

Cytotoxicity and radiosensitization effect of TRA-8 on radioresistant human larynx squamous carcinoma cells

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Abstract. TRAIL induces apoptosis in a variety of tumorigenic and transformed cell lines, but not in many normal cells. Recent studies have demonstrated that death receptor 5 (DR5), one of the two death receptors bound by TRAIL, showed expression in most malignantly transformed cells. This study evaluated effects of a monoclonal antibody (TRA-8) to human death receptor 5, combined with ionizing radiation, on radioresistant human larynx squamous carcinoma cell line (Hep-2R). Cells were treated with TRA-8 alone or in combination with radiation, cell viability inhibition was measured by MTT assay, and the induction of apoptosis was determined by Annexin V staining. Radiosensitivity of Hep-2R cells treated with TRA-8 were investigated with long-term clonogenic assays. Regulation of DR5 expression in cells after radiation was analyzed by indirect immunofluorescence using murine TRA-8 in combination with flow cytometry. The results suggested that TRA-8 enhanced radiosensitivity of Hep-2R cells, and that TRA-8 regulated Hep-2R cell cycle arrest at G2/M phase. Irradiation up-regulated the expression of DR5, and when combined with TRA-8 yielded optimal survival benefit. Therefore, TRA-8 can be used in combination with irradiation in radioresistant human larynx squamous carcinoma cells. Monoclonal antibodies such as TRA-8 may play an important role in the

development of an effective treatment strategy for patients with radioresistant cancers.

Introduction

Radiotherapy plays an important role in the treatment for cancers. Nevertheless, some radioresistant cancer cell lines have recently been discovered. Treatment for radioresistant cancers has long been a clinical challenge. There are two hypotheses explaining the phenotypic heterogeneity of tumor cells in proliferation capability and radiosensitivity. One is 'radiation sieved in subgroup cells' (1), and the other is 'cell mutagenesis by irradiation' (2). In our previous study, we established a radioresistant tumor cell line (Hep-2R) and a radiosensitivity comparison model, with results supporting the first hypothesis (3). Research on the radioresistant cell model induced by repeated ionizing radiation suggests an effective treatment strategy for patients with cancers that are resistant to radiotherapy, including recurrent tumors in a short period after radiotherapy.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) is a promising anticancer agent due to its ability to induce apoptosis in many cancer cell lines, with minimal adverse effects on most normal cells (4). TRAIL is expressed constitutively in many normal tissues and is a type 2 transmembrane protein that can be cleaved by specific proteases to release its soluble form. At least five membrane receptors for TRAIL have been identified, and two of them (DR4 and DR5) are death receptor (5-8); the remaining three receptors are decoy receptors that are capable of blocking TRAIL-mediated apoptosis (9-11). The initial optimism for TRAIL was tempered somehow because studies with early recombinant forms of TRAIL also induced apoptosis in normal human hepatocytes (12). Although newer zinc-optimized recombinant forms of TRAIL have avoided such hepatotoxicity (13,14), agonistic monoclonal antibodies to specific death receptors were developed in an effort to circumvent this observed toxicity (15,16). Ichikawa developed TRA-8, a mouse monoclonal antibody that specifically binds to human DR5, without binding to DR4 or decoy receptors, which induced apoptosis of tumor cell lines *in vitro*, with tumoricidal effects *in vivo* (15).

Various tumor cells have been noted to have increased levels of cell-surface expression of DR5, thereby making these

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Abbreviations: TRAIL, tumor necrosis factor related apoptosis-inducing ligand; DR, death receptor; DcR, decoy receptor; PI, propidium iodide; FITC, fluorescein isothiocyanate; DMSO, dimethyl sulphoxide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

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cells susceptible to DR5-mediated apoptosis (17-19). We hypothesize that DR5 on Hep-2R cells can be selectively targeted with TRA-8 to enhance the anti-tumor effects of ionizing radiation. The objective of this study was to determine the therapeutic potential of TRA-8 in combination with radiation therapy in Hep-2R cells.

Materials and methods

Reagents. Antibody against DR5 was purchased from Jingmei Biotech Co., Ltd. (R&D Systems, Inc.). LHK601-100/Annexin V FITC kit was purchased from Jingmei Biotech Co., Ltd. (Bender Medsystems). FITC-conjugated goat anti-mouse IgG1 was purchased from Wuhan Boster Biological Technology, Ltd.

Cells and culture conditions. Hep-2R cells were established in our previous work (3). All media were supplemented with 2 mM L-glutamine, 10% fetal calf serum and 1% penicillin-streptomycin and cells were cultured at 37°C with 5% CO₂.

Basal expression of DR5 in Hep-2R cells. Cells were grown in complete medium supplemented with 10% FBS. Cells in exponential growth phase were harvested, and were collected by centrifugation at 4°C for 5 min at 1,000 rpm, followed by resuspension in PBS at 4°C. Cells were counted and aliquots of 5x10⁵ cells were added to FACS tubes. Cells were recentrifuged and the pellet was resuspended in PBS. Cells were incubated with TRA-8 (2 µg/ml) for 40 min on ice followed by FITC-conjugated goat anti-mouse IgG antibody (Wuhan Boster Biological Technology, Ltd.). Cells were washed again with FACS buffer and were examined using flow cytometry (FACScan, Beckman Coulter). Data were analyzed using CellQuest software.

Assessment of apoptosis by Annexin-V staining. Hep-2R cells were plated in 6-well culture plates at 3x10⁵ cells/well and cultured overnight. Cells were treated with TRA-8 in different concentration, or an equal volume of media alone to serve as a negative control, incubated overnight at 37°C. Then TRA-8 was added, after 24 h cells were stained with annexin V and propidium iodide (PI) using an Annexin V-FITC apoptosis detection kit, according to the manufacturer's protocol. Cells were then analyzed using flow cytometry (FACScan Beckman Coulter). Data were analyzed with CellQuest software.

Synergistic cytotoxicity of TRA-8 in combination with ionizing radiation detected by MTT assay. Cells were seeded in 24-well plates at a concentration of 5x10⁴ cells/well overnight and irradiated in different doses with ⁶⁰Co γ-ray. After 24 h, TRA-8 was added, and another 24 h later, 50 µl/well of 5 mg/ml MTT solution (Amresco, USA) was added respectively. The medium was removed by centrifugation after 4 h of incubation at 37°C with 5% CO₂. The blue formazan product converted from MTT was dissolved by the addition of 150 µl/well DMSO and OD readings were obtained using a microplate reader (Tecan, Austria) at 570 nm. Cell viability was represented as a percentage of control cells [percent cell viability = (treated/control) x100].

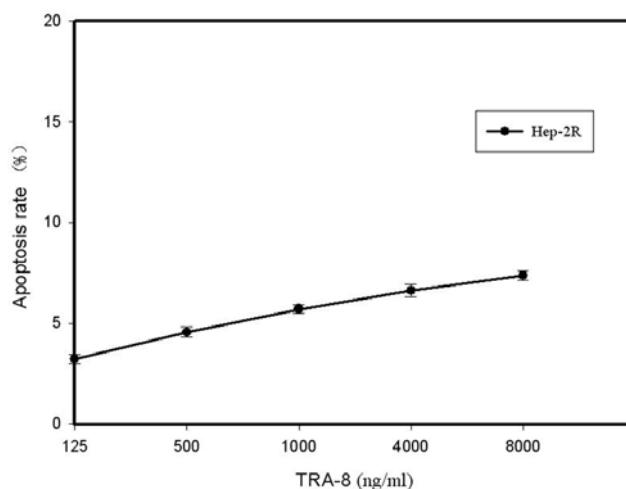


Figure 1. Cytotoxicity of TRA-8 against Hep-2R cells. Hep-2R Cells were plated in 6-well plates overnight, and were treated with TRA-8 in different concentrations. Cells were harvested 24 h after TRA-8 was added, and apoptosis was analyzed by Annexin-V-FITC staining. The mean in three independent experiments are shown.

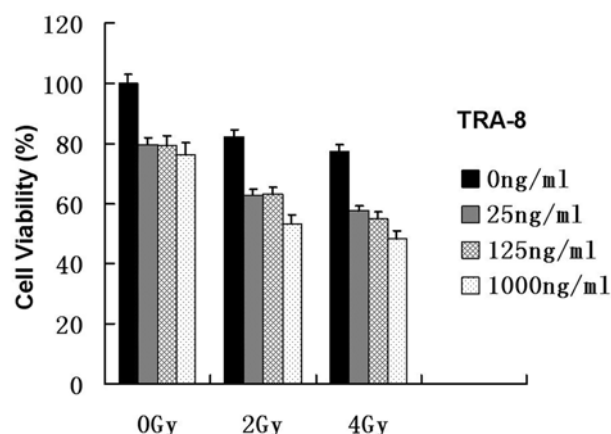


Figure 2. Cytotoxicity of TRA-8 in combination with radiation against Hep-2R cells. Cells were plated in 24-well plates and incubated overnight. Then cells were exposed to ⁶⁰Co irradiation (2, 4 Gy). TRA-8 was added 24 h after irradiation with the indicated concentrations. Cell viability was determined 24 h later by MTT assay. Cell viability was expressed as a percentage of untreated control cells, and there were 3 replicates in each assay. The results are derived from three experiments.

Determination of the clonogenic survival. Hep-2R cells were treated with 1000 ng/ml TRA-8 for 24 h. Cells plated at increasing densities up to 30,000 cells in culture flasks were irradiated with single dose of 0, 1, 2, 3, 4, 6, 8, 10 Gy with the ⁶⁰Co γ-rays (103.03 cGy/min). As a control, Hep-2R cells untreated with TRA-8 were irradiated. After being cultured for 12 days, the cells were stained with 1% crystal violet in absolute ethanol for 20 min, and then we counted the colonies with >50 cells. Colonies were counted by three independent observers using an inverted microscope. Data were fitted according to a second order polynomial model.

Expression of DR5 in Hep-2R cells after irradiation. Cells in exponential growth phase were seeded in 24-well plates at a concentration of 5x10⁵ cells/well overnight. Irradiation for

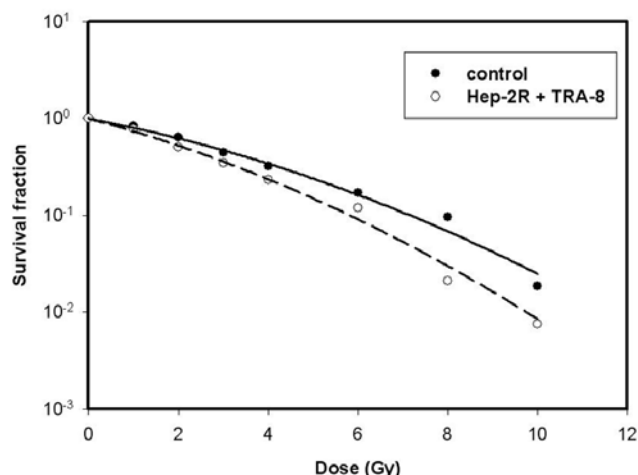


Figure 3. Survival curves of Hep-2R cells after γ irradiation. Hep-2R cells were inoculated into 100 ml culture flasks and treated with indicated doses of ^{60}Co γ -ray in presence or absence of 1000 ng/ml TRA-8 for 24 h before irradiation. Twelve days after irradiation, the cells were stained with 1% crystal violet in absolute ethanol for 20 min, and the colonies with >50 cells were counted. Data are expressed as mean values of three independent observations. Data were fitted according to a second order polynomial model.

2 Gy with ^{60}Co γ -ray, took cells untreated with irradiation as a control. After 24 h, cells were harvested by centrifugation at 4°C for 5 min at 1,000 rpm, followed by resuspension in PBS at 4°C. Cells were counted and aliquots of 5×10^5 cells were added to FACS tubes. Cells were recentrifuged and the pellet was resuspended in PBS. Cells were incubated with TRA-8 (2 $\mu\text{g}/\text{ml}$) for 40 min on ice followed by FITC-conjugated goat anti-mouse IgG antibody (Wuhan Boster Biological Technology, Ltd.). Cells were washed again with FACS buffer and were examined using flow cytometry (FACSscan Beckman Coulter). Data were analyzed using CellQuest software.

Distribution of cell cycle. Different cell cycle phases (G1, S or G2/M phase) are characterized by different DNA contents. Fluorescence dye propidium iodide (PI) binds to DNA strongly at a ratio of 1:1, and hence the DNA contents of cell cycle phases are reflected by varying PI fluorescent intensities. Hep-2R cells were harvested by trypsinization 24 h after 1000 ng/ml TRA-8 treatment, and fixed in 70% ice-cold (4°C) ethanol for 2 h. Cell pellets were resuspended in 1 mg/ml RNase solution (Sigma) for 30 min at 37°C, and then in 0.1 mg/ml PI solution (Sigma) at 4°C for 1 h in the dark. Cell cycle analysis was performed on a flow cytometer (Beckman Coulter).

Statistical analyses. Statistical analyses were performed using the one-way ANOVA, Chi-square test or Mann-Whitney t-test, contained in the SPSS software package (version 13.0) for Windows and the difference was regarded as significant at $p < 0.05$.

Results

TRA-8 induced cytotoxicity in Hep-2R cells. We assessed TRA-8 induced apoptosis on Hep-2R cells alone (Fig. 1).

Table I. The parameters of surviving Hep-2R cells after different dose of irradiation.^a

Group	$\alpha(\text{Gy}^{-1})$	$\beta(\text{Gy}^{-2})$	SF_2
Control	0.2204	0.0138	0.61 ± 0.0
TRA-8	0.2804	0.0193	0.53 ± 0.0

^aThe parameters (α , β , SF_2) of surviving of Hep-2R cells were from the survival curves (Fig. 3) according to a second order polynomial model. The SF_2 in different groups had a significant difference ($P < 0.05$).

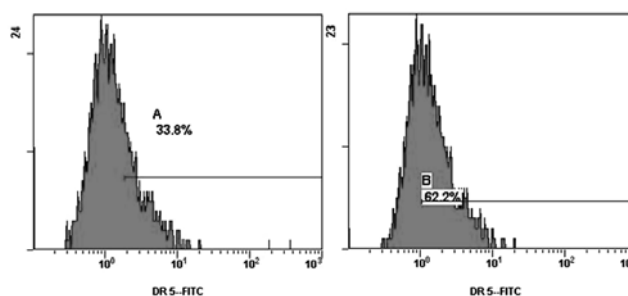


Figure 4. Effect of DR5 expression in Hep-2R cells after irradiation. (A) Expression of DR5 on Hep-2R cells. (B) Hep-2R cells were treated with 2 Gy ^{60}Co γ -ray irradiation. Twenty-four hours later, cells were harvested, and incubated first with 2 $\mu\text{g}/\text{ml}$ of TRA-8, then with goat anti-mouse IgG conjugated with fluorochrome. Cells were analyzed using FACSscan and analyzed with CellQuest software. The expression of DR5 protein in Hep-2R cells after irradiation was conspicuously different from the cells without ionizing radiation.

The cell viability assay suggested that Hep-2R cells were resistant to TRA-8, for $\text{IC}_{50} > 1000$ ng/ml, and there was a synergistic cytotoxicity in Hep-2R cells treated with ionizing radiation followed by TRA-8 administration (Fig. 2).

TRA-8 reduces clonogenic survival after ionizing radiation. To investigate the combined effects of TRA-8 and ionizing radiation on Hep-2R cell survival, we also used long-term clonogenic assays that incorporate effects on cell cycle activity, apoptotic and non-apoptotic death. The survival curves were constructed (Fig. 3), and the parameters of radiosensitivity in cells were assayed (Table I). The survival fraction at 2 Gy (SF_2) for Hep-2R cells was significantly decreased by TRA-8. The result indicated that the Hep-2R cells treated with TRA-8 before irradiation had a great enhancement in radio-sensitivity. The steeper slope of the curve representing the combined treatment indicates that TRA-8 reduced clonogenic survival.

Regulation of death receptors by ionizing radiation. We analyzed the expression of DR5 in Hep-2R cells 24 h after 2 Gy ^{60}Co γ -ray irradiation. The data suggested that the expression of DR5 protein was induced by irradiation in Hep-2R cell line (Fig. 4). After ionizing radiation, the expression of DR5 in Hep-2R cells increased to $(59.6 \pm 5.2)\%$ from $(36.9 \pm 3.5)\%$ ($p < 0.05$).

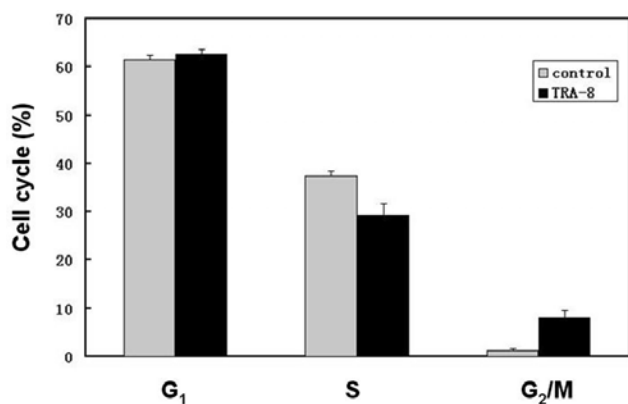


Figure 5. Change of cell cycle in Hep-2R after treatment with TRA-8. (Control) Hep-2R cells were not exposed to TRA-8; (TRA-8) Hep-2R cells were exposed to TRA-8 (1000 ng/ml) for 24 h. At the end of incubation period, cell cycle was measured by PI staining flow cytometry. Data are expressed as means \pm SD of three independent observations. G₁ phase $P > 0.05$; S-phase $P < 0.01$; G₂/M phase $P < 0.01$.

Effects of TRA-8 on cell cycle. We analysed whether there were any changes in the redistribution of cell cycle following treatment with TRA-8. The data suggested that there was no change in the percentage of G₁ phase after treatment with TRA-8 for 24 h, nevertheless, the cell cycle revealed an increase in the percentage of G₂/M phase and a decrease of S phase (Fig. 5).

Discussion

TRAIL-R monoclonal antibody-mediated treatment has been shown to be effective in some cancer cell lines (20-22). Phase I/II trials in patients with advanced tumors evaluated the efficacy, safety and tolerability as single agent (23-27), and a phase II clinical trial on DR5 mAb is ongoing. Additional evaluation of this novel apoptosis-inducing agent, particularly in combination with radiotherapy and chemotherapy agents, is warranted.

In this study, we examined the effects of TRA-8, a mouse monoclonal antibody binding to human DR5, combination with ionizing radiation on Hep-2R cells. We found that the greatest survival benefit in Hep-2R cells was from the combination of TRA-8 with radiation therapy. After ionizing radiation, the expression of DR5 in Hep-2R cells increased from (36.9 \pm 3.5)% to (59.6 \pm 5.2)%. As our radioresistant cell line was made by repeated irradiation to human laryngo-carcinoma Hep-2 cells, we examined the expression of DR5 on Hep-2 cells, which was 27.6 \pm 1.0% (data not shown). This result indicated that ionizing radiation induced expression of DR5 not only in the short-term, but also in the long-term. Ionizing radiation significantly up-regulated the expression of DR5 in human carcinoma cells, even under hypoxia (28-31). Unlike DR5, it has been reported that DR4 could not be induced by ionizing radiation (32). Thus, DR5 mAb might be a better choice for tumor therapy in combination with radiation therapy, as compared with DR4 mAb.

The clonogenic survival suggested that TRA-8 cut down the SF₂ and significantly enhanced the radiosensitivity of

Hep-2R cells. TRA-8 regulates G₂/M phase arrest, which could partially explain the mechanisms of enhancing radiosensitivity of Hep-2R cells by TRA-8. The changes in cell cycles indicated that cells in the radioresistant phase decreased and in radiosensitive phase increased. The regulation of the cell cycle observed in our study suggests that the complicated regulation mechanisms needed to be further investigated.

The TRA-8, murine monoclonal antibodies created from mice immunized with DR5, in combination with not only ionizing radiation, but with certain anti-tumor agents demonstrated synergistic anti-tumor activity, such as cisplatin, oxaliplatin, erlotinib, paclitaxel, CPT-11 (18,33-40). We will continue our study of TRA-8 on radioresistant cells in combination with other agents.

TRAIL may be a safer therapeutic agent than TNF and FasL (40-42), furthermore, TRAIL-R monoclonal antibody-mediated treatment has more prospects for targeting treatment of cancers that express death receptors. The present investigation enhances our understanding of the radiation mechanism of radiation and TRA-8-induced apoptosis. The observed synergy between TRA-8 and therapeutic doses of radiation can form the basis for developing strategies for pharmacological intervention, with potential for clinical application.

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