Autocrine Secretion of Fas Ligand Shields Tumor Cells from Fas-Mediated Killing by Cytotoxic Lymphocytes

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ABSTRACT

Mechanisms responsible for resistance of tumors to death receptor-mediated damage by cytotoxic lymphocytes are not well understood. Uveal melanoma cells expressed Fas but were insensitive to Fas triggering induced by bystander cytotoxic T lymphocytes or a Fas-specific agonistic antibody; this could not be ascribed to tumor counterattack against T cells or general resistance of the tumors to apoptosis. Treatment with inhibitors of metalloproteases rendered uveal melanomas sensitive to Fas-mediated cytotoxicity. Metalloprotease inhibitors did not affect the expression of Fas but increased the surface expression of Fas ligand (FasL), which correlated with the disappearance of soluble FasL, from culture supernatants of tumor cells. FasL eluted from the surface of uveal melanomas specifically inhibited cytotoxic T lymphocyte lysis of tumor cells pretreated with an inhibitor of metalloproteases. In addition to uveal melanomas, a number of other tumor cell lines of various cellular origins were sensitized to Fas-mediated cytolysis by metalloprotease inhibitors. Our results show that autocrine secretion of FasL shields tumor cells from Fas-mediated killing by cytotoxic lymphocytes. This defines a novel mechanism of tumor escape from immune surveillance.

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) induce apoptosis in tumor cells by two major mechanisms, exocytosis of granules containing perforin and granzymes or engagement of death receptors. The latter include a group of cell surface molecules of the tumor necrosis factor (TNF) receptor superfamily such as TNF receptor 1, Fas (CD95, APO-1), and TNF-related apoptosis-inducing ligand (TRAIL) receptor 1, which bind the relevant ligands, TNF-α, Fas ligand (FasL), and TRAIL, expressed on the cell surface of CTLs or secreted by activated CTLs.

Many malignancies escape CTL killing due to down-regulation of major histocompatibility complex (MHC) class I molecules at the cell surface. This can be achieved through a number of mechanisms extensively reviewed elsewhere (1, 2). However, many tumors expressing potentially immunogenic antigens and retaining high levels of MHC class I expression still appear to escape T-cell-mediated immune control. Therefore, analysis of alternative mechanisms allowing tumor escape from granule- or death receptor-mediated apoptosis becomes of special importance for developing new strategies of tumor-specific immunotherapy.

Uveal melanomas, the most common primary malignancy of the eye, possess a number of attributes necessary for MHC class I-restricted granule-mediated cytolysis, namely, the expression of tumor antigens that are capable of eliciting both humoral and cellular immunity, high levels of MHC class I complexes, and the presence of prominent lymphoid infiltration, both at the primary tumor site and in metastases. However, in an animal model, in vivo clearance of uveal melanomas by T cells was shown to be independent of MHC class I-restricted recognition and perforin (3). In agreement with this observation, Ericsson et al. (4) demonstrated that patients with high MHC class I expression in their primary uveal melanoma lesions had a significantly decreased survival, thus arguing against a major role of CTLs in the control of the disease. We have recently shown that interferon γ-treated uveal melanomas bind reduced amounts of granzyme B and are less sensitive to granule-mediated CTL lysis than untreated cells.3 These studies strongly suggest that uveal melanomas may escape granule-mediated CTL killing, but it remains unclear whether these tumors are subjected to immune control through death receptor-mediated damage by CTLs.

In this study we demonstrate that uveal melanomas are resistant to CTL- and natural killer (NK)-mediated killing via TRAIL and Fas. The latter, however, can be overridden by treatment with metalloprotease inhibitors and is not associated with changes in the levels of Fas receptor at the surface of tumor cells or counter-damage to the CTLs. We demonstrate that uveal melanomas produce soluble FasL, which, on its binding to the surface of the producer cells, protects them from Fas-induced cell death. Furthermore, we show that this phenomenon is not exclusively specific for uveal melanomas because the same effect was observed in a number of other tumor cell lines originating from different tissues. Our data demonstrate a novel mechanism of tumor escape from T-cell-mediated immune surveillance and broaden the potential applications of metalloprotease inhibitors in cancer therapy.

MATERIALS AND METHODS

Cell Lines and Culture Media. Uveal melanoma cell lines OCM1, OCM3, OCM8, Mel 285, Mel 290, and 92-1 were kindly provided by Dr. M. Jager (Department of Ophthalmology, Leiden University Medical Center, Leiden, the Netherlands). Cutaneous melanoma cell lines 1233 mel, DF1, Be mel, and 0505 were derived from metastatic lesions of patients treated at Radiumhemmetet, Karolinska Sjukhuset and established at the Microbiology and Tumor Biology Center at Karolinska Institutet. The renal cell carcinoma cell lines KRCY-5Y, RCC1852, and A234 were kindly provided by Dr. S. Inrehe (Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden). Neuroblastoma cell lines SK-N-FI, SK-N-BE(2), SK-N-SH, SH-SY5Y, and MC-IXC were obtained from American Type Culture Collection (Manassas, VA). The T-cell leukemia cell line Jurkat TIB 152 was from American Type Culture Collection. JAC-B2 is a HLA-A11–positive Epstein-Barr virus (EBV) nuclear antigen 4 (EBNA4)-expressing lymphoblastoid cell line obtained by transformation of B cells from a healthy donor with the B95.8 strain of the EBV. NK cell lines YT-Indy and Nishi were a kind gift of Dr. Jonas Sundbäck (Microbiology and Tumor Biology Center, Karolinska Institutet). All cell lines (except Nishi) were propagated in Iscove's modified Dulbecco's media (IMDM) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc., Grand Island, NY), 100 IU/mL penicillin, and 100 μg/mL streptomycin (complete medium). Nishi cells were cultured in complete medium with 3% human serum and 100 IU/mL interleukin 2. The CD8+ CTL clones BK289 and CAR13 are HLA-A1–restricted and specific for the EBNA4-derived peptide IVTDFSVK (IVT [5]).

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Chemicals. Complete Mini protease inhibitor tablets were from Roche Diagnostics Scandinavia AB (Bromma, Sweden), and 1,10-phenanthroline was purchased from Sigma (St. Louis, MO). The kit for annexin V staining was purchased from BD PharMingen (San Diego, CA), and tetramethylrhodamine ethyl ester permeable (TMRE) was from Molecular Probes Inc. (Eugene, OR).

Antibodies and Recombinant Proteins. The apoptosis-inducing mouse IgM anti-Fas antibody CH-11 and the Fas-blocking mouse monoclonal antibody B4 were from MBL (Nagoya, Japan). Recombinant soluble killer-TRAIL, Fc-fusion control (a fusion protein consisting of the extracellular domain of the mutated Ith-y1 and the Fc portion of human IgG1), and the mouse monoclonal antihuman FasL antibody 5G51 were from Alexis Corp. (Lausen, Switzerland). The FasL-specific neutralizing mouse monoclonal antibody NOK-2 and the anti-Fas-L mouse monoclonal antibody G247-4 was obtained from Pharmingen (San Diego, CA). Mouse IgG1 and IgG2a as well as the antihuman TNF-α antibody were from R&D Systems (Abingdon, United Kingdom). R-Phycoerythrin–conjugated rabbit antimouse IgG and horseradish peroxidase-conjugated rabbit antimouse IgG were from Dakopatts AB (Alvsjö, Sweden).

Treatment with Metalloprotease Inhibitors. Before all experiments (unless stated otherwise), tumor cells were treated overnight with the metalloprotease inhibitor 1,10-phenanthroline (0.1 mM/L in complete medium) or for 4–6 hours with Complete Mini protease inhibitor cocktail (referred to hereafter as PIC (one tablet in 10 mL of complete medium)). Untreated cells were used as a control. Viability of cells was assessed by trypan blue or propidium iodide (PI) staining in all experiments.

Chromium Release Assays and Blocking Experiments. Chromium release assays were performed as described previously (6). Briefly, target cells were labeled with Na251CrO4 for 1 hour at 37°C. After washing, cells were incubated with IVT-specific CTLs at an effector to target ratio of 5:1 or with the NK cell lines YT-Indy and Nishi at a 12:1 or 6:1 ratio, for 16 hours at 37°C. Release of chromium into the supernatants was measured by a gamma counter (Wallac Sverige AB, Upplands Väsby, Sweden). The anti-Fas antibody CH-11 and recombinant soluble TRAIL were used at final concentrations of 250 and 100 ng/mL, respectively. Mouse IgM and the Fc-fusion protein were used as controls at the corresponding concentrations. For blocking of FasL on effector cells, CTLs were preincubated with 10 μg/mL NOK-2 antibody for 45 minutes at room temperature before the assay. Mouse IgG2a antibodies were used as an isotype control. Blocking of Fas on the target cells was performed under similar conditions using 200 ng/mL ZB4 antibody or mouse IgG1 as a control. For analysis of CTL functions, BK289 cells were incubated with uveal melanoma cells, which were treated with either PIC or 1,10-phenanthroline or left untreated, at a 5:1 effector to target ratio for 16 hours at 37°C. To measure the cytolytic activity of CTLs, 31Cr-labeled JAC-B2 or Jurkat cells were subsequently added to achieve a 1:1 JAC-B2 cell to 5:1 (Jurkat cells) effector to target ratio. Release of chromium into the supernatants was measured after 4 hours for JAC-B2 targets and 16 hours for Jurkat targets.

Assessment of Apoptosis. Uveal melanoma cells were kept untreated or cultured in PIC-containing medium for 6 hours followed by a 24-hour incubation with either CTLs at an effector to target ratio of 2:1 or 250 ng/mL CH-11. Apoptotic changes were assessed by incubating cells in PBS containing 25 mM/L TMRE for 30 minutes at 37°C followed by incubation in 100 μL of annexin V binding buffer [10 mM/L HEPES/NaOH (pH 7.4), 140 mM/L NaCl, and 2.5 mM/L CaCl2] containing 25 mM/L TMRE and 5 μL of annexin V-FITC for 15 minutes in the dark at room temperature. A 300 μL of binding buffer were added before analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). For double staining with annexin V/PI, 5 μL of PI were added to the cells together with annexin V. Tumor cells were discriminated from CTLs on the basis of forward/side scatter characteristics.

Monitoring of Fas and Fas Ligand at the Cell Surface. Uveal melanoma cells were treated with either PIC or 1,10-phenanthroline for 6 hours or left untreated. To monitor the expression of FasL, at the cell surface, cells were washed extensively in ice-cold PBS and incubated with either 1 μg/mL of the FasL-specific antibody 5G51 or the mouse IgG1 isotype control for 1 hour on ice. To monitor the expression of surface Fas, cells were washed extensively in ice-cold PBS and incubated with 1 μg of Fas-specific antibody CH-11 or mouse IgM as the isotype control for 30 minutes on ice. After extensive washing in PBS, binding of Fas- and FasL-specific antibodies was visualized by R-phycocerythrin–conjugated rabbit antimouse antibodies. Cells were then fixed in 1% paraformaldehyde in PBS and analyzed by fluorescence-activated cell sorting.

Detection of Soluble Fas Ligand. Soluble FasL, in the culture supernatant of uveal melanoma cells was measured by Western blot. Three to four million adherent uveal melanoma cells were treated with PIC or 1,10-phenanthroline for 6 hours, the culture supernatants were discarded, and metalloprotease inhibitor-containing fetal calf serum-free X-VIVO15 medium was added to sufficiently cover the growing cells. After overnight incubation at 37°C, culture supernatants were collected, and recombinant TNF-α was added to a final concentration of 300 ng/mL. The culture supernatants were concentrated 10× using Amicon centrifuge centrifugal filter devices with a cutoff limit of 10 kDa (Millipore AB, Sundbyberg, Sweden). The resulting samples were separated on a 12.5% precast polyacrylamide gel using the Pharmacia Multiphor II electrophoresis system (Amersham Pharmacia Biotech, Uppsala, Sweden). After protein transfer, the polyvinylidene difluoride membrane (Millipore AB) was blocked in 5% milk/0.1% Tween 20 in PBS and subsequently probed with the FasL-specific antibody G247-4 (2 μg/mL) or the antihuman TNF-α antibody MAB210 (2 μg/mL) at 4°C overnight, followed by a horseradish peroxidase–conjugated rabbit antimouse antibody diluted 1:5,000. Specific bands were visualized by chemiluminescence (Pierce SuperSignal west femto maximum sensitivity substrate; Boule Nordic AB, Huddinge, Sweden) and digitally captured in a Fujifilm LAS-1000 Image Reader system (Science Imaging Scandinavia AB, Nacka, Sweden). Densitometry was performed using the Fujifilm ImageGauge software (Science Imaging Scandinavia AB).

Treatment with a Low pH Buffer and Preparation of Eluates. Uveal melanoma cells (1 × 107), untreated or treated with PIC, were removed from the plastics using a cell scraper, washed in PBS, and gently resuspended in 1 mL of a buffer containing 0.06 mol/L sodium dihydrogen carbonate and 0.113 mol/L citric acid (pH 3.0) or in PBS (pH 7.5). After 1 minute of incubation at room temperature, an equivalent volume of alkaline medium [IMDM (pH 9.0)] was added to the samples containing citric buffer, resulting in a pH of 7.2 to 7.5 in the samples. An equivalent volume of IMDM (pH 7.5) was added to the PBS-containing aliquots. All samples were pelleted down at 4°C for 5 minutes at 200 × g, and the supernatants were collected and subsequently cleared by centrifugation at 1,200 × g for 5 minutes. The resulting samples were concentrated 20× using Amicon centricrons (cutoff limit of 10 kDa) and adjusted to the initial volume (2 mL) with fresh IMDM. The samples were designated as follows: eluate 1, supernatant from PBS-treated tumor cells; eluate 2, supernatant from low pH-treated tumor cells; eluate 3, supernatant from PBS-treated tumor cells pretreated with PIC, and eluate 4, supernatant from low pH-treated tumor cells pretreated with PIC.

Monitoring the Effect of Eluates on CTL-Mediated Cytotoxicity. To analyze the effect of molecules eluted from tumor cells on CTL-mediated killing, untreated or PIC-treated uveal melanoma cells were labeled with 51Cr for 1 hour before treatment with low pH buffer (as described above). Next, 100 μL of each eluate (prepared as described in the previous section) were added to 75 μL of chromium-labeled tumor cells seeded in a 96-well plate. After incubation at 37°C for 1 hour, the cells were spun down, and 75 μL of the supernatant were removed. BK289 CTLs were added at an effector to target ratio of 5:1, and chromium release was measured after 16 hours.

Removal of Fas Ligand from Low pH Eluates. One milliliter of each eluate was incubated for 2 hours at 4°C with 10 μg/mL of either the anti-Fasl, NOK-2 antibody or the IgG2a isotype control antibody, followed by the addition of 50 μL of protein A-Sepharose [50% (v/v) suspension in IMDM]. After 2 hours of incubation at 4°C, the Sepharose beads were removed by centrifugation, and the remaining eluates were used in a 16-hour chromium release assay as described above.

Statistical Analysis. Results were analyzed by t test. Changes were considered significant at P < 0.05.

RESULTS

Treatment with Inhibitors of Metalloproteases Renders Uveal Melanoma Cells Sensitive to Non–MHC-Restricted Lysis by CD8+ CTLs. To analyze death receptor-mediated killing of uveal melanoma cells by CTLs, we developed a model that utilizes the human CD8+ HLA-A11–restricted CTL clones BK289 and CAR13, which are specific for the peptide epitope IVT (5), derived from...
EBNA-4. The epitope is naturally presented only by targets infected with EBV; therefore, IVT-specific CTLs did not exhibit any MHC class I-restricted antigen-specific cytolysis of EBV-negative tumor cell lines, as measured in standard 4-hour cytotoxicity assays (data not shown). However, substantial lysis of Jurkat cells was mediated in a TRAIL- and Fas-dependent manner by the same CTLs used as effectors in 16-hour cytotoxicity assays (Fig. 1A; data not shown). A panel of uveal melanoma cell lines was used to investigate the susceptibility of uveal melanoma tumors to non–MHC-restricted lysis by CD8+ CTLs. As shown in Fig. 1A, coincubation with BK289 CTLs resulted in a barely detectable level of 51Cr release from all of the tested uveal melanoma cell lines, although the same effectors efficiently killed Jurkat cells.

Several secreted factors were shown to contribute to the resistance of different targets to death receptor-mediated killing (7–12). We speculated that uveal melanoma cells may secrete factors inhibiting Fas- and/or TRAIL-mediated lysis, which could be prevented by inhibition of proteases acting at the cell surface. We used PIC, which contains a mixture of protease inhibitors including serine, cysteine, metalloprotease, and calpain I inhibitors. Indeed, 4 hours of treatment with PIC rendered the OCM1, OCM3, and OCM8 cell lines sensitive to CTL-mediated lysis (Fig. 1B). Inhibitors capable of blocking individual protease activities including calpain inhibitor I, aprotinin (an inhibitor of serine proteases), and E-64 (an inhibitor of papain and cysteine proteases such as cathepsin B and L) did not affect the lysis of uveal melanoma cells by CTLs when used alone or in combination (data not shown). However, when tumor cells were preincubated with an inhibitor of metalloproteases, 1,10-phenanthroline, an increase in CTL-mediated lysis was achieved (Fig. 1C), indicating that the activity of surface metalloproteases plays a role in the resistance of uveal melanoma cells to non–MHC-restricted lysis by T lymphocytes.

To verify that chromium release correlates with tumor cell apoptosis, PIC-treated tumor cells were incubated with CTLs and stained with annexin V and TMRE. As shown in Fig. 1D and E, only about 10% to 15% of untreated cells exhibited apoptotic changes, even after coincubation with CTLs. The same was true for PIC-treated tumor cells in the absence of effectors, although the same CTLs were able to induce apoptosis in about 30% to 40% of PIC-treated cells. This demonstrates that treatment with metalloprotease inhibitors renders
uveal melanoma cells more susceptible to CTL-mediated apoptosis induced in a non-MHC-restricted manner.

Inhibitors of Metalloproteases Render Uveal Melanomas Sensitive to Fas-Mediated Death but not TRAIL Receptor-Mediated Death. Given the time frame of our experimental procedures, FasL and TRAIL were likely to be the effector molecules mediating the non-MHC-restricted lysis of uveal melanomas by CTLs. To investigate the contribution of the two pathways in the lysis of uveal melanomas, agonistic anti-Fas antibody CH-11 or a recombinant TRAIL molecule was used as an inducer of cell death in 16-hour chromium release assays. As shown in Fig. 2A, the CH-11 antibody efficiently killed Fas-sensitive Jurkat cells but led to only marginal killing of untreated uveal melanoma cells. However, pretreatment of uveal melanoma cells with PIC induced a 2- to 3-fold increase in CH-11-induced 51Cr release (Fig. 2A), whereas recombinant TRAIL failed to induce killing of uveal melanoma cells under the same conditions (Fig. 2B). In agreement with these data, a substantial proportion of PIC-treated tumor cells exhibited apoptotic changes as measured by annexin V and TMRE staining, whereas only marginal alterations were detected in untreated uveal melanoma cells incubated with CH-11 (Fig. 2C).

To identify the molecular pathway responsible for the killing of uveal melanomas by CD8+ CTLs, we performed a set of experiments blocking either Fas on the tumor cells (Fig. 3A) or FasL on the effector cells (Fig. 3B). In a representative experiment shown in Fig. 3A, untreated OCM1 and OCM3 cells were largely resistant to CTL-mediated lysis, whereas some lysis of OCM8 cells was observed. Incubation of untreated or PIC-treated uveal melanoma cells with the Fas-neutralizing antibody ZB4 before their exposure to BK289 CTLs prevented tumor lysis (Fig. 3A). Similar effects were achieved by pre-blocking FasL on CTLs with the FasL-specific antibody NOK-2 (Fig. 3B). In conclusion, our results demonstrate that treatment with inhibitors of metalloproteases renders uveal melanoma cells susceptible to non-MHC-restricted CTL killing mediated primarily through Fas triggering.

To assess whether metalloprotease inhibitors modulate the sensitivity of tumor cells to NK-mediated Fas-dependent cytotoxicity, lysis of OCM8 uveal melanoma cells cultured in the absence or presence of either 1,10-phenanthroline or PIC was tested in 16-hour chromium release assays using the NK cell lines Nishi or YT-Indy as effectors (Fig. 4). The inhibitors substantially increased lysis of the uveal melanoma cell line OCM8 by both effectors (Fig. 4A and B). Notably, killing of the target cells was Fas-mediated as shown by virtually complete inhibition of cytotoxicity in the presence of FasL-blocking antibodies (Fig. 4C and D).
Inhibition of Metalloproteases Stabilizes Fas Ligand at the Cell Surface and Prevents the Release of Its Soluble Form. Both Fas and FasL exist in a membrane-bound and soluble form (reviewed in ref. 13). The enhanced sensitivity of uveal melanoma cells to killing via Fas on treatment with metalloprotease inhibitors could reflect an increase in the levels of Fas expression at the surface of tumor cells as a result of discontinued shedding. To address this question, we assessed the cell surface expression of Fas and FasL before and after inhibition of metalloproteases. Although >90% of OCM1 and OCM8 cells (data not shown) expressed Fas, expression levels of Fas did decrease on treatment with either PIC or 1,10-phenanthroline (Fig. 5A). The amount of FasL-positive cells increased 3- to 5-fold 6 hours after incubation with PIC, although only about 2% to 6% of untreated uveal melanoma cells stained positive for FasL (Fig. 5B). Similar results were obtained with the OCM3 cell line (data not shown).

To investigate whether uveal melanoma cells produce soluble FasL, culture supernatants from both untreated and PIC-treated cells were concentrated and analyzed by Western blot using antibodies recognizing the soluble variant of the molecule. Spiking with TNF-α, not produced by OCM cells,4 was performed to assess the accuracy of the concentration procedure and sample loading. A band of 26 kDa, corresponding to the size of soluble FasL (14), was detected in the control samples, whereas in the samples treated with either PIC or 1,10-phenanthroline, the intensity of this band was substantially decreased (Fig. 5C). Taken together, we show that uveal melanoma cells release soluble FasL that can be prevented by treatment with metalloprotease inhibitors. Moreover, this treatment increases the levels of membrane FasL and does not interfere with the levels of Fas at the surface of uveal melanoma cells.

Protection of Uveal Melanomas from Fas-Induced CTL Lysis Is Not a Result of Tumor Counterattack. It was proposed that tumors expressing FasL could escape immune surveillance by using the Fas-FasL pathway to counterattack the cells of the immune system (15, 16). To investigate whether or not the resistance of uveal melanomas to Fas-mediated CTL lysis was a result of this mechanism, CD8+ CTLs were first coincubated with uveal melanoma cells and then used as effectors against third-party targets in 4- (Fig. 6A) or 16-hour (Fig. 6B) chromium release assays. We found that the lymphoblastoid cell line JAC-B2, which expresses the IVT epitope at the cell surface in association with HLA-A11, was recognized with a similar efficiency by the IVT-specific CTL clone BK289 preincubated with either untreated or 1,10-phenanthroline-treated OCM1, OCM3, and OCM8 uveal melanoma cells (Fig. 6A). Similar results were

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4 A. De Geer, unpublished observation.
obtained when Jurkat cells were used as a target in a 16-hour $^{51}$Cr release assay. These data argue against the counterattack by uveal melanoma cells as a possible explanation for their escape from Fas-mediated lysis by CTLs.

**A Molecule Noncovalently Bound to the Cell Surface Protects Uveal Melanoma Cells from Fas-Mediated Killing.** Collectively, our results suggested that soluble FasL, produced by uveal melanoma cells could bind to and occupy Fas molecules on the tumor cells, thereby preventing induction of cell death via this receptor. To test this hypothesis, uveal melanoma cells were treated briefly with a low-pH citrate buffer to disrupt noncovalent protein-protein interactions at the cell surface and remove any FasL potentially bound to the cell surface, which was used to monitor the efficiency of the treatment (data not shown).

**KillerTRAIL, CH-11, or their respective control molecules, Fc-fusion protein and IgM, induced <10% lysis in untreated OCM1 and OCM8 cells (Fig. 7A and B).** After low-pH treatment, killing of these cells by CH-11 increased 5- to 7-fold, approaching the levels seen in PIC-treated cells. In contrast, exposure to low pH did not affect the sensitivity of PIC-treated cells to CH-11-mediated killing (Fig. 7A and B). In agreement with these data, surface elution with low-pH buffer increased the CTL-mediated killing of untreated OCM1 (Fig. 7C) and OCM8 (Fig. 7D) cells but did not affect the killing of PIC-treated cells. The sensitivity of OCM1 and OCM8 cells to killerTRAIL remained unaffected by treatment with PIC alone or in combination with exposure to low pH (Fig. 7A and B). In summary, this set of experiments suggested that a protein that is noncovalently bound to the cell surface protects uveal melanoma cells from Fas-mediated lysis.

**Release of Soluble Fas Ligand Protects Uveal Melanoma Cells from Fas-Mediated Lysis by CD8$^+$ CTLs.** If the protein mediating protection of uveal melanomas from Fas-induced killing was indeed soluble FasL, it should be possible to transfer its inhibitory effect onto a Fas-sensitive target. To test this hypothesis, $^{51}$Cr-labeled OCM8 cells were preincubated with eluates obtained by treatment with low-pH buffer and used as targets in a 16-hour chromium release assay. As shown in Fig. 8A, the eluates themselves did not induce a significant release of chromium from OCM8 cells and did not significantly alter the CTL lysis of untreated OCM8 cells. However, an approximately 50% reduction in CTL-mediated lysis of PIC-pretreated OCM8 cells was obtained with low-pH eluate from untreated (eluate 2), but not inhibitor-treated (eluate 4) OCM8 cells (Fig. 8A).

To test whether or not this inhibitory effect was mediated by soluble FasL, eluates of untreated tumor cells were incubated with either the FasL-specific antibody NOK-2 or the relevant isotype control antibody and then with protein-A Sepharose beads. As shown in Fig. 8B, preincubation of untreated OCM8 cells with eluates from tumor cells containing or devoid of soluble FasL did not affect their lysis by CTL ([]). Increased CTL lysis seen in PIC-treated OCM8 cells (■) was abolished by preincubating the tumor cells with low-pH eluate. However, immunodepletion of the eluate with NOK-2, but not the isotype control antibody, abolished the inhibitory activity of the eluate to a significant extent (Fig. 8B).

**Metalloprotease Inhibitors Render Tumors of Different Origins More Susceptible to Fas-Mediated Killing.** To understand whether or not the ability of metalloprotease inhibitors to potentiate Fas-
mediated killing is only applicable to uveal melanomas, we extended our analysis to a larger panel of tumor cell lines of different cellular origins, including cutaneous melanomas, neuroblastomas, and renal cell carcinomas. We found an increase in both CTL-mediated and CH-11-mediated lysis on treatment with PIC in two of four cutaneous melanomas. We found an increase in both CTL-mediated and CH-11-mediated cytotoxicity in a study using a mixture of protease inhibitors (Fig. 1B and C) which was based on experiments using a mixture of protease inhibitors (Fig. 1B), the components of which are not capable of penetrating the cell membrane. This led us to conclude that molecular events at the extracellular surface of the tumor cell membrane play a major role in the resistance to Fas-mediated death. It has been shown that cleavage of FasL is mediated by the matrix metalloprotease (MMP)-7 (36). In agreement with this finding, treatment with metalloprotease inhibitors led to the disappearance of the soluble form of FasL from the culture supernatant of uveal melanomas (Fig. 5C) and the concomitant enhancement of the expression of this molecule at the surface of tumor cells (Fig. 5B). These changes were accompanied by an increased susceptibility of uveal melanoma cells to lysis via Fas, prompting us to speculate that soluble FasL produced by tumor cells may bind in an autocrine fashion to Fas molecules on the producer and/or bystander cells and protect Fas from triggering by FasL or anti-Fas antibody.

Soluble FasL has been previously implicated as a negative regulator of Fas-mediated apoptosis. Tanaka et al. (10) used soluble FasL to inhibit killing of mouse hepatocytes by WR19L mouse cells expressing membrane FasL. Cleavage of FasL into soluble FasL by MMP-7 was shown to protect Ewing’s sarcoma cell lines from Fas-mediated apoptosis induced by doxorubicin (36). Here we present evidence for naturally occurring production of soluble FasL by tumor cells, which protects them from lysis via Fas by cytotoxic lymphocytes. Interestingly, we found that not more than 20% of uveal melanoma cells stained positive for membrane FasL even after treatment with metalloprotease inhibitors (Fig. 5B), suggesting that a relatively small

### DISCUSSION

A multitude of tumor escape mechanisms identified to date includes defects in antigen presentation, production of immunosuppressive cytokines, resistance to apoptosis by expression of antiapoptotic molecules, and alterations in death and/or decoy receptors (reviewed in refs. 17 and 18). The latter has been extensively studied for the Fas/FasL system, in which down-regulation or loss of Fas (19–22), expression of soluble Fas receptors with inhibitory functions (7, 23, 24), and tumor counterattack by FasL have been reported (15, 16, 25). Here we present evidence for a novel mechanism of tumor escape, demonstrating that tumors protect themselves from Fas-mediated killing through the release of soluble FasL.

The expression of FasL has been found in tumors of different origin and correlated with metastatic spread and poor prognosis (26–29). However, the role of FasL in tumor immune escape via counterattack remains a controversial issue (30–32). Whereas several reports showed that FasL-expressing tumors could kill effector cells (15, 25, 33, 34), others stressed a role for FasL expressed on T cells, which, on activation, underwent activation-induced cell death (31, 35). We could not detect any alterations in T-cell viability (data not shown) or cytotoxic activity (Fig. 6) induced by uveal melanomas, suggesting that their resistance to non-MHC-restricted T-cell–mediated lysis (Fig. 1A) is not due to tumor counterattack. This assumption is further strengthened by the fact that uveal melanoma tumors also resisted Fas-mediated killing induced in an effector cell-free system by the agonistic anti-Fas antibody CH-11 (Figs. 2A and 7A and B).

Our initial observation that treatment of uveal melanomas with inhibitors of metalloproteases restores their sensitivity to Fas-mediated death (Fig. 1B and C) was based on experiments using a mixture of protease inhibitors (Fig. 1B), the components of which are not capable of penetrating the cell membrane. This led us to conclude that molecular events at the extracellular surface of the tumor cell membrane play a major role in the resistance to Fas-mediated death. It has been shown that cleavage of FasL is mediated by the matrix metalloprotease (MMP)-7 (36). In agreement with this finding, treatment with metalloprotease inhibitors led to the disappearance of the soluble form of FasL from the culture supernatant of uveal melanomas (Fig. 5C) and the concomitant enhancement of the expression of this molecule at the surface of tumor cells (Fig. 5B). These changes were accompanied by an increased susceptibility of uveal melanoma cells to lysis via Fas, prompting us to speculate that soluble FasL produced by tumor cells may bind in an autocrine fashion to Fas molecules on the producer and/or bystander cells and protect Fas from triggering by FasL or anti-Fas antibody.

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#### Table 1

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<th>PIC</th>
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**NOTE.** Tumor cell lines of different origin, untreated or treated with PIC, were used as targets for non-MHC-restricted CTL-mediated and CH-11-mediated cytotoxicity in a 16-hour 51Cr release assay. Numbers indicate the percentage of specific lysis. Data from one representative experiment of two to four experiments performed for each cell line are shown.

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population of cells maintaining the production of soluble FasL in the tumor milieu can confer resistance of a potentially Fas-sensitive tumor mass to Fas-mediated lysis. This hypothesis is supported by the fact that we were able to transfer the inhibitory activity of soluble FasL with low-pH eluates from Fas-resistant uveal melanoma cells to their Fas-sensitive metalloprotease inhibitor-treated counterparts (Fig. 8).

It has been reported previously that soluble FasL can associate with membrane Fas but is >1,000-fold less efficient in the induction of apoptosis as compared with the membrane-bound trimeric complex (37). This was also valid for our experimental system because binding of soluble FasL released by tumors to self-Fas receptors or coinulation of soluble FasL-producing tumors with T-cells did not induce apoptosis in tumors themselves or in T cells (Fig. 6; data not shown).

Metalloproteases contribute to many biological events crucial for tumor development and progression of the disease, including remodeling of the extracellular matrix, vascularization, and cell migration (reviewed in ref. 38). Overexpression of MMPs has been shown in many tumors, including breast, colon, gastric, head and neck, prostate, and lung cancers (reviewed in ref. 39). The in vivo use of MMP inhibitors reduces the growth rate of both primary tumor and metastases (40, 41), due to inhibition of angiogenesis and the promotion of apoptosis in tumor cells. This study suggests yet another rationale behind the application of metalloprotease inhibitors in the therapy of cancer. Importantly, the ability of metalloprotease inhibitors to enhance the sensitivity of tumor cells to Fas-mediated killing is not an exclusive property of uveal melanomas, as we showed using a panel of tumor cell lines of different origins (Table 1). Therefore, our findings might prove useful for the design of immuno- or chemotherapeutic protocols for treatment of various cancers.

REFERENCES

Autocrine Secretion of Fas Ligand Shields Tumor Cells from Fas-Mediated Killing by Cytotoxic Lymphocytes

Kristian Hallermalm, Anna De Geer, Rolf Kiessling, et al.


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