

ORIGINAL ARTICLE

EXPRESSION OF POLY (ADP-RIBOSE) POLYMERASE AND P53 IN CULTURED MAMMALIAN CELLS EXPOSED TO ACCELERATED HEAVY IONS (IRON OR ARGON)

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Background – The possibility of using high-energy accelerated heavy-ion beams in radiotherapy of malignant tumors is an exciting development. Also, heavy ions comprise an important component of cosmic rays. The DNA molecule should be an important candidate target in heavy ion-induced cell lethality. Poly (ADP-ribose) polymerase (PARP) and tumor suppressor p53 are two important nuclear proteins that recognize heavy ions as a DNA damage signal. We studied the expression of PARP and p53 in cultured mammalian cells exposed to accelerated heavy ions (Fe or Ar).

Methods – Chinese hamster ovary cells (CHO) and Mongolian gerbil fetal (MGF) cells were exposed to iron (Fe) or argon (Ar) ions, which are heavy ions accelerated by a ring cyclotron in Riken, Japan. We studied PARP and p53 expression as DNA strand-break recognition signal markers by immunohistochemistry assay in exposed cells to heavy ions and control cells.

Results – The fraction of cells showing an elevated level of PARP in their nuclei compared to control cells after 1.5 hours of exposure to 4 Gy heavy ions was about 80%. The difference between PARP expression in CHO and MGF cells was negligible. In the immunohistochemistry assay for p53, which was performed in the Mongolian gerbil fetal cell line only, about 80% of cells showed an elevated level of p53 within the nucleus after 4 hours of exposure to 4 Gy heavy ions. However, the increase in PARP expression occurred earlier than p53 in nuclei of Mongolian gerbil fetal cells after exposure to heavy ions. About 20% of cells did not have increased PARP and p53 expression after exposure to either Fe or Ar ions. This 20% might represent a population of cells that did not receive nuclear DNA damage.

Conclusion – These findings suggest that the probabilities of DNA damage after exposure to heavy ions could be evaluated based on PARP or p53 expression. The variation in expression can be related to the traversal of heavy ions through the cell nucleus and induce DNA strand-break as well as key structural components during apoptosis.

Keywords • heavy ions (Fe or Ar) • PARP • p53

Introduction

High-energy heavy ions are components of cosmic rays. Heavy ions such as iron (Fe) ions are known to contribute

significantly to the estimated total radiation exposure during manned space missions.¹ For an accurate assessment of potential health risks to humans during such missions, it is necessary to understand the biological significance of such highly energetic particles. Also, the possibility of using high-energy accelerated heavy-ion beams in radiotherapy of malignant tumors is exciting. A

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higher biological effectiveness for cell killing and good localization are among the most important advantages of such particle beams.

Because the distribution of ionizing radiation is sparse and homogeneous for X or γ radiation, but is dense and concentrated for heavy-ion radiation, the probability that no ionization occurs within the cell target is much higher for heavy-ions radiation when compared at the same dose level. Heavy charged particles can produce clusters of ions during their traversal of the target cells, and kill cells more efficiently than X or γ rays.^{2, 3} In classical theory regarding radiobiological events, cell killing is viewed as an inevitable consequence of the accumulation of some given number of DNA strand breaks.⁴ In mammalian cells, one important cellular response after exposure to DNA damaging agents such as ionizing radiation is activation of poly (ADP-ribose) polymerase (PARP), which is a constitutive eukaryotic nuclear protein.^{5, 6} PARP participates in DNA strand break repair⁷ and induction of cell death or apoptosis.⁸ Also, PARP recognizes and binds to DNA strand breaks and synthesizes poly (ADP-ribose) from NAD substrate. This synthesis seems to depend on the number of DNA strand breaks.⁵

The above-mentioned properties suggest that PARP is a DNA strand-break signal generator. Another nuclear protein, p53, is also activated in response to DNA damage.⁹ Many lines of evidence implicate p53 as a central factor in the cellular response to ionizing radiation, leading to cell cycle checkpoint activation and apoptosis.^{10 - 12} As p53 can recognize the DNA damage terminus,^{13, 14} it might also be a direct DNA strand-break signal.

We performed immunohistochemical analysis with these two important proteins involved in DNA damage recognition by measuring the fraction of stained cells after exposure to accelerated Fe or Ar ions. The fraction of stained cells was related to the traversal of heavy ions through the cell nucleus and, presumably, induction of DNA strand breakage.

Materials and Methods

The Chinese hamster ovary cell line (CHO-K1) provided by the RIKEN Cell Bank, Japan and the Mongolian gerbil fetal cell line (MGF) established in our own laboratory (Dr H. Sasaki), were used in this study. The CHO and MGF cells were cultured in monolayers in media including Ham's F12 and Dulbecco modified eagle (DME) media, each

supplemented with 10% fetal calf serum. Cells were maintained in a 37°C incubator containing a humidified atmosphere of 5% CO₂. The generation times of CHO and MGF cells were 12 and 18 hours, respectively.

Irradiation

Cells were exposed to Fe ions with linear energy transfer (LET): 2000 keV/ μ m, Ar ions with LET: 1640 keV/ μ m, accelerated by the Ring Cyclotron in RIKEN (The Institute of Physical and Chemical Research, Japan). Control X-rays (200 kVp, Shimatzu Pantak, Japan) were used for comparison. LET was used to measure the energy of high-energy particles such as heavy ions. Details of the heavy ion beam delivery systems and dosimetry have been reported previously.^{15, 16}

Immunohistochemical assay

The heavy ion exposed cells were incubated for 2 or 4 hours at 37°C and fixed in 90% methanol for 15 minutes. Immunohistochemical detection was performed using an immunohistochemical staining kit (Non-Biotin Amplification (NAB),TM Zymed Laboratories, INC, USA), which includes: 1) blocking solution; 2) secondary antibody including fluorescence-conjugated fluorescein isothiocyanate (FITC); and 3) tertiary antibody including horseradish-conjugated peroxidase (HRP) and diaminobenzidine (DAB) chromogene. The monoclonal primary antibodies against PARP used was 10-H hybridoma (Trevigen, INC, USA) diluted 1:100 and against p53 protein was DO-1 (Santa Cruz Biotechnology, USA) diluted 1:50 with phosphate-buffered saline (PBS) containing 5% skimmed milk. The primary antibodies against PARP or p53 were incubated with cells at 37°C for 30 minutes and for 90 minutes at room temperature. The cells were then treated with secondary and tertiary antibodies for 30 minutes each at room temperature. After each antibody treatment, cells were washed three times for 5 minutes each in PBS. Positive controls were provided for both PARP and p53 immunohistochemical-staining procedures. Stained sections were observed on a light microscope and cell nuclei were counted to determine the PARP- and p53-positive and -negative cell fractions. This experiment was performed three times under the same conditions for reproducibility.

Results

The results of PARP and p53 protein expression

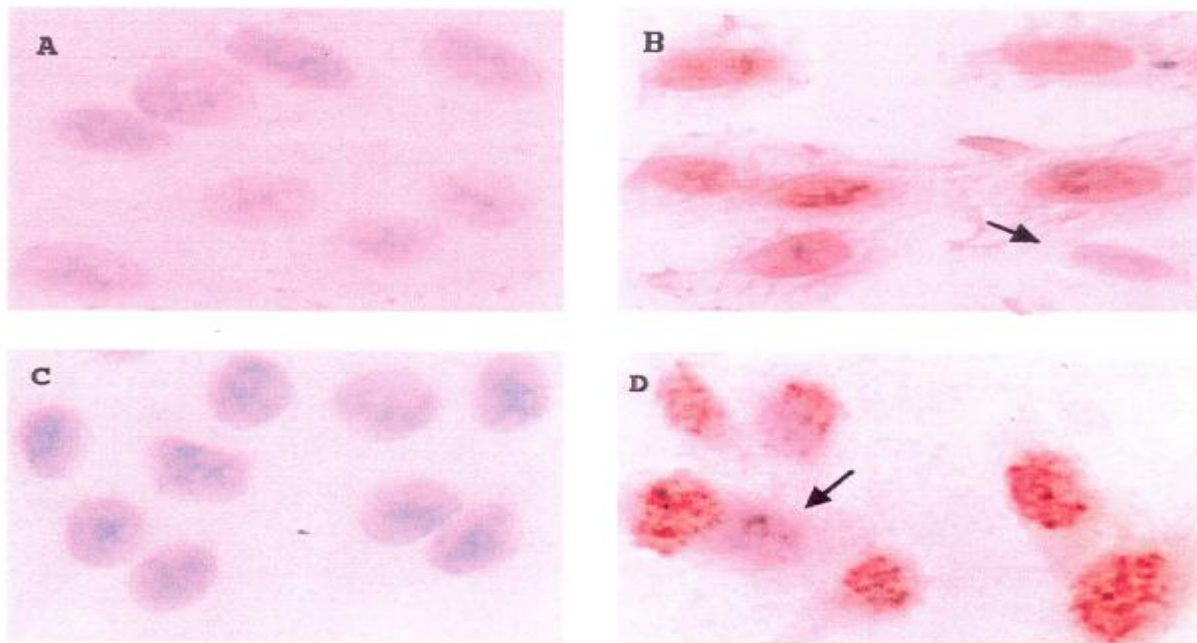


Figure. Photographs showing immunohistochemical staining of p53 in Mongolian gerbil fetal cells (A and B) and poly (ADP-ribose) polymerase in Chinese hamster ovary cells (C and D): A) nonirradiated cells; B) immunostained cells after 4 hours' exposure to 4 Gy Fe ions; C) nonirradiated cells, D) immunostained cells after 1.5 hours' exposure to 4 Gy Fe ions. The arrows show nonhit cells.

in CHO and MGF cells irradiated with heavy ions by immunohistochemical analysis are shown in Figure. Both CHO and MGF cell lines showed a strong positive immunostaining reaction for PARP. The p53 detection experiments were only performed in the MGF cell line, as CHO cells have a mutant p53 gene allele.

The Figure (part B) shows a population of MGF cells, which showed both cells with brown uniform staining of nuclei (p53-positive), and no staining of nuclei (p53-negative). The Figure presents the

results of PARP staining in (part D) CHO cells; there was brown, speckled staining in PARP-positive nuclei and no immunoreactivity in PARP-negative cells.

The calculated PARP-positive and PARP-negative fractions in both MGF and CHO cells are shown in Tables 1 and 2. In 300 cells observed after exposure to 2 and 4 Gy Fe ions, the PARP-positively stained fraction at 1.5 hours post-irradiation was about 43% and 79%, respectively (Table 1). We did not find significant differences

Table 1. Percentage of cells with immunohistochemical staining of poly (ADP-ribose) polymerase on exposure to accelerated Fe ions (LET: 2000 keV/μm).

Cell line	Dose	Incubation time					
		1.5 hr			3 hr		
		++	+	-	++	+	-
MGF	0 Gy	2	3	95 ± 2	3	2	95 ± 1
	2 Gy	9	34	57 ± 3	26	25	49 ± 1
	4 Gy	27	52	21 ± 3	53	27	20 ± 2
CHO	0 GY	3	3	94 ± 1	2	2	95 ± 2
	2 Gy	8	36	56 ± 2	25	26	50 ± 3
	4 Gy	54	28	19 ± 1	31	52	19 ± 1

Total observed cells: 300; (++) = diffusely positive; (+) = focally positive; (-) = negative. MGF = Mongolian gerbil fetal; CHO = Chinese hamster ovary.

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Table 2. Percentage of cells with immunohistochemical staining of poly (ADP-ribose) polymerase after exposure to accelerated Ar ions (LET: 1640 keV/μm).

Cell line	Dose	Incubation time					
		1.5 hr			3 hr		
		++	+	-	++	+	-
MGF	0 Gy	1	3	95 ± 2	3	2	95 ± 1
	2 Gy	9	32	59 ± 3	25	25	50 ± 1
	4Gy	24	52	24 ± 3	51	27	22 ± 2
CHO	0GY	2	4	94 ± 1	3	2	95 ± 2
	2 Gy	8	32	60 ± 2	22	26	52 ± 3
	4 Gy	54	25	21 ± 1	30	50	20 ± 1

Total observed cells: 300; (++) = diffusely positive; (+) = focally positive; (-) = negative. MGF = Mongolian gerbil fetal; CHO = Chinese hamster ovary.

in total (diffusely plus focally) PARP-immunostained cells with increased incubation time of 3 hours in either CHO or MGF cells. Almost the same as the 1.5 and 3 hours post-irradiation of Fe ion incubation were obtained after exposure to 2 and 4 Gy of Ar-ion for PARP-positive and -negative fraction (Table 2).

The immunohistochemical study of p53 in 300 MGF cells showed that about 43% and 78% of cells stained positively at 4 hr after exposure to 2 and 4 Gy Fe ions, respectively (Table 3). The p53-positive cell fraction after exposure to Ar ion was essentially the same as Fe ion results. We found that PARP-positive cell staining was detectable earlier than p53-positive staining of MGF cells in the same fraction.

MGF and CHO cells exposed to 4 Gy X-rays were also observed by immunohistochemical assay. Over 98% of cells were PARP-positive and

p53-positive, and unstained cells could not be identified.

Discussion

Ionizing radiation (IR) gives rise to a variety of cellular DNA lesions including double-strand breakage, single-strand breakage, and a wide variety of damage to the nitrogenous bases and ribose sugar of the DNA chain. DNA damage monitoring and signaling systems are responsible for cell cycle arrest control¹⁷ and repair signal pathways. The failure of these checkpoints leads to cell death.^{18, 19} If the cells sustained the high levels of DNA damage, which can activate in excess of survival factors. This process is important in facilitating DNA repair following low-to-moderate levels of DNA damage. In our previous study using time-lapse observation and CR-39 plastics in

Table 3. Percentage of Mongolian gerbil fetal cells with immunohistochemical staining of p53 after exposure to accelerated Fe ions (LET: 2000 keV/μm) or Ar ions (LET: 1640 keV/μm).

Heavy-ion	Dose	Incubation times					
		2 hr			4 hr		
		++	+	-	++	+	-
Fe	0 Gy	2	2	96 ± 1	3	5	92 ± 2
	2 Gy	9	36	55 ± 2	20	23	57 ± 2
	4Gy	23	51	26 ± 2	50	28	22 ± 3
Ar	0 Gy	3	2	95 ± 3	3	57	90 ± 1
	2 Gy	6	32	52 ± 2	20	23	57 ± 2
	4 Gy	21	55	24 ± 3	48	30	20 ± 3

Total observed cells: 300; (++) = diffusely positive; (+), focally positive; (-) = negative. MGF = Mongolian gerbil fetal; CHO = Chinese hamster ovary.

CHO cells exposed to heavy ions to evaluate hit or nonhit cells,²⁰ we showed that cell death or survival can be measured as a function of the number of particle traversals through the cell nucleus (hit cell) or cytoplasm (nonhit cell). A single hit of Fe or Ar ions to the cell nucleus was determined to be sufficient for a lethal hit event. We speculated in heavy-ions exposed cells in the nucleus, the DNA molecule should be an important candidate for the target of cell lethality induced by heavy ions; i.e. the cell killing effect of heavy ions is correlated with DNA damage. In particular, DNA strand breakage is thought to be the major structural change responsible for the reproductive death of heavy-ion irradiated cells.²¹ To elucidate this hypothesis, two important key players in DNA damage recognition—the tumor suppressor protein p53 and PARP were selected. However, generation of DNA strand interruption in cells exposed to heavy ions elicits a rapid stress response in mammalian cells, which involves attachment of PARP to the strand breaks and recognition of free DNA termini by p53.

The high, localized concentration of PARP and p53 in the cell nucleus could be a guide switch to the existence of perturbation in cell nuclei of DNA-damaged cells. However, high LET radiation induces high levels of DNA damage, which can activate PARP to a great enough extent so that utilization of the substrate NAD becomes the predominant biological effect. The high levels of PARP activation can cause rapid NAD and ATP depletion, leading to cell death before there is an opportunity to repair the DNA damage. This ATP depletion is a suicide mechanism that virtually eliminates the possibility of survival of highly damaged cells.^{22,23} We speculate that the estimated fraction of positively immunostained nuclei of cells is equal to the number of cells in which heavy ions traversed the nucleus.

P53 functions as a negative regulator of cell-cycle progression, especially at the G₁ checkpoint. There have also been reports that suggest that PARP is critical for the induction of G₁ arrest and is also involved in the regulation of G₂ arrest. It was confirmed that p53 stabilization is not altered by PARP inhibitors following γ -irradiation.²⁴

Therefore, it is suggested that there are two pathways for participation of PARP in G₁ arrest signal transfer. The first is through the modulation of WAF1/CIP/p21 pathways. Another possibility is that there is a p53-independent G₁ arrest pathway, in which PARP is involved. In agreement with the

above-mentioned phenomenon, our results indicate that a PARP-IHC assay could be used for detection of heavy ion hits to cell nuclei in p53 wild type (MGF) and also mutant type (CHO) cells. It seems that, during heavy ion induction of DNA damage, there is no requirement for functional interaction between PARP and p53 proteins.

Comparison of immunohistochemical analysis with DNA sequencing data for p53 protein accumulation has established a close correlation of results for the two methods.²⁵ Thus, immunostaining is a simple and fast method that permits not only the identification of the accumulated aberrant protein but also the subclassification of positively stained cells according to their characteristic staining pattern in the nucleus or cytoplasm.

In conclusion, we used immunohistochemical staining to measure the fractions of hit and nonhit cells using PARP and p53 expression as indicators of a hit by accelerated Fe or Ar ions.

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