Induction of apoptosis in malignant pleural mesothelioma cells by activation of the Fas (Apo-1/CD95) death-signal pathway

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Objective: Although well characterized in several solid tumors, the effects of Fas/Fas ligand interactions in malignant pleural mesothelioma cells have not been defined. The present study was undertaken to examine the functional status of the Fas/Fas ligand pathway in malignant pleural mesothelioma cells and to determine the feasibility of targeting this death-signal pathway for molecular intervention in patients with mesotheliomas.

Methods: Fas expression in primary normal human bronchial epithelial cells and 6 malignant pleural mesothelioma cell lines was quantified by means of flow cytometry. The caspase components of the Fas-mediated apoptotic pathway were evaluated by means of Western blot techniques. Soluble Fas ligand-mediated cytotoxicity and apoptosis were evaluated by means of MTS and TUNEL assays, respectively. Cisplatin (3 $\mu\text{g/mL}$) and lymphokine-activated killer cells were used to enhance mesothelioma sensitivity to soluble Fas ligand. An H2373 nude mouse xenograft model of malignant pleural mesothelioma was established to assess the in vivo effects of soluble Fas ligand.

Results: Four of 6 malignant pleural mesothelioma lines exhibited high levels of Fas expression, and 2 of 4 were inherently susceptible to soluble Fas ligand-mediated cytotoxicity (soluble Fas ligand 50% inhibitory concentration, $<15 \text{ ng/mL}$). Two soluble Fas ligand refractory cell lines (H2052 and H513) exhibited high levels of Fas receptor. Pretreatment with cisplatin resulted in a reduction of 50% inhibitory concentration from infinity to $4.17 \pm 0.14 \text{ ng/mL}$ and $10.23 \pm 1.58 \text{ ng/mL}$, respectively. Two additional soluble Fas ligand refractory cell lines (H2595 and REN) expressed low levels of Fas. Exposure of these cells to lymphokine-activated killer cells or lymphokine-activated killer cell-conditioned medium followed by a 24-hour treatment with cisplatin resulted in a significant reduction in 50% inhibitory concentration of soluble Fas ligand and pronounced induction of apoptosis. Intraperitoneally administered soluble Fas ligand mediated regression of H2373 xenografts.

Conclusion: The Fas/Fas ligand pathway in mesothelioma cells is either intrinsically intact or can be rendered functional with chemotherapeutic agents or immune effector cells. These preclinical data support further evaluation of strategies to enhance Fas-mediated apoptosis in mesotheliomas.

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Read at the Eighty-first Annual Meeting of The American Association for Thoracic Surgery, San Diego, Calif, May 6-9, 2001.

Received for publication May 3, 2001; revisions requested July 9, 2001; revisions received Aug 8, 2001; accepted for publication Aug 15, 2001.

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J Thorac Cardiovasc Surg 2002;123:295-302

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0022-5223/2002 \$35.00 + 0 12/6/119882

doi:10.1067/mtc.2002.119882

Malignant pleural mesothelioma (MPM) afflicts approximately 2500 Americans annually.¹ Although still relatively uncommon, the incidence of mesothelioma has risen dramatically during the past 2 decades as a result of asbestos exposure. More recently, simian virus 40 has been implicated as a cocarcinogen in the pathogenesis of this disease.² Because

Figure 1. Flow cytometric analysis of Fas expression in mesothelioma and control cells. Jurkat cells, which express high levels of Fas, were used as positive controls for subsequent experiments. These data are representative of 3 independent experiments that yielded similar results.

nearly 100 million Americans were exposed to simian virus 40 during polio vaccination, the number of mesothelioma cases is expected to increase for the next 20 to 30 years. Currently, the overall survival of patients with mesothelioma ranges between 3% and 6%, despite aggressive multimodality intervention.¹ These data highlight the need for a more fundamental appreciation of the pathogenesis of MPM and the rational design of novel therapies targeting molecular defects in this disease.³

Dysregulation of death-signal pathways, such as the one mediated by the Fas (Apo-1/CD95) receptor, have been described in a variety of malignancies.^{4,5} Fas is a member of the tumor necrosis factor receptor super family, which is widely expressed in normal and transformed cells of different histologic types.⁶ On binding with a specific ligand (Fas ligand [FasL]), Fas receptors form homotrimers that recruit intracellular adaptor molecules that activate caspase 8, thereby initiating the cleavage of downstream caspases and

resulting in mitochondrial disruption and apoptosis in susceptible cells.⁷⁻⁹

Fas/FasL interactions maintain homeostasis in normal tissues and are believed to contribute to immune surveillance, enhancing perforin/granzyme-mediated target cell lysis by lymphokine-activated killer (LAK) cells and natural killer cells, as well as cytolytic T lymphocytes.¹⁰⁻¹² Although a significant percentage of malignant cells express the Fas receptor, not all of them are intrinsically susceptible to FasL-mediated apoptosis.⁵ Furthermore, recent data suggest that expression of FasL expressed on tumor cells can engage Fas receptor on cytolytic T lymphocytes, resulting in the clonal deletion of tumor-reactive immune effector cells.^{13,14}

Although the Fas/FasL pathway has been extensively studied in several tumor histologic types, especially melanomas, limited information is available regarding the functional status of this pathway in MPMs. In the present study we sought to examine the role of the Fas/FasL pathway in mediating apoptosis in a panel of MPM cell lines. Herein we report that the majority of these lines express adequate levels of Fas receptor, yet only one third of them are inherently sensitive to soluble Fas ligand (sFasL). In the remaining cell lines the Fas/FasL pathway could be rendered functional by exposure to cisplatin (CDDP) alone or exposure to LAK cells or LAK-conditioned media, followed by CDDP. These data suggest that manipulation of the Fas/FasL pathway may be a novel strategy to induce apoptosis in MPMs.

Materials and Methods

Cells and Reagents

H28 (sarcomatoid histology), H2373 (sarcomatoid histology), H2052 (sarcomatoid histology), H513 (epithelioid histology), H2595 (sarcomatoid histology), and REN (sarcomatoid histology) MPM cell lines and Jurkat T-cell leukemia lines were available in tissue-culture banks at the National Institutes of Health and were maintained in RPMI medium supplemented with glutamine (1 mmol/L), streptomycin (100 mg/mL), penicillin (100 U/mL), and 10% fetal calf serum. Normal human bronchial epithelial (NHBE) cells and human umbilical vein endothelial cells (Clonetics Corp, San Diego, Calif) were cultured, as recommended by the vendor. Recombinant sFasL and enhancer protein were obtained from Alexis Corporation (San Diego, Calif) and reconstituted according to the manufacturer's instructions. Fluorescein isothiocyanate-conjugated anti-Fas monoclonal antibody and monoclonal antibodies recognizing caspases 3, 8, and 9 were purchased from PharMingen (San Diego, Calif).

Western Blot Analysis of Caspases 3, 8, and 9 in Human Umbilical Vein Endothelial Cells and MPM Cells

MPM cell lysates were electrophoresed and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, Calif). The PVDF membranes were then

Figure 2. Upregulation of Fas expression in H2595 (A) and REN (B) MPM cell lines after 24 hours' exposure to either LAK cells or cell-free LAK supernatant. Incubation of these lines with fresh basal LAK medium without (*control*) or with interferon γ (100 IU/mL) did not alter the basal expression of Fas. These data are representative of 3 independent experiments that yielded similar results.

TABLE 1. sFasL IC_{50} values for malignant plural mesothelioma cells and normal human bronchial epithelial cells

Cell line	SFasL alone	CDDP + sFasL	LAK/LAK supernatant	LAK supernatant/CDDP
H28	7.53 $\pm$ 2.87	8.54 $\pm$ 1.62	ND	ND
H2373	10.51 $\pm$ 1.75	9.28 $\pm$ 2.34	ND	ND
H2052	∞	4.17 $\pm$ 0.14	ND	ND
H513	∞	10.23 $\pm$ 1.58	ND	ND
H2595	∞	∞	∞	45.30 $\pm$ 3.2
REN	∞	∞	∞	26.80 $\pm$ 2.58
NHBE	80 $\pm$ 4.40	91.42 $\pm$ 9.87	81.63 $\pm$ 5.70	87.86 $\pm$ 7.89

These data are means $\pm$ SD of 3 independent experiments. *ND*, Not determined.

blocked with a 5% lowfat milk/Tris-buffered saline/Tween solution and exposed overnight to antibodies recognizing caspase 3, 8, and 9 (Pharmingen) or β -actin. The PVDF membranes were then washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Protein-antibody conjugates were detected with ECL techniques (Amersham Pharmacia Biotech Inc, Piscataway, NJ).

In Vitro FasL-mediated Cytotoxicity

Cells were seeded in flat-bottom, 96-well plates and, after overnight incubation, were treated with sFasL at concentrations ranging from 1 to 100 ng/mL for 24 hours. For CDDP-sFasL combination experiments, cells were exposed to CDDP (3 μ g/mL) for

24 hours before sFasL treatment. Cell viability was quantitated with the MTS assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay; Promega Corp, Madison, Wis). Soluble FasL-mediated cytotoxicity dose-response curves were plotted as the fraction of viable cells relative to untreated control cells versus sFasL concentrations. In CDDP/sFasL sequential experiments, cells treated with CDDP alone served as control cells to correct for minor growth inhibitory effects of this DNA damaging agent. The sensitivity of MPM cells to sFasL-mediated cytotoxicity was determined by estimating sFasL inhibitory concentration of 50% (IC_{50}) values from their respective dose-response curves. A lower IC_{50} value indicated increased susceptibility to the cytotoxic effects of sFasL. Induction of apoptosis after sFasL treatment,

Figure 3. Western blot analysis of caspases 3, 8, and 9 in human umbilical vein endothelial cells (HUVECs) and MPM cells.

Figure 4. Induction of apoptosis in Jurkat lymphoma, H28, and H2373 mesothelioma cells and NHBE cells after exposure to sFasL. These sFasL-sensitive tumor cells underwent significant apoptosis after treatment with 50 ng/mL sFasL. No apoptosis was detected in NHBE cells under similar conditions. These data are means $\pm$ SD of 3 independent experiments.

with or without prior exposure to CDDP, was evaluated with protocols and reagents contained in the Apo-BrdU kit (Pharmingen).

Exposure of MPM Cells to LAK Cells or Supernatant

Peripheral blood mononuclear cells from normal volunteers were cultured in basal LAK medium (RPMI supplemented with 2% human AB serum, 1000 U/mL interleukin 2, 1 mmol/L glutamine, 100 mg/mL streptomycin, and 100 U/mL penicillin) to generate LAK cells. LAK-conditioned medium was prepared by centrifuging 3-day-old LAK supernatants at 1500 rpm for 10 minutes to remove cellular components; conditioned medium was stored at -80°C for subsequent experiments.

Adherent MPM cells were either coincubated with LAK cells at an effector/target ratio of 10:1 or exposed to cell-free LAK-conditioned medium for 24 hours. Subsequently, MPM cells were washed 3 times with RPMI and either analyzed for Fas expression by means of flow cytometric techniques with a fluorescein isothiocyanate-labeled anti-Fas monoclonal antibody or used in FasL-mediated cytotoxicity assays.

MPM Human Xenograft in Nude Mice

In 2 independently performed experiments, 5 groups (5 animals/group) of athymic nude mice received intraperitoneal injection of 10^6 H2373 MPM cells suspended in 1 mL of Hanks buffered saline solution (HBSS). At either 1 or 2 weeks after tumor inoculation, mice in the treatment groups received intraperitoneal injections of sFasL (100 ng of sFasL dissolved in 1 mL of HBSS) 3 times per week for 3 weeks. Control animals received sham injections of HBSS only. One week after completion of treatment, all animals were killed, and intraperitoneal tumor burden was recorded by means of digital photography. All animals were treated in accordance with the Animal Welfare Act.

Results

Phenotypic Analysis of Fas and Caspase Expression in MPM Cells

Four MPM lines (H28, H513, H2052, and H2373) expressed levels of Fas receptor equal to or exceeding the

level noted in NHBE cells, whereas 2 lines (H2595 and REN) exhibited Fas expression that was lower than that seen in normal cells (Figure 1). Treatment with CDDP or interferon γ , both of which can induce Fas expression in some tumor cells,^{15,16} failed to upregulate surface Fas expression in H2595 and REN cells. On the other hand, 24-hour exposure to LAK cells or LAK-conditioned medium profoundly increased Fas expression in these 2 lines (Figure 2). Western blot analysis revealed that all of the MPM lines expressed abundant levels of caspases 3, 8, and 9 (Figure 3), which are essential for mediating apoptosis after Fas/FasL interaction.

In Vitro Functional Analysis of Fas-mediated Cytotoxicity

Treatment of either H28 or H2373 MPM cells, which express adequate levels of Fas receptor, with sFasL resulted in significant cytotoxicity (sFasL IC_{50} values of 7.53 ± 2.87 and 10.51 ± 1.75 ng/mL, respectively). H28 and H2373 cells were 8 to 10 times more sensitive to sFasL-mediated cytotoxicity relative to NHBE cells (sFasL IC_{50} , 80 ± 4.40 ng/mL). Interestingly, although expressing adequate levels of Fas, H513 and H2052 cells were refractory to sFasL-induced cytotoxicity, as evidenced by sFasL IC_{50} values approaching infinity (∞). H2595 and REN cells also were resistant to sFasL.

Figure 5. Induction of apoptosis in H2052 (A) and H513 (B) MPM cells after exposure to normal medium, sFasL alone (50 ng/mL for 24 hours), CDDP (3 μ g/mL for 24 hours) alone, or CDDP-sFasL. These data are representative of 3 independent experiments that yielded similar results.

Strategies to Enhance Susceptibility of Resistant MPM Cells to sFasL-mediated Cytotoxicity

Exposure of Fas-positive H2052 and H513 MPM cells to a sublethal dose of CDDP (3 μ g/mL) resulted in significant sensitization to the cytotoxic effects of sFasL, as evidenced by a reduction in sFasL IC_{50} from ∞ to 4.17 ± 0.14 and 10.23 ± 1.58 ng/mL, respectively; similar treatment of H2595 and REN cells failed to sensitize them to sFasL. Twenty-four-hour exposure to LAK cells or LAK-conditioned medium induced Fas expression in H2595 and REN cells to levels comparable with what was observed in unmanipulated NHBE cells; however, these mesothelioma cells remained resistant to sFasL. Additional experiments revealed that CDDP (3 μ g/mL) administered subsequent to upregulation of Fas expression markedly sensitized H2595 and REN cells to sFasL-mediated cytotoxicity. Similarly treated primary normal human epithelial cells were not further sensitized to sFasL-mediated cytotoxicity (Table 1).

Induction of Apoptosis as the Mechanism of sFasL-mediated Cytotoxicity

Significant apoptosis was noted in H28 and H2373 cells 24 hours after exposure to 50 ng/mL sFasL ($40.37\% \pm 6.55\%$ and $20.14\% \pm 8.85\%$ vs $3.05\% \pm 0.78\%$ and $3.80\% \pm 0.86\%$, respectively; Figure 4); in contrast, NHBE cells were quite resistant to this treatment. Soluble FasL-mediated apoptosis in MPM cells was completely inhibited by the caspase inhibitor zVAD (20 μ mol/L) or sFas (100

ng/mL, data not shown). CDDP alone exhibited limited proapoptotic effects in Fas-positive, sFasL-resistant H2052 and H513 cells. Interestingly, sFasL markedly enhanced apoptosis in CDDP-treated cells (Figure 5). Apoptosis induced by sequential CDDP/sFasL treatment was also blocked by zVAD or sFas, underscoring the essential role of Fas/FasL interaction in this process.

sFasL Mediates Tumor Regression in H2373 Nude Mouse Xenografts

Intraperitoneal inoculation of 10^6 H2373 MPM cells resulted in extensive carcinomatosis that was consistently observed within 6 weeks in untreated animals (Figure 6, A). Intraperitoneal injection of sFasL (100 ng in 1 mL of HBSS) 3 times per week for 3 weeks commencing 1 week after tumor inoculation (the time at which there was only microscopic tumor cell implantation; Figure 6, B) markedly inhibited the development of intra-abdominal carcinomatosis (Figure 6, C). Initiation of sFasL treatment 2 weeks after tumor cell inoculation (the time at which there were macroscopic tumor implants; Figure 6, D) also inhibited tumor growth (Figure 6, E), albeit to a lesser extent than when treatment was initiated for microscopic tumor burden.

Discussion

Induction of apoptosis through the Fas/FasL pathway has been examined in various histologic categories.¹⁷⁻¹⁹ However, limited information is available regarding the

Figure 6. In vivo response of H2373 MPM xenograft to locoregional delivery of sFasL: **A**, tumor burden 6 weeks after injection of H2373 MPM cells; **B**, tumor burden 1 week after injection of H2373 MPM cells; **C**, inhibition of tumor growth after treatment with sFasL commencing 1 week after tumor implantation; **D**, tumor burden 2 weeks after implantation of H2373 MPM cells; **E**, response to sFasL injections starting 2 weeks after tumor implantation. A similar magnitude of response was observed in all animals within each group. Representative digital photographs are shown here.

functional status of the Fas/FasL pathway in MPMs and the feasibility of targeting this pathway for the treatment of patients with this highly lethal disease.

Phenotypic and functional analysis of the Fas-mediated death-signal pathway in this small but representative panel of cultured MPM cells revealed 3 distinct subgroups with respect to Fas expression and FasL-mediated cytotoxicity. The first group (represented by H28 and H2373 cells) expressed adequate levels of Fas and appeared to be intrinsically sensitive to sFasL-mediated apoptosis. The second group (including

H2052 and H513) also expressed adequate levels of Fas but appeared refractory to sFasL; treatment of these 2 cell lines with a sublethal dose of CDDP for 24 hours significantly enhanced their susceptibility to sFasL-mediated apoptosis. The last group (represented by H2595 and REN cells) also appeared refractory to sFasL. Pretreatment with CDDP or interferon γ failed to upregulate Fas in these 2 mesothelioma lines. In contrast, LAK cells or LAK-conditioned medium did induce Fas expression in H2595 and REN cells, thereby enabling CDDP-mediated sensitization to sFasL.

The ability of CDDP to enhance responsiveness to sFasL in tumor cells has been described previously²⁰⁻²²; however, the mechanisms underlying this phenomenon have not been defined. CDDP is known to induce reactive oxygen species, which render cells susceptible to apoptotic signals.²³ Conceivably, reactive oxygen species resulting from DNA damage can prime mesothelioma cells to undergo apoptosis through Fas pathways. Interestingly, exposure to CDDP did not affect the intrinsic sensitivity of FasL-responsive mesothelioma cells.

A more interesting issue emanating from our study is the upregulation of Fas expression in Fas-negative cells after exposure to LAK cells or LAK-conditioned medium. Exposure to cytolytic T lymphocytes induces Fas expression in murine fibrosarcoma cells by mechanisms that are currently unclear.²⁴ In the present study induction of Fas in H2595 and REN cells cannot be attributable to interferon γ or interleukin 2 because interferon γ alone did not upregulate Fas expression, and interleukin 2 was present in LAK control culture medium, as well as in LAK supernatants. These findings suggest that LAK-mediated enhancement of Fas expression in MPM cells is regulated by currently unidentified cytokines in 3-day-old LAK-conditioned medium. The fact that CDDP was required to induce sFasL-mediated apoptosis in H2595 and REN cells after exposure to LAK cells or LAK-conditioned medium indicates that restoration of Fas expression to normal levels is necessary but not always sufficient to induce apoptosis in mesothelioma cells.

It is widely recognized that sFasL can induce apoptosis in a variety of tumor histologic types.²⁵ However, its therapeutic potential is hampered by the fact that normal tissues possess Fas receptor and therefore are sensitive to Fas-mediated apoptosis.²⁶ Although not practical for systemic administration, sFasL could prove efficacious for locoregional therapy of mesotheliomas. Our studies demonstrated that intraperitoneal administration of sFasL significantly inhibited the growth of MPM xenografts, without obvious regional or systemic toxicity (which could have occurred because of the fact that human sFasL can bind to murine Fas). Not surprisingly, tumor burden at the onset of sFasL administration determined the overall effectiveness of sFasL therapy; animals with macroscopic tumor implants had a less spectacular response compared with that seen in animals with microscopic disease. These results underscore the clinical potential for locoregional delivery of sFasL as adjuvant therapy after pleurectomy or pleuropneumectomy in patients with mesotheliomas.

Data presented in this article clearly indicate that Fas/FasL interactions can cooperate with cytotoxic agents and immune effector cells to deliver potent apoptotic signals to mesothelioma cells. Further evaluation of the mechanisms responsible for this phenomenon may enable the

development of novel treatment regimens designed to enhance apoptosis in mesotheliomas through activation of Fas and other related death-signal pathways.

We thank Sharyn Childs for her assistance in the preparation of this article. We also thank Arnold Mixon and Shawn Farid for performing flow cytometric studies.

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Discussion

Dr Ralph A. Schmid (*Berne, Switzerland*). Did you look at liver function in the animal studies? When you give FasL systemically, animals die of liver failure.

Dr Stewart. Actually, those studies came from intraperitoneal injection of Jo2 agonistic antibodies to Fas, and there was massive hepatic necrosis in those previous studies. However, with our intraperitoneal injections of Fas, we saw no changes in liver morphology, as evidenced by hematoxylin and eosin staining.

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