Effect of Ionizing Radiation on the Expression of p16, CyclinD1 and CDK4 in Mouse Thymocytes and Splenocytes[1]

GUI-ZHI JU, XIAO-MEI WANG, SHI-BO FU, AND SHU-ZHENG LIU

Department of Radiation Biology, Medical Center in Memorial of Norman Bethune, Jilin University, Changchun 130021, China

Objective To investigate the effect of ionizing radiation on the expression of p16, CyclinD1, and CDK4 in mouse thymocytes and splenocytes. **Methods** Fluorescent staining and flow cytometry analysis were employed for the measurement of protein expression. **Results** In time course experiments, it was found that the expression of p16 protein was significantly increased at 8, 24, and 48 h for thymocytes ($P<0.05$, $P<0.01$, and $P<0.05$, respectively) and at 24 h for splenocytes ($P<0.05$) after whole body irradiation (WBI) with 2.0 Gy X-rays. However, the expression of CDK4 protein was significantly decreased from 8 h to 24 h for thymocytes ($P<0.05$–$P<0.01$) and from 8 h to 72 h for splenocytes ($P<0.05$–$P<0.01$). In dose effect experiments, it was found that the expression of p16 protein in thymocytes and splenocytes was significantly increased at 24 h after WBI with 1.0, 2.0, and 4.0 Gy ($P<0.05$–$P<0.01$), whereas the expression of CDK4 protein was significantly decreased with 2.0Gy for thymocytes ($P<0.05$) and 0.5–6.0 Gy for splenocytes ($P<0.05$–$P<0.01$). Results also showed that the expression of CyclinD1 protein decreased markedly in both thymocytes and splenocytes after exposure. **Conclusion** The results indicate that the expression of p16 protein in thymocytes and splenocytes can be induced by ionizing radiation, and the p16-CyclinD1/CDK4 pathway may play an important role for G1 arrest of thymocytes induced by X-rays.

Key words: Ionizing radiation; CyclinD1; CDK4; Thymocytes and splenocytes

INTRODUCTION

It is well known that p16, encoded by Ink4a gene which has been identified as a tumor suppressor gene, is one of the cyclin dependent kinase (CDK) inhibitor proteins. It binds to and inhibits CDK4 and CDK6, thus preventing CDK4 and CDK6-dependent phosphorylation of Rb and resulting in a block during the G1-S phase transition of the cell cycle. It was reported that ultraviolet irradiation could induce the expression of p16 protein[1,2]. However, little is known about the effect of ionizing radiation on the induction of p16 protein. In the present study, it has been demonstrated that the expression of p16 protein in mouse thymocytes and splenocytes could be induced by X-rays, and the expression of CyclinD1 and CDK4 was reduced after irradiated by X-rays.

[1] This work was supported by a grant from the National Natural Science Foundation of China (No. 39770193).

Biographical note of the first author: Gui-Zhi JU, female, born in 1942, professor of radiation biology, majoring in the effect of ionizing radiation on cell cycle progression and gene control.

0895-3988/2003
CN 11-2816

MATERIALS AND METHODS

Animals

Male Kunming mice, 7-8 weeks old, from the Division of Experimental Animal of Jilin University were used. Standard laboratory chow and water were given. They were observed for one week before irradiation.

Irradiation

Philips therapeutic X-ray machine was used with 200 kV, 10 mA and filters with 0.5 mmCu and 1.0 mmAl. The dose rate was 0.278 Gy • min^{-1}. For dose-effect experiments, the mice were irradiated with 0.5, 1.0, 2.0, 4.0, and 6.0 Gy, respectively. For the time course experiments, the mice were irradiated with 2.0 Gy and the expressions of proteins was measured at 4, 8, 12, 24, 48, and 72 h after exposure. The shamly-irradiated mice were used as control.

Preparation of Cell Suspension

Following sacrifice of the mice by decapitation, thymus and spleen were dissected and squeezed between the mottled ends of two slides in RPMI 1640 medium in a petri dish. The single cell suspension was adjusted to a concentration of 1×10^7/mL and kept on ice after filtrated through nylon mesh and washed with Hank' s solution.

Fluorescent Staining and Flow Cytometry Analysis for Protein Expressions

Cells (1×10^6) fixed in ice-cold 75% ethanol were washed 2 times with ice-cold PBS before the first monoclonal antibodies (each 50 μL 1 50 dilution), mouse anti-p16 (Santa Cruz, USA), rabbit anti-CyclinD1 (Santa Cruz, USA), or rabbit anti-CDK4 (Santa Cruz, USA were added). After reaction at 4℃ for 45 min, cells were washed with PBS. Then the second antibodies (each 50 μL 1 100 dilution), goat anti-mouse IgG-FITC (Santa Cruz, USA), or goat anti-rabbit IgG-FITC (Santa Cruz, USA), were added and kept on ice for another 45 min, then washed. Before each sample was analyzed on a FACScan flow cytometer, the cells were suspended in 150 μL PBS. Ten thousand cells were analyzed for each sample using the software FACScan and the positive cell percentage of each protein was calculated with Lysis software.

Statistics

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

RESULTS

Time Course Changes in p16, CyclinD1 and CDK4 Protein Expression of Thymocytes After Irradiated by 2.0Gy

The protein expression of p16, CyclinD1 and CDK4 in mouse thymocytes was measured at 4, 8, 12, 24, 48, and 72 h after WBI with 2.0 Gy X-ray by flow cytometer. It

was found that p16 protein expression was significantly increased at 8 h (P<0.05), peaked at 24 h (P<0.01) and maintained its increase at 48 h (P<0.05). However, the expression of CDK4 protein was significantly decreased from 8 h to 24 h (P<0.05–P<0.01), and the expression of CyclinD1 was also slightly decreased from 4 h to 72 h. (Table 1)

TABLE 1

Changes of the Expression of p16, CyclinD1, CDK4 Proteins in Mouse Thymocytes at Different Time Intervals After WBI with 2.0 Gy X-rays ($\bar{x} \pm s$)

Time After Irradiation (h)	Percentage of Positive Cell (%)		
	p16	CyclinD1	CDK4
Control	29.89 ± 7.70	17.56 ± 4.47	34.96 ± 7.51
4	25.07 ± 4.25	15.50 ± 5.26	26.39 ± 4.89
8	40.17 ± 1.75*	12.93 ± 1.55	21.98 ± 3.18*
12	34.47 ± 3.59	16.61 ± 7.34	17.46 ± 4.12**
24	42.20 ± 3.82**	14.93 ± 1.67	23.10 ± 3.44*
48	39.21 ± 1.49*	16.31 ± 4.11	31.54 ± 4.60
72	24.66 ± 4.47	13.95 ± 3.60	26.25 ± 6.28

Note. n=5, *P<0.05, **P<0.01 vs control.

Time Course Changes in p16, CyclinD1 and CDK4 Protein Expressions of Splenocytes After Irradiated 2.0 Gy X-Ray

Changes of p16, CyclinD1 and CDK4 protein expressions in mouse splenocytes were also found as in thymocytes after 2.0 Gy exposure. The peak value of p16 protein expression was observed at 24 h (P<0.05). The expression of CDK4 protein was significantly decreased from 8 h to 72 h after exposure (P<0.05–P<0.01). The expression of CyclinD1 protein was also slightly decreased after exposure (Table 2).

TABLE 2

Changes of the Expression of p16, CyclinD1, CDK4 Proteins in Mouse Splenocytes at Different Time Intervals After WBI With 2.0 Gy X-rays ($\bar{x} \pm s$)

Time After Irradiation (h)	Percentage of Positive Cells (%)		
	p16	CyclinD1	CDK4
Control	22.45 ± 7.10	4.55 ± 1.98	31.64 ± 9.76
4	19.64 ± 7.00	3.56 ± 1.79	15.09 ± 4.48
8	24.08 ± 1.00	5.48 ± 1.55	14.80 ± 8.07*
12	29.33 ± 8.58	3.78 ± 1.20	13.39 ± 2.52*
24	32.37 ± 4.59*	3.31 ± 1.67	16.58 ± 2.73*
48	25.15 ± 4.25	1.64 ± 1.01	9.25 ± 2.43**
72	19.28 ± 2.73	3.90 ± 2.04	14.02 ± 2.59*

Note. n=5, *P<0.05, **P<0.01 vs control.

Dose-Effect Changes in p16, CyclinD1, and CDK4 Protein Expressions of Thymocytes

The expression of p16, CyclinD1, and CDK4 proteins in mouse thymocytes was analyzed by flow cytometry 24 h after WBI with 0.5, 1.0, 2.0, 4.0, and 6.0 Gy, respectively. It was found that the expression of p16 protein was significantly increased after exposure to

1.0, 2.0, and 4.0 Gy compared with the control ($P<0.05$, $P<0.01$, and $P<0.05$, respectively). It was also found that CDK4 protein expression was significantly decreased after 2.0 Gy exposure compared with the control ($P<0.05$). The CyclinD1 protein expression was also slightly decreased after exposure (Table 3).

TABLE 3

Changes of the Expression of p16, CyclinD1, CDK4 Proteins in Mouse Thymocytes at 24 h After WBI With Different Dose X-rays ($\bar{x} \pm s$)

Doses (Gy)	Percentage of Positive Cells (%)		
	p16	CyclinD1	CDK4
Control	29.89 ± 7.70	17.56 ± 4.47	34.96 ± 7.51
0.5	34.96 ± 5.77	12.37 ± 0.55	33.68 ± 3.16
1	36.29 ± 5.76*	14.53 ± 7.33	37.23 ± 4.95
2	42.19 ± 3.82**	13.61 ± 2.47	23.03 ± 1.91*
4	39.33 ± 5.04*	20.22 ± 1.96	33.65 ± 4.30
6	14.89 ± 1.24	15.36 ± 2.30	35.27 ± 1.55

Note. n=5, *P<0.05, **P<0.01 vs control.

Dose-Effect Changes in p16, CyclinD1, and CDK4 Protein Expressions of Splenocytes

The expression of p16, CyclinD1, and CDK4 proteins in mouse splenocytes were also measured at 24 h after exposure to different doses. The expression of p16 protein in mouse splenocytes was also found as in mouse thymocytes. P16 protein was significantly increased after irradiated by 1.0, 2.0 and 4.0 Gy ($P<0.05$, $P<0.05$, and $P<0.01$, respectively) of X-rays. However, the expression of CDK4 protein was significantly decreased after irradiated by 0.5 to 6.0 Gy ($P<0.05$–$P<0.001$) of X-rays, and the CyclinD1 protein expression was also markedly decreased after irradiated by 1.0–6.0 Gy of X-rays (Table 4).

TABLE 4

Changes of the Expression of p16, CyclinD1, CDK4 Proteins in Mouse Splenocytes at 24 h After WBI With Different Dose X-rays($\bar{x} \pm s$)

Doses (Gy)	Percentage of Positive Cells (%)		
	p16	CyclinD1	CDK4
Control	31.29 ± 3.78	7.11 ± 2.69	47.57 ± 3.11
0.5	34.57 ± 4.17	7.77 ± 3.54	36.81 ± 5.62*
1	37.63 ± 1.65*	5.59 ± 4.02	22.12 ± 1.03**
2	39.68 ± 3.19*	3.87 ± 1.62	17.28 ± 2.15***
4	41.11 ± 2.53**	2.95 ± 1.21	13.45 ± 5.93***
6	33.39 ± 2.37	1.96 ± 1.03	9.80 ± 3.44***

Note. n=5, *P<0.05, **P<0.01, ***P<0.001 vs control.

DISUSSION

Since the discovery of p16Ink4a[3], one of the cyclin-dependent kinase inhibitors, p16 protein has been intensively investigated. It has been demonstrated that p16 protein binds to

and inhibits the D-type cyclin-dependent kinases (CDKs) and is able to inhibit the activity of all CDKs. It has been found that enforced expression of p16 results in growth arrest in the G1 phase[4]. In recent years, several reports have been concentrated on the effect of UV radiation on the expression of p16 protein. It was reported that when human skin was irradiated by UV with suberythemal doses, p16 protein was expressed in melanocytes and keratinocytes within the epidermis. Levels of p16 protein were dramatically increased at 16 h post-irradiation, peaked at 24 h, and declined at 72 h[5]. Ahmed also reported that when normal human epidermis and cultured keratinocytes were irradiated by UVB, p16 protein increased in both UVB-irradiated epidermis and keratinocytes in a similar manner, in which p16 expression was induced at 24-48 h and peaked at 72-120 h after irradiation[6]. Gabrielli reported that in UV-irradiated HeLa and A2058 cells, the expression of p16 protein increased markedly[7]. Piepkorn reported that the level of p16INK4a protein in melanocytes was significantly increased after sublethal UVB irradiation *in vitro* as compared with that in nonirradiated cells[8]. However little is known about the effect of ionizing radiation on the expression of p16 proteins. In the present study, the effect of X-irradiation on the expression of p16 protein in mouse thymocytes and splenocytes was investigated. It was demonstrated that the expression of p16 protein in thymocytes was significantly increased at 8 h, peaked at 24 h and declined at 72 h, and that the expression of p16 protein in splenocytes was markedly increased at 12 h, peaked at 24 h and declined at 72 h after WBI with 2.0 Gy X-rays in mice. To compare our results induced by ionizing radiation with the others induced by UV radiation, the same tendency for the expression of p16 protein was obtained after exposure. But a certain extent of cell-type dependence was found for the induction of p16 expression induced by X-rays. In our experiments, the expression of p16 protein in mouse thymocytes and splenocytes was significantly increased at 24 h after WBI by 1.0, 2.0, and 4.0 Gy of X-rays.

It has been shown that p16 protein can specifically bind to CDK4 and CDK6 and inhibit their activity, and that CDK4 is redistributed from cyclinD-CDK4 complexes to p16-CDK4 complexes, and unbound D-type cyclin is rapidly degraded[9]. In our experiments, it was demonstrated that the expression of CDK4 protein in thymocytes was significantly decreased from 8 h to 24 h and that the expression of CDK4 protein in mouse splenocytes was also was significantly decreased from 8 h to 72 h after WBI with 2.0 Gy X-rays, while the expression of p16 protein was increased. It was also demonstrated that the expression of CyclinD1 protein in mouse thymocytes and splenocytes was markedly decreased from 4 h to 72 h after WBI with 2.0 Gy X-rays. In the present study, the expression of CDK4 protein was significantly decreased at 24 h after irradiated by 2.0 Gy of X-ray for thymocytes and 0.5 to 6.0 Gy for splenocytes.

It was reported that p16 protein is a negatively regulatory factor in G1-S transition[10, 11]. In our previous paper, we reported that G1 arrest of thymocytes could be induced by a single dose of 0.5, 1.0, or 2.0 Gy of X-rays[12]. It suggests that p16-CyclinD1/CDK4 pathway may play an important role in G1 arrest of thymocytes induced by X-rays.

REFERENCES

1. Wang, X.Q., Gabrielli, B.G., Milligan, A., Dickinson, J.L., Antalis, T.M., and Ellem, K.A.O. (1996). Accumulation of p16CDKN2A in response to ultraviolet irradiation correlates with a late S-G2 phase cell cycle delay. *Cancer Res*. **56**, 2510-2514.
2. Milligan, A., Gabrilli, B.G., Clark, J.M., Hayward, N.K., and Ellem, K.A.O. (1998). Involvenment of p16CDKN2A in cell cycle delays after low dose UV irradiation. *Mutation Res*. **433**, 43-53.
3. Serrano, M., Hannon, G.J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366**, 704-707.

4. Sherr, C.J. and Roberts, J.M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev*. **9**, 1149-1163.
5. Pavey, S., Conroy, S., Russell, T., and Gabrielli, B. (1999). Ultraviolet radiation induces p16CDKN2A expression in human skin. *Cancer Res*. **59**, 4185-4189.
6. Ahmed, N.U., Ueda, M., and Ichihashi, M. (1999). Induced expression of p16 and p21 proteins in UVB-irradiated human epidermis and cultured keratinocytes. *J. Dermatol Sci*. **19**, 175-181.
7. Gabrielli, B.G., Sarcevic, B., Sinnamon, J., Walker, G., Castellano, M., Wang, X.Q., and Ellem, K.A. (1999). A cyclin D-CDK4 activity required for G_2 phase cell cycle progression is inhibited in ultraviolet radiation-induced G_2 phase delay. *J. Biol. Chem.* **274**, 13961-13969.
8. Piepkorn, M. (2000). The expression of p16 (INK4a), the product of a tumor suppressor gene for melanoma, is upregulated in human melanocytes by UVB irradiation. *J. Am. Acad. Dermatol.* **42**, 741-745.
9. Diehl, J.A., Zindy, F., and Sherr, C.J. (1997). Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev.* **11**, 957-972.
10. Tam, S.W., Shay, J.W., and Pagano, M. (1994). Differential expression and cell cycle regulation of the cyclin dependent kinase 4 inhibitor p16INK4. *Cancer Res*. **54**, 5816-5820.
11. Shapiro, G.I., Edwards, C.D., and Rollins, B.J. (2000). The physiology of p16(INK4A)-mediated G1 proliferative arrest. *Cell Biochem. Biophys*. **33**, 189-197.
12. Ju, G.Z., Fu, H.Q., Fu, S.B., and Liu, S.Z. (2001). G1 Arrest and relative protein expressions in mouse thymocytes induced by WBI. *Biomed*. *Environ. Sci*. **14**, 27-31.

(*Received March* 23, 2002 *Accepted October* 19, 2002)