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Human Melanoma Cells Do Not Express Fas (Apo-1/CD95) Ligand

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Abstract

A recent report described the expression of Fas ligand (FasL) by melanoma cells as an important mechanism involved in the immune evasion by tumors [M. Hahne *et al.*, Science (Washington DC), *274*: 1363–1366, 1996]. To investigate the expression of FasL by melanomas, we screened a panel of early-passage cell lines by functional assay and reverse transcriptase-PCR. Using conditions designed to replicate those in the original report, we did not find functional FasL on any of the 19 human melanoma lines established at the National Cancer Institute. Furthermore, we additionally evaluated our melanoma lines using reverse transcriptase-PCR and found that 0 of the 26 human melanoma cell lines expressed FasL mRNA. FasL mRNA was abundantly expressed by antimelanoma T-cell lines after activation. These data do not support a role for FasL expression in the escape of melanoma cells from immune destruction.

Introduction

The molecular identification of antigens recognized by T cells on the surface of melanoma cells has led the way to the development of recombinant and synthetic anticancer vaccines. The first of these vaccines are now available for testing in clinical trials; some have shown objective clinical responses in patients with extensive metastatic melanoma (1–4). Despite advances in the development of new immunotherapies, many patients do not respond favorably to vaccination, leading many to speculate that tumors may actively evade immune destruction by selective loss of β_2-microglobulin, MHC class I expression, antigen processing capability, and antigen expression or by their induction of changes in T cells (5). An original view of FasL[2] (CD95L) function, which has since been retracted, was that FasL would engage Fas receptor on the surfaces of T lymphocytes that would stray into "immune-privileged" areas (6). Melanoma cells were reported to express FasL, enabling them to actively evade immune attack (7). To the best of our knowledge, there has been a single confirming follow-up report to this original study (8), but the only evidence presented was based on fluorescence-activated cell sorting analysis of melanoma cells using the C-20 polyclonal antibody (Santa Cruz Biotechnology), which has been shown to stain FasL-unrelated proteins (9, 10). Because the expression of FasL by melanoma cells could be a potential obstacle in the development of cancer vaccines and adoptive immunotherapies, we explored the expression of FasL by a random sampling of 26 of the >100 melanoma cell lines generated in our laboratory at the National Cancer Institute. In contrast to these earlier reports (7, 8), we were unable to demonstrate the expression of FasL by either sensitive functional assays or by RT-PCR.

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[2] The abbreviations used are: FasL, Fas ligand; RT, reverse transcriptase; FNA, fine needle aspiration; ICE, interleukin-1β-converting enzyme; IL-1β, interleukin 1β.

Materials and Methods

Melanoma Cells and Reagents. Melanoma cells were obtained from patients with histologically confirmed metastatic melanoma by either surgical resections or FNAs. All patients signed informed consent and were enrolled in trials approved by the Institutional Review Board of the National Cancer Institute. All cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. FNA cultures were passaged 5 (JM), 6 (JR), and 10 (FB) times to remove contaminating T lymphocytes and then tested for FasL expression by RT-PCR. Two freshly derived melanoma cultures from surgical specimens were also tested for FasL mRNA: 1448Y (passaged four times *ex vivo*) and 1182 (passaged eight times). Additionally, nine melanoma lines established from surgical specimens with <20 passages were tested by RT-PCR. The colon adenocarcinoma cell line, SW480, was purchased from American Type Culture Collection (Manassas, VA) and is derived from the same patient as SW620. The target cells L1210 and L1210-Fas and the T-cell hybridoma D11s were a gift from Pierre Henkart (National Cancer Institute). A20 was purchased from American Type Culture Collection.

The caspase inhibitor z-Val-Ala-Asp-CH_2F (z-VAD.fmk) and the control z-FA.fmk were reconstituted according to the manufacturer's protocol and used at a final concentration of 25 μM (Enzyme Systems, Dublin, CA).

Functional Assay for FasL. Target cells were labeled with 200 μCi of $Na^{51}CrO_4$ (Amersham) for 1 h at 37°C and then washed three times to remove excess chromium before they were added to 96-well plates in triplicate. The effector cells were plated at 5×10^4 cells/well and grown to confluence for 24 h before the addition of 10^5 chromium-labeled target cells. Maximum lysis of labeled cells was achieved with 2% SDS. After 16–20 h of incubation, the supernatants were collected, and the amount of released ^{51}Cr was determined by a γ-photon counter. The spontaneous lysis was always <30% of maximum lysis. Percentage specific lysis was calculated as follows: [(experimental cpm − spontaneous cpm)/(maximal cpm − spontaneous cpm)] × 100.

RT-PCR Detection of FasL mRNA. Total RNA from SW480 and Jurkat melanoma cell lines and antimelanoma cell line CTL 1143 was obtained by the TRIzol method (Life Technologies, Inc.). RNA was dissolved in diethyl pyrocarbonate-treated water and stored at −70°C. Using gene-specific, intron-spanning primers, we performed cDNA synthesis and PCR amplification in a single-step reaction using 1 μg of RNA per reaction (SuperScript One-Step RT-PCR System; Life Technologies, Inc.). In the minus RT reactions, PCR Supermix containing Taq (Life Technologies, Inc.) was substituted for the SuperScript II RT/Taq Mix. The intron-spanning primers used in these experiments were previously used to detect FasL mRNA (11) and had the following sequences: forward, 5′-GGATTGGGCCTGGGGATGTTTCA-3′; and reverse, 5′-TTGTGGCTCAGGGGCAGGTTGTTG-3′. These primers generated a 344-bp product from FasL mRNA and a 6.3-kb fragment from genomic DNA. Primers used to amplify β-actin were as follows (540-bp product): forward, 5′-GTGGGGCGCCCCAGGCACCA-3′; and reverse, 5′-CTCCTTAATGTCACGCACGATTTC-3′. The primers were used at a concentration of 20 pmol per reaction. The cDNA synthesis step was performed at 45°C for 20 min followed by 94°C for 2 min. After cDNA synthesis, PCR amplification was performed with 40 cycles of the following: 94° for 15 s, 55° for 30 s, and 72° for 1 min. PCR products were analyzed on a 1.5% agarose gel using ethidium bromide and UV illumination for detection of DNA fragments. For comparison, non-intron-spanning primers used by Tschopp *et al.* (7) were: forward, 5′-CTCTGGAATGGGAAGACACC-3′; and reverse, 5′-ACCAGAGAGAGCTCAGATACG-3′. These primers generated a 327-bp fragment from both FasL mRNA and genomic DNA.

Results and Discussion

Human Melanoma Cells Do Not Express Functional FasL. To investigate the expression of FasL by melanomas, a panel of early-passage cell lines derived from patients at the National Cancer Institute were screened by functional assay and RT-PCR. The assays used were designed, in part, to replicate those used by Hahne *et al.* (7). In the functional assay previously shown to detect FasL-mediated apoptosis, melanoma cells were incubated with cells sensitive to FasL-mediated killing (12). Two cell lines, A20 and L1210-Fas, expressed Fas, as measured by flow cytometry (Fig. 1*A*), and were sensitive to FasL-mediated cell death when they were incubated with D11s (Fas L^+; Fig. 1, *B–D*). This killing was blocked by the caspase-specific inhibitor, z-VAD.fmk, which blocks the processing of CPP32, but not with the control fluoro-methyl-ketone, z-FA.fmk (Fig. 1*B*). None of the19 human melanoma lines tested killed the Fas^+ targets in this functional assay, which was negative in six independent experiments (Fig. 1, *C* and *D*).

Human Melanoma Cells Do Not Express FasL by RT-PCR. Because the results from the functional assay were in direct contrast to those reported previously (7), we directly tested whether or not melanoma cells expressed FasL by RT-PCR using intron-spanning primers that have been reported previously to detect FasL expression by tumors (11). We randomly sampled 26 of the >100 melanoma cell lines generated in our laboratory to minimize sampling error. None of the 26 human melanoma cell lines expressed FasL mRNA, as evaluated by RT-PCR (Fig. 2). Furthermore, we grew early-passage melanoma cells obtained by FNA from our patients, and once T lymphocytes were eliminated, three of three FNA cultures (FB, JM, and JR) were negative for FasL expression by RT-PCR (Fig. 2*B*). Two freshly derived melanoma cultures from surgical specimens were also negative: 1448Y (passaged 4 times *ex vivo*) and 1182 (passaged 8 times), as were an additional nine lines with <20 passages (Fig. 2, *A* and *B*). In contrast, FasL mRNA was detected in antimelanoma T cells after growth in IL-2 or activation with OKT3 and in activated but not resting Jurkat cells (Fig. 2). One tumor cell line originating from a colon adenocarcinoma, SW480, expressed FasL mRNA, as reported previously (13). This finding served to verify the sensitivity of this assay in the detection of FasL mRNA in nonlymphoid cells.

Technical Considerations: PCR Primers and Caspase Inhibitors. Using a highly sensitive functional assay and RT-PCR, we did not detect FasL in metastatic melanoma cells obtained from patients

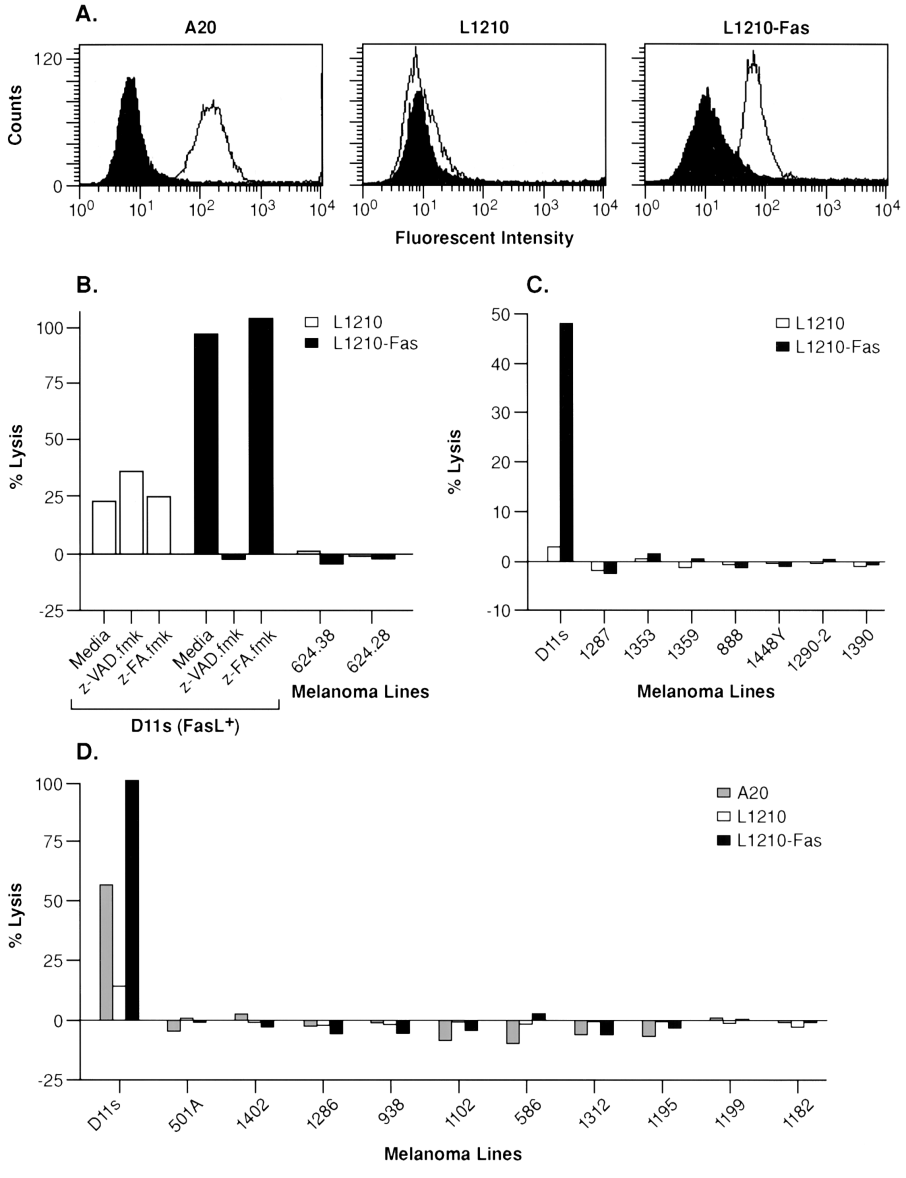

Fig. 1. Melanoma cell lines did not express functional FasL. *A*, expression of Fas (CD95) was confirmed for the targets by flow cytometry. *Closed histogram*, staining obtained with hamster immunoglobulin control; *open histogram*, staining obtained with the Jo2 anti-Fas antibody (PharMingen). The lymphoma lines A20 and L1210-Fas (Fas transfectant) expressed Fas. The parental L1210 demonstrated low levels of Fas expression. *B*, the lysis assay was found to detect apoptosis (12). The killing of L1210-Fas (■) by the $FasL^+$ D11s line was blocked by the caspase inhibitor z-VAD.fmk but not the control z-FA.fmk (25 μM). The low level of lysis of the parental L1210 (□) was not blocked by the caspase inhibitor, suggesting a nonapoptotic mechanism of cell death. In this assay, the melanoma lines 624.38 and 624.28 did not kill either L1210 or L1210-Fas. *C* and *D*, a panel of 19 human melanoma lines were tested in this functional assay. Two of six representative assays are shown. Melanoma lines did not lyse any of the targets. Additionally, functional FasL was not detected in the murine melanoma B16 (data not shown). L1210-Fas was found to be more sensitive than A20 as a target in this functional assay and was used as a target in all of the functional assays performed.

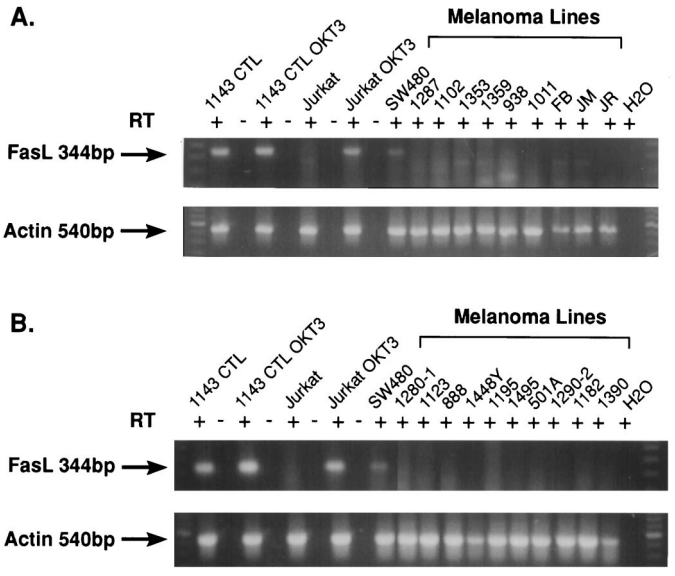

Fig. 2. Melanoma cell lines do not express FasL mRNA by RT-PCR. Total RNA was isolated from cultured melanoma lines, the antimelanoma T-cell line 1143, Jurkat cells, and the FasL$^+$ colon carcinoma SW480 cells (TRIzol; Life Technologies, Inc.). The RT reaction was followed by 40 cycles of PCR using intron-spanning primers. β-Actin was amplified as a positive control for each cell line. *Lanes* +, addition of SuperScript II to the RT reaction; *Lanes* −, absence of SuperScript II in the RT reaction. *A*, a 344-bp fragment corresponding to FasL mRNA was observed in the antimelanoma T cell line 1143 after growing in IL-2 and after TCR cross-linking with OKT3. FasL mRNA was observed in OKT3 activated Jurkat cells but not resting Jurkat cells. Additionally, the 344-bp fragment representing FasL mRNA was detected in the colon carcinoma SW480 but not in any of the 26 melanoma cell lines tested in four separate experiments. *B*, a nonoverlapping panel of melanoma lines demonstrating the absence of the 344-bp fragment that corresponds to FasL.

treated at the National Cancer Institute. These results are in direct contrast to those published previously (7). Hahne *et al.* (7) stated that melanoma cells expressed FasL by RT-PCR; however, their data were generated using non-intron-spanning primers, which will give the same-sized PCR product for both genomic DNA and mRNA (see "Materials and Methods"). Therefore, small quantities of DNA contamination in the RNA preparation may give a false-positive result. Other confounding variables need to be considered during the analysis of literature concerning FasL expression by tumors. Fresh tumor samples will have lymphocyte contamination, and detection of FasL cannot be attributed to the tumor cells unless precautions are taken to ensure the removal of lymphocytes and other nontransformed cells that may express FasL, such as vascular endothelial cells.

To avoid artifacts from DNA contamination and primer selection, intron-spanning primers that were previously reported to detect FasL in tumor cells were chosen and fresh tumor samples (either surgical or FNA) were passaged for short periods *in vitro* to remove lymphocytes. Although FasL expression was not detected in melanoma cells, we confirmed the expression of FasL mRNA by tumor-infiltrating lymphocytes and a colon adenocarcinoma that served to verify the sensitivity of the assay (Fig. 2, *A* and *B*).

The functional assay used in this report was designed to reproduce the conditions used by Hahne *et al.* (7) including the same target (A20) and incubation time. The results from our functional assay are in agreement with those recently published by Arai *et al.* (14), who demonstrated that none of the six human melanoma cell lines that they studied expressed functional FasL. Although Hahne *et al.* (7) demonstrated low levels of target cell lysis (at most, 30%), they did not show any ability to block this apparent lysis with anti-Fas antibodies or caspase inhibitors (7). We consistently observed nonspecific killing (~5–30%) of the L1210 parental cell line (which did not express Fas). However, the killing of the parental L1210 by the FasL$^+$ D11s was not blocked by z-VAD.fmk (Fig. 1*B*), suggesting a nonapoptotic form of cell death. These findings underscore the importance of using apoptosis inhibitors in the analysis of the expression of functional FasL. Furthermore, the specificity of death induced by purportedly FasL$^+$ tumors that has been reported previously has recently been questioned (15). Indeed, the SW480 colon line, which was positive for FasL expression by RT-PCR in our experiments, was recently shown to be incapable of inducing Fas-mediated lysis (15).

Theoretical Considerations: FasL and Inflammation. The paradigm summarized originally by Vaux (and recently retracted; Ref. 6) was one in which immune-privileged sites express FasL and destroy infiltrating T lymphocytes. A similar scenario was envisioned for tumor cells. The expression of FasL would make these tumor sites privileged as well. This hypothesis, however, may not fully take into account the immunological implications of the induction of apoptotic death of T cells, macrophages, and other Fas-expressing bystander cells. Specifically, cells killed through the Fas pathway are killed through a cascade of caspase proteases (16), including caspase-1 (also known as ICE). ICE may be a nexus of the ancient death pathway and the more recently evolved immune system. This enzyme cleaves pro-IL-1β into active IL-1β (16). The release of activated IL–1β by apoptotic cells in the tumor bed would likely attract a great deal of immunological attention.

A similar finding of inflammation as opposed to immune suppression has been reported when FasL is expressed in pancreatic β-cell transplants. Rather than conferring immune privilege to transplanted β islets, a result that would and represent a significant advance in the treatment of diabetes mellitus, FasL-expressing islets cells are targeted for rapid destruction (17–19). In another line of investigation, allogeneic islets of Langerhans cells wrapped with syngeneic myoblasts expressing FasL elicited a rapid inflammatory response, resulting in the formation of an abscess filled with granulocytes (20). Finally, generation of a recombinant vaccinia virus encoding FasL was not found to be more virulent. Instead, experiments done by Zinkernagel and coworkers (21) with such recombinant vaccinia virus demonstrated no direct role for CD95L in down-regulating antivaccinia CTLs.

Data from experimental animals confirm the potentially proinflammatory effects of the constitutive expression of FasL (14, 22). Tumor cells were transfected with functionally active FasL. Rather than granting immune privilege status to tumor cells, the experimental data demonstrate that FasL had the opposite effect. FasL-expressing tumors elicited a rapid influx of neutrophils, signaling the inflammatory microenvironment. The finding that constitutive FasL expression by tumors is inflammatory rather than immunosuppressive has been confirmed in three different experimental tumor models including B16, CT26, and RENCA (14, 22). Induction of FasL expression by human melanoma cells would likely enhance their immunogenicity through the activation of ICE-family proteases and the production of activated IL-1β, and it represents a novel immunotherapeutic strategy (14). Clearly, immune privilege is more than the mere expression of FasL and will likely involve other immunomodulatory factors, such as TGF-β (23).

Our recent success in vaccinating patients with metastatic melanoma has been tempered by our inability to determine what biological variables contribute to the clinical response (4). The hypothesis that some melanoma lesions express FasL offered an attractive explanation for the heterogeneity of responses seen in immunotherapy protocols. We were unable to establish any clinical correlate because all of the melanoma cells that we tested were found to be negative for FasL expression. A more complete understanding of the proinflammatory effects of FasL-mediated caspase activation may lead to the enhanced effectiveness of vaccines for infectious diseases and cancer.

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