Human Lung Carcinomas Express Fas Ligand

Gloria A. Niehans, Thomas Brunner, Sandra P. Frizelle, Jacob C. Liston, Christopher T. Salerno, Dennis J. Knapp, Douglas R. Green, and Robert A. Kratzke

Abstract

To reach a clinically detectable size, neoplasms must be able to suppress or evade a host immune response. Activated T cells may enter apoptosis in the presence of Fas ligand (FasL) (1), and tissue expression of FasL has been shown to contribute to immune privilege in the eye and testis (2, 3). We have demonstrated that all human lung carcinoma cell lines tested (16 of 16) express a Mr 38,000 protein consistent with FasL by immunoblotting, whereas the majority of resected tumors (23 of 28) show positive staining for FasL by immunohistochemistry. DNA sequencing of reverse transcription-PCR products from lung cancer cells and resected lung tumors confirms the presence of human FasL mRNA in these neoplastic tissues. Furthermore, lung carcinoma cells are capable of killing a Fas-sensitive human T cell line (Jurkat) in coculture experiments; this killing was inhibited by a recombinant form of the soluble portion of the Fas receptor (FasFc). FasL expression by neoplastic cells represents a potential mechanism for peripheral deletion of tumor-reactive T-cell clones.

Introduction

FasL and its receptor, Fas (also known as APO-1 and CD95), play a key role in the regulation of apoptosis within the immune system (1). Both proteins are highly expressed on activated T cells, with low levels of expression seen in resting T cells (2–5). Ligation of Fas by either activating antibody or by FasL transmits a “death signal” to the target cell, potentially triggering apoptosis (6–8). FasL-Fas interactions have been shown to be required for activation-induced cell death in peripheral T cells (9–13), which may normally be needed to terminate an immune response at the end of infection and/or to peripherally delete autoreactive clones. Mutations in either the Fas (lpr) or FasL (gld) loci in mice result in a syndrome of lymphoproliferation and autoimmune disease, accompanied by accumulation of CD4+CD8− T cells in lymph nodes and spleen (14, 15). Similar human lymphoproliferative disorders have been described in children with Fas gene mutations (16, 17).

Two recent reports have implicated FasL expression in ocular tissues and Sertoli cells as a critical factor in maintaining immune privilege in the eye and testis, respectively. T lymphocytes, which infiltrated the anterior eye chamber in response to a herpes viral infection, rapidly underwent apoptosis in normal but not gld mutant mice (18). Testis grafts derived from mice that express FasL survived indefinitely when transplanted under the kidney capsule of allogeneic mice, whereas testis grafts derived from gld mice were immediately rejected (19). Experimentally, cotransplantation of myoblasts engineered to express FasL has protected murine islet of Langerhans allografts from rejection (20). Because expression of FasL might similarly protect tumor cells from immune attack, we examined well-characterized human lung carcinoma cell lines as well as primary human lung neoplasms for evidence of FasL production.

Materials and Methods

Cell Culture and Tissue Collection. Cultured lung cancer cell lines used in this study were derived and maintained as described previously (51, 52) and cultured in RPMI supplemented with 10% FCS and 50 µg/ml of penicillin/streptomycin. Lung cancer cell lines used in this study are available from the ATCC (Rockville, MD) and were obtained from the ATCC or were a gift of Drs. Herb Oie, Gregory Ottersen, and Frederick Kaye from the National Cancer Institute-Navy Medical Oncology Branch (Bethesda, MD). Six NSCLC cell lines (H522, H1155, H2009, H2030, H2087, and H2172) and 10 SCLC cell lines (H69, H209, H417, H685, H689, H719, H774, H792, H865, and H1436) were tested in this study. Cell line H2373 is a mesothelioma cell line, as described previously (53). The Jurkat cell line and L1210 cell line were obtained from ATCC. Additional aliquots of Jurkat cells were obtained as gifts from Dr. Francine Foss (Boston, MA) and Dr. Gordon Ginder (Minneapolis, MN). Jurkat cells and L1210 cells were also cultured in RPMI supplemented with 10% FCS. Human tissues (tumors and adjoining nonneoplastic tissues) were collected from surgical resections performed at the Minneapolis Veterans Affairs Medical Center following a protocol approved by the Human Subjects subcommittee.

Immunoblotting of FasL in Lung Cancer Cells and Tumors. Whole-cell lysates from cultured cell lines were prepared from 5 × 10⁶ cells in 1 ml of lysis buffer [50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.1% NP40, 50 mM NaF, and 1 mM phenylmethylsulfonyl fluoride] as described previously (54). Approximately 100 µg of total protein were electroblotted onto nitrocellulose following separation on a 12.5% SDS-PAGE gel for FasL detection. Similar methods were used to prepare lysates of whole tissues. Nitrocellulose filters were blocked for 1 h using 5% dry milk/1% BSA in 1× PBS and incubated overnight at 4°C in a sealed bag with a 1:1000 dilution of the α-human FasL monoclonal antibody (Transduction Laboratories, Lexington, KY) in 10 ml of 5% dry milk/1% BSA in 1× PBS. The membranes were then sequentially incubated with a rabbit α-murine IgG secondary antiserum (PharMingen, San Diego, CA) and 2.5 × 10⁶ cpm of 125I-labeled protein A (Amersham Corp., Arlington Heights, IL) followed by autoradiography.

Immunofluorescent Staining of Lung Cancer Cells. The lung cancer cell lines H209, H2009, H522, and H841 were seeded at 1 × 10⁶ cells/ml into tissue culture chamber slides (Lab-Tek chamber slide, Naperville, IL) and grown for 2 days at 37°C. Cells were then washed twice with PBS and fixed with 4% formaldehde in PBS for 20 min at room temperature. Fixation solution was removed, and cells were permeabilized in 250 µl of 0.25% Triton X-100 in PBS for 20 min at room temperature. After two washes with PBS, nonspecific binding was blocked with 1% BSA in PBS for 10 min at room temperature. FasL was then stained with 5 µg/ml murine monoclonal α-human FasL antibody NOK-1 (PharMingen) in PBS/BSA overnight at 37°C. As a control, cells were treated the same way with normal mouse IgG (Jackson Laboratories, Westgrove, PA). Cells were then washed three times in PBS/1% calf serum and incubated with a 1:50 dilution of a goat α-mouse FITC-conjugate (Jackson Laboratories) for 30 min at room temperature in the dark. After three washes in PBS/1% calf serum, the well dividing structure was...
removed, cells were covered with anti-fade solution (SlowFade; Molecular Probes, Eugene, OR), coverslipped, and analyzed by fluorescent microscopy.

RT-PCR and DNA Sequencing of FasL. RT-PCR was performed as suggested by the manufacturer (Roche Molecular Systems, Branchburg, NJ) on total cellular RNA extracted from the H209 (SCLC), H417 (SCLC), and H522 (NSCLC) cell lines, as well as from a resected NSCLC tumor. RNA was extracted using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD), and following random hexamer priming, RNA was subjected to first-strand cDNA synthesis. First-round PCR was performed using a sense primer (CCAGCCTGGCATGAG-CAGCGCCTTCAATTACACCAT) corresponding to nucleotides 56 through 91 of the human FasL, open reading frame and an antisense primer (CTCTTATTAGGCTTATATAACGCGAAAAACTGCTGAGT) corresponding to nucleotides 914 through 875 of the human FasL sequence (21). A second round of PCR was carried out using a nested sense primer (GGTTCTGTTGCTTGGTAGGATT) corresponding to nucleotides 331 through 355 of the human FasL open reading frame and an antisense primer (AGCCGAAAACGCTGAGTTGCCTC-CTC) corresponding to nucleotides 872 through 896 of the FasL sequence, with a resulting PCR product representing 565 nucleotides of the 846 nucleotides of the human FasL protein coding sequence. Conditions for both rounds of PCR included a melting temperature of 95°C for 30 s, followed by an annealing temperature of 45°C for 1 min, and then 72°C for 2 min for a total of 35 cycles. PCR was carried out using a combination of Taq polymerase (Roche Molecular Systems, Branchburg, NJ) and pfu polymerase (Stratagene, La Jolla, CA) at a mix of 1000:1 to allow for 3′ to 5′ editing of incorrectly incorporated nucleotides. The resulting PCR-amplified products were subcloned into the pGEMT vector (Promega Corp., Madison, WI) and subjected to nucleotide sequencing (55). RNase protection assay was performed as described previously (12) using a 565-bp probe extending from nucleotides 331 through 896 of the human FasL cDNA.

Immunohistochemical Staining for FasL. Cell lines were pelleted at 2000 rpm in a collodion bag for 10 min, fixed in 10% buffered formalin for 4 h, dehydrated through graded alcohols and xylene overnight in a MVP tissue processor (Research and Manufacturing Company, Tucson, AZ), and embedded in paraﬃn. Primary tumors were inﬁltrated for 18—72 h with 10% formalin and processed as above into parafﬁn blocks. Sections were cut at 4 μm, mounted on glass slides coated with poly-L-lysine, and baked at 60°C for 1 h. Cooled slides were deparafﬁnized in xylene and rehydrated through graded alcohols into distilled water. Heat-induced epitope retrieval (56) was achieved by immersion of slides in Coplin jars ﬁlled with 10 ml uncitrate buffer (pH 6.0), which was heated to boiling. The slides were then placed in water. Heat-induced epitope retrieval (56) was achieved by immersion of slides in Coplin jars ﬁlled with 10 ml uncitrate buffer (pH 6.0), which was heated to boiling. After a hematoxylin counterstain, slides were dehydrated in graded alcohols and xylene, and coverslipped. Tumors were interpreted as positive for FasL by immunohistochemistry when at least weak to moderate cytoplasmic staining was seen in greater than 10% of neoplastic cells.

Co-culture Assays of Lung Cancer and Jurkat Cells. Two adherent NSCLC cell lines (H2009 and H522) were plated in triplicate in 24-multiwell tissue culture dishes and allowed to reach approximately 75% conﬂuency as a monolayer. Following removal of media and washing of the monolayers with 1X PBS, 2 × 10^5 Jurkat cells (nonadherent) were plated in 200 μl of tissue culture medium over a monolayer or into empty wells. Wells with NSCLC cells alone (without overlying Jurkat cells) were maintained as additional controls. Twenty-four h later, the nonadherent phase of the mixed culture was aspirated and assayed for viability using trypan blue exclusion. In experiments using recombinant FasFc as a competitor, similar experimental conditions were used with the following exception. Recombinant FasFc was prepared as described previously (12) and was added to the RPMI with 10% FCS at a concentration of either 8 or 12 μg/ml. The FasFc-containing medium was preincubated on the monolayer for a period of 1 h at 37°C, followed by the direct addition of Jurkat cells into the wells. Experiments using L1210 cells were carried out in a similar fashion.

Results

FasL Expression in Lung Cancer Cell Lines and Tumors. To test the possibility that FasL is expressed in lung carcinomas, we examined whole-cell lysates that were prepared from 10 SCLC lines, 6 NSCLC lines, and 1 mesothelioma line. These lysates were subjected to immunoblotting with a murine monoclonal antibody that recognizes human FasL. In all cell lines analyzed, a Mr of 38,000 protein was identified that comigrated with an extract of testicular tissue (19) used as a positive control (Fig. 1, A and B). The level of protein

![Image](https://cancerres.aacrjournals.org/figure/mam0065fig1a.png)

Fig. 1. Immunoblot analysis of FasL expression in lung cancer cells and tumors. Whole-cell lysates of six SCLC cell lines and six NSCLCs demonstrate expression of a Mr of 38,000 protein after immunoblotting with α-FasL antisera. A, Jurkat lung cancer (SCLC) is positive for FasL (Lane 1), as are SCLC cell lines H69, H209, H417, H719, H792, and H865 (Lanes 2—7). B, Jurkat lung cancer (Lane 2), mesothelioma cell line H2373 (Lane 3), and NSCLC cell lines H522, H1155, H2009, H2087, and H2172 (Lanes 4—8) are positive for FasL protein expression. C, whole tissue lysates of human testicle (Lane 2), NSCLC lung cancer (Lane 3), and cell lysate of H2009 (Lane 4) are positive for FasL. Nonmalignant human lung (Lane 4) tissue is negative for FasL expression, as is FCS alone (Lane 5).

1008
expression appeared relatively equal between the various cell lines, although densitometry was not performed (Fig. 1). Tissue samples from a resected NSCLC also demonstrated the presence of the \( M_r \) 38,000 protein, whereas corresponding tissue from uninvolved normal lung was negative (Fig. 1C). Immunofluorescent staining of two SCLC cell lines (H209 and H841) and two NSCLC cell lines (H522 and H2009) also demonstrated FasL expression by lung cancer cells (Fig. 2).

To confirm the expression of FasL by the lung cancer cells, nested RT-PCR was performed on total RNA extracted from four cell lines (two SCLCs and two NSCLCs) and one primary lung carcinoma. The first set of primers was selected to amplify the entire protein coding sequence of the human FasL cDNA extending from nucleotides 56 to 914, including both the consensus start and stop codons. No FasL product was visualized on ethidium bromide-stained agarose gels after the first round of amplification. Subsequently the first-round PCR products were subjected to further amplification using nested primers that encompass a fragment of the human FasL cDNA from nucleotides 331 to 896. A 565-bp product was identified in all lung cancer cell lines and in the primary tumor consistent with human FasL (Fig. 3). To confirm this result, a RNase protection assay was performed using the same 565-bp fragment as a probe against RNA from a different cell line (H841). A weak protected band of correct length

Fig. 2. Immunofluorescence of NSCLC cells with \( \alpha \)-FasL antisera. Immunofluorescence of a NSCLC cell line (H522) demonstrates expression of human FasL protein (A). Cells treated with control murine immunoglobulins are negative (B). Similar results were obtained with cell lines H209, H2009, and H841 (data not shown).

Fig. 3. RT-PCR of FasL from lung cancer cell lines and tumors. One NSCLC cell line (H522; Lane 1), two SCLC cell lines (H209, Lane 2; H417, Lane 3), and a resected NSCLC tumor (Lane 4) were subjected to nested RT-PCR, and the resulting products were electrophoresed in a 1% agarose gel and stained with ethidium bromide. Lane 5, negative control (no template).
Table 1 FasL expression by lung carcinomas

<table>
<thead>
<tr>
<th></th>
<th>Tested/Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoblotting</td>
<td></td>
</tr>
<tr>
<td>NSCLC cell lines</td>
<td>6/6</td>
</tr>
<tr>
<td>SCLC cell lines</td>
<td>10/10</td>
</tr>
<tr>
<td>Mesothelioma cell lines</td>
<td>1/1</td>
</tr>
<tr>
<td>NSCLC tumors</td>
<td>1/1</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td></td>
</tr>
<tr>
<td>NSCLC tumors</td>
<td>14/15</td>
</tr>
<tr>
<td>SCLC tumors</td>
<td>8/10</td>
</tr>
<tr>
<td>Pulmonary carcinoids</td>
<td>1/3</td>
</tr>
<tr>
<td>SCLC cell lines</td>
<td>2/2</td>
</tr>
<tr>
<td>NSCLC cell lines</td>
<td>1/1</td>
</tr>
<tr>
<td>RT-PCR</td>
<td></td>
</tr>
<tr>
<td>NSCLC tumors</td>
<td>1/1</td>
</tr>
<tr>
<td>NSCLC cell lines</td>
<td>2/2</td>
</tr>
<tr>
<td>SCLC cell lines</td>
<td>2/2</td>
</tr>
</tbody>
</table>

Fig. 4. FasL immunohistochemistry of human lung cancer. Resected NSCLC lung tumors were subjected to immunohistochemical analysis with α-FasL antibodies (A) or nonimmune mouse serum as a negative control (B). C, arrow, resected NSCLC with positive staining lymphocytes within tumor stroma. D, pelleted SCLC cells (H417) demonstrate FasL expression on immunohistochemical analysis.

was detected in total RNA extracted from cell line H841 (data not shown). To ensure that the PCR product was human FasL, the PCR product obtained from RT-PCR of the lung tumor and one of the cell lines (H209) was subcloned into the pGEMT vector and sequenced. In both cases, the cloned cDNA was found to be 100% homologous to human FasL (21).

Immunohistochemical Localization of FasL Protein in Tumors. To demonstrate the presence of FasL in vivo and avoid the detection of FasL from contaminating activated T cells, we performed immunohistochemistry on paraffin sections of 3 cell lines (two SCLC (H209 and H417) and one NSCLC (H2009)), 15 primary NSCLC tumors, 10 resected SCLC (7 primary tumors and 3 lymph node metastases), 3 primary pulmonary carcinoids, as well as nonneoplastic lung and bronchial tissue. Using an α-human FasL murine monoclonal antibody, immunoreactivity for FasL was seen in the 3 lung cancer cell lines; 14 of 15 NSCLCs; 8 of 10 SCLCs; and 1 of 3 pulmonary carcinoids (Fig. 4). Similar results were also obtained using an α-human FasL polyclonal antibody. Detectable cell surface staining was seen accompanied by strong granular cytoplasmic reactivity. Generally, staining was most intense at the periphery of the tumor and was present in the majority of neoplastic cells. Variable numbers of tumor-infiltrating lymphocytes and pulmonary macrophages also expressed FasL (Fig. 4C). Moderate cytoplasmic staining of normal bronchial epithelium was consistently noted. Normal alveolar lung tissue was negative for FasL, as seen previously on immunoblotting, but focal FasL immunoreactivity was identified in regenerating Type II pneumocytes. FasL staining in lung cancers and cell lines is summarized in Table 1.

Lung Cancer-mediated Killing of Jurkat Cells. To evaluate the functional significance of FasL expression in lung cancer cells and tumors, we performed coculture experiments using the Fas-positive Jurkat cell line (of human T-cell origin) as a target cell. These cells
six of six colon carcinomas, six of seven kidney carcinomas, seven of seven head and neck carcinomas, and five of seven melanomas. Interestingly, other investigators have reported recently that colon carcinoma, melanoma, and hepatocellular carcinoma cell lines may express FasL and kill Jurkat cells in a Fas-mediated manner (22—24). It will be important to investigate if the majority of human carcinomas, including lung cancer, possess the capacity to kill T cells via apoptotic pathways.

The outcome of interactions between FasL-bearing T lymphocytes and FasL-positive neoplasms may depend on their relative sensitivity to FasL-mediated apoptosis. Fas is widely expressed on epithelial cells as well as on subsets of WBCs (25). Studies of cytotoxic T cells have demonstrated that presentation of FasL to Fas-positive target cells constitutes one of the two major cytolytic effector mechanisms, with perforin-induced cell membrane damage accounting for the remainder of T-cell cytolytic activity (26—28). Thus, it is possible that inhibition of FasL activity by retinoic acid or glucocorticoids (29), or by soluble Fas antigen (30), would actually hinder T-cell-mediated tumor rejection. However, many carcinomas have lost surface Fas protein (30), and in vitro experiments have shown that even tumor cell lines with residual Fas expression are frequently resistant to Fas-mediated cell killing (31). Activation of certain oncogenes such as bcl-2 or bcr-abl may partially or completely inhibit the apoptotic cascade downstream from Fas (32—35). In contrast, activated T cells are known to have a window period of vulnerability to FasL-induced apoptosis (4, 5). It will be interesting to investigate if the small percentage of FasL-negative lung cancers represent, in part at least, a Fas (APO-1/CD95)-positive population.

FasL expression by lung carcinomas may have effects on the immune system beyond the tumor site. Soluble FasL has been identified in sera from individuals with certain types of FasL-positive leukemias and lymphomas (36). If circulating forms of FasL are also found in blood from lung cancer patients, this could contribute to the generalized depression of cellular immunity seen in patients with advanced neoplastic disease (37, 38). In addition, the effects of FasL expression by neoplastic cells may not be limited to immune interactions. For example, hepatocytes express Fas antigen (25, 39), and i.p. administration of activating α-Fas antibody induces massive hepatic apoptosis in mice (39). Thus, FasL expression might facilitate tumor invasion by inducing apoptosis in surrounding Fas-positive tissue, allowing the tumor to grow into the resulting space.

Our data indicate that a small subset of human lung tumors remain FasL-negative. Because there is now convincing experimental evidence that T lymphocytes can recognize certain tumor-associated peptides as aberrant (40, 41), this group of FasL-negative tumors confirms the importance of other previously defined mechanisms for evading T-cell-mediated cytotoxicity. Neoplastic cells have been shown to prevent a T-cell immune response by various means, including down-regulation of MHC class I molecules (42, 43), lack of costimulatory signals such as B7 (44, 45), secretion of immunoinhibitory proteins such as TGFB (46), loss of ζ+ signal transducing chains from tumor-infiltrating lymphocytes (47—49), and interaction of neoplastic cells with the inhibitory CTLA-4 receptor (50). Our results raise the strong possibility that tumor cells can also induce peripheral deletion of tumor-reactive T cell clones via a FasL-Fas interaction.

Acknowledgments

We thank M. Mescher for review of the manuscript.

References

2. Trauth, B. C., Klas, C., Peters, A. M. J., Matzku, S., Möller, P., Falk, W., Debain, K-M., and Krammer, P. H. Monoclonal antibody-mediated tumor regression by...
Human Lung Carcinomas Express Fas Ligand

Gloria A. Niehans, Thomas Brunner, Sandra P. Frizelle, et al.

Cancer Res 1997;57:1007-1012.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/6/1007

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.