Enhancement of Apo2L/TRAIL (tumor necrosis factor–related apoptosis-inducing ligand)–induced apoptosis in non–small cell lung cancer cell lines by chemotherapeutic agents without correlation to the expression level of cellular protease caspase-8 inhibitory protein

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Objective: Apo2L/tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a potential anticancer drug that promotes apoptosis specifically in tumor cells. Because not all cancer cells are susceptible to Apo2L/TRAIL, the aim of our study was to determine whether non–small cell lung cancer cells can be sensitized by chemotherapeutic agents for Apo2L/TRAIL-induced apoptosis. In addition, endogenous expression levels of the caspase-inhibiting cellular protease caspase-8 inhibitory protein (C-FLIP) were measured to investigate partial resistance to Apo2L/TRAIL.

Methods: Six human lung cancer cell lines (A549, NCI-H358, Calu1, Calu6, SkMes1, and SkLu1) were incubated with soluble Apo2L/TRAIL and two different concentrations each of cisplatin, paclitaxel, doxorubicin, 5-fluorouracil, and camptothecin. After 24 hours the rate of apoptosis was measured by annexin V/propidium iodide staining followed by FACScan analysis. Expression levels of C-FLIP in cell lines and lung cancer biopsy specimens were determined by Western blotting.

Results: Treatment of lung cancer cells with Apo2L/TRAIL alone resulted in apoptotic cell death in four cell lines ($P < .001$). Combining Apo2L/TRAIL and chemotherapeutic agents enhanced the rate of apoptosis significantly. Statistical analysis revealed a synergistic effect of Apo2L/TRAIL in combination with 1.8 mmol/L camptothecin and 100 μ mol/L cisplatin, each in four of the six cell lines ($P < .002$). Western blot analysis showed that sensitization to Apo2L/TRAIL did not correlate with the expression of cellular protease caspase-8 inhibitory protein. Furthermore, no increased cellular protease caspase-8 inhibitory protein levels relative to those in normal lung tissue could be found in non–small cell lung cancer specimens from 12 patients.

Conclusion: Apo2L/TRAIL-induced apoptosis in non–small cell lung cancer cell lines is significantly enhanced by chemotherapeutic agents. Resistance and sensitization to Apo2L/TRAIL are not correlated with the endogenous expression level of cellular protease caspase-8 inhibitory protein, implying that in non–small cell lung cancer other mechanisms are responsible for inhibition of the Apo2L/TRAIL pathway. Even though the molecular mechanism remains unclear, the combination of Apo2L/TRAIL with chemotherapy may be a promising treatment modality for non–small cell lung cancer.

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Lung cancer is the leading cause of cancer deaths in the United States among both men and women. The projected number of new cases of lung cancer in 1999 in the United States is 171,600, with more than 158,000 people dying from the disease during the same year. In fact, more people die each year of lung cancer than of breast, colorectal, prostate, and ovarian malignancies combined.¹

Eighty percent of all newly diagnosed lung cancers are non-small cell lung cancer (NSCLC). Current protocols for therapy of NSCLC include surgery, radiotherapy, and chemotherapy.² The aim of the latter two treatment modalities is the induction of apoptosis in cancer cells. Apoptosis is a morphologically distinct form of cell death that is associated with chromatin condensation and membrane blebbing. This type of cell death can be induced by cytokines of the tumor necrosis factor (TNF) family. A recently cloned member of this family is Apo2L/TNF-related apoptosis-inducing ligand (TRAIL).³ This cytokine, in contrast to other members of the TNF family, seems to induce apoptosis only in cancer cells and not in normal tissue.^{4,5} It thus could provide new therapeutic options for NSCLC.

The first aim of this study was to confirm that Apo2L/TRAIL does induce apoptosis in NSCLC cell lines. The second aim was to investigate whether this mechanism could be augmented by chemotherapeutic agents. The final aim was to evaluate the current hypothesis on the role of cellular protease caspase-8 inhibitory protein (c-FLIP) in resistance to Apo2L/TRAIL-induced apoptosis in NSCLC cell lines.

Material and Methods

Reagents

Soluble, nontrimerized Apo2L/TRAIL produced in *Escherichia coli* was kindly provided by Genentech, Inc, South San Francisco, Calif. Cisplatin, propidium iodide, cycloheximide (INN: cicloheximide), Bradford reagent, and the secondary goat antimouse and the monoclonal anti α -tubulin antibodies were purchased from Sigma Chemical Co, St Louis, Missouri. The 5-fluorouracil was from F. Hoffmann-La Roche Ltd, Basel, Switzerland. The protease inhibitor cocktail was produced by Roche Diagnostics GmbH, Mannheim, Germany. Paclitaxel, doxorubicin, camptothecin, and annexin V conjugated with fluorescein isothiocyanate were obtained from Alexis Corporation, San Diego, California. The monoclonal anti-c-FLIP (clone NF6) antibody was a kind gift from the group of Peter Krammer (German Cancer Research Center, Heidelberg, Germany). Culture media, penicillin, and streptomycin were purchased from Life Technologies Ltd, Paisley, United Kingdom. Fetal calf serum was purchased from Biochrom (Berlin, Germany). The reagents for enhanced chemoluminescence were obtained from Amersham Pharmacia plc, Little Chalfont, United Kingdom.

Tumor Cell Lines and Cell Culture

The human NSCLC cell lines (A549, nonspecified lung carcinoma; NCI-H358, bronchoalveolar carcinoma; Calu1, epidermoid

lung carcinoma; Calu6, anaplastic lung carcinoma; SkMes1, squamous cell carcinoma, and SkLu1, adenocarcinoma; American Type Culture Collection, Manassas, Va) were cultured at 37°C under 5% carbon dioxide in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and antibiotics.

Annexin V/Propidium Iodide Staining

Cells were plated at 5×10^4 cells/well in 24-well plates and allowed to attach overnight. Apo2L/TRAIL and chemotherapeutic agents were added at the indicated concentrations. After 24 hours cells were harvested by trypsinization, centrifuged, and resuspended in 50 μ L of annexin V in binding buffer (10-mmol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, pH 7.4; 150-mmol/L sodium chloride, 5-mmol/L potassium chloride; 1-mmol/L magnesium chloride; and 1.8-mmol/L calcium chloride). After 10 minutes of incubation at 4°C, 150 μ L of annexin V binding buffer and 20 μ L of propidium iodide (100 μ g/mL in phosphate-buffered saline solution) were added, and flow cytometry (FACScan; BD Immunocytometry Systems, San Jose, Calif) was performed. For every experiment a minimum of 1×10^4 cells were analyzed. All experiments were performed in triplicate ($n = 3$).

Western Blot

Cells were harvested and then lysed for 30 minutes on ice in a buffer containing 20-mmol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, pH 7.6, 120-mmol/L sodium chloride; 0.2-mmol/L ethylenediaminetetraacetic acid; 1% Triton X-100 (Sigma Chemical Co, St Louis, Mo), and protease inhibitors. Lysates were centrifuged at 14,000 rpm for 15 minutes and supernatants were collected. Protein concentration was determined with Bradford reagent by measuring the extinction at 595 nm and using bovine serum albumin dissolved in lysis buffer as a standard. Proteins were separated by denaturing gel electrophoresis with 12% polyacrylamide and loading the same amount of protein on every lane. The proteins were transferred to nitrocellulose membranes and incubated with blocking buffer (20-mmol/L tris[hydroxymethyl]aminomethane hydrochloride, pH 7.5, 500-mmol/L sodium chloride, 0.05% Tween 20, and 5% milk powder). After washing with buffer containing 20-mmol/L tris(hydroxymethyl)aminomethane hydrochloride, pH 7.5, 500-mmol/L sodium chloride, and 0.05% Tween 20, blots were incubated with the primary antibodies diluted in blocking solution. After a second washing the blots were incubated with horseradish peroxidase-coupled secondary antibody, washed, and developed with enhanced chemoluminescence reagent.

For stripping, membranes were incubated for 30 minutes at 50°C in a buffer containing 62.5-mmol/L tris(hydroxymethyl)aminomethane hydrochloride, pH 6.7, 2% sodium dodecyl sulfate, and 100-mmol/L β -mercaptoethanol. The blots were then washed and blocked again.

Statistical Analysis

For determination of synergy a statistical analysis was performed. For parameter estimates, t tests were calculated in a logistic regression model (quasiliikelihood approach according to McCullagh and Nelder⁶). The statistical software S-PLUS 2000 (MathSoft Engineering & Education, Inc, Cambridge, Mass) was used.

Figure 1. Apo2L/TRAIL induces apoptosis in NSCLC cell lines, but this is not correlated with expression of cFLIP. **A**, Cells were treated with 1-mg/mL TRAIL/Apo2L for 24 hours, and apoptosis was assessed by annexin V/propidium iodide staining followed by FACSscan analysis ($n = 3$). Bars represent mean; error bars represent SD. **B**, Expression of c-FLIP in NSCLC cell lines was determined by Western blot analysis. To confirm equal protein levels the same blot was stripped and developed with antitubulin antibody.

Results

Apo2L/TRAIL in NSCLC Cell Lines

To determine susceptibility of NSCLC cell lines to Apo2L/TRAIL, cells were treated with 1-mg/mL Apo2L/TRAIL. For assessment of apoptosis, cells were stained with annexin V/propidium iodide after 24 hours of incubation. A cell line was considered susceptible to Apo2L/TRAIL if more than 5% of the cells had positive staining for annexin V/propidium iodide compared with nontreated control cells. Four of six cell lines were susceptible to Apo2L/TRAIL-induced apoptosis. The highest rate of apoptotic cell death was noted in SkMes1 cells (33.2%), followed by NCI-H358 cells (19%), Calu6 cells (12.4%), and A549 cells (5.1%, $P < .001$, Figure 1, A).

Apo2L/TRAIL and Chemotherapeutic Agents

To investigate synergistic effects of Apo2L/TRAIL and chemotherapeutic agents, cell lines were incubated for 24

hours with 100 ng/mL Apo2L/TRAIL in combination with two different concentrations each of the chemotherapeutic agents doxorubicin, 5-fluorouracil, camptothecin, cisplatin, and paclitaxel. The doses of chemotherapeutic agents were determined in preliminary experiments so that at least one of the concentrations did not induce a high rate of apoptosis as a single treatment (data not shown). Apoptotic cell death was measured by annexin V/propidium iodide staining followed by flow cytometry (Figure 2). Synergistic effects could be determined with Apo2L/TRAIL and camptothecin (1.8 mmol/L) and Apo2L/TRAIL and cisplatin (100 μ mol/L), each in four of the six cell lines ($P < .002$, Table 1). Less pronounced effects were seen for the combination of Apo2L/TRAIL with doxorubicin and with 5-fluorouracil, whereas paclitaxel did not show a synergistic effect with Apo2L/TRAIL in any of the cell lines tested (Table 1).

Susceptibility of the NSCLC Cell Lines to Apo2L/TRAIL in Correlation With the Endogenous Expression of c-FLIP

Figure 1, B, indicates that the level of c-FLIP expression was not correlated with Apo2L/TRAIL-induced apoptosis. NCI-H358, a cell line susceptible to Apo2L/TRAIL, showed the highest expression of c-FLIP. Two other cell lines, Calu1 and SkLu1, which are not sensitive to Apo2L/TRAIL-induced apoptosis, clearly revealed less expression of c-FLIP. Rehybridization of the blot with an antitubulin antibody demonstrated comparable amounts of protein loaded on each lane (Figure 1, B).

Apo2L/TRAIL and Chemotherapeutic Agents Correlated With the Expression of c-FLIP

Because NSCLC cell lines can be sensitized to Apo2L/TRAIL-induced apoptosis by chemotherapeutic agents, we next evaluated whether this effect was inversely correlated with the expression of c-FLIP. NCI-H358 cells were treated with chemotherapeutic agents and then analyzed by Western blot for the expression of c-FLIP. Again, no correlation was noted between Apo2L/TRAIL-induced apoptosis and the expression of c-FLIP (Figure 3). Cycloheximide, which downwardly regulates the expression of c-FLIP,⁷ was used in negative control runs. Comparable loading of protein was demonstrated by rehybridization with an antitubulin antibody (Figure 3).

Expression of c-FLIP in Primary NSCLC

In primary tumors of 6 patients with adenocarcinoma and 6 patients with squamous cell carcinoma, the expression of c-FLIP was determined in comparison with normal lung tissue. The results did not show a difference in the expression of endogenous c-FLIP between normal lung tissue and tumor (Figure 4).

Figure 2. Apo2L/TRAIL-induced apoptosis is augmented by chemotherapeutic agents. Cells were incubated for 24 hours with 100ng/mL Apo2L/TRAIL (white bars), with chemotherapeutic agents (gray bars), and with combined Apo2L/TRAIL and chemotherapy (black bars). Apoptosis was measured by annexin V/propidium iodide staining followed by flow cytometry ($n = 3$). Bars represent mean; error bars represent SD. Concentrations of chemotherapeutic agents are indicated with numbers: 1 and 2, 0.5-mmol/L and 5-mmol/L doxorubicin; 3 and 4, 3-mmol/L and 30-mmol/L 5-fluorouracil; 5 and 6, 0.18-mmol/L and 1.8-mmol/L camptothecin; 7 and 8, 10- μ mol/L and 100- μ mol/L cisplatin; 9 and 10, 10-nmol/L and 100-nmol/L paclitaxel.

Interestingly, when we used another antibody for determination of c-FLIP (polyclonal antibody catalog number 1161; ProSci Incorporated, Poway, Calif), we observed significantly higher levels of this protein in the tumor than in normal tissue. However, further control runs revealed that this antibody was not specific (data not shown).

Discussion

Apo2L/TRAIL induced apoptosis in most of the NSCLC cell lines studied. The combination of Apo2L/TRAIL with

chemotherapeutic agents increased apoptotic cell death in a synergistic way.

Apoptosis may be induced by cytokines of the TNF family, as for example TNF- α and Fas ligand (FasL). Binding of TNF and FasL to their corresponding receptors on the cell membrane leads to trimerization of a conserved cytoplasmic receptor domain called *death domain*. Subsequently the adapter molecule FADD/MORT1 and the protease caspase-8 (FLICE) bind to the death receptor and form the death-inducing signaling complex.⁸ Recruitment of caspase-8

TABLE 1. Augmentation of Apo2L/TRAIL toxicity by chemotherapy in NSCLC cell lines

	NCI-H358	A549	Calu6	Calu1	SkMes1	SkLu1
TRAIL + 0.5- μ mol/L doxorubicin	—	—	—	—	+	—
TRAIL + 5- μ mol/L doxorubicin	+	—	—	—	—	+
TRAIL + 3-mmol/L 5-fluorouracil	—	+	—	—	+	—
TRAIL + 30-mmol/L 5-fluorouracil	—	+	+	—	—	—
TRAIL + 0.18-mmol/L camptothecin	+	—	—	—	—	+
TRAIL + 1.8-mmol/L camptothecin	+	+	—	+	—	+
TRAIL + 10- μ mol/L cisplatin	—	—	—	—	—	—
TRAIL + 100- μ mol/L cisplatin	+	—	+	—	+	+
TRAIL + 10-mmol/L paclitaxel	—	—	—	—	—	—
TRAIL + 100-mmol/L paclitaxel	—	—	—	—	—	—

Data represent the cell lines with and without significant augmentation of Apo2L/TRAIL-induced apoptosis by chemotherapeutic agents ($P < .002$).

activates its proteolytic properties, which further initiates a cascade of caspases and subsequent cleavage of death substrates, finally leading to apoptosis.^{9,10} Systemically administered TNF- α and FasL induce acute toxic effects, such as septic shock and acute hepatic failure, thereby limiting their potential clinical use for the treatment of cancer.^{11,12}

Another member of the TNF family, Apo2L/TRAIL, has been cloned recently.³ Similar to TNF- α and FasL, Apo2L/TRAIL induces apoptosis in various tumor cells but not in normal cell lines.⁵ Furthermore, it has been demonstrated that systemic administration of Apo2L/TRAIL in SCID mice or nonhuman primates results in antitumor activity without any toxic effects on normal tissue.⁴

Recent studies, however, demonstrate that not all tumor cell lines are susceptible to Apo2L/TRAIL-induced apoptosis. This was first thought to be due to the expression pattern of the four distinct Apo2L/TRAIL receptors. Two of Apo2L/TRAIL receptors (DcR1 and DcR2) lack a functioning death domain and were initially believed to act as “decoy” receptors. Subsequent studies could not confirm this hypothesis.¹³

To overcome the resistance of some tumor cell lines to Apo2L/TRAIL, two recently reported studies evaluated synergistic action of Apo2L/TRAIL with chemotherapeutic agents. Keane and colleagues¹⁴ demonstrated a synergistic effect of Apo2L/TRAIL with doxorubicin and 5-fluorouracil in breast cancer cell lines. Gliniak and Le¹⁵ noted synergy of Apo2L/TRAIL with camptothecin but not with other chemotherapeutic agents in colon carcinoma cell lines. The data from our study confirm these findings by showing a synergistic effect of camptothecin with Apo2L/TRAIL in four of six NSCLC cell lines studied. In addition, we noted synergy in four cell lines with the combination of Apo2L/TRAIL and cisplatin, an agent commonly used for chemotherapy of NSCLC.

Interestingly, no synergistic effects have been observed for the combination of paclitaxel and Apo2L/TRAIL.

Whereas all the other chemotherapeutic agents used in the study are DNA-damaging agents, paclitaxel exerts its apoptotic effects through stabilization of the microtubule system, thus interfering with the cell cycle.¹⁶ Our data therefore indicate that DNA damage, but not mitotic arrest, is needed for Apo2L/TRAIL-induced apoptosis.

To date we do not have much information on systemic toxicity of the combination of Apo2L/TRAIL with chemotherapy. In the study performed in a mouse model by Gliniak and Le,¹⁵ no toxic effect on normal tissue with the combination of Apo2L/TRAIL and camptothecin was observed. For possible clinical application, this point needs further evaluation.

The question of why some tumor cells are susceptible to Apo2L/TRAIL-induced apoptosis remains to be answered. When the decoy receptor hypothesis could not be proved, other groups suggested that resistance of some tumor cell lines to Apo2L/TRAIL-induced apoptosis is related to the expression of the intracellular antiapoptotic protein c-FLIP (FLICE-inhibitory protein).¹⁷ c-FLIP was cloned as the cellular homolog of a viral protein that was identified as an inhibitor of apoptosis. It is structurally similar to caspase-8, because it contains two death effector domains and a caspase-like domain, but it shows no caspase activity.¹⁸

To evaluate the role of c-FLIP in the resistance to Apo2L/TRAIL in our NSCLC cell lines, endogenous expression levels of c-FLIP were determined and compared with the susceptibility to Apo2L/TRAIL. A correlation between c-FLIP expression and susceptibility to Apo2L/TRAIL could not be demonstrated. Similar data have been demonstrated by Zhang and colleagues¹⁹ for melanoma cell lines. We further compared the expression of c-FLIP after treatment with chemotherapeutic agents with the enhanced susceptibility to Apo2L/TRAIL after treatment with chemotherapeutic agents. Again, there was no correlation between endogenous c-FLIP and susceptibility to Apo2L/TRAIL. Therefore, factors other than c-FLIP

Figure 3. Augmentation of Apo2L/TRAIL-induced apoptosis does not correlate with expression of c-FLIP. Cells were incubated with 5-mmol/L doxorubicin, 30-mmol/L 5-fluorouracil, 1.8-mmol/L camptothecin, 100- μ mol/L cisplatin, 100-nmol/L paclitaxel, and 1- μ g/mL cycloheximide for 24 hours. Expression of c-FLIP was determined by Western blot. Equal amount of protein was shown by reblotting against tubulin.

seem to be responsible for the resistance to Apo2L/TRAIL induced apoptosis. In contrast, Leverkus and colleagues⁷ suggested an inhibition of the Apo2L/TRAIL pathway by endogenous c-FLIP, as evidenced by experiments with cycloheximide, an unspecific inhibitor of protein synthesis. Treatment of cells with cycloheximide decreased levels of c-FLIP and enhanced susceptibility to Apo2L/TRAIL, but these findings were not confirmed with a specific inhibitor of c-FLIP.

Our data also suggest that downward regulation of c-FLIP, for example by an antisense approach, does not render NSCLC cell lines susceptible to Apo2L/TRAIL. This is in contrast to the Fas (CD95) pathway, for which Nagaraju and colleagues²⁰ showed that downregulation of c-FLIP enhances apoptotic cell death.

Other authors have reported that increased c-FLIP expression blocks apoptosis and so mediates the escape of tumors from the immune system by mediating resistance of tumor cells to apoptosis.^{21,22} Our data demonstrate that levels of c-FLIP in normal lung tissue and lung cancer tissue are equal and therefore indicate that c-FLIP does not seem to play a role in tumor immune escape in NSCLC.

In conclusion, Apo2L/TRAIL can efficiently induce apoptosis in NSCLC cell lines. Furthermore, we have shown for the first time that this effect can be augmented by chemotherapeutic agents in NSCLC cell lines and that c-FLIP does not modulate apoptotic cell death induced by Apo2L/TRAIL. Combined therapy of Apo2L/TRAIL with chemotherapeutic agents seems to be promising. Future in vivo studies are needed to confirm these findings.

We thank Dr P.H. Krammer for the c-FLIP monoclonal antibody and Genentech Inc for providing Apo2L/TRAIL.

Figure 4. Expression of cFLIP in primary NSCLC (C) and normal lung tissue (N). Protein lysates were obtained from frozen tissue from patients who underwent lung resection. c-FLIP expression was determined by Western blot. To confirm equal protein levels the same blot was stripped and developed with antitubulin antibody. A, Adenocarcinoma; B, squamous cell carcinoma.

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Discussion

Stephen G. Swisher, MD (*Houston, Tex*). Did you look at caspase-8 cleavage in your cells that were resistant? If you saw cleavage of caspase-8, then you would know that the inhibition is downstream from caspase-8, but if you do not see cleavage of caspase-8, most likely there is a block up higher near the receptor, maybe decoy receptors.

Dr Frese. Unfortunately, so far we have not looked at the cleavage of caspase-8. At the moment we are waiting for a caspase-8 antibody from the German Cancer Research Center.

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