Simultaneous Expression of Fas and Nonfunctional Fas Ligand in Ewing’s Sarcoma

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ABSTRACT

Fas-Fas ligand interactions play a central role in the regulation of the immune response. Fas ligand expression by tumors has been implicated in the abrogation of the host antitumor response by killing of Fas-positive effector lymphocytes. We have studied the presence and functional status of Fas and Fas ligand in Ewing’s sarcoma. All Ewing’s sarcoma cell lines tested expressed Fas on their surface. Three of the cell lines were readily killed after ligation of the Fas receptor. Four additional cell lines exhibited Fas-mediated apoptosis after preincubation with IFN-γ and/or cycloheximide, whereas two cell lines were resistant to Fas-mediated killing. With regard to Fas ligand, all cell lines examined were positive for protein by immunoblot, and specificity was confirmed by reverse transcription-PCR. However, using flow cytometric analysis, Fas ligand could only be detected in Ewing’s sarcoma cells after permeabilization. Furthermore, the cell lines were not capable of inducing apoptosis of Fas-sensitive Jurkat cells. In addition, Ewing’s sarcoma cell lines were able to serve as stimulators for the generation of cytotoxic effector lymphocytes and were susceptible to lysis by them. Therefore, Fas ligand is expressed in Ewing’s sarcoma but is not functional, suggesting that Ewing’s sarcoma is a potential target for immunotherapy.

INTRODUCTION

Ewing’s sarcoma (ES) is the second most common bone tumor in children (1). Unfortunately, the prognosis for patients with large primary tumors or metastatic disease treated with conventional therapy is poor (2, 3). Because of this, new biological-based therapies are presently under study, including attempts to develop immunotherapy that targets the tumor-specific translocation (4). Although the cell of origin of ES remains unclear, prevailing concepts hold that these tumors arise from genetic changes within primitive neuroectodermal tissues, which lead to clonal proliferation and a loss of normal differentiative capacity (5, 6). Because apoptosis is widespread during embryonal development and represents a critical component of normal tissue differentiation (7), it is possible that defects in apoptotic pathways might exist in ES. Indeed, blockades in the pathways responsible for programmed cell death can directly contribute to neoplastic transformation and have been implicated in resistance to chemo- and radiotherapy (8, 9).

The Fas-FasL system (also known as Apo-1/Apo1-Ligand or CD95/CD95L) has been recognized as a major pathway for the induction of apoptosis in lymphoid cells (10, 11). In activated lymphocytes, cross-linking of Fas via its natural ligand or an agonistic anti-Fas antibody rapidly induces apoptosis in sensitive cells by activating a death-signaling cascade (12, 13). Humans and mice with germ-line mutations in Fas have a profound defect in apoptosis of activated T cells, giving rise to a syndrome, which is characterized by lymphadenopathy and autoimmune manifestations (14–18). In malignant tissues examined thus far, there is variable expression of Fas on both lymphoid and nonlymphoid tumors (19, 20). Importantly, in many tumors examined, Fas-expressing cells are resistant to the induction of death by Fas cross-linking, implying alterations in the death-signaling cascade (21, 22).

FasL belongs to the tumor necrosis factor/nerve growth factor family of ligands (23) and was initially described on activated lymphocytes, where it can induce T cell and natural killer cytotoxicity in Fas-sensitive target cells (24, 25). Recently, FasL has also been described on a variety of tumors, including melanoma, astrocytoma, and lung cancer (26–31). Fasl-bearing tumors and tumor cell lines have been demonstrated to kill Fas-sensitive target cells in vitro. When Fasl-positive melanoma cells were injected into mice, tumors developed more rapidly in wild-type mice compared with Fas-deficient lrp mutants, providing evidence that functional Fasl-mediated deletion of Fas-positive lymphocytes can promote immune escape by these tumors (26).

To address the integrity of the Fas-dependent pathway of programmed cell death in ES and to study whether ES cells can evade the immune response by Fasl-mediated lysis of Fas-positive effector lymphocytes, we performed an analysis of the Fas/Fasl system in a series of ES cell lines.

MATERIALS AND METHODS

Cells and Cell Lines. ES cell lines A4573, CHP-100, JR, RD-ES, SK-N-MC, TC32, TC71, and 5838 were kindly provided by Dr. Jeff Toretsky (Pediatric Oncology Branch, National Cancer Institute). All cell lines, except JR and SB, which were derived from two patients with ES diagnosed at our institution, have been reported previously (32–36). Cell lines RD-ES and SK-N-MC are also available from American Type Culture Collection (Rockville, MD). The mouse/rat T-cell hybridoma D11s was kindly provided by Dr. Pierre Henkart (Experimental Immunology Branch, National Cancer Institute; Ref. 37). All cell lines were maintained in RPMI supplemented with 10% FCS and 100 units/ml penicillin, 100 μg/ml streptomycin, and 4 μM glutamine. Human PBMCs were obtained by Ficoll-Hypaque centrifugation of buffy coats from normal donors at the Department of Transfusion Medicine of the Clinical Center of the NIH. T-cell purification for analysis of FasL was performed via affinity selection as described by the manufacturer (R&D Systems, Minneapolis, MN).

Flow Cytometric Analysis. For staining of Fas, cells were suspended in FACS-buffer (HBSS containing 0.1% human albumin and 0.4% sodium azide) and incubated for 30 min at 4°C with FITC-labeled anti-human Fas antibody, clone DX2 (PharMingen, San Diego, CA). Cells were then washed and immediately analyzed on a FACScan analyzer (Becton Dickinson, Mountain View, CA). Dead cells were excluded via staining with propidium iodide (5 μg/ml).

For detection of surface Fasl, all cells were pretreated with the metalloprotease inhibitor KB8301 (PharMingen) for 4 h at 10 μM to prevent cleavage of Fasl. Purified T cells, used as a positive control, were stimulated with 1 μM ionomycin (Sigma Chemical Co., St. Louis, MO) for 4 h. Ewing’s sarcoma cells were detached with 5 mM EDTA. Cells were stained for 30 min at 4°C with a purified FasL antibody, clone NOK-1 (PharMingen), or mouse IgG1 as an isotype-specific control (Caltag, Burlingame, CA). Cells were then washed and incubated for 20 min with Streptavidin-PE (Caltag), washed twice in FACS-buffer, and immediately analyzed via flow cytometry. For analysis of intracellular Fasl, cells were detached with 5 mM EDTA and incubated in FACS-buffer (HBSS containing 0.1% human albumin and 0.1% sodium azide) for 4 h at 4°C with a purified FasL antibody, clone NOK-1 (PharMingen), or mouse IgG1 as an isotype-specific control. Cells were then washed and immediately analyzed on a FACScan analyzer. Dead cells were excluded via staining with propidium iodide (5 μg/ml).

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4 The abbreviations used are: ES, Ewing’s sarcoma; Fasl, Fas ligand; PBMC, peripheral blood mononuclear cell; FACS, fluorescence-activated cell sorter; RT-PCR, reverse transcription-PCR; MTt, 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenylterazolium bromide.

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EDTA, washed, then resuspended in FACS-medium, which was supplemented with 0.3% saponin (Sigma) and incubated with FasL antibody, clone G247-4 (PharMingen) or mouse IgG1 (Caltag) as an isotype-control for 30 min at 4°C. Cells were then washed, incubated with an FITC-labeled anti-mouse IgG (Caltag) for 20 min at 4°C, washed twice, and analyzed via flow cytometry.

RT-PCR Analysis and DNA Sequencing. RT-PCR was performed as suggested by the manufacturer (Perkin-Elmer, Foster City, CA). Total RNA was extracted from cells using the Trizol reagent (Life Technology, Inc., Gaithersburg, MD). For detection of FasL, mRNA was used as template and was isolated using the Poly(A)Purix kit (Ambion, Austin, TX) according to the manufacturer’s instruction. First-strand cDNAs were synthesized from 1 μg of RNA in 20-μl reactions using random primers in the presence or absence of reverse transcriptase. First-strand cDNA (1 μl) was amplified using Taq polymerase in a 100-μl reaction volume. For detection of FasL, a second, nested PCR was performed using 1 μl of the PCR product in a 100-μl reaction volume. The conditions and gene-specific primers have been published previously (29, 38). Briefly, for detection of Fas, the following primers were used: Fas sense, 5′-ACTGCGTGCCCTGGCAAGGAGT-3′; and Fas antisense, 5′-AAAGCACAAGGCCCCCAAGTTAGA-3′. The amplifying conditions included 30 cycles of the following: denaturation for 30 s at 94°C, annealing for 60 s at 60°C, and extension for 60 s at 72°C. Primers for FasL for the first-step amplification PCR were: FasL sense, 5′-CACGCTGCATGAGGGGCTCT-CTCAATTACCATAT-3′; and FasL antisense, 5′-CTTTATTGCCT- TATAAGGCAAAGCAGTGGATTTC-3′. For the second, nested round PCR, FasL sense, 5′-GGTTCTTGTTGGCCTTGAAGTTTGA-3′; and FasL antisense, 5′-AGGCGAATAACATCTGAGTTCCTC-3′. In both amplification procedures, 35 cycles of denaturation for 30 s at 95°C, annealing for 60 s at 45°C, and extension for 2 min at 72°C were used. Samples were analyzed on a 0.5% agarose-Tris-acetate-EDTA gel stained with ethidium bromide.

For confirmation, 1 μl of each RT-PCR product was treated with shrimp alkaline phosphatase and exonuclease 1 prior to direct sequence analysis using the Thermo Sequenase kit (Amersham, Cleveland, OH). Thermocycling conditions were 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Three primers were used to analyze the PCR products and confer sequence identity with the published sequence. These included the following: the FasL primer pair from the second round PCR, corresponding to nucleotides 331–355 and 882–896 of the human FasL; and the primer 5′-CAGCTCAAGGGGCTCTC-3′, corresponding to nucleotides 525–544.

Western Blot Analysis. Protein was extracted from cells by detergent lysis in a buffer containing 10% SDS. The lysate was boiled for 10 min, and cellular debris was then removed by centrifugation (10 min at 14,000 rpm). The protein concentration then was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Melville, NY). Forty μg of protein were boiled for 10 min before loading on a 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and then blocked for 30 min in PBS containing 5% dry milk and 0.05% Triton X-100 (Sigma). Membranes were incubated with primary antibodies (rabbit anti-human Fas, clone C-20, and rabbit anti-human FasL, clone N-20; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer for 1 h, washed three times with PBS/Triton, incubated for an additional 30 min with a goat anti-rabbit IgG-antibody conjugated to horse radish peroxidase at a 1:2000 dilution, washed three times with PBS/Triton, and then developed using enhanced chemoluminescence (Amersham, Arlington Heights, IL).

Chromium-Release Assay. Cell lines were labeled with 100 μCi (51Cr) sodium chromate (Amersham) for 2 h at 37°C, washed twice in RPMI/10% FCS, and then incubated for 30 min at room temperature to minimize spontaneous release. After a final wash, labeled cells were plated at 3000 cells/well. Where indicated, anti-human Fas antibody (clone ZB4 from Immunootech, Marseille, France; or clone DX2 from PharMingen) was added 30 min before adding the effector cells to block FasL-mediated cytotoxicity. Effector cell lines were detached using 5 mM EDTA, washed once in PBS, and then added to the target cells at the indicated E:T ratios in round-bottomed microtiter plates. For detection of lysis of Ewing’s sarcoma cell lines by D11s, the cells were cocultured for 4 h. For detection of lysis of Jurkat by ES cell lines, the incubation period was 20 h at 37°C in 5% CO2 incubator.

MTT Assay. To assess Fas-mediated cytotoxicity, cells were plated in triplicate in flat-bottomed wells at 2,500 cells/well (controls) and 10,000 cells/well (INF-γ treated) in 96-well microtiter plates and allowed to attach to the plate. After an overnight incubation, INF-γ (NCI Frederick, MD) was added where indicated at 2000 units/ml. Cells were incubated for 96 h before adding anti-Fas mouse monoclonal IgM antibody at 1 μg/ml (clone CH-11; MBL, Nagoya, Japan) and cycloheximide at 1 μg/ml (Sigma) where indicated. Sixteen h after application of anti-Fas antibody, cell viability was assessed by the MTT dye reduction assay as described previously (39).

Generation of Cytotoxic Effector Cells. ES cell line TC71 was irradiated at 13,000 Gy and plated at 2 × 106 cells/well in a 24-well plate. PBMCs from a healthy donor (Allol) were used as responder cells and added at 4 × 106 cells/well. As a control, PBMCs from an allogeneic donor (Allo2) were irradiated at 3000 Gy, plated as above, and cocultured with PBMCs from donor Allol. The cells were cocultured for 6 days at 37°C with 5% CO2. Allogeneic responder cells from the respective cocultures were then used as effector cells in a chromium release assay against labeled targets at the indicated E:T ratios.

RESULTS

Fas Expression in ES Cell Lines. Cell surface analysis using flow cytometry revealed Fas expression in all nine cell lines examined (Fig. 1A). However, the intensity of Fas expression was heterogeneous, with several of the cell lines showing low level expression. To confirm the variable level of expression and to provide further evidence for Fas expression in those cell lines showing minimal fluorescence above background, Western analysis of Fas protein expression and RT-PCR for detecting Fas mRNA were performed. As shown in Fig. 1, B and C, all cell lines tested expressed message for Fas receptor, with heterogeneity in the level of message present which generally correlated with the flow cytometry data. Similar results were seen using a polyclonal antibody to Fas in an immunoblot. These results show that Fas is expressed in all ES cell lines tested but at variable levels.

Fas-Induced Apoptosis in ES Cell Lines. Because Fas expression does not necessarily correlate with sensitivity to Fas-mediated lysis (21, 22), it was important to investigate whether ES cell lines were susceptible to Fas-mediated death and whether this correlated with the level of Fas expressed on the cell surface. To test Fas-mediated killing in ES cells, a chromium-release assay was performed using the mouse/rat T-cell hybridoma D11s as a FasL-positive effector cell. Three of the nine cell lines, TC32, RD-ES, and SK-N-MC, were killed in a 4-h incubation (Fig. 2A). The cytotoxicity could be inhibited when the target cells were incubated with the anti-human Fas antibody DX2. Chromium-release could also be inhibited when the target cells were incubated with caspase inhibitors ZVAD-fmk or BD-fmk (data not shown). Therefore, the signaling pathways required for induction of apoptosis via Fas are intact in at least some ES cell lines. Interestingly, the three cell lines susceptible to killing were among those cell lines showing the highest expression of Fas receptor by flow cytometry, suggesting that Fas sensitivity correlated with the level of Fas expression and that the remaining cell lines might be converted to become Fas-sensitive by up-regulating Fas. Alternatively, Fas-resistant cell lines may display a blockade in intracellular signaling pathways responsible for programmed cell death, independent of the level of surface Fas expression.

To study the degree to which surface expression levels and/or intracellular changes resulted in Fas resistance, ES cell lines were incubated with INF-γ for 96 h and then exposed to the anti-Fas antibody CH-11 in the presence or absence of cycloheximide. INF-γ has been shown to increase the susceptibility to Fas-mediated lysis in T lymphocytes and various tumor cell lines (40–42). This effect has been associated with both up-regulation of Fas receptor and increased expression of caspases. Cycloheximide has also been shown to render Fas-expressing tumor cells susceptible to Fas-mediated lysis, presumably by blockade of a short-lived inhibitor (43). As demonstrated in
Fig. 1. Expression of Fas in ES cell lines. A, flow cytometry for Fas surface expression. ES cell lines were stained with FITC-conjugated anti-Fas ab DX2 (open curve) or with FITC-conjugated isotype control antibody (filled curve). Net mean fluorescence intensities: TC32, 21.1; RD-ES, 9.8; SK-N-MC, 10.5; JR, 20.5; A4573, 10.0; CHP-100, 7.9; TC71, 3.1; 5838, 2.5; and SB, 1.9. B, expression of Fas mRNA by RT-PCR analysis. All nine ES lines expressed Fas mRNA. GAPDH is included as a positive control. C, detection of a M, 45,000 (45kD) protein by immunoblot corresponding to Fas. Jurkat cells were used as a positive control.

Fig. 2B, IFN-γ rendered the majority of cell lines (excluding RD-ES, JR, and SB) more susceptible to Fas-mediated cell death. Interestingly, with the exception of one cell line (TC71), this occurred despite a lack of up-regulation of surface Fas expression (data not shown), suggesting that Fas resistance in ES cell lines is related to attenuation within intracellular pathways rather than low level surface expression. Similarly, the addition of cycloheximide alone was sufficient to increase susceptibility to Fas-mediated death in TC32, SK-N-MC, RD-ES, and CHP-100. Finally, the combination of IFN-γ and cycloheximide in many cases enhanced susceptibility to death to a greater extent than either agent alone, suggesting that these agents modify different molecules within the intracellular Fas signaling pathways. No effect was seen in cell lines JR and SB with persistent resistance to Fas-mediated cell death, despite significant surface expression of Fas by JR.

Therefore, ES could be grouped according to three types: Fas-sensitive (RD-ES, SK-N-MC, and TC32); Fas-inducible (A4573, CHP-100, TC71, and 5838); and Fas-resistant (JR and SB). Interest-
Fig. 2. Killing of ES cell lines via the Fas pathway. A, 4-h chromium release assay with 51Cr-labeled ES as target and D1s cells as effectors in the absence (□) or presence (○) of the anti-Fas antibody DX2. The mean of triplicates (if >2%) is indicated; bars, SD. The same pattern was seen in three different experiments. B, MTT assay for cell viability. Cells were preincubated with or without IFN-γ (2000 units/ml) for 96 h and then incubated with anti-Fas antibody clone CH-11 (1 μg/ml) for 18 h in the presence or absence of 1 μg/ml cycloheximide. The viability of anti-Fas-treated cells was compared with respective controls that did not receive anti-Fas antibody treatment. The means of three replicates are indicated; bars, SD. The same pattern was seen in three different experiments.
FasL Expression in ES Cell Lines. FasL expression has been described recently in various different tumors and tumor cell lines (26–31). To examine FasL expression in ES, immunoblot analysis using a polyclonal anti-FasL antibody was performed. Using this approach, all cell lines were found to express FasL protein (Fig. 3A). However, because of reports of nonspecificity of anti-FasL antibodies (45), it was important to confirm FasL expression using molecular techniques. To do this, analysis for FasL mRNA was performed using a nested RT-PCR technique. All nine cell lines showed evidence of FasL expression; Fig. 3B shows five of them. The identity of the PCR products was confirmed by DNA sequencing. No mutation was found in the sequenced region, which encompasses nucleotides 355–872 and encodes a large part of the transmembrane portion and the extracellular domain of the human FasL (23, 46). Therefore, it is clear that ES cell lines express FasL simultaneously with expression of Fas. Furthermore, in the case of cell lines RD-ES, SK-N-MC, and TC32, cells remain viable despite the presence of an intact Fas-mediated programmed cell death pathway. These results suggested that FasL may be nonfunctional in these cell lines.

FasL Expressed by Ewing's Sarcoma Can Only Be Detected after Permeabilization of Cells. To address whether FasL was expressed on the cell surface, ES cell lines were stained with monoclonal anti-FasL antibodies and analyzed by flow cytometry. Calcium ionophore-treated T lymphocytes were used as a positive control. Because FasL has been shown to be readily cleaved by metalloproteinases (47), a metalloproteinase inhibitor was added to the cells 4 h before processing. No evidence for surface expression on ES cell lines was found using conventional surface staining, whereas FasL was readily detected on activated T lymphocytes with the NOK-1 antibody (Fig. 4). To detect intracellular expression of FasL, flow cytometry was performed after permeabilization of cells using saponin. Using this method, FasL was detected in all ES with the G247-4 antibody, whereas minimal intracellular expression of FasL was observed in T lymphocytes. Intracellular expression was also obtained with the antibody clone 33 (data not shown). Therefore, these results suggest that FasL in ES cells may be predominantly expressed intracellularly, resulting in functional incompetence. Interestingly, the NOK-1 antibody was able to detect surface FasL but not intracellular FasL, whereas the antibodies G247-4 and clone 33 stained only intracellular FasL. This could be explained if the NOK-1 antibody recognizes an epitope that might be sensitive to treatment with detergent, whereas the other two antibodies have higher affinity for FasL after treatment with detergent.
FasL Expressed by ES Cell Lines Is Functionally Inactive.
Although we could find no evidence for surface expression of FasL by ES cell lines, it was important to rule out whether either low level expression or secreted FasL could function to induce cell death. To do this, we investigated whether ES cell lines were able to kill Fas-positive Jurkat cells, as has been described for other tumor cell lines (27, 29). As shown, $^{51}$Cr-labeled Jurkat cells are susceptible to FasL-mediated death when incubated with D11s at an E:T ratio of 5:1 (Fig. 5). ES cell lines, preincubated with a metalloproteinase inhibitor to avoid cleavage by metalloproteases, however, were not able to kill Jurkat cells, even at an E:T ratio of 80:1. Furthermore, although Fas-mediated death by chromium release is readily seen within 4 h, in these assays no killing was detected despite coculture for 20 h. To test for activity of soluble FasL, which has been shown to be far less active than the membranous form and even has been demonstrated to inhibit apoptosis by the latter form (48, 49), a different experimental setting was chosen. ES cells and D11s were plated in a 96-well plate and grown to subconfluency before adding $^{51}$Cr-labeled Jurkat cells. Again, ES cells were not able to lyse labeled Jurkat cells (data not shown).

FasL-Expressing ES Cells Are Killed by Lymphocytes. The observation that some tumor cells express FasL on their surface and are able to kill sensitive cells in a Fas-dependent manner has led to the hypothesis that such tumors might escape the immune system by destroying tumor-specific lymphocytes (26–28). Although the ES cell lines were not able to kill Fas-sensitive Jurkat cells, we attempted to confirm their inability to abrogate the development of an immune response by normal T cells. To test this, ES cells were irradiated and cocultured with PBMCs from a healthy allogeneic donor for 6 days. As a control, PBMCs of the same donor were cocultured in a mixed lymphocyte reaction with allogeneic irradiated PBMCs. After 6 days, the proliferating cells were used as effector cells in a chromium release assay. As shown in Fig. 6, lymphocytes cocultured with the ES line TC71 (Alol@TC71) generated immune allospecific effectors capable of lysing the ES cell line TC71. Therefore, FasL expressing ES cell lines do not clonally delete alloreactive T cells during the development of an immune response and are susceptible to immune-mediated killing. Interestingly, this result also demonstrates the susceptibility of even the Fas-resistant ES cell line TC71 to immune-mediated killing, which presumably occurs via the perforin/granzyme-dependent pathway.

DISCUSSION
The data presented systematically address the expression and functional status of the Fas/FasL system in ES, using a panel of nine cell lines. Our results show that Fas and FasL are expressed uniformly in all ES cell lines studied. Importantly, primary ES tumors have also been shown to express both Fas and FasL by immunohistochemistry, suggesting that our observations are not unique to long-term cell cultures. These results point out, however, since functional data cannot be easily obtained from the study of primary tumors, that the significance of Fas/FasL expression in primary tumors in the absence of functional data must be interpreted with caution.

5 M. Tsokos, personal communication.

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**Fig. 5.** Absence of killing of Jurkat cells by FasL-positive ES cell lines. ES cell lines were used as effectors and cocultured with chromium-labeled, Fas-expressing Jurkat cells for 20 h at an E:T ratio of 80:1. FasL-expressing D11s cells were used as a positive control and were coincubated with Jurkat cells in the presence or absence of the Fas-blocking antibody clone ZB4 (0.5 μg/ml) at an E:T ratio of 5:1. The same pattern was seen in three different experiments. Bars, SD.

**Fig. 6.** Killing of ES by allogeneic cytotoxic effector lymphocytes. Cytotoxic effector lymphocytes were generated by coculture with irradiated ES cells for 6 days and then used as effectors (○, Alol@TC71) against fresh chromium-labeled targets of the same cell line. Cytotoxic effector cells (○, Alol@Allo2) from a mixed lymphocyte reaction were used as a control. Values are means of triplicates; bars, SD. Similar results were obtained in two different experiments.
Our results demonstrate that there is remarkable heterogeneity between the cell lines studied with regard to sensitivity to Fas-mediated cell death, thereby allowing us to divide the ES cell lines into three groups: (a) group 1, Fas-sensitive; (b) group 2, Fas-inducible; and (c) group 3, Fas-resistant. The three cell lines of group 1 could be killed directly by FasL-bearing D11s cells. Interestingly, these cell lines could not be killed by anti-Fas antibody in an MTT assay. Because the chromium release assay is performed with cells in suspension and cells are adherent in the MTT assay, a modulation of the Fas pathway dependent on adherence could account for this phenomenon. However, other factors such as differences in the potency of the rat FasL expressed by D11s versus the activity of the anti-Fas antibody CH-11 cannot be ruled out.

The second group of four cell lines required preincubation with IFN-γ and/or concomitant use of cycloheximide. IFN-γ has been shown to sensitize to Fas-mediated death in a variety of tumor cell systems, including glioblastoma, colon cancer, and breast cancer (40–42). Whereas this effect is associated with the up-regulation of Fas in some of the cell lines studied, the up-regulation of various proapoptotic proteins of the caspase and bcl-2 family has been incriminated in sensitization to Fas-mediated death in others. For example, it has been shown recently that sensitization of various breast cancer cell lines to IFN-γ correlated with increased expression of ICE (Caspase-1) but not Fas, and that transfection of ICE in these cells was able to replace the need for IFN-γ in sensitizing these cell lines to Fas-mediated death (41). Because no significant up-regulation of Fas with IFN-γ treatment is seen in our cell lines, with the exception of cell line TC71, we hypothesize that IFN-γ modulates the expression of downstream genes involved in the Fas pathway in a proapoptotic manner.

Cycloheximide has been required for Fas sensitization for a variety of tumors, including neuroblastoma and breast cancer (41, 50). It is presently unclear to what extent the mechanism of action of cycloheximide relies on its action as a protein synthesis inhibitor and to what extent on the activation of Jun kinases (51). Activation of the latter pathway has been shown to be required for Fas-mediated cell death (52). Importantly, however, these results show that resistance to Fas-mediated killing is not primarily related to Fas expression but rather to changes within the intracellular signaling pathways responsible for transmission of the death signal.

The heterogeneity observed among the ES cell lines studied in their sensitivity to Fas-mediated death could potentially correlate with the heterogeneous clinical responses seen in patients treated with chemotherapy for ES. For example, in cell lines JR and SB, resistance to Fas-mediated cell death is observed despite treatment with IFN-γ and cycloheximide. In addition, these cell lines also show resistance to Adriamycin and etoposide in vitro (data not shown) and were derived from two patients with chemoresistant disease, suggesting a defect common to both chemotherapy- and Fas-induced death. Further studies are necessary to elucidate downstream abnormalities in programmed cell death pathways that could impact upon sensitivity to both Fas- and chemotherapy-mediated cell death.

With regard to FasL, our data show that all ES cell lines studied were positive for FasL. Because the specificity of the anti-Fas ligand antibody, clone 33, has been questioned in a recent report (45), we conducted our studies using several anti-Fasl antibodies, primarily the rabbit polyclonal antibody N20 for immunoblot and the monoclonal antibodies NOK-1 and G247-4 for flow cytometry. In addition, using molecular techniques, we could detect message for FasL, which was confirmed by DNA sequencing. Despite the presence of FasL, however, we were unable to detect killing of Fas-sensitive Jurkat cells. FasL could not be detected by surface staining of the ES sarcoma cell lines but only after permeabilization of the cell membrane, suggesting exclusive intracellular distribution of FasL.

There have been recent reports describing intracellular distribution of FasL in various tumor cells (53, 54). Whereas the majority of FasL in pancreatic adenocarcinoma is expressed inside the cytoplasm, there is also membranous FasL, and these tumor cells have been shown to kill Fas-sensitive Jurkat cells (54). However, an exclusive intracellular distribution associated with the absence of FasL-mediated killing has been seen in keratinocytes (55). Interestingly, Jurkat cells, which are the classic target for Fas-dependent killing, have also been shown to have intracytoplasmic FasL but no surface FasL (56). Similar results have been observed in Burkitt’s lymphoma cell lines, where FasL could only be detected inside the cytoplasm, and no killing of Fas-sensitive target cells was noticed (57).

In our experiments, we were also able to generate cytotoxic lymphocytes in response to irradiated ES cells, which were able to lyse the ES cell line. This result not only confirms that ES-associated FasL is unable to kill cytotoxic lymphocytes or prevent the generation of an immune response, but it also precludes the possibility that ES cell lines might express other functional classes of death-inducing molecules, such as TRAIL (58).

In conclusion, we have demonstrated that ES expresses both Fas and FasL. Although the sensitivity to Fas-mediated death varies, none of the FasL expressed was shown to be functional. In addition, ES cell lines were able to serve as stimuliators for the generation of cytotoxic lymphocytes and were susceptible to killing by them. Based on these results, it is rational to hypothesize that ES may be a suitable target for immune therapy.

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FAS AND FAS LIGAND IN EWING'S SARCOMA


Simultaneous Expression of Fas and Nonfunctional Fas Ligand in Ewing's Sarcoma


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