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• ORIGINAL RESEARCH •

Fas counterattack in cholangiocarcinoma: a mechanism for immune evasion in human hilar cholangiocarcinomas

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Abstract

AIM: **To investigate FasL expression in hilar cholangiocarcinoma tissues and cultured cholangiocarcinoma cells, and to assess its ability to induce apoptosis.**

METHODS: **We studied the expression of FasL by human hilar cholangiocaroinomas tissues by immunohistochemistry, and the QBC939 cholangiocarcinoma cell line by RT-PCR, immunohistochemistry, and Western Blot. TUNEL and flow cytometry were used to detect apoptotic cells.**

RESULTS: **Prevalent expression of FasL was detected in 39 resected hilar cholangiocarcinoma tissues. TUNEL staining disclosed a high level of cell death among lymphocytes infiltrating FasL positive areas of tumor. FasL mRNA and protein expressions in cholangiocarcinoma cells could induce Jurkat cells.**

CONCLUSION: **Hilar cholangiocarcinomas may elude immunological surveillance by inducing, via Fas/FasL system, the apoptosis of activated lymphocytes.**

Subject headings cholangiocarcinoma/immunology; tumor cells, cultured/ immunology; membraneg lycoproteins/ biosynthesis; lymphocytes/immunology; apoptosis

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INTRODUCTION

The evasion, also called 'tumor escape', has been suggested to result from the inability of the immune system to react to the tumor, because of either non-recognition of tumor antigens or non-reactivity secondary to insufficient co-stimulation, anergy, tolerance, or immunosuppression. Recent reports showing the expression of FasL in Sertoli cells of the testis and ocular tissues[1,2], have provided new insights into the concepts of tolerance and immune-privilege. FasL triggers apoptotic cell death of sensitive lymphoid cells which express its cell surface receptor (Apo-1/CD95)[3-5]. FasL has been shown to confer immunological privilege in tissue transplantation experiments[6]. In rodents, successful allograft survival was obtained from FasL expressing tissues[2,7]. Moreover, FasL has been found to be expressed by non-lymphoid tumors as a mediator of immune evasion, which was initially raised by the finding that colon cancer cell lines express functional FasL[8]. Despite being immunogenic, cholangiocarcinoma overcomes antitumor immune responses by the mechanism that has not yet to be fully elucidated. The fact that many diverse tumors have been found to express FasL, suggests that a 'Fas counterattack' against antitumor immune effector cells may contribute to tumor immune escape[9-15]. We studied the expression of FasL on human hilar cholangiocarcinomas and the QBC939 cholangiocarcinoma cell line. We also studied apoptosis of lymphocytes (TILs) in filtrating into tumors. Jurkat cells were cocultured with cholangiocarcinoma cells.

MATERIAL AND METHODS

Human hilar cholangiocarcinoma tissue and cell

Thirty-nine human hilar cholangiocarcinomas of disparate pathological stages were collected from surgical resections performed at Qingdao municipal hospital. The patients were diagnosed as having hilar cholangiocarcinoma by histologic examination, and consisted of 17 males and 22 females. None of the patients have received chemo-, radio- or immuno-therapy before resection. The differentiati on of tumors were moderate (n = 12), poor (n = 18), or high (n = 9). The QBC939 cells (a human hilarcholangiocarcinoma cell line) were a generous gift from Professor Wang (Third Military Medical University, China)[16]. The human T cell line Jurkat was purchased from the American Type Culture Collection (Rockville, MD). QBC939 cells were cultured in DMEM supplemented with 100 mL·L^{-1} FBS. Jurkat cells were maintained in RPMI1640 nutrient medium supplemented with 100 mL·L^{-1} FBS, penicillin (100 KLI·L^{-1}) and streptomycin (100 mg·L^{-1}), and incubated at 37℃ in a 50 mL·L^{-1} CO_2 atmosphere.

Immunohistochemistry for FasL and CD45

Detection of FasL expression and CD45 positive cells was performed using a rabbit polyclonal anti-human FasL specific IgG and a mouse anti-human monoclonal antibody on paraffin sections of human cholangiocarcinoma respectively (Boster Biological Technology Company, Wuhan, China). Five μm thick sections on the slides were deparaffinized, rehydrated and blocked for removing endogenous peroxides activity with 3 mL·L^{-1} H_2O_2 in methanol. Then the sections were washed in PBS and pre-incubated with 50 mL·L^{-1} normal goat serum for 30 min. The slides were incubated with antibodies against FasL and CD45 for 1h at room temperature respectively. After washing, antibody binding was localized using a biotinylated secondary antibody with the ABC detection kit. The slides were counterstained with haematoxylin.

RT-PCR for FasL Mrna

Total RNA was prepared from the QBC 939 cell line with Trizol reagent (Gibco) according to the manufacturer's instructions. RT-

PCR was performed using RT-PCR kit (Promega) according to the manufacturer's protocol. cDNA synthesis was carried out with 2 μg of total RNA. The primers for PCR were 5'-TCCAACTCAAGGTCCATGCC-3' (forward) and 5'-CAGAGAGAGCTCAGATACGTTT-3'(reverse). PCR reactions mith total volume of 50 μL were processed in a MJ. PTC 100 Thermocycler under the following conditions: 94℃ for 2 min; 94℃ for 30 s, 57℃ for 45 s, and 72℃ for 1 min for 35 cycles; and 72℃ at 5 min. The RT-PCR products (342 bp fragments) were analyzed on 20 g·L^{-1} agarose gels. Amplication of human β-actin served as control for sample loading and integrity.

Western blotting

Immunoblotting was performed for detection of FasL. Cells (1×10^6) were scraped, centrifuged briefly, and lysed for 30 min on ice in 50 mmol ·L^{-1} Tris-HCl buffer (pH 8), containing 120 mmol·L^{-1} NaCl and 10 g·L^{-1} lyepal supplemented with the complete-TM mixture of proteinase inhibitors. T h e total protein was collected by centrifugation (14,000 r·min^{-1}, 30 min, 4℃) and assessed for protein concentration. SDS-PAGE (120 g·L^{-1}) was performed, an d the proteins were electroblotted onto nitrocellulose membranes. After 1h incubation in blocking solution (200 mL·L^{-1} IgG-free normal horse serum in PBS), the membrane was exposed to the primary antibody overnight at 4℃. After washing in PBS, the secondary peroxidase-labeled antibody was added at a 1:10 000 dilution for 40 min at room temperature. The proteins were visualized with the enhanced chemiluminescence technique.The primary anti-FasL antibody was the clone 33 (Jingmei Biotech Co. Ltd. China) mAb (1:1 000 dilution). The secondary antibody was peroxidase-labeled anti-mouse IgG antibody.

Cell death detection in situ by TUNEL

Cell death was detected *in situ* in resected tissues by enzymic labelling of DNA strand breaks using a TUNEL assay (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions. Only those cells with positive TUNEL staining and of apoptotic morphology were considered apoptotic.

T-cell apoptosis analysis

Cholangiocarcinoma cells were seeded in 6-well tissue culture plates and allowed to grow to 90% confluence. The cells were then washed twice with PBS and fixed with 20 g·L^{-1} paraformaldeyhde at 4℃ for 1 h. After the cells were washed 3 times with PBS, they were layered with 2 mL of Jurkat cell suspension (5×10^8 T cells·L^{-1}) in serum-containing media. After 48 h of coculture, Jurkat cells were collected from the 6-well plates, centrifuged, fixed in 700 mL·L^{-1} ethanol, and stored at -20℃ prior to analysis. Apoptotic cells were detected as a sub-G_1 fraction after propidium iodide staining and analysis using a FACScan.

RESULTS

Immunohistochemical localization of FasL

Paraffin sections from hilar cholangicarcinomas (n = 39) were stained for FasL. Positive staining for FasL was seen in the tissue of all 39 patients with hilar cholangiocarcinomas assessed (Figure 1). Positive staining of neoplastic tissue varied in both intensity and extent from individual tumor cell to cell, region to region in tumor and among tumors. Intensity of staining varied from weakly positive neoplastic areas to in tense regions and was stronger than that observed in local FasL positive TILs staining areas where staining was locally uniform with nests of tumor cells. All tumors examined were predominantly FasL positive (>70% of tumor area). To generate further evidence that FasL is expressed by human hilar cholangiocarcinoma cells, we also identified the protein expression of FasL in QBC939 cholangiocarcinoma cell line (Figure 2).

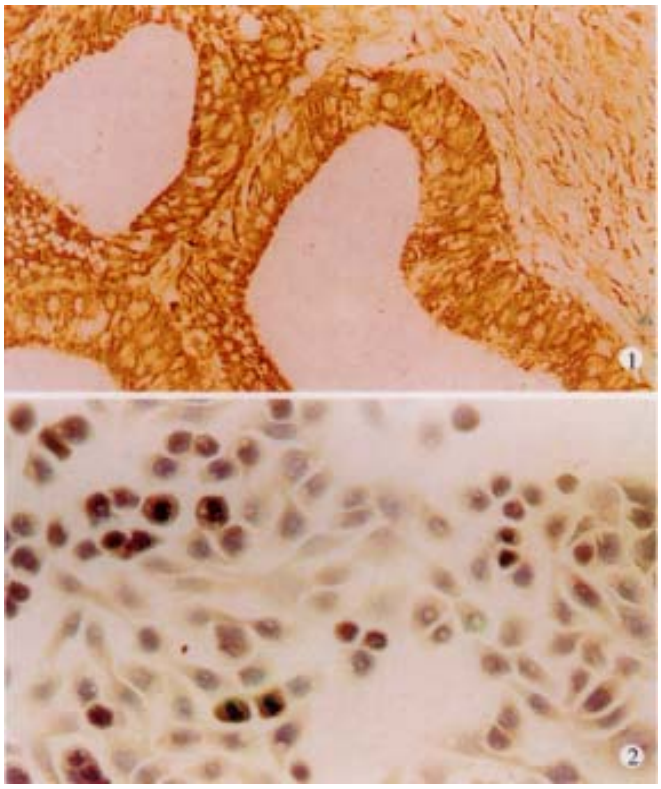

Figure 1 FasL positive in human hilar cholangiocarcinom as (brown). SABC×200
Figure 2 Expression of FasL in cholangiocarcinoma cell line. QBC939×200

Expression of FasL mRNA

To confirm the results obtained from the immunohistochemical studies, we evaluated the expression of FasL mRNA in the QBC939 human cholangiocarcinoma cell line. Total RNA was extracted and tested using RT-PCR. FasL mRNA was identified in QBC939 cells (Figure 3).

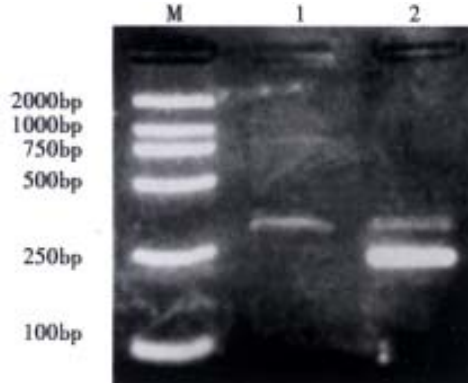

Figure 3 Expression of FasL mRNA in human cholangiocarcinoma cells QBC939. M: DL 2000 Marker; 1: FasL; 2: FasL+β-actin

Apoptosis of TILs

CD45 immunohistochemistry showed immunocyte infiltration in all 39 carcinomas (Figure 4). Most of the CD45 positive cells were of lymphoid morphology. Apoptosis was detected by TUNEL among TILs adjacent to FasL positive areas of the hilar cholangiocarcinomas. These

TUNEL positive TILs exhibited morphological features of apoptosis, including nuclear condensation and fragmentation (Figure 5). This was a consistent finding in all the tumors examined (n = 15).

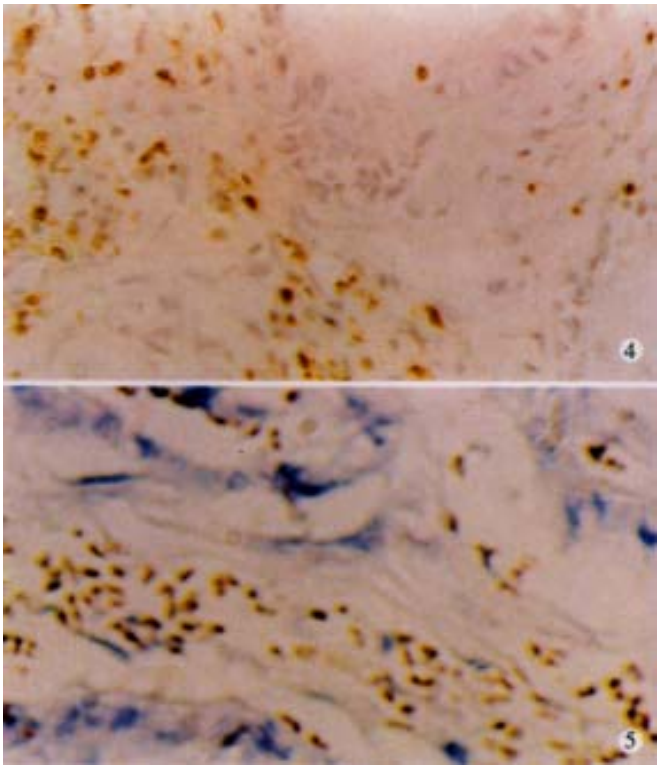

Figure 4 CD45 positive cells(brown) of lymphoid morphology adjacent to carcinoma. ×200

Figure 5 Positive apoptotic TUNEL stainig *in situ* (brown) with apoptotic morphology.

FasL by Western blot

Whole cell lysates from QBC939 cell cultures were electrophoresed in a polyacry lamide gel. We had previously obtained mRNA from the same samples, Mr 37 000 protein was recognized on line 06 clone 33 (Figure 6).

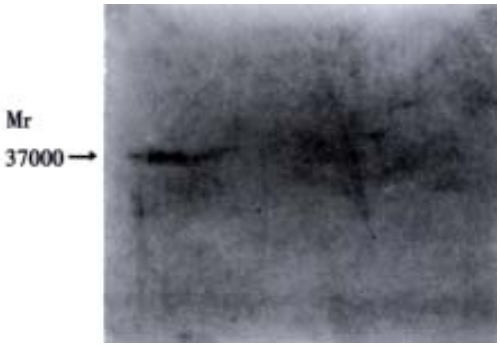

Figure 6 Western blotting of FasL protein with mAb from QBC939 cell cultures clone 33 from QBC939 cell cultures

Is functional FasL expression

To determine if FasL expressed by cholangiocytes in culture was capable of inducing cell death, we cocultured fixed cholangiocacinoma cells with FasL-sensitive (Jurkat) thymocytes. The QBC939 cholangiocarcinoma cell line induced cell death in FasL-sensitive T cells. At 48 h of coincubation, the cellline induced cell death in 50% of FasL-sensitive cells. These data show that the FasL expressed by the cholangiocarcinoma cells is capable of inducing T-cell apoptosis and therefore is functional.

DISCUSSION

It has been shown that hilar cholangiocarcinomas could express FasL, an inducer of immunocyte apoptosis in our study. The expression of FasL potentially enables hilar cholangiocarcinomas to counterattack and kill antitumor immune effector cells that seem Fas sensitive. As an established mediator of immune privilege and immunological tolerance in the eye and testis, functional FasL expressed in hilar cholangiocarcinomas was recognized as a contributor to the im mune evasion of hilar cholangiocarcinoma. Expression of FasL in human hilar cholangiocarcinomas was prevalent; all 39 samples resected from hilar cholangiocarcinomas were found to express FasL protein. FasL staining was variable in both intensity and extent within tumors. The fact that extensive expression (>70% of the tumor area) occurred in all tumors irrespective of tumor stage or degree of differentiation suggest that FasL may be expressed throughout hilar cholangiocarcinoma progression. In contrast with the extensive expression detected in the hilar cholangiocarcinomas, FasL expression was consistently restricted to only those epithelial cells at the luminal surface in control normal biliary epithelial sections (n = 6).It was estimated that a down-regulation and upregulation of FasL expression occurs during the transformation process. Using well-characterized, stable primary culture system of human cholangiocarcinoma cells, we found that cholangiocarcinomas in culture could express mRNA and protein of FasL and induce cell death in T cells. Apoptosis of TILs is an evidence *in vivo* which indicated that FasL expressed by the tumor is functional and can kill Fas sensitive anti tumor immune effector cells.

Several cancers have been reported to express FasL. The tumor derived cell lines in all of the cases could induce apoptosis in Fas sensitive, but not Fas resistant lymphoid target cells *in vitro*[17-30]. Tumors and cell lines themselves usually exhibit resistance to FasL mediated apoptosis because of various defects acquired in Fas signal transduction[31-37,44-48]. Immunogenic tumor cells are probably subjected to a barrage of c ell mediated cytotoxic antitumor immune assaults[38-43,49-52]. The fact that most tumor cells are efficiently killed by LAK cells *in vitro* suggests that cancer cells probably exhibit some degree of susceptibility to cell mediated cytotoxic mechanisms. Expression of a molecule to defuse antitumor immune challenge clearly offers a protective advantage to tumor growth and development. As an established mediator of immunological tolerance and privilege, FasL is such a molecule. Our findings have conclusively show that human hilar cholangiocarcinomas express functional FasL. Hilar cholangiocarcinoma may therefore be added to the growing list of malignancies that appear to be immunologically privileged through FasL expression. The high prevalence of FasL expression in the tumors suggests that this molecule may be critical to tumor immune privilege. In conclusion, the Fas counterattack appears to prevail as a potentially critical mechanism of immune privilege in human hilar cholangiocarcinoma.

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