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Effect of X-rays on Expression of Caspase-3 and p53 in EL-4 Cells and Its Biological Implications[1]

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Objective To investigate the effect of X-rays on expression of caspase-3 and p53 protein in EL-4 cells and its implications in induction of apoptosis and polyploid cells. **Methods** Mouse lymphoma cell line (EL-4 cells) was used. Fluorescent staining and flow cytometry analysis were employed for measurement of protein expression, apoptosis, cell cycle, and polyploid cells. **Results** The expression of caspase-3 protein increased significantly at 8 h and 12 h, compared with that of sham-irradiated control ($P<0.05$, respectively) and the expression of p53 protein increased significantly at 2, 4, 8, 12, and 24 h, compared with that of sham-irradiated control ($P<0.05$-$P<0.01$) in EL-4 cells after 4.0 Gy X-irradiation. Apoptosis of EL-4 cells was increased significantly at 2, 4, 8, 12, 24, 48, and 72 h after 4.0Gy exposure, compared with that of sham-irradiated control ($P<0.05$-$P<0.001$). G_2 phase cells were increased significantly at 4, 8, 12, 24, 48, and 72 h ($P<0.05$-$P<0.001$). However, no marked change in the number of 8 C polyploid cells was found from 2 to 48 h after 4.0 Gy exposure. **Conclusion** The expressions of caspase-3 and p53 protein in EL-4 cells are induced by X-rays, which might play an important role in the induction of apoptosis, and the molecular pathway for polyploid formation might be p53-independent.

Key words: X-rays; Caspase-3; p53; Apoptosis; Polyploid

INTRODUCTION

It is well known that both caspase-3 and p53 are involved in the induction of cell apoptosis[1-5]. However, little is known about the effects of ionizing radiation on the expression of caspase-3 and p53 protein. In this paper we report the changes in expression of caspase-3 and p53 protein in EL-4 cells at different time intervals following 4.0 Gy X-irradiation, and discuss its implications in the induction of apoptosis and polyploid cells by X-rays.

MATERIALS AND METHODS

Cell Line

Mouse lymphoma cell line (EL-4 cells) was used. The cells were cultured in RPMI 1640 medium (GIBCO, USA), supplemented with 20% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator containing 5% CO_2 at 37℃. The doubling time of EL-4 cells was 19 h.

Irradiation

X-rays machine (XSS 205 FZ, China) was used with 180 kV, 15 mA, and 66.5 cm focus-surface distance. The dose rate was 0.278 Gy·min^{-1}. EL-4 cells (1×10^6) were cultured in a six-well tissue culture plate (Nunc, Denmark) containing 2 mL of culture medium per well. After 12 h incubation at 37℃, cells were irradiated with 4.0 Gy X-rays with the parameters measured at 2, 4, 8, 12, 24, 48, and 72 h after exposure. At the same times, sham-irradiated cells were used as control.

Analysis of Caspase-3 and p53 Protein Expression

Expressions of caspase-3 and p53 protein was measured by flow cytometry with fluorescent staining. Cells (1×10^6) fixed in ice-cold 75% ethanol were washed twice with ice-cold PBS before the first monoclonal antibody (50 μL 1:50 dilution each), mouse anti-caspase-3 (Santa Cruz, USA) or rabbit anti-p53 (Santa Cruz, USA) was added. After reaction at 4℃ for 45 min, cells were washed with PBS. The second antibody (50 μL 1:100 dilution

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each), goat anti-mouse IgG- FITC (Santa Cruz, USA), or goat anti-rabbit IgG-FITC (Santa Cruz, USA), was added and kept on ice for another 45 min and washed. Before analysis of each sample on a FACScan flow cytometer, the cells were suspended in 150 μL PBS. Ten thousand cells were analyzed for each sample using the FACScan software and the percentage of positive cells of each sample was calculated with Lysis software.

Measurement of Apoptosis, Cell Cycle, and Polyploid Cells

Apoptosis, cell cycle, and polyploidy cells were measured with a flow cytometer. Cells (1×10^6) were stained with 200 μL propidium iodide (PI, 5 μg/mL, Sigma, Germany) and 50 μL RNase (10 μg/mL, Sigma, Germany) in dark at 4℃ for 30 min, and analyzed with a FACScan flow cytometer (Becton-Dickinson, USA). Ten thousand cells were analyzed for each sample. Apoptotic cells and cell cycle were analyzed using the Mod Fit software. Polyploid cells were analyzed using CELL Quest software.

Statistical Analysis

Student's *t* test was used to evaluate the results.

RESULTS

Changes in Expression of Caspase-3 and p53 Protein in EL-4 Cells After 4.0 Gy X-irradiation

The expression of caspase-3 and p53 protein in EL-4 cells was measured at 2, 4, 8, 12, 24, 48, and 72 h after 4.0 Gy X-irradiation with a flow cytometer. The results showed that the expression of caspase-3 protein increased significantly at 8 h and 12 h after 4.0 Gy exposure, compared with that of sham-irradiated control ($P<0.05$, respectively). The expression of p53 protein increased from 2 to 72 h after 4.0 Gy exposure and significantly at 2, 4, 8, 12, and 24 h, compared with that of sham-irradiated control ($P<0.05$ - $P<0.01$, Table 1).

TABLE 1

Changes in Expression of Caspase-3 and p53 of EL-4 Cells at Different Time Intervals After 4.0 Gy X-irradiation ($\bar{x} \pm s$)

Time After Irradiation (h)	Percentage of Positive Cells (%)			
	Caspase-3		p53	
	Sham-irrad	Irradiated	Sham-irrad	Irradiated
0	18.19±5.66	11.85±3.40	13.90±4.72	8.28±3.31
2	8.78±1.69	7.59±1.87	6.98±0.18	11.34±2.02*
4	13.81±1.70	10.53±2.94	9.66±0.89	13.76±0.58**
8	8.21±4.72	15.41±2.59*	5.49±1.22	12.14±0.71**
12	4.10±1.42	9.78±1.87*	6.81±1.63	13.19±0.83**
24	4.63±2.06	5.22±1.20	4.47±0.27	6.04±0.67*
48	2.26±1.46	2.61±1.66	3.11±1.64	4.46±2.66
72	4.51±1.89	4.81±3.35	2.99±2.56	4.95±2.47

Note. n= 4, *$P<0.05$, **$P<0.01$ *vs* sham-irradiated control.

Changes in Apoptosis of EL-4 Cells After 4.0 Gy X-irradiation

The number of apoptotic cells measured with a flow cytometer, increased significantly at 2, 4, 8, 12, 24, 48, and 72 h after 4.0 Gy exposure, compared with that of sham-irradiated control ($P<0.05$ - $P<0.001$, Table 2).

Changes in Cell Cycle of EL-4 Cells After 4.0 Gy X-irradiation

Cell cycle of EL-4 cells was measured at 2, 4, 8, 12, 24, 48, and 72 h after 4.0 Gy X-irradiation with a flow cytometer, the number of G_1 phase cells decreased from 4 to 72 h e and significantly from 8-72 h after 4.0 Gy exposure, compared with that of sham-irradiated control ($P<0.05$-$P<0.001$). The number of S phase cells also decreased significantly at 2 and 48 h after 4.0 Gy exposure, compared with that of sham-irradiated control ($P<0.05$ and $P<0.001$, respectively). However the number of G_2 phase cells increased significantly at 4, 8, 12, 24, 48, and 72 h after 4.0 Gy exposure, compared with that of sham-irradiated control ($P<0.05$-$P<0.001$, Table 3).

Changes in Polyploid Type of EL-4 Cells After 4.0 Gy X-irradiation

The polyploid type of EL-4 cells was measured at 2, 4, 8, 12, 24, and 48 h after 4.0 Gy X-irradiation with a flow cytometer. The results showed that no significant changes were found in the number of 8 C polyploid type cells from 2 to 48 h, even though a marked increase at 8 h. No significant changes were found in the number of 2 C cells from 2 to 24 h except at 48 h. However, the number of 4 C cells increased significantly at 2 h and 12 h ($P<0.05$, respectively) after exposure (Table 4).

TABLE 2

Changes in Apoptosis of EL-4 Cells at Different Time Intervals After 4.0 Gy X-irradiation ($\bar{x} \pm s$)

Time After Irradiation (h)	Percentage of Apoptotic Cells (%)	
	Sham-irradiated	Irradiated
0	5.96±2.34	5.71±5.69
2	7.73±1.04	11.35±1.18*
4	3.78±0.55	12.29±2.66**
8	7.86±1.15	12.80±1.95*
12	5.31±1.56	18.09±2.41***
24	2.68±2.38	18.65±1.58***
48	0.96±0.17	16.91±1.16***
72	1.39±0.89	10.37±0.23***

Note. n=5, $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs* sham-irradiated control.

TABLE 3

Changes in Cell Cycle of EL-4 Cells at Different Time Intervals After 4.0 Gy X-irradiation ($\bar{x} \pm s$)

Time (h)	Percentage (%)					
	G_1		S		G_2	
	Sham-irrad	Irradiated	Sham-irrad	Irradiated	Sham-irrad	Irradiated
0	61.96±1.99	62.34±7.68	32.00±1.31	27.41±4.02	6.05±3.30	10.25±3.66
2	51.80±1.64	61.94±0.61	37.59±6.77	26.72±1.66*	10.62±5.13	11.35±1.18
4	62.59±1.41	57.96±2.82	33.62±1.59	29.76±3.97	3.78±0.55	12.29±2.66**
8	70.32±2.61	61.09±3.58**	21.64±4.15	26.11±2.35	8.04±1.56	12.80±1.95*
12	70.76±1.09	59.61±1.97***	23.92±2.55	22.48±1.27	5.31±1.56	17.91±2.36***
24	86.60±4.27	74.03±4.49*	9.40±7.95	8.23±3.24	4.01±4.54	17.75±1.70*
48	92.28±0.21	80.54±1.12***	6.66±0.15	2.55±0.62***	1.07±0.06	16.91±1.16***
72	86.32±10.70	71.45±4.71*	12.29±11. 40	18.18±4.76	1.39±0.89	10.37±0.23***

Note. n = 4, $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs* sham-irradiated control.

TABLE 4

Changes in Polyploid of EL-4 Cells at Different Time Intervals After 4.0 Gy X-irradiation ($\bar{x} \pm s$)

Time (h)	Percentage (%)					
	2 C		4 C		8 C	
	Sham-irrad	Irradiated	Sham-irrad	Irradiated	Sham-irrad	Irradiated
0	70.49±5.07	66.49±3.14	14.33±2.19	14.08±117	14.14±2.50	15.39±2.11
2	69.45±1.12	67.50±4.58	14.45±0.88	16.52±1.38*	14.69±1.79	11.70±3.68
4	70.17±5.64	69.71±3.08	14.89±2.01	15.60±2.16	12.96±4.40	11.13±1.98
8	79.97±3.77	70.17±12.77	11.28±1.83	12.53±4.93	7.37±2.81	15.96±4.41
12	87.76±2.39	79.57±7.12	7.46±1.30	9.98±0.99*	5.01±2.75	4.30±1.24
24	71.38±9.88	73.61±10.88	10.67±2.24	10.26±1.24	4.58±0.63	5.84±1.69
48	57.04±12.12	78.71±5.57*	11.44±2.23	10.77±2.12	5.10±1.70	5.14±0.47

Note. n=4, $^{*}P$<0.05 *vs* sham-irradiated control.

DISCUSSION

It is well known that apoptosis can be induced by ionizing radiation both *in vivo* and *in vitro*. In recent years, more attention has been paid to the study of molecular pathway for induction of apoptosis involving caspase, cysteinyl aspartate-specific protease, and p53 protein. T. Hamasu reported that the formation of active fragment p20 of caspase-3 is increased after treatment with 5 Gy X-irradiation and subsequent induction of apoptosis in gastric cancer cell lines MKN45 and MKN28, lung cancer cell line A549, and prostate cancer cell line DU145[6]. Samuni reported that aerobic TK6 human lymphoblastoid

cells irradiated with 6 Gy and 18 Gy separately result in a similar and significant increase in the fraction of apoptotic cells and p53 and caspase-3 levels within 24 h post-irradiation[7]. Feng reported that MOLT-4 cells irradiated with 10 Gy X-rays present classical apoptotic morphology. Caspase-3 is activated and increased remarkably at 4 h after irradiation. The spatial shift of active caspase-3 in MOLT-4 cells induced by X-ray is one of the mechanisms of apoptosis[8]. Enns reported that human A549 lung carcinoma and T98G glioma cells show a marked hypersensitivity at doses <5.0 Gy and a low dose radiation influences cell cycle and apoptosis by caspase-3 activation[9]. Essmann reported that apoptosis resistance of MCF-7 breast carcinoma cells to ionizing radiation is independent of p53 but caused by the lack of caspase-3[10]. In the present study, the expression of caspase-3 protein increased significantly at 8 h and 12 h and the expression of p53 protein also increased significantly at 2, 4, 8, 12, and 24 h in EL-4 cells after 4.0 Gy X-ray exposure. Significantl apoptosis of EL-4 cells was induced at 2, 4, 8, 12, 24, 48, and 72 h after 4.0 Gy exposure, indicating that both caspase-3 and p53 are involved in the induction of apoptosis in EL-4 cells after X-irradiation, which is consistent with the reports mentioned above.

In the present study, the number of G2 phase cells increased significantly from 4-72 h after 4.0 Gy X-irradiation in EL-4 cells, suggesting that G2 arrest can be induced by exposure to X-rays. However, the number of 8 C polyploid cells did not change markedly in EL-4 cells after 4.0 Gy X-irradiation, indicating that S/M uncoupling cannot be induced by X-rays. S/M uncoupling is a new concept in radiation biology, meaning at G2 cells return to S phase before mitosis and continue DNA synthesis to form 8 C, 16 C, or 32 C polyploid cells which would die finally. As is well known, p53 is an important factor for monitoring cell cycle progression[11-12]. It was reported that S/M uncoupling can be induced by ionizing radiation. However, the molecular pathway of uncoupling remains controversial. Waldmen reported that $p53^-$ human colorectal cancer cells undergo DNA synthesis without intervening mitosis[13], While Wenz has reported lack of uncoupling of S/M in $p53^-$ human lymphoblast cells after irradiation[14]. In the present study, the expression of p53 protein increased significantly from 2 h to 24 h after 4.0 Gy X-ray exposure, whereas no marked changes were found in 8 C polyploid cells, suggesting that induction of uncoupling in EL-4 cells by X-rays is p53-independent . Further studies are needed to elucidate the molecular pathway for the induction of polyploid cells by X-rays.

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