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Contribution of Caspase-3 Differs by p53 Status in Apoptosis Induced by X-Irradiation

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We investigated the effect of p53 status on involvement of caspase-3 activation in cell death induced by X-irradiation, using rat embryonic fibroblasts (REFs) transduced with a temperature-sensitive mutant (mt) *p53* gene. Cells with wild-type (wt) p53 showed greater resistance to X-irradiation than cells with mt p53. In cells with wt p53, X-irradiation-induced apoptosis was not inhibited by the caspase-3 inhibitor acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspartyl-aldehyde (Ac-DMQD-CHO) and caspase-3 activity was not elevated following X-irradiation, although induction of p53 and p21/WAF-1 protein was observed. In contrast, irradiated cells with mt p53 showed 89% inhibition of cell death with Ac-DMQD-CHO and 98% inhibition with the antioxidant N-acetyl-L-cysteine (NAC). In cells with mt p53, caspase-3 activity was increased approximately 5 times beyond baseline activity at 24 h after irradiation. This increase was almost completely inhibited by NAC. However, inhibition of caspase-3 by Ac-DMQD-CHO failed to decrease production of reactive oxygen species by cells with mt p53. Differential involvement of caspase-3 is a reason for differences in sensitivity to X-irradiation in cells with different p53 status. Caspase-3 activation appears to occur downstream from generation of reactive oxygen species occurring independently of wt p53 during X-irradiation-induced cell death.

Key words: p53 — Caspase-3 — Reactive oxygen species — Apoptosis — X-irradiation

X-irradiation has been widely used to treat various malignant tumors such as cerebral glioblastoma, esophageal cancer, and lymphomas. Further improvement of the clinical effectiveness of X-irradiation will require understanding of differences in the contribution of target molecules leading to apoptosis in cells with different genetic backgrounds. Functional wild-type (wt) p53 induces apoptosis-promoting factors such as Fas, KILLER/DR5, and bax, leading to activation of caspases in cells treated with ionizing radiation (IR).[1–5] Thus, IR-induced apoptosis is believed to depend on p53 in many types of cells. Further, recent studies have shown that not only p53-dependent but also p53-independent signaling pathways contribute to the apoptotic response in IR-treated cells, particularly hematopoietic cells.[6, 7] However, the relationship in non-hematopoietic cells between p53 status and the X-irradiation-induced apoptotic response mediated via caspase-3 activation is not known. We therefore investigated the caspase-3-mediated signaling pathway during X-irradiation-induced cell death using genetically matched rat embryonic fibroblasts (REF-112) transduced by a temperature-sensitive (t.s.) mutation of *p53*. This cell line was established by Michalovitz *et al.* and this mutant (*p53* val 135) elicits transformation at 37.5°C while it suppresses transformation and behaves like authentic wt p53 at 32.5°C.[8, 9] This simple system enables us to study the effect of the p53 mutation on signaling cascades in an identical cell line. Previous studies have implicated reactive oxygen species (ROS) in apoptosis in various systems,[10, 11] but the relationship between ROS production and caspase-3 activation in the signal transduction pathway of X-irradiation-induced apoptosis is not well known. Therefore, we further examined the sequence of caspase-3 activation and ROS production in X-irradiated REF-112 cells, especially those harboring mutant (mt) p53, in which the influence of molecules interacting with caspase-3 activation could be minimized.

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MATERIALS AND METHODS

Cell culture Rat embryonic fibroblasts of clone 112 (REF-112) were cultured in Dulbecco's modified Eagle's medium (DMEM; Nipro, Osaka) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biological Industries, Kibbutzbeit Haemek, Israel). The cells were pre-cultured at 32.5 or 37.5°C for 24 h before the experiments for wt p53 and mt p53, respectively.[8, 9]

***In vitro* assay for cell survival** Cells (5×10^3/100 μl) were placed in the wells of a 96-well microculture plate (Costar, Tokyo) and incubated for 18 h in 5% CO_2. Next, the plates were X-irradiated at various doses (1–30 Gy), followed by incubation for 48 h in 5% CO_2. X-irradiation was carried out using an MBR-1520A-TW device (20 mA,

150 kV; Hitachi Medical, Tokyo) at a dose rate of 2.089 Gy/min. Cell survival was assessed by a colorimetric method using the new tetrazolium reagent WST-8, "Cell counting kit 8" (Dojindo, Kumamoto). Briefly, 1/10 volume of WST-8 was added to cells and the plates were incubated for 1 h at 37°C. Absorbance (indicating viable cell number) was measured by an EAR 400 reader (SLT-Labinstruments GmbH, Salzburg, Austria; measurement at 450 nm).

Analysis of apoptosis Apoptosis was assessed by fluorogenic staining. Briefly, cells in a 100 mm culture dish (Costar) were X-irradiated with or without pretreatment with a specific caspase-3 inhibitor, acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspartyl-aldehyde (Ac-DMQD-CHO; Peptide Institute Inc., Osaka) or antioxidant N-acetyl-L-cysteine (NAC; Sigma, St. Louis, MO). Then additional incubation was performed at the appropriate temperature for 48 h, and cells were collected. Each sample was then stained with Hoechst 33258 (Sigma) according to the reported method,[12] and the apoptotic cells were determined morphologically under a fluorescence microscope by counting 1000 cells per sample.

Assay for endogenous p53 and p21/WAF-1 expression Total amounts of p53 and p21/WAF-1 protein were determined by an indirect method using a fluorescein-labeled second antibody, according to the method described earlier.[13] Briefly, 1×10^6 cells/100 mm culture dish (Costar) were irradiated and incubated for 24 h, and washed twice with phosphate-buffered saline (PBS). The cells were collected and fixed with paraformaldehyde solution (4% v/v in PBS) for 20 min at room temperature. Next they were treated with Triton X-100 (0.2% v/v in PBS) to permeabilize the cell membrane. For blocking, 1% goat or serum in PBS was added to each tube for 30 min at 37°C. As a first antibody, Ab-1 or Ab-3 (Oncogene Research Products, Cambridge, MA) for wt or mt p53, or Ab-5 (Oncogene Research Products) for p21/WAF-1, diluted 1/20 and 1/100 with PBS containing 1% bovine serum albumin (BSA; Sigma), was added to each tube. Following incubation, the cells were washed and treated with "Alexa-488" labeled goat anti-mouse IgG and goat anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands) as a second antibody for detection of p53 and p21/WAF-1, respectively. Following an additional incubation, cells were washed and the intensity of cell fluorescence was measured in a Spectrafluor (SLT-Labinstruments; excitation 485 nm, emission 538 nm). Relative fluorescence intensity (FI) was determined compared to the FI of non-treated cells.

Measurement of ROS Endogenous amounts of ROS were measured by fluorometric assay using dihydrorhodamine 123 (DHR 123; Molecular Probes). Briefly, cells in a 100 mm dish (Costar) were incubated with 50 μM DHR 123 in the medium at 37°C for 30 min, and then detached from the dish with 0.25% trypsin in 0.02% EDTA PBS. Next, cells were washed with PBS twice, and the FI of resuspended cells was measured with a Spectrafluor (SLT-Labinstruments; excitation 485 nm, emission 538 nm).

Measurement of caspase-3 activity Enzymatic activity of caspase-3 was measured by a fluorometric assay according to the instruction manual for apoptosis published by Pharmingen (2nd edition, December 1998, San Diego, CA). Cells were treated with lysis buffer (10 m*M* Tris-HCl, 10 m*M* NaH_2PO_4/Na_2HPO_4 pH 7.5, 130 m*M* NaCl, 1% Triton X-100, 10 m*M* sodium pyrophosphate) and the lysate was centrifuged at 12 000*g* for 15 min. The supernatant was collected and the protein concentration was measured by BioRad (Hercules, CA) DC-protein assay. Next the lysate, fluorogenic substrate acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid-α (4-methylcoumaryl-7-amide) (Ac-DEVD-MCA; Peptide Institute) with or without caspase-3 inhibitor Ac-DMQD-CHO (Peptide Institute), and reaction buffer (40 m*M* HEPES pH 7.5, 20% glycerol, 4 m*M* dithiothreitol) were mixed in a 96 well microtiter plate (Costar). The substrate cleavage was monitored in terms of AMC liberation in a Spectrafluor (SLT-Labinstruments; excitation 360 nm, emission 465 nm) at 37°C. The fluorescence of produced amino-4-methylcoumarin (AMC) was measured every 1 min during a 60-min period, and the caspase-3 activity was calculated as FI/min/mg protein.

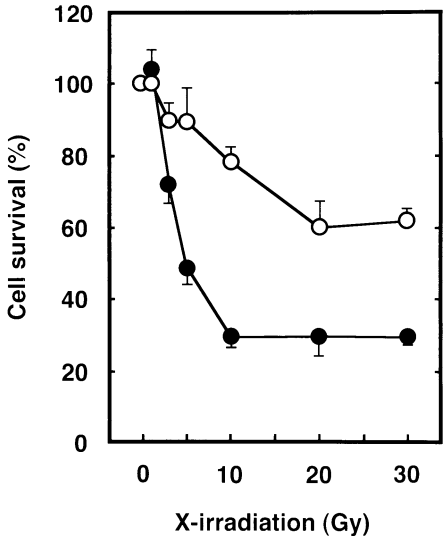

Fig. 1. Cell survival curve of X-irradiated REF-112 cells with wild type (wt) *p53* (open circles) and mutant (mt) *p53* (closed circles). Cells were exposed to indicated doses of X-irradiation followed by incubation for 48 h. Cell survival was estimated by a colorimetric assay as described in "Materials and Methods." The data are the mean±SD of three independent experiments.

RESULTS

Cell survival and apoptosis at different p53 status We first assessed cell sensitivity to X-irradiation after 48 h, because we needed to determine cell survival and apoptosis in the early phase as opposed to the clonogenic late phase. Cells with wt p53 were more resistant than cells with mt p53 (Fig. 1). This difference also was evident when apoptosis was assessed microscopically (Table I). In subsequent experiments, an irradiation dose of 20 Gy was chosen for cells with wt p53; a dose of 5 Gy was chosen for cells with mt p53, since it caused a similar frequency of apoptosis.

Expression of p53 and p21/WAF-1 according to p53 status Protein expression of p53 and that of p21/WAF-1 were examined by an indirect fluorescent antibody method after X-irradiation of cells with differing p53 status. Induction of p53 protein followed X-irradiation in cells with wt p53, but not mt p53 (Fig. 2A). P21/WAF-1 expression also was increased in cells with wt p53, but not mt p53 after X-irradiation (Fig. 2B). Expression of p21/WAF-1 in cells with mt p53 was not altered even following 20 Gy of X-irradiation (data not shown).

Involvement of caspase-3 in X-irradiation-induced cell death according to p53 status To investigate involvement of caspase-3 in X-irradiation-induced apoptosis, we first examined the effect of Ac-DMQD-CHO, a specific inhibitor of caspase-3, on apoptosis. No inhibitory effect of Ac-DMQD-CHO on apoptosis was seen in cells with wt p53 (Fig. 3A). In contrast, in cells with mt p53, Ac-DMQD-CHO prevented X-irradiation-induced apoptosis in a dose-dependent manner; 89% inhibition was seen with 200 μM Ac-DMQD-CHO (Fig. 3B). We next examined directly whether enzymatic caspase-3 activity is elevated in X-irradiated cells with mt p53. AMC release from the fluorogenic substrate Ac-DEVD-MCA increased in a time-dependent manner (data not shown). Almost complete inhibition of AMC release resulted from inclusion of the specific caspase-3 inhibitor Ac-DMQD-CHO, indicating that fluorescence detected in this assay truly represented caspase-3 enzymatic activity (Fig. 4). In cells with wt p53, no induction of caspase-3 activity was evident at 24 h after 20 Gy of irradiation. In contrast, caspase-3 activity was increased after X-irradiation of cells with mt p53. At 24 h after irradiation, activity was five times that seen in cells not irradiated.

The relationship between ROS and caspase-3 in X-irradiated cells with mt p53 We next examined whether production of ROS and activation of caspase-3 occurred sequentially in X-irradiated cells with mt p53. The effect of the antioxidant NAC on X-irradiation-induced caspase-3 activity was examined. Induction of caspase-3 activity by X-irradiation was almost completely inhibited by addition of NAC, while without irradiation NAC had no effect on baseline activity (Fig. 5). Furthermore, NAC treatment

Table I. Induction of Apoptosis by X-irradiation in REF-112 Cells

p53 status	5 Gy	10 Gy	20 Gy
Wild type	3.6±2.3	23.4±1.3	42.6±3.7
Mutant type	46.1±4.8	61.3±2.7	66.6±2.3

The percentage of apoptotic cells was determined by evaluating nuclear morphologic change. Each value is the mean±SD of three independent samples.

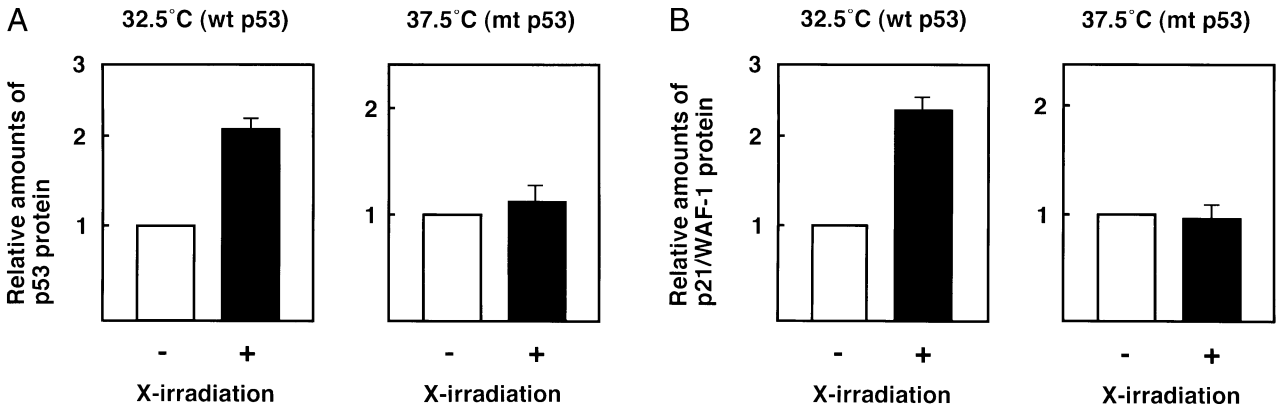

Fig. 2. Analysis of p53 (A) and p21/WAF-1 (B) protein expression in X-irradiated REF-112 cells according to p53 status (wild type, wt; mutant, mt). Cells were irradiated and then incubated for 24 h. Amounts of each protein were measured by a fluorometric assay described in "Materials and Methods." Protein amounts are shown relative to those in untreated cells, and the data are the mean±SD of three independent experiments.

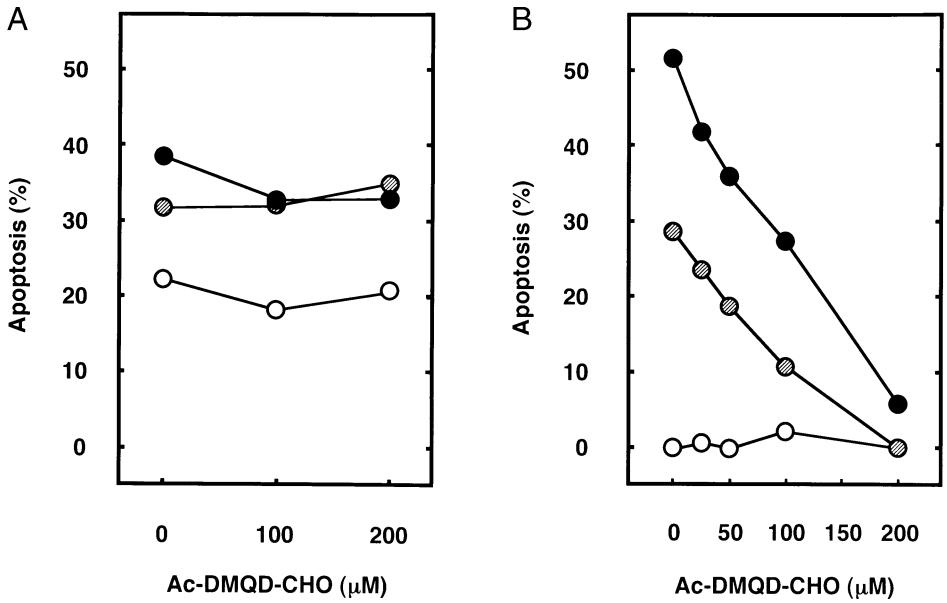

Fig. 3. Effect of a specific caspase-3 inhibitor (Ac-DMQD-CHO) on X-irradiation-induced apoptosis in REF-112 cells with wt *p53* (A) or mt *p53* (B). Increasing concentrations of Ac-DMQD-CHO were added to cultures 3 h before irradiation. In A, cells were irradiated at 10 Gy (open circles), 20 Gy (hatched circles), or 30 Gy (closed circles) followed by incubation at 32.5°C for 48 h. In B, cells were irradiated at 1 Gy (open circles), 3 Gy (hatched circles), or 5 Gy (closed circles) and incubated at 37°C for 48 h. Apoptotic cells were detected by a fluorometric assay using Hoechst 33258. The data are the mean values for three independent experiments. Ac-DMQD-CHO, acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspartyl-aldehyde.

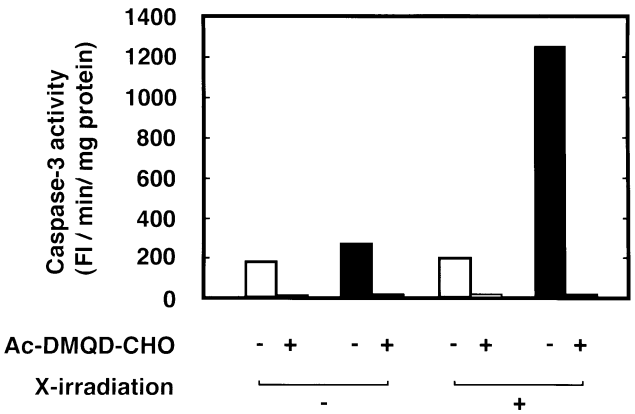

Fig. 4. Caspase-3 activity in cells according to p53 status. Cells were X-irradiated and cell lysates were assayed with or without the caspase-3 inhibitor Ac-DMQD-CHO. Cell lysates were incubated at 37°C for 1 h, and the amount of AMC released by cleavage of the fluorogenic substrate Ac-DEVD-MCA (100 *μM*) with or without 100 *μM* Ac-DMQD-CHO was measured. The experiments were performed in duplicate, and the data are mean values. Open columns represent cells with wt *p53*; closed columns, cells with mt *p53*. Ac-DMQD-CHO, acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspartyl-aldehyde; Ac-DEVD-MCA, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid-α (4-methylcoumaryl-7-amide); mt, mutant type; wt, wild type; FI, fluorescence intensity.

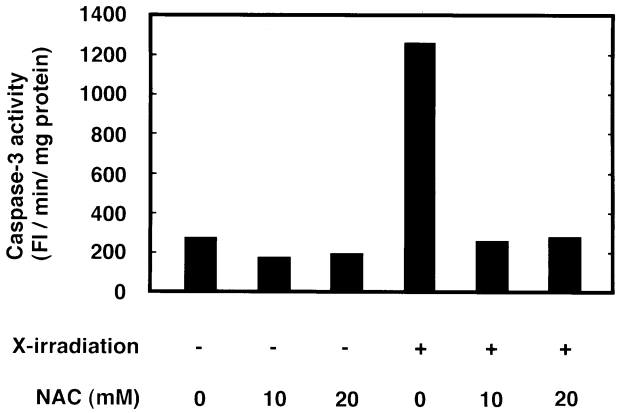

Fig. 5. The effect of NAC on caspase-3 activity in REF-112 cells with mt p53. Cells were treated with or without indicated concentrations of NAC 2 h before 5 Gy of X-irradiation followed by incubation at 37°C for 24 h. Cell lysates were incubated at 37°C for 1 h and the amount of AMC released by cleavage of the fluorogenic substrate Ac-DEVD-MCA (100 *μM*) was measured at each time point. The data are the mean values for triplicate experiments. NAC, N-acetyl-L-cysteine; mt, mutant type; AMC, 7-amino-4-methylcoumarin; Ac-DEVD-MCA, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid-α (4-methylcoumaryl-7-amide); FI, fluorescence intensity.

Table II. Effect of a Caspase-3 Inhibitor and an Antioxidant on X-irradiation Induced ROS Production in REF-112 Cells with Mutant p53

Irradiation (5 Gy)	−	+	+	+
Ac-DMQD-CHO (100 μM)	−	−	−	+
NAC (10 m*M*)	−	−	+	−
ROS production	5206±173	6159±158	2158±85	6387±257

ROS production is reported as fluorescence intensity per 30 min, and the data are the mean value±SD of three independent experiments. NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species.

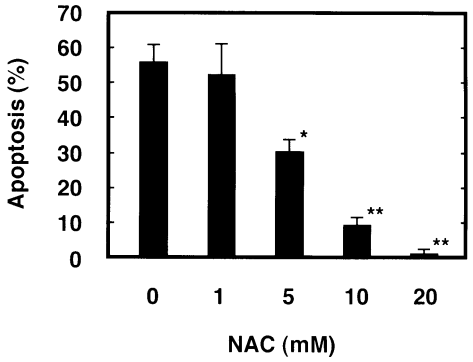

Fig. 6. Effect of the antioxidant N-acetyl-L-cysteine (NAC) on X-irradiation-induced apoptosis in REF-112 cells with mt *p53*. Increasing concentrations of NAC were added to cultures 2 h before irradiation. Cells were irradiated at 5 Gy followed by incubation at 37°C for 48 h. Apoptotic cells were detected by a fluorometric assay using Hoechst 33258. The data are the mean±SD of three independent experiments. ∗ $P<0.05$, ∗∗ $P<0.01$ by Student's *t* test.

dramatically prevented X-irradiation-induced apoptosis in a dose-dependent manner (Fig. 6). We next investigated whether ROS production occurred upstream or downstream from caspase-3 activation, and found that Ac-DMQD-CHO had no inhibitory effect on the amounts of ROS in X-irradiated cells, while NAC treatment decreased ROS production. ROS production, then, occurred upstream from caspase-3 activation (Table II).

DISCUSSION

Previous studies have demonstrated that not only p53-dependent, but also p53-independent apoptosis contributes to cell death induced by IR.[6, 14] Further, a recent study indicated that γ-ray-induced apoptosis in lymphoblastic cells was mediated by a mechanism involving caspase-3 activation that was shared by both p53-dependent and -independent pathways.[7] However, whether p53 status affected the contribution of caspase-3 activation to the apoptotic response to X-irradiation was not known. In the present study, we first found that following X-irradiation cell survival was less and the apoptotic response was greater in cells harboring mt *p53* than in cells harboring wt *p53*. This unexpected result was at odds with the general understanding of the apoptosis-inducing function of p53 and with numerous studies demonstrating that a *p53* mutation confers resistance to IR, especially γ-rays, in many types of cells.[15, 16] However, other investigators have found *p53* mutation to be associated with greater radiosensitivity, as judged from longer survival in patients with glioblastoma treated by X-irradiation and from results of experiments with γ-irradiated cell lines derived from breast cancers and fibroblasts.[14, 17, 18] Consequently, the biologic response to IR may differ by p53 status, but it is not wholly dependent on p53. The exact reason for the inverse relationship between p53 status and apoptotic response that we observed remains to be delineated. However, these findings and our study suggest that the therapeutic modality can not be selected only by p53 status in all cases, and that treatment targeting p53, such as gene therapy, may result in unexpected therapeutic effects in some cases.

Apart from the paradoxical nature of our results with respect to the *p53* mutation and sensitivity to IR, the difference in apoptotic response according to p53 status found in this study is consistent with and clearly explained by differences in caspase-3 activation and p21/WAF-1 induction. Accordingly, the caspase-3 activity increase following X-irradiation was greater in cells with mt p53 than in cells with wt p53, although basal activity did not vary with p53 status. In addition, a specific inhibitor for caspase-3 dramatically prevented apoptosis only in cells with mt p53. This study presents the first evidence that p53-independent caspase-3 activation is involved in X-irradiation-induced apoptosis and that caspase-3 represents a critical X-irradiation-sensitive target leading to apoptosis in cells with mt p53. Recently, other investigators have demonstrated that the apoptotic response to γ-rays is closely related to caspase-3 activation.[7] Their experiments included an interesting result that caspase-3 activation also occurred after γ-irradiation in cells harboring mt p53 or completely lacking the gene, although these cells were

relatively resistant to γ-rays. Thus, the biologic sensitivity to IR due to apoptosis seems to be determined by induced caspase-3 activity, and p53 protein is not always required for caspase-3 activation. In the present study, p53-dependent induction of p21/WAF-1 was detected only in cells with wt p53. Recent studies have demonstrated that p21/WAF-1 induction protects cells from apoptosis induced by ultraviolet and X-irradiation and that mutant p21/WAF-1 protein fails to prevent apoptosis induced by X-irradiation[19, 20]; these findings strongly support our results.

Our present experiments demonstrated essentially no decrease in survival of cells with wt p53 at a low dose of X-irradiation, a dose that produced apoptosis in nearly 50% of cells with mt p53. This result may reflect the protective function of both baseline and induced expression of p21/WAF-1. At a high dose of irradiation lethal for cells with mt p53, cells with wt p53 showed decreased survival due to apoptosis. In the cells carrying wt *p53*, functional p53 protein accumulates and induces various factors such as Fas and KILLER/DR5 that activate caspase-3 in various apoptotic systems.[3–5] This function of wt p53 leading to caspase-3 activation has been thought to act also in IR-induced apoptosis of the cells carrying wt *p53*.[2] However, in the cells used in this study, caspase-3 activity was not elevated even at 20 Gy of irradiation. Further, a caspase-3 inhibitor showed no effect on apoptosis, even though p53-dependent p21/WAF-1 induction was observed. This result suggests that an uncharacterized caspase-3-independent mechanism is involved in X-irradiation-induced apoptosis in cells with wt p53.

Mitochondrial production of ROS is considered an important step in common pathways leading to apoptosis induced by various genotoxic and nongenotoxic stresses.[10, 11] Numerous studies have indicated that several mitochondrially related molecules such as Apaf-1 and cytochrome *c* act upstream of the caspase cascade in cells exposed to genotoxic stresses including IR.[21, 22] However, any role of ROS as a mediator of caspase activation in IR-induced apoptosis was unclear. We therefore examined the relationship between ROS and caspase-3 in signal transduction during X-irradiation-induced apoptosis using cells with mt p53, where caspase-3 activation was apparent. The antioxidant NAC almost completely prevented increases in caspase-3 activity and apoptosis induced by X-irradiation. A recent study demonstrated that the antioxidant Trolox, which inhibits lipid peroxidation, also reduced caspase-3-dependent apoptosis in X-irradiated MOLT-4 cells with nonfunctional p53,[23] a result consistent with the presently observed effect of NAC. Further, we found that caspase-3 activation occurs downstream rather than upstream of ROS generation in X-irradiated cells. Our findings suggest the possibility of an alternative pathway of caspase-3 activation downstream of ROS production. A recent study demonstrated that peroxynitrite, a potent inducer of apoptosis, directly induces caspase-3 activity.[24] The individual radical species among possibilities such as superoxide, hydroxyl radicals, and peroxynitrite that induce caspase-3 in X-irradiated cells remain to be determined.

Taken together, our findings suggest that caspase-3 activation occurs downstream of ROS generation in p53-independent apoptosis induced by X-irradiation, and that differential involvement of caspase-3 contributes to p53-related differences in sensitivity of non-hematopoietic cells to X-irradiation.

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