Enhanced Induction of Apoptosis by Combined Treatment of Human Carcinoma Cells with X Rays and Death Receptor Agonists

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**Apoptosis/DR5/Fas/Redox/X irradiation.**

The death receptors Fas and DR5 are known to be expressed not only in immune cells but also in various tumor cells. The aim of the present study was to determine whether X irradiation enhanced induction of apoptosis in Tp53 wild type and Tp53-mutated tumor cell lines treated with agonists against these death receptors. We showed that 5 Gy of X irradiation significantly up-regulated the expression of death receptors Fas and DR5 on the plasma membrane in gastric cancer cell lines MKN45 and MKN28, lung cancer cell line A549, and prostate cancer cell line DU145, and that subsequent treatments with agonistic molecules for these death receptors, Fas antibody CH11 and TRAIL, increased the formation of active fragment p20 of caspase 3 followed by the induction of apoptosis. This death-receptor-mediated apoptosis was independent of Tp53 status since MKN28 and DU145 were Tp53-mutated. The post-irradiation treatment of the cells with N-acetyl-L-cysteine (NAC) abolished the up-regulation of the expression of Fas and DR5 on the plasma membrane. NAC also attenuated the increase in the formation of p20 and the induction of apoptosis by agonistic molecules. These results suggested that the increase in the induction of apoptosis by combined treatment with X irradiation and CH11 or TRAIL occurred through a change of the intracellular redox state independent of Tp53 status in human carcinoma cell lines.

**INTRODUCTION**

The death receptors such as Fas (also Apo1 or CD95) and death receptor 5 (DR5; also Apo2, TRAIL-R2, TRICK2, or KILLER), are classified into the tumor necrosis factor (TNF) receptor superfamily and have the ability to induce apoptosis. These receptors are activated through trimerization of each receptor's intracellular death domain by ligation with its ligand, followed by the formation of death-inducing signaling complex (DISC) by recruitment of FADD as an adaptor protein and caspase 8, leading to the direct activation of caspase 3. In some cell lines, the death receptor-mediated activation of caspase 8 is known to activate mitochondrial apoptotic pathways such as the cytochrome C/Apaf-1/caspase 9/caspase 3 cascade through production of the proapoptotic fragment of Bid. Fas and DR5 have been reported to be expressed not only in immune cells but also in various tumor cells. FasL (Fas ligand), agonistic Fas antibody CH11 and TRAIL (TNF-related apoptosis-inducing ligand) are able to induce apoptosis in tumor cells with diverse origins. Therefore, treatment of tumor cells with these ligands is recently considered to have a therapeutic potential against tumors.

Ionizing radiation is reported to induce the overexpression of Fas and DR5 in prostate cancer cell lines DU145 and LNCaP, which leads to the enhancement of apoptosis induction by their agonists. Chemotherapeutic agents also induce the overexpression of Fas and DR5 and enhance agonist-induced apoptosis in breast cancer cell lines. In recent studies, we demonstrated that X irradiation induced the overexpression of Fas on the plasma membrane and subsequently led to ligand-independent DISC formation and caspase-8/3-dependent apoptosis in leukemia cell line MOLT-4. The radiation-induced Fas expression and apoptosis were attenuated by post-irradiation treatments with the antioxidant Trolox and N-acetyl-L-cysteine (NAC) in MOLT-4 cells, suggesting that the expression of Fas was regulated by the post-irradiation redox state. Therefore, it is of significance to study the redox regulation of radiation-induced and death receptor-mediated apoptosis in solid tumor cells. For this purpose, we exposed human carcinoma cell lines MKN45, A549, MKN28 and DU145 to 5 Gy of X rays and subsequently treated them with death

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receptor agonists (CH11 and TRAIL). The expression of death receptors, the formation of the active fragment of caspase 3 and the induction of apoptosis were then examined. Furthermore, the effects of NAC, which increases the intracellular glutathione (GSH) level, on the expression of death receptors and the induction of apoptosis were also examined in these tumor cells.

**MATERIALS AND METHODS**

**Materials**
Anti-Fas monoclonal antibody CH11 and FITC-conjugated anti-Fas antibody UB2 were obtained from MBL Co., LTD (Nagoya, Japan). 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). Anti-DR5 polyclonal antibody was obtained from Cayman Chemical (Ann Arbor, MI). Alexa Fluor 594 anti-rabbit antibody was obtained from Molecular Probes (Eugene, OR). Anti-caspase 3 antibody was obtained from Transduction Laboratories (Lexington, KY). Anti-Fas and anti-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The chemiluminescence detection kit was from Boehringer Mannheim GmbH (Mannheim, Germany). The protein assay kit was obtained from Bio-Rad (Brea, CA). NAC and other reagents were obtained from Wako Pure Chemical Co. (Tokyo).

**Cell culture**
The human lung carcinoma cell line A549 (Tp53 wild type) and human prostate carcinoma cell line DU145 (Tp53 mutation [codon 274, valine to phenylalanine]) were generously provided by the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). The gastric carcinoma cell lines MKN28 (Tp53 mutation [codon 251, isoleucine to leucine]) and MKN45 (Tp53 wild type) were provided by Dr. A. Matsuda (Hokkaido University, Sapporo, Japan). These cells were grown in RPMI 1640 medium containing 10% fetal calf serum at 37°C for 24 or 48 h immediately after X irradiation.

**X irradiation and drug treatments**
X irradiation was performed under ice-cold conditions with an X ray generator (2.0 mm Al filter, 200 kVp, 20 mA, Shimazu HF-320, Kyototo). The dose rate was 3.0 Gy/min, which was determined by Fricke’s chemical dosimeter. Treatments of cells with CH11, TRAIL and NAC were carried out in growth medium at 37°C for 24 or 48 h immediately after X irradiation.

**Fluorescence microscopic observation of apoptotic cells**
Cells incubated for 48 h after X irradiation were collected by centrifugation at 1,000 rpm for 5 min at 4°C. The pellet was washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline, PBS(–), and fixed with 1% glutaraldehyde/PBS(–) solution. For fluorescence microscopy, the fixed cells were washed and resuspended in PBS(–). An aliquot was stained with PBS(–) containing 40 µg/ml PI for 15 min in the dark. The stained cells were placed on a microscope slide and gently covered with a coverslip. Fluorescence microscopic observation was performed with a microscope (BX-50, Olympus Optical Co., Ltd, Tokyo) with reflected light fluorescence to count cells with chromatin condensation and nuclear fragmentation as apoptotic cells. In each case, at least 300 cells were evaluated for apoptosis.

**Detection of Fas and DR5 on the cell surface**
The expression of Fas on the cell surface was examined by incubating cells with 100 µl of IF buffer (PBS[–], 1% BSA, 0.1% sodium azide) containing an FITC-conjugated anti-Fas antibody for 30 min on ice. In the detection of expression of DR5 on the cell surface, cells were incubated with 100 µl of IF buffer containing the anti-DR5 antibody for 45 min on ice. After a wash with PBS(–), the cells were incubated with the Alexa Fluor 594 anti-rabbit antibody for 45 min on ice. Each sample was washed and resuspended in 1 ml of PBS(–) and analyzed using an EPICS ALTRA flow cytometer (Beckman Coulter, Inc., Fullerton, CA).

**SDS-PAGE and Western blotting**
Western blotting for caspase 3 and actin was performed as previously described. Briefly, collected cells were washed with PBS(–) and resuspended in 50 µl of cell-lysis buffer (20 mM HEPES, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 2 µg/ml aprotinin, 1 mM Na3VO4, 2 µg/ml pepstatin A and 1 mM DTT) and sonicated twice for 30 sec at ice-cold temperature. The cell lysate was incubated for 30 min at ice-cold temperature, and then centrifuged at 15,000 rpm for 30 min at 4°C. The protein concentration of the supernatant was determined using a Bio-Rad protein assay kit. The samples for SDS-PAGE were prepared by adding 25 µl of 3 x Laemli’s buffer (187.5 mM Tris-HCl, pH 6.8, 30% glycerol, 6% SDS, 15% β-mercaptoethanol and 0.003% bromophenol blue) to 50 µl of 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 an anti-caspase 3 or anti-actin antibody in TBST buffer (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl and 0.1% Tween-20) containing 5% non-fat skim milk or 5% BSA. Proteins were detected by a method combining HRP-conjugated secondary antibodies and a chemiluminescence detection kit.

**Data analysis**
The data were compared using Student’s t-test, and values of p < 0.05 were regarded as significant.
RESULTS

Effects of X irradiation and CH11- or TRAIL-treatment on the induction of apoptosis in carcinoma cell lines MKN45, A549, MKN28 and DU145

When four carcinoma cell lines were treated with CH11 (0–500 ng/ml) or TRAIL (0–100 ng/ml) after exposure to 5 Gy of X rays, the induction of apoptosis judged by typical morphological alterations characteristic of apoptosis (chromatin condensation and nuclear fragmentation) was observed in a concentration-dependent manner (Fig. 1). The incubation time was 48 h. The number of apoptotic cells in each cell line exposed to X irradiation alone was not significantly different from that of untreated control cells. In cells treated with CH11 or TRAIL alone, apoptosis was induced in MKN45, A549 and MKN28 cells in a dose-dependent manner to a certain extent, but not in DU145 cells. Combined treatment of cells with X irradiation and CH11 or TRAIL significantly enhanced the induction of apoptosis in all cell lines. Tp53 is widely recognized as a molecule linked to genotoxic agent-induced apoptosis, but no difference in the enhancement of CH11- or TRAIL-induced apoptosis between Tp53 wild-type cells (A549 and MKN45) and Tp53 mutant cells (MKN28 and DU145) was observed.

Effects of X irradiation on the expression of Fas and DR5 in carcinoma cells

The amounts of Fas and DR5 in X-irradiated cells at 0, 24 and 48 h was examined by Western blotting. As shown in Fig. 2A, a significant time-dependent increase in Fas was induced in MKN45, A549 and MKN28 cells exposed to X rays and a radiation-induced increase of Fas in DU145 was detected only at 24 h after irradiation. A radiation-induced increase of DR5 was observed in MKN28 but not in MKN45 and A549 cells. In DU145 cells, high spontaneous expression of DR5 protein was observed, but the radiation-induced increase of DR5 protein was small. Next, the expression of Fas and DR5 on the cell surface was examined by flow cytometry using specific antibodies (Fig. 2B). The flow cytometric profiles of all cell lines were shifted rightward in a dose-dependent manner at 24 h after X irradiation. These results indicated that the expression of Fas and DR5 dose-dependently increased on the plasma membrane in all cell lines. Since radiation did not increase Fas synthesis in DU145 cells or DR5 synthesis in MKN45, A549 and DU145 cells, it was suggested that radiation promoted the redistribution of death receptors from the cytosolic pool to the plasma membrane in these cell lines.

Effects of NAC on the induction of apoptosis by CH11- or TRAIL-treatment of X-irradiated carcinoma cells

To examine whether the redox regulation was associated with the enhancement of CH11- or TRAIL-induced apoptosis in X-irradiated cells, effects of post-irradiation treatment of NAC, which increases the intracellular GSH level, on the induction of apoptosis were examined. As shown in Fig. 3, the CH11-induced apoptosis in all carcinoma cell lines exposed to 5 Gy of X rays at 48 h was clearly decreased by post-irradiation treatment with 5 mM NAC. The TRAIL-induced apoptosis was also significantly decreased by NAC treatment in all carcinoma cell lines exposed to 10 Gy of X rays.
Effects of NAC on X-ray-induced expression of Fas and DR5 in carcinoma cells

To clarify whether the radiation-induced expression of death receptors was regulated by the intracellular redox state, the expression of Fas and DR5 on the cell surface in X-irradiated carcinoma cell lines was examined without or with the post-irradiation NAC treatment. Flow cytometric analysis showed that post-irradiation treatment of cells with NAC induced a decrease in the expression of Fas and DR5 in all carcinoma cells (Fig. 4), whereas the treatment with NAC alone did not affect the basal expression levels of these receptors. These results indicated that the radiation-induced increase of death receptors was strongly associated with radiation-induced changes in the intracellular redox state.

Effects of NAC on the activation of caspase 3 induced by CH11- or TRAIL-treatment of carcinoma cells exposed to X rays

To examine whether the radiation-induced change in the redox state affected the activation of caspase 3, the effect of NAC on the formation of active fragment p20 of caspase 3 was examined. X irradiation alone produced faint bands of p20 in MKN45 and DU145 cells and no bands in A549 and MKN28 cells (Fig. 5). Combined treatment with X irradiation and agonists (Fas or TRAIL) for death receptors enhanced p20 bands of all cell lines, indicating that the activation of caspase 3 was enhanced. The post-irradiation treatment with 5 mM NAC considerably attenuated the formation of the active fragment. These data showed that X irradiation enhanced the CH11- and TRAIL-induced activation of caspase 3 through a change of the intracellular redox state.
Fig. 3. Effect of NAC on apoptosis induced in carcinoma cells treated with X irradiation and CH11 or TRAIL. Cells were treated with 5 mM NAC and 250 ng/ml CH11 (left panels) or 100 ng/ml TRAIL (right panels) immediately after irradiation with 5 or 10 Gy of X rays. The induction of apoptosis at 48 h was detected by PI staining. In each case, at least 300 cells were evaluated for apoptosis. Data are expressed as mean ± SEM for three experiments.

Fig. 4. Effect of NAC on the X-ray-induced expression of Fas and DR5. Flow cytometric profiles of Fas (left panels) and DR5 (right panels) expression on the cell surface of carcinoma cell lines MKN45, A549, MKN28 and DU145 incubated with or without 5 mM NAC for 24 h after exposure to 0 Gy, 5 Gy and 10 Gy of X rays. Data are expressed as mean ± SEM for three experiments.
DISCUSSION

Ionizing radiation has been reported to induce overexpression of Fas or DR5 and enhances agonist-induced apoptosis in human prostate cancer cell lines LNCaP, PC-3 and DU145, whereas no radiation-induced expression of TRAIL occurs in normal prostate cell line PrEC. In this study, in addition to the DU145 cells, we proved that combined treatments of human gastric cancer cell lines MKN45, A549, MKN28 and DU145 with X irradiation and CH11 or TRAIL also induced caspase 3-dependent apoptosis through radiation-induced expression of Fas and DR5 on the plasma membrane (Figs. 1, 2 and 5). The data suggested the possibility of using combined treatment with X rays and agonistic reagents for Fas or DR5 as a novel anticancer strategy. Ogasawara et al. showed that systemic treatment with Fas antibodies or recombinant FasL in the mouse caused severe damage to the liver and other organs, suggesting a limitation of their clinical utility. However, in recent studies reported by Aoki et al., expression vectors for a FasL cleavage mutant, with low toxicity for liver and high toxicity for tumor cells, was developed for gene therapy. In addition, a new agonistic Fas-targeted antibody, HFE7A, with

|          | MKN45 | A549 | MKN28 | DU145 |
|----------|-------|------|-------|-------|
| X rays   | -     | -    | -     | -     |
| agonist  | -     | -    | -     | -     |
| NAC      | -     | -    | -     | 5 mM  |
|          | 5 Gy  | 5 Gy | 5 Gy  | 10 Gy |
|          | 10 Gy | 10 Gy| 10 Gy |
| Incubation time: 24 h |          |

![Fig. 5. Effect of NAC on the activation of caspase-3 by CH11- or TRAIL-treatment of carcinoma cells exposed to X rays. Carcinoma cell lines MKN45, A549, MKN28 and DU145 were treated with 5 mM NAC and 250 ng/ml CH11 (left panels) or 100 ng/ml TRAIL (right panels) immediately after exposure to 5 or 10 Gy of X rays. The active fragment of caspase-3 (p20) at 24 h was detected by Western blotting.](image-url)
low hepatotoxicity was demonstrated in the mouse, suggesting that the development of less toxic Fas-targeted antibodies is possible.\textsuperscript{17} While TRAIL, which induces apoptosis in human hepatocytes, might cause substantial liver toxicity,\textsuperscript{18} an anti-human DR5 monoclonal antibody, TRA-8, was reported to induce DR5-mediated apoptosis in tumor cells but not significant cell death in normal human hepatocytes.\textsuperscript{19} Thus, various agonistic antibodies for death receptors with low hepatotoxicity have recently been developed. These antibodies seem to have therapeutic potential and be useful for cancer treatment. Since ionizing radiation enhanced the expression of functional Fas and DR5 in various tumor cells as shown in Fig. 2, the administration of lower doses of agonistic antibodies for death receptors without significant hepatotoxicity may be specifically effective against tumors exposed to ionizing radiation. Further \textit{in vivo} experiments on combined treatments with X rays and death receptor agonists on xenografts will be necessary to evaluate the antitumor efficacy and the side effects of this method.

As shown in Fig. 1, the treatment with CH11 alone for MKN45 cells and the treatment with TRAIL alone for MKN45, A549 and MKN28 cells resulted in dose-dependent increases in apoptosis, whereas treatment with CH11 alone for A549 and MKN28 cells caused a slight increase in apoptosis. In the case of DU145 cells, treatment with CH11 or TRAIL alone induced no apoptosis although Fas and DR5 proteins were clearly detected as shown in Western blotting in Fig. 2A. Furthermore, X irradiation was shown to significantly enhance apoptosis in all cell lines treated with CH11 or TRAIL although X irradiation alone induced no apoptotic cell death in any cell line. It seems to be difficult to explain at the present time why X irradiation enhanced apoptosis in all cell lines treated with CH11 and TRAIL, although the sensitivity against agonists for death receptors was different in the cell lines. X Irradiation seems to increase the number of functional death receptors on the plasma membrane even in cell lines such as DU145.

In the expression of radiation-induced death receptors, DNA damage might trigger their up-regulation, because genotoxic agents such as paclitaxel, Adriamycin, etoposide and camptothecin, have been reported to induce the expression of Fas and DR5 in various tumor cell lines.\textsuperscript{7,21,22} Liu \textit{et al.}\textsuperscript{23} and Sheard \textit{et al.}\textsuperscript{23} reported that genotoxic stimuli such as etoposide, doxorubicin and ionizing radiation induced the expression of Fas and DR5 in a Tp53-dependent manner.\textsuperscript{22,23} However, as shown in Fig. 2B, X-ray-induced Fas and DR5 expression on the plasma membrane was observed in not only MKN45 and A549 cells with wild-type Tp53 but also in MKN28 and DU145 cells with mutant Tp53. These data suggested that a Tp53-independent mechanism for the death receptor expression existed in the tumor cells. Our recent studies demonstrated that the radiation-induced expression of Fas was associated with the SAPK(JNK)/c-Jun cascade in MOLT-4 cells.\textsuperscript{11} Furthermore, Shimada \textit{et al.} showed that the inhibition of SAPK(JNK) activation by overexpression of dominant negative MKK7 suppressed the etoposide-induced expression of Fas and the induction of apoptosis in PC-3 and DU145 prostate cancer cell lines with Tp53 mutation.\textsuperscript{20} From these reports it is considered that the SAPK(JNK) pathway also plays an important role in the radiation-induced expression of death receptors in tumor cells.

In human peripheral T cells, the most abundant intracellular thiol antioxidant, GSH, was shown to negatively regulate Fas- and CD2-mediated apoptosis,\textsuperscript{24} and treatment with glutamine, a precursor of GSH, also inhibits TRAIL-induced apoptosis in intestinal epithelial cells.\textsuperscript{25} Furthermore, oxidative stress due to H\textsubscript{2}O\textsubscript{2} induces the expression of Fas in several tumor cells.\textsuperscript{26} Judging from these studies, the expression mechanism of Fas and DR5 seems to involve redox regulation dependent on the concentration of intracellular GSH. In our recent study, a significant decrease in the concentration of intracellular GSH and an increase in the expression of Fas were observed in X-irradiated MOLT-4 cells, and post-irradiation treatment of cells with NAC attenuated radiation-induced apoptosis, the expression of Fas and the activation of SAPK(JNK).\textsuperscript{11} Since, in the present study, X irradiation induced overexpression of Fas or DR5 on the plasma membrane and enhanced Fas- or DR5-mediated apoptosis and this apoptosis was attenuated by post-irradiation NAC treatment in carcinoma cell lines (Figs. 3 and 4), a redox mechanism similar to that of MOLT-4 cells may exist in these cell lines. On the other hand, it has been reported that ionizing radiation enhances intracellular reactive oxygen species (ROS) from mitochondria in human hepatocellular carcinoma cell line HLE\textsuperscript{27} and that H\textsubscript{2}O\textsubscript{2} induces the expression of Fas in murine intestinal epithelial cells.\textsuperscript{26} Therefore, as another mechanism for radiation-induced expression of death receptors, the radiation-induced ROS from mitochondria, which could be inhibited by the post-irradiation NAC treatment, might be responsible for expression of Fas and DR5 in tumor cell lines.

In summary, the present study demonstrated that X irradiation sensitized Fas- and DR5-mediated apoptosis independently of the Tp53 status through a change in the intracellular redox state in several carcinoma cell lines derived from solid tumors. Combined treatments with ionizing radiation and low doses of recombinant agonistic antibodies might be useful for tumors with Tp53 mutation as a novel anticancer strategy without side-effects like hepatotoxicity.

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