Abnormal G1 Arrest in the Cell Lines from LEC Strain Rats after X-Irradiation

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ABSTRACT. The effect of X-irradiation of cell lines from LEC and WKAH strain rats on a progression of cell cycle was investigated. When WKAH rat cells were exposed to 5 Gy of X-rays and their cell cycle distribution was determined by a flow cytometer, the proportion of S-phase cells decreased and that of G2/M-phase cells increased at 8 hr post-irradiation. At 18 and 24 hr post-irradiation, approximately 80% of the cells appeared in the G1 phase. On the contrary, the proportion of S-phase cells increased and that of G1-phase cells decreased in LEC rats during 8–24 hr post-irradiation, compared with that at 0 hr post-irradiation. Thus, radiation-induced delay in the progression from the G1 phase to S phase (G1 arrest) was observed in WKAH rat cells but not in LEC rat cells. In the case of WKAH rat cells, the intensities of the bands of p53 protein increased at 1 and 2 hr after X-irradiation at 5 Gy, compared with those of unirradiated cells and at 0 hr post-irradiation. In contrast, the intensities of the bands were faint and did not significantly increase in LEC rat cells during 0–6 hr incubation after X-irradiation. Present results suggested that the radioresistant DNA synthesis in LEC rat cells is thought to be due to the abnormal G1 arrest following X-irradiation. — **KEY WORDS:** cell cycle, G1 arrest, LEC strain rat, p53 protein, X-irradiation.

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LEC strain rats have been established at the Center for Experimental Plants and Animals, Hokkaido University [22]. Rats of this strain suffered from spontaneous fulminant hepatitis associated with severe jaundice at about 4 months of age. Other characteristics of LEC rats are a high incidence of spontaneous liver cancer in long-surviving individuals [28] and an increased sensitivity to whole-body irradiation [4, 5]. We have reported that the hypersensitivity of LEC rats to whole-body irradiation is controlled by a single autosomal recessive gene, *xhs* [6], and that the frequencies of all types of chromosome aberrations induced by X-irradiation in the bone marrow cells of LEC rats are approximately 2- to 3-fold higher than those of WKAH rats [17]. Recently, we reported that the fibroblast cell lines established from LEC rats show a high sensitivity to X-irradiation and that the DNA synthesis is resistant to X-irradiation in LEC rat cells [7].

Ataxia telangiectasia (AT) is a human autosomal recessive disorder characterized clinically by progressive cerebellar degeneration, ocular telangiectasia, immunodeficiency and proness to cancer [8, 20, 24, 25]. Patients with AT are unusually sensitive to ionizing radiation, and cultured cells derived from AT patients show an increased sensitivity to ionizing radiation [19, 26]. This disorder is also associated with increased spontaneous and induced chromosome aberrations [3]. The cells from AT patients are also characterized by radioresistant DNA synthesis [18]. Thus, LEC rat cells show the resemble characteristics to AT cells. The radioresistance of DNA synthesis in AT cells is primarily due to the abnormality of many or all of the transient cell cycle arrests which occur in normal cells following ionizing radiation [10, 21].

In the present study, we examined the effect of X-irradiation on the cell cycle progression from the G1 phase

to S phase in the cell lines derived from lung fibroblasts of LEC rats and showed an abnormal G1 arrest following X-irradiation.

MATERIALS AND METHODS

Culture of the cells and X-irradiation: The rat fibroblast cell lines were established from lungs of LEC and WKAH rats by SV 40 immortalization as described previously [7]. Cells were grown in monolayer culture in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS). Cell cultures were kept at ambient humidity and 37°C in an atmosphere containing 5% CO₂.

X-irradiation was carried out utilizing a Hitachi MBR-1520R X-ray generator operated at 150 kV and 15 mA with a 0.5 mm Cu + 1.0 mm Al filter at a dose rate of 0.95 Gy/min.

Flow cytometry: The cell cycle phase distribution of a cell population was determined by measuring the DNA content of individual cells by flow cytometry. In preparation for flow cytometry, cells (1×10^6 to 5×10^6) were exposed to 5 Gy of X-rays. After X-irradiation, the cells were incubated at 37°C for 0–24 hr, harvested by trypsinization, washed with PBS and then pelleted by centrifugation at 500 × g. The cells were fixed in 5 ml of cold 70% ethanol for 30 min at room temperature and then stored at -20°C. Just prior to flow cytometric analysis, individual samples were treated with RNase (1 mg/ml) for 30 min at room temperature and then stained with propidium iodide according to the manufacturer's instructions for the cellular DNA flow cytometric analysis reagent set (Boehringer Mannheim). Fluorescence was measured with a Coulter EPICS Elite flow sorter using a 610-nm filter. Cell cycle distribution was determined using multicycle software.

Immunoblotting: After exposure of the cells (1×10^6 to 5×10^6) to 5 Gy of X-rays, the cells were incubated at 37°C for 0–6 hr, harvested by trypsinization, washed with PBS and then pelleted by centrifugation at $500 \times g$. The cells were counted and solubilized in Laemmli sample buffer as described by Kastan *et al.* [9], and Nelson and Kastan [16]. A modified Lowry assay was used to quantitate relative protein levels in the samples, and extracts were loaded so that equivalent cell numbers and/or equivalent protein amounts were loaded in each comparable set of lanes. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and wet electrotransfer (Mini TransBlot Cell, BioRad) to polyvinylidene fluoride (PVDF) paper (Immobilon-P, Millipore), equivalent protein loading was confirmed by staining the PVDF paper with amido black. Monoclonal antibody to p53 (Pab240) was obtained from Oncogene Science, Inc. Peroxidase-labeled anti-mouse immunoglobulin antibody was obtained from Zymed Laboratories, Inc. The proteins were detected with ECL-Western blot reagents (Amersham Co.) according to the protocols provided by manufacturers. Densitometric analysis was carried out using imaging software (NIH Image).

RESULTS

Cell lines established from lung fibroblasts of LEC and WKAH rats were exposed to 5 Gy of X-rays. After X-irradiation, the cells were incubated at 37°C for 0–24 hr, and their cell cycle distribution was determined by a flow cytometer. Typical sets of data from WKAH and LEC rat cells are shown in Figs. 1 and 2. In the case of WKAH rat cells, at 8 hr post-irradiation, the proportion of S-phase cells in the total cell population decreased from 22.7% (at 0 hr post-irradiation) to 12.6% and the percentage of G2/M-phase cells increased from 13.1% (at 0 hr post-irradiation) to 35.4% (Fig. 1 and Table 1). At 18 and 24 hr post-irradiation, approximately 80% of the cells appeared in the G1 phase. On the contrary, at 8 hr post-irradiation, the proportion of S-phase cells in the LEC rat cell population increased from 23.3% (at 0 hr post-irradiation) to 34.4% (Fig. 2 and Table 1). The percentage of G2/M-phase cells in the LEC rat cell population increased during 8–24 hr post-irradiation compared with that at 0 hr post-irradiation, while the proportion of cells in the G1 phase decreased. Thus, radiation-induced delay in the progression from the G1 phase to S phase (G1 arrest) was observed in WKAH rat cells but not in LEC rat cells.

Expression of the p53 protein in LEC and WKAH rat cells was analyzed by Western blotting after X-irradiation at 5 Gy (Fig. 3a). Results of densitometric analysis of the Western blotting was shown in Fig. 3c. In the case of WKAH rat cells, the intensities of the bands increased at 1 and 2 hr post-irradiation, compared with those of unirradiated cells and at 0 hr post-irradiation. No difference was observed between intensities of the bands of p53 proteins at 0 hr and at 6 hr post-irradiation. In the case of LEC rat cell extracts, only faint bands were observed. The intensity of the bands showed a tendency to increase at 1

Fig. 1. Cell cycle progression of WKAH rat cells following exposure to 5 Gy of X-rays. After X-irradiation, WKAH rat cells were incubated at 37°C for 0, 6, 18 and 24 hr, and the cell cycle distribution was analyzed by a flow cytometer at each incubation time.

Fig. 2. Cell cycle progression of LEC rat cells following exposure to 5 Gy of X-rays. After X-irradiation, LEC rat cells were incubated at 37°C for 0, 6, 18 and 24 hr, and the cell cycle distribution was analyzed by a flow cytometer at each incubation time.

Table 1. Cell cycle distribution of LEC and WKAH rat cells after exposure to 5 Gy of X-rays

Cells	Incubation time after X-irradiation (h)	% of total cell cycle population in:		
		G1	S	G2/M
WKAH	0	64.3 $\pm$ 0.7	22.7 $\pm$ 0.6	13.1 $\pm$ 0.1
	8	52.0 $\pm$ 3.6	12.6 $\pm$ 2.1	35.4 $\pm$ 1.5
	18	82.0 $\pm$ 3.7	10.2 $\pm$ 2.9	7.9 $\pm$ 0.8
	24	79.1 $\pm$ 6.1	11.6 $\pm$ 4.0	9.4 $\pm$ 2.0
LEC	0	58.0 $\pm$ 5.6	23.3 $\pm$ 1.3	18.7 $\pm$ 4.8
	8	33.0 $\pm$ 4.2	34.4 $\pm$ 0.6	32.6 $\pm$ 4.5
	18	39.9 $\pm$ 1.9	33.1 $\pm$ 1.1	26.9 $\pm$ 2.0
	24	36.7 $\pm$ 1.8	34.9 $\pm$ 1.4	28.4 $\pm$ 2.9

Each value represent the average of four separate experiments with the standard deviation.

and 2 hr post-irradiation, but the increase was not significant, compared with that at 0 hr (Fig. 3c).

DISCUSSION

In previous report [7], we have shown that the fibroblast cell lines of LEC rats are hypersensitive to X-irradiation and that the DNA synthesis in LEC rat cells is more radioresistant than that in WKAH rat cells. The dose-response curves for inhibition of DNA synthesis in LEC rat cells are similar to those in AT cells that show a hypersensitivity to ionizing radiation [18, 19]. The radioresistance of DNA synthesis in AT cells is primarily due to the abnormality of many or all of the transient cell cycle arrests which occur in normal cells following ionizing radiation [10, 21]. The present results showed that radiation-induced delay in the progression from the G1 phase to S phase (G1 arrest) was observed in WKAH rat cells but not in LEC rat cells. Therefore, the radioresistant DNA synthesis in LEC rat cells is thought to be due to abnormal G1 arrest.

It has been shown that levels of the tumor suppressor protein p53 transiently increase in temporal association with the transient decrease in replicative DNA synthesis that follows exposure of cells to ionizing radiation, and that the

Fig. 3. Expression of the p53 protein in WKAH and LEC rat cells after X-irradiation. After WKAH rat cells (lanes 1–4) and LEC rat cells (lanes 6–9) were exposed to 5 Gy of X-rays, the cells were incubated at 37°C for 0 (lanes 1 and 6), 1 (lanes 2 and 7), 2 (lanes 3 and 8) and 6 (lanes 4 and 9) hr. Lane 5 represents unirradiated WKAH rat cell extracts. (a) Immunoblots of p53 protein. (b) Total protein loading illustrated by amide black staining of the gel. Dalton mark VII-L (Sigma Chemical Co.) was used as a molecular weight marker (lane M). (c) Densitometric analysis of the filter. Relative intensities of the individual bands of p53 protein to that of WKAH rat cells at 0 hr post-irradiation. Error bars represent the standard deviation of the mean value (n=3).

p53 protein plays a critical role in radiation-induced G1 arrest [9]. Although Western blotting is not a precise quantitative analysis, the present study showed that the expression level of the p53 protein increased in WKAH rat cells at 1–2 hr post-irradiation. In contrast, the intensities of the bands were faint and no significant increase in the level of the p53 protein was observed in LEC rat cells following X-irradiation. This might be responsible for the abnormal G1 arrest following X-irradiation in LEC rat cells. However, the role of p53 in cell cycle arrest and radioresistant DNA synthesis is controversial now. There have been suggestions that p53 abnormalities may be found in AT cells post-irradiation [10, 11], but others have shown that there is significant overlap in p53 responses between AT cells and the cells from normal individuals [2, 14]. Furthermore, it has been reported that the resistance of DNA synthesis in AT cells is dependent on the dose of X-rays [12]. Therefore, further investigation is required to clarify a relationship between the expression of p53 and G1 arrest after X-irradiation in LEC rat cells.

The AT gene is a human phosphatidyl inositol-3'-kinase (PI-3'K) homologue gene [23]. It has been suggested that the products of the AT gene function in the regulation of the expression of the p53 protein in upstream of a damage-responsive pathway that leads to G1 arrest [10, 15]. In LEC rat cells, it remains unknown yet whether the p53 gene *per se* is mutated or whether an abnormality in the upstream gene that regulates the expression of p53 protein occurs. A study on the PI-3'K homologue gene and the p53 gene in LEC rat cells is now in progress.

It is well known that large T antigen of SV 40 virus binds to cellular protein such as p53 protein and affects functions of the cellular protein [13]. It has been reported that SV 40 immortalization itself increases the cellular radioresistance of AT cells [1]. All cell lines established from LEC rats are more sensitive in cell killing and more resistant in DNA synthesis to X-irradiation than those from WKAH rats [7]. Furthermore, DNA synthesis in untransfected lung fibroblasts of LEC rats is more radioresistant than that in untransfected fibroblasts of WKAH rats [7]. Therefore, radiosensitivity and radioresistant DNA synthesis of cells from LEC rats might be unaffected by SV 40 immortalization. Since G1 arrest was observed in WKAH rat cell lines following X-irradiation, it has been suggested that G1 arrest in rat cells also might be unaffected by SV 40 immortalization.

LEC rats exhibit a characteristic radiosensitivity of the whole animal, radioresistant DNA synthesis, and an abnormality in the transient cell cycle arrest just like as ataxia telangiectasia mutation in humans. In the case of AT cells, it has been reported that the radiosensitivity in cell killing is segregated from the radioresistant phenotype of DNA synthesis [26]. Recently, we suggested that two phenotypes, radiosensitivity and radioresistant DNA synthesis, might be also segregated in LEC rat cells [27]. Thus, LEC rat could provide a useful animal model to assist in an understanding of the mechanism of AT, and the study

on the sensitivity and cellular response of LEC rat cells to ionizing radiation may bring to light some of the biological processes involved repair process of radiation-induced DNA damage and regulation of the cell cycle.

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