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Fas Ligand Is Expressed on Human Squamous Cell Carcinomas of the Head and Neck, and It Promotes Apoptosis of T Lymphocytes[1]

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ABSTRACT

Recent reports have variously described expression of Fas ligand (FasL) or its absence in human tumors. The importance of the Fas-FasL mechanism for the immune evasion by tumors provided a strong rationale for the examination of FasL expression and function in squamous cell carcinoma of the head and neck (SCCHN), which is one of the most immunosuppressive human cancers. Using immunostaining or immunoblotting, SCCHN cell lines and tumor biopsies were examined for the presence of the components of the Fas-FasL pathway and found to express Fas, as well as both the full-length and cleaved forms of FasL. By reverse transcription-PCR, mRNA for FasL and Fas were detected in all SCCHN tested, and cross-hybridization to radioactive Fas and FasL cDNA probes confirmed the specificity of amplification. To demonstrate that FasL expressed on cell surface of SCCHN cells was biologically active, various SCCHN lines were coincubated with the Fas-sensitive Jurkat T-cell lines or activated peripheral blood mononuclear cells. Tumor-induced apoptosis of T cells was dependent on the ratio of tumor cells: lymphocytes. It was significantly but only partially inhibited by neutralizing antibodies to FasL and antagonistic antibodies to FasR. Tumor-induced apoptosis was enhanced by the pretreatment of tumor cells with metalloproteinase inhibitors, which increased expression of FasL on tumor cells. Supernatants of tumor cells transduced with FasL also induced apoptosis of Jurkat cells. Thus, coincubation of SCCHN with Fas-sensitive lymphocytes can induce apoptosis of these lymphocytes, and the Fas/FasL pathway appears to be responsible, at least in part, for tumor-induced lymphocyte death. The data suggest that the Fas/FasL pathway is potentially immunosuppressive and may be involved in the escape of human carcinoma cells from immune destruction.

INTRODUCTION

Many studies have demonstrated that lymphocytes isolated from human solid tumors are functionally deficient in comparison to those in the peripheral blood (reviewed in Refs. 1 and 2). Functional deficiencies observed with freshly isolated TIL[3] range from a complete lack of responsiveness to T-cell mitogens to a partial inhibition of proliferation, antitumor cytotoxicity, or cytokine production (3–8). The mechanisms responsible for these defects are unknown, but considerable evidence has accumulated that indicates that tumor-derived inhibitory factors are responsible for functional defects of TIL (9–11). In addition, similar defects, although generally less extensive, are detectable in the circulating lymphocytes of patients with cancer, including those with head and neck cancer (12, 13). More recently, identification of changes associated with proteolysis in TILs *in situ* focused attention on the possibility of apoptosis as explanation for TIL dysfunction. The presence of TUNEL$^+$ T lymphocytes in tumor tissues, in the absence of detectable apoptosis in tumor cells, indicated that many of the TILs were dying in the tumor microenvironment (14, 15).

Host antitumor immunity has been considered to play a role in protection against the development of malignancy. However, tumor evasion of the immune system, including low levels or lack of expression of tumor-associated antigens, various costimulatory or MHC molecules, defective antigen processing, or production of immunosuppressive factors, is accompanied by dysfunction of immune effector cells and accelerated death. More recent evidence has suggested that Fas (Apo-1/CD95) ligand (FasL), one of the TNF family members, may be involved in the destruction of immune cells and in maintaining a state of tumor immune privilege (5, 13–16). Whereas expression of FasL protein and its message in a variety of tumor cell lines and tumors *in situ* has been reported (13, 17–21), its role in apoptosis of TIL observed in human tumor biopsies is unclear. Also, the mechanism(s) by which FasL engagement might cause death of human lymphocytes has not been extensively investigated. A controversy has recently developed about FasL expression in melanomas, with Chappell *et al.* (16, 22) suggesting that FasL has no role in the escape of melanoma cells from immune destruction. In addition, substantial concerns have been raised about the specificity of antibodies used for detection of FasL in human tissues (23–26), requiring additional evidence for FasL expression and function in tumors. We therefore examined the presence of FasL on human SCCHN cell lines and tumor *in situ* and proceeded to confirm in a series of *ex vivo* coincubation experiments that tumor-associated FasL is indeed involved in inducing apoptotic signals in activated T lymphocytes.

MATERIALS AND METHODS

Cell Lines. Cell lines of human SCCHN were established and maintained in our laboratory, as described previously (27). The cell lines currently in culture were selected for *ex vivo* experiments and included PCI-1, PCI-13, PCI-4A, PCI-4B, SCC-68, and SCC-74. In addition, an oral carcinoma (OSC-19) cell line obtained as a gift from Dr. Etsuhide Yamamoto (Kanazawa University, Kanazawa, Japan) was used. The lines were routinely tested for the presence of *Mycoplasma* (Gen Probe, San Diego, CA) and found to be negative. Jurkat cells were obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% (w/v) FCS and antibiotics (all from Life Technologies, Inc., Grand Island, NY).

Isolation and Activation of PBMC. Venous blood samples were obtained from normal donors and collected in heparinized tubes. PBMC were separated by Ficoll-Hypaque gradient centrifugation, as described earlier (5), washed in the culture medium, counted in a trypan blue dye to assess their viability, and adjusted to a concentration of 1×10^6 cells/ml for culture. The cells were incubated in the presence of 10 μg/ml of concanavalin A at 37°C for 3 days in complete medium in 25-cm^2 tissue culture flasks. Prior to staining, the cells were stimulated for 4 h with PMA (10 ng/ml) and 1 μM ionomycin in either the

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[3] The abbreviations used are: TIL, tumor-infiltrating lymphocyte; SCCHN, squamous cell carcinoma of the head and neck; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; Ab, antibody; RT, reverse transcription; PMA, phorbol 12-myristate 13-acetate; FasL, Fas ligand; rFasL, recombinant Fas ligand.

[4] B. R. Gastman, D. E. Johnson, T. L. Whiteside, and H. Rabinowich, Activation of caspase-3 and cleavage of Bcl-2 in tumor-induced apoptosis of T lymphocytes, submitted for publication.

presence or absence of metalloproteinase inhibitor, BB94 (Kanebo, Osaka, Japan), used at a concentration of 10 μM.

Staining and Flow Cytometry. Activated PBMCs, nonactivated and activated Jurkat cells, and carcinoma cells were stained by the indirect method with the following pretitered anti-FasL or anti-Fas Abs: C-20 (Santa Cruz Biotechnology Co., Santa Cruz, CA) at 0.5 μg/ml; NOK-1, NOK-2, or G247-4 (PharMingen, San Diego, CA) at 10 μg/ml; and A-33 (Transduction Technology, Lexington, KY) at 10 μg/ml. NOK-3 anti-FasL Ab was kindly provided by Dr. H. Yagita (Tokyo, Japan) and used at a concentration of 10 μg/ml. For detection of Fas, N-18 Ab from Santa Cruz and ZB4 Ab (Upstate Biotechnology, Lake Placid, NY) were used. FITC-labeled antimouse IgG or IgM and antirabbit IgG were purchased from Caltag (South San Francisco, CA) and Santa Cruz, respectively. Isotype Ab controls mouse IgG or IgM or rabbit IgG were obtained from DAKO (Carpinteria, CA) and Sigma Chemical Co. (St. Louis, MO), respectively.

Primary and secondary Abs were titered on activated PBMC and tumor cells. The optimal working dilution of each Ab was determined by performing "checkerboard" titrations, in which cells were initially incubated with increasing dilutions of each primary Ab (0.1–25 μg/ml). For titrations, each Ab was used at five concentrations. The highest Ab dilution that gave positive staining relative to the accompanying isotype control was used for titration of the secondary (FITC-labeled) reagent. The optimal working dilutions determined individually for each Ab were used in all subsequent experiments.

For cell surface staining, tumor cell monolayers were dissociated using cell dissociation solution (Sigma) and washed. The primary Ab was added and the cells incubated at 4°C for 30 min and washed in PBS containing 0.1% FCS. The secondary Ab labeled with FITC was added, and the cells were incubated at 4°C for 30 min. Following extensive washing in the PBS-sodium azide buffer, the cells were analyzed by flow cytometry using FACScan (Becton Dickinson). To determine intracytoplasmic expression of Fas or FasL, lymphocytes or tumor cells were prefixed in 2% (w/v) paraformaldehyde in PBS for 20 min at 4°C and washed. The cells were resuspended in the permeabilization solution [0.1% (w/v) saponin and 0.1% (w/v) BSA in PBS], and the primary Ab was added. The cells were incubated for 30 min at 4°C, and following several washes in the saponin buffer, they were stained with the secondary Ab, washed, and analyzed by flow cytometry.

To confirm Ab C-20 specificity, blocking experiments were performed, in which an excess (0.5 μg) of the FasL peptide (COOH-terminal 260–279 amino acids, Santa Cruz) was added to primary Ab (0.1 μg) as a direct, internal competitive control. Following 30 min of incubation, the C-20Ab was used for surface staining of tumor cells, and the immunizing peptide was found to completely block this staining. Additionally, a 10-fold excess of recombinant FasL (Alexis Corp., San Diego, CA) was used to block surface staining of tumor cells with C-20 Ab. The Ab (0.1 μg) was incubated with 1 μg of rFasL in a total volume of 10 μl prior to staining of tumor cells. Blocking was also performed with a concentrated (7×) supernatant of cells transduced with the *FasL* gene and secreting FasL, which was shown to induce apoptosis of lymphocytes in JAM assays. In these experiments, 40 parts of $FasL^+$ supernatant were added to 1 part of C-20 Ab, and the inhibition of surface staining on tumor cells was measured by flow cytometry.

Patients and Tumor Tissues. Tumor tissues were collected from 16 patients with squamous cell carcinoma of the oral cavity (6, floor of the mouth; 5, tongue; 3, gingiva of the mandible; 1, retromolar trigone; 1, buccal mucosa). The patients underwent surgery at the University of Mainz (Mainz, Germany). All patients gave informed consent. Of the patients, 13 were male and 3 were female. None had received chemo-, radio- or immunotherapy prior to tissue collection. The surgical specimens of the primary tumors were routinely fixed in 4% formalin and embedded in paraffin, and blocks were stored until sectioned for this study. The tumor-node-metastasis staging categories were determined according to the criteria proposed by the International Union Against Cancer. Clinical definitions of the tumor stage were confirmed by histopathology, and 4 patients were defined as stage II and 12 as stage IV.

Immunohistochemical Detection of FasL and Fas in SCCHN Tissues. Consecutive paraffin sections (5 μm thick) were obtained from each specimen and mounted on electrostatically precharged slides (Superfrost Plus, Menzel-Glaser, Germany). Sections were deparaffinized in xylene, rehydrated, and washed twice for 5 min in a wash buffer containing 50 mM Tris-Cl, pH 7.6, 50 mM NaCl, and 0.0001% saponin. After endogenous peroxidase quenching in 3% H_2O_2 in methanol for 5 min, slides were washed as before, except that the wash buffer for this and all subsequent steps included 1% normal goat serum. Slides were then blocked for 1 h in wash buffer containing 5% normal goat serum. Slides were washed and incubated overnight at 4°C with a rabbit polyclonal antihuman FasL Ab recognizing an N-terminal peptide (amino acids 2-21; Q-20, Santa Cruz Biotechnology, Heidelberg, Germany) at a 1:200 dilution made in the wash buffer. Ab binding was localized using a biotinylated secondary Ab and streptavidin-conjugated horseradish peroxidase LSAB-2 kit (DAKO, Glostrup, Denmark), and 3,3′-diaminobenzidine substrate. Fas was detected using the same protocol, except that rabbit polyclonal antihuman Fas Ab (Santa Cruz Biotechnology, Heidelberg, Germany) was used. In all experiments, rabbit nonimmune serum purchased from DAKO was used as a control. Slides were counterstained with hematoxylin and examined in a light microscope.

Transduction of SCCHN Cells with the Human *FasL* Gene. Two different human carcinomas cell lines, PCI-13 (SCCHN) and HR (gastric carcinoma) were transduced with the DFG-FasL gene. The retroviral vector was produced by inserting cDNA for human FasL (generously provided by Dr. Skigekazu Nagata, Osaka, Japan in the form of PBX-hFL1 plasmid) into the MFG vector, so that the translational initiation codon ATG of the gene was fused to the ATG of the retroviral Env gene containing the IRES-Zeo cassette (Fig. 1).

This vector was transduced into a BOSC amphotropic packaging cell line by the calcium phosphate method. Retroviral supernatants were generated and used for infection of SCCHN cells. Following selection in zeomycin-containing medium, the FasL transfectants were characterized for expression of FasL by flow cytometry, Western blots, and RT-PCR and for biological activity in JAM assays, using Jurkat cells as targets.

RT-PCR for Fas, FasL, or TRAIL mRNA. Total cellular RNA was extracted from 3×10^6 parental or transduced tumor cells or from lymphocytes. The method for RNA extraction and conditions used for RT-PCR in our laboratory were described previously (28). Conventional or hot PCR with [α-^{32}P]dCTP was performed, as described previously (28), using cDNA obtained by RT of 100 ng of cellular RNA for each template. To show that the use of 100 ng of RNA was not limiting amplification of weakly expressed genes, initial titrations were performed using various quantities of cellular RNA (from 50 ng to 1 μg). The following sense/antisense primers were used for PCR. FasL: sense, 5′-GGATTGGGCCTGGGGATGTTCA-3′ (344 bp); antisense, 5′-TTGTGGCTCAGGGGCAGGTTGTTG-3′. FasR: sense, 5′-CAGAACTTGGAAGGCCTGCATC-3′ (682 bp); antisense, 5′-TCTGTTCTGCTGTGTCTTGGAC-3′. TRAIL: sense, 5′-TGCACTTGAGGAATGGTGAACTGG-3′ (717 bp); antisense, 5′ -TGTCCCTTAAGGAAACCTGGAGGC-3′. β-Actin: sense, 5′ -GGGTCAGAAGGATTCCTATG-3′ (237 bp); antisense, 5′ -GGTCTCAAACATGATCTGGG-3′. A solution (25 μl) consisting of 2 mM $MgCl_2$, 0.5 μM of each primer, 1 μCi [α-^{32}P]dCTP, 5 μl of each RT reaction, and 1 unit of Ampli *Taq* DNA polymerase was added to each amplification tube. Preliminary titrations of the number of PCR cycles (between 25 and 40) were performed to select that on the ascending (linear) portion of the amplification curve. Amplification (35 cycles) was performed with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Aliquots (25 μl) of the amplified products were separated on 5% polyacrylamide gels. The gels were either stained with ethidium bromide and examined under UV light or were dried for analysis in a PhosphorImager. The ratios of cDNA for each product against the β-actin cDNA were calculated from the radioactive signals. For Southern analyses, the amplified products were separated on agarose gels, transferred to nylon transfer membrane (Hybond-N^+, Amersham Pharmacia Biotech) and hybridized with ^{32}P-labeled cDNA probes for Fas or FasL generously provided by Dr. Shigekazu Nagata (Osaka, Japan). Cross-hybridization experiments, in which Fas cDNA was hybridized with FasL amplicon and *vice versa*, were performed to confirm specificity. Finally, DNA sequencing of the PCR products corresponding to FasL in Jurkat (as control) and PCI-13 tumor cells was performed.

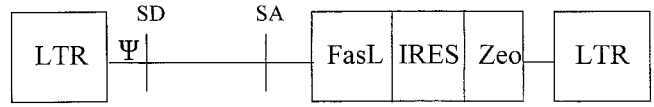

Fig. 1. Schematic representation of the retroviral vector used for transduction of the human *FasL* gene into PCI-13 cells.

PCR bands were cut out from 1.8% agarose gel after electrophoresis, and DNA was purified using JETsorb gel extraction kit (Genomed Inc., Research Triangle Park, NC). Aliquots of 400 mg of the PCR product and 3.5 pmol of the sense primer for FasL were submitted for automatic sequencing to the DNA Sequencing Facility at the University of Pittsburgh Cancer Institute.

Coculture of SCCHN Cells with Lymphocytes. SCCHN cell lines were coincubated with Jurkat cells at the tumor to lymphocyte ratios ranging from 10:1 to 80:1 for 16–18 h. For these experiments, confluent SCCHN monolayers were dissociated with a trypsin solution (Life Technologies, Inc.), washed, counted, plated at a desired cell number/well in U-shaped wells of 96-well microculture plates, and incubated for 24 h prior to addition of lymphocytes. Jurkat cells suspended in medium were added, and the plates incubated at 37°C for 16–18 h. In some experiments, tumor cells were preincubated for 2 h in the presence of neutralizing anti-FasL Ab 4H9 (MBL International Corp., Watertown, MA) and used at a concentration of 200 ng/ml. To block Fas expression on Jurkat cells, antagonistic Ab (ZB4, mouse IgG) was purchased from Upstate Biotechnology and used at concentrations of 200-1000 ng/ml. Jurkat cells were incubated with ZB4 for 2 h prior to coculture with SCCHN cells. Isotype controls (IgM or hamster IgG, respectively) were purchased from Sigma and DAKO and used in all blocking experiments.

Apoptosis Detection Assays. To detect DNA fragmentation in lymphocytes the JAM (29) and TUNEL (30) assays were used. Briefly, DNA labeling of Jurkat cells (targets) for JAM assays was performed by incubating the cells in the presence of 5 μCi/ml of [^{3}H]dThd (NEN, Boston, MA) for 18 h. Tumor cells were coincubated with labeled Jurkat cells for 16–18 h at 37°C at the range of tumor to lymphocyte cell ratios of 10:1 to 80:1. At the end of the coincubation period, the cells were harvested onto glass fiber filters, as described previously. In this assay, the radioactivity of intact chromosomal DNA retained on each filter is measured, after fragmented DNA from target cells is washed through the filter. Specific DNA fragmentation was calculated as follows:

$$\% \text{ apoptosis} = \frac{S - E}{S} \times 100$$

where S = cpm of DNA retained in the absence of tumor cells (spontaneous fragmentation) and E = cpm of DNA retained on the filter in the presence of tumor cells (experimental).

To identify fragmented DNA in Jurkat cells, the TUNEL assay was performed according to directions supplied by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). The cells were washed in PBS and fixed in 2% (w/v) paraformaldehyde for 30 min at room temperature. After fixation, the cells were washed twice in PBS containing 0.01% (w/v) BSA and resuspended in TUNEL reaction mixture containing fluorescein dUTP and terminal deoxynucleotidyl transferase. Control cells were incubated in the absence of terminal deoxynucleotidyl transferase. The fluorescein label incorporated into DNA strand breaks was detected by flow cytometry.

Western Blot Analysis. Cell lysates were prepared and proteins were separated by SDS-PAGE, using 7.5–12% polyacrylamide gels as described previously (5). In some of the experiments, 6 M urea was included in the sample buffer and separating gels. Following immunoblotting with FasL-specific Abs, including Ab-3 (Oncogene Science, Cambridge, MA), the protein bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL).

Statistics. Statistical significance of results was determined by the Mann-Whitney nonparametric U tests. Differences between experimental groups were considered significant at $P < 0.05$.

RESULTS

Expression of FasL on SCCHN Cell Lines. Both immunostaining and flow cytometry were used to evaluate expression of FasL on SCCHN cell lines available in our laboratory. These studies were performed with several commercially available anti-FasL Abs and the NOK-3 Ab generously provided by Dr. Yagita. Before their use for staining of tumor cells, all primary and secondary Abs were titrated to determine their optimal working dilutions. This was considered to be an essential step for comparison of various anti-FasL Abs by flow cytometry, using activated normal PBLs, untreated Jurkat cells, and activated Jurkat cells, as well as tumor cell lines. Surface FasL expression was detectable in a very small proportion (<1%) of *ex vivo* activated normal PBMCs (data from at least 10 separate determinations are not shown) or Jurkat cells (Fig. 2). In contrast, surface expression of FasL on various SCCHN cell lines was observed on a large proportion of tumor cells (see, *e.g.*, PCI-13 staining in Fig. 2).

Generally, a shift of the entire peak to the right was observed, indicating that tumor cell subpopulations characterized by negative, weak, or strong surface expression of FasL could be distinguished by flow cytometry. All of the tested commercially available and NOK-3 anti-FasL Abs, with the exception of G247-4 Ab and NOK-2 Ab, detected FasL expression on the surface of a considerable proportion of tumor cells, which ranged from 10 to 80%, depending on the SCCHN cell line. For example, fewer OSC-19 cells than PCI-13 cells expressed FasL on the cell surface, but the proportion of FasL + tumor cells varied from experiment to experiment, possibly because of effects of cell dissociation, which was necessary for flow cytometry. The C-20 Ab generally stained a higher proportion of tumor cells than did other anti-FasL Abs. To confirm its specificity, competitive blocking with the COOH-terminal FasL peptide was performed, and the peptide was found to completely inhibit surface staining of tumor cells. Blocking with sFasL-containing supernatants of tumor cells transduced with the *FasL* gene partially (30%) decreased surface staining (Fig. 2). Blocking with rFasL (Alexis) partially (30%) reduced surface staining of tumor cells with anti-FasL Abs (Fig. 2). However, it is important to note in Fig. 2 that blocking of C-20 Ab staining with rFasL resulted in a complete inhibition of staining in a subset of tumor cells. In some experiments, SCCHN cells were preincubated with BB-94, a metalloproteinase inhibitor, to prevent cleavage of FasL from the cell surface, and this preincubation was found to increase expression of FasL by 10–30% (MFI shift; data not shown).

When monolayers of SCCHN cell lines were stained with anti-Fas Abs (C-20 or NOK-1) surface-associated expression of FasL was detected (Fig. 3*A*). Surprisingly, expression of FasL on the surface of *FasL*-transduced carcinoma cells was either not increased or only slightly increased relative to that seen with nontransduced cells (data not shown), possibly because FasL was being cleaved and secreted. Immunocytochemical staining of these transfectants with Ab N-20 (Fig. 3*B*) showed the presence of FasL in the Golgi. As reported previously (2), nontransduced, parental SCCHN cell lines also expressed intracytoplasmic FasL, which was consistently detectable in >80–90% of permeabilized tumor cells by flow cytometry, using NOK-1, NOK-2, or NOK-3 Abs. However, FasL protein was not localized to the Golgi compartment in parental cells but diffusely distributed in the cytoplasm (not shown), indicating that SCCHN cells contain but do not actively secrete FasL.

Expression of FasL and Fas in SCCHN *in Situ*. Paraffin-embedded SCCHN biopsy tissues were sectioned and stained by immunoperoxidase to determine FasL and Fas expression in tumor cells *in situ*. This series of experiments was performed to confirm that FasL expression is not an artifact induced by culture of tumor cells. FasL expression was detected in all SCCHN *in situ*. However, regions that were FasL-positive and regions that were FasL-negative were observed in all specimens (Fig. 4*A*). We determined that FasL expression was particularly strong in areas of the tumors closest to infiltrating lymphocytes and in tumors that contained substantial lymphocytic infiltrates (Fig. 4*B*). In addition, we observed that expression of Fas was heterogeneous in human SCCHN. As shown in Fig. 4, uniformly moderate staining for Fas was observed in some tumors (Fig. 4*C*), but other tumors contained few Fas+ tumor cells (not shown), and these cells were largely infiltrating lymphocytes.

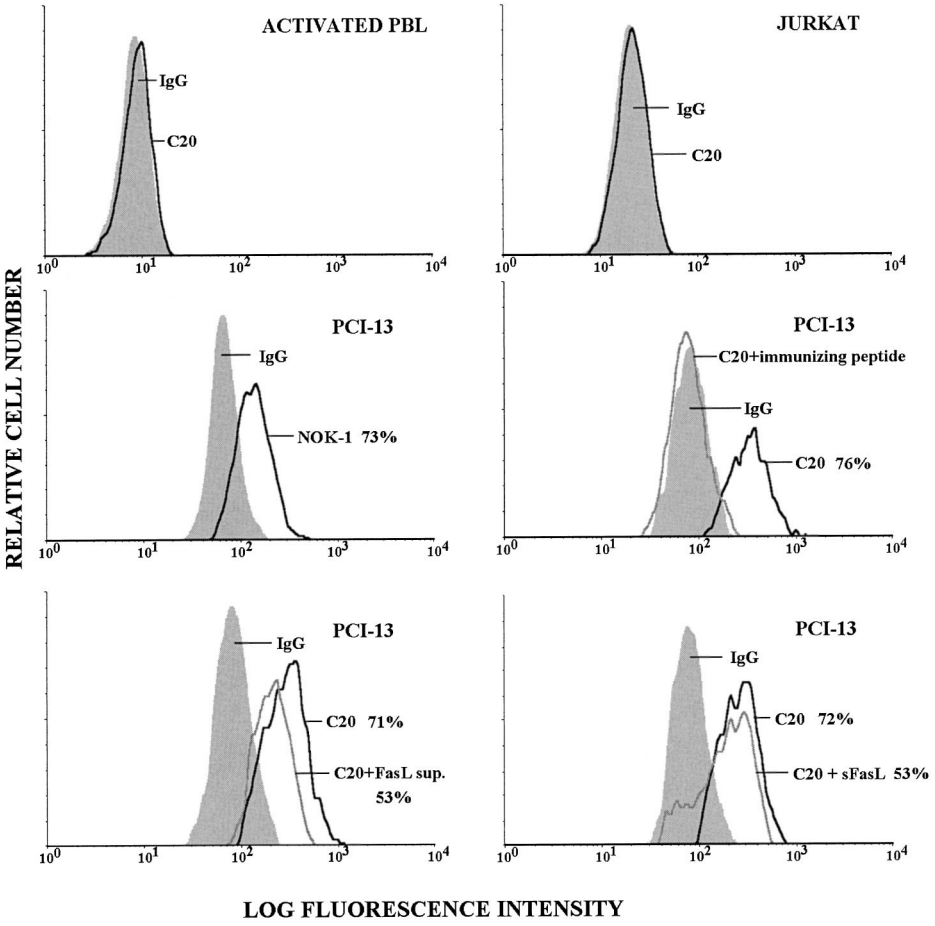

Fig. 2. Surface expression of FasL on normal activated PBMCs, Jurkat cells, and PCI-13 cells. Tumor cells were dissociated, stained with pretitered anti-FasL or isotype control Abs by the indirect immunofluorescence method, and examined by flow cytometry. With C-20 Ab, blocking experiments using rFasL, FasL-containing supernatants, or the immunizing peptide were performed as described in "Materials and Methods." All Abs were tested in the same experiment. The data are representative of at least four experiments. The percentages of positive PCI-13 cells are indicated in each panel.

Expression of mRNA for FasL. The SCCHN cell lines and activated PBLs as a control were studied by RT-PCR for expression of mRNA for FasL and Fas. Both mRNAs were detected, and the identity of the messages was confirmed by Southern hybridization using FasL cDNA or Fas cDNA. Cross-hyrbidization experiments confirmed the specificity of the cDNA probes for the FasL and Fas amplicons. Automatic DNA sequencing performed with the PCR products obtained from mRNA in PCI-13 cells and in Jurkat cells (control) confirmed the sequence identity to that of FasL (data not shown). Comparatively less mRNA for FasL than for Fas was present in cellular RNA obtained from the various SCCHN cell lines (Fig. 5). This was a consistent observation in all human tumor cell lines studied in our laboratory (5). In the presence of cycloheximide (10 μg/ml for 6 h), carcinoma cells were shown to express up to 100-fold more FasL mRNA by semiquantitative RT-PCR (data not shown). Thus, the low mRNA levels for FasL normally seen in SCCHN may be due to a rapid cleavage and utilization of this protein. In addition to mRNA for FasL, we also detected mRNA for TRAIL in all of the SCCHN cell lines tested. Untreated Jurkat cells, PMA/ionomycin-activated Jurkat cells, and parental as well as FasL-transduced cells lines were also tested for expression of FasL or Fas mRNA and found to be positive.

Immunoblots for FasL. Initially, to verify that anti-FasL Abs recognize rFasL protein, Western analyses were performed that showed that Abs C-20 and G247-4 immunoreacted with the rFasL (Fig. 6*A*, 35-kDa band), which is longer by 20 amino acids than natural sFasL. Next, lysates of SCCHN and Jurkat cells were sepa-

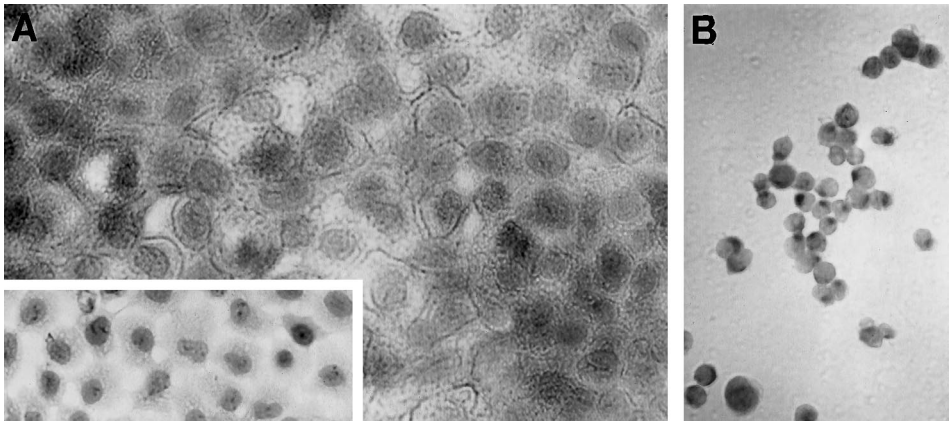

Fig. 3. Immunocytochemical staining of PCI-13 monolayers for expression of FasL with C-20 polyclonal Ab, which decorates cell surfaces of tumor cells (*A*). *Inset*, isotype control. *A*, ×600 (*inset*, ×300). FasL is localized to the Golgi in tumor cells transduced with the *FasL* gene and secreting FasL (*B*). *B*, ×100.

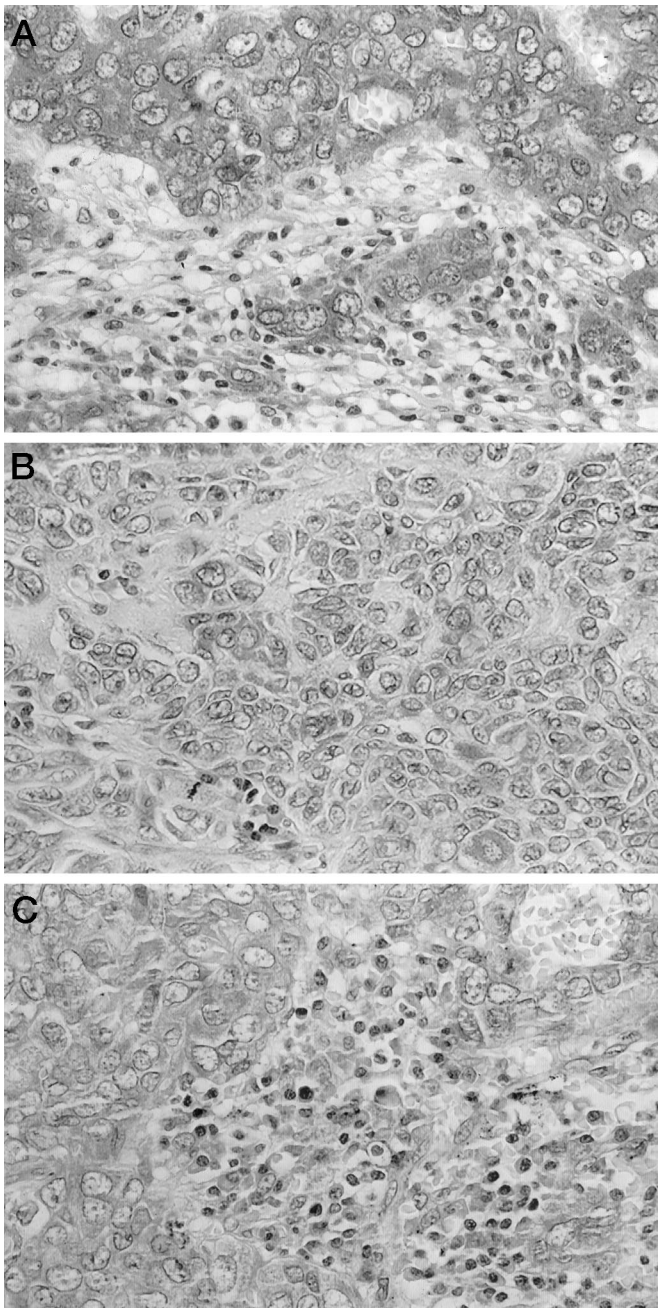

Fig. 4. Immunocytochemical staining of representative SCCHN tissues for expression of FasL (*A* and *B*) or Fas (*C*). Note strong, uniform expression of FasL in *A*, but weak and uneven FasL expression in *B*. In *C*, note Fas^+ mononuclear cells infiltrating the tumor. ×1500.

rated on polyacrylamide gels and immunoblotted using anti-FasL G 247-4 or Ab-33 monoclonal Abs, as well as C-20 or Ab-3 polyclonal Abs, all of which are known to work in protein immunoblots. G 247-4 Abs gave a strong positive signal (27-kDa band) in SCCHN cells and in Jurkat cells. A weak band at 42 kDa was also detected (Fig. 6*B*). In contrast, Ab-3 and Ab-33 only gave a 42-kDa signal, as confirmed by reblotting of stripped G 247-4-treated membranes (not shown). Because Ab-3 or Ab-33 did not immunoblot with rFasL, we interpreted these data as consistent with the possibility that FasL was present in Jurkat or tumor cell lysates in two forms: (*a*) the 42 kDa full-length form; and (*b*) the smaller, 27-kDa cleaved form, also detected in supernatants of *FasL*-transduced cells (Fig. 6*C*). To confirm that C-20 Ab recognized both the full-length and cleaved forms of FasL in carcinoma cells, Western blots were performed with lysates and supernatants of FasL-transduced carcinoma cells. As shown in Fig. 6*C*, C-20 Ab blotted both the 42- and 27-kDa FasL forms in cell lysates and only the cleaved 27-kDa FasL in supernatants of carcinoma transduced with FasL.

Coincubation of SCCHN Lines with T Cells. In preliminary experiments, coincubation of SCCHN cells with activated T lymphocytes (either PMA/ionomycin-activated normal PBMC or Jurkat cells) for 18 h resulted in death of a proportion of T cells, as measured in JAM assays (Fig. 7). To further investigate this interaction and the role of FasL in inducing apoptosis in T cells, an *in vitro* model was established in which various SCCHN cell lines were coincubated with Jurkat cells under strictly defined conditions. This model proved to be extremely useful for evaluating the mechanisms of tumor-induced apoptosis in lymphocytes coincubated with tumor cells (10). Initially, we observed that killing of Jurkat cells or activated PBMC by SCCHN cells was dependent on the ratio of tumor cells to lymphocytes (Fig. 7), as measured in JAM assays following 18 h of coincubation. Apoptosis was optimally induced in the presence of a considerable excess of tumor cells, generally, at a 40:1 or 80:1 tumor:Jurkat cell ratio. At a tumor (PCI-13) to Jurkat ratio of 40:1, the percentage of Jurkat cells killed (range, 30–40%) was comparable to that induced by incubation of Jurkat cells with CH-11 Ab at 200 ng/ml. Also, kinetics of tumor-induced apoptosis were investigated by coincubating tumor cells with Jurkat cells for periods ranging from 4 to 48 h (data not shown). The optimal coincubation period for inducing apoptosis was determined to be 18 h in this model.

Involvement of FasL and Fas in Lymphocyte Apoptosis. To confirm the participation of FasL expressed on the tumor in apoptosis of lymphocytes coincubated with SCCHN cells, blocking of apoptosis with anti-FasL Abs was performed. As shown in Table 1, preincubation of PCI-13 cells with various concentrations of anti-FasL Ab (4H9) for 2 h resulted in a partial inhibition (40%) of tumor-induced apoptosis in Jurkat cells at an Ab concentration of 5 μg/ml On the other hand, with another SCCHN cell line, OSC-19, anti-FasL Ab used at the same time concentration almost completely inhibited apoptosis in Jurkat cells at tumor:lymphocyte ratios of 80:1 and 40:1 (Fig. 8*A*). These experiments suggested that FasL expressed on the tumor might mediate apoptosis of lymphocytes coincubated with tumor cells, although the engagement of FasL on Jurkat cells could not be excluded.

To determine whether blocking of Fas on Jurkat cells with antagonistic anti-Fas Ab (ZB4) also inhibits their apoptosis, we preincubated these cells for 2 h with various dilutions of ZB4 Ab prior to coculture with PCI-13 cells. At a concentration of 200 ng/ml, ZB4 anti-Fas Ab, but not isotype control Ab, significantly inhibited apoptosis ($P < 0.001$) induced by tumor cells in lymphocytes, as determined in JAM assays (Fig. 8*B*). As a positive control for this experiment, agonistic anti-Fas Ab (CH-11) was used to induce apoptosis of Jurkat cells. Apoptosis induced by CH-11 Ab was also significantly inhibited when Jurkat cells were preincubated with anti-Fas ZB4 Ab (Fig. 8*B*). Importantly, tumor cells used at an 80:1 ratio induced a higher level of apoptosis in Jurkat cells after 18 h of coincubation than did agonistic CH11 Ab, used as a positive control. Together, these Ab blocking experiments support the involvement of Fas expressed on lymphocytes and FasL expressed on SCCHN cells in tumor-induced apoptosis but do not rule out the possibility that tumor induces expression of FasL on Jurkat cells, leading to activation-induced cell death.

Tumor Cell Supernatants and Death of Jurkat Cells. Supernatants collected from 24–72-h cultures of SCCHN did not induce killing of Jurkat cells, as determined in JAM or TUNEL assays. However, unconcentrated supernatants of carcinoma cells transduced

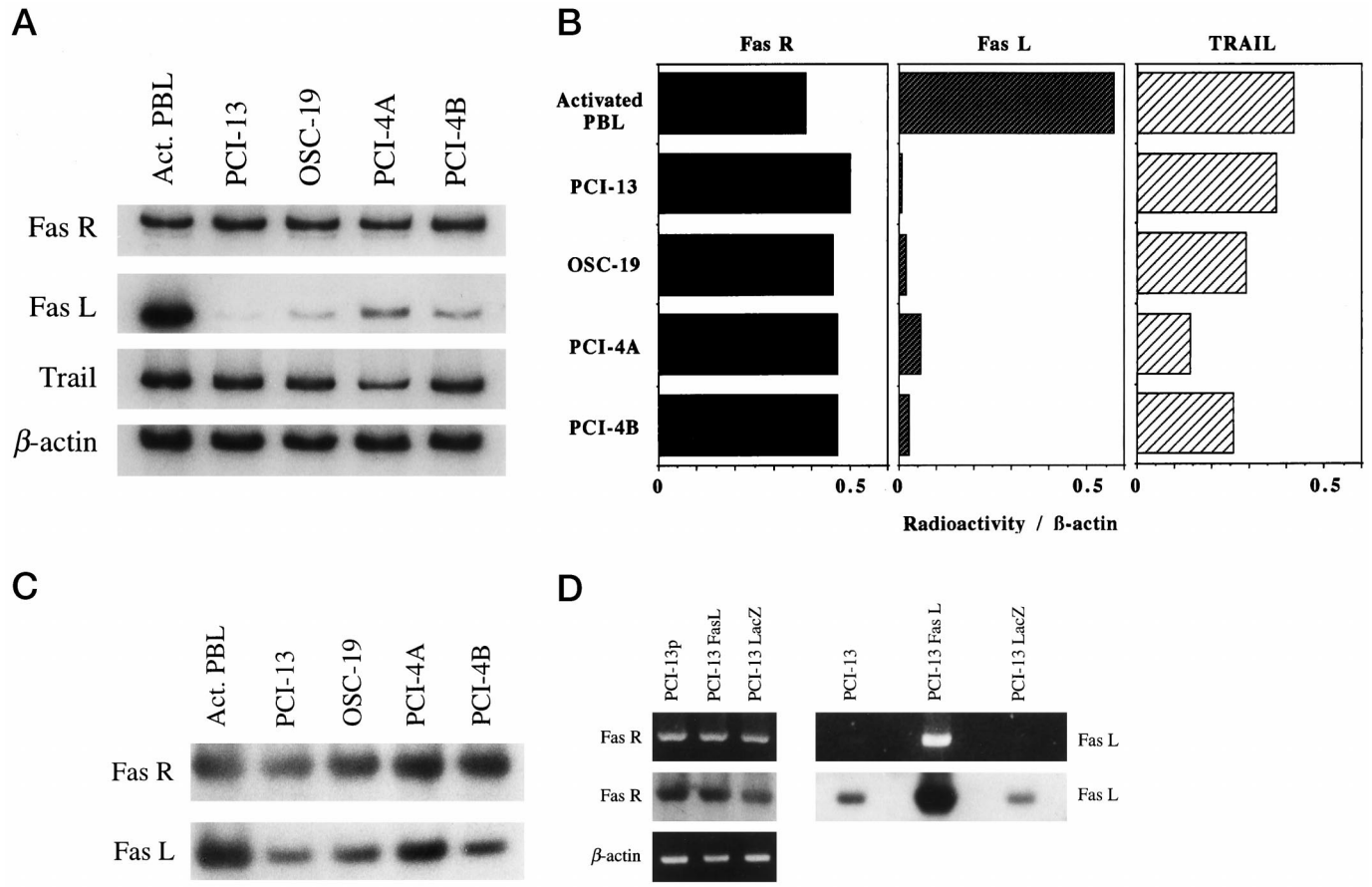

Fig. 5. RT-PCR for expression of mRNAs for FasR, FasL, and TRAIL in various SCCHN cell lines and activated PBLs used as control (*A*). In *B*, ratios of cpm for each of the messages relative to cpm for β-actin message in each of the tumor cell lines or PBLs. Radioactivity counts were determined in a PhosphorImager. In *C*, PCR products for FasR and FasL shown in *A* were hybridized to cDNA probes for FasR or FasL, respectively, to confirm the product identity. In *D*, RT-PCR for expression of FasR and FasL messages in PCI-13p (parental) and PCI-13 transduced either with the hFasL gene or with the LacZ gene as control. Ethidium bromide gels show the PCR products for FasR or FasL (*top row*), the products hybridized to the respective cDNAs (*middle row*), and β-actin used as control (*bottom row*).

with the FasL gene induced death of Jurkat cells at the level of 39% ± 1 (mean ± SD of three experiments; Fig. 9) as measured in JAM assays, which was comparable to the level of 30–40% killing induced in the same cells by agonistic CH-11 Abs. Similarly, 24-h coincubation of FasL-transduced tumor cells with Jurkat resulted in 66% killing at a tumor:Jurkat cell ratio of 20:1, in comparison to 20% or less killing induced by parental cells in Jurkat targets under the same experimental conditions (Fig. 9). These levels of killing of Jurkat targets were substantially higher than those observed with soluble rFasL purchased from Alexis.

Effects of Metalloproteinase Inhibitors on Tumor-induced Cell Death. FasL expression on the cell surface is thought to be regulated by metalloproteinases (31). We observed that expression of FasL on SCCHN cell lines was up-regulated by pretreatment of tumor cells with the metalloproteinase inhibitor, BB-94, as determined by flow cytometry (data not shown). When PCI-cell lines pretreated with BB-94 were coincubated with Jurkat cells, they induced DNA fragmentation in a greater proportion of lymphocytes than did tumor cells not pretreated with the inhibitor (Fig. 10). This finding suggests that the level of FasL expression on the surface of tumor cells is an important factor in tumor-induced apoptosis of Jurkat cells.

DISCUSSION

Tumor-associated lymphocytes often display changes consistent with apoptosis, as was recently demonstrated by the use of TUNEL assays in several different tumor types (5, 13). Thus, human tumors appear to be able to evade the host immune response by inducing death of immune cells present in the tumor microenvironment, and particularly those immunocytes that are localized adjacent to or within tumor cell nests (18). The mechanism responsible for *in situ* apoptosis of TIL is not clear, but it is possible that the Fas/FasL pathway participates, at least in part, in this tumor-induced apoptosis, as suggested previously (5, 13–16). FasL is a type 2 membrane protein, which upon cross-linking its receptor, Fas, induces apoptosis of target cells, including activated lymphocytes (32). Whereas FasL expression on tissue cells, especially at the immunologically privileged sites, is considered to be essential for elimination of potentially tissue-destructive, activated T cells (33), its presence on all types of human tumor cells and its role in tumor-induced immune cell death has not been universally accepted (16).

The nominal "counterattack" of tumor against immune cells, as proposed by O'Connell *et al.* (14), depends on expression on the tumor surface of functional FasL, which may in turn induce apoptosis of Fas-sensitive antitumor effector cells, thus rendering the tumor an immunologically privileged site. Tumor cell lines and tumors of various histological origin, including SCCHN, have been reported to express FasL (5, 13–21, 34, 35). However, expression of FasL on the surface of various cell types is highly variable, in part because the molecule is actively cleaved by cell membrane-associated metalloproteinases (21, 31). Our studies show that SCCHN cell lines, as well as SCCHN in tissues, express FasL protein, as well as mRNA for this protein, and that it is detectable on the cell surface of SCCHN cells.

As FasL is a death-inducing ligand, which is normally cleaved from the cell surface, we were somewhat surprised at its expression on the surface of a considerable proportion of SCCHN cells by flow cytometry. In contrast, only few activated PBMC or Jurkat cells expressed FasL on the surface. Whereas expression of FasL on the surface of tumor cells, as detected by several anti-FasL Abs, varied in different cell lines, there was a reasonable agreement among these Abs for each individual tumor cell line. The C-20 Ab from Santa Cruz was reported previously to detect FasL-associated protein(s) instead of FasL (23–26). However, after this Ab was optimally titered, it was shown to detect FasL on the surface of tumor cells, as confirmed by blocking experiments. Also, Western blotting showed that C-20 Ab detects rFasL and both the 42-kDa and cleaved (27-kDa) FasL protein in cell lysates (Fig. 6). Not all anti-FasL Abs are suitable for surface staining, and in our hands, Ab G247-4 and NOK-2 Abs from PharMingen failed to stain activated PBMC, Jurkat cells, or tumor cells for FasL by flow cytometry. The presence of FasL on the tumor cell surface was always confirmed by intracytoplasmic staining for FasL, as reported previously (5) and by RT-PCR for FasL mRNA, followed by Southern hybridization with FasL cDNA. All SCCHN cell lines examined thus far in our laboratory abundantly expressed FasL in the cytoplasm and expressed mRNA for this protein, and FasL was detectable on the surface of a variable but substantial proportion of these tumor cells by flow cytometry, despite the need for cell dissociation prior to flow cytometry.

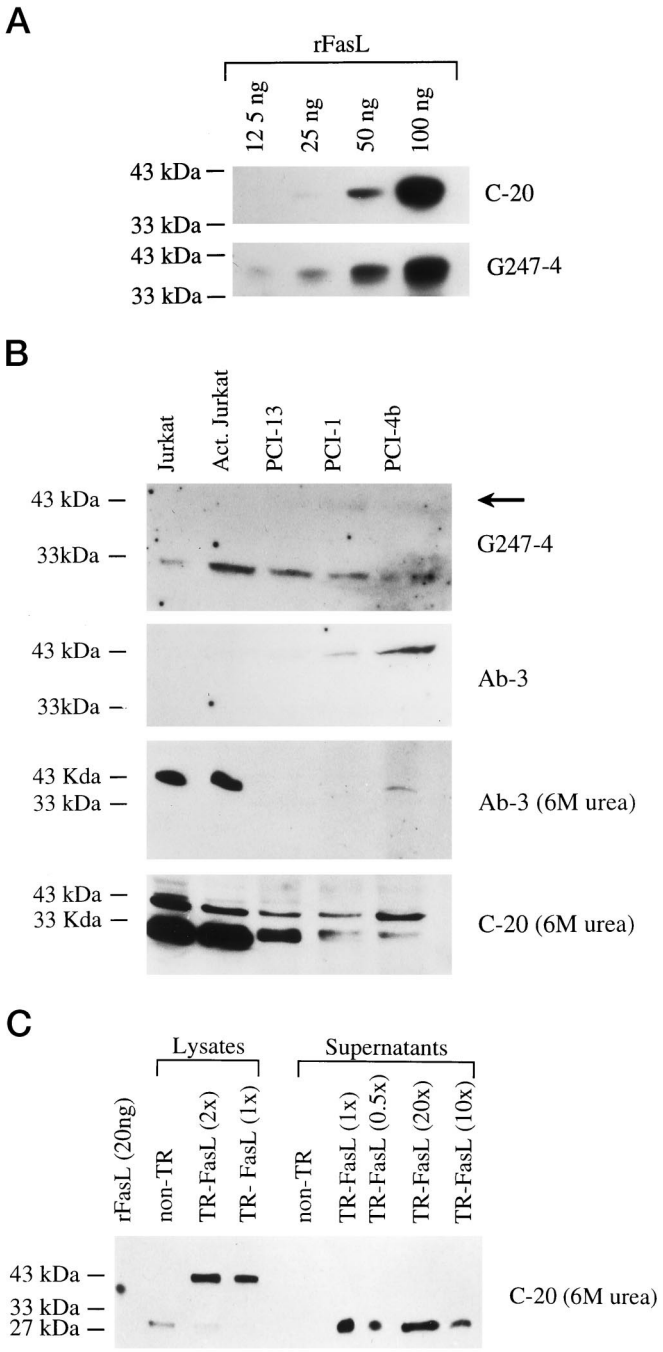

Fig. 6. Immunoblots of soluble recombinant FasL (rFasL, Alexis) in *A* and of tumor cell lysates in *B* developed with various anti-FasL Abs. Abs C-20 (Santa Cruz) and G 247-4 (PharMingen) immunoreact with a 37-kDa rFasL (*A*). In tumor cell lysates (*B*), Ab G 247-4 recognizes a 33-kDa form and a larger 43-kDa form of FasL (*arrow*). Ab-3 (Oncogene) and Ab-33 (not shown) react only with the 43 kDa form (*B*). These two Abs do not immunoreact with rFasL or sFasL in the supernatants of FasL-transduced cells. Ab-3 gives strong bands with Jurkat cells only in 6 M urea (*B*). Ab C-20 detects both the 43- and 33-kDa bands only in 6 M urea (*B*). AbC-20 detects both 42- and 27-kDa forms of FasL in cell lysates, and only the cleaved 27-kDa FasL in supernatants of tumor cells transduced with the *FasL* gene (C).

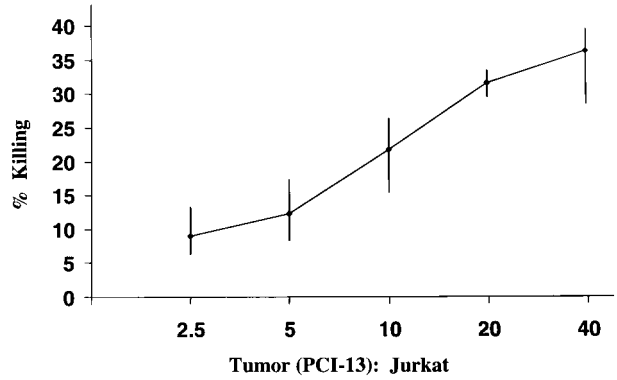

Fig. 7. Dose-dependent killing of Jurkat cells by PCI-13 tumor cells, as measured in JAM assays. *Bars*, SDs of the mean cpm values from six wells. Shown is a representative experiment of five performed with Jurkat cells. Similar results were obtained when PBMCs of different donors were used as targets.

Table 1 *Tumor cell-mediated apoptosis in Jurkat cells: blocking of FasL-induced apoptosis with anti-FasL antibodies*[a]

Ab concentration (μg/ml)	% Apoptosis inhibition
0	None[b]
0.2	4
0.5	22
1.0	25
2.5	30
5.0	40

[a] Tumor cells (PCI-13) were pre-incubated with the blocking Ab (4H9) for 2 h, washed, and then coincubated with Jurkat cells for 18 h. Apoptosis of Jurkat cells was tested in JAM assays. Isotype control Ab used at the same concentrations as 4H9 Abs showed no effects on apoptosis of Jurkat cells.

[b] In the absence of Ab, the level of apoptosis in Jurkat cells was 40% at the 80:1 ratio of tumor:Jurkat cells.

Expression of both FasL and Fas in SCCHN *in situ* was heterogeneous, in that FasL-positive and -negative regions of the tumor were observed. This observation is similar to the data reported by Bennett *et al.* (18) for esophageal carcinoma *in situ*. Furthermore, similar to Bennett *et al.* (18), we observed that the number of infiltrating TIL were significantly higher in the SCCHN regions with strong FasL expression, as opposed to a lower number of TIL in tumor areas with low FasL expression. These observations suggest that *in situ* FasL expression in tumor cells might be up-regulated by the presence of infiltrating lymphocytes. We and others have proposed that apoptosis of lymphocytes interacting with tumor cells might be responsible for functional impairment of TIL. In fact, this might be one of the mechanisms potentially responsible for immunosuppression observed in the tumor microenvironment.

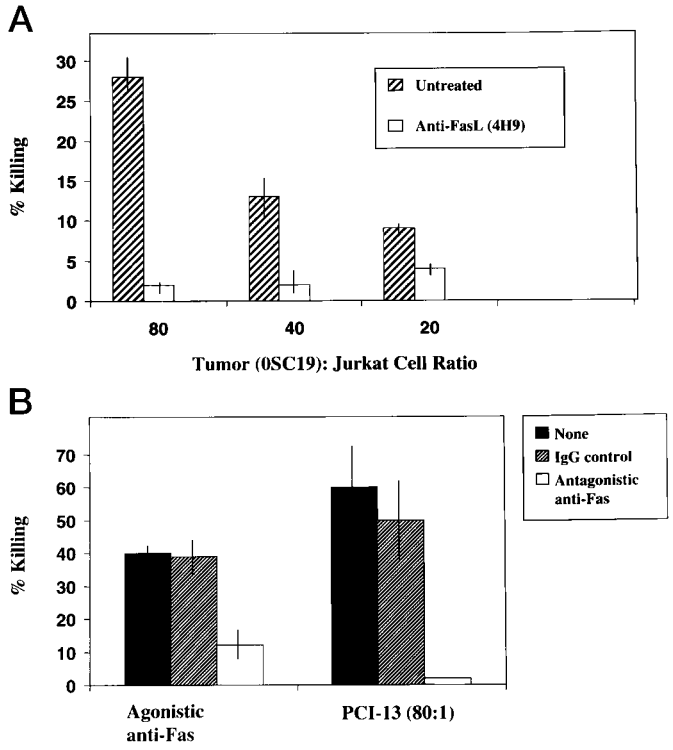

Fig. 8. Inhibition of tumor-induced apoptosis by preincubation of tumor cells with anti-FasL Ab (4H9; *A*) or by blocking of Fas on Jurkat cells. Apoptosis of Jurkat cells was measured in JAM assays. In *A*, inhibition by 4H9 Ab was significant of $P < 0.001$. Isotype control (not shown) was not different from untreated tumor cells. In *B*, as a control for tumor cells, agonistic anti-Fas Ab (CH-11) was used to induce Jurkat cell killing (*left*). Killing of Jurkat cells by PCI-13 or CH-11 Ab was significantly inhibited ($P < 0.0001$) in the presence of ZB4 anti-Fas Ab (200 ng/ml) but not of isotype control Ab. The data are mean percentages ± SD.

To confirm that FasL expressed on the surface of SCCHN cells can induce apoptosis of T cells, we coincubated Jurkat cells or *in vitro* activated PBMC (data not shown; Ref. 36) with various SCCHN cell lines for 18 h. As a result of direct contact of SCCHN cells with Jurkat cells and of FasL-Fas interaction, apoptotic signal was induced in lymphocytes and resulted in DNA fragmentation detectable in both JAM and TUNEL assays. Blocking with neutralizing anti-FasL or antagonistic anti-Fas Abs confirmed that apoptosis was induced, in part, by FasL-Fas interactions. Our experiments demonstrated that SCCHN can directly induce death of activated lymphocytes. However, it is also possible that tumor activates the immune cells, induces up-regulation of FasL on these cells, and thus facilitates activation-induced cell death of lymphocytes, which then kill each other, as proposed by others (16). Three findings strongly suggest that the tumor may have a role in inducing lymphocyte cell death in this setting: (*a*) expression of FasL on tumor cells was found to be considerably higher than that in activated lymphocytes; (*b*) incubation of tumors with anti-FasL Ab or FasFc chimeric protein (10) partially blocked apoptosis in lymphocytes; and (*c*) supernatants of FasL-transduced tumor cell were able to induce apoptosis in Jurkat cells. Furthermore, in preliminary experiments, we showed that in the presence of metalloproteinase inhibitors, which prevent cleavage of FasL from SCCHN cell surface (31), a higher percentage of Jurkat cells was apoptotic after coincubation with the inhibitor-treated tumor cells than with the untreated cells. Together, these data suggested that the Fas-FasL pathway may be involved in T-cell apoptosis induced by SCCHN. Because all SCCHN cell lines examined also strongly expressed mRNA for TRAIL, it is likely that interactions mediated via TRAILR on Jurkat cells may also play an important role in the process of tumor-induced lymphocyte apoptosis.

Overall, our experiments verify the presence of biologically active FasL in human carcinoma cells and cell lines. Considerable effort expanded in demonstrating by several different methodologies and reagents *ex vivo* and *in situ* of FasL involvement in killing of T lymphocytes was necessary in view of persisting doubts and discrepancies reported from various laboratories (11, 16). The controversy about the biological significance of FasL in the tumor counterattack has to be tempered by a better understanding of the biology of FasL itself and of diversity of FasL forms recognized by different Abs.

In tumor cells coincubated with Jurkat or activated PBMC *ex vivo* or in tumor cells *in situ*, no apoptosis was observed. Because considerable numbers of tumor cells express FasL as well as Fas, it is likely that the tumor is relatively resistant to FasL expressed by itself or to FasL expressed on lymphocytes. We demonstrated earlier that SCCHN cells are sensitive to such apoptosis inducers as VP-16, but not to agonistic CH-11 Ab (2). This type of evidence suggests that tumor cells are largely Fas-resistant, possibly to protect themselves from apoptosis mediated via FasL expressed on other tumor cells or present in plasma/serum, as suggested earlier (11). Such Fas resistance of SCCHN might also contribute to tumor evasion from immune effector cells, and additional protective mechanisms, *e.g.*, expression of Bcl-2 in SCCHN (37), might further reinforce these evasion tactics. Immune cells, on the other hand, can be also protected from tumor-induced cell death by different strategies, including blocking of the

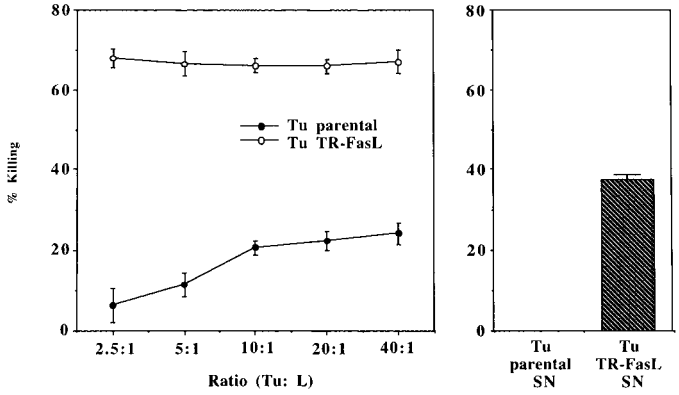

Fig. 9. Apoptosis of Jurkat cells incubated in the presence of parental tumor cells or tumor cells stably transduced with the *FasL* gene. Apoptosis was measured in JAM assays. The data are mean ± SE of six wells. A representative experiment of three performed is shown on the *left*. *Right*, apoptosis of tumor cells incubated in the presence of unconcentrated supernatant of tumor cells transduced with the *FasL* gene. The data are presented as mean percentages ± SD from six experiments.

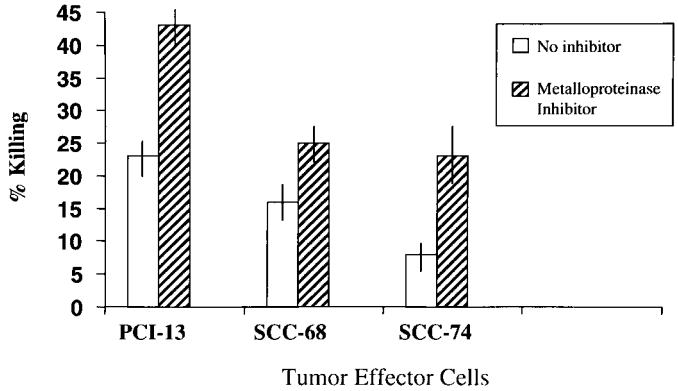

Fig. 10. A metalloproteinase inhibitor (BB-94) enhances tumor-induced apoptosis of Jurkat cells. Apoptosis was measured JAM assays. The ratio of tumor:Jurkat cells was 40:1. Increases in the presence of BB-94 were significant at $P < 0.01$. The data are mean percentages ± SD.

apoptotic pathway by inactivation of caspases or pretreatment with cytokines IL-2 or IL-12, as indicated by our preliminary data (not shown), and perhaps other cytokines alone or in combinations (38). The process of TIL apoptosis in the tumor microenvironment is likely to be dependent on the ratio of tumor to effector cells, the state of TIL activation, and the cytokine profile determined by tumor-effector cell interactions. The mechanisms involved in the protection from and induction of apoptosis in TIL are under intense investigation in our and other laboratories. A better understanding of these mechanisms is necessary to devise and use therapeutic strategies for protection of effector cells from tumor-induced cell death in the tumor microenvironment.

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