Cytotoxic Effects of Camptothecin and Cisplatin Combined with Tumor Necrosis Factor-related Apoptosis-inducing Ligand (Apo2L/TRAIL) in a Model of Primary Culture of Non-small Cell Lung Cancer

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Abstract. *Background: The cytokine tumor-necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) has been shown to preferentially induce apoptosis in cancer cells. A previous study of our group demonstrated that non-small cell lung cancer cell lines can be sensitized to Apo2L/TRAIL-induced apoptosis by chemotherapeutic agents. The aim of the present study was the evaluation of these results in a model of primary culture of non-small cell lung cancer. Materials and Methods: Lung cancer tissue and normal lung tissue obtained from 8 patients who underwent operation were cultured and treated with Apo2L/TRAIL alone and in combination with cisplatin and the topoisomerase I inhibitor camptothecin for different periods. Metabolic activity of the tissue was measured by alamar blue. Markers for apoptosis were determined by immunohistochemistry and Western blot. Expression of Apo2L/TRAIL receptors in primary lung cancer and normal lung tissue was evaluated by semi-quantitative RT-PCR. Statistics were performed using nonparametric repeated measures Anova with Dunnett's correction. Results: Treatment with cisplatin, camptothecin and the combination of camptothecin and Apo2L/TRAIL demonstrated significant reduction of metabolic activity in tumor and normal lung tissue. In addition, the combination of camptothecin plus Apo2L/TRAIL revealed greater cytotoxic activity in cancer tissue compared with normal lung tissue. Importantly, no toxic activity of Apo2L/TRAIL alone in normal lung tissue was observed. Conclusion: Our results obtained in a model of primary culture of lung cancer suggest that the combination of Apo2L/TRAIL and camptothecin might present an effective strategy for the treatment of non-small cell lung cancer.*

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Lung cancer is the leading cause of cancer death in the United States among both men and women. The projected number of new lung cancer cases in 2008 in the United States was 215,020, accounting for 15% of all new cancer cases and for 29% of all cancer deaths. In fact, more people die each year from lung cancer than from breast, colorectal, prostate and ovarian malignancies combined (1). There is a similar situation in Europe, with 292,200 lung cancer cases and 253,300 patients who died from lung cancer deaths in 2006 (2). Therefore, new treatment strategies are needed for this disease.

A potential new anticancer drug might be the cytokine tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Apo2L/TRAIL). Apo2L/TRAIL is closely related to TNF-α and FasL, members of the tumor necrosis factor family (3). Apo2L/TRAIL induces apoptosis by interacting with death receptor 4 (DR4, TRAIL-R1) and death receptor 5 (DR5, TRAIL-R2), leading to the formation of the death-inducing signaling complex (DISC) with subsequent binding of caspase-8 (FLICE). Recruitment of caspase-8 to the DISC activates its proteolytic properties, which initiates a cascade of protease activation involving enzymes such as caspase-3, promoting the cleavage of death substrates and finally resulting in apoptosis (4). Apo2L/TRAIL can also bind to three other receptors, namely TRAIL-R3 (DcR1 or TRID), TRAIL-R4 (DcR2 or TRUNDD) and the osteoprotegerin receptor OPG. Since these receptors contain no functional cytoplasmic death domain they are presumed to primarily operate as competitive decoy receptors for Apo2L/TRAIL (5).

Different studies have shown that Apo2L/TRAIL induce apoptosis only in tumor not in normal cells (6, 7), providing

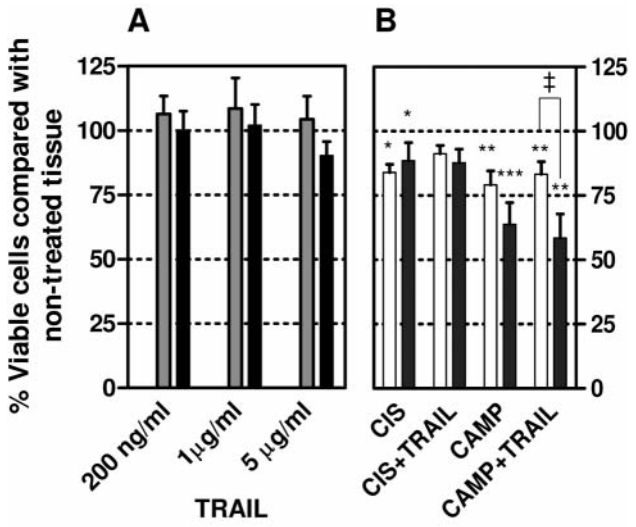

Figure 1. *Cell viability evaluated in patient-derived tissue treated with Apo2L/TRAIL alone or in combination with cisplatin and camptothecin. A, Treatment with 200 ng/ml, 1 μg/ml and 5 μg/ml Apo2L/TRAIL, respectively for two different times (grey bar=6 days, closed bar=11 days). B, Combined treatment with 200 ng/ml Apo2L/TRAIL and 15 μg/ml cisplatin or 1 μg/ml camptothecin, respectively for 11 days in normal lung tissue (open bar) and tumor tissue (closed bar). Data are expressed as percentage of levels in non-treated normal or tumor tissue, respectively. Statistical comparisons were made between treated tissue; using various agents and concentrations vs. non-treated tissue. *p<0.05, **p<0.01, ***p<0.001. ‡Significant effect of combined treatment with 200 ng/ml Apo2L/TRAIL+camptothecin in normal tissue vs. tumor tissue at p<0.05. Mean values±SEM (n=8).*

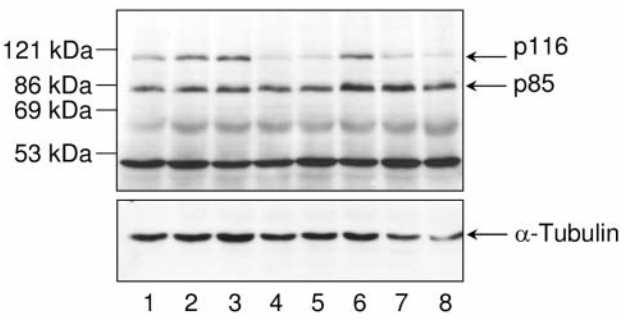

Figure 2. *Cleavage of PARP in cultivated lung cancer tissue. Tumor tissue was treated as indicated for 72 hours then 50 μg protein of cell lysates were analyzed for cleavage of the 116 kDa full length form of PARP by Western blotting. To show equal amounts of protein, the plot was stripped and re-probed with an antibody against α-tubulin. Tissue treatments: lane 1: non-treated, lane 2: 200 ng/ml Apo2L/TRAIL, lane 3: 1 μg/ml Apo2L/TRAIL, lane 4: 5 μg/ml TRAIL, lane 5: 15 μg/ml cisplatin, lane 6: cisplatin+200 ng/ml Apo2L/TRAIL, lane 7: 1 μg/ml camptothecin, and lane 8: camptothecin+200 ng/ml Apo2L/TRAIL.*

evidence that Apo2L/TRAIL can be used as a new cancer-selective treatment option. However, some cytotoxic effects of Apo2L/TRAIL against certain types of normal cells (8, 9) have been reported. Moreover, agonistic anti-DR5 antibodies have been demonstrated to be involved in the development of cholestatic liver disease (10).

Another problem for the use of Apo2L/TRAIL as anti-cancer agent is the fact that approximately 50% of tumor cells possess resistance to Apo2L/TRAIL-induced apoptosis. Therefore, different groups are searching for strategies as how to sensitize cancer cells but not normal cells to Apo2L/TRAIL-mediated cell death. In previous studies, our group was able to show that chemotherapeutic agents such cisplatin and the topoisomerase I inhibitor camptothecin (11), the Chinese herb *Tripterygium wilfordii* PG490 (12) as well as cardiac glycosides such as digoxin (13) might be applied in combination with Apo2L/TRAIL to induce apoptosis in lung cancer cells.

In order to find preclinical evidence as to whether Apo2L/TRAIL might be used for the treatment of lung cancer, in the present study we examined the combination of cisplatin and camptothecin with Apo2L/TRAIL in a model of primary culture of lung cancer. Additionally, we investigated the expression pattern of DR4, DR5, DcR1 and DcR2 in freshly isolated tumor tissue and adjacent normal tissue from lung cancer patients.

Materials and Methods

Reagents. Soluble, nontrimerized Apo2L/TRAIL was kindly provided by Genentech (South San Francisco, CA, USA). Camptothecin was obtained from Alexis Corporation (San Diego, CA, USA) and cisplatin was purchased from Sigma (St Louis, MO, USA).

Primary culture of lung cancer and normal lung tissue. All patients reported here gave their written consent to be included in this study. Primary culture was performed as described elsewhere (12, 14). Briefly, on the morning of resection, 1×1 cm^2 pieces of gelfoam (Pharmacia and Upjohn, Kalamazoo, MI, USA) were placed in a 24-well plate. The gelfoam was allowed to soak with Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and antibiotics). Lung tumors and adjacent normal lung tissues of patients with non-small cell lung cancer (NSCLC) were taken from the operating room immediately after resection. The specimens were dissected into 2-3 mm cubes. Per well, 50-55 μg of tissue was placed on the gelfoam and plates were incubated at 37˚C with 5% CO_2. The next day, tumor tissue and adjacent normal lung tissue were treated with 200 ng/ml, 1 μg/ml and 5 μg/ml Apo2L/TRAIL for 6 and 11 days. Combined treatment was performed with 200 ng/ml Apo2L/TRAIL and 15 μg/ml cisplatin or 1 μg/ml camptothecin, respectively, for 11 days. For 11-day cultures, at day 5 the medium was changed by adding new treatment solution. The viability of the tissue was determined by metabolism of the dye alamar blue (Serotech, Raleigh, NC, USA) using a Spectramax Gemini Fluorometer (Molecular Devices, Sunnyvale, CA, USA) at 590 nm emission wavelength.

Immunohistochemistry. Two micrometer-thick paraffin sections, placed on SuperFrost Plus slides (Roth, Karlsruhe, Germany), were dewaxed before antigen retrieval with 15% acetic acid for 8 min at room temperature. After blocking of endogenous peroxidases with

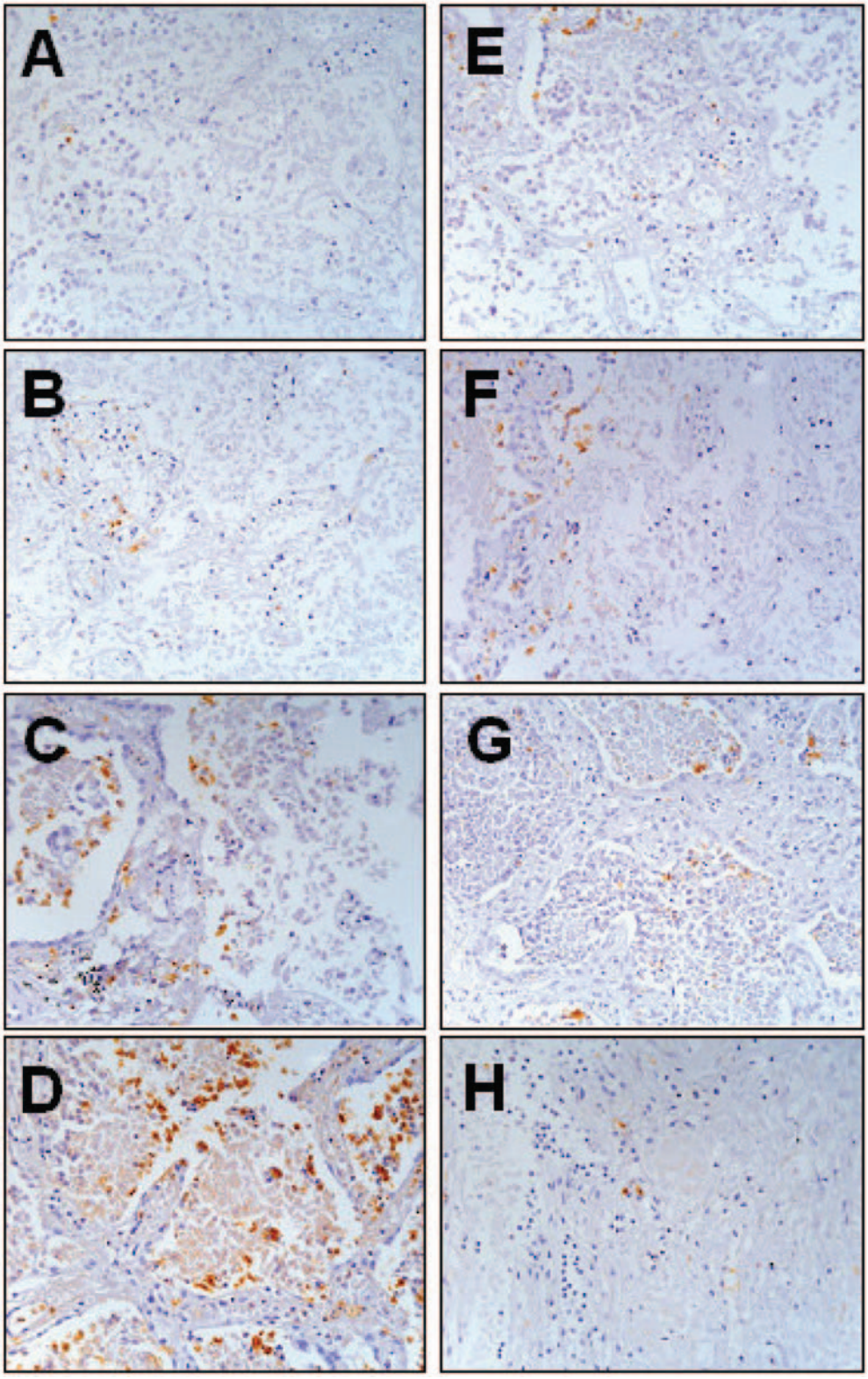

Figure 3. *Cleavage of lamin A in lung cancer tissue was determined by immunohistochemistry on paraffin-embedded tissue with an antibody specifically recognizing the cleaved form of this protein. A, Non-treated tissue. Tissue treated for 72 hours with: B, 200 ng/ml Apo2L/TRAIL; C, 1 μg/ml Apo2L/TRAIL; D, 5 μg/ml Apo2L/TRAIL; E, 15 μg/ml cisplatin, F, cisplatin+200 ng/ml Apo2L/TRAIL; G, 1 μg/ml camptothecin; H, camptothecin+200 ng/ml Apo2L/TRAIL. Sections were examined by light microscopy, and representative areas were photographed using a ×20 objective.*

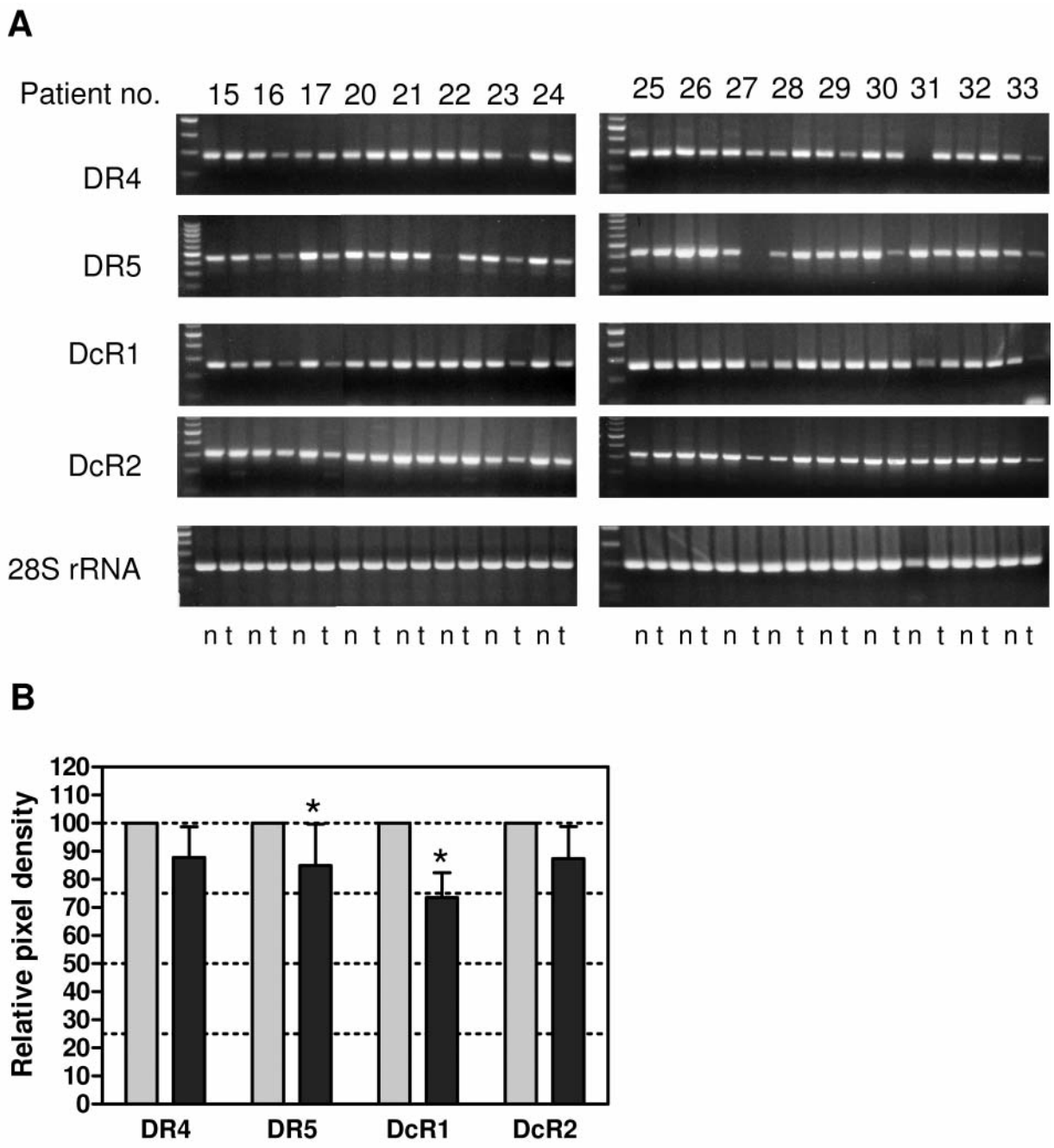

Figure 4. *Apo2L/TRAIL receptor mRNA expression in freshly isolated lung cancer and adjacent normal tissue from 17 NSCLC patients. A, DR4, DR5, DcR1 and DcR2 mRNA expression was detected in total RNA isolates by RT-PCR. Amplification of 28S rRNA mRNA was carried out as an internal control. The data are representative of three independent experiments. Lung cancer tissue (t); normal tissue (n). B, Band intensities were evaluated densitometrically and are expressed as percentage relative to normal tissue and normalized to 28S rRNA. Results are expressed as mean±SEM. *p<0.05.*

3% H2O2 for 5min, slides were incubated with anti-lamin A antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) diluted in 1:1 Tris-buffered saline/phosphate-buffered saline, containing 0.5% bovine serum albumin for 3 hours. For detection of the immunosignal, the PicTure-Plus kit (mouse; Zymed, South San Francisco, CA, USA) was used. Sections were counterstained with hematoxylin.

Immunoblot analysis. Fifty micrograms of protein were separated by polyacrylamide gel electrophoresis under reducing conditions and transferred onto nylon membranes (BioRad Laboratories, Hercules, CA, USA) as described elsewhere (15). Protein detection was performed using Immunoblot Chemiluminescence Reagent Plus (New England Nuclear, Life Science Products,

Boston, MA, USA). As primary antibody for the detection of PARP, clone C-2-10 (Alexis) was used. For stripping, membranes were incubated for 30 min at 50˚C in a buffer containing 62.5 mM TRIS-HCl, pH 6.7, 2% SDS and 100 mM β-mercaptoethanol. Subsequently, blots were washed, blocked and re-probed again.

Polymerase chain reaction (PCR). Total RNA was isolated using the GeneElute Mammalian total RNA miniprep kit (Sigma). After DNAse digestion using a Deoxyribonuclease I kit from Sigma, cDNA was synthesized by standard methods using reverse transcriptase and oligo(dT) primer from Roche (Rotkreuz, Switzerland). For the semiquantitative PCR reaction, 5 μl cDNA-template were mixed with 2.5 μl 10× PCR-buffer, 0.5 μL 10 mM dNTPs, 0.25 μl Taq polymerase and 0.25 μl of each primer (50 μM; Invitrogen Custom Primers, Basel, Switzerland) in a total volume of 25 μl for each probe. PCR was carried out in an Eppendorf Mastercycler (Vaudaux-Eppendorf, Schönenbuch, Switzerland) using the following primers (sense primers are indicated first): *DR4* 5'-TTGTGTCCACCAGGATCTCA-3', 5'-GTCACTCCAGGGCG TACAAT-3'; *DR5* 5'-ACTCCTGGAATGA CTACCTG-3', 5'-ATCCCAAG-TGAACTTGAGCC-3' *DcR1* 5'-GCTGTGTTCCCACAGACAGA-3', 5'-CTCACCCTTGTCACCC AGTT-3' and *DcR2* 5'-AGGCTGTTTACATGGGTTGC-3', 5'-AAACCAGACACATG GCTTCC-3'. Amplification of *28S* rRNA served as internal control. The *28S* rRNA primers were 5'-GTGG AATG-CGAGTGCCTA-3' and 5'-GTTGATTCGGCAGGTGAGTT-3'. Negative controls were performed for each set of primers. After amplification, PCR products were separated by electrophoresis on 1.5% agarose gels containing ethidium bromide and visualized by UV light illumination. PCR conditions were as follows: 1 cycle, 3 min/95˚C; 22-26 cycles, 30 s/95˚C, 30 s/58˚C and 1 min/72˚C.

Statistical analysis. Data were analyzed by using Prism 2.0 (GraphPad Software Inc., San Diego, CA, USA). Comparisons between two groups were made using paired *t*-test. Comparisons between three or more groups were carried out by nonparametric repeated measures ANOVA followed by Dunnett *post hoc* test. A two-tailed *p*-value <0.05 was considered statistically significant.

Results

Using a primary culture model of lung cancer, 8 patient-derived lung tumors (NSCLC) were evaluated for their sensitivity to Apo2L/TRAIL. To confirm histology of NSCLC, all tumor samples were assessed by the pathologist. The results shown in Figure 1A demonstrate that Apo2L/TRAIL alone was unable to significantly suppress tumor viability, neither in a time- nor in a dose-dependent manner. Importantly, treatment of adjacent normal lung tissue with Apo2L/TRAIL alone using similar conditions demonstrated no obvious cytotoxic activity (data not shown). Treatment with chemotherapeutic agents for 11 days resulted in significant reduction of metabolic activity for both cisplatin and camptothecin. However, while cisplatin was not able to sensitize lung cancer tissue to TRAIL-induced apoptosis, the combination of camptothecin and Apo2L/TRAIL reduced cell viability significantly more in tumor cells than in cultures of normal lung tissue (Figure 1B).

Previous studies have described cellular poly(ADP-ribose)polymerase (PARP) as a nuclear apoptotic marker, which is cleaved by caspase-3 during apoptosis. To analyze the degree to which our treatments induced apoptosis, Western blot experiments were performed using an antibody which recognizes both the 116 kDa pro-form and the 85 kDa fragment of cleaved PARP. Although inhibition of cell viability in tumor tissue by Apo2L/TRAIL was not statistically significant, enhanced cleavage of PARP was found in the tumor tissue treated for 11 days with 5 μg/ml Apo2L/TRAIL (Figure 2, lane 4) compared to tumor tissue that was either non-treated or treated with a lower concentration of Apo2L/TRAIL. In line with the cell viability experiments, there was enhanced PARP cleavage in single treatments with cisplatin (lane 5) and camptothecin (lane 7), as well as for the combination of camptothecin and Apo2L/TRAIL (lane 8). Combined treatment with cisplatin and Apo2L/TRAIL (lane 6) did not lead to pronounced PARP cleavage. To further support these results, immunohistochemical experiments were performed using an antibody that detects the small fragment of the structural protein lamin A when cleaved by caspase-6 (16). An increased level of cleaved lamin A was found in tumor tissue treated for 11 days with Apo2L/TRAIL compared to non-treated tumor tissue. Cleavage of lamin A was dose dependent, with the highest amount detected in tumor tissue treated with 5 μg/ml Apo2L/TRAIL (Figure 3A-D). Slightly elevated staining of cleaved lamin A compared to that in non-treated tumor tissue was also detected in tissue treated with cisplatin (Figure 3E) and camptothecin (Figure 3G) alone and for both treatments in combination with Apo2L/TRAIL (Figure 3F, H). However, staining for cleaved lamin A in these sections was much weaker than in tissue treated with Apo2L/TRAIL alone at the highest concentration, suggesting that the apoptotic effect of cisplatin and camptothecin was not caspase-6 dependent.

In order to evaluate the correlation between apoptosis and expression of TRAIL receptors, *DR4, DR5, DcR1* and *DcR2* mRNA expression was examined by RT-PCR in freshly isolated lung tumor tissue as well as adjacent normal lung tissue from 17 patients. The results indicate that receptor expression of *DR5* and *DcR1* was significantly lower in tumor tissue *vs*. normal tissue (Figure 4). There were no significant differences observed between normal and tumor tissue for the expression of DR4 and DcR2 receptors.

Discussion

Since its discovery in 1995, Apo2L/TRAIL has been considered a new promising anticancer agent. Some of the first studies with this cytokine demonstrated that subcutaneous tumors generated by injection of clonal

cancer cell lines into immunodeficient mice could be successfully treated with Apo2L/TRAIL (6, 7). However, the model of subcutaneously implanted clonal tumor cell lines does not reflect the situation of non-clonal tumor cells in patients and cannot answer the question of whether Apo2L/TRAIL has any clinical benefit. Therefore, for our experiments we used a model of primary culture utilizing small pieces of lung tumors and normal lung tissue. In preliminary experiments, we also applied a model of primary culture where tumor tissue was digested with the enzymes collagenase and hyaluronidase producing a single cell suspension culture. Similar protocols were used by other groups demonstrating both Apo2L/TRAIL-sensitivity of primary human colon cancer cells (17) and the resistance of freshly isolated melanoma cells against Apo2L/TRAIL (18). However, at least in our hands, such obtained cultures were quickly overgrown by proliferating fibroblasts and we, therefore, changed to the model utilizing intact tumor pieces.

With this model, we were able to show that Apo2L/TRAIL in combination with the topoisomerase I inhibitor camptothecin efficiently induce apoptosis in human lung cancer cells. Importantly, while the combination of Apo2L/TRAIL and the chemotherapeutic agent cisplatin was demonstrated previously to be toxic toward normal human hepatocytes and resting lymphocytes (19), our results clearly suggest that the combination of Apo2L/TRAIL and camptothecin was significantly more cytotoxic to lung cancer than to normal lung cells. To conclude from our data that cancer cells in general are more susceptible to Apo2L/TRAIL and topoisomerase I inhibitors might be difficult but is supported by data from Gliniak and colleagues demonstrating the successful treatment of colon carcinoma tumors in mice with this combination (20, 21).

The mechanism of how inhibition of topoisomerase I sensitizes cancer cells to Apo2L/TRAIL-induced apoptosis is most likely to be found at the Apo2L/TRAIL receptor level. Different groups showed that inhibition of topoisomerase increase the expression of DR4 and -5 at the cell surface (21, 22). We demonstrated in the present study that the two functional Apo2L/TRAIL receptors DR4 and DR5 were equally or less expressed in lung cancer tissue compared to normal lung tissue. This might explain why Apo2L/TRAIL alone was not cytotoxic and why camptothecin-mediated up-regulation of DR4 and/or DR5 was needed to sensitize lung cancer tissue to Apo2L/TRAIL-induced cell death.

In conclusion, our data clearly demonstrate that combined treatment with Apo2L/TRAIL and camptothecin is significantly more cytotoxic to primary lung cancer tissue than to normal lung tissue. Therefore, the combination of Apo2L/TRAIL and camptothecin might be used as new strategy for the treatment of lung cancer.

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