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**BIOLOGY CONTRIBUTION**

# TARGETING PRO-APOPTOTIC TRAIL RECEPTORS SENSITIZES HELA CERVICAL CANCER CELLS TO IRRADIATION-INDUCED APOPTOSIS

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**Purpose:** To investigate the potential of irradiation in combination with drugs targeting the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor (DR)4 and DR5 and their mechanism of action in a cervical cancer cell line.

**Methods and Materials:** Recombinant human TRAIL (rhTRAIL) and the agonistic antibodies against DR4 and DR5 were added to irradiated HeLa cells. The effect was evaluated with apoptosis and cytotoxicity assays and at the protein level. Membrane receptor expression was measured with flow cytometry. Small-interfering RNA against p53, DR4, and DR5 was used to investigate their function on the combined effect.

**Results:** rhTRAIL and the agonistic DR4 and DR5 antibodies strongly enhanced 10-Gy–induced apoptosis. This extra effect was 22%, 23%, and 29% for rhTRAIL, DR4, and DR5, respectively. Irradiation increased p53 expression and increased the membrane expression of DR5 and DR4. p53 suppression, as well as small-interfering RNA against DR5, resulted in a significant downregulation of DR5 membrane expression but did not affect apoptosis induced by irradiation and rhTRAIL. After small-interfering RNA against DR4, rhTRAIL-induced apoptosis and the additive effect of irradiation on rhTRAIL-induced apoptosis were abrogated, implicating an important role for DR4 in apoptosis induced through irradiation in combination with rhTRAIL.

**Conclusion:** Irradiation-induced apoptosis is strongly enhanced by targeting the pro-apoptotic TRAIL receptors DR4 or DR5. Irradiation results in a p53-dependent increase in DR5 membrane expression. The sensitizing effect of rhTRAIL on irradiation in the HeLa cell line is, however especially mediated through the DR4 receptor. © 2008 Elsevier Inc.

**Irradiation, Death receptors, Tumor necrosis factor-related apoptosis-inducing ligand, TRAIL, Cervical cancer, Apoptosis.**

## INTRODUCTION

Currently, locally advanced cervical cancer is treated with irradiation combined with chemotherapy. Despite the improvement with this combination compared with irradiation alone, the overall survival rate is still around 52% (1). In addition, this treatment results in considerable morbidity (2). Additional improvement in survival rates by intensifying the standard treatment (irradiation with platinum-based chemotherapy) is limited because of tumor resistance to irradiation and/or chemotherapy and the expected increase in short- and long-term side effects.

Irradiation-induced cell death is considered to be the result of DNA damage linked to mitosis. However, in preclinical models resistance to irradiation can be overcome by inducing apoptosis with molecular targeted agents (3–5). Targeting molecular pathways involved in apoptosis are therefore tentative options in the effort to improve the therapeutic effect in cervical cancer (6).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a tumor necrosis factor family member cytokine that can induce apoptosis by binding to the agonistic death receptor 4 (DR4) or DR5. Binding of TRAIL to its DRs results in activation of the DR, which activates caspase-8. Activation of caspase-8 can directly lead to apoptosis through caspase-3 activation (extrinsic pathway). In addition, cleavage of the Bcl-2 family member Bid by caspase-8 can activate the mitochondrial (intrinsic) apoptotic pathway (7, 8). Several stress signals, including radiation-induced DNA damage can also activate the intrinsic apoptotic pathway. Pro-apoptotic proteins are then released from the mitochondria into cytosol-enhancing caspase activation. In addition to preclinical data, which have demonstrated that DRs are interesting targets

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for treatment, recent Phase I studies have proved that recombinant human TRAIL (rhTRAIL) and agonistic antibodies directed against the DR4 and DR5 can be safely administered in humans (9, 10).

Combining ionizing radiation with rhTRAIL in preclinical studies can render tumor cells more sensitive to apoptosis (11–17). Depending on the model used, increased DR4 and DR5 expression, induction of Bax or Bak, and decreased Bcl2 expression have been suggested to explain the increase in cytotoxicity of the combination of irradiation with rhTRAIL (11–13, 18). A molecular basis for the synergy between irradiation and rhTRAIL in these studies might be p53-dependent upregulation of DR5 and DR4, because p53 transactivation sites are present in the DR gene coding sequences (19–21). p53 is frequently functionally inactivated in cervical cancer because of the presence of high-risk human papillomaviruses (HPVs), especially HPV16 and HPV18. These HPVs play a crucial role in the pathogenesis of cervical cancer (22). The HPV-encoded oncogenes E6 and E7 can inactivate the tumor suppressor gene product p53 and pRb, respectively, by targeting them to the ubiquitin-proteasome system for degradation (23).

Little is known about the combined effect of irradiation and rhTRAIL in HPV-positive cervical cancer models or the role of p53, DR4, and DR5 in this setting. Irradiation induces p53 transcriptional activation in cervical cancer cell lines (19), and increased expression of the DR4 and DR5 is found in cervical tumors compared with normal cervical tissue (24). The objective of this study was, therefore, to explore the potential of irradiation in combination with drugs targeting the TRAIL DRs. As a model we used the HPV18-positive human cervical cancer cell line HeLa S3, which contains wild-type p53 and is intermediately sensitive to rhTRAIL (25). Emphasis was put on the role of DR4 and DR5 in relation to irradiation-induced p53 expression.

## METHODS AND MATERIALS

### *Reagents and chemicals*

Dulbecco's minimum essential medium and Nutrient Mixture F-12 (HAM) were obtained from Invitrogen-Life Technologies (Merelbeke, Belgium) and fetal calf serum from Bodinco (Alkmaar, The Netherlands). 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands), dimethyl sulfoxide from Merck (Amsterdam, The Netherlands), trypsin stock (10×) solution and ethylenediaminetetraacetic acid from Invitrogen-Life Technologies, and skim milk powder from Merck (Darmstadt, Germany).

Recombinant human TRAIL was produced noncommercially in cooperation with IQ-Corporation (Groningen, The Netherlands) following a protocol described previously (9). The agonistic anti-TRAIL-R1 (mapatumumab, HGS-ETR1) and anti-TRAIL-R2 (HGS-TR2J) antibodies were a gift from Human Genome Sciences (Rockville, MD).

### *Cell line and cell culture*

The human cervical carcinoma cell lines HeLa S3 (HeLa; HPV18-positive, wild-type p53), CaSki, and SiHa (both HPV16-positive, wild-type p53) were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown at 37°C in a humidified atmosphere with 5% carbon dioxide in 1:1 Dulbecco's minimum essential medium/HAM medium supplemented with 10% fetal calf serum. The cells were detached with 0.05% trypsin/0.5 mM ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 M KCl, 6.4 M $Na_2HPO_4$, $2H_2O$, 1.5 M $KH_2PO_4$, pH 7.4).

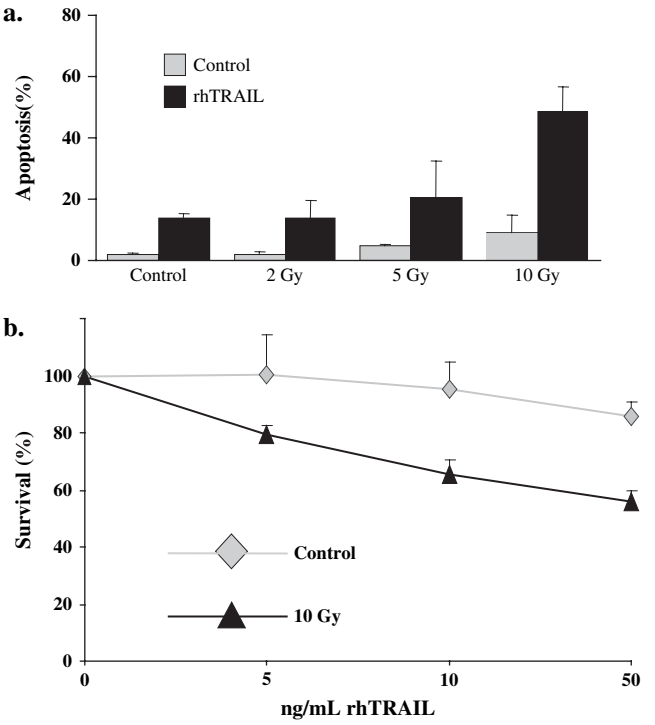


Fig. 1. Induction of recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL)-mediated apoptosis and reduced survival after irradiation. (a) Apoptosis determined by acridine orange staining, 48 h after irradiation and 24 h after 0.1 $\mu$g/mL rhTRAIL. Data presented as mean ± standard deviation of three independent experiments. (b) Reduced cell survival by rhTRAIL combined with 10 Gy irradiation measured with 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide cell survival assay. Percentages normalized for effect caused by irradiation alone. Data presented as mean ± standard deviation of three independent experiments.

### *Irradiation*

The cells were irradiated with a $^{137}Cs$ $\gamma$-ray machine (IBL 637, CIS Bio International Gif/Yvette, France) with a dose rate of 0.9 Gy/min.

### *Apoptosis assay*

In a 96-well culture plate, 5,000 cells/well were seeded in 100 $\mu$L culture medium. The next day, the cells were treated with irradiation and/or rhTRAIL in different combinations, varying in treatment order, concentration, and duration in 200 $\mu$L/well. At the end of the experimental period, acridine orange was added, and apoptosis quantified using a fluorescence microscope. Apoptosis was defined by the appearance of apoptotic bodies and/or chromatin condensation. The results are expressed as the percentage of apoptotic cells in culture (by counting ≥300 cells/well). This assay was performed three times.

### *Cytotoxicity assay*

The MTT assay was used to determine the cytotoxic activity of rhTRAIL in combination with irradiation. In a 96-well culture plate,