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Potential role of DNA-dependent protein kinase in cellular resistance to ionizing radiation

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Abstract In this paper, we study the ability of DNA-PK-deficient (M059J) and -proficient (M059K) cells to undergo the rate of cellular proliferation, cell cycle distribution and apoptosis after 10 Gy X-ray irradiation, and the role of DNA-PK in radiosensitivity. The results showed that M059J cells exhibited hyper-radiosensitivity compared with M059K cells. A strong G2 phase arrest was observed in M059J cells post irradiation. Significant accumulation in the G2 phase in M059J cells was accompanied by apoptosis at 12 h. Altogether, the data suggested that DNA-PK may have two roles in mammalian cells after DNA damage, a role in DNA DSB repair and a second role in DNA-damaged cells to traverse a G2 checkpoint, by which DNA-PK may affect cellular sensitivity to ionizing radiation.

Key words DNA-dependent protein kinase (DNA-PK), Cell cycle checkpoint, Apoptosis, Radiosensitivity

1 Introduction

The DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine kinase, and its activity is stimulated by DNA double-strand breaks (DSB)[1], which are particularly dangerous, among various types of DNA damages within the cell, as DSB may cause cell death or cancer if improperly repaired. Eukaryotic cells repair DNA DSB by at least two pathways: homologous recombination (HR) and non-homologous end joining (NHEJ). DNA-PK plays a critical role in DSB repair through NHEJ[2-4]. DNA-PK consists of an approximately 470-kDa catalytic subunit (DNA-PKcs) and a DNA end-binding component, the Ku70/80 heterodimer[5]. DNA-PKcs, by virtue of its C-terminal kinase domain, belongs to a family of phosphatidylinositol 3-kinase-like protein kinases (PIKKs), which also includes ATM and ATR[6]. In response to ionizing radiations, DNA-PKcs is autophosphorylated *in vivo*, and phosphorylated DNA-PKcs co-localizes with γ-H2AX and 53BP1[7,8]. Cells lacking one of these subunits exhibit high radiosensitivity and V(D)J recombination defect. Thereby, DNA-PK is considered as a critical enzyme in the recognition and/or repair of DSB. DNA-PK has been suggested to regulate p53-mediated apoptosis[9,10]. However, results from different studies are conflicting, and some data indicate that irradiated cells deficient in DNA-PK exhibit p53-mediated apoptosis[11,12].

Apoptosis, or programmed cell death, by which unwanted cells in multicellular organisms are eliminated[13,14], plays a major role in counteracting tumor growth and, therefore, acquired defects in apoptotic signaling pathways can be hallmarks of cancers[15,16]. DNA-damaging agents, such as chemotherapeutic drugs or ionizing radiations, are known to induce apoptosis *via* the intrinsic mitochondrial death pathway, in which a class of cysteine proteases termed as caspases plays a crucial role[17,18]. Mechanisms causing radioresistance have not been fully understood, however, recent evidence suggests that aberrant apoptosis may contribute to the phenomenon.

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M059J and M059K are two glioma cell lines, established from different areas of the same tumor[19]. DNA-PK activity was assayed using a synthetic peptide (EPPLSQEAFADLWKK)[20]. Because of a frameshift mutation in exon 32 of the DNA-PKcs gene, M059J cells fail to express DNA-PKcs protein and activity, but M059K cells express normal levels of DNA-PKcs. Studies on two cells deficient or proficient in the DNA-PK complex have provided understanding of the significance of DNA-PK in biologic processes. In this work, we used the two cell lines to investigate the role of DNA-PK in radiosensitivity.

2 Materials and methods

2.1 Culture conditions and irradiation of the cell lines

Two human malignant glioma cell lines, M059K (DNA-PKcs-proficient) and M059J (DNA-PKcs-deficient) were provided by Dr. ZHOU Guangming of Columbia University. The cells were maintained in a medium containing a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM; Gibco) and Ham's F12 medium (Gibco) with 2.5 mM L-glutamine adjusted to contain 15 mM HEPES, 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate supplemented with 0.05 mM non-essential amino acids and 10% fetal bovine serum.

The cells were plated into T25 flask 24 h before 10 Gy X-ray irradiation at a dose rate of 1.0 Gy/min at Gansu Tumor Hospital in Lanzhou.

2.2 In vitro cell proliferation analysis

The rate of cellular proliferation was analyzed with a real-time cell electronic sensing (RT-CES) system (ACEA Bioscience, CA) [21]. The cells were grown on the microelectronic sensors, which consisted of circle-on-line electrode arrays and were placed in the bottom of the microtiter plate. Changes in cell number were monitored and quantified by detecting electrical impedance of the sensor. Cell index (CI) values obtained on the RT-CES system were quantitatively correlated with the cell numbers. The cells were harvested after irradiation and seeded into a 16-well strip at a density of 2×10^3 cells/well for M059K and M059J, respectively. The sensors were placed into a 5% CO_2 incubator, and the CI value was determined every 15 min by the RT-CES system for up to 48 h.

2.3 Cell cycle distribution analysis

Cells were collected at 0, 1, 6, 12, 24, 36 and 48 h after exposure, washed twice with cold phosphate buffered solution (PBS), fixed with 70% ethanol in PBS for 30 min, and stained in dark for 30 min at room temperature with a solution containing 5μg/mL propidium iodide (PI), 10 kU/mL RNase (Sigma), and 0.005% Triton-100, before flow cytometric analysis. The samples were assessed with FACSCalibur (Becton, Dickinson and Company, USA). A minimum of 10 000 cells analyzed in each sample served to determine the percentages of cells in each phase of the cell cycle using Flowjo 5.7.2 software. Three independent experiments were performed.

2.4 Western blot analysis

At 0, 1, 6, 12, 24, 36 and 48 h post-irradiation, the cells were washed twice with cold PBS and lysed in RIPA buffer (50 $mmol\cdot L^{-1}$ Tris-HCl, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate). Protein concentrations were determined with the BCA assay (Thermo Scientific). The samples were electrophoresed through SDS-PAGE and electrophoretically transferred to a Hybond membrane (Amersham). The membrane was blocked in TBS containing 3% BSA and 0.1% Tween 20 and probed with a primary antibody and secondary antibody. Primary antibodies used were rabbit polyclonal antibody to poly (ADP-ribose) polymerase (PARP-1) (Santa Cruz Biotechnology, Inc.) and β-actin (Santa Cruz Biotechnology, Inc.). Secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (Millipore Corp.). Secondary probes were detected by ECL Western blot detection reagents and analysis system (Amersham).

2.5 Statistical analysis

The means of three independent experiments (triplicate samples) ±SD are shown, and statistical calculation was done using Microsoft Excel. Statistical analyses were performed followed by the Student *t* test. $P<0.05$ was considered to be statistically significant.

3 Results

3.1 Effects of exposure to X-rays on cell growth in M059K and M059J cells

The rate of cellular proliferation after irradiation was dynamically monitored by RT-CES system. As shown in Fig.1, cell viability was significantly inhibited in M059J cells, but slightly promoted in M059K cells after irradiation. The data indicate that M059J cells show the sensitivity to X-rays, but M059K cells appear radioresistant phenotype.

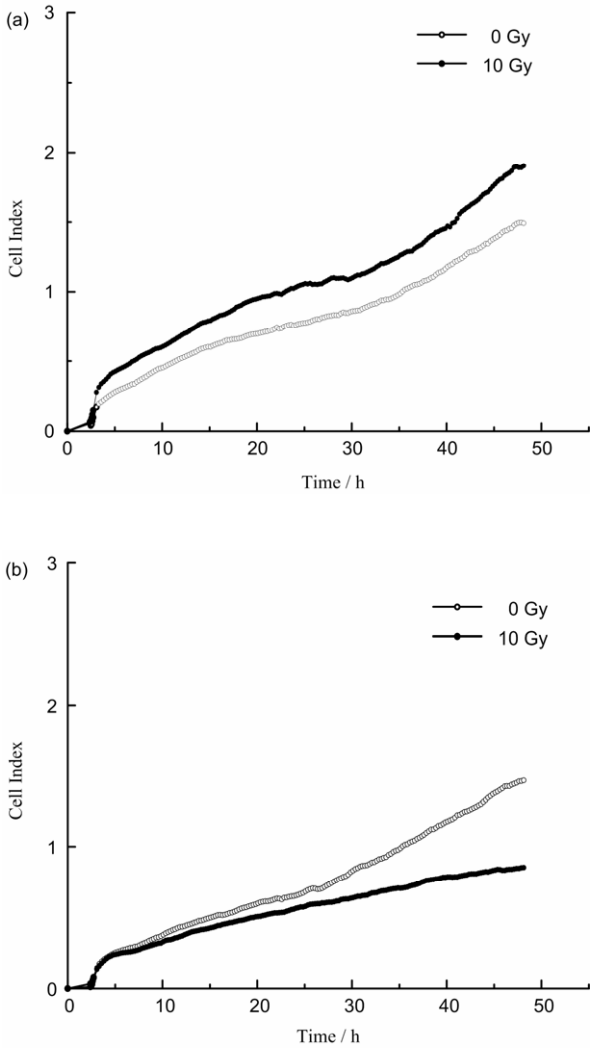

Fig.1 The rate of cellular proliferation of M059K (a) and M059J (b) after 10 Gy X-ray irradiation. Cell index (CI) values obtained on the RT-CES system were correlated with the cell numbers. The CI values were determined every 15 min by the RT-CES system for up to 48 h.

3.2 Effects of exposure to X-rays on cell cycle distribution in M059K and M059J cells

To determine effects of the X-ray exposure on cell cycle progression, cell cycle distribution was assessed by flow cytometry (Fig.2). M059K cells were found to accumulate in the S and G2 phase at 24 h after irradiation, when the percentage of cells was 44.2% (±3.0%) for S phase and 59.5% (±7.5%) for G2 phase, significantly greater than that of the control group(p<0.001). At 36 h, the percentage of cells in G2 phase decreased sharply and, accordingly, the proportion of cells in G1 phase increased from 18.2% (±2.1%) to 49.3% (±2.3%). These indicate that the release of cells from the G2 phase block results in an accumulation of cells in G1 phase.

In contrast, exposure of M059J cells to X-rays resulted in S and G2 phase arrest. Particularly, a substantial increase of G2 phase cells was detected for M059J cells in 48 hours. At 1 h after irradiation, the percentage of G2 cells increased from 22.3 (±3.4%) to 29.0% (±2.3%), which was significantly greater than the control group (p<0.05). At 36 h, the level increase of G2 cells became more obvious and the number of cells in G2 phase reached a maximum (68.2±2.2%). In contrast, the G1 phase population was on the decrease in 48 hours.

3.3 Effects of exposure to X-rays on apoptosis in M059K and M059J cells

It is known that PARP-1 is cleaved during apoptosis by caspase-3 and cleaved PARP-1most likely contributes to apoptosis in various ways[22, 23]. Thus, the presence of cleaved PARP-1 has been considered a characteristic hallmark of apoptosis, and measurement of PARP-1 cleavage is used as a direct measure of apoptosis[24]. To confirm the effect of 10 Gy exposure to X-rays on apoptosis in the M059K and M059J cells, the expressions of native and cleaved PARP-1 were determined by Western blot (Fig.3). PARP-1 precursor (116 kDa) was cleaved to an 89 kDa product in M059J cells at 12 h post-irradiation. In contrast, cleaved PARP-1 was not detectable in M059K cells in 48 hours.

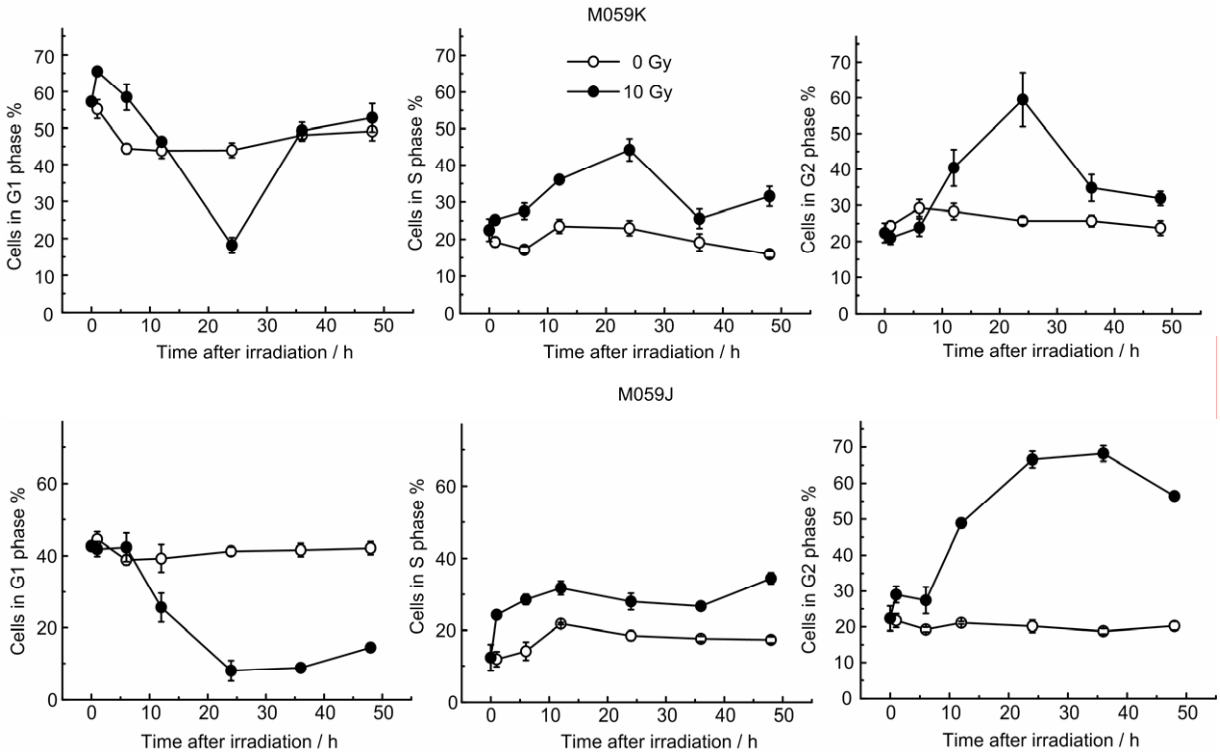

Fig.2 Cell cycle distribution in M059K and M059J cells after 0 Gy (○) and 10 Gy (●) X-rays exposure. The error bars indicate the standard error of three independent experiments.

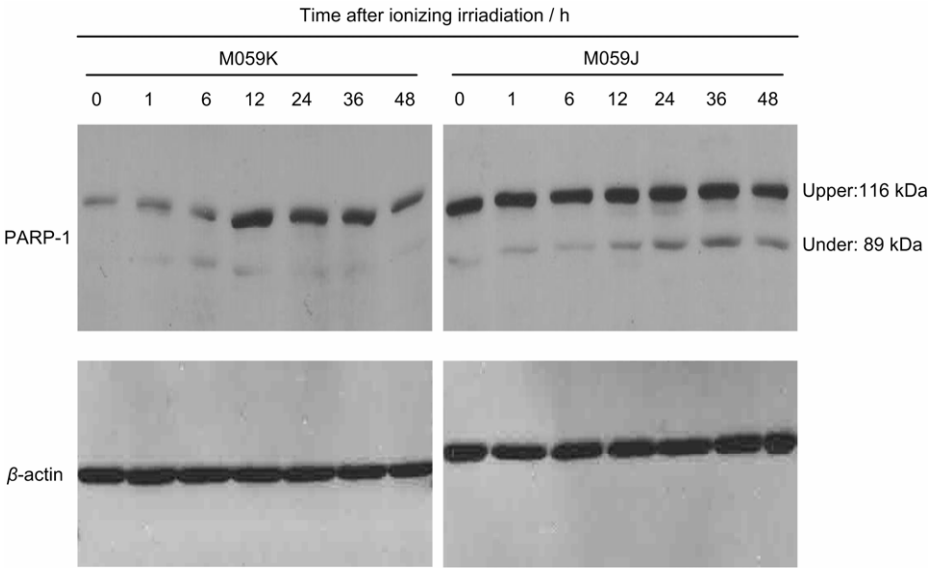

Fig.3 PARP-1 protein expression in M059K and M059J cells after 10 Gy X-ray exposure. Equal amounts of whole cell extracts were separated on SDS-PAGE gels, and protein were detected by Western blot analysis. *β*-actin was used as the control.

4 Discussion

Intrinsic or acquired resistance of tumor cells to chemotherapy or radiotherapy remains a major obstacle to successful cancer management. Mechanisms causing resistance are diverse and poorly defined. It applies especially to the interfaces between DNA-damage recognition, DNA repair, cell cycle arrest and apoptosis signaling. DNA-PK is involved in diverse cellular processes including DNA repair,

V(D)J recombination and apoptosis[1]. In this work, we investigated the potential role of DNA-PK in cellular resistant to X-rays.

The DNA-PKcs–deficient M059J cells were sensitive to X-rays, but the DNA-PKcs–proficient M059K cells displayed radioresistant phenotype. These are consistent with the results in Ref.[25]. DNA-PK plays a critical role in DSB repair and is considered a critical enzyme in the recognition and/or repair of DSB. Thus, a lack of DNA-PKcs activity results in decreased ability to repair DNA DSB in M059J cells and increased sensitivity to ionizing radiations. M059K cells express normal levels of DNA-PKcs and exhibit faster kinetics for DNA DSB repair compared with M059J cells, leading to increase resistance to DNA damage[3]. Moreover，this difference in capacity for DNA DSB repair may somehow contribute to the difference in radiation-induced apoptosis between the two cell lines. Our data showed that the expression of cleaved PARP-1fragment, the characteristic hallmark of apoptosis, was detected in M059J cells rather than M059K cells after irradiation.

DNA damage by X-rays triggers cell cycle checkpoint control signaling and subsequent cell cycle arrest, augmenting the ability of cells to repair damaged DNA. DNA-PK activity is regulated in a cell cycle-dependent manner. Lee S E, *et al*.[26] and Nilsson A, *et al*.[27] reported that wild-type cells exhibit two distinct peaks of DNA-PK activity, one at the G1/early S phase and another at the G2 phase. However, the function of DNA-PK activity in DNA DSB repair at the G1/early S phase or G2 phase is still unclear. Our data indicate that M059J cells arrest in the S and G2 phases after irradiation. This was also reported in Ref.[28]. Particularly, a substantial increase of G2 phase cells was detected for M059J 48 h after irradiation. In contrast, M059K cells were released from the G2 block after irradiation. This demonstrates a role for DNA-PKcs in G2 checkpoint exit after DNA DSB induction. This observation agrees well with Lee S E, *et al*.[26] and Holgersson A, *et al*.[29], who concluded that G2 phase DNA-PK activity is important for G2 exit. The release of M059K cells from the G2 phase block results in an accumulation of cells in G1 phase at 36 h. It is well known that DNA-PK is involved primarily in NHEJ, where the ends of DSB are joined together regardless of the sequences at the two ends, and that this type of DNA repair occurs primarily during G1/early S [30]. Thus, it might be argued that M059K cells released from the G2 checkpoint entered into G1 phase to repair DNA damage through NHEJ. Consequently, it is reasonably argued that M059J cells arrested in G2 phase because of DNA-PK activity deficiency in these cells. Moreover, our data showed that significant accumulation in the G2 phase of the cell cycle in M059J cells was accompanied by apoptosis at 12 h post-irradiation. Thus, we postulated that a persistent arrest in the G2 phase induced the generation of apoptotic signal and the M059J cells with unrepaired DNA damage are eliminated through apoptosis pathway. So, these differences in cell cycle response between M059J and M059K cells indicate that the propensity and ability to deal with the induced DNA damage are also involved.

Altogether, the data support the notion that the inability of the M059J cells to repair DNA DSB results in increased sensitivity to DNA DSB-inducing agents, such as IR, compared with the M059K cells[31,32]. These observations argue that DNA-PK may have at least two roles in mammalian cells after DNA damage, a well-characterized role in DNA DSB repair and a second role in DNA-damaged cells to traverse a G2 checkpoint. These functions of DNA-PK may somehow lead to the difference in apoptotic response between the two cell lines and eventually affect the cellular sensitivity to ionizing radiations. However, the more molecular mechanisms for how DNA-PK is involved in cell cycle progression and how this further affects sensitivity to ionizing radiations, remains to be investigated.

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