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Identification of Interleukin 8 as an Inhibitor of Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis in the Ovarian Carcinoma Cell Line OVCAR3[1]

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is known to trigger apoptosis in many malignant cells. Whereas cancer cells are responsive to TRAIL-induced cell death when used alone or in combination with other agents, normal cells are known to be relatively less sensitive to the ligand, making it a desirable therapeutic compound to target a variety of cancers. TRAIL induces apoptosis through its interaction with its two proapoptotic death receptors (DRs), DR4 and DR5. In addition, it may also bind the decoy receptors (DcRs), DcR1 and DcR2, which lack an intracellular signaling domain, thus negatively regulating TRAIL-induced apoptosis. Previously, it has been shown that interleukin (IL)-8 is elevated in the ascites of patients with ovarian cancer. Therefore, we examined the role that IL-8 may play in modulating sensitivity to TRAIL-mediated apoptosis. We treated the TRAIL-sensitive cell line OVCAR3 with TRAIL over a period of time with or without pretreatment with IL-8. Here we show the novel findings that IL-8 blocks TRAIL-induced cell death and was able to turn the TRAIL-sensitive cell line into a TRAIL-resistant one. We hypothesized that decreased expression of DRs DR4 and DR5 may contribute to TRAIL resistance. Both reverse transcription-PCR and flow cytometry revealed a decrease in DR4 expression after pretreatment of OVCAR3 cells with IL-8. We have also shown that TRAIL was able to induce caspase-8 cleavage in these cells, whereas pretreatment with IL-8 blocked this caspase cleavage. Through array analysis and confirmation with other techniques, we have determined that IL-8 regulates the expression of a member of the mitogen-activated protein kinase superfamily, p38γ. These findings provide important insights into the modulation of apoptosis by TRAIL and IL-8 in ovarian cancer. The data suggest a potentially important role of IL-8 in protecting ovarian cancer cells from TRAIL-mediated apoptosis and signify a new potential chemotherapeutic target to augment TRAIL therapy.

INTRODUCTION

Ovarian cancer is the most fatal gynecological malignancy and is the fourth leading cause of cancer-related deaths in the United States (1). The American Cancer Society estimates as many as 23,000 new cases and 14,000 deaths from ovarian cancer in the United States each year (2). About 90% of ovarian cancer originates as a result of malignant transformation of the ovarian surface epithelium and is often diagnosed only after the disease has reached an advanced stage (3). The advanced stage of ovarian cancer is characterized by metastases outside of the pelvic region to the peritoneum or even to tissues beyond the peritoneum. Currently, common treatment for ovarian cancer includes surgery followed by chemotherapy. There are high response rates (60–80%) after treatment; however, most of these responsive patients develop resistance to the chemotherapeutic drugs to which they are exposed (4). This multidrug resistance is a major clinical problem in ovarian cancer, thus requiring new strategies to combat the disease (5–6).

The TRAIL[3] or Apo2 ligand is a member of the tumor necrosis factor superfamily of cell death-inducing ligands (7). One of the most remarkable features of TRAIL is its ability to induce programmed cell death or apoptosis in a variety of tumor cell lines but not typically in normal or nontransformed cells (8). Also, animal studies have demonstrated that TRAIL can induce regression of cancer xenografts without detrimental effects to normal cells (9). These features make TRAIL a desirable therapeutic agent to fight cancer. TRAIL induces apoptosis in malignant cells by interacting with the DRs DR4 and DR5. These receptors induce the apoptotic signal through interaction of their intracellular death domains with adaptor proteins. This event leads to the proteolytic activation of caspases and initiation of a proteolytic cascade that ultimately results in the demise of the cell (10). Two other cell surface TRAIL receptors, DcR1 and DcR2, lack a functional death domain and compete with DR4 and DR5 for cell surface TRAIL binding and cannot transmit the apoptotic signal, therefore they are commonly known as the DcRs (11). Activation of the DR pathways is thought to be a possible new method of treatment for cancer. It has been reported that the combination of chemotherapy and TRAIL could induce apoptosis in some chemoresistant and TRAIL-resistant ovarian cancer cells (12).

Patients with ovarian cancer produce large amounts of ascitic fluid. The ascites fluid contains many growth factors (13, 14) and provides an excellent environment conducive to the growth of ovarian cancer cells (15–17). The concentrations of various cytokines have been examined in the ascites from patients with ovarian cancer. Compared with patients with benign gynecological disorders, ascites of patients with ovarian cancer showed significantly higher levels of IL-8 (18). IL-8, a member of the C-X-C chemokine family, has been identified as a potent chemoattractant for T cells, neutrophils, and basophils (19–21). Studies have demonstrated that IL-8 is a potent proangiogenic and proinflammatory factor implicated in tumor growth and metastasis (22–27).

Because the biological function of IL-8 in ovarian tumors is not yet entirely evident, the purpose of this study was to determine the effects of IL-8 on TRAIL-induced apoptosis of the surface epithelium ovarian cancer cell line OVCAR3. The rationale for using OVCAR3 cells was that it has previously been determined that this cell line is sensitive to TRAIL-induced apoptosis (12). We therefore sought to determine the viability of these cells after treatment with IL-8 and TRAIL over time. Several reports have proposed that differential expression of TRAIL receptors may establish a cell's sensitivity to TRAIL (28). We hypothesized that decreased expression of the

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[3] The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MAPK, mitogen-activated protein kinase; IL, interleukin; DcR, decoy receptor; DR, death receptor; RT-PCR, reverse transcription-PCR; ERK, extracellular signal-regulated kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; NFDM, nonfat dry milk; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SAPK, stress-activated protein kinase; AB, antibody.

TRAIL DRs may contribute to prolonged survival of OVCAR3 cells after IL-8 pretreatment. To test this, we evaluated the regulation of TRAIL receptor protein and mRNA expression in OVCAR3 cells after IL-8 treatment. We provide evidence here that IL-8 is capable of blocking TRAIL-mediated apoptosis through the regulation of TRAIL receptor expression.

We also provide evidence for possible regulation of other apoptotic mechanisms due to IL-8 and TRAIL treatments of this system. With the goal of identifying some of the molecular mechanisms that may be involved in TRAIL-mediated apoptosis and the role of IL-8 in blocking this apoptosis, we performed an array analysis. We discovered that a member of the MAPK superfamily, *p38γ*, was among the genes regulated in this system by TRAIL and IL-8. There are five isoforms of the p38 group of MAPKs that have been identified to date: p38α; p38β1; p38β2; p38γ; and p38δ (29–33). Many of the cellular controls are regulated by a complex system of interactive networks or signal transduction pathways, such as the MAPKs. Two other important members of the human MAPK superfamily, other than p38, are the ERK and c-Jun NH_2-terminal kinase (34). Diverse extracellular stimuli such as physiological stress, lipopolysaccharide, osmotic stress, UV exposure, and proinflammatory cytokines such as tumor necrosis factor α have been shown to trigger the stress-regulated protein kinase cascade, p38 MAPK pathway (35–39). The importance of this information led us to also study whether there was any significant evidence for regulation of the p38 MAPK member after IL-8 treatment.

MATERIALS AND METHODS

Cell Culture. The surface epithelial ovarian cancer cell line OVCAR3 was maintained in a humidified 5.0% CO_2, 37°C incubator in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 mg/ml streptomycin sulfate, and 1% L-glutamine. All tissue culture reagents were obtained from Invitrogen (Gaithersburg, MD).

***In Vitro* Viability and TUNEL Assay.** OVCAR3 cells were cultured in 6-well plates at a concentration of 1×10^6 cells/ml and allowed to adhere to the plate overnight. The cells were incubated with either TRAIL (1 μg/ml; Biomol, Plymouth Meeting, PA) or IL-8 (10 ng/ml; R&D Systems, Minneapolis, MN) for 16 h or pretreated with IL-8 for 16 h before the addition of the TRAIL. All of the experiments performed throughout this paper used the same concentrations of IL-8 and TRAIL stated above. Each treatment was conducted at 37°C for 6, 24, or 48 h. The cells were trypsinized and washed with 1× PBS, and 5×10^5 cells were mounted on the slides using a Cytospin centrifuge. Cells were fixed with 4.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature and permeabilized for 2 min at 0°C with 0.1% Triton X-100 in 0.1% sodium citrate. The slides were incubated with the TUNEL reagent (Roche, Indianapolis, IN) for 1 h at 37°C and counterstained with 0.05 mg/ml propidium iodide in PBS. The cells were mounted in Vectashield (Vector Laboratories) and examined using confocal laser scanning microscopy.

All viability data for each time point are based on the evaluation of 200 cells/slide in triplicate for each experimental condition. Results of multiple experiments are given as the mean ± SE.

SDS-PAGE and Western Blotting. Total protein was extracted by lysing the cells in 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM sodium vanadate, and 0.1% NP40. Cell debris was removed by centrifugation at $8000 \times g$ for 5 min, and protein concentration was determined by a Bradford assay. The cellular proteins (50 μg/lane) were resolved by 10% SDS-PAGE and transferred to nitrocellulose. The membranes were blocked overnight at 4°C in 10% NFDM/PBS/0.1% Tween 20 and incubated for 1 h with either primary monoclonal AB or primary polyclonal AB diluted in 5% NFDM/PBS/0.1% Tween 20. The membranes were then incubated for 1 h with either antimouse or antirabbit AB (diluted 1:2500 in 5% NFDM/PBS/0.1% Tween 20). Proteins were detected using enhanced chemiluminescence. Equal protein loading was confirmed by tubulin detection. The primary ABs used were caspase-8 (BD Biosciences, San Diego, CA), caspase-3 (Cell Signaling Technology), and p38γ (Upstate Biotechnology, Lake Placid, NY).

Semiquantitative RT-PCR Assays. RNA was isolated from whole cell extracts of OVCAR3 cells after treatments using the procedure of the Qiagen RNeasy kit (Qiagen, Valencia, CA.). One-step RT-PCR was performed on 3 μg of RNA using the Clontech Titanium One-Step RT-PCR kit (Clontech, Palo Alto, CA) protocol. Samples were amplified simultaneously for *GAPDH* and *DR4, DR5, DcR1, DcR2,* or *ERK6/p38γ/SAPK3* with the following primers: F-GAPDH, 5′-CCACCCATGGCAAATTCCATGGCA-3′; R-GAPDH, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′; F-DR4, 5′-TGCAACCATCAAACTTCA-3′; R-DR4, 5′-GATGTTTGCAAACTTGTC-3′; F-DR5, 5′-TGAGTCTGCTGCTCTGATCAC-3′; R-DR5, 5′-CAAGTCTGCAAAGTCATC-3′; F-DcR1, 5′-ATGCAAGGGGTGAAGG-3′; R-DcR1, 5′-GAGGTAATGAGAAGAGGC-3′; F-DcR2, 5′-GAAGGACATGAACGCCGCCGAAAAG-3′; R-DcR2, 5′-CACTACCTTATCATCATAGTGGTTTT-3′; F-ERK6/p38γ/SAPK3, 5′-ATGAGCTCTCCGCCGCCCACCCGCAGT-3′; and R-ERK6/p38γ/SAPK3, 5′-CCCAGTCATCACACTGACTGCCTGCCTGCC-3′. The cycling conditions for PCR consisted of the following: 94°C for 1 min; 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and extension at 72°C for 2 min. Ten percent of the PCR products were analyzed on 1.5% agarose gels containing ethidium bromide (0.1 μg/ml). The fold induction for each of the TRAIL receptors after treatment was determined using the Kodak 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY), and levels were normalized for GAPDH expression.

Northern Blot Analyses. RNA was isolated from whole cell extracts of OVCAR3 cells after treatments using the procedure of the Qiagen RNeasy kit (Qiagen). Our procedure for Northern analysis has been described elsewhere (40). Blots were probed with cDNA probes prepared using the TRAIL receptor primers and GAPDH primers listed above, labeled by the random primer method.

Analysis of Surface TRAIL Receptors by Flow Cytometry. Clones were analyzed for the surface expression of TRAIL receptors DR4, DR5, DcR1, and DcR2 by indirect staining with primary mouse antihuman TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), and TRAIL-R4 (DcR2) IgG (all obtained from Alexis Biochemicals, San Diego, CA) followed by phycoerythrin-conjugated rabbit antigoat IgG secondary AB (Sigma). OVCAR3 cells were treated with IL-8, TRAIL, or IL-8 + TRAIL for 24 or 48 h. The cells were then harvested after treatments, and staining for cell surface receptors was performed on 1×10^6 cells. Cells were resuspended in 10 μg of primary AB prediluted in 5% human plasma. After incubation with the primary AB, cells were washed with 0.1% gelatin/PBS and incubated with a secondary AB. Cells were then fixed in 10% buffered formalin and analyzed in 1% buffered formalin. The experiments were performed in duplicates, and mean values were calculated. Analysis was performed on a Beckman Coulter XL Analyzer.

Expression Analysis of Gene Regulation in OVCAR3 Cells. The Atlas cDNA Apoptosis Expression Array (Clontech) was performed following the manufacturer's protocol. A brief overview of the procedures used is as follows. The cells were incubated with either TRAIL or IL-8 for 16 h or pretreated with IL-8 for 16 h before the addition of the TRAIL. After 16 h of treatment, cells were harvested, and RNA was collected from each group. Probe mixtures were then synthesized by reverse transcribing each RNA population using the CDS Primer Mix specific for the Atlas Apoptosis Array and labeling with α-^{32}P. Each of the radioactively labeled probe mixes prepared was then hybridized to a separate Atlas Array. The hybridization pattern after autoradiography was analyzed to determine changes in gene expression levels using Atlas Image 2.1 software (Clontech). Only differences of ≥5 in total intensity and fold change of intensity of ≥1.5 between two of the membranes were considered significant.

RESULTS

Effect of TRAIL and IL-8 on Ovarian Cancer Cell Viability *in Vitro*. Apoptosis is recognized as playing a pivotal role in the regulation of tumorigenesis; however, the factors triggering apoptosis or survival in ovarian cancer remain unclear. IL-8 is present in elevated levels in ascites fluid of ovarian cancer patients; therefore, we examined the role that IL-8 may play in modulating survival of ovarian cancer cells *in vitro*. We conducted initial studies to examine the effects of TRAIL and IL-8 on cell viability of the surface ovarian epithelial cancer cell line OVCAR3 (Fig. 1). We show the kinetics of cell survival after treatment of cells with TRAIL (1 μg/ml) and/or

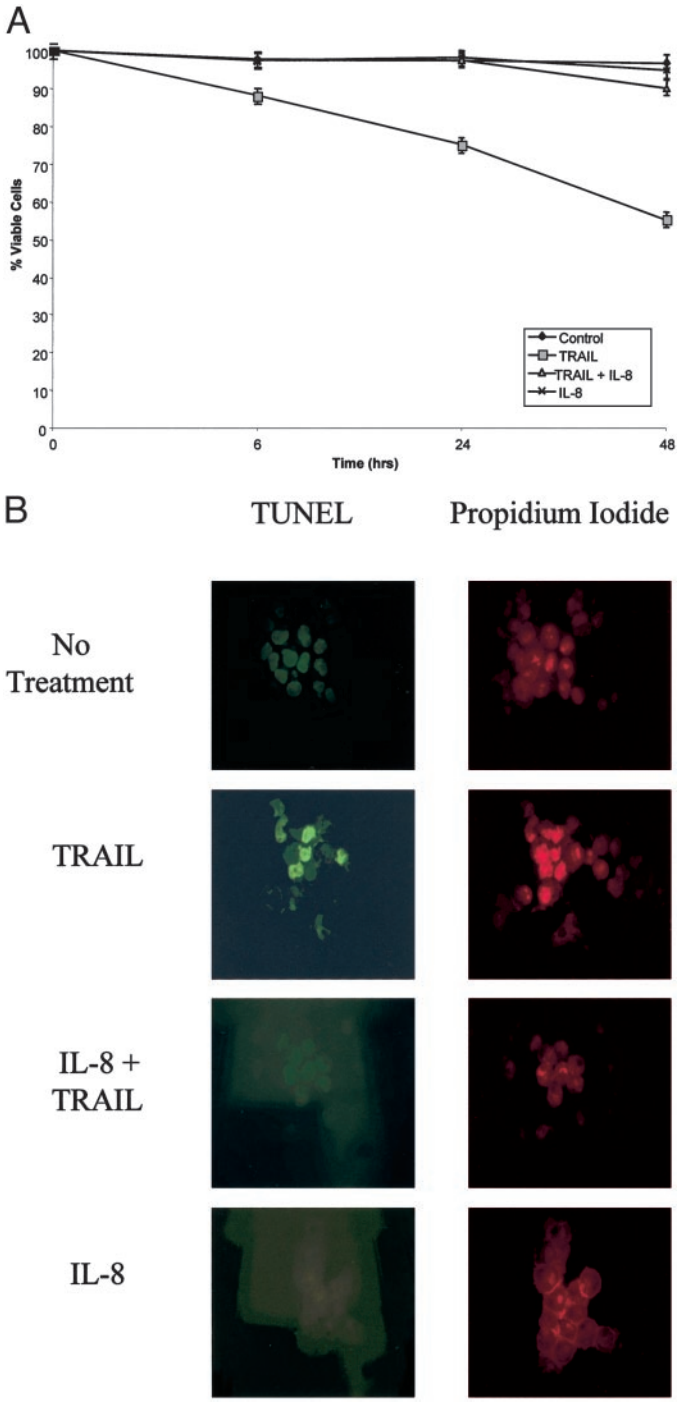

Fig. 1. IL-8 blocks TRAIL-induced apoptosis of OVCAR3 cells. *A*, survival curve based on TUNEL assay was generated for OVCAR3 cell cultures for a 48-h time course with recombinant TRAIL (1 μg/ml) and/or IL-8 (10 ng/ml). Data are mean ± SE; $n = 3$. *B*, representative TUNEL assay for 48-h treatment. Cells were processed using the TUNEL assay and counterstained with propidium iodide. TRAIL was able to induce apoptosis in these cells; however, IL-8 blocked this apoptosis from occurring.

IL-8 (10 ng/ml; Fig. 1). OVCAR3 cells treated with TRAIL exhibited ~55% cell death within 48 h (Fig. 1*A*). In contrast, IL-8 treatment was able to significantly block the TRAIL-mediated cell death. The cells coincubated with TRAIL + IL-8 showed only ~9% cell death within 48 h of the treatment, similar to the control and the IL-8 alone treatments (6% and 8%, respectively; Fig. 1*A*). Taken together, these findings demonstrate that the presence of the IL-8 was able to dramatically decrease TRAIL-mediated cell death.

To confirm that the modulation of cell death by TRAIL was apoptotic, we evaluated the cells for morphological changes (Fig. 1*B*). The TRAIL-induced apoptosis was assessed in OVCAR3 cells by examining cellular DNA for nuclear fragmentation and internucleosomal cleavage. The cells stimulated with TRAIL, IL-8 + TRAIL, or IL-8 alone were subjected to *in situ* labeling of the DNA by terminal deoxynucleotidyl transferase using the TUNEL assay. As shown, cells treated over time with TRAIL exhibited the typical morphological changes associated with apoptosis, such as shrinkage and nuclear blebbing, compared with untreated cells after 48 h of treatment (Fig. 1*B*). In addition, their nuclei exhibited fluorescence, indicating the presence of the labeled 3′-OH ends characteristic of apoptotic cells. Within 24 h (data not shown) and 48 h, the OVCAR3 cells undergo apoptosis when treated with TRAIL (Fig. 1*B*). In contrast, the survival of these cells is markedly prolonged by IL-8 (Fig. 1*B*).

Regulation of TRAIL Receptors DR4, DR5, DcR1, and DcR2 by TRAIL and IL-8 Treatments. We hypothesized that regulation of TRAIL receptor expression may be involved in the ability of IL-8 to make the previously TRAIL-sensitive cells resistant. OVCAR3 cells were treated with TRAIL, IL-8, or IL-8 + TRAIL, as specified in "Materials and Methods," for a period of 6, 24, or 48 h. The cells were harvested after the treatments, and RNA was prepared from the whole cell lysates and analyzed by RT-PCR for the TRAIL receptors. When the OVCAR3 cells were treated for 6 h, we saw no differences in DR4 expression. However, we observed that after IL-8 treatment for 24 h, expression levels of DR4 were reduced (~6.5 fold) compared with cells that were not treated with IL-8, even in the presence of TRAIL (Fig. 2*A*). Similar results (DR4 reduced ~7-fold) were observed for these cells when they were treated over 48 h with IL-8. DR5 expression increased upon 6 h of TRAIL treatment (1.2-fold) and TRAIL + IL-8 treatment (1.6-fold). For 24-h treatment, there was no observed change in DR5 expression. When the OVCAR3 cells were treated for 48 h, DR5 expression increased 2.0-fold upon treatment with TRAIL alone, 2.5-fold upon treatment with TRAIL + IL-8, and 2.2-fold upon treatment with IL-8 alone.

We also observed variable expression for the DcRs with different treatments. The expression levels for DcR1 went up 2.8-fold and 2.0-fold when cells were treated with TRAIL alone for 6 and 24 h, respectively. Interestingly, after 48 h of treatment with IL-8 + TRAIL or IL-8 alone, the expression of DcR1 was down ~5-fold. There were minimal changes in DcR2 expression over 24 h of treatment. However, after 48 h of treatment with IL-8, even in the presence of TRAIL, we observed ~2-fold decrease in expression.

We went on further to confirm the RT-PCR results through the use of Northern blot analyses (Fig. 2*B*). The OVCAR3 cells were treated as described previously for 24 and 48 h. Northern blot analyses were performed on the TRAIL receptors and GAPDH as described in "Materials and Methods." The results were able to confirm the effects of IL-8 on TRAIL receptor expression in OVCAR3 cells. These results suggest that the TRAIL receptors may play a significant role in the ability of IL-8 to block TRAIL-mediated apoptosis.

Flow Cytometry Analysis of Surface TRAIL Receptors after IL-8 and TRAIL Treatments. To further substantiate the role of TRAIL receptor regulation by IL-8, we examined the receptor profile on the cell surface by flow cytometry. The ovarian cancer cells were treated for 48 h with TRAIL, IL-8, or IL-8 + TRAIL to determine the surface protein expression levels of the specific TRAIL receptors (Fig. 3). Surface expression of DR4 was decreased by approximately 60% when cells were stimulated with IL-8 for 24 and 48 h of treatment, even when TRAIL was included in the treatment with IL-8 (Fig. 3, *A* and *B*). IL-8 also reduced surface expression of DcR1 approximately 50% and 32% after 24 and 48 h of treatment, respectively, and reduced surface expression of DcR2 approximately 20%

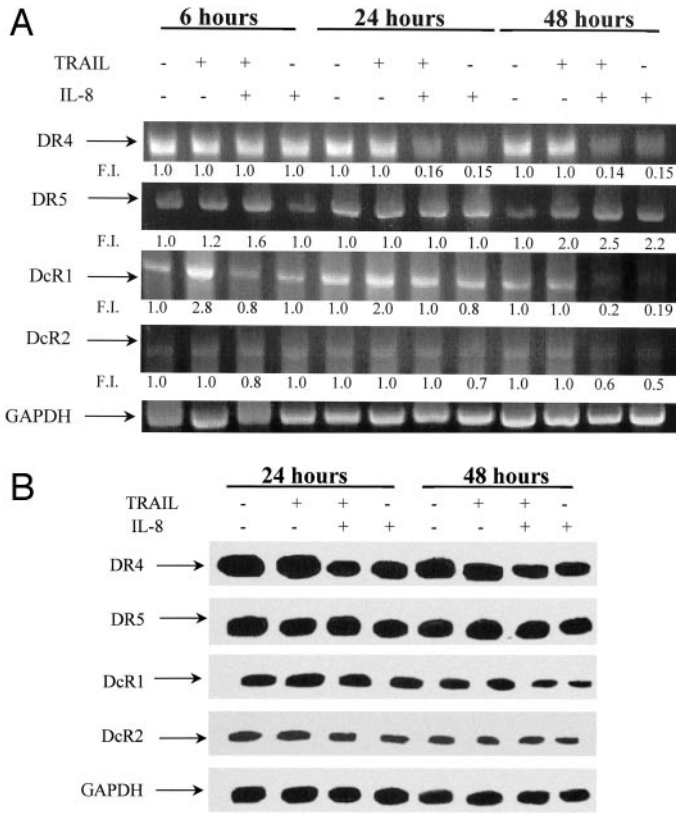

Fig. 2. TRAIL receptor expression of OVCAR3 cells is modulated by IL-8 and TRAIL. *A*, cells were treated with TRAIL (1 μg/ml) and/or IL-8 (10 ng/ml) for 6, 24, and 48 h. The RNA was collected, and RT-PCR was performed. The fold induction was measured with Kodak 1D Image Analysis Software and normalized for GAPDH expression levels to compare expression levels due to treatment with those of the control. Variable regulation of TRAIL receptors was observed after TRAIL and IL-8 treatments. *B*, Northern analysis of OVCAR3 cells exposed to TRAIL and/or IL-8. OVCAR3 cells were treated with TRAIL (1 μg/ml) and/or IL-8 (10 ng/ml) for 24 and 48 h. The RNA was collected and detected by Northern blot analysis using ^{32}P-labeled GAPDH or TRAIL receptor fragments of the cDNA as described in "Materials and Methods." The data shown are representative of three separate experiments.

and 72% at 24 and 48 h of treatment, respectively (Fig. 3, *A* and *B*). There was only an approximately 5% decrease in DR5 surface expression when cells were stimulated with IL-8 (Fig. 3, *A* and *B*). These data confirm that the receptor protein expression, as well as the RNA levels, was modulated by IL-8 treatments of these cells. These data further confirm that regulation of the TRAIL receptor expression may be involved in the ability of IL-8 to make the previously TRAIL-sensitive cells resistant.

IL-8 Treatment Prevents TRAIL-induced Caspase Activation in OVCAR3 Cells. A variety of death commitment signals in mammalian cells converge to activate the caspase cascade. Caspases are cysteine proteases that cleave proteins at aspartic acid residues contained within a tetrapeptide recognition motif (41). These enzymes are produced as inactive proteins and are activated during apoptosis. Apoptotic signals, such as TRAIL, can activate upstream caspases, such as caspase-8, which in turn activate the downstream caspases, such as caspase-3 (42). They are divided into two classes, effector (downstream) caspases and initiator (upstream) caspases; for example, caspase-8 is an initiator caspase, and caspase-3 is an effector caspase. When activated, effector caspases assist in carrying out the processes of cell death, either by directly cleaving proteins or by activating other degradation enzymes. The initiator caspases transmit apoptotic signals by promoting the cleavage and activation of the effector caspases. To assess the role that caspases play in OVCAR3 apoptosis, the cells were cultured first in the presence or absence of IL-8 for 16 h, followed by the addition of TRAIL and/or IL-8 for another 16 h, and whole cell extracts were examined by Western blot analysis (Fig. 4). Activation of the proform of apical caspase-8 and effector caspase-3 was observed after TRAIL treatment of OVCAR3 cells, as indicated by the proteolytic processing of the two caspases (Fig. 4). In contrast, cotreatment with IL-8 + TRAIL inhibits proteolysis of caspase-8 and caspase-3 (Fig. 4). These data indicate that IL-8 blocks the TRAIL apoptotic signaling pathways through a mechanism that involves inhibition of caspase activation. Therefore, we observed that after stimulation with IL-8, OVCAR3 cells are rendered resistant to TRAIL-induced apoptosis.

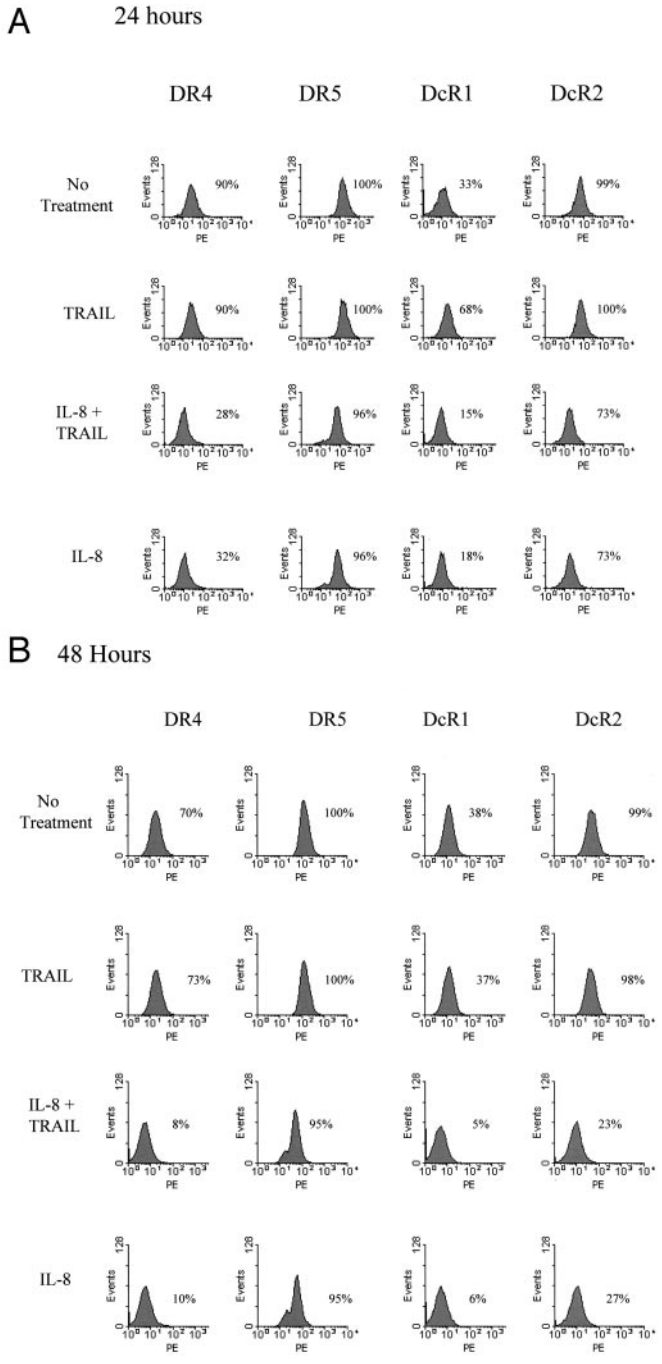

Fig. 3. Flow cytometry analysis of TRAIL receptor protein levels after treatments. Analysis of cell surface expression of DR4, DR5, DcR1, and DcR2 by flow cytometry as described in "Materials and Methods." The results are shown in fluorescence histograms representing receptor expression and isotype IgG control after treatments of (*A*) 24 and (*B*) 48 h. These results revealed confirmation of earlier RT-PCR results.

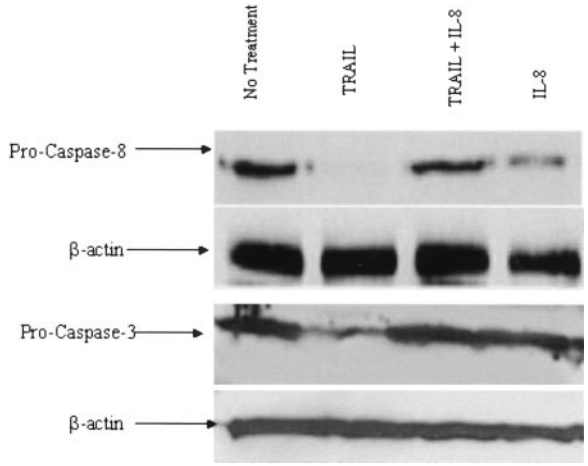

Fig. 4. Activation of endogenous caspases during TRAIL-induced apoptosis. OVCAR3 cells were treated with TRAIL (1 μg/ml) and/or IL-8 (10 ng/ml) for 16 h. Samples containing whole cell extracts were subjected to SDS-PAGE followed by immunoblotting with caspase-8 or caspase-3 AB. TRAIL activated endogenous caspase-8 and caspase-3; however, IL-8 blocked this activation. β-Actin was used as the loading control.

TRAIL and IL-8 Regulation of p38γ Expression. To further identify the molecular mechanisms involved in OVCAR3 cell survival and apoptosis, we used the Human Apoptosis cDNA Expression Array (Clontech). One of the genes found to be regulated by the treatments was p38γ. RT-PCR was performed on the RNA isolated from the 16-h treatments using primers for p38γ and GAPDH as a loading control (Fig. 5*A*). The results indicated that p38γ expression was decreased with IL-8 pretreatment and that it was slightly increased with TRAIL treatment. Western blot was also performed on p38γ to identify whether the results from the message were translated to protein; actin was used as the loading control (Fig. 5*B*). We found that 24 h after treatment periods, the p38γ protein levels were decreased when OVCAR3 cells were pretreated with IL-8 (Fig. 5*B*). These findings suggest that the role of IL-8 in this system may be mediated through the regulation of p38γ or other isoforms.

DISCUSSION

This study was performed to evaluate the effects of IL-8 on TRAIL-induced apoptosis in ovarian carcinoma cells. IL-8 has previously been shown to be important in the growth and metastasis of tumors (22–27). We show for the first time that IL-8 stimulation of OVCAR3 cells successfully protects these cells from TRAIL-induced apoptosis. The TUNEL assays demonstrated that TRAIL is able to induce apoptosis in OVCAR3 cells, but the effect can be reversed by IL-8. However, it was not clear through which mechanisms IL-8 achieves this protection. To determine possible mechanisms by which IL-8 promotes survival of these cells, we first began examining the expression of the TRAIL receptors. Through the use of RT-PCR (Fig. 2), as well as flow cytometry (Fig. 3), we have shown that IL-8 pretreatment of these cells results in down-regulation of the DR4 TRAIL receptor, suggesting a possible involvement of this receptor in blocking TRAIL-induced apoptosis. TRAIL DR activation transmits a death signal to the cell. The downstream effectors of these signals have not yet been completely identified; however, it is known that one mechanism by which TRAIL receptors induce cell death is through caspase activation. Although the DcRs also seem to be modulated by IL-8, studies of cancer cell lines and tumors have not revealed correlations between DcR expression and TRAIL resistance (43). Using Western blot analyses, we have shown that caspase activation occurs in OVCAR3 cells when they undergo treatment with TRAIL. We have also observed that TRAIL-induced caspase activation in these cells is blocked when they are treated with IL-8, even in the presence of the TRAIL.

To further determine the molecular mechanisms that may be involved in the ability of IL-8 to block TRAIL-induced apoptosis in this system, we performed the array analysis. The results of the array suggested that p38γ expression in OVCAR3 cells is modulated by treatment with cytokines. We determined from the array that p38γ expression in OVCAR3 cells could be down-regulated by treatment with IL-8. We were able to confirm that p38γ expression was down-regulated by IL-8 treatment using RT-PCR. We were also able to further confirm that protein expression of p38γ is down-regulated by treatment of cells with IL-8 through use of Western blot analysis. At this point, we have been able to demonstrate that one of the five p38 MAPK isoforms, p38γ, is regulated in OVCAR3 cells by TRAIL and IL-8 treatments. Studies have shown that certain cellular stresses, such as hypoxia, can specifically activate p38γ (44). It has also been shown that p38γ is activated by γ irradiation and that it is necessary for G_2 arrest after DNA damage due to the irradiation (45). Lee *et al.* (46) have found, in human adenocarcinoma HeLa cells, that after TRAIL treatment there was an accumulation of reactive oxygen species, which in turn activated p38, which subsequently activated caspases. Our results suggest that p38γ may be involved in TRAIL-induced apoptosis in these cells and that the ability of IL-8 to overcome this apoptosis may be dependent on regulation of p38γ. Additional studies are necessary to clarify the role of p38γ in TRAIL-induced apoptosis of OVCAR3 cells as well as the ability of IL-8 to inhibit this apoptosis.

In summary, this work demonstrates that pretreatment with IL-8 can desensitize OVCAR3 cells to TRAIL-induced apoptosis. TRAIL has been proposed as therapy against some cancers due to its ability to effectively kill the malignant cells effectively, without known detrimental effects to normal cells (47). Given that IL-8 has been shown to be involved in tumor progression and to be present at high levels in ascites of ovarian cancer patients, these malignancies might be further resistant to the use of TRAIL as a therapeutic. Conse-

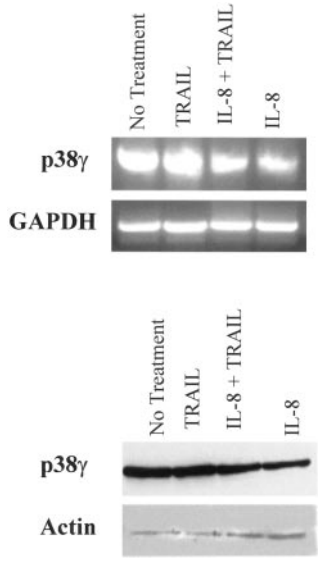

Fig. 5. Regulation of p38γ expression in OVCAR3 cells by IL-8 treatment. OVCAR3 cells were exposed to TRAIL (1 μg/ml) and/or IL-8 (1 ng/ml). *A*, the cells were treated for 16 h, after which they were lysed, and expression of p38γ was determined by RT-PCR. *B*, OVCAR3 cells were treated for 24 h as described previously. The cell lysates were obtained as described above, but total protein was analyzed through Western blot with anti-p38γ AB. Decreased expression of p38γ was observed after IL-8 treatments of OVCAR3 cells.

quently, inhibition of the antiapoptotic role of IL-8, combined with the proapoptotic properties of TRAIL, may provide an effective regimen for inducing apoptosis and eventual use as treatment for ovarian carcinomas. Although TRAIL has been suggested as a prospective therapeutic agent for many malignant tumors, our results suggest that the role that IL-8 may play in blocking the therapeutic capabilities of TRAIL may pose a serious problem for the use of TRAIL to treat ovarian carcinomas. Therefore, it is of considerable interest to identify and characterize the mechanisms that are regulated in response to IL-8 and TRAIL in ovarian cancer. Additional studies to define mechanisms underlying these processes will be crucial in our understanding of these events in carcinogenesis.

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