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Delayed Expression of Apoptosis in X-irradiated Human Leukemic MOLT-4 Cells Transfected with Mutant *p53*

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Apoptosis/Leukemia/p53/Radiation/Transfection.

The effects of X-rays on cell survival, apoptosis, and long-term response in the development of cell death as measured by the dye exclusion test were studied in human leukemic MOLT-4 cells (p53 wild-type) stably transfected with a mutant p53 cDNA expression vector. Cell survival, as determined from colony-forming ability, was increased in an expression level dependent manner, but the increase was partial even with the highest-expressing clone (B3). This contrasts with the prior observation that cell death and apoptosis in B3 are completely inhibited at 24 h after irradiation with 1.8 Gy of X-rays[1)]. The examination of B3 cells incubated for longer than 24 h after X-irradiation showed a delay in the induction of cell death and apoptosis. Western blot analysis revealed that the time required to reach the highest level of wild-type p53 protein in B3 was longer than the time in MOLT-4 and that the p53 may be stabilized by the phosphorylation at Ser-15. These results suggest that the introduction of mutant p53 into MOLT-4 merely delays the development of apoptosis, during which the cells could repair the damage induced by X-rays, and results in the partial increase in cell survival.

INTRODUCTION

Apoptosis is a form of controlled cell death. It is observed naturally in cells during a course of development and often in damaged cells when cells are exposed to agents such as X-rays, anticancer drugs, and mutagens[2–4)].

When cells are exposed to γ-rays, apoptosis is induced *via* a signal transduction process such as a p53- or a ceramide-mediated pathway[5)]. Thymocytes are a typical example to undergo p53-dependent apoptosis after ionizing irradiation[6,7)]. Human leukemic MOLT-4 cells are a good *in vitro* model system of thymocytes and die by p53-dependent apoptosis when the cells are exposed to X-rays[1,8)].

Previously, we demonstrated that X-ray-induced apoptosis in MOLT-4 cells is inhibited by the expression of murine mutant p53[1)]. Because this inhibitory effect on apoptosis was observed in the cells during postirradiation incubation, it is of interest to study if this block means the recovery from radiation-induced cell death. In the present work, we studied the effects of X-rays on cell survival as determined from the colony-forming ability of MOLT-4N1 transfectants having the different expression levels of the mutant p53. We found that the block by mutant p53 did not greatly change the effects of X-rays on cell survival, though almost complete inhibition of cell death as measured by the dye exclusion test was observed at 24 h after 1.8 Gy of X-rays[1)]. The present results suggest that the effects of X-rays on the colony-forming ability of cells transfected with mutant *p53* may be explained by the delayed response of a p53-dependent apoptotic pathway in these cells.

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MATERIALS AND METHODS

Cell lines and culture

A MOLT-4N1 (p53 wild-type) clone, which is isolated by selecting a colony from an unirradiated single MOLT-4 cell[9,10)], was previously transfected with murine mutant p53 cDNA (TGC→CGC of codon 173, corresponding to codon 176 in human *p53*), and a variety of stable transfectants were generated and termed F, A, B1, B2, and B3, which express various levels of mutant p53 protein[1)]. The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 U penicillin and 0.1 mg streptomycin per ml). The doubling times of these cell lines were 18 h. COS-7 cells (SV40 transformed kidney cells derived from African green monkey) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics. MCF-7 cells (derived from human breast adenocarcinoma) were grown in Dulbecco's mod-

ified Eagle's medium with 10% fetal bovine serum, 0.1mM MEM nonessential amino acids (Invitrogen), and antibiotics. The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

X-irradiation and colony formation assay

X-irradiation was performed at a dose rate of 0.64–0.66 Gy/min by 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 (the effective energy was 48 keV). Exponentially growing cells were irradiated in a plastic tissue culture flask[9].

A colony formation assay was performed as described previously[9]. In brief, the cells were mixed with 2 ml of culture medium containing 0.3% agarose, and plated on top of 1.5 ml of the culture medium containing 0.5% agarose. The plating efficiencies of the control cells were 89.5 ± 4.3, 82.4 ± 10.2, 73.0 ± 5.3, 69.4 ± 9.6, and 65.5 ± 9.1 % for the F, A, B1, B2, and B3 transfectants, respectively.

Dye exclusion test and morphological analysis

The cells irradiated with X-rays were incubated in a humidified 5% CO_2 atmosphere for various intervals and stained with erythrosine B, as described previously[9].

Morphological features were studied as follows: The cells were fixed in a solution of ethyl alcohol : acetic acid (3 : 1). The fixed cells were placed on a glass slide, stained with 1% Giemsa solution, and observed under an optical microscope.

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 phenyl methyl sulfonyl fluoride (PMSF), then boiled for 3 min. The protein contents were measured with a Bio-Rad protein assay kit (Bio-Rad Lab., Inc.). The COS-7 cells were treated with 400 J/m^2 of UV by using STRATALINKER UV Crosslinker 2400 (Stratagene, USA) and incubated at 37°C for 30 min. The MCF-7 cells were irradiated with 50 J/m^2 of UV and incubated at 37°C for 12 h.

Equal protein amounts (10 or 20 μg) were loaded into each lane of a 10% (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.) and polyclonal antibodies against phospho-p53 at Ser-15, Ser-20, or Ser-46 (Cell Signaling Technology, Inc.) were used as primary antibodies. The pAb1801 is specific to human p53 and does not recognize murine p53. The secondary antibody was horseradish peroxidase conjugated antimouse IgG. The proteins were detected by the use of enhanced chemiluminescence reagents (ECL™) and Hyperfilm™ (Amersham Pharmacia Biotech, Inc.).

RESULTS

Partial recovery of cell survival as observed by the colony-forming ability

Figure 1 shows the cell-killing effects of X-rays on F, A, B1, B2, and B3 cells. These cells were obtained by the transfection of mutant p53 cDNA into MOLT-4N1 cells[1]. The expression levels of the mutant p53 were F<A<B1<B2<B3 for B3 as the highest. It should be noted that in the F cells, mutant cDNA is transfected, but the mutant p53 protein is not expressed; therefore F cells serve as a good reference for a series of experiments[1]. The sensitivity of these cell lines was decreased in relation to the level of mutant p53 expressed. The radiation dose required to reduce the surviving fraction of cells to 10% (D_{10}) was 1.04, 1.00, 1.06, 1.15, and 1.42 Gy for F, A, B1, B2, and B3 cells, respectively. Table 1 shows the inhibited fraction of these cell lines irradiated with X-rays at 0.9 Gy or 1.8 Gy in cell death as measured by the dye exclusion test at 24 h of postirradiated incubation and the inhibited fraction of them in cell death as determined from the colony-forming ability. The significant difference in the effects was clearly seen between the results obtained by the two methods.

Mutant p53-mediated delay of cell death and apoptosis

Time course studies on the development of dead cells as measured by the dye exclusion test and of apoptosis as determined from morphological changes in the nucleus were shown in Table 2. The development of dead cells and apoptosis was delayed in a

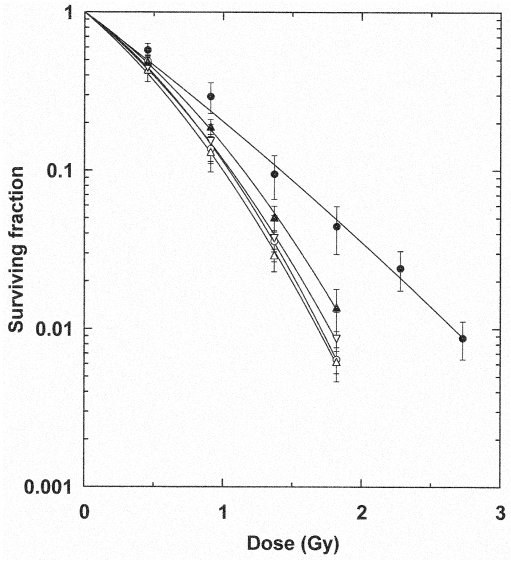

Fig. 1. Survival curves determined by the colony formation assay. F (open squares), A (open triangles), B1 (inverted open triangles), B2 (solid triangles), and B3 (solid circles) cells are transfectants of MOLT-4N1 expressing different levels of mutant p53. The data were expressed as average ± standard deviations.

Table 1. Comparison of cell death as measured by the dye exclusion test and the fraction of dead cells as determined from the colony-forming ability.

Dose (Gy)	Clones	Dye exclusion test*		Colony-forming ability	
		Dead cells (%)	Inhibited fraction**	Dead cells (%)	Inhibited fraction**
0.9	F	33.1 ± 4.6	–	86.3	–
	A	26.7 ± 5.6	6.4	86.9	–
	B1	22.8 ± 3.5	10.3	84.7	1.6
	B2	9.4 ± 1.2	23.7	81.1	5.2
	B3	9.0 ± 0.3	24.1	70.6	15.7
1.8	F	73.4 ± 3.7	–	99.4	–
	A	62.4 ± 8.5	11.0	99.4	0
	B1	52.2 ± 5.9	21.2	99.1	0.3
	B2	21.6 ± 3.1	51.8	98.6	0.8
	B3	11.1 ± 1.0	62.3	95.6	3.8

The data were shown as average ± standard deviation. *The cells were incubated at 37°C for 24 h after X-irradiation. **The inhibited fraction was estimated as the difference in the percentage of dead cells between F and other clones.

Table 2. Time course for the development of cell death as measured by the dye exclusion test and apoptosis.

Clones	Dose (Gy)	Control*	Incubation time (h)			
			15	24	48	72
			Percentage of dead cells			
MOLT-4N1	1.8	7.4 ± 2.2	63.8 ± 1.3	80.8 ± 8.6	83.1 ± 8.2	–
F	1.8	4.5 ± 1.2	–	73.4 ± 3.7	–	–
	4.5		73.4	94.1 ± 0.5	–	–
	9.1		92.9	99.2 ± 0.3	–	–
A	1.8	4.1 ± 1.6	–	62.4 ± 8.5	–	–
B3	1.8	6.0 ± 2.0	–	10.5 ± 0.6	32.0 ± 3.9	59.4 ± 7.1
	4.5		11.6	29.2 ± 2.2	–	–
	9.1		29.9	69.9 ± 3.4	–	–
			Percentage of apoptosis			
MOLT-4N1	1.8	5.6 ± 1.5	69.5 ± 0.6	89.3 ± 2.5	–	–
F	1.8	3.0 ± 2.0	50.5 ± 1.3	83.7 ± 1.5	–	–
B3	1.8	3.6 ± 1.4	4.1 ± 0.5	10.0 ± 3.0	29.1 ± 8.9	64.7

The data were shown as average ± standard deviations. Control means the result for unirradiated cells.

mutant-p53 level–dependent manner in transfectants expressing mutant p53. It is interesting to note that the development of apoptosis was also delayed in coincidence with that of cell death as measured by the dye exclusion test, as it was for MOLT-4N1 or F cells. The results suggest that the fundamental mechanism underlying the induction of apoptosis was not affected by the presence of mutant p53 protein.

Mutant p53 affects persistence of high-level p53 following X-irradiation

Figure 2 shows the Western blot analysis of p53 after X-irradiation. In MOLT-4N1 cells, the level of endogenous wild-type human p53 was increased at first, then decreased slightly for 24 h during postirradiation incubation, but in B3 cells, the level was increased continually up to 24 h or longer and persisted at least up to 48 h (Fig. 2a). Phosphorylation of p53 at Ser-15 was increased before the increase in the level of p53, then decreased in MOLT-4N1, but the level of phosphorylated p53 was kept for longer than 48 h in B3 (Figs. 2a and 2b). It should be noted that the phosphorylation of p53 at Ser-20 was not detected in either cell line (Figs. 2a and 2b) and that no significant increase in the phosphorylation of p53 at Ser-46 was observed in MOLT-4N1 cells irradiated with 9.1 Gy of X-rays in the present study (Fig. 2c).

DISCUSSION

It has been demonstrated that the expression of mutant p53 blocks the activity of coexpressed wild-type p53 through a dominant-negative effect[11–13]. An example of this phenomenon is X-ray-induced apoptosis in MOLT-4 cells[1], which expresses wild-type p53[1,14–16]. X-ray-induced apoptosis in MOLT-4 cells is p53-dependent and has been previously shown to be inhibited by the introduction of a murine mutant p53 cDNA (TGC→CGC of

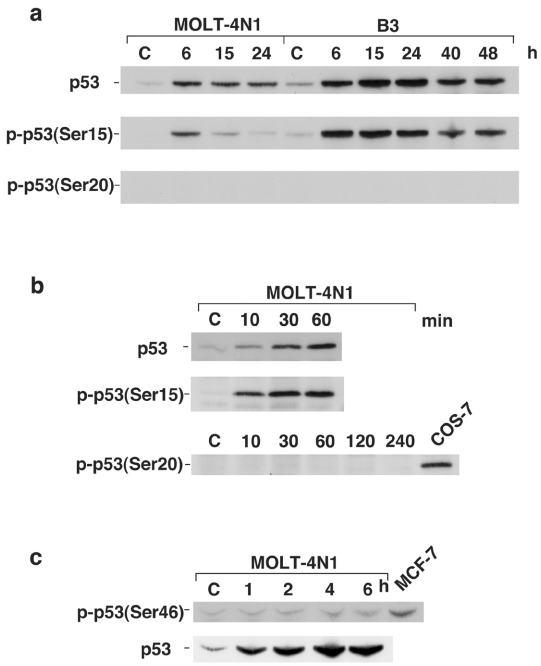

Fig. 2. Western blot analysis of p53 and phosphorylated p53 at Ser-15, -20, or -46. (a) Time course of expression and phosphorylation of p53 at Ser-15 or -20 after irradiation with 1.8 Gy of X-rays in MOLT-4N1 cells and B3 cells. (b) Phosphorylation of p53 at Ser-15 or -20 after irradiation with 9.1 Gy of X-rays in MOLT-4N1 cells. (c) Phosphorylation of p53 at Ser-46 after irradiation with 9.1 Gy of X-rays in MOLT-4N1 cells. The phosphorylation of p53 in COS-7 and in MCF-7 was shown as positive control.

codon 173, corresponding to codon 176 in human *p53*) expression vector into the cells[1].

To examine whether this mutant p53-mediated inhibition of apoptosis saves irradiated MOLT-4N1 cells from dying, we determined the colony-forming ability of MOLT-4N1 cells transfected with a mutant p53 cDNA expression vector. The results showed that the inhibition by mutant p53 was partial (Fig. 1), even in B3, in which X-ray–induced cell death was almost completely blocked for 24 h after 1.8 Gy-irradiation (Table 1). Time course studies revealed that the expression of mutant p53 merely delayed the induction of cell death and apoptosis (Table 2). There are conflicting reports regarding the correlation between radiosensitivity as measured by colony-forming ability and p53 status. Some studies have demonstrated that no alteration in radiosensitivity was found in certain cell lines transfected with mutant *p53*[17,18], but in others, other cell lines transfected with mutant *p53* have been shown to be more radioresistant[19,20]. The present results suggest that an introduction of mutant p53 into MOLT-4 cells may not completely save these cells from dying, but merely delays the development of the cell death and apoptosis. The observed partial recovery in cell survival determined from the colony-forming ability may be attributable to the effects of this delay, since opportunity for the repair of X-ray-induced damage will increase.

Two possible mechanisms for the delayed expression of apoptosis are as follows: the induction of a p53-independent pathway leading to apoptosis and the delayed appearance of a wild-type p53-dependent pathway. It has been demonstrated that there is a late-expressing apoptosis independent of p53 in contrast to an early-expressing one dependent on p53[20,21]. Therefore it is possible to propose a p53-independent pathway for the induction of this delayed expression of apoptosis. In the present case, however, a more probable mechanism would be the latter case, i.e., a delayed appearance of wild-type p53-dependent apoptosis for the following reasons: (a) The increase in cell survival determined from the colony-forming ability was dependent on the expression level of the mutant p53; (b) in X-irradiated B3 cells, wild-type p53 was maintained at a high level for at least 48 h, during which time apoptosis was induced; and (c) the delayed expression of apoptosis coincided with that of cell death, measured by the dye exclusion test, as it did for the early-expressing p53-dependent apoptosis observed in MOLT-4N1 (wild-type p53) cells (Table 2). The mechanism may be explained as follows: In B3 cells, a p53-dependent pathway is activated by X-rays, but it is at first blocked by the mutant p53. Then during the course of incubation, this block begins to leak at a higher level of wild p53.

Recent studies have shown that Ser-15 and/or Ser-20 of p53 are phosphorylated in response to DNA damage caused by ionizing radiation[23,24], that phosphorylation at Ser-15 and/or Ser-20 is important for apoptosis[25], and that, in some cases, phosphorylation at Ser-20 is required for p53 stabilization after DNA damage[26,27]. In the present study, phosphorylation of Ser-20 was not detectable (Fig. 2). Therefore the high level of p53 protein observed in X-irradiated B3 may be due to the stabilization of wild-type p53 *via* Ser-15, but not Ser-20, phosphorylation.

The mechanisms involved in wild-type p53 for the induction of apoptosis have not been clarified yet. One possible explanation is the phosphorylation of p53 at Ser-46[28], which may be blocked by mutant p53 through a dominant negative effect. However, this possibility was not the case for X-ray-induced apoptosis in MOLT-4 cells, since there was little change in Ser-46-phosphorylation, even in nontransfected MOLT-4N1 (Fig. 2c).

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