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Enhancement of Cell Death by TNF α-related Apoptosis-inducing Ligand (TRAIL) in Human Lung Carcinoma A549 Cells Exposed to X Rays under Hypoxia

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TRAIL/Radiation/Caspase/Hypoxia.

Our previous study showed that ionizing radiation induced the expression of death receptor DR5 on the cell surface in tumor cell lines and that the death receptor of the TNF α-related apoptosis-inducing ligand TRAIL enhanced the apoptotic pathway (Hamasu *et al.,* (2005) *Journal of Radiation Research,* **46**:103–110). The present experiments were performed to examine whether treatment with TRAIL enhanced the cell killing in tumor cells exposed to ionizing radiation under hypoxia, since the presence of radioresistant cells in hypoxic regions of solid tumors is a serious problem in radiation therapy for tumors. When human lung carcinoma A549 cells were irradiated under normoxia and hypoxia, respectively, radiation-induced enhancement of expression of DR5 was observed under both conditions. Incubation in the presence of TRAIL enhanced the caspase-dependent and chymotrypsin-like-protease-dependent apoptotic cell death in A549 cells exposed to X rays. Furthermore, it was shown that treatment with TRAIL enhanced apoptotic cell death and loss of clonogenic ability in A549 cells exposed to X rays not only under normoxia but also under hypoxia, suggesting that combination treatment with TRAIL and X irradiation is effective for hypoxic tumor cells.

INTRODUCTION

Ionizing radiation has been widely used as a tool for tumor treatment. When tumors are irradiated, induction of apoptosis is generally considered to occur in tumor cells through a mitochondria-dependent signaling pathway and radiation-induced double-strand breaks of DNA are known to induce loss of clonogenic ability. Recently, however, genotoxic anti-tumor reagents, *e.g.*, camptothecin,[1)] cisplatin,[2)] ultraviolet light,[3)] 2-chlorodeoxyadenosine[4,5)] and etoposide,[6)] etc., have been reported to induce Fas clustering or aggregation without Fas ligand (FasL), followed by apoptosis through the formation of death-inducing signaling complex (DISC) containing trimerized Fas to activate procaspase-8.

Our recent reports have shown that ionizing radiation also induces increased expression of Fas, and that FasL-independent formation of DISC leads to apoptosis in human leukemia cell line MOLT-4. Treatment with the antioxidant N-acetyl-L-cysteine (NAC) inhibits this radiation-induced increase of the expression of Fas and apoptosis, suggesting that modulation of the intracellular redox state regulates this radiation-induced FasL-independent apoptosis in MOLT-4.[7,8)] Moreover, in solid tumor cell lines, we demonstrated that exposure to ionizing radiation induced cell surface expression of not only Fas but also DR5.[9)] Distinct from the case of MOLT-4, treatments using agonists for the death receptors, *i.e.*, FasL, an agonistic antibody for Fas and TNF α-related apoptosis-inducing ligand (TRAIL), enhanced the radiation-induced apoptosis in solid tumor cell lines. This enhancement of radiation-induced apoptosis by treatment with a death receptor activator or ligand is observed in not only p53 wild-type cell lines MKN45 and A549, but also p53-mutated cell lines MKN28 and DU145, suggesting the

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Abbreviations: TRAIL, TNF α-related apoptosis-inducing ligand; TNF α, tumor necrosis factor α; NF-κB, nuclear factor-κB; Z-Asp-CH_2-DCB, benzyloxycarbonyl-L-Asp-1-yl-[(2,6-dichlorobenzoyl)oxy]methane; Ac-DEVD-CHO, acetyl-Asp–Glu–Val–Asp-aldehyde; Ac-IETD-CHO, acetyl-Ile–Glu–Thr–Asp-aldehyde; Ac-LEHD-CHO, acetyl-Leu–Glu–His–Asp-aldehyde; TPCK, N^{α}-tosyl–Phe– chloromethylketone; TLCK, N^{α}-tosyl–Lys–chloromethylketone; AEBSF, 4-(2-aminoethyl)benzenesulfonylfluoride.

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possibility of tumor therapy against p53-mutated tumors.[9,10] This phenomenon may be explained by the expression of DR5, which is regulated not only by p53[11] but also NFκB, AP-1 and SP-1.[12,13]

Recent studies have identified four distinct cell surface TRAIL receptors on the cell surface: DR5, DR4, DcR1 and DcR2.[14–16] While TRAIL binds to all of the receptors, the functions of their intracellular domains are not uniform. It was reported that the ligation of TRAIL with DR5 and DR4 was essential for induction of apoptosis, although DcR1 and DcR2, which lack a cytoplasmic domain, might serve as 'decoys' that competed with DR5/DR4 for binding to TRAIL. The sensitivity of TRAIL in regulating apoptosis is hypothesized to be due to the higher expression of decoy receptors in normal cells and the absence of or lower expression of these receptors in tumor cells.[11,17–19]

These reports lead us to postulate that stimulation by DR5 of tumor cells exposed to ionizing radiation may be an ideal treatment for enhancement of cell killing without adversely affecting normal tissue. However, the presence of radioresistant cells in hypoxic regions of solid tumors is well-known to be a serious problem in tumor radiotherapy.[20,21] Therefore, it is important to determine whether treatment with TRAIL enhances cell killing in tumor cells exposed to ionizing radiation under hypoxia. In this work, the effect of TRAIL treatment on apoptotic and reproductive cell death in human lung carcinoma A549 cells exposed to X rays under hypoxia was compared with that under normoxia.

MATERIALS AND METHODS

Chemicals

TRAIL was obtained from Alexis Biochemicals (Montreal, Canada). Propidium iodide (PI) was purchased from Sigma (St Louis, MO). Nitrocellulose membranes were from ADVANTEC Toyo (Tokyo, Japan). An anti-DR5 polyclonal antibody was obtained from Alexis Biochemicals (Montreal, Canada). An Alexa fluor 488 anti-goat antibody was obtained from Molecular Probes (Eugene, OR). Anti-caspase-3 was obtained from Cell Signaling Technology (Beverly, MA). Anti-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The protein assay kit was obtained from Bio-Rad (Brea, CA). Other reagents were obtained from Wako Pure Chemicals Co. (Tokyo, Japan).

Cell culture

Human lung carcinoma cell line A549 was generously provided by the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). The cells were grown in RPMI 1640 medium containing 10% fetal bovine serum at 37°C in 5% CO_2.

X Irradiation under hypoxia and treatment with TRAIL

X irradiation was performed under ice-cold temperature with an X ray generator (200 kVp, 20 mA, Shimazu HF-350, Kyoto). A 2.0 mm aluminum (Al) filter was used and the dose rate was 3.0 Gy/min, which was determined by Fricke's chemical dosimeter. In the case of irradiation under hypoxia, cells on a 6-cm plastic dish were placed in a specially designed gas-exchangeable chamber in which hypoxia (oxygen concentration = 3 mmHg, measured by YSI Model 5300 oxygen monitor) was achieved by continuously passing 99.999% nitrogen gas for 30 min at ice-cold temperature before and during irradiation. Treatment of cells with TRAIL was carried out in growth medium in 5% CO_2 in air at 37°C for the indicated time immediately after X irradiation.

Fluorescence microscopic observation of apoptotic cells

Cells incubated for 48 h after X irradiation were collected by centrifugation at ×200 for 5 min at 4°C. The pellet was washed with PBS(–), and fixed with 70% ethanol at –20°C. The fixed cells were washed, resuspended in PBS(–) and stained with 1 mg/ml PI for 30 min in the dark. The stained cells were placed on a microscopic slide and gently covered with a coverslip. Fluorescence microscopic observation and capture of photographs were performed with a microscope (BX-51, Olympus Optical Co., Ltd, Tokyo) with reflected light fluorescence to take photographs of cells with chromatin condensation and nuclear fragmentation as apoptotic cells.

Measurement of apoptotic cells by flow cytometry

Cells incubated for 10, 24, 48 and 72 h after X irradiation were collected by centrifugation at ×200 for 5 min at 4°C. The pellet was washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, PBS(–), and fixed with 70% ethanol at –20°C. The fixed cells were washed once and resuspended in PBS(–) with 10 mg/ml RNase (DNase-free) and incubated for 30 min at 37°C. The incubated cells were stained with 1 mg/ml PI for 30 min in the dark. The stained cells were resuspended in PBS(–) and analyzed using an EPICS XL flow cytometer (Beckman Coulter Inc., Fullerton, CA).

Detection of DR5 on the cell surface

The expression of DR5 on the cell surface was examined by flow cytometry. The X-irradiated cells were incubated with 100 μl of IF buffer (PBS(–), 1% BSA and 0.1% sodium azide) containing the anti-DR5 antibody for 45 min on ice. After a wash with PBS(–), the cells were incubated with the Alexa Fluor 488 anti-goat antibody for 45 min on ice. Each sample was washed and resuspended in PBS(–) and analyzed using an EPICS XL flow cytometer.

SDS-PAGE and western blotting

Western blotting for each protein was performed as described previously.[5,9] Briefly, collected cells were washed with PBS(–). The cells were resuspended in 50 μl of cell lysis buffer (20 mM HEPES, 2 mM EGTA, 1% Triton X-100, 10% glycerol, and 1 mM DDT) and centrifuged at

15,000 rpm for 10 min at 4°C. The protein concentration of the supernatant was determined using a Bio-Rad protein assay kit. The sample for SDS-PAGE was prepared by adding 3 × SDS loading buffer (187.5 mM Tris-HCl, pH 6.8, 30% glycerol, 6% SDS, 15% β-mercaptoethanol and 0.003% bromophenol blue) to the cell lysate. Proteins were separated by SDS-PAGE after boiling for 3 min and electrically transferred onto a nitrocellulose membrane. The membrane was probed with each antibody in TBST buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.1% Tween-20) containing nonfat skim milk or BSA at a suitable concentration. Proteins were detected by a method combining HRP-conjugated secondary antibodies and a chemiluminescence detection kit.

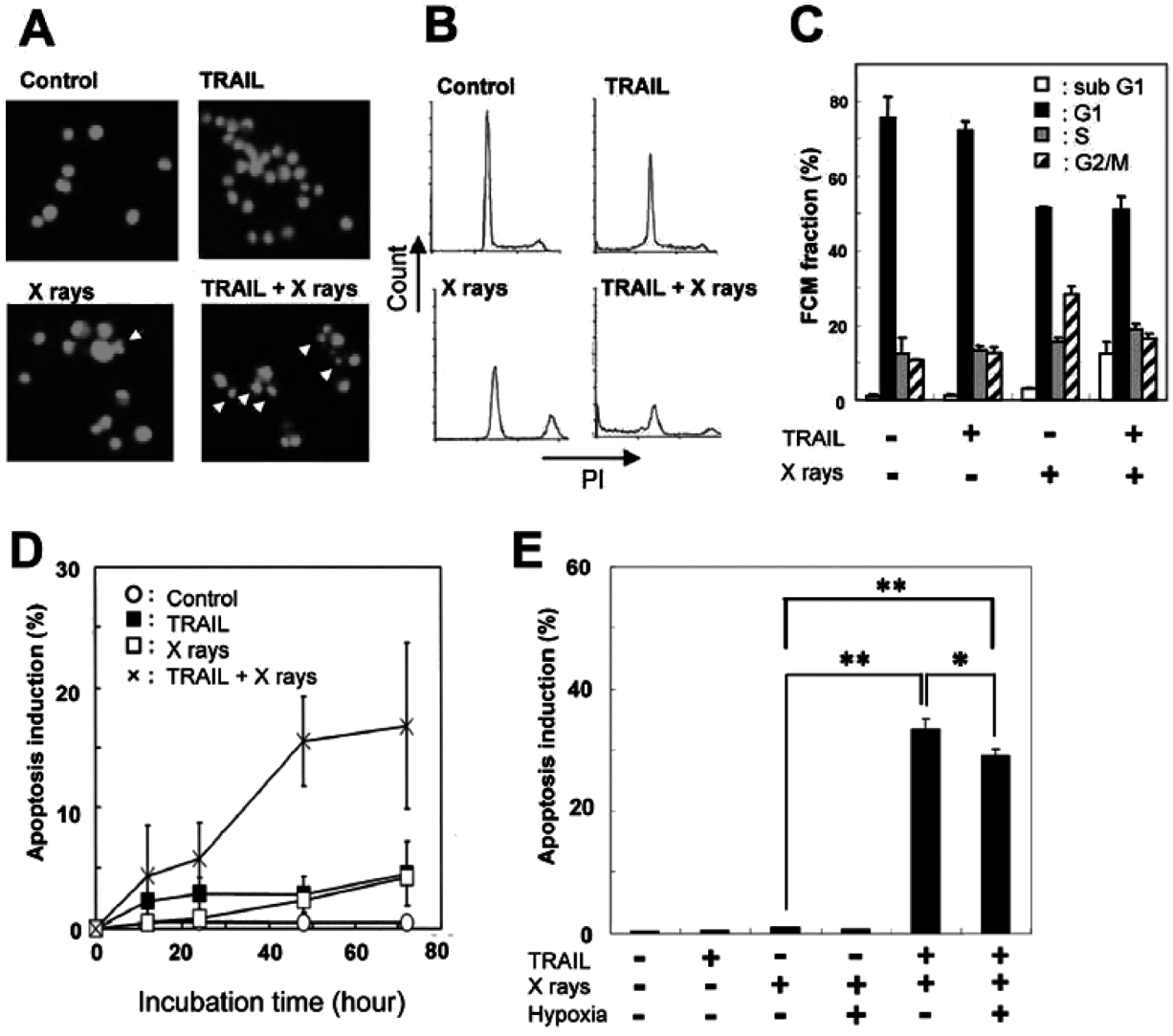

Fig. 1. TRAIL-induced enhancement of apoptotic cell death in A549 cells exposed to X rays. **(A)** Morphological changes in A549 cells induced by TRAIL and/or irradiation. The cells were treated with TRAIL (100 ng/ml), X rays (10 Gy) and TRAIL (100 ng/ml) + X rays (10 Gy), respectively. After incubation for 48 h, the cells were stained with propidium iodide (PI) and the morphological changes of nuclei were detected by fluorescence microscopy. Arrows indicate the cells with typical apoptotic morphological changes, chromatin condensation and fragmentation. **(B)** Flow cytometric profiles for cell cycle and apoptosis in A549 cells at 48 h after treatment with TRAIL, X rays and TRAIL + X rays, respectively. After the cells were fixed and stained by propidium iodide (PI), 10^4 cells were analyzed by flow cytometry. **(C)** Summarized data of the flow cytometric analysis in A549 cells at 48 h after treatment with TRAIL, X rays and TRAIL + X rays, respectively. The FCM fraction in the vertical axis is indicated as a percent of total cells. Data are expressed as mean ± standard deviation (SD) for three independent experiments. **(D)** Time dependent of apoptosis induction in A549 cells after treatment with TRAIL, X rays and TRAIL + X rays, respectively. **(E)** Effect of hypoxia on apoptosis induced by the combination treatment with TRAIL and X rays in A549 cells. Amounts of apoptosis in **(D)** and **(E)** were evaluated using the sub-G1 fraction obtained by the flow cytometric analysis. Data are expressed as mean ± SD for three independent experiments. *: $P < 0.05$; **: $P < 0.01$ significant difference by Student's *t*-test.

Clonogenic assay

Fifty to 3,000 A549 cells were seeded in 6-cm plastic dishes and irradiated with X rays at doses of 0 to 15 Gy. Immediately after irradiation under normoxia or hypoxia at ice-cold temperature as described above, medium in each well was changed to fresh medium with or without 100 ng/ml TRAIL; then the cells were incubated for 8 to 14 days in 5% CO_2 at 37°C. The cells were fixed with methanol and stained with Giemsa. Only colonies containing more than 50 cells were scored as surviving cells. The surviving fraction at each dose was calculated with respect to the plating efficiency of the control and the dose response curves were plotted.

RESULTS

TRAIL-induced increase of apoptosis in A549 cells exposed to X rays under normoxia and hypoxia

After A549 cells were exposed to 10 Gy of X rays alone or 100 ng/ml TRAIL alone and incubated for 24 h, morphological changes such as chromatin condensation and fragmentation of nuclei were hardly observed as shown in Fig. 1A, indicating that there were few apoptotic cells induced by X rays alone or TRAIL alone. However, the combined treatment with TRAIL and X rays (TRAIL + X rays) significantly induced typical apoptotic morphological changes with chromatin condensation and fragmentation. Figure 1B shows flow cytometoric profiles of A549 cells treated with X rays, TRAIL and TRAIL + X rays, respectively. The flow cytometric profile obtained from the cells treated with TRAIL alone shows a slight decrease of the sub-G1 fraction, but there was no significant difference from the untreated control as shown in Fig. 1C. X Irradiation increased the fraction of G2/M-phase cells, indicating the occurrence of radiation-induced G2/M arrest in the A549 cells. Interestingly, the TRAIL treatment reduced this radiation-induced increase of the G2/M fraction, and increased the sub-G1 fraction, indicating that the treatment converted the G2/M phase cells arrested by X irradiation to apoptotic cells. A large amount of the sub-G1 fraction induced by the treatment with TRAIL + X rays was observed at 48 h after irradiation as shown in Fig. 1D, whereas TRAIL alone or X rays alone induced a marginal increase of this fraction. Moreover, we tested whether the TRAIL treatment enhanced the apoptotic fraction in the cells exposed to X rays under hypoxia. As shown in Fig. 1E, the amount of apoptosis in the cells exposed to TRAIL + X rays under hypoxia was significantly higher than that of X rays alone under hypoxia, although it was significantly smaller than that of TRAIL + X rays under normoxia.

Expression of DR5 in A549 cells exposed to radiation under normoxia and hypoxia

Since our previous study showed that ionizing radiation induced DR5 expression on plasma membranes of various solid tumor cell lines,[9] we confirmed the radiation-induced enhancement of DR5 expression in A549 cells. As shown in Fig. 2A, flow cytometric analysis showed that X irradiation

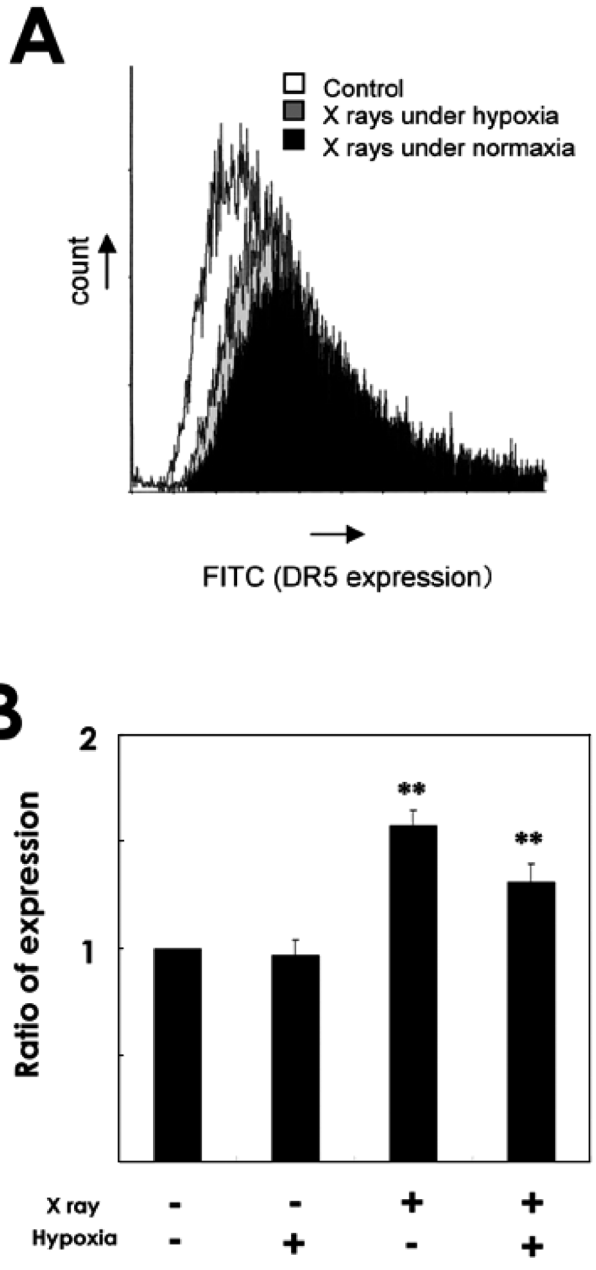

Fig. 2. X Irradiation induces the expression of death receptor DR5 in A549 cells. **(A)** Flow cytometric analysis of expression of DR5 on the cell surface of A549 cells exposed to 10 Gy of X rays under normoxia (Black) or hypoxia (Gray). Flow cytometric profile indicated by the white area shows the constitutive expression level of DR5 in A549 cells without irradiation (control). **(B)** The expression of DR5 in A549 cells. Each data was calculated from average of fluorescence intensity, representing the amount of DR5 expression on cell surface. Data are expressed as mean ± SD for three independent experiments. **: $P < 0.01$ significant difference by Student's *t*-test.

under normoxia increased DR5 expression on the cell surface on the plasma membrane in A549 cells. In addition, X irradiation also increased DR5 expression on the cell surface under hypoxic conditions in comparison with the unirradiated control, but this DR5 expression under hypoxia did not reach the expression level induced by X irradiation under normoxia. As shown in Fig 2B, the DR5 expression under normaxia and hypoxia was 1.6 ± 0.08 and 1.3 ± 0.09 fold increase in comparison with that of control without X rays and hypoxia, respectively.

Involvement of caspase- and chymotrypsin-like serine protease in apoptosis induced by the combination of TRAIL and X rays.

To explore the involvement of proteases in apoptosis induced by TRAIL + X rays, we used various protease ihibitiors, Z-Asp-CH_2-DCB for global caspase, Ac-DEVD-CHO for caspase-3, Ac-IETD-CHO for caspase-8, Ac-LETD-CHO for caspase-9, TPCK for chymotrypsin-like serine protease, TLCK for trypsin-like protease and AEBSF for general serine protease. As shown in Fig. 3A, apoptosis induced by the treatment with TRAIL + X rays was significantly inhibited by Z-Asp-CH_2-DCB, Ac-DEVD-CHO, Ac-IETD-CHO and TPCK. In contrast, Ac-LETD-CHO did not inhibit this apoptosis, and TLCK and AEBSF enhanced the apoptosis induced by TRAIL + X rays in A549 cells. Treatment with an inhibitor alone did not influence induction of apoptosis in A549 cells (data not shown).

Furthermore, involvement of caspase-3 in apoptosis was also confirmed by western blotting as shown Fig. 3B. Although the treatment with TRAIL alone induced cleavage of procaspase-3 to the p20 subunit, TRAIL + X rays induced further processing of p20 to the p17 subunit. These findings suggested that cleavage to p17 subunits is necessary for apoptosis.

Effects of TRAIL on clonogenic ability in A549 cells exposed to X rays under normoxia and hypoxia

To clarify the effectiveness of the TRAIL + X ray treatment for proliferative cell death under normoxia and hypoxia, we tested the clonogenic ability of A549 cells exposed to X rays under normoxia and hypoxia. In the experiment without exposure to X rays, TRAIL alone, hypoxia alone and TRAIL + hypoxia did not affect colony-forming ability (Fig. 4A). The survival cuuves in A549 cells exposed to X rays under normoxia with and without TRAIL are shown in Fig. 4B. The results showed that the clonogenic ability in the presence of TRAIL was significantly decreased in comparison with that without TRAIL. The survival curves in A549 under hypoxia also showed that the treatment with TRAIL significantly decreased clonogenicity in comparison with that without TRAIL (Fig. 4C). The values of D_0 calculated from survival curves of -TRAIL/normaxia, +TRAIL/normaxia, -TRAIL/hypoxia and +TRAIL/hypoxia were 4.9 Gy, 3.2 Gy, 9.1 Gy and 5.5 Gy, respectively. These results clearly show that TRAIL significantly enhances loss of clonogenic ablity of tumor cells exposed to radiation not only under normoxia but also hypoxia.

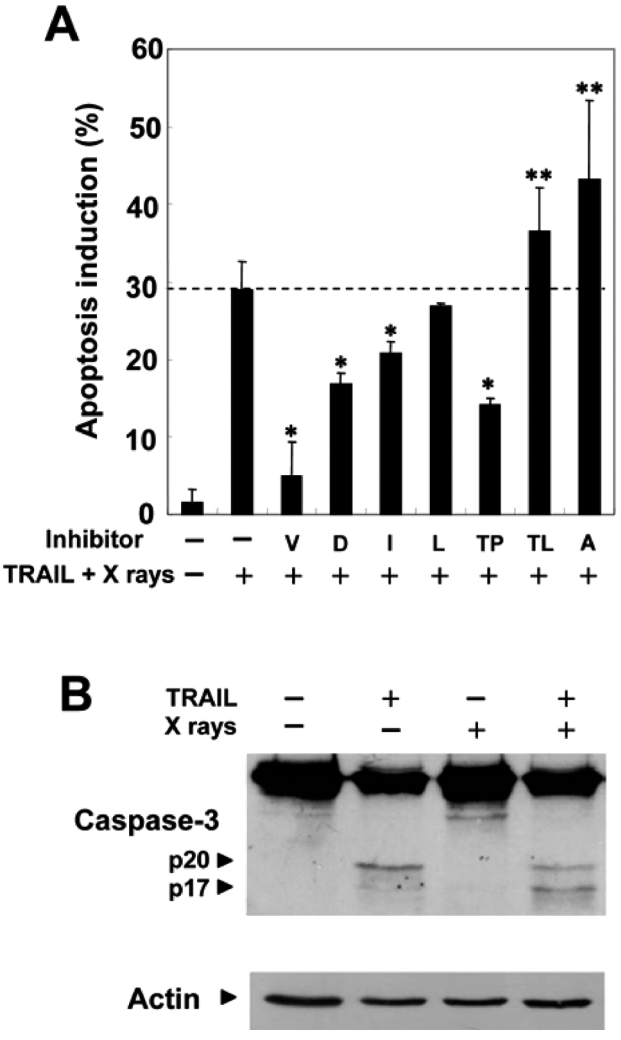

Fig. 3. Apoptosis induced by the combination of TRAIL and X rays is dependent on caspase-8/-3 and chymotrypsin-like serine protease. **(A)** Inhibition of apoptosis in A549 cells by specific caspase inhibitors or serine protease inhibitors. The cells irradiated with 10 Gy of X rays were incubated with 100 ng/ml TRAIL and a protease inhibitor, **V:** Z-Asp-CH_2-DCB, **D:** Ac-DEVDCHO, **I:** Ac-IETD-CHO, **L:** Ac-LEHD-CHO, **TP:** TPCK, **TL:** TLCK and **A:** AEBSF. Each inhibitor was used in this experiment at 50 μM. At 48 h after incubation, the apoptotic cells were evaluated by the sub-G1 fraction obtained from flow cytometric analysis. Data are expressed means ± SD for three independent experiments. *: $P < 0.05$: significant difference by Student's *t*-test. **(B)** Western blot analysis for the active fragments of caspase-3 in A549 cells at 48 h after treatment with 100 ng/ml TRAIL alone, 10 Gy of X rays alone and the combination of TRAIL and X rays.

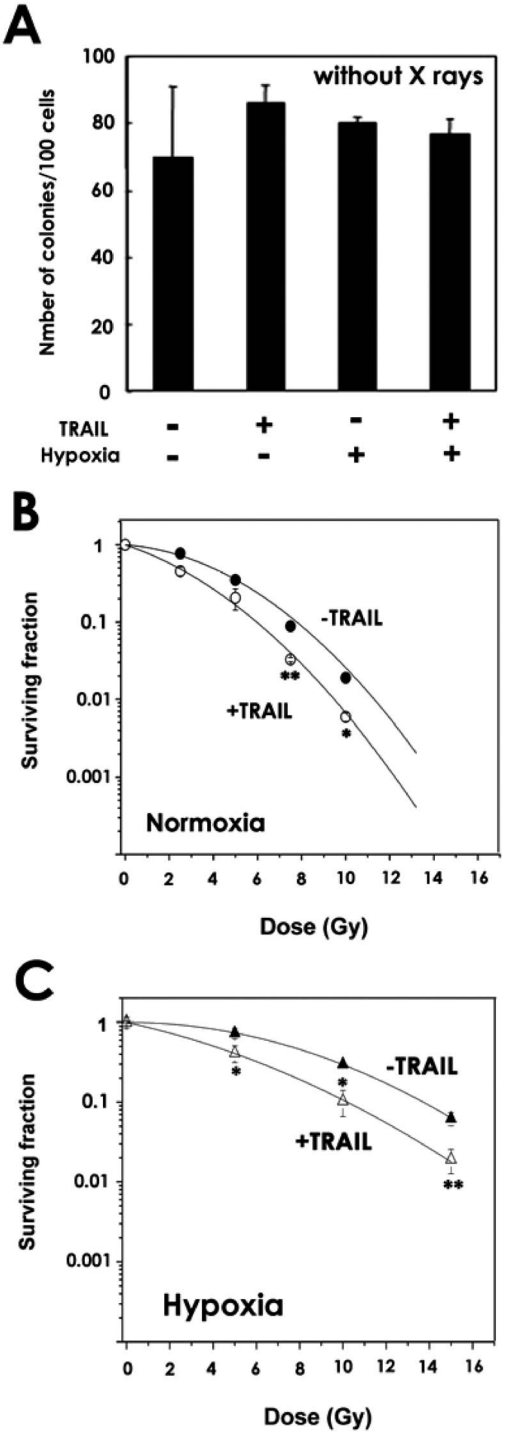

Fig. 4. The TRAIL-induced enhancement of loss of clonogenic ability in A549 cells exposed to X rays under normoxia and hypoxia. Hypoxia was as described in Materials and Methods. **(A)** The colony-forming ability of A549 cells under normoxia or hypoxia without irradiation. Survival curves of A549 cells exposed to X rays under **(B)** normoxia and **(C)** hypoxia, with or without TRAIL (100 ng/ml). Data are expressed as mean ± SD for three independent experiments. *: $P < 0.05$; **: $P < 0.01$, significant difference by Student's *t*-test.

DISCUSSION

Our recent study showed that X irradiation induced Fas and DR5 on the cell surface in not only the p53 wild-type cell lines MKN45 and A549, but also in the p53-mutated cell lines MKN28 and DU145, and apoptosis was induced by incubation of the irradiated cells in the presence of the agonistic antibody CH11 for Fas or TRAIL for DR4/5. The present experiments also confirmed the enhancement of apoptosis by TRAIL treatment in X-irradiated A549 cells. Furthermore, we clearly demonstrated that TRAIL abolished the radiation-induced G2/M checkpoint and converted this fraction to the sub-G1 fraction as shown in Fig. 1A–C. Since decoy receptors against DR5 such as DcR1 and DcR2 were reported to be highly expressed in normal cells but not tumor cells,[15] the DR5 in tumor cells was recently recognized as an ideal target for tumor therapy.[15,17] In radiation therapy, the presence of radioresistant cells in hypoxic regions of solid tumors is a serious problem. Therefore, we investigated whether enhancement of apoptosis by TRAIL treatment occured in tumor cells exposed to X rays under hypoxia. As shown in Fig. 1E, the TRAIL treatment significantly induced apoptotic cell death even in the cells exposed to X rays under hypoxia. To explore the mechanism of this enhancement of apoptosis, we measured the expression of DR5 on the cell surface by flow cytometry. Increased expression of DR5 was significantly observed in cells exposed to X rays under hypoxia although this expression level was slightly lower than that under normoxia as shown in Fig. 2A. This result indicated that induction of apoptosis by TRAIL in the cells irradiated under hypoxia was due to the radiation-induced increase of DR5 expression.

Furthermore, we examined whether TRAIL affected clonogenic ability in irradiated cells. It was clearly demonstrated to increase the sensitivity against X irradiation under normoxia and hypoxia (Fig. 4B and 4C), but did not induce any toxicity in unirradiated cells (Fig. 4A). In this experiment, it seemed to be important that TRAIL did not induce any change in apoptosis or clonogenic formation in unirradiated cells (Fig. 4A), because this meant that only irradiated cells would become targets of TRAIL. As shown in lane 2 of Fig. 3B, the treatment of unirradiated cells with TRAIL produced immature p20 fragments, not the full activated fragment p17 of caspase-3 as an executioner for apoptosis[22] and therefore this condition did not induce apoptosis (Fig. 1) and loss of colony-forming ability (Fig. 4A). This means that DR5 was constitutively expressed in A549 cells, suggesting that this DR5 is partly functional. In fact, we also observed the constitutive DR5 expression by western blot analysis (data not shown). This indicated that the signal transduction pathway for apoptosis through constitutive DR5 was partly activated by TRAIL without X rays. The combination of TRAIL and X rays further produced p17

fragments of caspase-3, followed by apoptotic cell death. These results suggested that radiation-induced enhancement of DR5 expression facilitated apoptotic signaling associated with TRAIL/DR5/caspase-8/caspase-3. In addition, TRAIL treatment may enhance an unknown apoptotic signal transduction pathway linked with other proteases, since TPCK, widely used as an inhibitor of chymotrypsin-like proteases, inhibited the apoptosis induced by combination treatment with TRAIL and X rays (Fig. 3A).

The degree of hypoxia in solid tumors has been shown to be a negative prognostic indicator in a variety of cancers. For radiotherapy, one obvious contributing factor is that hypoxic cells are up to threefold more resistant to ionizing radiation than cells irradiated under well-oxygenated conditions.[21,23] The search for better methods to kill hypoxic tumor cells is therefore an important ongoing endeavor. The present study has clearly demonstrated that ionizing radiation, even under hypoxic conditions, enhanced the expression level of functional DR5 on the plasma membrane and the subsequent TRAIL treatment induced not only apoptotic cell death but also enhanced loss of clonogenic ability. The tumor microenvironment, which is characterized by hypoxia, a low glucose concentration, high lactate concentration and low extracellular pH, is generally considered to be able to alter the therapeutic response in the tumor. Recently, it was shown that incubation under hypoxia (0.001% O_2) for 4 h and with low glucose (0–17 mM) enhanced TRAIL-induced apoptosis in human prostate adenocarcinoma DU-145 cells.[24] Moreover, Weinmann *et al*. demonstrated that severe hypoxia (< 0.1% O_2) did not alter TRAIL-induced apoptosis in four cell lines, human Jurkat E6 T-lymphoma cells, A549 lung carcinoma, HCT 15 colon carcinoma and NCI H 460 lung carcinoma, and that moderate hypoxia (1% O_2) enhanced TRAIL-induced apoptosis in these cell lines.[25] Low extracellular pH (pH6.8) was also demonstrated to induce augmentation of TRAIL-induced apoptotic cell death in DU-145 cells, which was inhibited by overexpression of BCL-2.[26] From these results, we can speculate that expression of DR5 in the tumor microenvironment *in vivo*, especially under hypoxia, is similar to or slightly higher than that in well-oxygenerated normal tissue and the combination treatment using radiation with TRAIL may be effective for hypoxic regions *in vivo*. The findings in the present *in vitro* experiments provide a cellular basis for future *in vivo* studies, though further studies using xenograft models combined with double visualization techniques for hypoxia region, and apoptotic regions are necessary.

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