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 anti-melanoma 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 recombiant 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 β2-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 An original view of FasL (CD95L) function, which has since been retracted, was that FasL would engage Fas receptor on the surfaces of T lymphocytes that would stray into “immune-privileged” areas (6).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.

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 D11 were a gift from Pierre Henkart (National Cancer Institute). A20 was purchased from American Type Culture Collection.

The caspase inhibitor z-Val-Ala-Asp (zv-Val-Ala-Asp)-CH$_2$F (z-VAD.fmkk) and the control z-FA.fmkk 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$_2$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 antimeelanoma 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 amplification was performed with 40 cycles of the following: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min. PCR products were analyzed by ethidium bromide and UV illumination for detection of DNA fragments. For comparison, non-intron-spanning primers used by Tsopp et al. (7) were: forward, 5'-TTGTTGCTTCAGGGCAGGTTGTTG-3'; and reverse, 5'-GGATTGGGCCTGGGGATGTTTCA-3'.

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2The abbreviations used are: FasL, Fas ligand; RT, reverse transcriptase; FNA, fine needle aspiration; ICE, interleukin-1β-converting enzyme; β-1/β, interleukin-1β.
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. 1A), and were sensitive to FasL-mediated cell death when they were incubated with D11s (FasL+; 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. 1B). None of the 19 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. 2B). 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 antinevomelanoma 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...
344-bp fragment representing FasL mRNA was detected in the colon carcinoma SW480 observed in OKT3 activated Jurkat cells but not resting Jurkat cells. Additionally, the 1143 after growing in IL-2 and after TCR cross-linking with OKT3. FasL mRNA was fragment corresponding to FasL mRNA was observed in the antimelanoma T cell line within the study.

FNA) were passaged for short periods to remove 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).
Fas LIGAND IS NOT EXPRESSED BY MELANOMA CELLS

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References

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