Natural Immunity Enhances the Activity of a DR5 Agonistic Antibody and Carboplatin in the Treatment of Ovarian Cancer

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Abstract

The tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) induces apoptosis specifically in cancer cells with little effect on normal cells. We have previously shown that TRAIL signaling is altered in most ovarian cancer patients and that resistance to TRAIL contributes to ovarian cancer progression. In this study, we investigated whether resistance to TRAIL may be overcome by a monoclonal TRAILR2 (DR5) agonistic antibody (AD5-10). We found that the joint presence of AD5-10 with TRAIL and natural killer (NK) cells expressing TRAIL resensitizes ovarian cancer cells to apoptosis in vitro and in vivo, respectively. The combination of AD5-10 with carboplatin exerts a more than additive effect in vitro, which may at least partially be explained by the fact that TRAIL triggers DR5 expression on ovarian cancer cells. Moreover, AD5-10 restores the sensitivity of platinum-resistant ovarian cancer to carboplatin in vitro. In addition, we found that TRAIL expression and NK cells are abundant in the tumor microenvironment and that depletion of NK cells abolishes the antitumor activity of AD5-10. This indicates that NK-mediated immunosurveillance against ovarian cancer might be mediated by TRAIL and that apoptosis induced by AD5-10 requires the presence of NK cells. In conclusion, this study indicates a key role and strong antitumorigenic effect of DR5 and highlights a novel link between NK-mediated immunosurveillance and activation of DR5-mediated apoptosis in ovarian cancer. Mol Cancer Ther; 9(4); 1007–18. ©2010 AACR.

Introduction

Ovarian cancer is the most lethal gynecologic cancer and the fifth leading cause of cancer-related deaths among women in western industrialized countries (1, 2). Ovarian cancer originating from the ovarian surface epithelium is the most common form and displays a range of histologic subtypes (3). Whereas most ovarian cancers are sensitive to platin-based chemotherapy at the time of diagnosis, recurrence of the disease is frequent, and ultimately, platinum-resistant disease develops in all patients.

Apoptosis is important for maintaining cellular homeostasis in normal tissues by eliminating disordered cells, and defects in the apoptosis pathway may lead to cancer (4). The apoptotic cascade can be stimulated by death receptors, resulting in activation of caspases (5). Trimerization of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) functional receptors TRAILR1 (DR4) or TRAILR2 (DR5) by their ligands leads to the assembly of death-inducing signaling complex, which initiates apoptotic cascade (6). DR4 and DR5 are characterized by an extracellular cystein-rich domain and an intracellular death domain, giving them the ability to trigger the assembly of the death-inducing signaling complex. We have previously found that TRAIL is highly expressed in the human ovarian cancer microenvironment, but that tumor tissues display a reduced number of TRAIL functional receptors (7).

One major physiologic role of TRAIL is the mediation of natural immunity and the elimination of developing tumors (8, 9). Previous studies have shown that soluble TRAIL or agonistic monoclonal antibodies specific for functional TRAIL receptors exhibit tumoricidal activities, a phenomenon that has been tested in clinical trials (10). The agonistic human DR5-specific monoclonal antibody AD5-10 used in this study was reported to mediate antitumor effects in various tumor cells and, due to its unique binding site, does not compete with TRAIL for binding to DR5 in contrast to other agonistic DR5 antibodies (11).

In the current study, we identified the functional role of DR5 in ovarian cancer progression and shed light on a novel strategy to eliminate ovarian cancer in a preclinical mouse model. Moreover, we show for the first time that the function of natural killer (NK) cells is necessary for...
the activation of DR5-mediated apoptosis and that the presence of NK cells is highly correlated with a longer life span in xenograft mice.

Materials and Methods

Drugs

Carboplatin, paclitaxel (both EBEWE), bevacizumab (Roche), lapatinib (GlaxoSmithKline), agonistic DR5 monoclonal antibody (AD5-10) prepared as described previously (11), and recombinant human and mouse soluble TRAIL (Alexis) were used for stimulation of ovarian cancer cell lines at various concentrations.

Cell culture

The human ovarian cancer cell line MDAH-2774 (ovarian endometroid adenocarcinoma–derived cell line, originating from the ascitic fluid of a patient; ref. 12) and a platin-resistant subline of the ovarian cancer cell line A2780 (2780CP8, human epithelial ovarian cancer cell line established from tumor tissue; ref. 13) were cultured in RPMI 1640 (Invitrogen). The ovarian cancer cell line ES-2 (human ovarian clear cell carcinoma cell line taken from a 47-y-old woman; ref. 14) was cultured in McCoy’s medium (Invitrogen). Medium was supplemented with 10% FCS (PAA Laboratories GmbH), 1 mmol/L glutamine, and 1% penicillin/streptomycin (PAA Laboratories GmbH). MDAH-2774 and ES-2 cell lines were obtained from American Type Culture Collection, and the cell line 2780CP8 was obtained from the European Collection of Animal Cell Cultures.

Determination of apoptosis

MDAH-2774, 2780CP8, and ES-2 ovarian cancer cell lines were plated at a density of 5 × 10^5 cells per well in 24-well plates and incubated for 24 h before stimulation. Then cells were treated with either AD5-10 (1 μg/mL), carboplatin (100 μg/mL), paclitaxel (0.05 μmol/L), bevacizumab (100 ng/mL), lapatinib (4 μmol/L), or an antibody-drug combination for 24 h. For inhibition of caspase activation, the caspase-8 inhibitor N-benzylloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (zIETD.fmk; Bachem) was used. Cells were harvested by trypsinization using 0.05% trypsin and 0.02% EDTA without Ca^2+ and Mg^2+ (PAA Laboratories). The membrane proteins were incubated with the following primary antibodies: mouse anti-caspase-8 monoclonal antibody, 12F5 (recognizes human procaspase-8 and active human caspase-8, Alexis), mouse anti-caspase-3 monoclonal antibody, 31A1067 (recognizes human procaspase-3 and active human caspase-3, Imgenex), mouse anti-p53 monoclonal antibody (Cell Signaling), mouse monoclonal anti-human cellular Fas-associated death domain–like interleukin-1β–converting enzyme–like inhibitory protein (c-FLIP; NF6, Alexis), and goat anti-actin polyclonal antibody (Santa Cruz). Detection was done with the appropriate horseradish peroxidase–conjugated secondary antibody (Santa Cruz). The membranes were developed using enhanced chemiluminescence (Amersham).

Detection of TRAIL ligand and its functional receptor expression by flow cytometry

MDAH-2774, 2780CP8, and ES-2 ovarian cancer cell lines were collected and washed in PBS. The cells were then incubated with mouse monoclonal antibodies against human TRAILR1, HS101 (Alexis), human TRAILR2, HS201 (Alexis), or mouse IgG1 isotype control (Ansell) for 1 h at 4°C. Afterwards, cells were washed in PBS, incubated with goat anti-mouse IgG-FITC (Santa Cruz) for 1 h at 4°C in the dark, washed with PBS, and analyzed by flow cytometry. TRAIL intracellular staining was done as described previously (15). Briefly, cells were washed with PBS and resuspended in 2% formaldehyde and incubated for 10 min at 4°C. Afterwards, cells were washed with PBS and resuspended in blocking solution (0.1% saponin and 20% serum) and incubated for 20 min at room temperature. Cells were then stained with mouse monoclonal antibody against human TRAIL, III6F (Alexis), or mouse IgG2b isotype control (Ansell) for 30 min at room temperature in staining buffer (0.1% saponin, 2% FCS serum). After that, cells were washed thrice with staining buffer, resuspended in secondary antibody goat anti-mouse IgG-FITC (Santa Cruz), and incubated for 1 h at 4°C in staining buffer. Then, cells were washed thrice with staining buffer and analyzed by flow cytometry. Flow cytometric analysis was done using FACScan (Becton Dickinson), and the resulting data were analyzed with CELLQuest Pro software.

Immunohistochemistry

Immunohistochemical analysis was done using paraffin-embedded sections as described previously (7). Briefly, tissue sections were deparaffinized and rehydrated. For epitope retrieval, specimens were incubated in 96°C prewarmed 10 mmol/L citrate buffer (pH 6.0) for 20 min.
Slides were then incubated with 0.3% H2O2/PBS for 10 min at room temperature to block endogenous peroxidase. After blocking the secondary antibody (diluted 1:200 in PBS), tissues were incubated for 1 h at room temperature with primary antibody diluted in serum/PBS. The following primary antibodies were used: rabbit anti-human cleaved caspase-3 monoclonal antibody Asp175 (dilution 1:50, Cell Signaling); mouse anti-human Ki-67 monoclonal antibody (dilution 1:200, Dako); goat anti-mouse TRAIL polyclonal antibody AF1121 (dilution 1:100, R&D Systems); rabbit anti-human TRAILR1 polyclonal antibody H130 (dilution 1:200, Santa Cruz); and rat anti-mouse Ly-49G2 monoclonal antibody 4D11 (ref. 16; dilution 1:400, eBioscience). The appropriate secondary biotinylated antibodies (Vector Laboratories) were diluted with the serum/PBS buffer (dilution 1:200) and incubated for 30 min at room temperature. Tissue sections were incubated with StreptABComplex/HRP (Dako) for 45 min at room temperature, then visualized with 3,3’-diaminobenzidine (Dako), and counterstained with Mayer’s hematoxylin. Tissue sections were analyzed on an Olympus BX50 upright light microscope (Olympus Europe) equipped with the soft imaging system CC12.

Apoptosis was measured by an in situ cell death detection kit (Roche) according to the manufacturer’s instructions. Afterward, the cells were analyzed on a fluorescence microscope (Nikon Eclipse 800) equipped with a Nikon DS-R1 camera, using the NIS-Elements software.

Negative controls were done by excluding incubation with primary antibody and yielded negative results. The percentage of positive cells was determined by a blinded operator.

Xenograft mouse model

Four- to six-week-old female athymic nude-Foxn1nu/nu mice were obtained from Harlan (Italy) and maintained under specific pathogen-free conditions at the animal resource service of the Medical University of Vienna. Mice were s.c. inoculated with 2780CP8 ovarian cancer cells on both sides. Treatment started on day 2 by i.p. injection of PBS, AD5-10, carboplatin, or the latter two in combination. Tumor size was measured every second day by calipers. The tumor volume was calculated according to the formula $V = \frac{4}{3} \pi \left(\frac{L}{2} \times W \times \frac{W}{2}\right)$, $L$, length; $W$, width (17). At the end of the experiment, tumors were recovered, weighed, and then prepared for histologic and pathologic analysis. Animal experiments were done according to protocols approved by the Austrian Federal Ministry for Education, Science, and Art. For NK depletion experiments, mice were treated with 20 μl anti-asialo GM1 antibody (Wako) and 100 μg NK1.1 antibody (prepared as described previously; ref. 18) 3 d before inoculation and then every 4 d.

Generation of splenocytes and analysis of NK cells

The spleen was removed from sacrificed mice and placed into 60-mm tissue culture dishes. A single-cell sus-

pension was made by passing the tissue through a 70-μm nylon cell strainer (BD Bioscience) in PBS containing 2% FCS. Cells were centrifuged, and hypotonic lysis of RBC was done using ACK lysis buffer [0.15 mol/L NH4Cl, 1 mmol/L KHCO3, and 0.1 mmol/L Na2EDTA (pH 7.2)], followed by resuspension in an appropriate volume of PBS containing 2% FCS. Cells were incubated at 4°C with Fc-block (BD Bioscience) for 5 min and then stained with PerCP hamster anti-mouse CD3ε, APC mouse anti-mouse NK-1.1, FITC rat anti-mouse CD49b (BD Bioscience), and PE rat anti-mouse TRAIL (Santa Cruz) antibodies for 30 min at 4°C in PBS. After that, cells were washed thrice with PBS and analyzed by flow cytometry. Flow cytometric analysis was done on FACS-Calibur (BD Biosciences). Data were analyzed using the CellQuest Pro software.

Statistical analysis

Two-sided Student’s $t$ tests were used to detect statistically significant differences between study groups and controls, using R and StatSoft’s Statistica software. Where appropriate, a one-way ANOVA was used. Data were visualized with box plots or bar plots. $P$ values below 0.05 were considered statistically significant, and $P$ values below 0.005 were considered highly significant. The correlation between variables was estimated using the Pearson correlation coefficient.

Results

In vitro effect of combining AD5-10 with cytotoxic drugs

We have previously shown that the TRAIL signaling pathway plays a fundamental role in ovarian cancer progression (7, 19). In this study, we addressed the functional role of human DR5 using three distinct ovarian cancer cell lines (MDAH-2774, 2780CP8, and ES-2). The selected ovarian cancer cell lines have accumulated different mutational and epigenetic changes that alter normal cell growth and survival pathway, e.g., mutated p53 in MDAH-2774 and ES-2, epigenetic silencing of DR4 in 2780CP8, as well as upregulation c-FLIP in most of them (19–22). Moreover, they express different levels of DR5 (Fig. 1A), and all of them were resistant to TRAIL-induced apoptosis. We found that AD5-10 triggered tumor cell apoptosis to a detectable extent and had an additive effect when combined with different pharmacologic and cellular anticancer agents (paclitaxel, bevacizumab, lapatinib; Supplementary Fig. S1). In combination with carboplatin, AD5-10 showed a more than additive effect (Fig. 1B), illustrated by comparing the sum effect of AD5-10 and carboplatin to a combination of both. This particular effect was observed in three different carboplatin-resistant ovarian cancer cell lines and was confirmed by detection of active caspase cleavage fragments (Fig. 1B and C, top). To prove whether DR5-enhanced caspase activation leads to apoptosis, we used caspase-8 inhibitor zIETD.fmk. Apoptosis in
Figure 1. Effect of combining AD5-10 with carboplatin in vitro. A, histogram showing expression of the TRAIL ligand as well as its functional receptors DR4 and DR5 on the surface of selected ovarian cancer cell lines as determined by flow cytometry. Selected ovarian cancer cell lines were stained with either TRAIL ligand (III6F, Alexis), anti-DR4 (HS101, Alexis), anti-DR5 (HS201, Alexis) monoclonal antibody (colored histogram), or isotype control IgG1 monoclonal antibody (unfilled histogram). Data represent three independent experiments. B, top, MDAH-2774, 2780CP8, and ES-2 ovarian cancer cell lines were either left untreated or treated with either AD5-10 (1 μg/mL), carboplatin (100 μg/mL), or both for 24 h. Apoptosis was determined by Annexin V and propidium iodide (PI) staining. Numbers in dot plot quadrants represent the percentage of stained apoptotic cells. Bottom, quantitative evaluation of top panel as well as the apoptosis rate of two additional experiments. Columns, mean of three independent experiments; bars, SEM. Statistically significant (*, *P < 0.05) or highly significant (**, **P < 0.005) differences were obtained by comparing the sum effect of AD5-10 and carboplatin to a combination of both. C, top, expression levels of activated caspase-8 and caspase-3 were determined by immunoblotting in untreated or treated ovarian cancer cells using AD5-10 (1 μg/mL), carboplatin (100 μg/mL), or both. Data represent two independent experiments. CF, cleavage form. Bottom, selected ovarian cancer cell lines were either untreated or treated with AD5-10 (1 μg/mL) and carboplatin (100 μg/mL) in absence or presence of 20 μmol/L zIETD.fmk (40 μmol/L for MDAH-2774) for 24 h, and apoptosis was determined. Columns, mean of three independent experiments; bars, SEM. Statistically highly significant (**, **P < 0.005) differences obtained by comparing the apoptotic effect of AD5-10 and carboplatin in absence or presence of zIETD.fmk. D, left, dose-response curve of carboplatin is presented using MDAH-2774 cells. MDAH-2774 ovarian cancer cell line was incubated with the specified concentration of carboplatin, AD5-10 (1 μg/mL), or both for 24 h, and apoptosis was determined. Columns, mean of four independent experiments; bars, SEM. Statistically highly significant (**, **P < 0.005) differences obtained by comparing the sum effect of AD5-10 and carboplatin in absence or presence of zIETD.fmk. D, right, dose-response curve of AD5-10 is presented using MDAH-2774 cells. MDAH-2774 ovarian cancer cell line was incubated with the specified concentration of AD5-10, carboplatin (100 μg/mL), or both for 24 h, and apoptosis was determined. Data are represented as indicated above in D (left).
response to combination of AD5-10 and carboplatin was almost completely blocked in the presence of zIETD.fmk in all three selected ovarian cancer cell lines (Fig. 1C, bottom). The dose-response curves for carboplatin in the presence of AD5-10 and the dose-response curves for AD5-10 in the presence of carboplatin confirmed the observed effects (Fig. 1D). Taken together, these findings suggest that AD5-10-mediated stimulation of DR5 in the presence of carboplatin efficiently sensitizes ovarian cancer cell lines to apoptosis.

**Carboplatin cooperates with AD5-10 to trigger apoptosis in ovarian cancer cells by upregulation of DR5**

We then focused on the mechanism by which carboplatin cooperates with AD5-10 signaling to mediate enhanced tumor cell death. DR5 is a transcriptional target of p53 (23), and carboplatin has been shown to induce the p53 tumor suppressor pathway (24). To identify whether carboplatin cooperates with AD5-10 via upregulation of DR5 expression, we treated MDAH-2774 cells (overexpressing a mutated form of p53, Arg273His; ref. 22), 2780CP8 cells (expressing wt p53; ref. 20), and ES-2 cells (expressing one mutant allele of p53, S241F, and the other wild type; ref. 21; Supplementary Fig. S2A) with carboplatin. Consequently, we analyzed the expression of p53 and DR5 by immunoblotting and flow cytometry, respectively (Fig. 2A and B). The p53 status was confirmed by sequencing p53 (data not shown). DR5 expression levels were increased in all cell lines after carboplatin treatment, irrespective of the p53 status (Fig. 2A and B). Notably, DR5 expression (after treatment with carboplatin) and the apoptosis rate (after treatment with a combination of AD5-10 with carboplatin) in wild-type p53 cells were higher than in cells with mutant p53 (Figs. 1B and 2B, respectively). These observations indicate that carboplatin forces expression of DR5 on ovarian cancer cells, thereby enhancing their susceptibility to AD5-10-mediated apoptosis. In addition, MDAH-2774 harboring a mutant p53 allele also reacted with apoptosis in a clear dose-dependent manner (Fig. 1B and D). This illustrates that carboplatin cooperates with AD5-10 regardless of the p53 status in ovarian cancer cells. Because carboplatin treatment induced an increase in DR5 expression irrespective of the p53 status, p53 mutations do not limit the therapeutic usefulness of combining AD5-10 with carboplatin. Interestingly, we found no change in DR5 expression in MDAH-2774 and ES-2 ovarian cancer cell lines upon treatment with paclitaxel (Supplementary Fig. S2B). On the other hand, an increase in DR5 expression on 2780CP8 was detected (Supplementary Fig. S2B). These data are in line with our observation that...
the combination of paclitaxel with AD5-10 in MDAH-2774 and ES-2 has no effect (Supplementary Fig. S1). In contrast, there is a more than additive effect in 2780CPS ovarian cancer cell line.

c-FLIP can prevent the recruitment and activation of caspase-8 (25, 26). We previously showed that the long isoform of c-FLIP (c-FLIP<sub>L</sub>) is highly expressed in more than two third of ovarian cancer patients (7). To determine whether c-FLIP<sub>L</sub> expression, as DR5 modulators, is affected in our experimental system, we analyzed its expression before and after treatment with carboplatin by immunoblotting. Notably, ES-2 has a very low basal expression of c-FLIP<sub>L</sub>, indicating that c-FLIP<sub>L</sub> is of no importance in this cell line (Fig. 2C). Significant c-FLIP<sub>L</sub> expression was detected in MDAH-2774 and 2780CPS cells. Carboplatin treatment does not alter c-FLIP<sub>L</sub> expression in MDAH-2774 but decreases c-FLIP<sub>L</sub> expression in 2780CPS cells (Fig. 2C), which is inline with the fact that active p53 inhibits c-FLIP expression (27). Altogether, our data indicate that combination of carboplatin-induced DR5 expression with AD5-10 potentiated activation of caspase-dependent cell death in a c-FLIP<sub>L</sub>-independent manner.

Combination of AD5-10 and carboplatin eradicates tumors in a xenograft ovarian cancer mouse model

To assess the antitumor efficacy of AD5-10 alone and/or in combination with carboplatin, we applied a tumor model of ovarian cancer in xenograft-bearing mice. In this model, mice bearing s.c. established tumors were randomly grouped for treatment with vehicle, AD5-10, carboplatin, or a combination of AD5-10 and carboplatin. Treatment started on day 2, and tumor size was measured every second day after tumor inoculation. After 2 weeks, all tumor-bearing mice were sacrificed and tumor-free mice were maintained for another 2 weeks (Table 1).

Mice treated with AD5-10 showed a clear reduction in tumor progression compared with untreated mice (Fig. 3). As expected, the treatment with carboplatin alone had no significant effect on tumor growth. In contrast, the combination of AD5-10 with carboplatin significantly suppressed tumor growth. Six of seven treated mice (86%) remained tumor-free at the time of sacrificing (day 14). After 28 days, >50% of mice (n = 4) remained tumor-free following combined AD5-10 and carboplatin treatment (Table 1). Overall, our data suggest that AD5-10 in combination with carboplatin induces a high apoptosis rate in ovarian cancer cells in vitro and ovarian cancer rejection in a significant number of mice in a xenograft model, whereas carboplatin alone has no significant effect (Figs. 1B and 3; Table 1).

Significant differences in tumor volume between control and AD5-10–treated mice were also observed. The largest reduction in tumor volume compared with the control group of untreated mice was observed in the group treated with AD5-10 in combination with carboplatin (Fig. 3A and C; Table 1). Similar results were obtained for tumor weight (Fig. 3B). In contrast, mice receiving carboplatin alone did not display any statistically significant differences in tumor volume or tumor weight. Clearly, the tumor suppressive effect of AD5-10 alone and the elimination effect of AD5-10 in combination with carboplatin suggest that DR5 plays a strong antitumor role in carboplatin-resistant ovarian cancer.

AD5-10 enhances antitumor activity against ovarian cancer in vivo

We then evaluated the cytotoxic effect of given substances on tumor tissues by immunohistochemical analysis. We observed a significant increase in caspase-3 activation and consequently in the apoptosis rate [measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay] for AD5-10, but

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<th>Group</th>
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<td>Carboplatin (n = 6)</td>
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<td>1&lt;sup&gt;†&lt;/sup&gt;</td>
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<td>1 (16.7%)</td>
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<td>AD5-10 + carboplatin (n = 7)</td>
<td>1</td>
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<td>1</td>
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<td>4&lt;sup&gt;†&lt;/sup&gt; (57.2%)</td>
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NOTE: (a) Two mice in the AD5-10 and one mouse in the carboplatin-treated group were excluded from the statistical analysis due to inoculation resulting in i.p. tumor. (b) On day 14, all tumor-bearing mice were sacrificed; the remaining mice were sacrificed upon reaching the final tumor volume of ∼4,000 mm<sup>3</sup>.

<sup>*</sup>One tumor-bearing mouse died before sacrificing.

<sup>†</sup>Sacrificed due to subcutaneous tumor development and ascites.

<sup>†</sup>One mouse without tumor was sacrificed for the photograph in Fig. 3C on day 14.
not for carboplatin, when compared with a control group (Fig. 4A and B). No significant differences in proliferation were observed for AD5-10 or carboplatin treatment (Supplementary Fig. S3). These data indicate that AD5-10 treatment induces apoptosis, consequently inhibiting the growth of ovarian tumor tissue.

DR4 is epigenetically silenced in the 2780CP8 cells in vitro (19). To determine whether the observed effects are due to DR5 and not to DR4, e.g., by demethylation of the DR4 promoter and reconstitution of DR4 expression in vivo, we analyzed the expression of DR4 in tumor tissues. Immunohistochemical and immunoblotting analysis showed no reconstitution of DR4 expression in vivo (Fig. 4C), indicating that TRAIL-induced apoptosis is only mediated by DR5. Furthermore, we analyzed the immunohistochemical expression of murine TRAIL using anti-mouse TRAIL antibody. Interestingly, we found murine TRAIL to be highly expressed in tumor tissues (Fig. 4C). Remarkably, selected human ovarian cancer cells do not express human TRAIL (Fig. 1A), and murine TRAIL was shown to have some cross-reactivity with human tumor cells (Fig. 5A; ref. 28). This finding might support our previous observation in humans that TRAIL is highly expressed in the tumor microenvironment of ovarian cancer patients (7). TRAIL is a key mediator of cytotoxic activity of activated NK cells and CD8+ lymphocytes (29) and is at least in part responsible for antimetastatic activities in vivo (30, 31). Immunohistochemical analysis revealed a dense infiltration of the tumor tissues with NK cells (Fig. 4C). Moreover, flow cytometric analysis for mouse TRAIL surface positivity of NK cells within the tumor showed that ~60% of NK cells express mouse cell-bound TRAIL (data not shown). Together, our data suggest that NK cells mediate cytotoxicity in ovarian cancer by TRAIL-induced cell death.

Figure 3. Combination of AD5-10 with carboplatin eradicates large, established tumors in a xenograft ovarian cancer mouse model. A, 28 nude/nude mice were inoculated s.c. with 1.5 × 10⁷ 2780CP8 ovarian cancer cell line into the left and right hind ventral flank (day 0). Random groups of seven mice each were then treated i.p. with either saline (control tumor) or AD5-10 (10 mg/kg) on days 2, 6, 9, 13, 16, and 20; carboplatin (100 mg/kg) on day 2; or a combination of AD5-10 and carboplatin at the respective doses and time as indicated above. Tumor volumes were measured every second day, and on day 14, all tumor-bearing mice were sacrificed. Each measurement in each group is the sum of both left and right tumor volumes per animal and is included in the box plots. Box plots depict median values and the interquartile range, whereas dotted lines depict mean values in each group. Statistically significant differences (*) between tumor volume and the control group. B, tumor weight (after 14 d) in the four different groups; the median is indicated by a horizontal line. Each measurement in each group is the sum of both left and right tumor weight per animal. C, representative mice and tumors of the indicated groups were assessed on day 14.

Natural Immunity Enhances Activity of AD5-10

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AD5-10 cooperates with NK cells to suppress tumor growth of established ovarian cancer in a xenograft mouse model

To gain more insight on the role of NK-mediated immunosurveillance mediated by TRAIL, we further explored the antitumor effect of AD5-10 in combination with mouse TRAIL. Combination of human or mouse TRAIL (at different concentrations) with AD5-10 enhances 2780CP8 cellular sensitivity to TRAIL-induced apoptosis in vitro (Supplementary Figs. S4A and S5A). Notably, AD5-10 antibody has been shown to bind to a different binding site than TRAIL (11). Therefore, AD5-10 does not compete with the TRAIL-binding site, which may explain a significantly increased rate of apoptosis observed after treatment with the combination of AD5-10 and mouse soluble TRAIL (1,000 ng/mL; Fig. 5A).

Finally, we determined whether NK cell-mediated cytotoxicity is involved in AD5-10-induced effects. To do so, we made use of NK cell–depleting antibodies. Four groups of nude mice were s.c. inoculated with 2780CP8 cells and were either left untreated or treated with AD5-10 and/or NK1.1- and anti-asialo–depleting antibodies (Supplementary Fig. S4B). Tumor development was observed for a period of 4 weeks. In NK cell–depleted mice, AD5-10 had only a minimal effect on tumor growth (Fig. 5B), whereas AD5-10 significantly reduced tumor volume in the presence of NK cells (Figs. 3A and 5B). This suggests that TRAIL expressed by active NK cells is required for AD5-10–induced apoptosis. This finding is in line with the fact that DR5 is more efficiently activated by secondary cross-linked trimers of TRAIL (32, 33). NK cell depletion alone had only a small effect on tumor growth, which might reflect the...
fact that 2780CP8 cells are resistant to TRAIL-induced apoptosis (expressed via NK cells). Moreover, we found that NK cell numbers correlate with the survival of the animal, with a high NK cell number being protective (Fig. 5C). In summary, these data suggest that the antitumor activity of AD5-10 depends on NK in ovarian cancer.

Discussion

The TRAIL pathway has been extensively studied in vitro and in vivo, and molecules targeting this pathway have become attractive candidates for anticancer treatment (10). Preclinical studies in mice provided the first evidence that the soluble form of recombinant TRAIL suppresses the growth of human tumor xenografts with no apparent systemic toxicity (34, 35). More recently, recombinant TRAIL has also entered clinical trials for the treatment of various malignancies (36). Although published phase 1 and phase 2 studies have indicated tolerated toxicity, the therapeutic efficiency is variable. In addition to the soluble ligand, several agonistic antibodies to the TRAIL functional receptors (DR4 or DR5) have been developed and entered into clinical trials in parallel (10). These agonistic antibodies may be more effective than the ligand at eradicating tumors for several reasons, one of them being the prolonged half-life time in vivo when compared with the recombinant proteins. Furthermore, the decoy receptors, which have been implicated in modulating the response to TRAIL, are not targeted by these ligands. Cross-linking therapeutic

Figure 5. Depletion of NK cells suppresses the tumoricidal effect of AD5-10 on tumor outgrowth. A, 2780CP8 ovarian cancer cell line was either untreated or treated with the specified concentration of mouse soluble TRAIL, AD5-10 (1 μg/mL), or both for 24 h, and the apoptosis rate was measured. Columns, mean of three independent experiments; bars, SEM. Statistically significant (*, P < 0.05) difference obtained by comparing the sum effect of AD5-10 and mouse soluble TRAIL to a combination of both. B, two groups of mice were depleted of NK cells by administration of polyclonal rabbit anti-asialo GM1 antibody at a dose of 20 μl i.p. every 4 d starting on day 3 and NK1.1 antibody at a dose of 100 μg i.p. every third day starting at day 3. A total of 1 × 10⁷ 2780CP8 cells were inoculated s.c. into the left and right dorsal sides of the mice. Mice were treated i.p. with AD5-10 or saline as indicated in Fig. 3A. Tumor volumes were measured every second day. Mice were sacrificed when tumor size (at least in one flank) reached the required volume (∼4,000 mm³). Each measurement in each group is the sum of both left and right tumor volumes per animal. Data are represented as indicated in Fig. 3A. C, correlation between NK cells (%) in depleted mice and life span.
antibodies might also overcome the resistance mechanisms to TRAIL-induced apoptosis observed in ovarian cancer (5, 13), according to the data in our study, which provides a novel link between NK-mediated immunosurveillance and activation of DR5-mediated apoptosis.

DR5 was identified in 1997 (23, 37), and several DR5 agonistic antibodies exhibit a potent antitumor effect against TRAIL-sensitive tumor cells but not against TRAIL-resistant tumor cells. These are currently in phase II evaluations in patients with advanced malignancies (38, 39). In the present study, we used a DR5 agonistic antibody AD5-10 against TRAIL-resistant ovarian cancer cells, and our results shed light on the postulated critical role of DR5 and NK-mediated immunosurveillance in ovarian cancer progression. As expected, we observed limited cytotoxic effects on ovarian cancer cells resistant to TRAIL-induced apoptosis in vitro upon stimulation with AD5-10 alone (Fig. 1B and C). We hypothesized that combined therapy with anti-DR5 and different pharmacologic anticancer agents might induce more than additive antitumor activity. Our results showed that out of a number of agents used in the standard treatment of ovarian cancer only the combination of AD5-10 with carboplatin displayed more than additive effect in vitro (Supplementary Fig. S1A and B). This phenotype stimulated our interest in the nature of this mechanism. Consequently, we determined the cytotoxic effect of AD5-10 alone or in combination with carboplatin in a platin-resistant ovarian cancer mouse model. Whereas there was, as expected, no effect of carboplatin alone, an effect of AD5-10 was observed. Importantly, the combination of AD5-10 with carboplatin eventually eliminated ovarian cancer (Fig. 3; Table 1) in a significant number of animals. Chemotherapeutic agents have been shown to cooperate with the TRAIL ligand to enhance apoptosis (40). However, the molecular basis behind such cooperation has not yet been elucidated. One mechanism is based on our observation that carboplatin cooperates with AD5-10 by upregulating DR5 expression regardless of the p53 status (Fig. 2A and B). As a consequence, this DR5 upregulation in the presence of AD5-10 leads to increased induction of caspase-dependent cell death (Fig. 1C, top), because the resulting apoptosis is completely blocked in the presence of a caspase-8 inhibitor (Fig. 1C, bottom). The observed apoptotic effect upon combination of AD5-10 and carboplatin seems to be independent from the DR5 modulator c-FLIP (28). It has been recently shown that a modified TRAIL ligand that specifically binds to DR5 has a more pronounced apoptotic effect dependent on cisplatin-induced DR5 expression than TRAIL in vitro as well as in an orthotopic bioluminescent ovarian cancer mouse model (41). Collectively, the described data support the importance of drug-induced DR5 upregulation in the enhanced sensitivity of ovarian cancer cells to TRAIL-induced apoptosis.

As postulated previously, the TRAIL pathway plays a key role in tumor surveillance (7, 29–31). Understanding the activity of a potential agent targeting this pathway requires putting a spotlight on the activity of the hosts’ immune system as reflected in the tumor microenvironment. Our previously published data support a key role of TRAIL and the functional TRAIL receptors in ovarian cancer, showing increased survival in patients expressing a high level of TRAIL in tissue adjacent to the tumor (7, 19). However, the origin of this expression has not been fully evaluated to date. In our current study we addressed the role of NK cell-mediated effects of DR5-induced apoptosis. We recorded that AD5-10 alone did not have a significant effect on apoptosis. However, it had a more than additive effect when combined with human or mouse soluble TRAIL in vitro to enhance TRAIL-mediated cytotoxicity (Supplementary Figs. S4A and S5A). Membrane-bound TRAIL is more active than soluble TRAIL in inducing apoptosis in tumor cells, which might explain the strong inhibitory effects we observed in mice treated with AD5-10 (Fig. 3; Table 1). Moreover, we observed that TRAIL expression and NK cells are abundant in the tumor microenvironment, and ~60% of tumor infiltrating NK cells in tumors expressed cell-bound TRAIL (Fig. 4C; data not shown). Furthermore, we determined that depletion of NK cells from mice bearing tumors led to increased tumor growth and also abolished the cytotoxic effect of AD5-10 (Fig. 5B and C). Taken together, these findings indicate that TRAIL expressed on active NK cells might cross-link AD5-10, suggesting that DR5 is more efficiently activated by secondary cross-linked trimers of soluble TRAIL than by non–cross-linked molecules (Fig. 5A; refs. 32, 33). Also, our above-mentioned observations might support a role for TRAIL in mediating NK immunosurveillance against ovarian-transformed cells in vivo. Nevertheless, further experiments are still needed to fully prove that TRAIL alone or in combination with other molecules like perforin and granzymes mediates spontaneous and activated NK-induced cytotoxicity against ovarian cancer (42, 43). Notably, AD5-10 antibody binds to a different binding site than TRAIL (11), and murine TRAIL was shown to have some cross-reactivity with human tumor cells (Fig. 5A; ref. 28). However, other mechanisms like upregulation of DR5 on ovarian cancer cells by carboplatin and targeting by AD5-10 efficiently activated cell killing without the need for secondary cross-linking (Fig. 1B and C). In fact, according to our data, forced expression of DR5 in the presence of TRAIL-expressing NK cells provides an ideal environment for highly efficient activation of DR5 by AD5-10. This fact is supported by eradication of implanted tumors in an NK-competent xenograft ovarian cancer mouse model (Fig. 3; Table 1) and by the abolished cytotoxic activity of AD5-10 in NK-depleted mice (Fig. 5B). This finding clearly confirms the importance of NK-mediated immunosurveillance in ovarian cancer in a preclinical mouse model. Agonistic DR5 antibodies probably act together with naturally occurring TRAIL.
ligand to overcome the resistance mechanisms that develop during tumorigenesis.

Our study highlights the lack of knowledge regarding the interplay between therapeutic agents, host immunity, and the evasion mechanisms developed by individual tumors. Only an improved understanding of these basic mechanisms will ultimately lead to tailored patient selection and the choice of combination partners for drugs targeting the TRAIL pathway. Carboplatin and AD5-10 seem to be a promising regimen for future clinical trials in plat-in-resistant ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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