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• *BRIEF REPORTS* •

Role of cytokines in promoting immune escape of FasL-expressing human colon cancer cells

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Abstract

AIM: To investigate the potential role of cytokines in promoting Fas ligand (FasL)-expressing colon cancer cells.

METHODS: Immunohistochemical SABC method was used to observe the expression of Fas receptor and ligand in SW620 colon cancer cell line and Jurkat T cells in order to provide the morphological evidence for the functions of Fas receptor and ligand. To examine the cytotoxicity of effector cells, CytoTox96® non-radioactive cytotoxicity assay was adopted to measure the lactate dehydrogenase-releasing value after SW620 cells were co-cultured with Jurkat T lymphocytes.

RESULTS: The FasL of colon cancer SW620 cells was positive. The positive substances were distributed in the cell membrane and cytoplasm. The Fas receptor of colon cancer SW620 cells was negative. The Fas receptor and ligand of Jurkat T lymphocytes turned out to be positive. The positive substances were distributed in the cell membrane. After phytohemagglutinin (PHA)-stimulated Jurkat T lymphocytes were co-cultured with phorbol 12-myristate 13-acetate (PMA)-plus-ionomycin-stimulated (for 48 h) SW620 cells or tumor necrosis factor-alpha (TNF-α)-stimulated (for 48 h) SW620 cells or unstimulated SW620 cells for 4 h, the cytotoxicity of SW620 cells to PHA-stimulated Jurkat cells at effector-to-target ratios of 10:1, 5:1, 2.5:1, and 1.25:1 was 74.6%, 40.8%, 32.4%, and 10.9% ($F = 8.19$, $P<0.05$); or 54.9%, 35.3%, 22.0%, and 10.3% ($F = 11.12$, $P<0.05$); or 14.9%, 10.5%, 6.9%, and 5.8% ($F = 3.45$, $P<0.05$). After PHA-stimulated Jurkat T lymphocytes were co-cultured with unstimulated SW620 cells for 8 h, the cytotoxicity of SW620 cells to PHA-stimulated Jurkat cells at effector-to-target ratios of 5:1, 2.5:1, and 1.25:1 from the experiment was 83.9%, 74.1%, and 28.5% ($F = 137.04$, $P<0.05$) respectively. Non-radioactive cytotoxicity assay showed that the apoptotic rate of Jurkat cells remarkably increased with the increase of planting concentration of SW620 cells and co-culture time after the SW620 cells were co-cultured with the Jurkat T lymphocytes. The cytotoxicity was significantly enhanced by PMA+ionomycin or TNF-α.

CONCLUSION: The FasL expressed in human colon cancer cells may be regulated by endogenous factors in the microenvironment of the host and facilitate the escape of tumor cells from the host immune system.

Key words: Cytokines; Fas/Fas ligand; Colon cancer; Apoptosis; Immune escape

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INTRODUCTION

The Fas/Fas ligand (FasL) system plays an important role in the transduction of apoptotic signal into cells. In recent years, numerous studies have demonstrated that Fas is expressed on the surface of cells, whereas FasL expression is restricted to a small number of cell types, such as lymphocytes, cells of the immune-privileged organs and many types of malignant tumor cells[1]. Evidence has pointed to an abnormal increase in apoptosis among activated Fas-positive lymphocytes, mainly in the periphery of the FasL-expressing tumors[2]. On the other hand, the occurrence of tumor is due to the fact that the converted cells cannot undergo a normal process of apoptosis. Resistance to apoptosis through the Fas receptor pathway coupled with expression of the FasL might enable many cancers to deliver a pre-emptive strike or counterattack against the immune system[2-4]. This study aimed to observe the interaction *in vitro* between T cells expressing Fas and tumor cells expressing FasL, and to investigate the potential role of FasL-expressing colon cancer cells *in vitro* and the effect of endogenous cytokines on tumor cells counterattacking T lymphocytes.

MATERIALS AND METHODS

Reagents, antibodies, and apparatus

Ionomycin, PMA, and phytohemagglutinin (PHA) were purchased from Sigma Chemical Co., USA. Tumor necrosis factor-alpha (TNF-α) and CytoTox96® non-radioactive

cytotoxicity assay kits were purchased from Promega Co., USA. RPMI1640 and DMEM were obtained from Gibco Co. Fetal bovine serum (FBS) was purchased from Hyclone Co. Monoclonal mouse anti-human CD95/FAS and monoclonal mouse anti-human FasL were purchased from Zhongshan Co., Beijing, China. SABC detection kit was purchased from Bosden Co., Wuhan, China. A-5082 Sunrise automated ELISA reader was purchased from Tecan, Austria.

Cell lines and cell culture

The human colon cancer cell line SW620 from American Tissue Culture Collection (ATCC) was kindly donated by Dr. Joe O'Connell, Cork University Hospital, Ireland. The acute T cell leukemia cell line Jurkat (ATCC) was provided by Institute of Hematology, Chinese Academy of Medical Sciences. The human glioma cell line TJ905 was kindly donated by Dr. Zhang WZ, Tianjin Huanhu Hospital, China. SW620 and TJ905 cells were cultured in DMEM supplemented with 100 mL/L FBS. Jurkat T lymphocytes were cultured in RPMI1640 medium (with 100 mL/L FBS) and stimulated with 4 mg PHA/L[3]. All cell lines were incubated at 37 ℃ in a humidified 50 mL/L CO_2 atmosphere.

Immunocytochemical procedures for detection of FasL and Fas protein

Jurkat, TJ905, and SW620 cells were cultured on glass chamber slides respectively. After fixation in 4% paraformaldehyde for 60 min respectively, slides were washed twice for 5 min in a wash buffer containing 50 mmol/L Tris-Cl, pH 7.6, 50 mmol/L NaCl, and 0.001% saponin. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol for 5 min. Slides were washed as before except that the wash buffer contained 1% normal goat serum, and then blocked for 1 h in wash buffer containing 5% normal goat serum[5]. Slides were washed and incubated overnight at a dilution of 1:200 with monoclonal mouse anti-human primary antibodies at 4 ℃ in a high-humidity chamber. Antibody binding was localized using biotinylated secondary antibody, avidin-conjugated horseradish peroxidase, and diaminobenzidine substrate. Slides were counterstained with hematoxylin, washed for 1 h with PBS, air-dried and covered with mounting medium. Negative control slides were processed with PBS in place of primary antibodies.

Fas-mediated cytotoxicity assay

To examine the cytotoxicity of effector cells, CytoTox96® non-radioactive cytotoxicity assay[6] was adopted to measure lactate dehydrogenase (LDH)-releasing value after SW620 or TJ905 cells (control) were co-cultured with Jurkat T lymphocytes. Jurkat cells were incubated with PHA (4 mg/L)[3] in RPMI1640 (50 mL/L FBS) for 24 h and counted with a hemocytometer before cytotoxicity assay. SW620 or TJ905 cells (control) were seeded on 96-well U-bottom tissue culture plates (Falcon) at a density of 2×10^9/L, 1×10^9/L, 5×10^8/L, and 2.5×10^8/L respectively, and kept under controlled conditions or stimulated with 10 μg/L PMA+500 μg/L ionomycin or with 20 μg/L TNF-α for 48 h. For a further 24 h of culture PHA-stimulated Jurkat cells (2×10^4 in 100 μL) were added, keeping effector-to-target cell ratios at 10:1, 5:1, 2.5:1, and 1.25:1. PHA-stimulated Jurkat cells were co-cultured with effector cells in 100 μL RPMI1640 medium (50 mL/L FBS) each well. Tissue culture plate was centrifuged at 250 r/min for 5 min at 4 ℃ to ensure cell-cell contact[6]. In each experiment triplicate wells were analyzed. At the same time, the values of effector cell spontaneous LDH release, target cell spontaneous LDH release, target cell maximum LDH release, volume correction control, and culture medium background were measured. Then the following formula was applied in the calculation of percent cytotoxicity:cytotoxicity (%) = (experimental LDH release-effector cell spontaneous LDH release-target cell spontaneous LDH release)/(target cell maximum LDH release-target cell spontaneous LDH release)×100.

Statistical analysis

Results were compared by analysis of variance (ANOVA) using SPSS10.0 software. $P<0.05$ was considered statistically significant.

RESULTS

Immunocytochemical detection of FasL and Fas protein in SW620, Jurkat and TJ905 cells

FasL expression in colon cancer SW620 cells was strongly positive. The positive substances were distributed in the cell membrane and cytoplasm (Figure 1A), while Fas expression in SW620 cells was negative (Figure 1B). Fas and FasL expression in Jurkat T lymphocytes turned out to be positive. The positive substances were distributed in the cell membrane and cytoplasm, while the nuclei of the cells were negative (Figures 1C and D). Fas expression in TJ905 cells was positive. The positive substances were distributed in the cell membrane and cytoplasm (Figure 1E). FasL expression in TJ905 cells was weakly positive (Figure 1F).

Results of cytotoxicity assay

After the SW620 cells were co-cultured with the PHA-stimulated Jurkat T lymphocytes for 4 h, the cytotoxicity (Figure 2) of SW620 cells to PHA-stimulated Jurkat cells at different effector-to-target ratios had significant differences ($F = 3.45$, $P<0.05$). After stimulation with 10 μg/L PMA+500 μg/L ionomycin for 48 h, the SW620 cells were co-cultured with the PHA-stimulated Jurkat T lymphocytes for 4 h. The cytotoxicity (Figure 2) of SW620 cells to PHA-stimulated Jurkat cells at different effector-to-target ratios had significant differences ($F = 8.19$, $P<0.05$). After stimulation with 20 μg/L TNF-α for 48 h, the SW620 cells were co-cultured with the PHA-stimulated Jurkat T lymphocytes for 4 h. The cytotoxicity (Figure 2) of SW620 cells to PHA-stimulated Jurkat cells at different effector-to-target ratios had significant differences ($F = 11.12$, $P<0.05$). After the SW620 cells were co-cultured with the PHA-stimulated Jurkat T lymphocytes for 8 h, the cytotoxicity of SW620 cells to PHA-stimulated Jurkat cells at effector-to-target ratios of 5:1, 2.5:1, and 1.25:1 was 83.9%, 74.1%, and 28.5% respectively and had significant differences ($F = 137.04$, $P<0.05$). The cytotoxicity of Jurkat cells after co-culture with SW620 (different effector-to-target ratios) for 8 h was much higher than that after being co-cultured with SW620 (different effector-to-target ratios) for 4 h ($P<0.05$, Table 1).

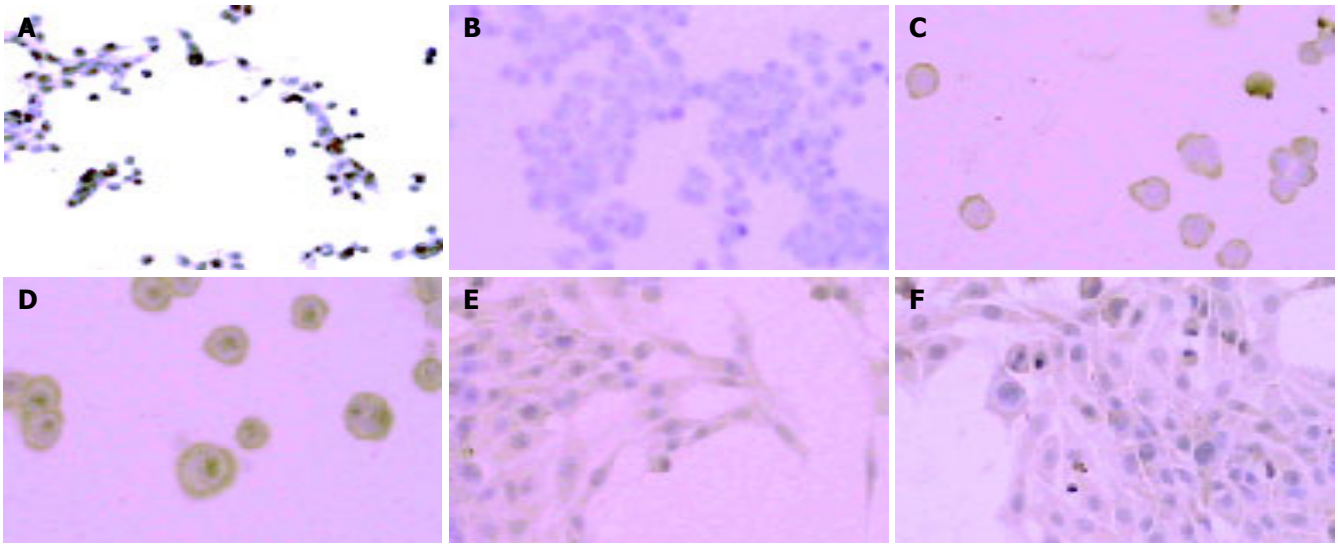

Figure 1 Expression of FasL and Fas in SW620 cells (**A** and **B**), Jurkat cells (**C** and **D**), and TJ905 cells (**E** and **F**).

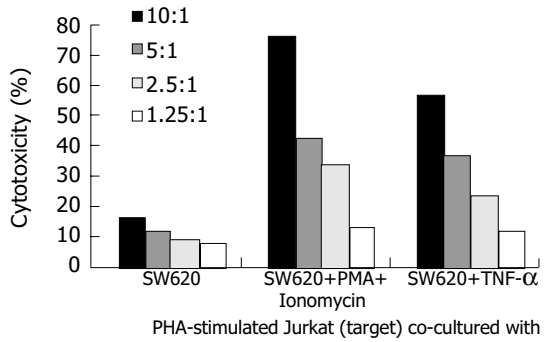

Figure 2 Comparison of cytotoxicity assay among three groups.

After PMA-plus-ionomycin-stimulated (for 48 h) SW620 cells or unstimulated SW620 cells were co-cultured with PHA-stimulated Jurkat T lymphocytes for 4 h, the cytotoxicity of PMA-plus-ionomycin-stimulated SW620 cells was much higher than that of control. The cytotoxicity had significant differences between the two groups ($P<0.05$, Table 1). After TNF-α-stimulated (for 48 h) SW620 cells or unstimulated SW620 cells were co-cultured with PHA-stimulated Jurkat T lymphocytes for 4 h, the cytotoxicity of TNF-α-stimulated SW620 cells was much higher than that of control. The cytotoxicity had significant differences between the two groups ($P<0.05$, Table 1).

After the TJ905 cells were co-cultured with the PHA-stimulated Jurkat T lymphocytes for 4 h, the cytotoxicity of TJ905 cells to PHA-stimulated Jurkat cells at different effector-to-target ratios had no differences ($F = 0.25$, $P>0.05$). TJ905 cells were not cytotoxic to Jurkat cells. After the TJ905 cells were co-cultured with the PHA-stimulated Jurkat T lymphocytes for 8 h, the cytotoxicity of TJ905 cells to PHA-stimulated Jurkat cells at different effector-to-target ratios had no differences ($F = 2.92$, $P>0.05$). TJ905 cells were not cytotoxic to Jurkat cells. After the TJ905 cells were stimulated with 10 μg/L PMA+500 μg/L ionomycin for 48 h, the TJ905 cells were co-cultured with the PHA-stimulated Jurkat T lymphocytes for 4 h. The cytotoxicity of TJ905 cells to PHA-stimulated Jurkat cells at different effector-to-target ratios had no differences ($F = 0.04$, $P>0.05$), TJ905 cells were not cytotoxic to Jurkat cells. After the TJ905 cells were stimulated with 20 μg/L TNF-α for 48 h, the TJ905 cells were co-cultured with the PHA-stimulated Jurkat T lymphocytes for 4 h. The cytotoxicity of TJ905 cells to PHA-stimulated Jurkat cells at different effector-to-target ratios had no differences ($F = 0.97$, $P>0.05$), TJ905 cells were not cytotoxic to Jurkat cells. Cytotoxicity assay showed that no cytotoxicity to human glioma TJ905 cells was observed in the PHA-stimulated Jurkat T lymphocytes.

DISCUSSION

Fas is constitutively expressed in lymphocytes of normal subjects. After lymphocytes are triggered by inflammatory cytokines or tumor antigen, Fas expression is significantly augmented. Since the expansion of tumor-specific CD4 and CD8 cells is the current goal of many promising immunotherapeutic strategies, it is important to understand the factors that may influence the fate of such specific cells. Moreover, uncloned CD4 cell line originally isolated from human tumor tissue[7]

Table 1 Comparison of cytotoxicity assay after co-culturing PHA-stimulated Jurkat T lymphocytes with SW620 cells

Sources of deviations	*F* (8 h)	*P*	*F* (PMA-plus-ionomycin)	*P*	*F* (TNF-α)	*P*
Sample	475.99	<0.05	27.71	<0.05	57.87	<0.05
Column	77.62	<0.05	10.29	<0.05	129.92	<0.05
Intercept	51.09	<0.05	5.14	<0.05	15.75	<0.05

Two-way ANOVA.

as well as tumor-specific human cytotoxic T lymphocytes (CTLs) clones[8] are also reported to express Fas *in vitro* and to be sensitive to its ligation. Recent evidence shows that tumor-infiltrating lymphocytes (TILs) exhibit significantly increased expression of Fas relative to peripheral blood lymphocytes[9]. There is evidence that the lack of co-stimulatory signals such as B7.1, a feature of many tumors, promotes T-cell sensitivity to FasL in the tumor microenvironment[10]. Since TILs are difficult to be isolated and expanded *in vitro*, we chose Jurkat T lymphocytes instead of TILs in our study.

Fas is expressed in each colonocyte of normal colon mucosa, and downregulated or lost in the majority of colon cancers. Immunohistology revealed that the majority of colon cancers express Fas at abnormally low levels or entirely lack Fas[11]. Our results are compatible with these findings. Further more data have confirmed that colon cancer cell line is constitutively or at least relatively resistant to Fas-mediated apoptosis[12,13]. Resistance to Fas-mediated apoptosis is a common feature of cancers, irrespective of cell surface expression of Fas[14]. Thus, the downmodulation or abrogation of Fas on tumor cells and/or acquirement of relative resistance to Fas ligation might be a selection advantage, and constitute a mechanism of immune evasion to Fas-mediated killing by T cells[15,16].

The FasL-expressing SW620 cell line is derived from a lymph node metastasis of primary colon cancer. The present study further confirmed that the expressed FasL was demonstrated to be functional, since co-culture experiments using FasL-expressing SW620 cells resulted in the apoptosis of Jurkat T leukemia cells that are sensitive to Fas-mediated apoptosis, which consequently may facilitate metastatic development. Our findings and other data suggest that tumor cells can evade immune attack by downregulating the Fas and inducing apoptosis in activated T lymphocytes through the expression of FasL. Furthermore, the constitutive expression of FasL in hepatic metastatic tumors suggests that FasL may also be important in their colonization in the liver through induction of apoptosis in the surrounding Fas-expressing hepatocytes[17]. FasL expression in human colon cancers is associated with apoptotic depletion of TILs *in vivo*[2]. In addition, upregulation of FasL expression probably induces killing of Fas-bearing tumor cells by promoting the selection of malignant tumor variants, because its Fas pathway has become insensitive to FasL binding. In the tumor microenvironment, IFN and other potentially relevant cytokines may be provided endogenously by immune system interaction, such as $CD4^+$ T cells, following interaction with MHC class II^+ antigen-presenting cells. Ag-specific $CD8^+$ CTLs may then lyse cytokine-modified tumor cells through Fas-dependent and/or Fas-independent pathways, depending upon the intrinsic susceptibility of the tumor population to one or more immune effector mechanisms. Zeytun *et al*.[18], reported a mutual killing model in which $FasL^+$, Fas^+ tumor cells, LSA and EL-4, kill Fas^+ tumor-specific CTLs and are also killed by tumor-specific $FasL^+$ CTLs. They concluded that the survival of the tumor or the host might depend on the cells which can accomplish FasL-based killing more efficiently. It is clear that the highly sophisticated and flexible adaptive immune response of higher vertebrates requires subtle regulation, particularly of receptors that can deliver such a devastating outcome as cell death. Ding *et al*.[19], reported that PMA+ionomycin and endogenous cytokines such as interleukin-18 (IL-18), TNF-α and IFN-γ upregulate the expression of FasL protein in human colon cancer cell lines DLD-1 and SW620. We further showed that the cytotoxicity was significantly enhanced by PMA+ionomycin and TNF-α. Since IFN-γ, TNF-α, and other potentially relevant cytokines are mainly secreted by activated T cells and macrophages, the upregulation of FasL in cancer cells in response to some cytokines may thus counterselect activated TILs and favor a microenvironment of T-cell anergy and the immune escape of cancer cells. Our data suggest that the FasL expressed in human colon cancer cells may be regulated by endogenous factors in the microenvironment of the host and facilitate the escape of tumor cells from the host immune system. Pages *et al*.[20], observed that synthesis of IL-18 decreases or is abolished in colon adenocarcinomas compared to that in normal mucosa, thus resulting in decreasing IFN-γ production and impairing FasL-dependent cytotoxicity of immune cells. This feature is correlated with the existence of distant metastasis and an unfavorable outcome. Xu *et al*.[21], reported that IFN-γ upregulates the expression of Fas and FasL in HT29 cells, a human colon adenocarcinoma cell line, and subsequently induces apoptosis of these cells in an autocrine and paracrine manner. However, it is important to note that many mechanisms of Fas-resistance can also occur, such as Fas-associated phosphatase-1, overexpression of bcl-2 and secretion of soluble Fas from tumor cells in a variety of human tumor cell lines that express Fas[22,23]. TNF-α and IFN-γ are potent immunostimulatory cytokines with tumoricidal effects in a variety of cancers. But at the same time these cytokines might facilitate escape of tumor cells from the host immune system.

Taken together, we also considered a new mechanism of immune evasion, namely, the active destruction of T lymphocytes by tumor cells expressing CD95 ligand. It may provide insights into the processes of both tumor immunity and tumor escape for at least a potential subset or fraction of malignancies. Disarming the Fas counterattack is a conceptually appealing and exciting potential goal for tumor immunotherapy. Ongoing studies are aimed at further understanding the basis of Fas resistance and counterattack, thus determining how to restore tumor cell sensitivity to Fas or block expression or function of FasL in tumor cells.

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