Ionizing radiation can overcome resistance to TRAIL in TRAIL-resistant cancer cells

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Abstract Although the majority of cancer cells are killed by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand treatment), certain types show resistance to it. Ionizing radiation also induces cell death in cancer cells and may share common intracellular pathways with TRAIL leading to apoptosis. In the present study, we examined whether ionizing radiation could overcome TRAIL resistance in the variant Jurkat clones. We first selected TRAIL-resistant or -sensitive Jurkat clones and examined cross-responsiveness of the clones between TRAIL and radiation. Treatment with γ-radiation induced significant apoptosis in all the clones, indicating that there seemed to be no cross-resistance between TRAIL and radiation. Combined treatment of radiation with TRAIL synergistically enhanced killing of TRAIL-resistant cells, compared to TRAIL or radiation alone. Apoptosis induced by combined treatment of TRAIL and radiation in TRAIL-resistant cells was associated with cleavage of caspase-8 and the proapoptotic Bid protein, resulting in the activation of caspase-9 and caspase-3. No changes in the expressions of TRAIL receptors (DR4 and DR5) and Bcl-2 or Bax were found after treatment. The caspase inhibitor z-VAD-fmk completely counteracted the synergistic cell killing induced by combined treatment of TRAIL and γ-radiation. These results demonstrated that ionizing radiation in combination with TRAIL could overcome resistance to TRAIL in TRAIL-resistant cells through TRAIL receptor-independent synergistic activation of the cascades of the caspase-8 pathway, suggesting a potential clinical application of combination treatment of TRAIL and ionizing radiation to TRAIL-resistant cancer cells. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Tumor necrosis factor-related apoptosis-inducing ligand resistance; Ionizing radiation; Synergistic cell killing; TRAIL receptor-independent

1. Introduction

Apoptosis of tumor cells can be initiated by triggering cell death receptors, leading to activation of the intracellular apoptotic machinery. Chemotherapeutic drugs used in cancer treatment may exert their therapeutic effects by activating these pathways. On the other hand, it is known that defects in the apoptotic pathways or activation of antiapoptotic machineries can confer resistance to chemotherapy. Thus, control of the balance between pro- and antiapoptotic processes within the cell has been recognized as an important target for therapeutic intervention [1,2].

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family, which also includes TNF, Fas ligand (FasL), and lymphotoxin-α. Previous studies suggest that unlike TNF and FasL, TRAIL is capable of inducing apoptosis of various transformed or cancer cells but not of normal cells [3]. Because of this unique ability, TRAIL may have a potential use in cancer treatment. TRAIL can induce apoptosis by interacting with two receptors: death receptor (DR) 4 (TRAIL-R1) and DR5 (TRAIL-R2) [4–7]. TRAIL also binds two additional receptors, decoy receptor (DcR) 1 (TRAIL-R3) and DcR2 (TRAIL-R4), which are highly expressed in normal cells and believed to protect apoptosis [6,8–10].

Apoptosis signal transduction and execution require the coordinated action of the cascades of caspases, which are aspartate-specific cysteine proteases, and human caspases 1 through 11 have been described [11–13]. It has been shown that caspase-8 and caspase-3 became activated when HeLa cells were treated with TRAIL [14] and also caspase-3 cleavage was observed in TRAIL-sensitive breast cancer cells [15]. Therefore, although there is a missing link between TRAIL DR and caspase activation, it is clear that TRAIL induces apoptosis through the activation of caspases [5,6,8].

DNA damaging agents including etoposide and γ-radiation often trigger the endogenous suicide machinery of cells, and ionizing radiation-induced apoptosis requires activation of multiple genes. In certain cancer cell types, radiation induces apoptosis through the p53-directed de novo synthesis of the death agonist Bax [16,17], indicating involvement of the mitochondrial caspase pathway. However, it is still unclear how the signal is transmitted to these enzymes. Recently, several

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Abbreviations: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; DR, death receptor; DcR, decoy receptor; PARP, poly(ADP-ribose) polymerase; z-VAD-fmk, benzyloxycarbonyl-Phe-Val-Ala-Asp (Ome)-fluoromethylketone

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reports demonstrated that DNA damaging chemotherapeutic agents, such as doxorubicin, cisplatin or etoposide, and ionizing radiation enhanced TRAIL sensitivity in certain cancer types [18–20]. However, conflicting results for the correlation between apoptosis mediated by DNA damaging agents and by TRAIL have been presented. Gibson et al. [21] and Gong et al. [22] suggested that activation of TRAIL receptor was directly involved in etoposide- and radiation-mediated cell death in certain cancer cells. On the other hand, Walczak et al. [23] demonstrated that the pathway induced by etoposide was distinct from those induced by TRAIL. In the present study, therefore, we examined whether TRAIL-resistant cancer cells showed cross-resistance to γ-radiation and also whether combination treatment of TRAIL and γ-radiation showed a synergistic cell killing effect in TRAIL-resistant cancer cells. The results obtained showed that TRAIL-resistant Jurkat clones did not have cross-resistance to ionizing radiation and combined treatment of radiation with TRAIL synergistically increased cell death through the activation of the caspase pathway in these clones resistant to TRAIL-induced apoptosis. These results suggest that combined treatment of ionizing radiation with TRAIL may be useful in the clinical treatment of TRAIL-resistant human cancer.

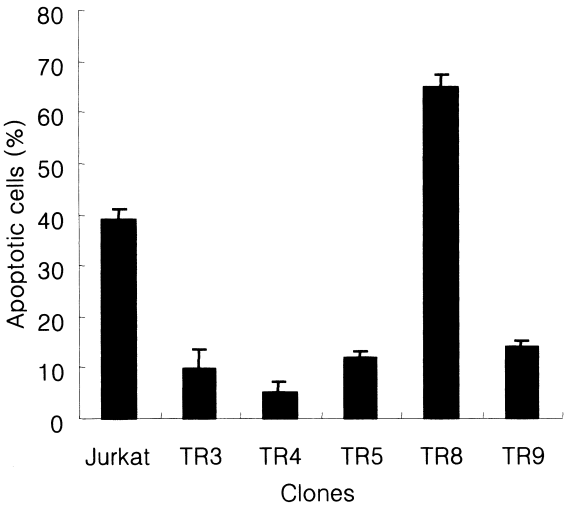

Fig. 1. Selection of TRAIL-resistant and -sensitive Jurkat clones. Cells were treated with or without 100 ng/ml soluble human recombinant TRAIL and harvested after 24 h of incubation. Cells were then fixed and stained with Hoechst 33258, and were examined by fluorescence microscopy. Apoptotic cells were identified by condensed and fragmented nuclei. The data represent average values of triplicate experiments with S.D.

2. Materials and methods

2.1. Cell culture

Jurkat human T cell lymphoma was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin.

2.2. Materials

Recombinant TRAIL was a gift from Dr. Byung-Ha Oh in POSTECH, South Korea. Polyclonal antibodies to caspase-8, caspase-3 and poly(ADP-ribose) polymerase (PARP) were purchased from Upstate Biotechnology, and monoclonal antibodies to Bcl-2, Bax and Bid were obtained from Santa Cruz Biotechnology.

2.3. Hoechst 33258 staining

Hoechst 33258 staining was performed as described previously [24]. Briefly, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed once with phosphate-buffered saline (PBS). 50 ng/ml Hoechst 33258 was added to the fixed cells, and they were incubated for 30 min at room temperature and washed with PBS. Cells were mounted and examined by fluorescence microscopy. Apoptotic cells were identified by condensation and fragmentation of nuclei. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted $\times 100$. A minimum of 500 cells was counted for each treatment.

2.4. Flow cytometric analysis

The expression of TRAIL-R1 (DR4) through TRAIL-R4 was studied in the TRAIL-resistant cells by flow cytometry. Cells were incubated with appropriate concentrations of antibodies (diluted at 1:1000) for 1 h at room temperature, FITC-conjugated secondary antibodies were added, after washing twice with PBS, and further incubated for 1 h. Cells were then analyzed by FACScan flow cytometry (Becton Dickinson).

2.5. Western blot analysis

Western blot analysis was performed as described [25]. Briefly, cell lysates were prepared by extracting proteins with lysis buffer (40 mM Tris–HCl, pH 8.0, 120 mM NaCl, 0.1% Nonidet-P40) supplemented with protease inhibitors. Proteins were separated by SDS–PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence procedures (NEN) according to the manufacturer's recommendation.

3. Results and discussion

3.1. Selection of TRAIL-resistant and -sensitive Jurkat clones

Earlier reports demonstrated that normal cells were resistant to TRAIL treatment, while cancer cells showed variable responses to TRAIL: certain types of cancer cells showed resistance to TRAIL [15,26–29], although the majority of cancer cells were killed by TRAIL treatment. In this study, therefore, we selected variant clones from parental Jurkat cells for sensitivity or resistance to TRAIL-mediated apoptosis. As shown in Fig. 1, clones TR3, TR4, TR5 and TR9 showed resistance to TRAIL, whereas clone TR8 was relatively sensitive to TRAIL, compared with parental Jurkat cells. When treated with 100 ng/ml TRAIL, less than 15% of cells in the TRAIL-resistant clones TR3, TR4, TR5 and TR9 showed apoptotic morphology, as judged by Hoechst 33258 staining. However, treatment of TRAIL-sensitive clone TR8 with 100 ng/ml of recombinant TRAIL led to progressive apoptosis, over 60% of the cells displaying apoptotic morphology within 24 h of treatment.

3.2. TRAIL-resistant clones do not show cross-resistance to γ-radiation

Recently, it has been demonstrated that CD95-resistant Jurkat clones show cross-resistance with ionizing radiation and etoposide [30], suggesting that CD95 and DNA damaging stimuli require common events to induce apoptosis. To determine whether TRAIL and ionizing radiation also have cross-responsiveness in Jurkat clones, we examined the radiosensitivity in TRAIL-sensitive and TRAIL-resistant clones (Fig. 2). Treatment with 8 Gy γ-radiation caused significant apoptosis of all the clones, indicating that these clones did not show cross-sensitivity or -resistance to ionizing radiation. The fact

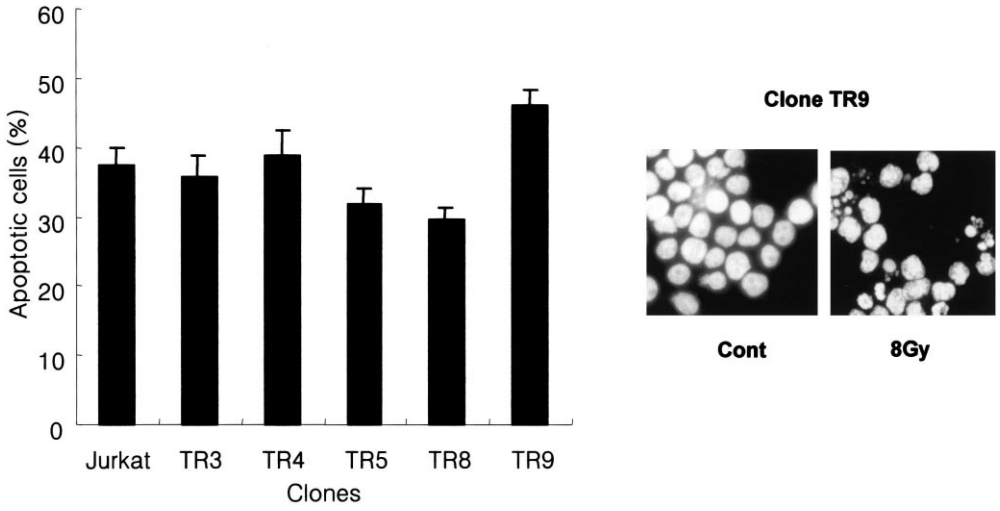

Fig. 2. TRAIL-resistant clones do not show cross-resistance to γ-radiation. Cells were treated with or without 8 Gy γ-radiation, and were harvested after 24 h. Then, cells were stained with Hoechst 33258, and apoptotic cells were identified by condensed and fragmented nuclei. The data represent average values of triplicate experiments with S.D.

that TRAIL-resistant cells did not have cross-responsiveness to ionizing radiation suggested potential circumvention of TRAIL resistance by γ-radiation in certain cancer cells.

3.3. *Ionizing radiation sensitizes TRAIL-resistant cells to TRAIL-induced apoptosis*

Earlier studies demonstrated that DNA damaging chemotherapeutic agents such as doxorubicin, cisplatin or etoposide enhanced TRAIL sensitivity in certain cancer types [18–20], therefore we next examined whether combined treatment of radiation with TRAIL also had any synergistic effect on apoptosis of TRAIL-resistant cells. As shown in Fig. 3, combination treatment indeed synergistically enhanced the killing of all TRAIL-resistant cells. Similarly, γ-radiation also potentiated the TRAIL effect on parental Jurkat cells as well as TRAIL-sensitive cells (data not shown). These findings suggested that γ-radiation in combination with TRAIL modulated the responsiveness to TRAIL from resistance to sensitivity in TRAIL-resistant cells, resulting in synergistic enhancement of the cell death.

3.4. *Radiation in combination with TRAIL does not affect the expression of TRAIL receptors, Bcl-2 and Bax in TRAIL-resistant cells*

In certain cancer types, activation of TRAIL receptor is directly involved in etoposide- and radiation-mediated cell death [21,22]. In order to examine whether combined treatment increased expression of TRAIL receptors (DR4 and DR5) in TRAIL-resistant cells, we performed flow cytometric analysis with anti-DR4 and -DR5 antibodies. No comparable changes were observed when TR9 cells were treated with radiation or TRAIL alone or together (Fig. 4A). We also could not observe any effect on the expression of DcR1 and DcR2 by these treatments (data not shown). The transcriptional reg-

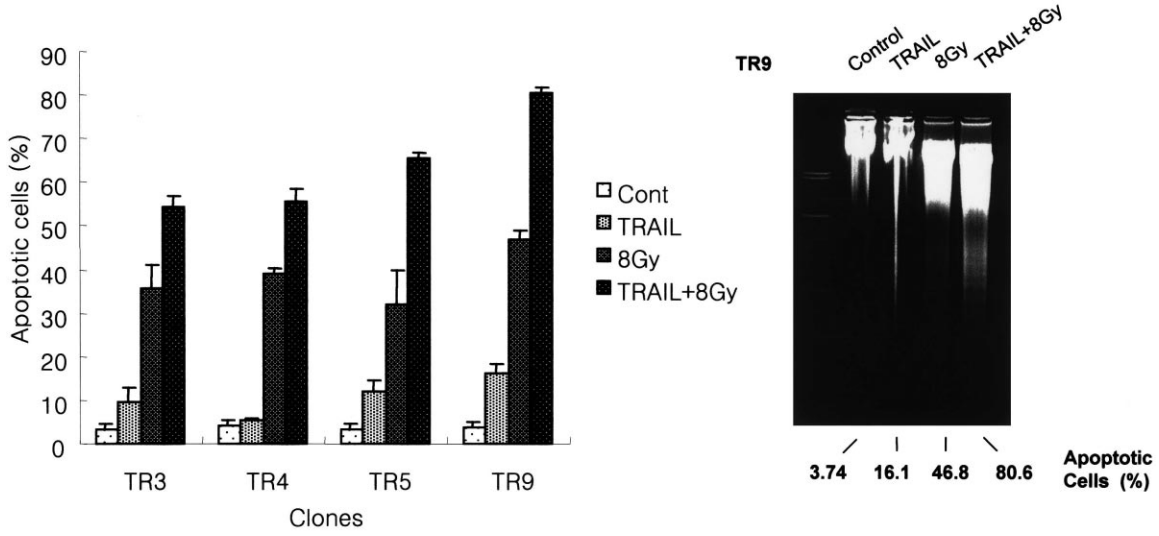

Fig. 3. Ionizing radiation sensitizes cells to the TRAIL-induced apoptosis in variant Jurkat clones. TRAIL-resistant Jurkat clones were treated with 100 ng/ml recombinant TRAIL alone, 8 Gy radiation alone, or a combination of both for 24 h. Then, cells were stained with Hoechst 33258 and examined by fluorescence microscopy. Apoptotic cells were identified by condensed and fragmented nuclei. The data represent average values of triplicate experiments with S.D.

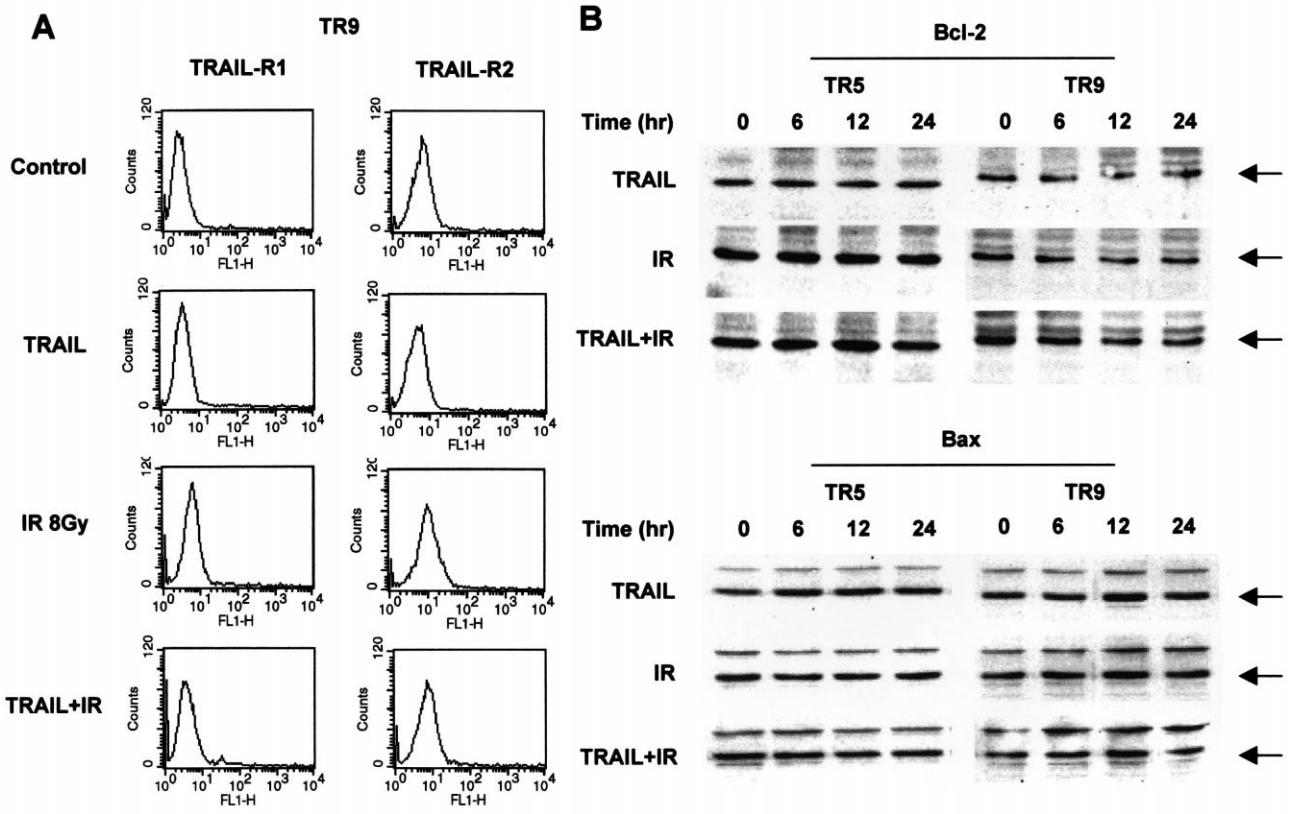

Fig. 4. Radiation in combination with TRAIL does not affect the expression of TRAIL receptors (DR4 and DR5), Bcl-2 and Bax in TRAIL-resistant cells. A: TR9 cells were treated with 100 ng/ml recombinant TRAIL alone, 8 Gy radiation alone or a combination of both for 24 h. Expression of DR4 and DR5 was examined by flow cytometry after staining the cells with anti-DR4 and -DR5 antibody (diluted at 1:1000). B: TR5 and TR9 cells were treated with 100 ng/ml recombinant TRAIL alone, 8 Gy radiation alone, or a combination of both for for the times indicated. Cell lysates were prepared and Western blot analysis was performed using anti-Bcl-2 and -Bax antibodies. The data represent a typical experiment conducted three times with similar results.

ulation of Bcl-2 and Bax seems to be a key element of apoptosis and to be related to mitochondria-mediated caspase activation. In order to determine possible correlation existing between enhancement of apoptosis by combination treatment and Bcl-2 family expression, we performed Western blot analysis with antibodies against Bcl-2 and Bax proteins. As shown in Fig. 4B, however, changes in Bcl-2 and Bax expression were not observed in TRAIL-resistant clones after combined treatment. One of the other molecules that possible interfere with sensitivity to TRAIL is FLIP, FLICE-inhibitory protein. In TRAIL-resistant cells, however, we did not observe any changes in FLIP expression after treatment with TRAIL and radiation alone or their combination (data not shown). All of the above results suggested that the induction of apoptosis by combined treatment of radiation and TRAIL was TRAIL receptor-, Bcl-2- or Bax-independent.

3.5. *Combination of radiation and TRAIL enhances cell death by increasing caspase-8, Bid, caspase-9 and caspase-3 activation*

The combined effect of TRAIL and ionizing radiation on cell killing in TRAIL-resistant clones suggested that proapoptotic molecules were induced and they might reset the responsiveness of the cells from resistant to sensitive. To find a possible convergence in apoptosis signaling by ionizing radiation, we first tested whether ionizing radiation activated the caspase pathway in TRAIL-resistant clones. Therefore we determined which caspases were activated and their order of activation by radiation through Western blot analysis of total cell lysates with antibodies against initiator caspase-8, pro-apoptotic Bid protein, caspase-9, and their effector caspase-3 and its substrate PARP. As shown in Fig. 5, treatment of clones TR5 and TR9 with TRAIL or radiation alone caused only slight activation of caspase-8, -9 and -3. However, combination treatment with radiation and TRAIL resulted in much greater activation of caspase-8, Bid, caspase-9 and caspase-3. Recently it has been demonstrated that DNA damaging agents such as etoposide and doxorubicin can augment TRAIL-induced apoptosis in breast cancer cells, perhaps by inducing DR4 and/or DR5 expression and subsequent activation of caspase-8 and caspase-3 [15,21,26]. On the other hand, conflicting results showing that the pathways induced by etoposide are distinct from those induced by TRAIL have also been presented [23]. In this study, we observed that radiation-mediated apoptosis was independent of the death receptors DR4 and DR5, and therefore appeared to activate caspase-8 in a death receptor-independent fashion.

To clarify the role of caspase-8 and -3 activation in the enhanced cell death by combination treatment, we examined the effect of a caspase inhibitor, benzyloxycarbonyl-Phe-Val-Ala-Asp (Ome)-fluoromethylketone (z-VAD-fmk). Pretreatment of clones TR5 and TR9 with z-VAD-fmk completely blocked apoptosis induced by subsequent combined treatment of γ-radiation with TRAIL as well as γ-radiation alone (data

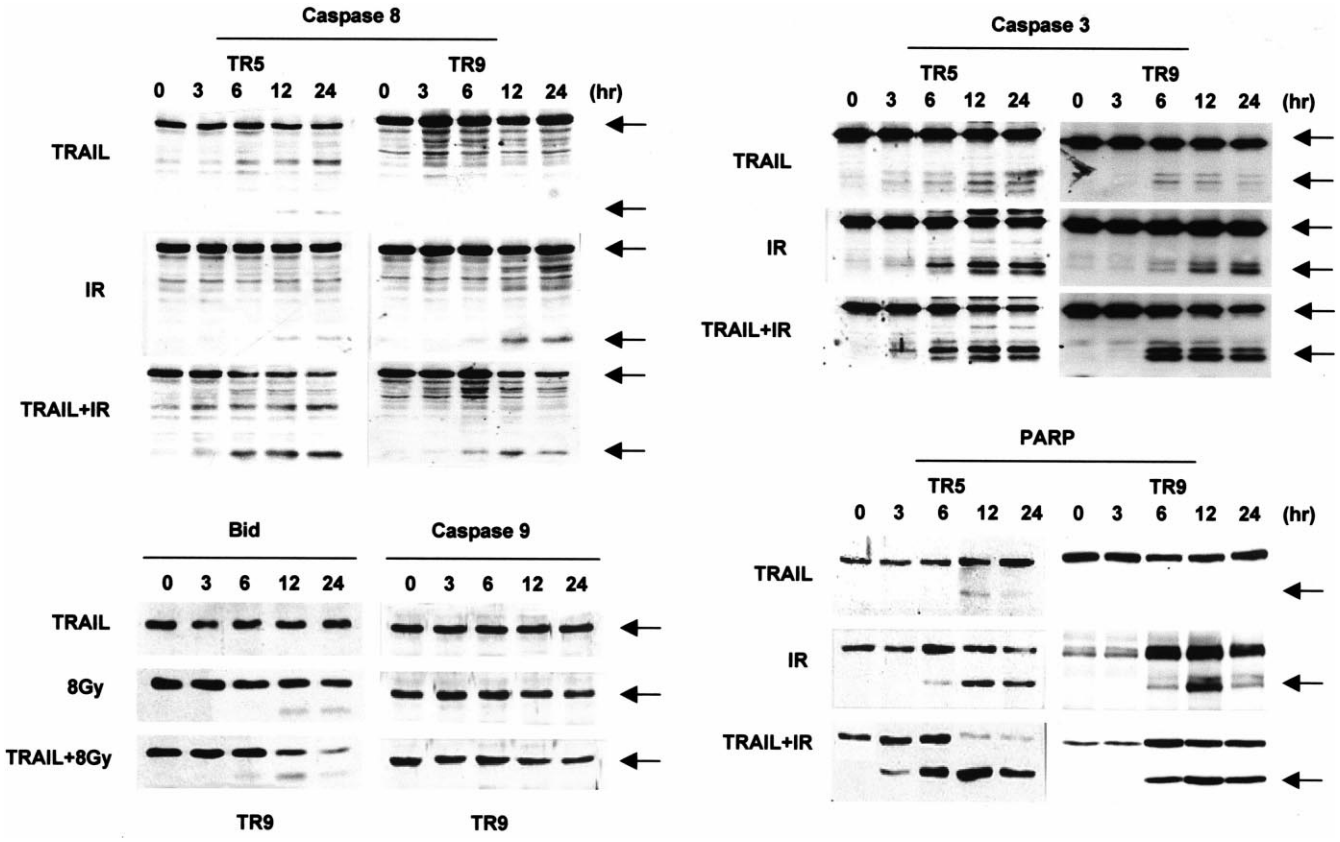

Fig. 5. Combined treatment of radiation and TRAIL enhances cell death by increasing caspase-8, Bid, caspase-9 and caspase-3 activation. Cells were treated with 100 ng/ml recombinant TRAIL alone, 8 Gy radiation alone, or a combination of both for the times indicated. Cell lysates were prepared and Western blot analysis was performed using anti-caspase-8, Bid, caspase-9 and caspase-3 and PARP antibodies. The data represent a typical experiment conducted three times with similar results.

not shown). These findings suggested that treatment with radiation enhanced the TRAIL-mediated death-inducing signaling complex without induction of TRAIL receptor expression, which in turn caused enhanced activation of caspase-8 and Bid, and resulted in the engagement of the mitochondrial pathway in apoptosis.

In conclusion, in the present study, we have demonstrated that ionizing radiation in combination with TRAIL can overcome TRAIL resistance in selected Jurkat clones through the TRAIL receptor-independent activation of the cascades of the caspase-8 pathway. The results suggest that combined treatment of TRAIL and ionizing radiation would be a promising tool for the clinical therapy in TRAIL-resistant cancer cells.

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