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# X Irradiation Combined with TNF α-related Apoptosis-inducing Ligand (TRAIL) Reduces Hypoxic Regions of Human Gastric Adenocarcinoma Xenografts in SCID Mice

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**Radiation/Solid tumor/TRAIL/Hypoxia/Xenograft.**

Our previous study showed that X irradiation induced the expression of death receptor DR5 on the cell surface in tumor cell lines under not only normoxia but also hypoxia. X irradiation combined with TNF α-related apoptosis-inducing ligand (TRAIL), which is the ligand of DR5, induced apoptosis *in vitro* (Takahashi *et al.,* (2007) *Journal of Radiation Research,* 48: 461–468). In this report, we examined the *in vivo* antitumor efficacy of X irradiation combined with TRAIL treatment in tumor xenograft models derived from human gastric adenocarcinoma MKN45 and MKN28 cells in SCID mice. X irradiation combined with TRAIL synergistically suppressed the tumor growth rates in the xenograft models derived from MKN45 and MKN28 cells, which have wild type Tp53 and mutated Tp53, respectively, indicating that the antitumor effects occurred in a Tp53-independent manner. Histological analysis showed that the combination of X irradiation and TRAIL induced caspase-3-dependent apoptotic cell death. Moreover, the immunohistochemical detection of hypoxic regions using the hypoxic marker pimonidazole revealed that caspase-3-dependent apoptosis occurred in the hypoxic regions in the tumors. These results indicated that X irradiation combined with TRAIL may be a useful treatment to reduce tumor growth in not only normoxic but also hypoxic regions.

## INTRODUCTION

In solid tumors, hypoxic regions are known to exist as a result of excessive tumor growth with a deficiency of blood vessels.[1,2] It is well known that the low oxygen concentrations in the regions reduce the cell killing efficacy induced by ionizing radiation.[1,2] Moreover, several studies have shown the resistance of hypoxic regions in solid tumors to antitumor drugs such as bleomycin[3] and etoposide.[4] Thus, it is very important to develop a cancer therapy technique to conquer the resistance in the hypoxic regions in solid tumors to genotoxic agents.[5,6] Recently, we have shown that the combination of TNF α-related apoptosis-inducing ligand (TRAIL) and ionizing radiation induces both apoptosis and reproductive cell death in lung adenocarcinoma A549 cells under not only normoxic but also hypoxic conditions *in vitro.*[7] Exposure to ionizing radiation under these conditions was demonstrated to induce the expression of DR5 on the cell surface in various solid tumor cell lines.[7,8] TRAIL is one of the TNF α family members and it has five receptors, including two functional receptors, DR4 and DR5, and three decoy receptors, DcR1, DcR2, and osteoprotegrin.[9] Because of the DR5 expression in tumor cells, but not in normal cells, treatment with TRAIL was demonstrated to induce apoptosis mostly in carcinoma cells rather than in normal cells.[10,11] TRAIL, because of these characteristics, could be expected for be useful as a novel sensitizing reagent of hypoxic regions for radiotherapy.

In the present study, we examined whether TRAIL treatment decelerated the growth of solid tumor *in vivo*. For this purpose, we employed tumor xenograft models in SCID mice bearing human gastric adenocarcinoma MKN45 (wild type Tp53) and MKN28 (mutated Tp53) cells and evaluated

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; HIF-1α, hypoxia inducible factor-1 α; NAC, N-acetyl-L-cysteine; SCID, severe combined immunodeficient; TNF α, tumor necrosis factor α; TRAIL, TNF a-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

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the effects of local injection of TRAIL into the tumors on the radiation-induced growth delay and the activation of caspase-3. Since the binding of 2-nitroimidazole derivative such as pimonidazole, which was widely used as hypoxic marker *in vivo*, has been shown to increase dramatically below an $O_2$ concentration about 1.3% (10 mmHg),[12–15] we employed pimonidazole as a hypoxic marker and examined whether TRAIL treatment was capable of enhancing the radiation-induced reduction of hypoxic regions.

## MATERIALS AND METHODS

### *Materials*

TRAIL and anti-DR5 were obtained from Alexis Biochemicals (Montreal, Canada) and Peprotech EC (London, UK) for *in vitro* and *in vivo* use, respectively. Terminal deoxynucleotidyl transferase and biotin-deoxyuridine triphosphate were purchased from Invitrogen (Carlsbad, CA) and Roche Diagnostics (Tokyo, Japan). Horseradish peroxidase and streptavidin were from Vector Laboratories (Burlingame, CA). Diaminobenzidine was from Dojindo Laboratories (Kumamoto, Japan). Hypoxyprobe™-1 kits were purchased from Chemicon International (Temecula, CA). Anti-cleaved caspase-3 was purchased from Cell Signaling Technology (Beverly, MA). Other reagents were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

### *Animals and tumor models*

Male severe combined immunodeficient (SCID)/jcl mice aged 5–7 weeks were purchased from Japan CLEA (Tokyo, Japan). The transplantation of tumor cells into SCID mice was performed as previously described.[16] Briefly, human gastric carcinoma cell lines MKN45 and MKN28 were grown in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% $CO_2$. MKN45 cells were cultured to confluence and harvested with trypsin/EDTA and washed twice with PBS (–). Collected cells were suspended in medium without serum and $5 \times 10^6$ cells were subcutaneously injected into footpads of mice. About 5 to 7 days after inoculation, when the tumor had grown to at least 70 $mm^3$, measurement of tumor volume started. At least 5 mice were included in each group: 1) control, 2) TRAIL alone, 3) X irradiation alone and 4) X irradiation and TRAIL. Tumor volume (length × $width^2$) was measured per day. When the average tumor volume in all mice was about 300 $mm^3$, X irradiation and drug treatments were carried out.

### *Animal welfare*

This study was conducted according to the "Guidelines Concerning Animal Experiments in Hokkaido University" and the "Law for The Care and Welfare of Animals" and approved by the Animal Experiment Committee of Graduate School of Veterinary Medicine, Hokkaido University. The observation of tumor volume was stopped when the tumor volume of untreated group reached the endpoint was in accord with the National Institutes of Health Office of Animal Care and Use's "Guidelines for Endpoints in Animal Study Proposals."

### *X Irradiation and drug treatment*

X Irradiation and drug treatment of SCID mice were performed as previously described.[16] Briefly, X irradiation was performed with an X ray generator (200 kV, 20 mA, Shimadzu HF-350, Kyoto). A 0.5 mm Cu filter with 0.5 mm Al was used and the dose rate was 0.8 Gy/min, which was determined using Fricke's chemical dosimeter as described previously.[7] The treatment of cells with TRAIL was carried out immediately after X irradiation. An aqueous solution containing 2.5 μg of TRAIL was directly inoculated into solid tumors on the mouse footpads. The solution without TRAIL was used as a control for TRAIL.

### *Immunohistochemical analysis of death receptor 5 (DR5) and caspase-3*

Immunohistochemical analysis of death receptor 5 and caspase-3 was performed as previously described.[16] Briefly, each tumor tissue was excised from mice and fixed in 4% buffered formaldehyde. The excised tumors were embedded in paraffin and sectioned 5 μm thick. After deparaffinization and rehydration, antigen retrieval was performed with a 1 mM EDTA buffer (pH 8.0) or 10 mM sodium citrate buffer (pH 6.0) for 3 min with boiling under high pressure. After quenching endogenous peroxidase activity and blocking nonspecific binding sites, the sections were incubated with primary antibodies overnight at 4°C. Anti-DR5 and anti-cleaved caspase-3 were found to produce optical staining. The sections were reacted with a biotinylated secondary antibody for 1 h at 37°C and then incubated with avidin biotin complex (Vectastain Elite ABC Kit; Vector Laboratories). Immunoreactivity was visualized by incubation with diaminobenzidine. Each stained slide was lightly counterstained with hematoxylin. The sections were analyzed under an Olympus BX51 microscope (Tokyo, Japan).

### *Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining*

TUNEL-staining was performed as previously described.[16] Briefly, each section was incubated with 20 μg/ml proteinase K for 15 min. Endogenous peroxidase was quenched by 1% hydrogen peroxide for 5 min. The sections were incubated with 0.15 U/μL terminal deoxynucleotidyl transferase and 10 μM biotin-deoxyuridine triphosphate for 1 h at 37°C. The sections were covered with 3.3 μg/ml active conjugate of horseradish peroxidase and streptoavidin at the molar ratio of 1.5:1.0 for 30 min. Nuclei of apoptotic cells were detected with diaminobenzidine. The sections were analyzed under an Olympus BX51 microscope (Tokyo, Japan).

*Visualization and numerical analysis of hypoxic regions in tumors in vivo*

To detect hypoxic regions in solid tumors a Hypoxyprobe™-1 kit was used. The staining was done according to the manufacturer's procedures. Numerical analysis of stained tissue was performed using Image J 1.37v software and the percentage of hypoxic regions against total regions in each sample was calculated.

*Immunohistochemical double-fluorescence staining of tumor sections with pimonidazole and caspase-3*

To detect the activation of caspase-3 in hypoxic regions, immunohistochemical analysis with double staining was performed. Briefly, 16 days after inoculation of MKN45 cells, X irradiation and drug treatments were carried out. After 2 days of each treatment, a tumor sections obtained from pimonidazole-treated mice were deparaffinized and rehydrated, and antigen retrieval was performed with 10 mM sodium citrate buffer (pH 6.0) for 3 min with boiling under high pressure. After quenching endogenous peroxidase activity and blocking nonspecific binding sites, the sections were incubated with a mixed solution of anti-cleaved caspase-3 and anti-pimonidazole antibodies overnight at 4°C. The sections were reacted with a mixture of fluorescent secondary antibodies, Alexa fluor 488 anti-mouse IgG and Alexa fluor 546 anti-rabbit IgG from Molecular Probes™ (Carlsbad, CA), for 1 h at 37°C. The sections were analyzed using an Olympus BX51 microscope with fluorescence light (Tokyo, Japan).

*Statistical analysis*

Differences among different groups were analyzed by Student's $t$ test. The minimum level of significance was set at $p < 0.05$.

## RESULTS

*Effects of TRAIL on tumor growth in MKN45 and MKN28 tumor xenografts exposed to X rays*

The tumor growth curves obtained from mice with no treatment (referred to as controls), TRAIL alone, X irradiation alone and X irradiation combined with TRAIL are shown in Fig. 1. The irradiation doses of 2 Gy for MKN45 and 1 Gy for MKN28 and 2.5 μg of TRAIL for both MKN45 and MKN28 were chosen to evaluate the antitumor effects of the combined treatment, because these doses were examined as maximum doses without statistically significant effects on tumor growth (data not shown). As shown in Fig. 1, when Tp53 wild type MKN45 tumors and Tp53 mutated type MKN28 tumors were exposed to 2 Gy and 1 Gy, respectively, the growth curves showed slight deceleration but there were no significant differences at any point on the growth curves between the X-irradiated and unirradiated groups (control). Similarly, in the case of local injection of

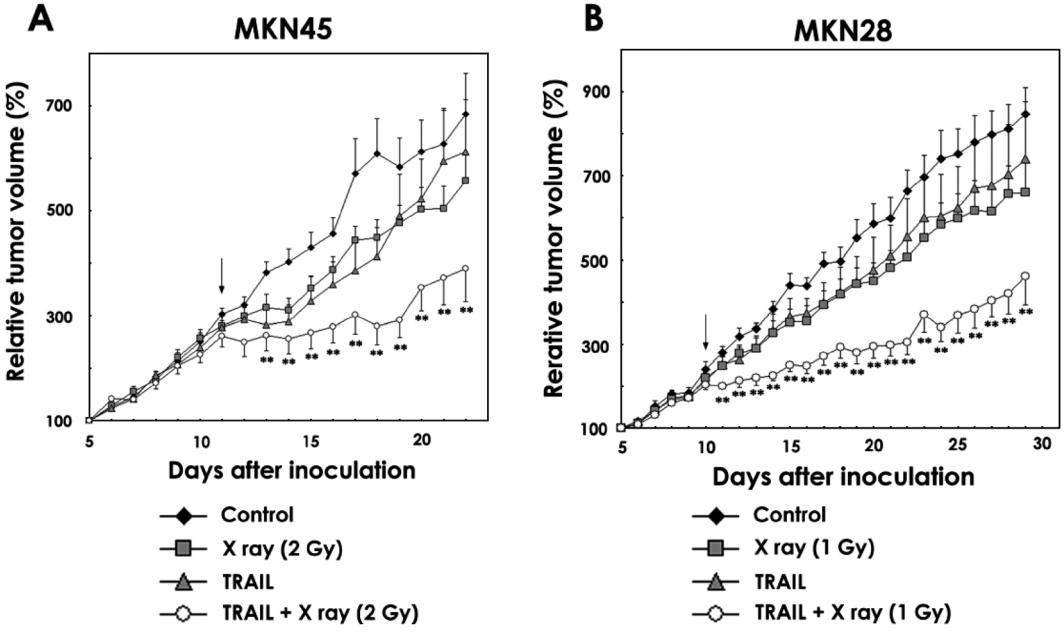



**Fig. 1.** Growth curves of MKN45 tumor xenograft (A) and MKN28 tumor xenograft (B). Tumor volumes were monitored every day, as described in Materials and Methods. Arrows indicate the day of treatment. Vertical bars show standard error (SE). A; Animals were treated with 2 Gy of X rays (n = 9), 2.5 μg of TRAIL (n = 5), or TRAIL (2.5 μg) + X rays (2 Gy) (n = 5), or received no treatment (controls) (n = 10). **: p > 0.01 control vs. TRAIL (2.5 μg) + X rays (2 Gy). B; Animals were treated with 1 Gy of X rays (n = 9), 2.5 μg of TRAIL (n = 5), or TRAIL (2.5 μg) + X rays (1 Gy) (n = 5), or received no treatment (controls) (n = 10). **: p > 0.01 control vs. TRAIL (2.5 μg) + X rays (1 Gy).

2.5 μg of TRAIL into each tumor, we found no significant differences between the TRAIL-injected and untreated groups (control) at any point on the growth curves. X irradiation combined with TRAIL of both MKN45 and MKN28 tumor xenografts, significantly decelerated the tumor growth in comparison with each untreated group (control) as shown in Fig. 1.

### *X Irradiation-induced expression of DR5 in MKN45 tumor xenografts*

Since we have demonstrated that radiation induces the expression of DR5 in *in vitro*,[7,8] we next examined whether radiation also induced its expression *in vivo.* As shown in Fig. 2, the immunohistochemical detection of DR5 revealed that X irradiation induced an increase of accumulation of strong brown deposits in the tumor cells, indicating the expression of DR5 protein, whereas there were few DR5-positive untreated tumor cells (control). These results indicated that radiation induced expression of DR5 protein in the tumor xenograft model as well as in an *in vitro* culture system.

### *Effects of TRAIL on induction of apoptosis and activation of casapse-3 in MKN45 tumor xenografts exposed to X rays*

To obtain evidence for the involvement of apoptotic cell death in the delay of tumor growth by X irradiation combined with TRAIL, we examined its induction in MKN45 tumors using the TUNEL assay. As can be seen in Fig. 3, an increase of apoptotic cells was observed in the MKN45 tumor treated with X irradiation combined with TRAIL. Quantitative analysis (Fig. 3B) revealed that 15% of the cells treated with 2 Gy of X irradiation combined with TRAIL were apoptotic, whereas no increased response of TUNEL-positive cells was found in MKN45 tumors treated with TRAIL alone, and a small increase of apoptotic cells (about 5% of all cells) was found in the case of X irradiation alone. These results indicated that growth delay induced by X irra-

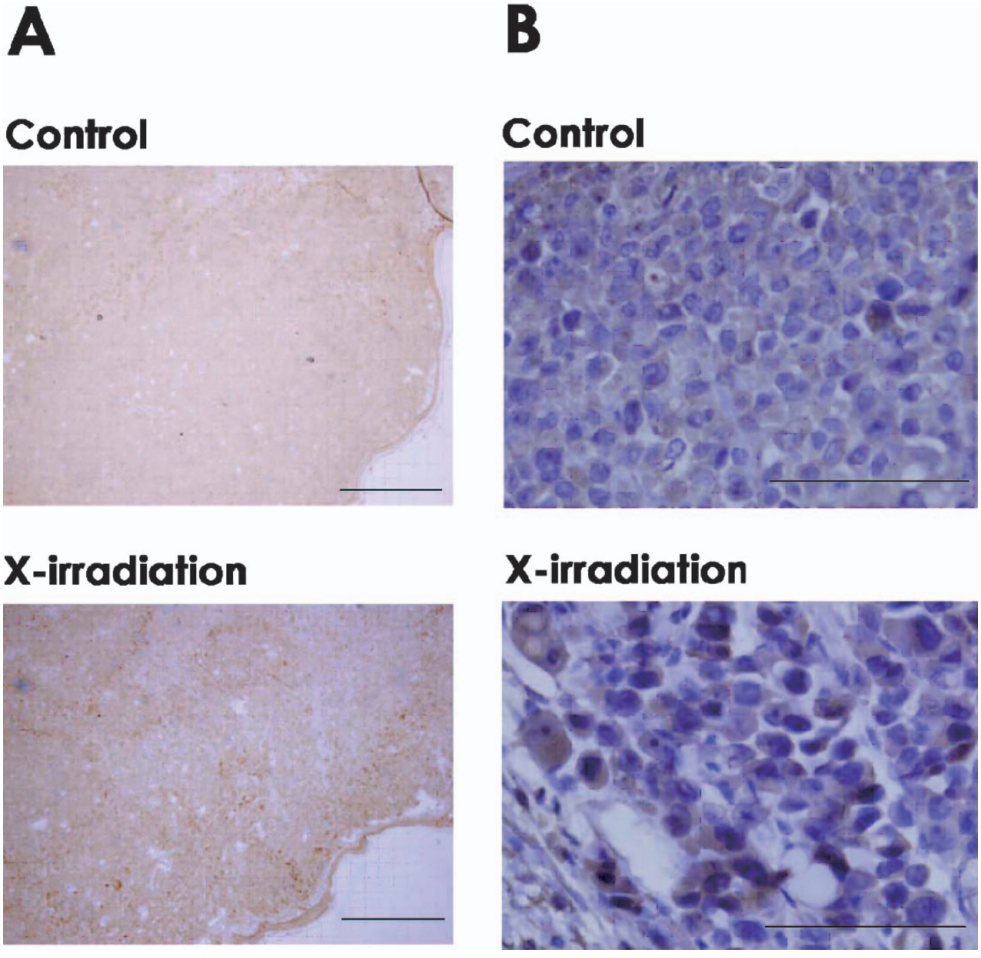



**Fig. 2.** Immunohistochemistry for the expression of DR5 in MKN45 tumors. Tumors were excised 2 days after X irradiation. A, microscopic photographes at 40-fold magnification. Bar = 500 μm. B, microscopic photographes at 200-fold magnification. Bar = 100 μm. In this case, each stained slide was lightly counterstained with hematoxylin.

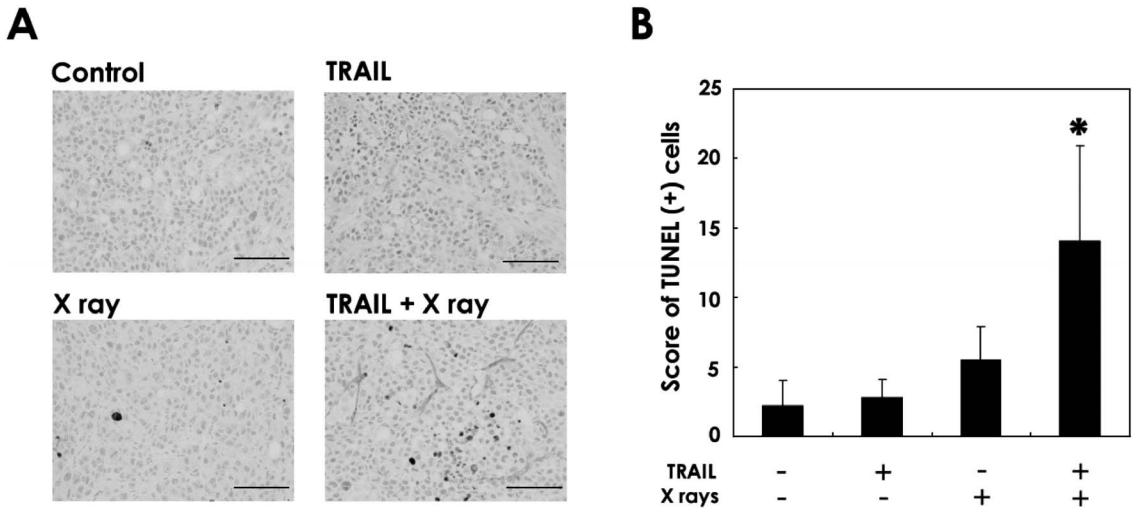



**Fig. 3.** TUNEL assay in MKN45 tumor xenografts treated with TRAIL and/or irradiation. Tumors were excised 2 days after the treatment. A: TUNEL staining on sections of MKN45 tumor tissue. The cells with dark deposits are TUNEL-positive cells. Bar = 100 μm. B: Quantitative analysis of TUNEL-positive cells in MKN45 tissue. The number of TUNEL-positive cells in more than five random fields of each section was calculated and is presented as mean ± SD. *: $p < 0.05$ compared to control.

diation combined with TRAIL was at least partly associated with induction of apoptosis. Furthermore, immunohistochemical detection using a cleaved caspase-3 antibody confirmed the activation of apoptotic signaling in tumor cells exposed to X irradiation or/and TRAIL. In fact, a significant increase in cleaved caspase-3-positive cells was observed in the tumor treated with X irradiation combined with TRAIL, whereas TRAIL alone or X irradiation alone induced no cleaved caspase-3 positive cells (Fig. 4). These results indicated that apoptosis induced by treatment with X irradiation and TRAIL was due to the activation of caspase-3.

*Effects of TRAIL on hypoxic area in the MKN45 tumor xenograft exposed to X rays*

The effects of the combination of X irradiation and TRAIL on the hypoxic region in tumor tissue were exam-

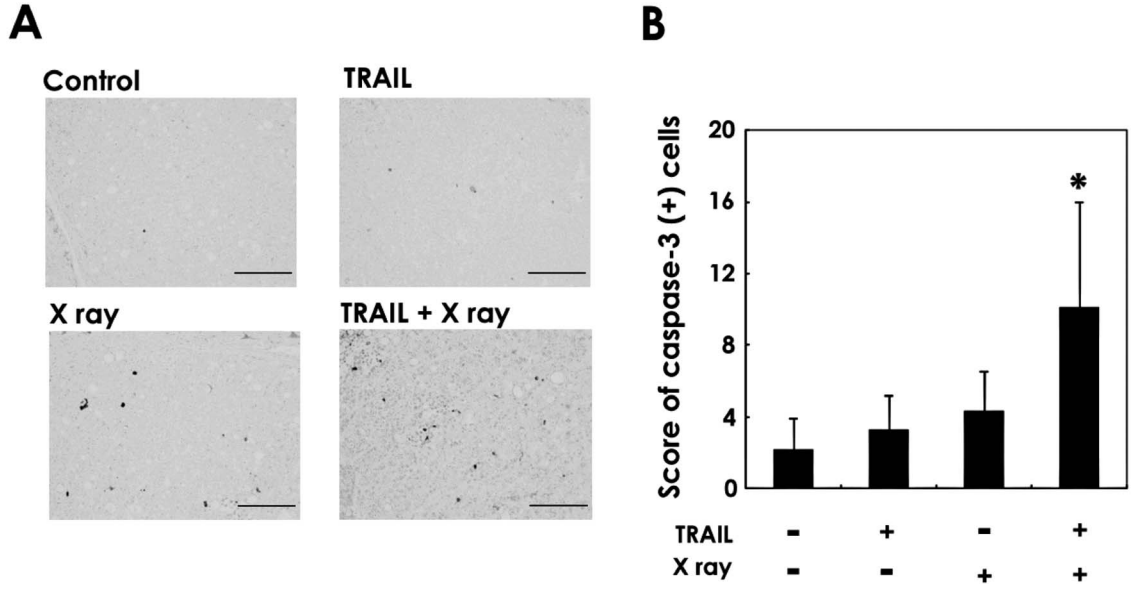
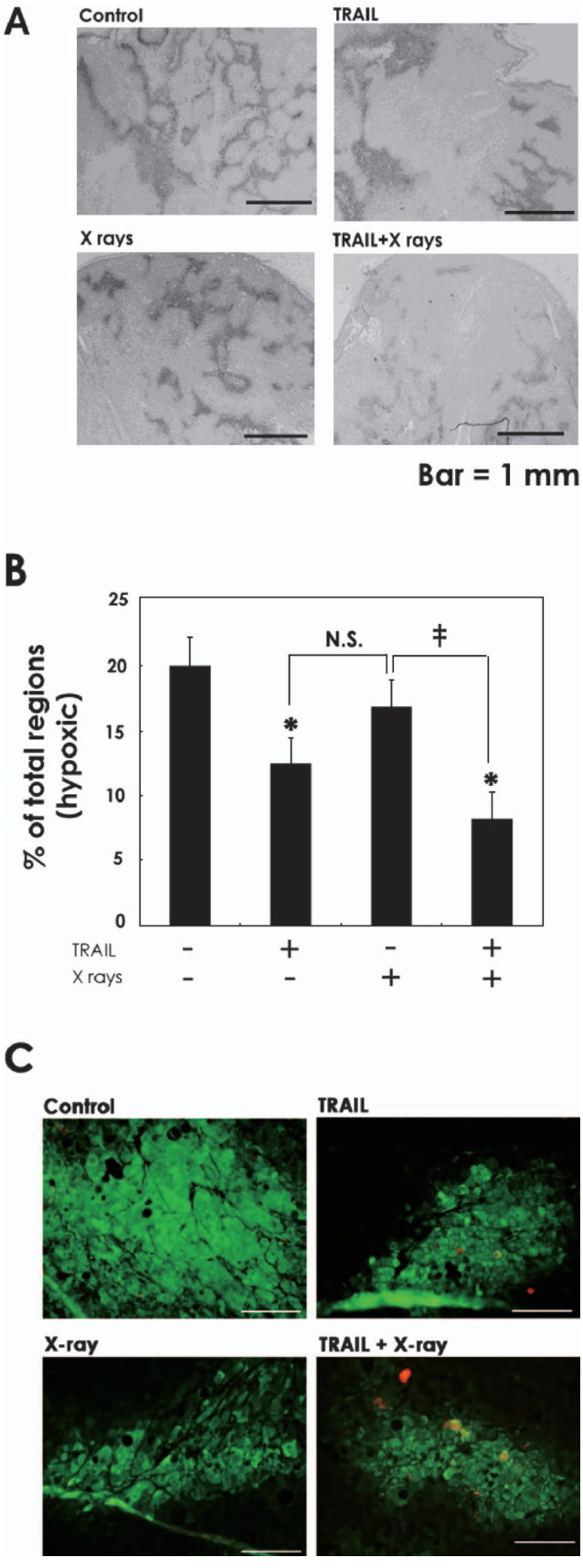



**Fig. 4.** Immunohistchemistry for caspase-3 in MKN45 tumors. Tumors were excised 2 days after the treatment. A: Immunohistochemical staining on section of MKN45 tumor tissue. Bar = 100 μm. B: Quantitative analysis of cells with activated caspase-3 in MKN45 tissue. The number of caspase-3-positive cells in more than five random fields of each section was calculated and is presented as mean ± SD. *: $p < 0.05$ to control.




ined. As shown in Fig. 5, immunohistochemical staining for the hypoxic region using the hypoxic marker pimonidazole showed that the combination of X irradiation and TRAIL reduced the area of this region in the MKN45 tumor compared with that of untreated one (control). Interestingly, the tumor treated with TRAIL alone showed a significant reduction of the hypoxic area compared with the untreated one (control), although this reduction induced by TRAIL alone did not reach that induced by X irradiation combined with TRAIL (Fig. 5B). Furthermore, to examine whether apoptosis occurred in hypoxic regions in tumors exposed to X irradiation and TRAIL, immunohistochemical double-fluorescence staining with pimonidazole (green) and cleaved caspase-3 (red) was performed. Figure 5C clearly shows that caspase-3 positive cells in hypoxic regions (yellow) were mainly observed in tumors treated with X irradiation combined with TRAIL rather than in those treated TRAIL alone or X irradiation alone. These results proved that the combination of X irradiation and TRAIL brought about the efficient induction of apoptosis in hypoxic regions.

## DISCUSSION

In lymphoblast MOLT-4[17–20] and Jurkat cells[21] exposed to ionizing radiation, not only the mitochondria-mediated Tp53-dependent apoptotic pathways (intrinsic pathways) but also death-inducing signaling complex (DISC)-mediated apoptotic pathways (extrinsic pathways) were reported to be activated. Recently, X irradiation was shown to induce the expression of death receptors such as Fas and DR5 on the plasma surface in solid tumor cell lines, *i.e.*, gastric cancer cell lines MKN45 and MKN28, lung cancer cell line A549 and prostate cancer cell line DU145, and subsequent treatment with the Fas agonistic antibody CH11 and TRAIL enhanced radiation-induced apoptosis of these cell lines.[8] Since MKN28 and DU145 are tumor cells with mutated Tp53, the enhancement of radiation-induced cell killing by TRAIL was independent of Tp53 status in the tumors.[8] Furthermore, we showed that treatment with TRAIL enhanced the loss of clonogenic ability in X-irradiated A549 cells.[7] In

**Fig. 5.** Effect of the combination of TRAIL and X rays on hypoxic regions of MKN45 tumors. Tumors were excised 13 days after the treatment. A: immunohistochemical staining with pimonidazole on sections of MKN45 tumor tissue. B: Quantitative analysis of hypoxic regions in MKN45 tissue. The percentage of hypoxic regions in whole section was calculated as described in Materials and Methods and is presented as mean ± SD. *: $p < 0.05$ compared to control. ‡: $p < 0.05$ compared to X rays. N.S.: Not significant. C: Tumors were excised 2 days after X rays, TRAIL and TRAIL + X rays, respectively. Immunohistochemical analysis was performed by using a double-fluorescence technique with pimonidazole (green), cleaved caspase-3 (red) and double-positive cells (yellow) in MKN45 tissue. Each bar indicates 100 μm.

the present study, we demonstrated that X irradiation combined with TRAIL induced deceleration of tumor growth followed by the induction of capsase-3-dependent apoptosis in gastric adenocarcinoma tumor xenografts, whereas X irradiation alone or TRAIL alone did not significantly reduce tumor growth. Since this TRAIL-induced delay of growth in X-irradiated tumors was observed in not only Tp53 wild type MKN45 tumors but also in Tp53 mutated MKN28 tumors, the TRAIL-induced growth delay occurred in a Tp53-independent manner. This means that X irradiation combined with TRAIL has an advantage in therapy for Tp53-mutated tumors because radioresistance in many tumors is considered to be partly caused by loss of Tp53 functions.

In previous *in vitro* experiments, we have reported that the expression of Fas and DR5 on the plasma membrane increases in X-irradiated A549, MKN45, MKN28 and DU145 cells and that the mechanism for the radiation-induced expression of these death receptors is associated with redox regulation because treatment with N-acetyl-L-cysteine (NAC) blocked the radiation-induced expression of death receptors.[8,20] In the present study using tumor xenografts, the expression of DR5 after irradiation was observed as shown in Fig. 2, and the caspase-3-dependent induction of apoptosis in tumors exposed to X irradiation combined with TRAIL was observed as shown in Figs. 3 and 4, similar to that in *in vitro* experiments. From these results, it was suggested that the enhancement of radiation-induced caspase-3-dependent apoptosis by TRAIL was associated with an increase of susceptibility to TRAIL due to the radiation-induced overexpression of functional DR5 on the plasma membrane.

In various tumor xenograft models, *i.e.*, Ewing's sarcoma,[22] prostate cancer[23] and non-small cell lung cancer,[24] TRAIL treatment was reported to be able to induce growth delay of tumors. Our results also showed that X irradiation and local injection of TRAIL significantly decelerated tumor growth in gastric tumor xenografts of MKN45 and MKN28, as shown in Fig. 1. These results suggested that TRAIL was a useful substance to reduce the tumor volume on various tumors. However, it was still unclear whether TRAIL was effective in hypoxic regions of tumor xenografts. Therefore, we measured the hypoxic areas in tumor xenografts after treatment with X irradiation alone, TRAIL alone and X irradiation combined with TRAIL with hypoxia marker pimonidazole. The results presented in Fig. 5 provided evidence that TRAIL alone significantly reduced hypoxic area and X irradiation further promoted TRAIL-induced reduction of the area, though X irradiation alone exert no influence on the hypoxic region. In untreated control, rapid tumor growth is generally considered to produce the hypoxic area that oxygen was not insufficiently supplied because of relative low density in blood vessels. Contrast to this, it seems that the combination of X-irradiation and TRAIL significantly inhibited tumor growth as shown in Fig. 1A and the percentage of hypoxic region in total tumor area was relatively small as shown in Fig. 5A and 5B. Unexpectedly, we observed that the treatment with TRAIL alone significantly reduced hypoxic area. This may be explained by the hypoxia-induced expression of DR5, since Cao *et al.*[25] showed the increased DR5 mRNA in a hepatocyte cell line after long-term hypoxia. Lee *et al.* also demonstrated that hypoxia-related situations such as low extracellular pH[26] and a low concentration of glucose[27] influenced TRAIL-induced apoptotic cell death in human prostate adenocarcinoma DU145 cells and LNCaP cells. However, the treatment of TRAIL alone did not significantly inhibit the cell growth in Fig. 1, although this treatment significantly reduced hypoxic area as mentioned above. This may be due to insufficient expression of DR5 in tumor cells without X irradiation as shown in Fig. 2.

The existence of hypoxic regions in solid tumors is well recognized as one of the causes of radioresistance.[1,2,5,6] Recently, as a mechanism for the radioresistance of apoptosis, it was reported that chronic hypoxia protected human head and neck squamous cell carcinoma against the inhibition of mitochondrial translocation of bax protein induced by γ-irradiation.[28] Furthermore, the combination of radioiodide and angiogenic inhibitors has been shown to enhance apoptosis in tumor cells through the HIF-1α-regulated apoptotic pathway.[29] As shown in Fig. 5, the TRAIL treatment overcame the radioresistant hypoxic region by the induction of caspase-3-dependent apoptosis in tumors exposed to X rays. Our observations described here are the first report providing evidence that TRAIL treatment efficiently induces apoptosis and reduces hypoxic area in xenografts. Recently, fractionated stereotactic radiotherapy has attracted increasing interest, because the use of multiple fractions improves the oxygenation status of the hypoxic cells and reduces the damage to the critical late-responding normal tissues.[30] The present study shows that a single irradiation with a single injection of TRAIL decreased tumor growth and increased apoptotic cell death. Therefore, the treatment of fractionated irradiation and repetitive injection of TRAIL may be useful to conquer the resistance in the hypoxic regions in solid tumors in practical tumor therapy.

In summary, we clearly demonstrated that X irradiation combined with TRAIL-induced decreases of tumor volumes in gastric adenocarcinoma xenograft models. This occurred in a Tp53-independent manner, since this growth delay was observed in Tp53 mutated MKN28 tumors. Moreover, this treatment reduced the hypoxic area in the xenograft. These results indicate that X irradiation combined with TRAIL might be a useful treatment to reduce tumor growth in not only normoxic but also hypoxic regions in radiotherapy.

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