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• *CLINICAL RESEARCH* •

Action and mechanism of Fas and Fas ligand in immune escape of gallbladder carcinoma

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Abstract

AIM: To study the role of Fas and Fas ligand (FasL) in biological behaviors of gallbladder carcinoma, and their correlated action and mechanism in tumor escape.

METHODS: Streptavidin-biotin-peroxidase immunohistochemistry technique was used to study the expression of Fas and FasL protein in 26 gallbladder carcinoma tissues, 18 gallbladder adenoma tissues, 3 gallbladder dysplasia tissues and 20 chronic cholecystitis tissues. Apoptosis of the infiltrating lymphocytes in these tissues was studied by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method. Expression of both proteins and apoptosis of the tumor infiltrating lymphocytes in cancer tissues of primary foci was compared with clinicopathological features of gallbladder carcinoma.

RESULTS: The positive rates of Fas were not significantly different among carcinoma, adenoma, dysplasia and chronic cholecystitis. The positive rate of FasL in carcinoma was significantly higher than that in chronic cholecystitis ($\chi^2 = 4.89$, $P<0.05$). The apoptotic index (AI) in carcinoma was significantly higher than that in adenoma ($t' = 4.19$, $P<0.01$) and chronic cholecystitis ($t' = 8.06$, $P<0.01$). The AI was significantly lower in well-differentiated carcinoma and Nevin I-III carcinoma than that in poorly-differentiated carcinoma ($t' = 2.63$, $P<0.05$) and Nevin IV-V carcinoma ($t' = 3.33$, $P<0.01$). The confidence interval (CI) of infiltrating lymphocytes in adenoma, chronic cholecystitis, well-differentiated carcinoma and Nevin I-III carcinoma was very significantly lower than that in carcinoma ($t' = 6.99$, $P<0.01$), adenoma ($t' = 3.66$, $P<0.01$), poorly-differentiated carcinoma ($t' = 5.31$, $P<0.01$) and Nevin IV-V carcinoma ($t' = 3.76$, $P<0.01$), respectively. The CI of apoptosis of infiltrating lymphocytes in well-differentiated carcinoma was significantly lower than that in poorly-differentiated carcinoma ($t = 2.52$, $P<0.05$), and was not significantly lower in Nevin I-III carcinoma than in Nevin IV-V carcinoma ($t = 1.42$, $P>0.05$). Apoptosis of infiltrating lymphocytes was not discovered in adenoma and chronic cholecystitis.

CONCLUSION: FasL expressed in gallbladder carcinoma cells permits tumor cells to escape from immune surveillance of organism by inducing apoptosis in infiltrating lymphocytes of carcinoma tissues. Up-regulation of FasL expression plays an important role in invasive depth, histological classification and metastasis of gallbladder carcinoma.

Key words: Fas; Fas ligand; Immune escape; Gallbladder carcinoma

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INTRODUCTION

Gallbladder carcinoma is one of the most common malignancies of the biliary tract[1-5]. It is difficult to diagnose in its early phase and its prognosis is poor[6,7]. Previous studies suggested that some carcinomas may escape from immune surveillance of organism[8-11]. Apoptosis, or programmed cell death, as a special type of physiologic cell death, is involved in immune escape. Fas is called death molecular receptor, FasL is called death molecule. When FasL crosslinks with Fas, Fas-associated death domain (FADD) is activated, after which cellular apoptosis is induced. The Fas/FasL system has been suggested to play an important role in the establishment of immune privilege status for tumors by inducing Fas-mediated apoptosis in tumor-specific lymphocytes[14-16]. The apoptosis of infiltrating lymphocytes in tumor tissues permit cancer cells to escape from elimination[8-13].

In this study, 26 cases of gallbladder carcinoma confirmed by surgery and pathology were analyzed. The aim of the prospective study was to evaluate the role of Fas and FasL in biological behaviors of gallbladder carcinoma, and their correlated actions and mechanisms in tumor escape.

MATERIALS AND METHODS

Subjects

Surgical specimens of 26 gallbladder carcinomas (11 well-

differentiated carcinomas and 15 poorly-differentiated carcinomas, 13 Nevin I-III carcinomas and 13 Nevin IV, V carcinomas), 18 gallbladder adenomas and three gallbladder dysplasiae were studied in Tongji Hospital of Huazhong University of Science and Technology from 1991 to 2002. Surgical specimens of 20 chronic cholecystitis were randomly selected. None of the patients underwent radiotherapy or chemotherapy before operation. All specimens were fixed in 4% formaldehyde solution, embedded in paraffin and cut into 4-μm-thick sections.

Immunohistochemistry

The representative and consecutive sections from each specimen were immunostained with streptavidin-biotin-peroxidase immunohistochemistry technique. Anti-Fas antibody (Santa Cruz) and anti-FasL antibody (Santa Cruz) were both diluted in phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4) at the dilution ratio of 1∶50. All procedures were implemented according to the product illustrations. For negative controls, sections were processed as above with PBS instead of the primary antibodies. Colon tissue of Fas^+ and $FasL^+$ tested were selected for positive controls.

TUNEL

In order to examine the apoptosis of cancer cells and infiltrating lymphocytes in cancer tissues of primary foci, the representative and consecutive sections from each specimen were immunostained with TUNEL technique (TUNEL kit, Zhongshan Biotech). All procedures were implemented according to the product illustrations.

Evaluation of Fas, FasL staining and apoptosis

The immunoreactivity to Fas, FasL and apoptosis was localized in cytoplasm and cell membrane, which were stained brown. One hundred tumor cells of each section were counted from randomly selected representative fields, and then their apoptotic index (AI) of tumor cells of primary foci was calculated. Apoptosis quantity of the infiltrating lymphocytes in cancer tissues of primary foci was counted from 10 randomly selected representative fields (×400) of each section, and then their confidence interval (CI) of the mean was calculated.

Statistical analysis

Statistical evaluation was performed using χ^2-test to determine the rates of different groups and using *t*-test to detect the quantities of different groups. The data of apoptosis were expressed as mean±SE. $P<0.05$ was considered statistically significant.

RESULTS

Fas and FasL expression in tissues of gallbladder carcinoma, adenoma, dysplasia and chronic cholecystitis

The positive rates of Fas in carcinoma, adenoma, dysplasia and chronic cholecystitis of gallbladder were 46.2% (12/26), 72.2% (13/18), 100% (3/3) and 80% (16/20), respectively, there was no significant difference among them. The positive rates of FasL in carcinoma, adenoma and dysplasia were 84.6% (22/26), 83.3% (15/18) and 100% (3/3), respectively. The positive rate of FasL in chronic cholecystitis was 55% (11/20), which was significantly lower than that in carcinoma ($\chi^2 = 4.89$, $P<0.05$) (Table 1, Figures 1A and B).

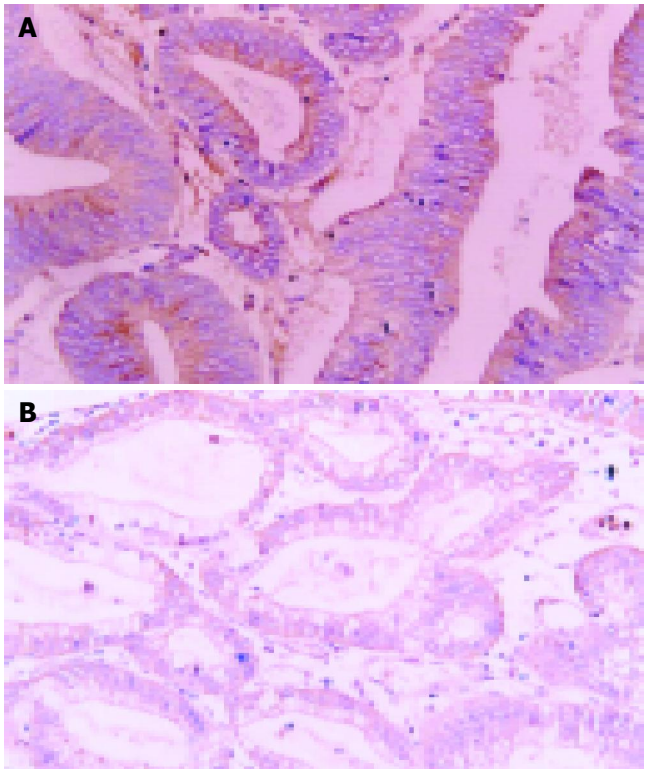

Figure 1 Positive expression in Fas (**A**) and FasL (**B**) in gallbladder carcinoma (S-P×200).

Table 1 Fas and FasL expression in tissues of gallbladder carcinoma, adenoma and chronic cholecystitis (mean±SE)

Pathological types	*n*	Fas^+	$FasL^+$	Apoptosis index
Gallbladder carcinoma	26	12	22[a]	3.88±0.88[b]
Grading				
Well-differentiated	11	7	9	2.73±0.75[c]
Poorly-differentiated	15	5	13	4.73±1.29
Staging				
Nevin I–III	13	5	10	2.62±0.68[d]
Nevin IV–V	13	7	12	5.15±1.33
Gallbladder adenoma	18	13	15	1.67±0.55
Gallbladder cholecystitis	20	16	11	0.20±0.18

[a]$P<0.05$ ($x^2 = 4.89$ *vs* chronic cholecystitis tissues); [b]$P<0.01$ ($t' = 4.19$ *vs* gallbladder adenoma tissues; $t' = 8.06$ *vs* chronic cholecystitis tissues); [c]$P<0.05$ ($t' = 2.63$, *vs* poorly-differentiated gallbladder carcinoma tissues); [d]$P<0.01$ ($t' = 3.33$ *vs* Nevin IV-V gallbladder carcinoma tissues).

Apoptosis of tissue cells in carcinoma, adenoma, dysplasia and chronic cholecystitis of gallbladder

AI of tissue cells in carcinoma, adenoma and chronic cholecystitis of gallbladder were 3.88±0.88, 1.67±0.55 and 0.20±0.18, respectively, there was no apoptosis in dysplasia. AI of tissue cells in well- and poorly-differentiated and Nevin

I-V carcinomas was 2.73±0.75, 4.73±1.29, 2.62±0.68, and 5.15±1.33, respectively. AI of tissue cells in carcinoma was significantly higher than that in adenoma (t' = 4.19, P<0.01) and chronic cholecystitis (t' = 8.06, P<0.01), while it was significantly lower in well-differentiated and Nevin I-III carcinomas than that in poorly-differentiated carcinoma (t' = 2.63, P<0.05) and Nevin IV-V (t' = 3.33, P<0.01) (Table 1 and Figure 2).

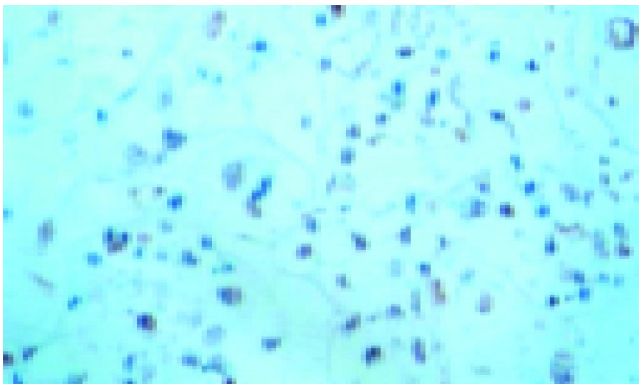

Figure 2 Apoptosis in gallbladder carcinoma (TUNEL ×400).

Quantities of infiltrating lymphocytes in tissues of carcinoma, adenoma, dysplasia and chronic cholecystitis of gallbladder

The CI of infiltrating lymphocytes in carcinoma, adenoma, dysplasia and chronic cholecystitis of gallbladder was 20.31±4.46, 3.89±1.13, 15.30±5.10, and 1.55±0.54, respectively. The CI of infiltrating lymphocytes in well- and poorly-differentiated carcinoma, and Nevin I-V carcinoma was 11±2.98, 27.13±5.16, 13.38±4.28, and 27.23±5.81, respectively. The CI of infiltrating lymphocytes in adenoma, chronic cholecystitis, well-differentiated carcinoma, Nevin I-III carcinoma significantly lower than that in carcinoma (t' = 6.99, P<0.01), adenoma (t' = 3.66; P<0.01), poorly-differentiated carcinoma (t' = 5.31, P<0.01), Nevin IV-V carcinoma (t' = 3.76, P<0.01) (Table 2 and Figure 2), respectively.

Table 2 Apoptosis quantities and total quantities of infiltrating lymphocytes in tissues of gallbladder carcinoma, adenoma and chronic cholecystitis (mean±SE)

Pathological types	*n*	Total quantities	Apoptosis quantities
Gallbladder carcinoma	26	20.31±4.46[b]	3.96±0.84
Grading			
Well-differentiated	11	11±2.98[d]	2.82±1.15[i]
Poorly-differentiated	15	27.13±5.16	4.8±1.02
Staging			
Nevin I–III	13	13.38±4.28[f]	3.38±1.23
Nevin IV–V	13	27.23±5.81	4.54±1.10
Gallbladder adenoma	18	3.89±1.13	0
Gallbladder cholecystitis	20	1.55±0.54[h]	0

[b]P<0.01 (t' = 6.99 *vs* gallbladder adenoma tissues); [d]P<0.01 (t' = 5.31 *vs* poorly-differentiated gallbladder carcinoma tissues; [f]P<0.01 (t = 3.76 *vs* Nevin IV–V gallbladder carcinoma tissues); [h]P<0.01 (t' = 3.66 *vs* gallbladder adenoma tissues); [i]P<0.05 (t = 2.52 *vs* poorly-differentiated gallbladder carcinoma tissues).

Apoptosis of infiltrating lymphocytes in tissues of carcinoma, adenoma, dysplasia and chronic cholecystitis of gallbladder

The CI of apoptosis of infiltrating lymphocytes in gallbladder carcinoma and dysplasia was 3.96±0.84 and 3.67±2.35, respectively, apoptosis of infiltrating lymphocytes was not discovered in gallbladder adenoma and chronic cholecystitis. The CI of apoptosis of infiltrating lymphocytes in well- and poorly-differentiated carcinoma and Nevin I-V carcinoma was 2.82±1.15, 4.8±1.02, 3.38±1.23, and 4.54±1.10, respectively. The CI of apoptosis of infiltrating lymphocytes in well-differentiated carcinoma was significantly lower than that in poorly-differentiated carcinoma (t = 2.52, P<0.05), and was not significantly lower in Nevin I-III carcinoma than in Nevin IV-V carcinoma (t = 1.42, P>0.05) (Table 2 and Figure 2).

DISCUSSION

Fas (Apo-1/CD95) and FasL belong to tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor/ligand kindred[14-16], which are comprehensively expressed in various histocytes. Fas, a type I membrane protein, has 80 amino acid sequences homologous to TNF-R1. This section induces cell death, and is named death domain. FasL, a type II membrane protein, is Fas's natural ligand. It induces cell apoptosis which expresses Fas through crosslinks with Fas[17]. Fas can bind to FasL and change the constitution of cell surface. Therefore, it can transmit signals in cells to switch on apoptosis mechanism, leading to apoptosis of cells expressing Fas. The function of Fas and FasL is to maintain immune stability of body and balance of body's apoptosis. The Fas/FasL system is likely to play an important role in the regulation of apoptosis including apoptosis of tumor cells.

Studies have demonstrated that tumor can induce apoptosis of activated lymphocytes in tumor tissues, the apoptosis of infiltrating activated lymphocytes in tumor tissues permits tumor cells to escape from elimination. In certain tumors, such as lung cancer[18], gastric cancer[19,20], pancreatic cancer[21], ovarian cancer[22], colon cancer[23,24], and others[25], tumor cells may express apoptosis-inducing molecules, FasL, on the cell surface that can bind to Fas receptor (FasR) expressed on activated lymphocytes, thus inducing apoptosis of lymphocytes. It has been proposed that the expression of FasL in tumors may play an important role in immune escape[26-31]. Experiments *in vitro* and *in vivo* indicate that FasL expressed in various cancer cell strains can induce apoptosis of lymphocytes, expressing Fas and that tumor can express some antigens which can offset the function of tumor infiltrating lymphocytes[13]. The quantities of apoptotic lymphocytes and tumor infiltrating lymphocytes increase with advanced tumor malignant degree, and the ratio of apoptotic lymphocytes to tumor infiltrating lymphocytes is gradually elevated. In this way, tumor tissues protect tumor cells from lymphocyte attack, consequently decrease the chance of being killed by lymphocytes, inducing super-proliferation of tumor cells. In these processes, the mechanism protecting normal tissues from immune attack may be used by tumor tissues[32,33].

In our study, the positive rate of FasL in gallbladder

carcinoma was significantly higher than that in chronic cholecystitis ($\chi^2 = 4.89$, $P<0.05$), the apoptosis of infiltrating lymphocytes in well-differentiated carcinoma was significantly lower than that in poorly-differentiated carcinoma ($t = 2.52$, $P<0.05$), apoptosis of infiltrating lymphocytes was not discovered in adenoma and chronic cholecystitis. These results indicate that expression of FasL protein in malignant gallbladder tissue is significantly higher than that in benign gallbladder tissue, and the quantities of apoptotic lymphocytes of infiltrating lymphocytes in malignant tissues is significantly higher than that in benign tissues. Up-regulation of FasL expression permits tumor cells to escape from immune surveillance of organism by inducing apoptosis of infiltrating lymphocytes in gallbladder carcinoma tissues. FasL participates in immune escape of gallbladder carcinoma, and gallbladder carcinoma has the same immune escape mechanism as other tumor tissues. In our study, the effect of Fas on immune escape of gallbladder carcinoma was not confirmed.

The quantities of infiltrating lymphocytes increases with malignant degree of the tumor, indicating that tumor has immunogenicity[31]. Recent experimental and clinical studies have made it clear that tumor infiltrating lymphocytes play a role in controlling tumor development, and many tumor vaccines enhance the effect of tumor infiltrating lymphocytes[33]. This mechanism has been proved in bile duct carcinoma, hepatocarcinoma[8], bladder carcinoma[9], *etc*.

Our study indicates that there is no apoptosis of infiltrating lymphocytes in tissues of gallbladder adenoma and chronic cholecystitis, accordingly lymphocytes may not be correlated with the mechanism of immune escape. We observed not only infiltration of lymphocytes but also apoptosis of infiltrating lymphocytes in specimens of atypical gallbladder hyperplasia of cholecyst epithelia, indicating that the organism may present different responses to different pathological conditions of gallbladder, including chronic inflammation, adenoma, atypical hyperplasia and carcinoma. The response of organism to these pathological conditions is a gradually accumulated process, which finally results in malignant pathological changes. Formation of gallbladder carcinoma is a complex multi-stage process, which finally induces cell proliferation out of control.

In conclusion, FasL expressed in gallbladder carcinoma cells permits tumor cells to escape from immune surveillance of organism by inducing apoptosis of infiltrating lymphocytes in gallbladder carcinoma tissues. Up-regulation of FasL expression plays an important role in invasive depth, histological classification and metastasis of gallbladder carcinoma.

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