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WJG, 1999 February; 5(1):12-14
World Journal of Gastroenterology
 ISSN 1007-9327

In situ detection of tumor infiltrating lymphocytes expressing perforin and fas ligand genes in human HCC *

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Subject headings carcinoma, hepatocellular; lymphocytes; fas ligand genes; perforin; *in situ* hybridization; liver neoplasms

Abstract

AIM **To investigate the expression of perforin and fas-ligand (fas-L) of tumor infiltrating lymphocytes (TILs) in human hepatocellular carcinoma (HCC).**

METHODS **By *in situ* hybridization and immunohistochemistry, the perforin and fas-L gene expression of TILs was studied in 20 HCC cases.**

RESULTS **Positive expression of perforin and fas-L genes was detected in 16 HCC cases. One patient had expression of perforin and fas-L genes in the majority of TILs and survived 1.5 years after tumor resection without HCC relapse. This seems that the presence of a large number of activated T cells might be beneficial for the antitumor immunity. In other cases, less than 10% o f TILs were able to express perforin and fas-L genes.**

CONCLUSION **Although there were a number of T cells in HCC, only few of them were immunoactive and able to kill tumor cells. It seems important to promote further proliferation of these activated T cells *in vitro* or *in vivo*.**

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***Project supported by the Key National Natural Science Foundation of China, No.39730440 and the National Natural Science Foundation of China, No.39500082.**

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Received 1998-11-09

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) play a major role in killing tumor cells. Two pat hways have been described by which a cytotoxic cell may induce lysis of its target[1,2]. The first pathway is called perforin pathway. T cell receptors (TCRs) of CTLs binding with MHC antigens on the tumor cells induce the release of granules filled with perforins and granzymes, and perforins then attack the target cell surface, followed by granzymes entering into the cell and killing it. The second is fas-L pathway. Fas-L from activated T cell binds with Fas antigen on the tumor cell surface, directly causing cell apoptosis. Although we applied TILs to treat tumor several years ago[3], this is the first domestic report studying whether activated T cells around the tumor express the two killing genes, perforin gene and fas-L gene. We studied the expression of perforin and fas-L genes of TILs in the HCC specimens using *in situ* hybridization and immunohistochemistry to find out whether there are T cells with killing activities in HCC tissues.

MATERIALS AND METHODS

Materials

Specimens were obtained from 20 HCC patients (16 men and 4 women; ranging in age from 25 to 64 years with a mean of 47 years) who underwent tumor resection from January to June, 1994 in our hospital. Among these patients, 17 were associated with liver cirrhosis. The control specimens were from the normal liver tissues of 3 patients with hepatic angioma. The normal liver tissues and HCC tissues were quickly frozen in liquid nitrogen within half an hour after removed from the bodies of the patients, and stored at -70℃. The specimens cut from the marg in between tumor and paratumor areas were embedded with O. C. T, fixed with 40mL/L-paraformaldehyde, gradiently dehydrated with ethanol, and then stored at -70℃. Human fas-L cDNA was kindly presented as a gift by Dr. Nagata of J apanese Bioscience Institute, and human perforin cDNA by Dr. Kevin Y.T. Thia of Australia Austin Institute. Rabbit-anti-human fas-L polyclonal antibodies were purchased from Santa Cruz Biotech Company of USA. Rat-anti-human

perforin monoclonal antibody was donated by Dr. Eckhard. R. Padack of the Medical Academy of Miami University, USA. Digoxin labeling and detection kit was purchased from Boehringer Mannheim Company of Germany. The immunohistochemistry kit of streptavi din-biotin amplification system was a-product of WAK Company of Ger many.

Methods

HE staining

In situ hybridization Probe labeling proceeded according to the method of random primer labeling presented by the digoxin labeling kit of Boehringe r Mannheim Company. In situ- hybridization was performed following a previously published protocol[3]. The concentration of the probe was 1×10^3 μg/L and cells with blue granules under microscope were regarded as positive.

Immunohistochemistry The specimens were processed according to the ABC method, and visualized with DAB. Cells with brown granules under microscope were regarded as positive.

RESULTS

HE staining and immunohistochemistry

A few lymphocytes with negative perforin and fas-L expression were seen in the 3 normal liver tissues, while in the 19 HCC specimens, there was a various number of TILs, most in the mesenchyma of the tumor and a few in the parenchyma. Furt hermore, in another HCC case (No.14) which had not-experienced relapse for 1.5 years after tumor resection, there were a large number of TILs with positive expression of perforin and fas-L not only in the mesenchyma of the tumor, but also extensively in the parenchyma of the liver (Figures 1-3). TILs of 15 patients had positive perforin and fas-L expression with a positive rate below 10%. In the other four cases, although there was a various number of TILs infiltrating in the tumor mesenchyma, no TILs expressed perforin and fas-L. There was no relationship between the number of TILs in the liver tissue and the positive rate of perforin and fas-L expression.

In situ hybridization

There were no perforin and fas-L positive-hybridization signals in the 3 normal liver specimens. In HCC specimens, perforin and fas-L expression of TILs showed strong positivity in one case (No.14), mild positivity in 15 cases and negativity in 4 cases, indicating that perforin and fas-L expression in transcriptive level was parallel to that in protein level in HCC.

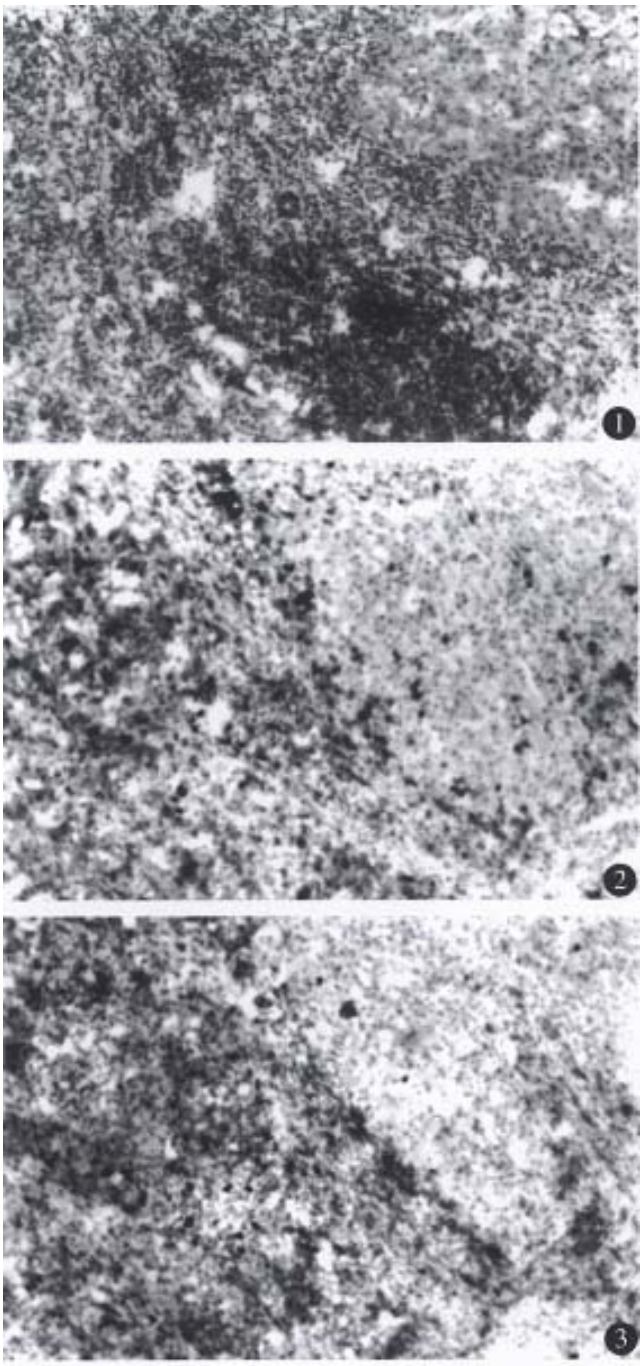

Figure 1 Most infiltrating TILs in the mesenchyma of the tumor, few in the parenchyma. HE×100

Figure 2 Strong positive signals of perforin in the TILs of hepatoma tissue. ABC×100

Figure 3 Strong positive signals of fas-L in the TILs of hepatoma tissues. ABC×100

DISCUSSION

TILs, a heterogeneous group of lymphocytes consisting largely of T lymphocytes, are located mainly in the tumor mesenchyma. As early as 1907, TILs were found in tumor tissues and the phenomenon that lymphocytes infiltrated in tumor tissues was supposed to be the result of the resistance of the

host to the tumor. Later, it was found that the greater the number of TILs in the tumor tissue, the better outcome the patient would get[4].

Early studies on TILs focused on their phenotypes. Recent investigations showed that T lymphocytes killed the tumor cells mainly under the perforin and fas-L pathways. Perforin and fas-L expression in TILs of tumor tissues is directly related to the killing activity of TILs in tumor tissues. In 1994, Leger-Ravet *et al*[5] found perforin and granzyme B expression in TILs in 10 follicular lymphoma cases. Of all 20 HCC cases we studied, TILs expressed perforin a nd fas-L in varing degrees in 16 patients, and negatively in 4. This indicates that cytotoxic T lymphocytes existed in most of the HCC patients. Furthermore, in one case there was a large number of TILs expressing fas-L and perforin in both the mesenchyma and the parenchyma of the tumor tissues. This patient did not sustain relapse within the 1.5 year follow-up period. This implies that large quantities of T lymphocytes with killing activities existing in the tumor tissues are beneficial for the prognosis of HCC patients. Except the case presen ted above, TILs expressing perforin or fas-L in the other 19 cases of HCC were below 10%, showing that most of the TILs were in an immunosuppressive state. The mechanism of this phenomenon might be ①the HCC could not activate T lymphocytes due to its deficient expression of the second signal B7; ② T lymphocytes expressing high levels of fas and fas-L resulted in self-apoptosis[6,7]. Therefore, amplification of the T lymphocytes with killing activities *in vivo* or *in vitro* may improve the therapeutic effect for the patients with tumors.

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Edited by MA Jing-Yun