Evaluative Significance of GrB Positive Cells in Hepartocellular Carcinoma for the Biological Characters and Prognosis

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Abstract **Objective** Granzyme B (GrB) is a kind of proteinase expressed characteristically in activated natural killer(NK) cells and cytotoxic T lymphocytes(CTLs), which plays an important role in tumor immune. The aims of the study were to investigate the location, quantity and evaluative significance of GrB positive cells in hepatocellular carcinoma (HCC), adjacent non-tumorous liver and normal liver tissues and their relationship with the biological characters and prognosis of human HCC. **Methods** Surgical specimens from 60 cases of HCC and 23 cases of normal liver tissues were investigated by immunohistochemical staining of GrB with streptavidin-horseradish peroxidase detective system, and the results were compared with the histologic grades and clinicopathological parameters. **Results** The quantity of GrB positive cells in the HCC (5.5±4.6) and the adjacent non-tumorous liver tissues(6.7±3.9) were both significantly higher than that in the liver of normal controls (3.6±2.4, t=2.38, P<0.05; 3.6±2.4, t=4.32, P<0.01, respectively). The quantity of GrB positive cells in the adjacent non-tumorous liver was significantly higher than that in HCC (t=4.32, P<0.05). There was no relationship between the quantity of GrB positive cells in HCC tissues and their differentiation grades. The quantity of GrB positive cells in HCC of stage Ⅰ, Ⅱ was significantly higher than that of stage Ⅲ, Ⅳ (6.8±5.3 VS 4.1±3.2, t=2.32, P<0.05). The quantity of GrB positive cells in HCC in the cases with metastasis in 15 months was significantly lower than that without metastasis (2.5±1.6 VS 7.0±4.3, t=5.02, P<0.01). **Conclusion** The quantity of GrB positive cells decreased with the process of the HCC patients in clinic. The quantity of GrB positive cells might be important markers to estimate the local immune status, also be useful factors to predict the biological characters and prognosis of HCC patients.

Key words Granzyme B; Hepatocellular carcinoma; Prognosis

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide[1,2], especially in Guangxi, China. The major etiologies and risk factors for HCC development are well defined and some of the multiple steps involved in hepatocarcinogenesis have been elucidated in recent years. Despite these scientific advances and the implementation of measures for early HCC detection in patients at risk, patient survival has not improved during the last three decades. The causes and the mechanism of the formation and development of HCC have either not been clarified very clear yet [3]. The immune function of the host was believed to be responsible for the control of the development of HCC [4]. Data also revealed that apoptosis or programmed cell death, and the elimination of apoptotic cells were crucial factors in the maintenance of liver health. Apoptosis allows hepatocytes to die without provoking a potentially harmful inflammatory response[5,6].

Granzyme B (GrB) is the prototypic member of a family of serine proteases localized to the cytolytic granules of cytotoxic lymphocytes. Granule-mediated cytotoxicity requires a combination of both perforin and GrB. Perforin polymerizes to form transmembrane channels and presumably allows GrB access to target cell substrates. One clue to the identity of the physiological substrates activated by GrB comes from its unusual specificity for cleaving synthetic substrates after aspartate residues[7]. Together with perforin, GrB is capable of in-

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ducing all aspects of apoptotic death in target cells. A number of GrB substrates had been identified and it had been demonstrated that GrB is responsible, directly or indirectly, for the morphological nuclear changes observed in target cell apoptosis, including DNA fragmentation [8]. Thus, it is important to understand the precise molecular mechanisms whereby cytotoxic lymphocytes destroy susceptible target cells. The aims of the present study were to investigate the expression of GrB in HCC , its adjacent non-tumorous liver and normal liver tissues by immunohistochemical staining with streptavidin-horseradish peroxidase detective system to access the function of the cellular immunocytes, the relationship between these positive cells, the differentiation and prognosis of HCC patients and to explore their roles in the tumourgenesis and development in HCC.

MATERIALS AND METHODS

Patients

Sixty HCC patients (57 males and 3 females, the mean age was 47 years, ranged 23-73 years) who had undergone curative hepatectomy from May 2002 to January 2003 at the Hepato-Biliary-Pancreatic Surgery Department, First Affiliated Hospital, Guangxi Medical University, China, were included in the study. Patients who had previously a hepatectomy or hepatic arterial chemoembolization (TACE) were excluded. HCC tissues were obtained from all patients. Its adjacent non-tumorous liver tissues were taken at least 2cm far away from the cancerous node. Among the 60 cases , 46 cases went with cirrhosis, 14 without, and 38 cases were AFP positive. The patients were strictly followed up. According to WHO standard , their pathology grades were: G1 3 cases, G2 38 cases, G3 19 cases; According to clinic TNM standard, their clinical stages were: stage Ⅰ 3 cases, stage Ⅱ 28 cases, stage Ⅲ 11 cases, stage Ⅳ 18 cases. Among 60 patients, 25 cases had metastasis in 15 months and 29 had no metastasis(with 6 cases being lost touch). Normal liver tissues were obtained from twenty-three patients with the liver cavernous heamangioma (10 male and 13 female, mean age was 45 years, ranged 26-67 years). Histopathological diagnosis and classification were made by the same pathologist.

Methods

Specimens were fixed by 4% neutralized mult-assemble formaldehyde, embedded by paraffin. The sections were continuously cut in 3-4micrometer thick and used to routine hematoxylineosin (HE) staining and immunohistochemical staining. Immunohistochemical reagent box with streptavidin-horseradish peroxidase detective system and DAB staining reagent were purchased from Maxim Co.. Mouse-anti-human monoclonal GrB antibody was purchased from Beijing Zhongshan Jinqiao Biotechnology Co. Ltd..

Immuhistochemistry

The staining steps were: slides were dewaxed, treated with 0.3% hydrogen peroxide at room temperature for 10 min to inhibit the activity of endogenous peroxidase. Then heated for 20 min at 95℃ to repair antigens and then rinsed in PBS. Nonspecific protein staining was blocked by goat serum. The slides were incubated with anti-human GrB polyclone antibody at 37℃ for 1h, with biotinylated secondary antibody for 20 min at 37℃, with streptavidin-horseradish peroxidase for 30 min, and detected with DAB. For negative control, PBS was used. The known positive lymph node sections were used to be the positive controls. After immunoperoxidase staining, the slides were counterstained with hematoxylin, redehydrated and mounted.

Evaluation of immunohistochemical staining

Positive expression of GrB was buff, light brown , brown-yellow or dark brown in cell cytoplasm with the shape of granule. The positive cells were recognized and counted in 10 randomly high-power fields (HPF 400×) by a light optic microscope.

Statistical analysis

The quantity of the positive cells were expressed as mean ± standard deviation (Mean ±SD). According to the applicable condition, ANOVA and Student t test were employed to analyze the results of experiment by SPSS 12.0 software for windows. $P<0.05$ was considered statistically significant.

RESULTS

Table 1 The quantity of GrB positive cells in various tissues (Mean ± SD)

Tissues	Cases	GrB
HCC	60	5.5±4.6
Adjacent	60	6.7±3.9
Control	23	3.6±2.4

Table 2 The relationship between the quantity of GrB positive cells and the pathological grades of HCC. (Mean ± SD)

Histological grades	Cases	GrB
Ⅰ	3	3.8±4.1
Ⅱ	38	5.9±4.8
Ⅲ	19	4.8±4.3

The location and quantity of the GrB positive cells in different tissues

GrB positive cells had no intensive trend, which were generally confined in normal liver sinusoids, blood space in HCC tissues, fibrous tissues and portal areas of the adjacent non-tumorous liver tissues.

The quantitys of GrB positive cells in various tissues

The quantity of GrB positive cells from highest to lowest was: adjacent non-tumorous liver tissues , HCC and normal control liver tissues. The quantity of GrB positive cells in the liver of HCC patients was significantly higher than that in the liver of normal controls ($P<0.05$). The quantity of GrB positive cells in the adjacent liver was significantly higher than that in normal controls ($P<0.01$).The quantity of GrB positive cells in the adjacent liver was significantly higher than that in HCC($P<0.05$, Table 1).

The relationship between the quantity of GrB positive cells and the pathological grades of HCC

The quantity of GrB positive cells had no relationship with the histological grades either in HCC and its adjacent non-tumorous liver tissues ($P>0.05$, Table 2)

The relationship between the quantity of GrB positive cells and clinical TNM stages

The quantity of GrB positive cells in the cases of stage Ⅰ and Ⅱ was significantly higher than that in stage Ⅲ and Ⅳ of HCC tissues ($P<0.05$).But in its adjacent non-tumorous liver tissues, no significant differences($P>0.05$,Table 3).

The relationship between the quantity of GrB positive cells and metastasis of HCC

The quantity of GrB positive cells in HCC tissues with metastasis within 15 months were significantly lower than that without metastasis ($P<0.01$), whereas no significant difference for GrB positive cells in the adjacent non-tumorous liver tissues of the cases with metastasis and without metastasis($P>0.05$, Table 3).

DISCUSSION

Granzymes, a family of serine proteases, are expressed exclusively by CTLs and NK cells, components of the immune system that protect higher organisms against viral infection and cellular transformation[9,10]. Fol-

Table 3 The relationship between the quantity of GrB positive cells and clinical TNM stages (Mean ± SD)

Clinical parameters	Cases	GrB	
		HCC	Adjacent
Stage Ⅰ, Ⅱ	31	6.8±5.3	6.0±3.7
Stage Ⅲ, Ⅳ	29	4.1±3.2	7.4±4.1
Metastasis	25	2.5±1. 6	5.4±4.0
No metastasis	29	7.0±4.3	7.4±3.6

lowing receptor-mediated conjugate formation between a granzyme-containing cell and an infected or transformed target cell, granzymes enter the target cell via endocytosis and induce apoptosis [11]. GrB has the strongest apoptotic activity of all granzymes [12-16], as a result of its caspase-like ability to cleave substrates at key aspartic acid residues. Other granzymes may serve additional functions, and some may not induce apoptosis. Granzymes have been well characterized only in human and rodents, and can be grouped into three subfamilies according to substrate specificity: members of the granzyme family that have enzymatic activity similar to the serine protease chymotrypsin are encoded by a gene cluster termed the "chymase locus"; granzymes with trypsin-like specificities are encoded by the " tryptase locus"; and a third subfamily cleaves after unbranched hydrophobic residues, especially methionine, and is encoded by the "Met-ase locus". All granzymes are synthesized as zymogens and, after clipping of the leader peptide, maximal enzymatic activity is achieved by removal of an amino-terminal dipeptide. They can all be blocked by serine protease inhibitors, and a new group of inhibitors has recently been identified - serpins, some of which are specific for granzymes. Future studies of serpins may bring insights into how cells that synthesize granzymes are protected from inadvertent cell suicide[17].

"Granule enzymes" or "granzymes" comprise about 90% of the mass of cytolytic granules [18], specialized "secretory" lysosomes, of both CTLs and NK cells. The granzymes are closely related structurally to chymotrypsin, with a triad of key residues - histidine, aspartic acid and serine - conserved at the catalytic site, and they are genetically linked to other leukocyte serine proteases, especially those of mast cells and monocytes. A total of eight granzymes (A-G and M) have been identified in the mouse, but only five of them are known in humans (A, B, H, M and tryptase-2, which is also known as granzyme 3). No human equivalents of mouse granzymes C-G are known, and granzyme H appears to be specifically human.

The expression of granzymes is restricted to activated T lymphocytes, immature T cells in the thymus (thymocytes), γδ T cells (a small population of specialized T cells mainly found in the gut) and NK cells. Of these, NK cells and γδ T cells constitutively express and store granzymes, whereas in T lymphocytes the production of granzyme mRNA and proteins must be induced following exposure to antigen or following other types of stimulation. Granzymes are expressed by most CD_8 positive and a smaller proportion of CD_4 positive T cells sensitized in vitro by antigen or lectin [19]. But it needs further research to confirm that GrB positive cells in our study are T lymphocytes or NK cells .The gene for granzyme M is only expressed in NK cells, whereas other members of the subfamily encoded by the Met-ase locus have a much wider expression. In this case, the density of GrB positive cells can reflect the local immune state of tumor directly, which is more accurate than the immune index of peripheral blood to reflect the host`s local antitumor immune response level. After the killer cells discern the target cell, it discharges its peculiar killer granules and leads the target cell to apoptosis.

The cell-death-inducing properties of GrB have recently been studied in detail. GrB can cleave, and therefore activate, several procaspases directly, and can also directly cleave downstream caspase substrates, including the inhibitor of caspase-activated DNase (ICAD). It can thus contribute in a major way to DNA fragmentation in the target cell. Over-expression of the anti-apoptotic Bcl-2 protein in mitochondria inhibits GrB completely, however, the mitochondrial disruption is an indispensable feature of granzyme-mediated cell death [20-22]. Recently, studies have shown that in human and mouse cells the pro-apoptotic Bcl-2-family member Bid is cleaved specifically and rapidly by GrB distal to the aspartic acid residue at position 75, and that the truncated Bid molecule inserts into the mitochondrial membrane to induce the release of other pro-apoptotic mediators, including cytochrome c and Smac/Diablo [23]. In addition to caspase-dependent mechanisms, there are also caspase-independent pathways: cells in which caspase activity is abolished are nevertheless killed by granzymes, although the caspase-independent mechanisms are poorly understood but probably involve cytoskeletal disruption[7].

In the present study, the quantity of GrB positive

cells in its adjacent non-tumorous liver tissues and HCC were higher than that in normal liver tissues, which proved that in the tumour tissues, the activated CTLs and NK cells obviously increase and may be related to the stimulus of the tumor antigen. GrB positive lymphocytes in its adjacent non-tumorous liver tissues were higher than that in the HCC tissues, which may be because the tumor necrosis influenced the expression of GrB.

The histology grade has no obvious relationship with the distribution of GrB positive cells in HCC and its adjacent non-tumorous liver tissues, which indicates that there is no obvious correlation between these lymphocytes and the differentiation degree of the tumor .

The quantity of GrB positive lymphocytes in the cases of stage Ⅰ and Ⅱ were significantly higher than those in stage Ⅲ and Ⅳ ($P<0.05$) of HCC, which meant that GrB positive cells in HCC had degressive current with the clinical TNM stage developing ($P<0.05$). GrB positive lymphocytes can be regarded as the index of judging HCC patient`s prognosis, meanwhile, while the cells of HCC transform malignantly, GrB may have certain influence and function in the course of progress of the tumor. It has been inferred that during the clinical progress in HCC, some sensitive sub-clone has been killed in tumor immunity, while the remaining sub-clone may resist the cytotoxic attack by CTLs and NK cells. It can even be able to bear the attack of the apoptotic signal and gain stronger survival ability to escape the immunity surveillance . This is helpful to the genesis and development of the HCC. The mechanism is similar with immunologic escape through FAS/FASL, and it may be another molecular mechanism for the tumor to escape the immunity surveillance.

The quantity of GrB positive cells in HCC tissues with metastasis were significantly lower than that without metastasis($P<0.01$), which delicated that the cellular immune status in the group with metastasis was much poorer than that without metastasis. Activated T lymphocytes and NK cells have the function of killing tumor cells and can suppress the metastasis of the HCC cells. The decreasing of immunocytes might be a risk factor for predicting tumor metastasis.

To sum up, as the condition of HCC patient worsens, the quantity of GrB positive cells decreased gradually in clinic. The quantity of GrB positive cells might be important markers to estimate the special anti-tumor immune status, also be useful factors to predict the biological characters and prognosis of HCC patients.

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