.

Immunohistochemistry

Tissue samples collected from different locations (spleen, hepatocellular carcinoma tissue) were taken as soon as possible after mice executed and immediately fixed with 4% polyphosphate formaldehyde.The specimens embedded in paraffin following routine methods. Then the embedded tissues were cut into 4-um thick sections for immunohistochemical staining of Foxp3 and granzyme B. The sections were initially deparaffinized in xylene and rehydrated through ethanol to water. For antigen retrieval, sections were heated in citrate buffer for 30 minutes at 100 °C and then treated with 3% hydrogen peroxide for 15 minutes to abolish endogenous peroxidase activity. The sections were respectively covered with rabbit anti- mouse Foxp3 and anti- mouse granzyme B monoclonal antibody for 60 minutes at 37°C. After washing with PBS, the sections were incubated with the secondary antibody for 30 minutes at 37°C. Subsequently the sections were washed with PBS for three times,every time for 5 minutes. Colouration with 3,3-diaminobenzidin, kept at room temperature without light for 2 minutes. Finally the sections were washed with distilled water and counterstained for nuclei with hematoxylin and dehydrated and mounted with neutral gums. The negative control group was carried out with the same steps as described abovely, but the anti- Foxp3 and anti- granzyme B monoclonal antibody were replaced by PBS. The evaluation standard was chosen with respect to previous studies (Zhou et al., 2009; Berbic et al., 2010; Junginger et al., 2012). The Foxp3+ or granzyme B+

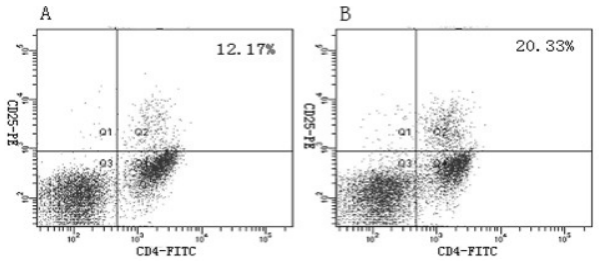

Figure 2. The Proportions of CD4+CD25+Treg/ CD4+ in Spleen Lymphocytes from Normal BALB/C Mice (A) and from tumor bearing mice (B). Spleen single-cell suspensions were prepared, and stained with FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD25 antibodies and analyzed by flow cytometry

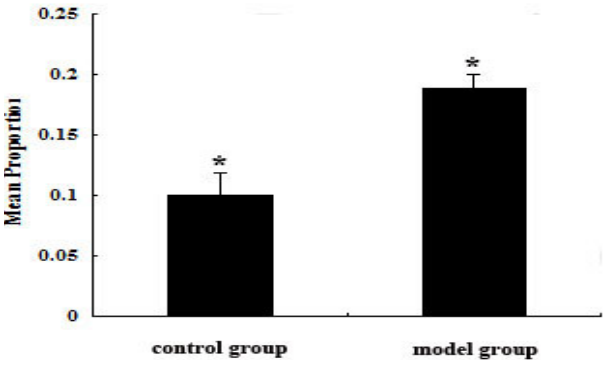

Figure 3. Mean CD4+CD25+ Frequency in CD4+ Population from Normal Mice and Hepatocellular Carcinoma Bearing Mice. The proportion of CD4+CD25+Treg /CD4+ T cells from hepatocellular carcinoma mice (18.8% ±1.26%) was significantly higher than the control group(9.99%±1.90%) (*P<0.01)

lymphocyte infiltration was evaluated by two researchers, each counting the cells in 10 representative fields at 400× magnification (0.146 mm^2/field) per sample using a light microscope. Tregs were characterized by the brown nuclear staining of Foxp3 antibody in specimens. The positive standard for Granzyme B was the brown cytoplasmic staining. Each average counting from each observer was calculated and a mean was calculated.

Statistical Analysis

Data are expressed as box plots and mean±sd.Each group was assessed for normal distribution using a histogram, Q-Q plot, and Shapiro Wilk test. Statistical analysis was performed using the Mann-Whitney U test with SPSS18.0 program (SPSS, Chicago) . All P values <0.05 were considered statistically significant.

Results

Increase in the number of spleen Treg from hepatocellular carcinoma mice

To evaluate the normal proportion of CD4+CD25+Treg/ CD4+ in spleens of mice, the control mice (n=10) were sacrificed to test the proportion.by flow cytometry. The proportion of CD4+CD25+Treg/CD4+ in spleens was 9.99% ±1.90% ,which was in accordance with the results reported by others (Liu et al., 2005). The representable figures were shown in Figure 2. To investigate the changes of the proportion in tumor bearing mice, 104

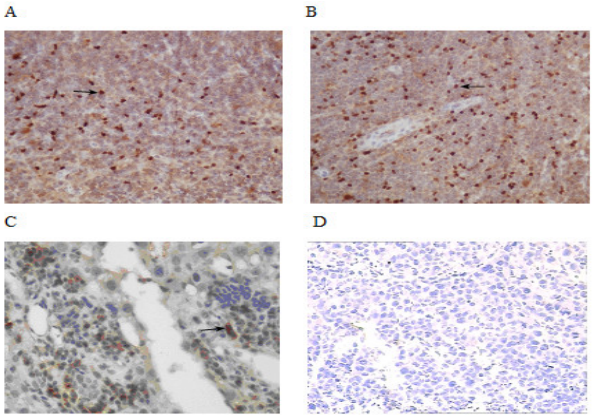

Figure 4. The Immunohistochemical Expression of Foxp3+ Regulatory T Cells (Tregs) and Granzyme B. Picture A, B, and C were under 400× Magnification and Picture D was Under 200× Magnification. Foxp3+ Tregs are characterized by brown spots in nucleus. Representative images of immunohistochemical staining of Foxp3+ Tregs in spleens from normal mice (A) and from mice with hepatocellular carcinoma (B). The median count of Foxp3+ cells was 90.6 cells/ field in normal mice and 181.6 cells/field in tumor bearing mice. The picture C showed the expression of Foxp3+ in carcinoma tiusses. Tregs were mainly distributed in the lymphocytes gathered places. The picture D showed the expression of granzyme B in carcinoma tiusses. But it were rarely observed in carcinoma tiusses

live H22 hepatoma cells were inoculated to hepar to build mice HCC in situ model (n=10). About 25 days later, the models were formed.The spleen lymphocytes were prepared for double staining with anti-mouse CD4 and CD25 antibodies. We found a prominent increase in CD4+CD25+Treg/CD4+ in tumor bearing mice, compared with that in control mice . The proportion of CD4+CD25+Treg /CD4+ T cells in HCC mice (18.8% ±1.26%) was significantly higher than the control group (9.99%±1.90%) (P<0.01) (Figure 3) .

Immunohistochemical staining of FoxP3 in spleen

To observe Foxp3+ Treg in situ,we performed immunohistochemical staining of Foxp3+ in paraffin-embedded tissues. Positive cells were stained brown in nucleus but unstained cytoplasm. In spleens, Tregs were mainly clumps distribution in white pulp but rare visible in red pulp. Treg counts were divided along the median value of Foxp3+ cells/field. The number of Foxp3+ cells in spleens from HCC mice (median, 181.6 cells/ field; range, 120—280/field) (n=6) was obviously higher than the control group (median, 90.6 cells/field; range, 55-130 cells/field) (n=6), and there were statistically significant differences among the two groups (p<0.05). The representable figures were shown in Figure 4.

Foxp3 and Granzyme B expression in carcinoma tissues

Granzyme B can mediate cytotoxic T lymphocytes and natural killer cells to induce apotosis in target cells and play a very important role in antineoplastic immune. Instead, Tregs can impair the anti-tumour immune and help tumor cells to escape from immunological surveillance. So, to investigate the tumor local immune status, we performed immunohistochemical staining of

Granzyme B and Foxp3 in carcinoma tissues. There were scattered lymphocytes gathered places in tumor tissues, and Tregs were mainly distributed in these lymphocytes gathered places.

But granzyme B was rarely observed in carcinoma tiusses. The antineoplastic immune was inhibited.

Discussion

CD4+CD25+T cells, known as regulatory T cells (Tregs), have potent immunoregulatory functions and could control self-tolerance. In the past, there were only two main types of Treg: naturally occurring Treg (nTreg) and induced Treg (iTreg). But by now many new subsets of Treg have been discovered, such as CD8+Tregs, CD4+CD25–Tregs, etc (Kiniwa et al., 2007; Han et al., 2009). The nTregs, as a major Treg subsets, develop in the thymus and migrate to the periphery, playing an important role in keeping immune homeostasis and regulating the immune responses. The iTregs develop in the periphery from conventional CD4+ T cells following antigenic stimulation under a variety of conditions (Workman et al., 2009). Two main subsets of iTregs have been described: type 1 regulatory T cells (Tr1), which are induced by IL-10 (Groux et al., 1997) and T helper 3 (Th3), which are induced by TGF-ß (Weiner et al.,2001). Both nTregs and iTregs share the similar phenotypic and functional characteristic, such as CD25,CTLA-4,GITR,CD62L,CCR4 and so on (Zheng et al., 2008). Foxp3, a forkhead family transcription factor, as the most specific maker of Treg , is a critical regulator of Treg development, function, and homeostasis (Hori et al.,2003).

It has been shown there is a close relationship between Treg cells and cancer. Many researchs have found an increase number of Treg in various cancers . The increasing number of Treg in patients with cancer indicates the poor prognosis. Tregs play an important role in helping tumor cells to escape from immune monitoring and the occurrence, development, final outcome of the cancer.Studies indicated that Tregs maybe an effective therapy target to improve anti-tumour immune. Although a higher proportion of Treg has been found in peripheral blood and in tumor infiltrating lymphocytes of HCC patients (Ormandy et al., 2005), but almost no researchs have described the change of Treg in spleens and the tumor local immune status in HCC. In addition, spleen is a professional immune organ, and it can better reflect the body'immune status when problems occur. Previously, people routinely inoculated cancer cells subcutaneously at axilla to build mice tumor-bearing model. But in order to more close to the occurrence process of HCC, we directly injected H22 hepatocellular carcinoma cell suspension to the liver to build BABL/C mice HCC in situ model. We used flow cytometry and immunohistochemistry to demonstrate an obvious increase of CD4+CD25+Treg/CD4+ in spleen. We also found that there were Treg cells expression in tumor microenviroment. Tumor antigen and cytokine in tumor microenvironment maybe important for the collection , amplification and induction of Tregs. Cytotoxic T lymphocytes and natural killer cells induce apotosis in target cells using a variety of mecheanisms including secretion of proapoptotic cytokines (e.g TNF-α and IFN-γ), engagement of cell death receptors (e.g. Fas), and granule exocytosis of perforin and granzymes which is a major mediator of the cytotoxic immune response by inducing target cell death. When a CTL recognizes a target cell such as a tumor cell, an immunological synapse is formed between the two cells and then cytotoxic granules containing perforin and granzymes release into synaptic cleft. Perforin inserts the membrane of the target cell and forms a "passageway" which facilitates the delivery of granzymes into the cytosol of the target cell causing the DNA fracture (Trapani et al., 2002). But granzyme B-positive cells were almost not detected in carcinoma tissues in our study. It maybe because that Treg with its unique immune inhibition inhibits the anti-tumour immune or what we used is artifical tumor model, not spontaneous tumor model, and the tumor grew so quickly that the anti-tumour immune did not build.

There are sevral explanations for the mechanism Treg mediating the immunosuppressive effects: inhibitory cytokines, cytolysis, metabolic disruption and modulation of APC function.. Treg can secret suppressive IL-10 and TGF-ß which can suppress the function of effect T lymphocytes (Huang et al., 2009). Tregs may lead to apoptotic or necrotic T cell death throgh granzyme A in humans and granzyme B in mice (Cao et al., 2007). The mechanisms of metabolic disruption include the generation of pericellular adenosine by CD39 and CD73 and the subsequent activation of the adenosine receptor 2A on conventional Tcells, and the transfer of the inhibitory second messenger cyclic AMP into conventional T cells via gap junctions (Vignali et al., 2008). Treg can also suppress target cells by down-regulation the expression of CD80/CD86 on APC to reduce the ability of APCs to activate convention T cells (Cederbom et al., 2000; Herman et al., 2012). Recently, people have found another new mechanism that soluble CD25 in the serum of patients with HCC could suppress Teff proliferation to inhibit immune (Cabrera et al., 2010). Although we have known the above mechanisms, futher efforts are still needed to discover the mechanism how Treg cells restrain antineoplastic immune and how to restore the immune function in cancer patients.

In conclusion, our study demonstrated that, compared with normal mice, The proportion of CD4+CD25+Treg/CD4+ in spleens in HCC mice was obviously higher. An increased Treg frequency and no expression of granzyme B in tumor microenvironment showed the suppression of the beneficial antitumor response. So targeting the number and function of Tregs in patients with tumor may be an effective strategy to induce immunity to the tumor. We have begun to isolate Tregs from tumor mice to better understand the mechanisms of the Treg increase in HCC and the exact relationship between Tregs and tumour immunity. Efforts may help for future immunotherapeutic in patients with tumor.

Acknowledgements

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