Role of Wild-type p53 in Apoptotic and Non-Apoptotic Cell Death Induced by X-irradiation and Heat Treatment in p53-mutated Mouse M10 Cells

Atsushi ITO1*, Hisako NAKANO2 and Kunio SHINOHARA3

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The sensitizing effects of wild-type p53 on X-ray-induced cell death and on heat-induced apoptosis in M10, a radiosensitive and Trp53 (mouse p53 gene)-mutated lymphoma cell line which dies through necrosis by X-irradiation, were investigated using three M10 derived transfectants with wild-type TP53 (human p53 gene). Cell death was determined by colony formation and/or dye exclusion test, and apoptosis was detected as the changes in nuclear morphology by Giemsa staining. Expression of wild-type p53 protein increased radiosensitivity of cell death as determined by both clonogenic and dye exclusion assays. This increase in radiosensitivity was attributable largely to apoptosis induction in addition to a small enhancement of necrosis. Interestingly neither pathway to cell death was accompanied by caspase-3 activation. On the other hand, heat-induced caspase-3 dependent apoptotic cell death without transfection was further increased by the transfection of wild-type p53. In conclusion, the introduction of wild-type p53 enhanced apoptotic cell death by X-rays or heat via different mechanisms that do or do not activate caspase-3, respectively. In addition, p53 also enhanced the X-ray-induced necrosis in M10 cells.

INTRODUCTION

M10, a radiosensitive mouse leukemia cell line, provides a unique tool for investigating p53 contribution in the mechanism of cell death caused by various external stimuli, since it has mutated p53 status in spite of having comparable high radiosensitivity to human leukemia MOLT-4 (p53; wild-type) which has been frequently used in the study of apoptosis via a p53 pathway. The extremely high sensitivity of M10 is shown to result from the lack of XRCC4 in the repair of DNA double strand breaks.1) We previously found that M10 undergoes necrosis in contrast to MOLT-4.2) Although there have been literatures describing p53-independent apoptosis induction by radiation,3,4) no cell lines have been reported that exhibited very high radiosensitivity due to necrosis independent of p53. In order to investigate further critical role of p53 in radiation sensitivity, the present report examines the responsiveness or enhancing effect of wild-type p53 transfection on the radiosensitivity using M10 system where radiosensitivity seems to be fully raised to a pathway of p53-independent necrosis.

Another interesting feature of M10 (p53; mutated) is the completely different cell death response to X-rays and heat treatment: necrosis to X-rays5) and apoptosis to heat.6) The mechanism of this apoptosis has not been clarified yet, though it is clear that the apoptosis is not dependent of p53. Therefore, the question arises if transfection of wild-type p53 to M10 enhances the heat induced cell death additionally.

The purpose of this study is to examine a possible role of p53 in cell death pattern in addition to necrotic cell death pathway in X-irradiation and apoptotic cell death pathway in heat treatment. We found that wild-type p53 protein induced additionally not only apoptosis but also necrosis for the transfected cells irradiated with X-rays, and induced a large enhancement of apoptosis for the cells with heat treatment.

MATERIALS AND METHODS

Cell line and culture

M10 is a radiosensitive mutant of mouse leukemia cell line L5178Y5) and is mutated in p533) and XRCC4.1) The cells were grown in culture medium containing RPMI 1640 supplemented with 10% horse serum and antibiotics (100 U
penicillin and 0.1 mg streptomycin per ml). Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. The doubling time was 11.5 hrs.

**Plasmid**

A human wild-type p53 expression vector termed pCAGp53 was constructed as follows: Total RNA was extracted from human leukemia MOLT-4 cells with normal p53 using an RNeasy Mini Kit (QIAGEN, Inc.), and the coding region of human wild-type TP53 was amplified by a reverse transcription-polymerase chain reaction (RT-PCR) system (SUPERSCRIPT ONE-STEP™ RT-PCR System, Life Technologies, Inc.). The 5’-primer (5’-CCG TTC GAG ACT GCC TTC CGG GTC ACT -3’) and 3’-primer (5’-CCG CTC GAG GCT GTC AGT GGG GAA CAA -3’) were synthesized to contain a XhoI site. RT-PCR was conducted as the following sequence: 50°C for 30 min, 94°C for 2 min followed by 40 cycles of 94°C for 15 sec, 56°C for 30 sec and 72°C for 2 min, and then 72°C for 10 min. The XhoI-XhoI fragment was next cloned into a XhoI site of the pCAG-PURO vector (6.1 kbp), a derivative of pCAGGS expression vector (4.8 kbp),7 in the correct orientation, and the sequencing vector was confirmed. The vector was verified using an ABI PRISM™ 310 Genetic Analyzer (Perkin-Elmer, Corporation). pCAGp53 contains a human TP53 cDNA.

**Transfection**

M10 cells were transfected with pCAGp53 (7.4 kbp) using DMRIE-C reagent (Invitrogen), and stable transfectants (termed as #1, #5, #6) were cloned by the colony formation incubated for 12 days in the presence of 3 μg/ml puromycin. The growth rate of the cells was not changed by the transfection.

Transfectants were examined by PCR analysis and Western blot analysis. DNA for PCR analysis was isolated from cells using a DNA extraction kit (SepaGene™, Sanko Junyaku Co. Ltd., Japan). PCR was run with the above primers and AmpliTaq Gold polymerase (Perkin Elmer, Corporation) for 9 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at 65°C and 2 min at 72°C, and then 10 min at 72°C. PCR products were analyzed on a 1.0% agarose gel and stained with 1.0 μg/ml ethidium bromide.

**Chemical treatment**

Ac-DEVD-CHO (Ac-Asp-Glu-Val-Asp-H), which is an inhibitor for caspase-3, -7 and -8,8 was purchased from the Peptide Institute (Osaka, Japan) and dissolved in dimethyl sulfoxide (DMSO). Cells in culture medium were mixed with Ac-DEVD-CHO giving a final concentration of 300 μM immediately after X-irradiation or 1 hour prior to heat treatment. Irradiated cells were incubated for 24 hrs in the presence of Ac-DEVD-CHO or DMSO. Heated cells were incubated at 37°C for 2 hrs after the treatment in the presence of Ac-DEVD-CHO or DMSO.

**Western blot analysis**

Cells were lysed by sonication in SDS-sample buffer (1% SDS; 0.04 M Tris-Cl, pH 6.8; 7.5% glycerol; 0.05 M dithiothreitol) containing 1 mM PMSF, and then boiled for 3 min. Protein contents were measured with a Bio-Rad protein assay kit (Bio-Rad Lab., Inc.).

Equal amounts of proteins (20 μg) were loaded into each lane of a 12.5% (w/v) polyacrylamide gel and separated by SDS-PAGE at 25 mA/gel. The proteins were then electrophoretically transferred to a nitrocellulose membrane (Hybond™-C extra, Amersham Pharmacia Biotech, Inc.). A monoclonal antibody against human p53 (pAb1801, Novocastra Laboratories, Ltd.) was used as a primary antibody. It should be noted that this antibody (pAb1801) is human-specific.9 The secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG. Proteins were detected using the enhanced chemiluminescence reagents (ECL™) and Hyperfilm™ (Amersham Pharmacia Biotech, Inc.).

**X-irradiation and heat treatment**

X-irradiation was performed at a dose rate of 0.62–0.65 Gy/min using a 150 kV X-ray generator unit operating at 5 mA with an external filter of 0.1 mm Cu and 0.5 mm Al (M-150WE, Softex, Japan). Exponentially growing cells, the density of which is 2–3 × 10⁵ cells/ml, were irradiated in a plastic tissue culture flask.10 Heat treatment was carried out in a test tube at the temperature of 44°C for 30 min.

**Dye exclusion test and morphological analysis**

After irradiation with X-rays or heat treatment, cells were incubated in a humidified 5% CO₂ atmosphere for various intervals and stained with an equal volume of 0.4% erythrosine B in phosphate-buffered saline (PBS). Stained cells were counted under an optical microscope at 4–10 min after mixing.

Morphological features were observed as follows: Cells were fixed in a solution of 3 volume of ethanol and 1 volume of acetic acid. Fixed cells were stained with 1% Giemsa solution or 2 μg/ml DAPI solution and observed under an optical microscope. Cells showing typical apoptotic morphology as indicated by arrows in Fig. 4(b), in which condensed nuclei are scattered, were counted as apoptotic cells.

** Colony formation assay**

M10 cells and the transfectants were mixed with 2 ml RPMI medium containing 15% horse serum and 0.3% SeaPlaque agarose, plated on the top of 1.5 ml of medium containing 15% horse serum and 0.5% agarose in a 35 mm well of six-well cell culture plate (Linbro®, Flow Laboratories, Inc.), and incubated at 37°C for 15 days. Visible colonies were counted to determine the colony-forming ability.
Flow cytometric analysis

Cells were washed in PBS, fixed, and then permeabilized with IntraPrep permeabilization reagent (Beckman Coulter, Inc.). Cells were treated with PE-conjugated monoclonal active caspase-3 antibody apoptosis kit I (clone C92-065; BD Biosciences) overnight at 4°C, washed twice in cold PBS with 0.1% sodium azide and 0.1% bovine serum albumin (BSA; Sigma-Aldrich Co. A-7030), and resuspended in 0.4 ml of PBS containing 0.5% formaldehyde. Ten thousand cells stained with fluorescent monoclonal antibody per sample were analyzed by flow cytometry (FACSCalibur; Becton, Dickinson and Co.) using Cell Quest software (Becton, Dickinson and Co.).

RESULTS

Expression of externally introduced wild-type p53

M10 cells were transfected with pCAGp53, a human wild-type p53 expression vector. The stable transfectants were selected by colony formation in the presence of puromycin. PCR analysis confirmed that these clones carried the wild TP53 cDNA from the vector (data not shown). Those were further demonstrated by Western blot analysis for the expression of wild-type p53 protein as shown in Fig. 1. In contrast to the absence of wild-type p53 in M10, clones #1, 5 and 6 exhibited significant expression level of wild-type p53 in this order. As a reference, expression levels in MOLT-4 cells (p53; wild type) before and after irradiation with 9 Gy of X-rays were displayed for comparison.

Clonogenic assay for wild-type p53 transfected cells with X-irradiation

Figure 2 shows survival curves upon X-irradiation on M10, and clones #1, 5 and 6. The survivals were significantly decreased in parallel with the expression level of wild-type p53. The radiation dose required to reduce the surviving fraction by 10% (D10) was 1.01, 0.91, 0.84, and 0.80 Gy for M10, and clones #6, 5 and 1, respectively. The results show that M10 cells defective in nonhomologous end-joining are further sensitized to X-rays by the expression of wild-type p53 protein.

Dye exclusion test for X-irradiated cells

Figures 3(a) and (b) show dose dependence of cell death at the incubation time of 24 hrs (panel (a)) and 48 hrs (panel (b)) after irradiation. The percentage of dead cells showed a significant increase in the transfected cells at 24 hrs and further increase at 48 hrs after irradiation, depending on the level of wild-type p53 protein. These results are in agreement with those of clonogenic survival.

Apoptosis induction and morphological changes after irradiation

Apoptosis induction determined by the Giemsa staining method was shown in Fig. 4(a) at 24 hrs after irradiation. The experiments with the higher dose of 10 Gy were added for the clone #1, the highest wild-type p53 expressed clone. Figure 4(b) shows morphological change of irradiated cells, indicating the typical morphological feature of apoptosis including the fragmentation of cell nuclei as indicated by arrows. Apoptosis was hardly detected in M10 cells, which supports our previous results that M10 dies via necrosis. As apparent from Fig. 4(a) apoptosis was exhibited in the order of clones #5 and 1, the order of the expression level of wild-type p53, suggesting that the incorporated p53 acted as an inducer of apoptosis. In addition, the enhanced total cell death in clones #5 and 1 as measured by the dye exclusion test may mainly result from the development of apoptosis.
accompanied by the small increase in non-apoptotic cell death attributable to transfected wild-type p53.

Taken the results of Figs. 3 and 4 together, exogenously introduced p53 appears to accelerate the development of cell death.

**Participation of caspase-3 in X-irradiated cells**

Flowcytometric patterns of X-irradiated MOLT-4, M10 and clone #1 cells are shown in Fig. 5 to examine the participation of caspase-3 in the process of cell death. In MOLT-4 cells at 7 hrs after irradiation, a peak assigned to the active caspase-3 appeared, which is consistent with our previous results\(^1\),\(^2\) that MOLT-4 cells undergo apoptosis via the p53 pathway followed by caspase-3 activation. On the other hand, irradiated M10 and clone #1 cells exhibited no peak corresponding to active caspase-3. These results indicate that clone #1 expressing wild-type p53 dies via caspase-3 independent pathway, which differs from cell death in MOLT-4 with wild-type p53.

Figures 6(a) and (b) show effects of an inhibitor of caspase-3, Ac-DEVD-CHO, on the cell death detected by dye exclusion test (panel (a)) and apoptosis (panel (b)). Similar to Fig. 4(a) 10 Gy irradiation was attempted only for clone #1. Ac-DEVD-CHO provided no suppressive effect not only on total cell death as measured by the dye exclusion test but also on apoptosis by Giemsa staining, in contrast to the large suppression in the MOLT-4 case.\(^12\) These results also confirm that apoptosis induced by the transfection of wild-type p53 occurs via caspase 3 independent pathway.

**Fig. 3.** Cell death upon X-irradiation in M10 and wild-type p53 transfected clones as measured by the dye exclusion test. Panel (a) and (b) show dose dependence of cell death at the incubation time of 24 hrs and 48 hrs after irradiation, respectively. The error bars mean standard deviation derived from several experiments.

**Fig. 4.** Apoptosis induction in M10 and wild-type p53 transfected clones exposed to X-rays at 24 hrs after irradiation. Panel (a) shows dose dependence of apoptosis as determined by nuclear morphological changes stained with Giemsa solution, along with cell viability by the dye exclusion test. Panel (b) represents typical morphological changes of clone #1 cells irradiated with 10 Gy at 24 hrs after irradiation observed by staining with DAPI. The error bars mean standard deviation derived from several experiments.
Heat-induced cell death and apoptosis in wild-type p53 transfected cells
Heat treatment at 44°C for 30 min to clones #5 and 1 significantly enhanced both total cell death and apoptosis evaluated as the similar morphological feature after 2 hrs incubation at 37°C (Fig. 7). The nearly identical percentage
of stained cells in both methods indicates that the enhancement of cell death by p53 introduction is due to the induction of apoptosis. Caspase dependency was observed by the inhibition of a caspase inhibitor as shown in the panel (b) of Fig. 7.

Figure 8 shows the activation of caspase-3 in the heat induced cell death (apoptosis). In accordance with the occurrence of apoptosis in M10 and clone #5 as shown in Fig. 7, a peak for active caspase-3 on a flow cytometric profile was slightly observed in M10 (panel (b)), while clone #5 exhibited a significant peak for active caspase-3 (panel (c)). These results demonstrated that the enhancement of heat-induced cell death by TP53 transfection is ascribed to apoptosis via p53 and caspase-3 dependent pathway.

DISCUSSION

Enhancement of cell death by the incorporation of wild-type p53

The modification of cell death by the expression of wild-type human p53 in mouse radiosensitive M10 cells with mutated p53 was studied upon necrosis by X-irradiation and upon apoptosis by heat treatment. Transfection of wild-type p53 demonstrated significant enhancement in both radiation-,
and heat-induced cell death as determined by the colony formation assay and by the dye exclusion test, respectively. However, the enhanced cell death is likely to result from different mechanisms: For the X-ray case, transfection of wild-type p53 induced largely apoptosis along with partial contribution of necrosis (non-apoptotic cell death), both of which are independent of caspase-3, indicating that p53 enhancement of radiosensitivity in M10 is still executed via pathways that do not use caspase-3 as well as in the p53 independent case. While for the heat induced apoptosis, transfected wild-type p53 seems to enhance the same apoptotic pathway via caspase activation in the downstream of p53 participation. Thus additional enhancement by p53 seems to stick to the same pathways irrespective of the final death mode, apoptosis or necrosis. The present study could also contribute to the understanding of therapeutic effect by adenovirus-mediated wild-type p53 on p53 mutated cancer cells\(^\text{4,14}\) with special concern in the further enhancement of radio-therapeutic effect even for radiosensitive cancer cells.

**Caspase independent cell death by X-irradiation in wild-type p53 transfected M10**

For the p53-dependent and caspase-independent apoptosis induced by the transfection of wild-type p53 the most frequently discussed pathway is probably the AIF (apoptosis-inducing factor) release from mitochondria to nucleus resulting in caspase-independent chromatin condensation.\(^\text{15-20}\) Regulation of AIF by p53 seems to be well established from several investigations.\(^\text{21-25}\) There exist a few reports describing p53-dependent and caspase-independent apoptosis with X-irradiation. Johnson *et al.*\(^\text{26}\) reported that caspase inhibitors such as zVAD-fmk, zDEAD-fmk and BAF did not affect cell death although caspase was activated upon irradiation. Recently Herzog *et al.*\(^\text{27}\) examined radiation-induced apoptosis in retinal progenitor cells, which was not suppressed by a pan-caspase inhibitor in spite of the occurrence of caspase activation upon irradiation. We have also observed a caspase-3 independent pathway to apoptosis and/or apoptosis-related programmed cell death by the treatment of caspase-3 inhibitors upon p53 dependent and caspase-3 activated apoptosis in MOLT-4 cells.\(^\text{22}\) The relevance of radiation-induced caspase-independent apoptosis to the AIF activation should remain to be studied.

The enhancement of necrosis by p53 introduction deserves some explanation. To our knowledge no investigations have appeared to demonstrate p53 enhancement of necrosis. An explanation may be possible that cells under the fate of the death driven by transfected wild-type p53 manage to find out other routes available for cell death even if caspasas, the efficient way to cell death, are blocked for some reason. However since our method to identify necrosis is the subtraction between cell death measured by the dye exclusion test and that by the Giernsa staining, non-apoptotic cell death other than necrosis cannot be excluded in the clasification of necrosis. Lockshin and Zakeri\(^\text{26,29}\) proposed the importance of the participation of proteases other than caspase and the possibility of resultant other types of cell death including autophagy. In fact autophagic cell death has been reported to be induced by radiation.\(^\text{30,31}\) Reports demonstrating p53 participation in autophagy\(^\text{32-34}\) may be in accord with our observation of p53 enhancement of non-apoptotic cell death.

It should be noted the role of Bcl-2 family in the caspase independent cell death including AIF participating apoptosis, necrosis and autophagy. The participation of Bax was reported in the caspase-independent apoptosis\(^\text{35}\) and Bcl-2 family also regulates autophagy.\(^\text{35}\) Furthermore necrosis was suggested to be protected by Bcl-2.\(^\text{35}\) In addition, in the caspase-independent apoptosis in human promyelocytic HL-60 with null p53, Bax activation was detected.\(^\text{36}\) In this respect, Bcl-2 family proteins may play an important role for the enhancement of cell death by the transfection of wild-type p53 protein.

The whole view of the X-ray induced cell death in M10 may be speculated as follows by considering the enhancement by wild-type p53 transfection: X-ray irradiated cells cannot help using p53-independent pathways with no caspase participation because of mutated p53. Since even the introduction of wild-type p53 could not compensate for the inhibition of caspase-dependent apoptotic pathway by mutated p53, wild-type p53 chooses other routes leading to apoptosis or other type of cell death.

**Caspase dependent cell death by heat treatment in wild-type p53 transfected M10**

Since heat treatment induced apoptosis in p53 mutated M10 cells,\(^\text{51}\) the heat is likely to interact with downstream of p53 in the apoptotic pathway, which is distinct from the case for X-rays. The report in which heat treated HL-60 with null p53 exhibited caspase dependent apoptosis would support this notion.\(^\text{30}\) This interaction is probable at the part of mitochondrion participation. In accord with this explanation, p53 independent apoptosis by heat treatment has been reported to be accompanied with Bax activation.\(^\text{36,37}\) Transfection of wild-type p53 probably leads to the same process as reported by Ota *et al.*\(^\text{38}\) in which heat activates p53 followed by Bax and caspase activation. Since caspase participates in the p53 independent apoptosis in M10 by heat treatment as indicated by panel (b) in Fig. 8, it is plausible that the introduction of p53 enhances the same apoptotic pathway.

Finally the possible down-regulation of a surviving signal such as the Akt/survivin pathway by p53 needs some discussion. The Akt/survivin pathway has been frequently referred to in anticancer chemotherapy for acute myeloid leukemia.\(^\text{39,40}\) On the contribution of p53 in this pathway several studies were reported: Using cell lines differing in their p53 status, the Akt/survivin pathway seems to be independent of p53 in heat-induced apoptosis\(^\text{41}\) or in the apop-
tosis by geranylgeranyltransferase I inhibitors, a new class of anticancer drug,\textsuperscript{40} while the case has been reported that a p53-induced route certainly exists, in which p53 down-regulates Akt/survivin pathway via a down-regulation of PTEN.\textsuperscript{41} Considering that survivin has a capability to inhibit caspase-3,\textsuperscript{42} induction of caspase independent apoptosis by wild-type p53 observed in the present results for X-ray case is not likely to be mediated by the Akt/survivin pathway. However the case for the enhancement of heat induced apoptosis via caspase-3 could not exclude the possible involvement of the Akt/survivin pathway, although only p53-independent apoptosis via this pathway was reported in the case of heat treatment.\textsuperscript{41}

In conclusion, transfection of wild-type p53 enhanced cell death induced by both radiation and heat. This enhancement to heat was probably due to the induction of p53-dependent and caspase-3 dependent apoptotic pathway in contrast to the case for X-rays, where necrosis and caspase-3 independent apoptosis was increased. These results suggest that M10 (p53; mutated) has a potential to develop a certain process of p53 dependent and caspase-3 dependent apoptotic pathway which is able to be activated by the heat treatment and that this potential did not work upon X-irradiation and was not recovered by the transfection of wild-type p53.

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