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EXPRESSION OF FAS LIGAND IS NOT A MAIN MECHANISM USED BY TUMORS TO COUNTERACT ANTITUMOR IMMUNITY

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1. ABSTRACT

The role of Fas ligand (FasL) in tumor immune privilege is controversial. In this study, 22 human tumor cell lines reported to be $FasL^+$ were reevaluated by Western blot analysis, ELISA, and a functional assay. None of the cells lines expressed FasL. To assess whether human tumors express FasL *in vivo*, susceptibility to FasL-mediated killing was evaulated. About 75% of the 22 tumors tested were sensitive to FasL-mediated apoptosis, suggesting, therefore, that only about 25% could possibly express FasL. To investigate whether "$FasL^+$" human tumor cells could suppress the proinflammatory effects of FasL *in vivo*, FasL transfectants were generated from two prototype "$FasL^+$" tumor cell lines. The transfectants expressing FasL were rejected by SCID mice. In contrast, all the mice inoculated with parental tumor cells developed large tumors. These results suggested that human tumor cells that express FasL and resist both FasL-mediated apoptosis and inflammation are rare or nonexistant. We concluded that FasL expression is not a main mechanism that tumors use to counteract antitumor immunity.

2. INTRODUCTION

The ability of tumors to escape immune surveillance *in vivo* is central to their pathogenicity. The discovery that various tumors express CD95 ligand (FasL) suggested a new mechanism by which tumors may kill lymphocytes, and thus evade immune attack (1, 2). CD95 (Fas) is a cell surface receptor that mediates apoptosis when engaged by its natural ligand, FasL (3–10). FasL expression has been reported in a broad spectrum of human tumors including colon cancer, melanoma, pancreatic cancer, hepatocellular carcinoma, esophageal cancer, lung cancer, breast cancer, neuroblastoma, glioma, prostate cancer, choriocarcinoma, and sarcoma (11–34). Research has also revealed that FasL stimulates a proinflammatory reaction (35–37). $FasL^+$ grafts and $FasL^+$ tumor transfectants were rapidly rejected by inflammatory cells *in vivo* (35–40). In addition, our research has shown that TGF-ß suppresses the proinflammatory effect of FasL and facilitates the growth of $FasL^+$ tumor transfectants *in vivo* (37). These results suggest that human tumors either do not express FasL or they are capable of suppressing its proinflammatory effect (41–43).

Some investigators have reported that FasL expression could not be confirmed in melanoma and colon cancer cells (36, 44, 45). In addition, some anti-FasL antibodies have been reported to be nonspecific for human FasL (46–48). The polyclonal rabbit anti–human FasL antibody C-20 was determined to be inappropriate for flow cytometric analysis of human FasL expression (46). The specificity of another frequently used antibody, mAb33, also is in doubt (47–49). These findings demand the reevaluation of the concept that tumors express FasL to counteract antitumor immunity (41, 42).

Using a transfectant that expresses intact human FasL as a positive control, we studied 22 human tumor cell lines that have been reported to be $FasL^+$. Using a FasL-specific antibody, FasL expression was not detected in any of the 22 cell lines. Furthermore, none of the tumor cells killed Fas^+ Jurkat cells, suggesting that functional FasL was not expressed either. Most of the "$FasL^+$" tumor cell lines

Table 1. Characteristic of human tumor cell lines

Cell	Type	Western Blot	ELISA (ng/ml)	Cytotoxic Effect to Jurakt (%)	Killing by CT26-hFasL (%)
CT26-hFasL	Murine colon Ca	+	33±7.5	77±12	ND
CT26-neo	Murine colon Ca	-	0	0.3±0.42	ND
SW620 [1]	Human colon Ca	-	0	0	34.9±9
SW480 [1]	Human colon Ca	-	0	0.9±0.56	68.75±1.14
DLD-1 [1]	Human colon Ca	-	0	0	50.54±3.6
LoVo [1]	Human colon Ca	-	0	0	43.85±2.7
WiDr [1]	Human colon Ca	-	0	0	58.12±15
HT-29 [1]	Human colon Ca	-	0	0	0
HCT 116 [1]	Human colon Ca	-	0	0	33.6±17.1
LS 174T [1]	Human colon Ca	-	0	0	66.8±33.7
Hep G2 [1]	human hepatoma	-	ND	0	71.2±14
AsPc-1 [1]	human pancreatic Ca	-	0	0	5.6±3.6
BxPc-3 [1]	human pancreatic Ca	-	0	0	52.2±4.4
MDA-MB-231 [1]	human breast Ca	-	0	0	ND
SK-BR-3 [1]	human breast Ca	-	0	0	0
LNCAP [1]	human prostate Ca	ND	0	0	ND
DU 145 [1]	human prostate Ca	-	0	0	65±20
SHEP-1 [1]	human neuroblastoma	-	0	0.5±0.7	74±4.9
IMR-32 [1]	human neuroblastoma	-	0	0	0
NCI-H522 [1]	human lung Ca	-	0	0	35.6±5.5
JEG-3 [1]	human choriocarcinoma	-	0	0	0
SK-N-MC [1]	human neuroepithelioma	-	0	0	64±25
U-87 [1]	human glioblastoma	-	0	0.55±0.77	65±19
U-118 [1]	human glioblastoma	-	0	0	82.5±16

[1] Cell lines have been previously reposted to express FasL ND: experiments were not performed

tested were susceptible to FasL-mediated apoptosis, further confirmation that these cells lack FasL, because otherwise they would have been killed. In addition, forced expression of FasL in apoptosis-resistant human tumor cells induced rejection in SCID mice. These results suggest that human tumors do not express FasL nor are they able to suppress the proinflammatory effect of FasL *in vivo*.

3. MATERIALS AND METHODS

3.1. Vectors

A retroviral vector expressing human FasL, pPGSciteneo-hFasL, was kindly provided by Dr. Gary Nabel. This vector is generated by cloning the human FasL gene into the vector pPGSciteneo, which expresses a neomycin-resistant gene (50).

3.2. Cell lines

Human tumor cell lines (see Table 1) were maintained in media as recommended by American Type Culture Collection. CT26-hFasL, a murine colon cancer cell line expressing intact human FasL was kindly provided by Dr. Gary Nabel (50). The control cell line, CT26-neo, was established as previously described (36). SW620-hFasL, HT-29-hFasL, SW620-neo, and HT-29-neo cell lines were established by infecting the human colon cancer cell lines SW620 and HT-29 with the retroviral vector expressing the intact human FasL gene, pPGSciteneo-hFasL, or the control vector, pPGSciteneo. Stable cell lines were established after G418 selection.

3.3. Antibodies and reagents

Antibody G247.4, antibody clone 33 (mAb33), and antibody N20 were purchased from Pharmingen (San Diego, CA), Transduction Laboratories (Lexington, KY), and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Monoclonal antibody specific for α-tubulin was purchased from Lab Vision Corporation/NeoMarkers (Fremont, CA), and antibody specific for β-catenin, from Transduction Laboratories. The metalloproteinase inhibitor KB8301 was purchased from Pharmingen and recombinant human FasL was obtained from Upstate Biotechnology (Waltham, MA).

3.4. Immunoblot analysis of FasL expression

Tumor cells were harvested by scraping to avoid the digestion of surface protein. Cells were washed twice in 1 × PBS containing proteinase inhibitors (Roche, Indianapolis, IN). The cell pellets were dissolved in 2 × Laemmli buffer (100 mM Tris, pH 6.8, 4% SDS, 1.4 M mercaptoethanol), and the protein concentrations were determined using a Bio-Rad protein assay reagent (Hercules, CA). Equal amounts of total protein (50 μg each lane) were loaded and analyzed by SDS PAGE using a 4% to 15% polyacrylamide gel. After blocking with 5% nonfat dry milk in TBS, the membranes were incubated with anti-human FasL antibodies as described in the figure legends. The membranes were washed in 1 × TBS supplemented with 0.05% Tween 20 and incubated with HRP-conjugated goat antibody to mouse IgG_1 (1:2000, Santa Cruz Biotechnology). After washing in TBST, antibody binding was detected using chemiluminescence (Lumi-Light, Roche). The same membranes were then stripped and immunoblotted with α-tubulin-specific monoclonal antibody (1 μg/ml) or β-catenin-specific antibody.

3.5. ELISA

Plates were coated with purified mouse anti–human FasL (Pharmingen cat. no. 65431A, 250 ng/well), stored overnight at 4ºC, and then blocked with PBS

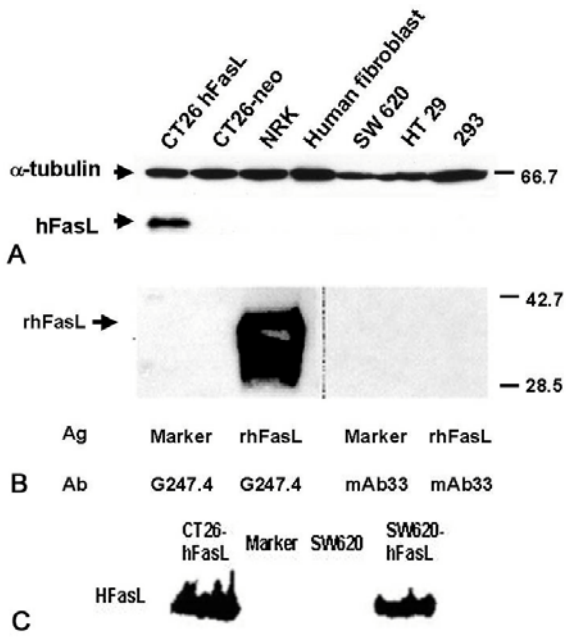

Figure 1. Specificity of anti-FasL antibody G247.4. *A. Antibody G247.4 recognized human FasL (hFasL) expressed in murine cells.* Total cell lysates from different cell types were prepared and detected by antibody G247.4 (2 μg/ml) or α-tubulin antibody. hFasL transfectant (CT26-hFAsL) was used as a positive control. *B. Antibody G247.4 recognized recombinant hFasL.* Recombinant hFasL was loaded in lanes 2 and 4 and detected by antibodies G247.4 (lane 2) and mAb33 (lane 4). *C. Antibody G247.4 recognized hFasL expressed in human tumor transfectant.* A human colon cancer cell line transfected with intact hFasL (SW620-hFasL) and the parental cell line SW620 were analyzed by Western blot.

containing 5% Tween 20, 5% sucrose, and 5% NaN_3. Supernatants from the cultured cell lines at confluence (200 μl/well) were added and incubated for 2 h at room temperature. Biotinylated mouse anti–human Fas ligand (Pharmingen cat. no. 65322A, 100 ng/well) was added subsequently. After washing, diluted HRP-straptavidin (Zymed [South San Francisco, CA], 1:20,000, 100 μl/well) was added and the color was read on an ELISA reader at 450 nm vs 570 nm.

3.6. Susceptibility of human tumor cells to human FasL

FasL-mediated cytotoxicity was assayed as previously described (36). The human tumor cells (10^6) were labeled with with sodium chromate Cr 51 (^{51}Cr) (Amersham Biosciences, Piscataway, NJ) for 2 h at 37ºC in 100 μl of medium containing 10% FCS. The ^{51}Cr-labeled target cells (10^4 cells) were mixed with CT26-hFasL or CT26-neo (effector - target ratio, 10 : 1) and cultured for 16 h at 37ºC in 5% CO_2/95% air. After centrifugation, the supernatants were collected with a harvesting frame (Skatrib, Sterling, VA) and assayed for radioactivity. The spontaneous ^{51}Cr release was determined by incubating the target cells with the medium alone, and the maximum ^{51}Cr release was determined by incubating the cells in 0.1% Triton X-100. The percentage of specific lysis was calculated as follows: experimental ^{51}Cr release – spontaneous ^{51}Cr release/maximum ^{51}Cr release – spontaneous ^{51}Cr release)

3.7. Functional Assay for FasL

To test whether human tumor cells express functional FasL, human tumor cells (10^5) were incubated with ^{51}Cr-labeled Jurkat cells (10^4) for 16 h. The supernatants were collected and assayed for radioactivity as described in previous paragraph.

3.8. Animal experiments

Animal experiments were carried out in accordance with both University of Michigan and National Institutes of Health animal care regulations. Eight-week-old female SCID and nude mice were obtained from Charles River (Wilmington, MA) and kept in a specific-pathogen-free environment. To examine FasL expression by SW620 tumor cells *in vivo*, SW620 tumor cells (5×10^6) were injected (s.c.) into nude mice. Tumors were collected 2 wk later for immunoblot analysis.

To investigate whether "FasL$^+$" human tumor cells can resist FasL-mediated inflammation *in vivo*, SCID mice were inoculated (s.c.) with SW620-hFasL (5×10^5) or SW620-neo (5×10^5) tumor cells. Tumors were measured after 28 days — the cross-sectional area was calculated from two-dimensional calipered measurements.

4. RESULTS

4.1. Specificity of anti-FasL antibodies

To verify the expression of FasL by human tumors, the specificity of anti-FasL antibodies for human FasL had to be confirmed. CT26-hFasL, a mouse colon cancer cell line that expresses intact human FasL, was used as a positive control. We determined that antibody G247.4 recognized both intact human FasL expressed in murine colon cancer cells and recombinant soluble human FasL (Figure 1*A*, *B*). We generated SW620-hFasL, a human colon cancer cell line infected with retroviral vector expressing human FasL, and confirmed that G247.4 also recognized intact human FasL when it was expressed in human tumor cells (Figure 1*C*). In contrast, antibody clone 33 (mAb33) could bind to proteins from all cells tested including CT26-neo, human fibroblast, and 293 cells that are susceptible to FasL (Figure 2*A*). Furthermore, mAb33 did not recognize recombinant soluble human FasL (Figure 1*B*). These results confirmed the poor antigen specificity of mAb33 for human FasL (45, 46). We also examined the specificity of antibody N20, and determined that it not only binds weakly to human FasL, but it also reacts with proteins of a similar molecular weight (Figure 2*B*). Because this nonspecific binding could be interpreted as FasL detection in the absence of intact human FasL as a positive control, we reevaluated FasL expression in human solid tumors, using human FasL-specific antibodies G247.4.

4.2. Human tumor cells do not express functional FasL

Twenty-two human solid tumor cell lines reported to be FasL$^+$ were used in this study (see Table 1)

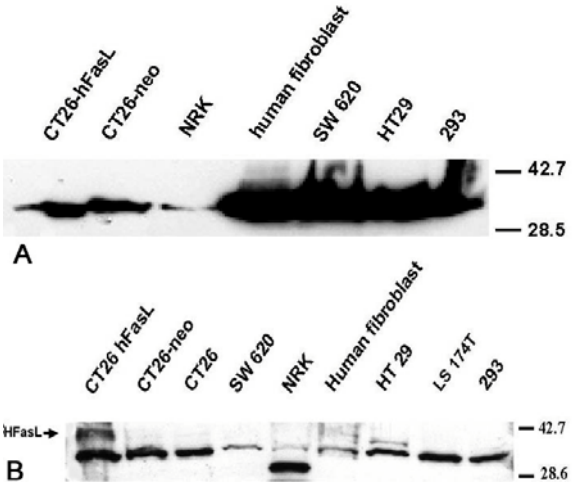

Figure 2. Specificity of anti-FasL antibodies mAb33 and N20. Total cell lysates from different cell lines were prepared and detected by antibody mAb33 (*A*) or by rabbit polyclonal anti-FasL antibody N20 (*B*). The poor antigen specificity of antibody mAb33 is evident. Antibody N20 binds to human FasL, but also has additional binding specificity. CT26-hFasL and CT26-neo were used as positive and negative controls.

(11–34). The tumors were colon cancer, pancreatic cancer, hepatocellular carcinoma, lung cancer, breast cancer, neuroblastoma, glioma, choriocarcinoma, and sarcoma. Western blot analysis of the total cell lysates using antibody G247.4 revealed that none of the 21 human tumor cell lines tested expressed FasL (Figure 3*A*, *B* and Table 1). Neither was human FasL detected in the cell lysates derived from tumors cultured with the metalloproteinase inhibitor KB830, which prevents the shedding of FasL from the cell membrane (data not shown). Considering that tissue culture may not be the optimal condition for FasL expression, SW620, a prototype $FasL^+$ tumor, was inoculated (s.c.) into nude mice. Analysis of the tumors 2 wk later revealed an absence of FasL expression (Figure 3*C*). Furthermore, the soluble form of FasL was not detected in any of the culture supernatants examined by ELISA. (Figure 3*D*, Table 1). To confirm that these human tumor cells do not express FasL, a functional assay was performed. Human tumor cells were incubated with Jurkat cells, which are sensitive to FasL-mediated killing. CT26-hFasL and CT26-neo cells were used as positive and negative controls. None of the 22 human tumor cell lines tested were able to kill Jurkat cells in two independent experiments (Figure 4, Table 1). In addition, SW620 tumor tissues derived from nude mice had no cytotoxic effect on Jurkat cells (data not shown). These results suggested that human tumors do not express functional FasL.

4.3. Majority of human tumor cells were susceptible to FasL-mediated killing

Although we demonstrated that the human tumor cell lines did not express functional FasL *in vitro*, expression in FasL *in vivo* had not been ruled out. To better evaluate this possibility, we examined the susceptibility of human tumor cells to FasL, since any tumor cell sensitive to FasL-mediated killing would be incapable of expressing FasL. CT26-hFasL was used to test the susceptibility to FasL, because a recent study revealed that only membrane-bound FasL consistently triggered apoptosis, whereas anti-Fas antibodies did not reliably mimic FasL (50). In this assay, ^{51}Cr-labeled human tumor cells were incubated with CT26-hFasL or CT26-neo (36). Greater than 77% of the tumors were susceptible to FasL, with greater than 30% specific lysis (Figure 5*A*, Table 1). To confirm that tumors sensitive to FasL cannot express FasL, the cell line SW620, which demonstrates 35% specific lysis, was infected with a retroviral vector expressing human FasL. As expected, a high rate of cell death was observed postinfection compared to control retroviral infection (data not shown). Nevertheless, we were able to establish a stable cell line expressing human FasL, SW620-hFasL (Figure 1*C*). SW620-hFasL was completely resistant to FasL-mediated killing (Figure 5*B*), suggesting that only those tumors completely resistant to FasL could express FasL. These results indicated that about 77% of those tumors previously described as $FasL^+$ could not express FasL.

4.4. Expression of FasL induced rejection of human tumor cells in SCID mice

To investigate whether "$FasL^+$" human tumor cells can resist FasL-mediated inflammation *in vivo*, SCID mice were inoculated (s.c.) with SW620-hFasL tumor cells. Tumors did not grow in 8 of 8 mice. In contrast, all the mice inoculated with SW620-neo tumor cells developed large tumors (Figure 6*A*). Histologic analysis revealed a destruction of SW620-FasL tumor cells and inflammation within 48 h after inoculation, in contrast to the robust tumor growth of SW620-neo cells (data not shown). Because tumors that are spontaneously resistant to FasL-mediated apoptosis are more likely to express FasL, an HT-29-hFasL cell line was established by transducing HT-29 cells with a retroviral vector expressing human FasL. HT-29-hFasL cells were rejected in SCID mice, while the parental tumor cells transduced with control virus grew well (Figure 6*B*). These results suggested that human tumors cannot suppress FasL-mediated inflammation *in vivo*.

5. DISCUSSION

The expression of FasL to counteract antitumor immunity is an important and novel concept in tumor immunology. More than 70 articles supporting this hypothesis have been published to date (1, 2). However, recent studies have also revealed that FasL stimulates a proinflammatory reaction (35, 36). Tumors transfected with FasL interact directly with neutrophils to mediate tumor destruction (37). The expression of FasL on myotubes or pancreatic islets of transgenic mice induces a granulocytic response that accelerates graft rejection (38, 39, 52). Our previous research had revealed that some cytokines such as TGF-β suppress the proinflammatory effect of FasL, thus facilitating the growth of the $FasL^+$ tumor transfectant *in vivo* (37). These results suggested that human tumors either do not express FasL or they have the ability to suppress the proinflammatory effect of FasL *in vivo*.

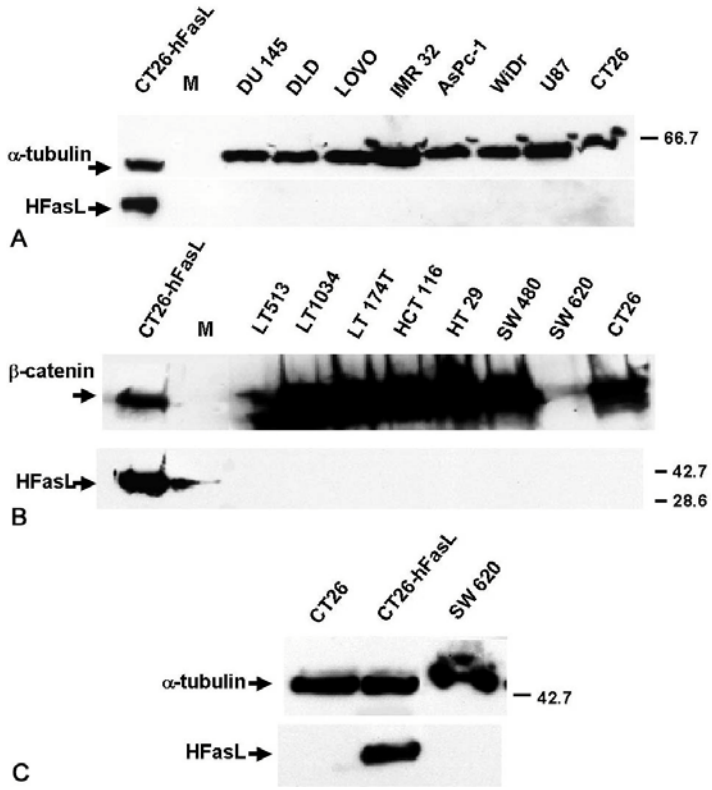

Figure 3. Human tumor cell lines do not express human FasL. *A. Western blot analysis did not detect human FasL (hFasL) expression in any of the 21 human tumor cell lines studied.* Total cell lysates from the human tumor cells were prepared and analyzed by antibody G247.4 (lower panel) and α-tubulin antibody (upper panel). A representative assay is shown. *B. hFasL expression not detected in human colon cancer cells.* Total cell lysates of human colon cancer cells were prepared and analyzed by antibody G247.4 (lower panel) and β-catenin antibody (upper panel). *C. FasL was not detected in human tumor mass derived from nude mice.* Human colon cancer cells SW620 (5×10^6) were injected (s.c.) into nude mice. The tumor tissues were collected and analyzed by antibody G247.4 (lower panel) and α-tubulin antibody (upper panel). *D. Soluble hFasL was not detected in the supernatants of the human tumor cell lines.* Supernatants from 22 human tumor cell cultures were collected and analyzed by ELISA. The tumor cells were cultured in the absence of metalloproteinase inhibitor. A representative assay is shown.

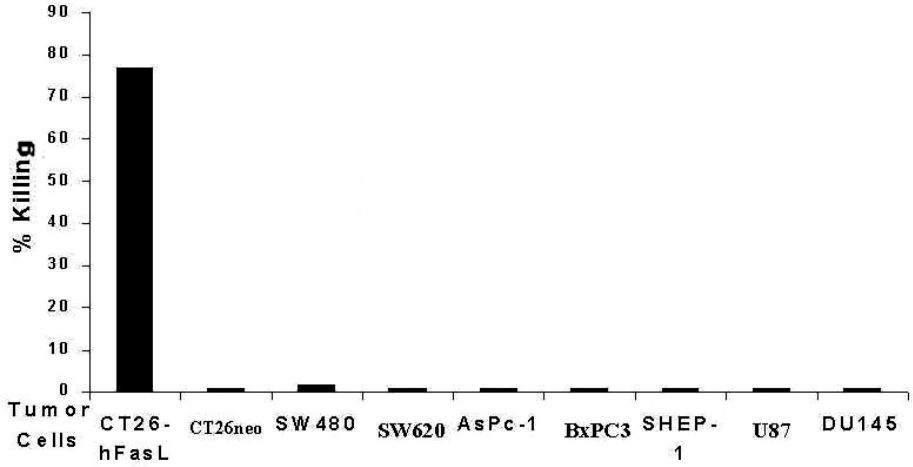

Figure 4. Functional assay for FasL. Human tumor cells do not have cytotoxic effects on Fas^+ Jurkat cells. Human tumor cells (10^5) were incubated with of ^{51}Cr-labeled Jurkat cells (10^4) for 16 h as described in *Materials and Methods*. Twenty-two human tumor cell lines were tested at least twice (see summary of results, Table 1). A representative assay is shown. CT26-hFasL and CT26-neo were used as positive and negative controls.

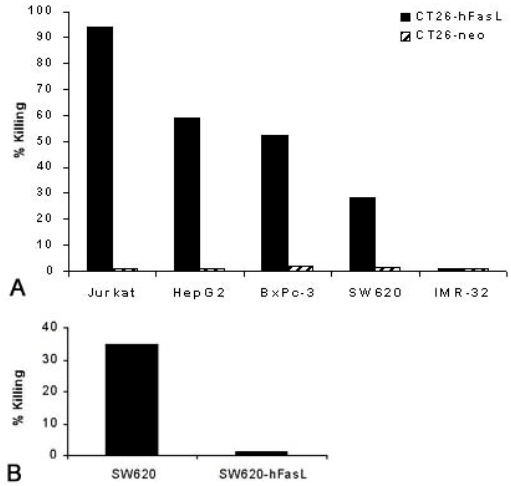

Figure 5. Susceptibility of human tumor cells to human FasL. *A. Most of the human tumor cells were sensitive to FasL-mediated killing.* ^{51}Cr-labeled human tumor cells (10^4) were incubated with 10^5 of CT26-hFasL or CT26-neo cells for 16 h as described in *Materials and Methods*. Each cell line was tested at least twice (see summary of results, Table I). A representative assay is shown. *B. SW620-hFasL cells resisted FasL-mediated killing.* The human colon cancer cell line SW620 expressing human FasL (SW620-hFasL) was established as described in *Materials and Methods*. SW620-hFasL and control cells SW620 (SW620-neo) were labeled with ^{51}Cr and incubated with CT26-hFasL or CT26-neo cells for 16 h.

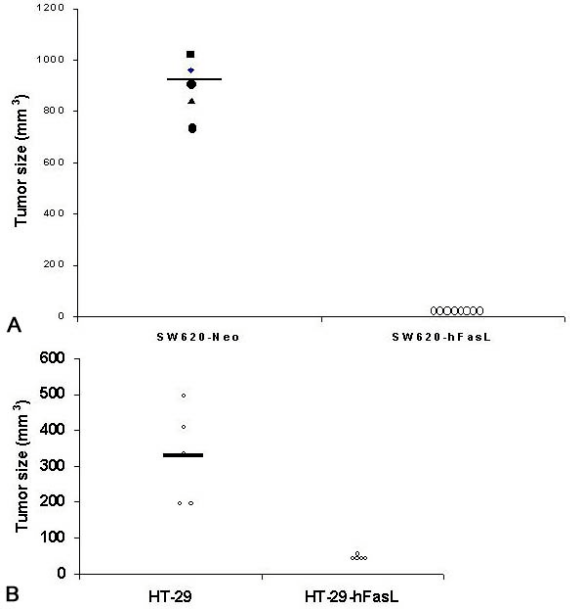

Figure 6. Inability of tumors reported as FasL$^+$ to suppress proinflammatory effects of FasL *in vivo*. *A. SW620-hFasL cells rejected in SCID mice.* SCID mice were inoculated (s.c.) with SW620-hFasL (5×10^5) or SW620-neo (5×10^5) tumor cells. The tumor sizes were measured 28 days after tumor inoculation. The tumor cross-sectional area was calculated from two-dimensional calipered measurements. *B. HT-29-hFasL cells rejected in vivo.* SCID mice were inoculated (s.c.) with HT-29-hFasL (2×10^6) or HT-29-neo (2×10^6) tumor cells. The tumor sizes were measured 17 days after tumor inoculation.

We were unable to detect FasL protein or demonstrate the function of FasL in 22 human tumor cell lines previously reported to express FasL. The discrepancy between our results and previous studies is most likely due to the poor specificity of antibodies used and the lack of appropriate positive controls. The use of non–intron-spanning PCR primers without proper controls may also have led to false-positive results (44). Furthermore, the JAM test, a widely used functional assay, is prone to false-positive detection of apoptosis when adherent tumor cells are used as effectors (53).

The assessment of *in vivo* expression of FasL by human tumors has proven to be difficult. Contamination of lymphocytes in the tumor samples and the poor specificity of antibodies can produce false-positive results. The expression of FasL mRNA may not correlate well with protein expression. In the current study, we used a different approach to test FasL expression *in vivo*. Spontaneous expression of FasL *in vivo* requires resistance to FasL-mediated apoptosis, and resistance to rejection mediated by FasL-induced inflammation. We found that most human tumor cells are sensitive to FasL-mediated apoptosis, and only tumors completely resistant to FasL could survive in the presence of FasL. Our results suggested that less than 25% of tumors could possibly express FasL *in vivo*. In addition, we found that FasL expression induced rejection of human colon cancer cells SW620 and HT-29 in SCID mice, which suggests that these tumors cannot suppress the proinflammatory effect of FasL *in vivo*. The inability of human tumor cells to suppress the proinflammatory effect of FasL further decreases the possibility that human tumors express FasL *in vivo*.

Although we could not totally exclude the possibility that some human tumors express FasL *in vivo*, our findings strongly suggest that tumors able to express FasL and resist both FasL-mediated apoptosis and inflammation are rare or nonexistant. Recently, Kurooka et al.(54) reported FasL was detected in tumors in vivo at levels up to 10^4-fold higher than those in cell culture and predominantly in host tumor-infiltrating macrophages, but not in tumor cells, consistent with the findings reported herein. We conclude that the expression of FasL is not a main mechanism that tumors use to counteract antitumor immunity.

6. ACKNOWLEDGMENTS

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