E1A Sensitizes Cancer Cells to TRAIL-Induced Apoptosis through Enhancement of Caspase Activation

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) has been shown to induce apoptosis of cancer cells. Sensitization of cancer cells to TRAIL, particularly TRAIL-resistant cancer cells, could improve the effectiveness of TRAIL as an anticancer agent. The adenovirus type 5 E1A that associates with anticancer activities including sensitization to apoptosis induced by tumor necrosis factor is currently being tested in clinical trials. In this study, we investigated the sensitivity to TRAIL in the E1A transfectants ip1-E1A2 and 231-E1A cells and the parental TRAIL-resistant human ovarian cancer SKOV3.ip1 and TRAIL-sensitive human breast cancer MDA-MB-231 cells. The results indicated that the percentage of TRAIL-induced apoptotic cells was significantly higher in the E1A transfectants of both cell lines than it was in the parental cell lines. To further investigate the cellular mechanism of this effect, we found that E1A enhances TRAIL-induced activation of caspase-8, caspase-9, and caspase-3. Inhibition of caspase-3 activity by a specific inhibitor, Z-DEVD-fmk, abolished TRAIL-induced apoptosis. In addition, E1A enhanced TRAIL expression in ip1-E1A2 cells, but not in 231-E1A cells, and the anti-TRAIL neutralizing antibody N2B2 blocked the E1A-mediated bystander effect *in vitro*. Taken together, these results suggest that E1A sensitizes both TRAIL-sensitive and TRAIL-resistant cancer cells to TRAIL-induced apoptosis, which occurs through the enhancement of caspase activation; activation of caspase-3 is required for TRAIL-induced apoptosis; and E1A-induced TRAIL expression is involved in the E1A-mediated bystander effect. Combination of E1A and TRAIL could be an effective treatment for cancer. (Mol Cancer Res 2005;3(4):219–26)

Introduction

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), also known as Apo-2L, belongs to the tumor necrosis factor family and is distributed in a wide range of tissue types (1, 2). It has been shown to induce apoptosis in a broad range of cancer cell types but not in normal cells and tissues, suggesting that it could be a potential therapeutic agent for the treatment of cancer (3-5). TRAIL suppresses the growth of human tumors inoculated into mice and improves the survival rate of tumor-bearing mice (3, 4). Gene therapy of xenografted human colon cancer with TRAIL inhibits tumor growth and lacks toxicity to normal tissues. The *TRAIL* gene transferred *in vitro* induces apoptosis and bystander effect in cancer cells (6). The combination of TRAIL and chemotherapeutic drugs or ionizing radiation also substantially increases killing of cancer cells (7-9). Inactivation of TRAIL increases tumor development, liver metastases, and the mortality of tumor-bearing mice (10-13). Certain cancer cell lines are known to be resistant (such as SKOV3) or sensitive (MDA-MB-231) to TRAIL-induced apoptosis. However, the mechanism responsible for the resistant or sensitive phenotype is not yet clear (4, 14).

TRAIL initiates death signaling through association with the death receptors DR4 (TRAIL-R1 and APO-2A) and DR5 (TRAIL-R2, KILLER, and TRICK2; refs. 15, 16). DR4 and DR5 contain a cytoplasmic death domain that is required for recruitment of the adaptor molecule Fas-associated death domain/MORT1 and the apoptosis initiator caspase-8 (17, 18). Activation of caspase-8 induces mitochondrial-dependent apoptotic pathway including Bid cleavage, Bax and Bak dimerization, and cytochrome *c* release (19-21). In the presence of dATP/ATP, cytochrome *c* associates with apoptotic protease activating factor 1 (Apaf-1) and recruits caspase-9 to form a complex known as the "apoptosome," which subsequently activates caspase-9 (22). Two additional TRAIL receptors, DcR1 (TRAIL-R3, LIT, and TRID) and DcR2 (TRAIL-R4 and TRUNDD), are designated as decoy receptors because they lack the functional death domain and cannot mediate death signaling on ligation. Like DR4 and DR5, DcR1 and DcR2 are also distributed in a variety of tissue types (23, 24). The adenovirus

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type 5 E1A associates with anticancer activity through multiple molecular mechanisms including down-regulation of receptor tyrosine kinases, human epidermal growth factor receptor 2, Axl, and epidermal growth factor receptor; down-regulation/inactivation of signal transducers, Akt, FLICE-like inhibitory protein, and nuclear factor κB; and up-regulation of cell cycle inhibitor p21 (25-35). The E1A transfectants induce bystander effect by killing the target cancer cells (36). Routes et al. (37) reported that expression of *E1A* gene products enhances TRAIL-dependent killing in melanoma and fibrosarcoma cell lines. However, the mechanism of E1A-enhanced TRAIL-induced apoptosis has not been addressed. In this study, we evaluated the effect of E1A on TRAIL-induced apoptosis in breast and ovarian cancer cells and the mechanism of E1A sensitization of TRAIL-induced apoptosis.

Results

E1A Sensitization of Human Ovarian Cancer Cells and Breast Cancer Cells to TRAIL-Induced Apoptosis

To assess the effect of E1A on TRAIL-induced apoptosis, we treated the ovarian cancer cell line SKOV3.ip1 and the breast cancer cell line MDA-MB-231 and their E1A stable transfectants with recombinant human TRAIL. SKOV3.ip1, a subline of SKOV3, was found to be resistant to TRAIL-induced apoptosis, but four independent E1A transfectants (ip1-E1A2, ip1-E1A3, ip1-E1A10, and ip1-E1A15) became sensitive to TRAIL. TRAIL induced 29% to 40% apoptosis in the E1A stable ip1 transfectants but only 4% apoptosis in the parental cells (Fig. 1A), indicating that E1A sensitized TRAIL-resistant SKOV3.ip1 cells to TRAIL-induced apoptosis. In contrast, MDA-MB-231 cells were relatively sensitive to TRAIL treatment and E1A expression further sensitized TRAIL-induced apoptosis in four independent 231 E1A transfectants (231-E1A, 231-E1A-10, 231-E1A-25, and 231-E1A-36; Fig. 1B). Thus, the results suggest that E1A is able to sensitize TRAIL-induced apoptosis in both TRAIL-sensitive and TRAIL-resistant cancer cells.

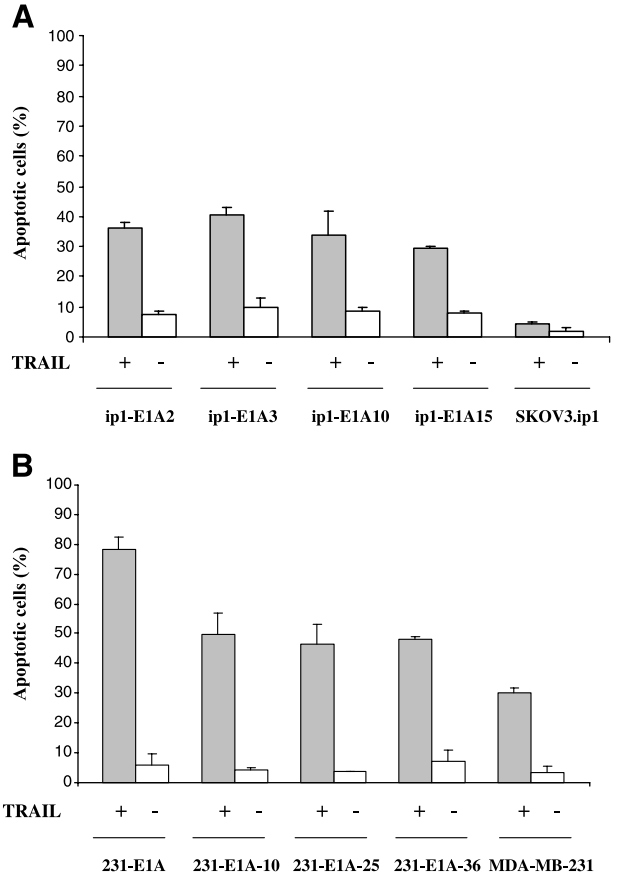

FIGURE 1. E1A sensitizes cancer cells to TRAIL-induced apoptosis. TRAIL-induced apoptosis was analyzed by flow cytometry. The cells were treated with (+) or without (−) recombinant human TRAIL (250 ng/mL) for 6 hours before they were harvested and stained with propidium iodide and analyzed by flow cytometry. **A.** Apoptotic cells were detected in E1A stable transfectants (ip1-E1A2, ip1-E1A3, ip1-E1A10, and ip1-E1A15) and parental human ovarian cancer SKOV3.ip1 cells. **B.** E1A stable transfectants (231-E1A, 231-E1A10, 231-E1A25, and 231-E1A36) and parental human breast cancer MDA-MB-231 cells. Bars, SD determined from three independent experiments.

E1A-Mediated Enhancement of TRAIL-Induced Caspase Activation

Previous studies have indicated that activation of caspase-8 is essential for TRAIL-induced apoptosis (17, 38). In the TRAIL-induced apoptotic signaling pathway, caspase-8 is recruited to the receptors/adaptor protein complex and subsequently activated by autocleavage (17, 39). To examine the effect of E1A on TRAIL-induced caspase-8 activation, immunoblotting for caspase-8 was done in the TRAIL-treated E1A transfectants (ip1-E1A2 and 231-E1A) and parental cells. The results indicated that cleavage of caspase-8 was higher in the TRAIL-treated ip1-E1A2 and 231-E1A cells than in the parental cells (Fig. 2B). TRAIL induced slight cleavage of caspase-8 in MDA-MB-231 cells but no cleavage was observed in SKOV3.ip1 cells (Fig. 2A). TRAIL-induced caspase-8 activation was confirmed by measuring caspase-8 activity using a peptide substrate. TRAIL induced caspase-8 activity in ip1-E1A2, 231-E1A, and MDA-MB-231 cells but not in SKOV3.ip1 cells (Fig. 2B).

In the mitochondrial-dependent apoptotic pathway, activation of caspase-8 induces cytochrome *c* release. The cytochrome *c* associates with Apaf-1, which in turn recruits and activates caspase-9. We evaluated TRAIL-induced activation of caspase-9 in the E1A transfectants and parental cells by immunoblotting and found that TRAIL-induced caspase-9 activation was higher in the E1A transfectants than it was in the parental cells (Fig. 2A). It has been reported that upon caspase-9 activation and apoptosis induction, Apaf-1 undergoes proteolytic degradation (40, 41). We examined the changes of Apaf-1 level in TRAIL-treated E1A transfectants and parental cells. Indeed, the results show a TRAIL-induced Apaf-1 decrease, and the reduction of Apaf-1 is correlated with the caspase-9 activation (Fig. 2A). This phenomenon was even more profound in the E1A transfectants. It is well established that caspase-9 activates the downstream caspase-3; thus, we further evaluated the activation of caspase-3 in cells treated with TRAIL (42). Again, TRAIL-induced caspase-3 activation was higher in the E1A transfectants than in the parental cells (Fig. 2A). The procaspase-3 level was reduced in the TRAIL-treated MDA-MB-231 cells. In a longer exposure, TRAIL-induced caspase-3

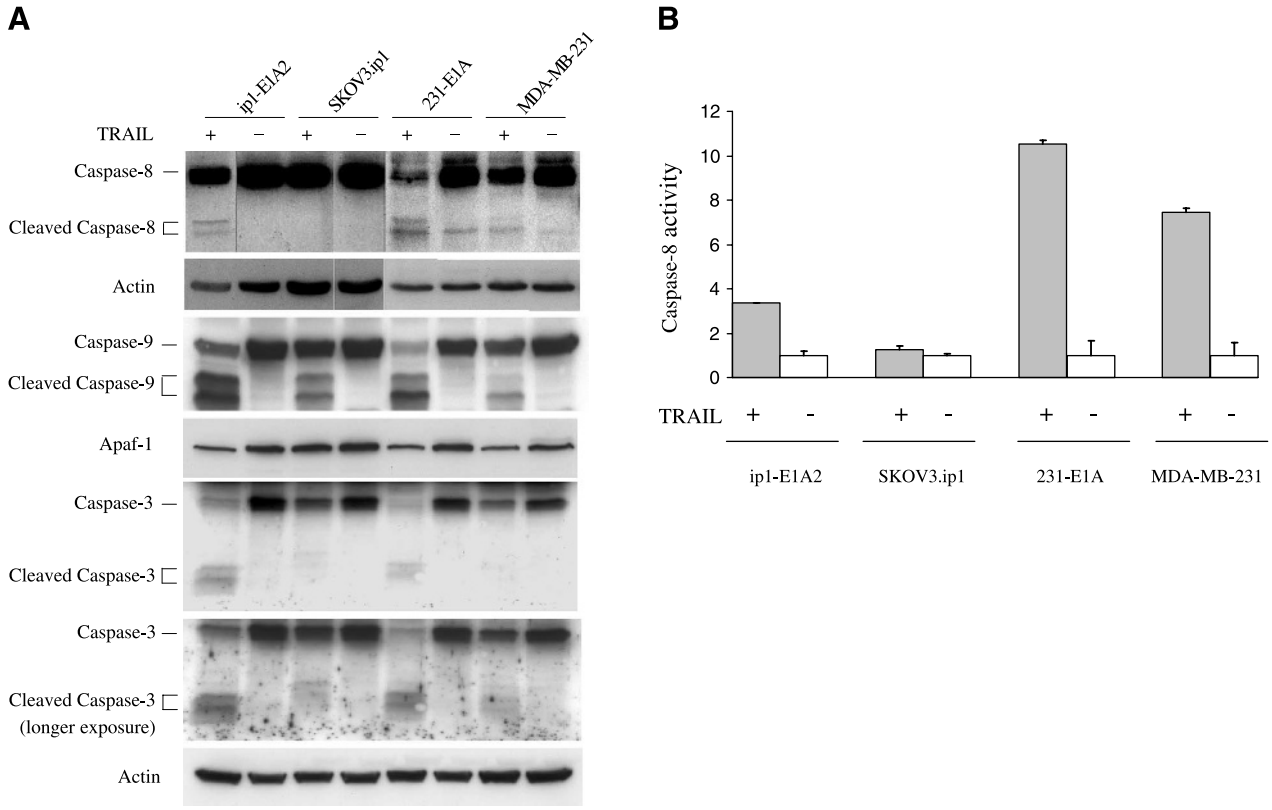

FIGURE 2. E1A enhances the activation of caspase induced by TRAIL. **A.** TRAIL-induced activation of caspase-8, caspase-9, Apaf-1, and caspase-3 in E1A transfectant ip1-E1A2, parental ovarian cancer SKOV3.ip1, E1A transfectant 231-E1A, and parental breast cancer MDA-MB-231 cells was analyzed by immunoblotting. Cells were treated with (+) or without (−) recombinant human TRAIL (250 ng/mL) for 4 hours before being harvested. Equal amounts of total cell lysates were subjected to SDS-PAGE (12% gel), and the activation of caspase-8, caspase-9, Apaf-1, and caspase-3 was detected by immunoblotting with the corresponding antibodies. As a loading control, the same blots were probed with an anti-actin antibody. **B.** Caspase-8 activity of cells treated with (+) or without (−) TRAIL was determined as described in Materials and Methods. Bars, SD of triplicate samples.

cleavage can also be observed in the parental MDA-MB-231 cells (Fig. 2A). Together, these results indicate that E1A mediates enhancement of TRAIL-induced activation of caspase-8, caspase-9, and caspase-3.

Blockage of Caspase-3 Activity Leading to Abolishment of TRAIL-Induced Apoptosis

Activation of the apoptosis effector caspase-3 is essential for various stimuli–induced apoptosis, including TRAIL-induced apoptosis (43, 44). To address whether activation of caspase-3 is required for E1A sensitization to TRAIL-induced apoptosis, the caspase-3 specific inhibitor Z-DEVD-fmk was used to block caspase-3 activity during TRAIL treatment. Z-DEVD-fmk treatment resulted in abolishment of most or all TRAIL-induced apoptosis (Fig. 3A and B). Enhancement of caspase-3 cleavage and the active products p20 and p17 was observed in the TRAIL-treated E1A cells (Fig. 3C and D). To further confirm the inhibitory effect of Z-DEVD-fmk on caspase-3 activity, the caspase-3 proteins and the caspase-3 substrate poly(ADP-ribose) polymerase (PARP) were analyzed by immunoblotting. TRAIL induced PARP cleavage in the E1A-transfected cells and TRAIL-sensitive MDA-MB-231 cells, and this cleavage was suppressed by the Z-DEVD-fmk treatment (Fig. 3E and F). The PARP protein level was lower and no cleavage was observed in the TRAIL-resistant SKOV3.ip1 cells (Fig. 3E). The degree of PARP cleavage indicates the relationship between caspase-3 activity and the degree of apoptosis, suggesting that caspase-3 activity is essential for TRAIL-induced apoptosis. Notably, an increase in the level of PARP protein was observed in the E1A transfectants. The cause of this protein elevation is not yet clear.

E1A-Mediated Increase of TRAIL *mRNA Level*

Increasing TRAIL concentration within a certain range is related to an increase in sensitivity. To evaluate whether E1A mediated changes in TRAIL expression, the levels of *TRAIL* mRNA were evaluated by Northern blotting. The results showed that *TRAIL* mRNA was expressed at an increased level in ip1-E1A2 cells (Fig. 4A). Similar results were also observed in the other three E1A transfectants (Fig. 4B). However, the *TRAIL* mRNA level is virtually nondetectable in MDA-MB-231 and 231-E1A cells.

Abrogation of E1A-Mediated Bystander Effect by Anti-TRAIL Neutralizing Antibodies

E1A has been previously shown to induce bystander effect in SKOV3.ip1 cells (i.e., ip1-E1A2 cells secret factor(s) to

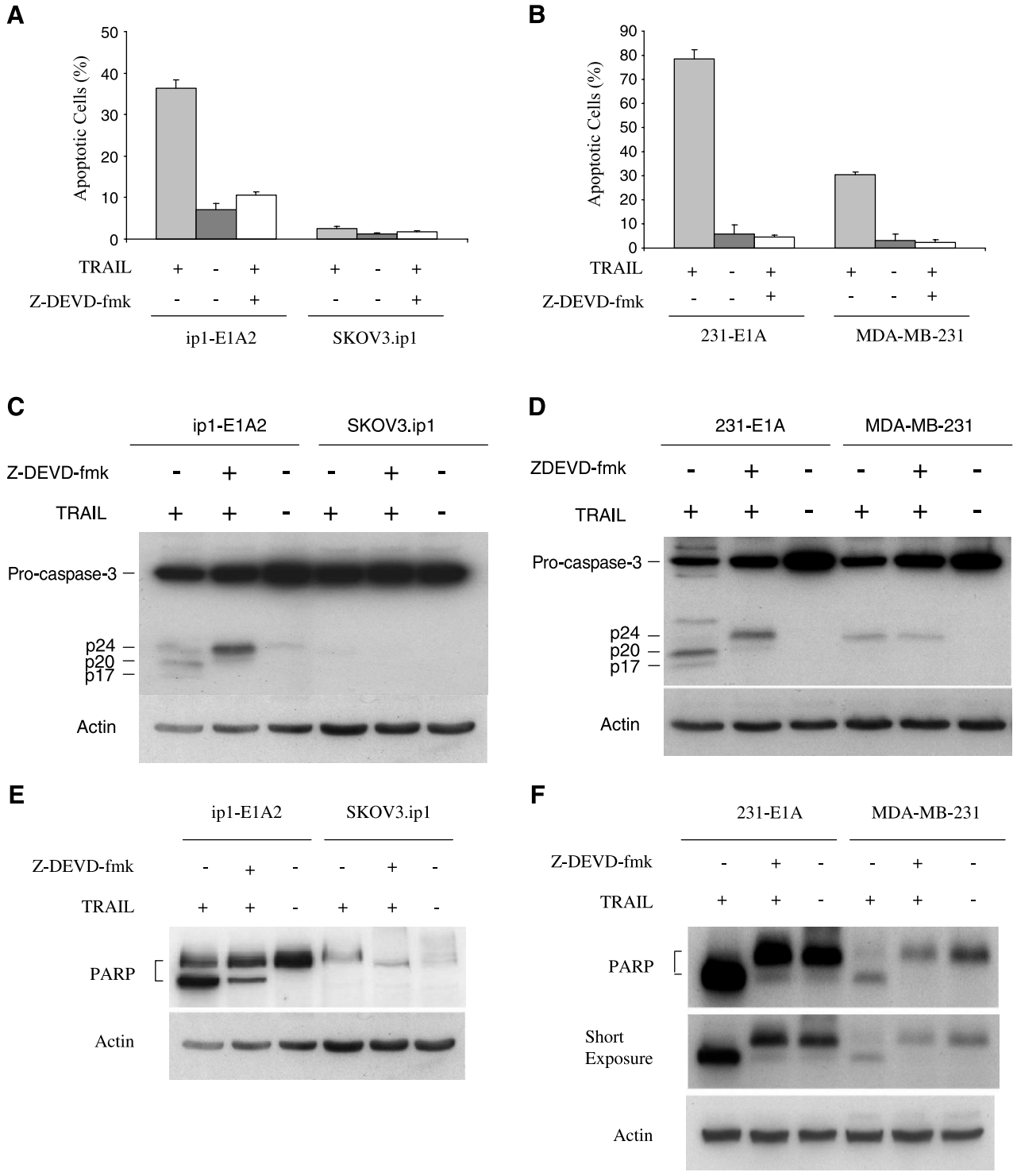

FIGURE 3. The caspase-3 inhibitor Z-DEVD-fmk inhibits TRAIL-induced apoptosis and cleavage of caspase-3 and PARP. Apoptosis was analyzed by flow cytometry. The ip1-E1A2, SKOV3.ip1, 231-E1A, and MDA-MB-231 cells were treated with (+) or without (−) 250 ng/mL recombinant human TRAIL for 6 hours or cultured with (+) or without (−) 20 μmol/L Z-DEVD-fmk for 1 hour and then cultured with TRAIL. Apoptotic cells were analyzed as described in Fig. 1. **A.** Percentage of apoptotic cells in ip1-E1A2 and SKOV3.ip1 cells. **B.** Percentage of apoptotic cells in 231-E1A and MDA-MB-231 cells. Columns, mean of three independent experiments; bars, SD. **C-F.** The cleavages of caspase-3 and PARP were analyzed by immunoblotting. The cells were treated with (+) or without (−) TRAIL for 4 hours or were treated with (+) or without (−) 20 μmol/L Z-DEVD-fmk for 1 hour before being cultured with TRAIL. The total cell lysates were subjected to SDS-PAGE (12% gel), and caspase-3 and PARP were detected with anti-caspase-3 and anti-PARP antibodies. **C.** Caspase-3 was detected in ip1-E1A2 and SKOV3.ip1 cells and (**D**) in 231-E1A and MDA-MB-231 cells. (**E**) PARP was detected in ip1-E1A2 and SKOV3.ip1 cells and (**F**) in 231-E1A and MDA-MB-231 cells. As a control, the same blots were probed with an anti-actin antibody (*bottom*).

induce apoptosis of neighboring cells; ref. 36). Because E1A significantly enhances TRAIL expression in ip1-E1A2 cells and TRAIL is suggested to contribute to the Stat3β-induced bystander effect (45), we investigate whether TRAIL expression may also be involved in the E1A-induced bystander effect. The anti-mouse TRAIL and the anti-human TRAIL neutralizing antibodies N2B2 and RIK-2 were used in the coculture of E1A transfectants and target cells. The results showed that ip1-E1A2 cells induced more apoptosis in the neighboring MDA-MB-231 cells than in the neighboring SKOV3.ip1 cells, and the N2B2 antibody abolishes apoptosis of the target SKOV3.ip1 and MDA-MB-231 cells induced by the coculture of ip1-E1A2 (Fig. 5). Similar results were observed with the RIK-2 antibody (data not shown). Interestingly, when 231-E1A cells were subjected to the bystander assay in the coculture system using SKOV3.ip1 and MDA-MB-231 cells as target cells, there is virtually no apoptotic cells detected in the target cells (i.e., 231-E1A dose not enhance apoptosis for its neighboring cells and thus does not induce bystander effect; data not shown). Taken together, the E1A-induced TRAIL expression in ip1-E1A2 cells is involved in the E1A-induced bystander effect, and 231-E1A cells do not enhance TRAIL expression and therefore do not induce bystander effect.

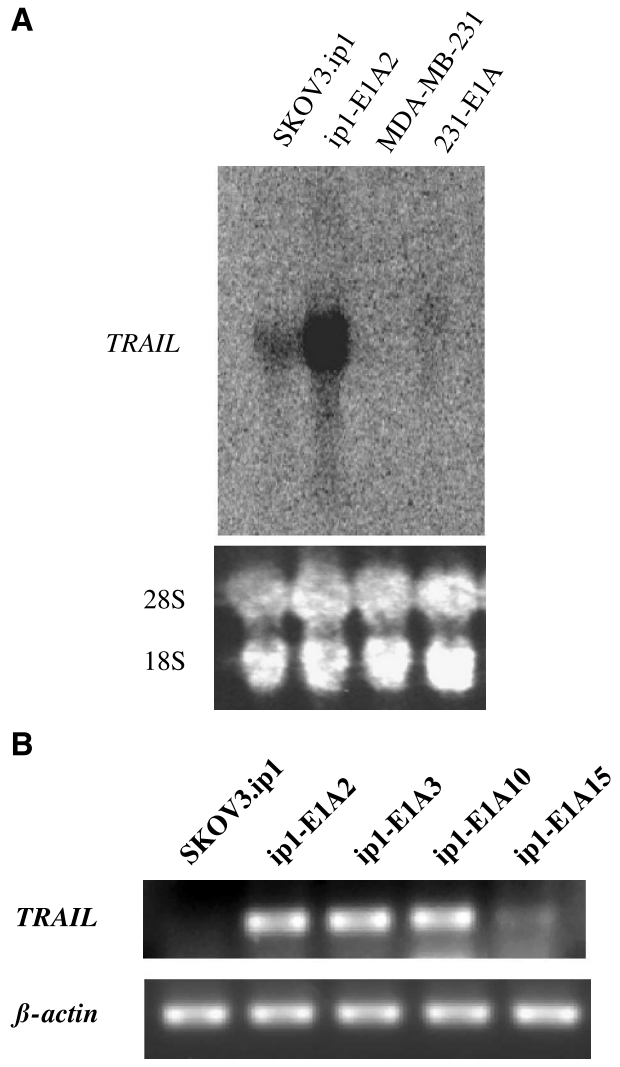

FIGURE 4. E1A increases *TRAIL* mRNA level in ip1-E1A transfectants. **A.** *TRAIL* mRNA in ip1-E1A2 and SKOV3.ip1 cells was analyzed by Northern blot. The total RNA of the cells was isolated with the Trizol reagent, and 10 mg of total RNA were loaded into the denaturing formaldehyde agarose gel and blotted onto a Hybond-N Nylon membrane. The ^{32}P-labeled *TRAIL* DNA was used as the probe to detect the *TRAIL* mRNA. *Bottom,* 28S and 18S ribosome RNAs as loading control. **B.** *TRAIL* mRNA expressions in multiple SKOV.ip1 E1A stable transfectants were measured by reverse transcription-PCR. β-Actin is used as an internal control.

Discussion

Recombinant TRAIL or transfer of TRAIL expression vectors has been used to kill cancer cells both *in vitro* and *in vivo* (4, 6). The combination of TRAIL and chemotherapeutic agents or radiation can increase TRAIL-induced cell killing, particularly TRAIL-resistant cancer cells. It is interesting to note that E1A sensitizes the highly resistant SKOV3.ip1 human ovarian cancer cells to TRAIL-induced apoptosis. The expression of E1A has made MDA-MB-231 cells more sensitive to TRAIL.

In this study, no correlation was observed between E1A-induced TRAIL sensitization and levels of death receptors DR4 and DR5 (data not shown). Other reports have also shown that there is a lack of correlation between TRAIL sensitivity and DR4 and DR5 levels (46, 47). In the TRAIL-induced death signaling pathway, caspase-8 is a key caspase activated by TRAIL (17, 48). Activation of caspase-8, in turn, activates the mitochondrial-dependent cytochrome *c* pathway, including caspase-9 and caspase-3 activation. This pathway has been shown to be a conventional one for TRAIL-induced apoptosis (20, 49). Our data show that E1A enhances TRAIL-induced caspase-8, caspase-9, and Apaf-1 activation, suggesting that activation of mitochondrial-dependent apoptotic pathway is the main pathway for E1A sensitization of cancer cells to TRAIL-induced apoptosis.

Caspase-3 is a critical effector caspase in apoptosis induced by TRAIL and a variety of other stimuli (44, 50). E1A has been shown to induce expression of multiple caspases (51). Inhibition of caspase-3 activity with the specific inhibitor Z-DEVD-fmk resulted in blockage of TRAIL-induced apoptosis, indicating that caspase-3 activity is essential for TRAIL-induced apoptosis. This result is consistent with that of a recent report (44). Because activation of caspase-3 by caspase-8 can also be mitochondrial independent (43, 44), the E1A-mediated enhancement of TRAIL-induced caspase-3 activity may also be involved in the mitochondrial-independent pathway (34, 52).

The adenovirus E1A protein in virus-infected cells can control and reprogram the cellular gene expression. The E1A transfectant ip1-E1A2 cells have been shown to induce bystander effect (36). In this study, the data showed that the TRAIL mRNA level is highly increased in ip1-E1A2 cells, and the anti-TRAIL antibodies block the bystander effect of ip1-E1A2 cells, suggesting that TRAIL is involved in the bystander effect. In support of this notion, 231-E1A, which does not stimulate TRAIL expression, also does not induce bystander effect. The reason causing the difference in E1A-mediated TRAIL expression between ip1-E1A2 and 231-E1A

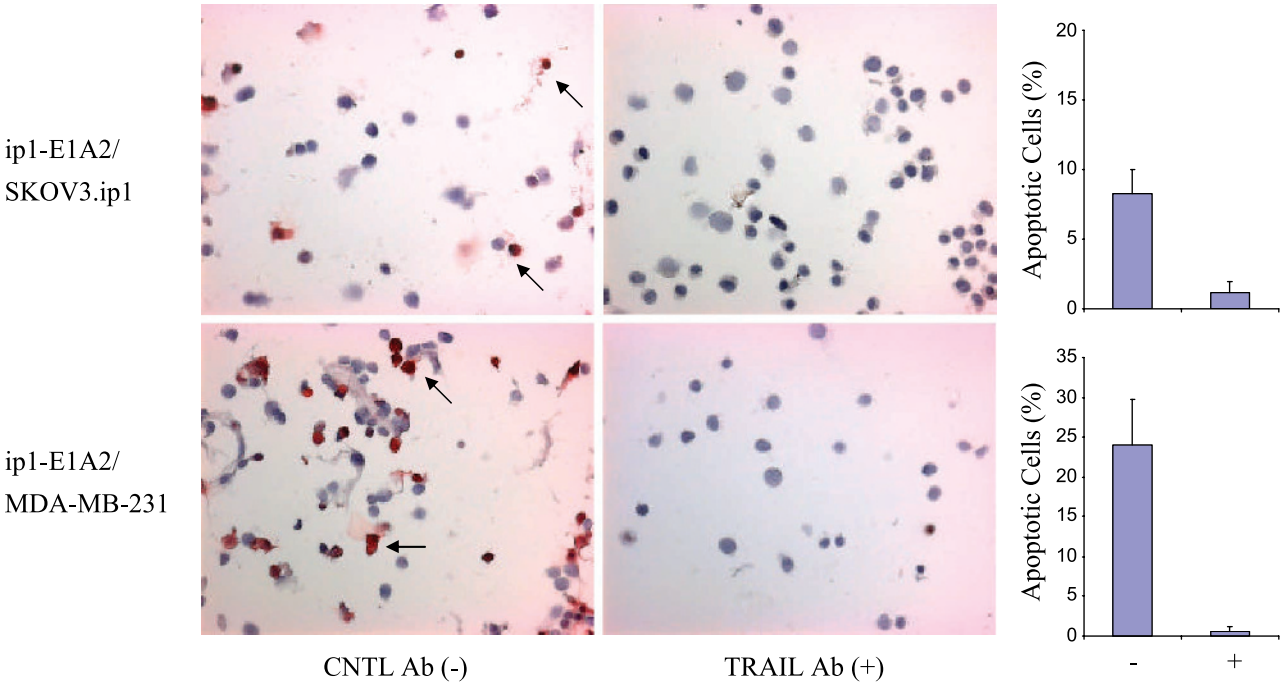

FIGURE 5. Anti-TRAIL antibody N2B2 blocks apoptosis of the target cells mediated by the bystander effect of ip1-E1A2. The ip1-E1A2 cells were cultured in the upper chamber and the SKOV3.ip1 or MDA-MB-231 cells were cultured in the lower chamber of the transwell unit (ip1-E1A/SKOV3.ip1 or ip1-E1A2/MDA-MB-231) with the N2B2 antibody (+) or control antibody (−) as described under Materials and Methods. The cells were harvested after 4 days in culture and the apoptotic cells in the lower chamber were analyzed by the terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling assay. *Arrows,* positive-staining cells. Bars, SD of three independent experiments.

cells is not yet clear. It may be due to the different nature of the parental cells. E1A induces cancer cell apoptosis under various conditions, including serum starvation and cytotoxic stress (31). Here we showed that E1A sensitizes the TRAIL-sensitive and TRAIL-resistant cancer cells to TRAIL-induced apoptosis, and the mechanism of this sensitization involves the activation of caspase. The E1A-mediated TRAIL expression is contributed to the bystander effect. Combination therapy of TRAIL and E1A may provide an effective way for cancer therapy.

Materials and Methods

Cell Lines and Culture

The human ovarian cancer cell line SKOV3.ip1 and its E1A stable transfectant ip1-E1A2 and the human breast cancer cell line MDA-MB-231 and its E1A stable transfectant 231-E1A were used. The establishment of the E1A stable transfectants and the condition for the cell culture have been described previously (36, 53, 54).

Propidium Iodide Staining and Flow Cytometry Assay

Cells (2.5×10^5) were cultured in a six-well plate in 2 mL of F12/DMEM medium. After 24 hours, the medium was replaced with 1 mL of fresh F12/DMEM medium, medium containing 250 ng of recombinant human TRAIL (Chemicon, Temecula, CA), or medium containing 20 mmol/L Z-DEVD-fmk (R&D Systems, Minneapolis, MN) for 1 hour before the addition of TRAIL. The cells were cultured with TRAIL for 6 hours and then harvested for fluorescence-activated cell sorting analysis as described previously (31). Briefly, floating and trypsinized cells were collected together. The cells were washed once with PBS and fixed with 70% ice-cold ethanol and stored at −20°C overnight. The fixed cells were washed once with PBS and stained with propidium iodide. Flow cytometry was used to determine the percentage of sub-G_1 cells.

Immunoblotting

Cells (7.5×10^5) were cultured in a 60-mm dish. After 24 hours, the medium was replaced with 2 mL of fresh F12/DMEM medium or medium containing 20 μmol/L Z-DEVD-fmk or DMSO as a control. The cells were cultured for 1 hour before 250 ng/mL of TRAIL was added to the culture. The cells were grown with TRAIL for an additional 4 hours and then harvested. Total cell lysates were prepared, subjected to 12% SDS-PAGE, and blotted onto nitrocellulose membranes. Polyclonal antibodies against caspase-8, Apaf-1 (PharMingen, San Diego, CA), caspase-9, caspase-3 (Cell Signaling Technology, Beverly, MA), and PARP (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

Caspase-8 Activity Assay

SKOV3.ip1, ip1-E1A2, MDA-MB-231, and 231-E1A cells were treated with 250 ng/mL TRAIL for 4 hours, harvested, and lysed with lysis buffer (25 mmol/L HEPES, 5 mmol/L $MgCl_2$, 5 mmol/L EDTA, 2 mmol/L DTT, 0.1% CHAPS). The lysates were cleared of debris by centrifugation. Protein

concentration was measured using the DC Protein Assay Kit (Bio-Rad, Hercules, CA) and equal amounts were incubated with 200 μmol/L caspase-8 substrate Ile-Glu-Thr-Asp-pNA (AnaSpec, San Jose, CA) for 4 hours at 37°C in water bath. Release of pNA was determined by reading the absorbance at 405 nm.

Northern Blot Analysis

Total RNA was purified from E1A transfectants and parental cells with Trizol (Life Technologies, Gaithersburg, MD) according to the protocol of the manufacturer. For Northern blotting, 10 μg of total RNA were size fractionated on a denaturing formaldehyde agarose gel and blotted onto a Hybond-N nylon membrane (Amersham, Piscataway, NJ). A 0.85-kb *Nhe*I/*Nco*I fragment from the pORF-hTRAILvector (InvivoGen, San Diego, CA) was used as a probe and labeled with a random primed DNA labeling kit (Roche, Mannheim, Germany). Hybridization was done as described previously (55).

In vitro *Apoptosis Assay*

To evaluate the effect of anti-TRAIL neutralizing antibody on E1A-induced bystander effect, the E1A transfectants and the target cells were cocultured in the transwell unit as described previously (36). In brief, 4×10^5 ip1-E1A2 or 231-E1A cells, in a volume of 200 μL, were seeded in the upper chamber and 3×10^5 SKOV3.ip1 or MDA-MB-231 cells, in a volume of 1 mL, were cultured in the lower chamber of the transwell unit. The anti-mouse TRAIL antibody N2B2 or the anti-human TRAIL antibody RIK-2, 12 μg in 12 μL, was added to the lower chamber on days 2, 3, and 4 (56). At the end of day 4, the cells in the lower chamber were collected for the terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling assay as described previously (57).

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