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Role of Ku80-dependent end-joining in delayed genomic instability in mammalian cells surviving ionizing radiation

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ABSTRACT

Ionizing radiation induces delayed destabilization of the genome in the progenies of surviving cells. This phenomenon, which is called radiation-induced genomic instability, is manifested by delayed induction of radiation effects, such as cell death, chromosome aberration, and mutation in the progeny of cells surviving radiation exposure. Previously, there was a report showing that delayed cell death was absent in Ku80-deficient Chinese hamster ovary (CHO) cells, however, the mechanism of their defect has not been determined. We found that delayed induction of DNA double strand breaks and chromosomal breaks were intact in Ku80-deficient cells surviving X-irradiation, whereas there was no sign for the production of chromosome bridges between divided daughter cells. Moreover, delayed induction of dicentric chromosomes was significantly compromised in those cells compared to the wild-type CHO cells. Reintroduction of the human Ku86 gene complimented the defective DNA repair and recovered delayed induction of dicentric chromosomes and delayed cell death, indicating that defective Ku80-dependent dicentric induction was the cause of the absence of delayed cell death. Since DNA-PKcs-defective cells showed delayed phenotypes, Ku80-dependent illegitimate rejoining is involved in delayed impairment of the integrity of the genome in radiation-survived cells.

1. Introduction

It is generally accepted that DNA repair pathways are indispensable for the survival of cells exposed to DNA damaging agents, such as ionizing radiation [1–5]. However, DNA repair, by itself, may threaten the stability of the genome in the cells surviving DNA damaging agents [6–9]. For example, non-homologous end-joining (NHEJ), which is the primary DNA repair pathway functions in G1 phase, is error-prone. It sometimes causes loss or rearrangement of the genetic information through mis-rejoining of DNA double strand breaks. Processing of DNA broken ends by exonucleases and endonucleases also provide another chance to alter DNA sequences. These events result in a loss of heterozygosity as well as gross genome rearrangements. In contrast, homologous recombination is a faithful repair in general, as homologous sister chromatids are used to restore the gap of the genetic information. Although most genome rearrangements are generated directly by the initial radiation exposure [10], recent findings have demonstrated that the integrity of the genome is also endangered eventually, if the cells were survived exposure to DNA damaging agents.

It is well described that ionizing radiation induces delayed effects in the progeny of surviving cells [11–14]. This phenomenon is now called radiation-induced genomic instability, which is manifested as the expression of various delayed effects, such as delayed reproductive death or lethal mutation, delayed chromosomal instability, and delayed mutagenesis in the progenies of cells surviving radiation. Radiation-induced genomic instability results in accumulating gene mutations and chromosomal rearrangements, therefore, it has been thought to play a pivotal role in radiation-induced carcinogenesis [15–18]. Because radiation-induced genomic instability is induced in a certain fraction of the progenies stem from a single survived cell, not a single gene mutation but some epigenetic changes may be involved in the initiation of radiation-induced genomic instability. Although oxidative stress and altered chromatin structure have been proposed as the mechanisms of perpetuation of radiation-induced genomic instability [19–24], the mechanism of manifestation has not been fully understood yet. We have shown that delayed unscheduled induction of DNA double strand breaks is involved in the manifestation of delayed phenotypes [25]. In fact, our previous study indicated that increased phosphorylated histone H2AX foci, which correspond to DNA double strand breaks, are frequently detected in the progeny of normal human diploid cells surviving X-rays. Moreover, delayed reactivation of p53 in response to DNA damage was manifested in

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the surviving clones [25]. Delayed induction of DNA double strand breaks was also confirmed by delayed induction of chromosomal aberrations [26]. Thus, it is evidenced that induction of DNA double strand breaks is induced indirectly in surviving cells from exposure to radiation, suggesting that DNA repair pathways could play roles in protecting the genome of surviving cells from harmful effects of radiation.

Previously, Chang and Little reported that radiation-induced genomic instability was abrogated in xrs5 cells, which are NHEJ-deficient Chinese hamster cells defective in Ku80 protein [27]. It was found that delayed reproductive death was not observed in these cells, however, the reason for the absence of delayed reproductive death in xrs5 cells has not been elucidated yet. We have hypothesized that defective NHEJ in xrs5 cells decreases the chance of mis-rejoining of the broken ends, which result in the formation of dicentric chromosomes involved in division halt. Therefore, we examined delayed chromosomal instability in two NHEJ-defective cells, xrs5 and xrs6 cells, and compared the frequency with the wild-type CHO cells. Firstly, we found that delayed induction of DNA double strand breaks in those cells, determined by DNA repair foci formation, was similar. Furthermore, delayed induction of chromatid breaks showed no defect in Ku80-deficient cells. However, delayed induction of dicentric chromosomes was significantly compromised in both xrs5 and xrs6 cells. These results demonstrate that Ku80-dependent mechanism is involved in delayed induction of dicentric chromosomes, and that dicentric chromosomes caused by the mis-rejoining of broken ends are associated with the induction of delayed cell death through the inhibition of cell division. This conclusion was confirmed by the experiment, in which the reintroduction of human Ku80 gene into xrs5 cells restored both delayed dicentric formation and delayed reproductive death. Furthermore, delayed induction of dicentric chromosomes was observed in cells defective in the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). In summary, our present study clearly indicates that the progenies of surviving cells induce delayed DNA damage several generations after the initial insult, and DNA repair capacity is an important determinant for the integrity of the genome in the cells surviving radiation exposure.

2. Experimental procedure

2.1. Cell culture and irradiation

Chinese hamster ovary (CHO) cells, XRCC1-defective EM-9 cells, and Ku80-defective xrs5 and xrs6 cells were cultured in αMEM supplemented with 10% fetal bovine serum (FBS) (TRACE Bioscience PTY Ltd., Australia). Severe combined immunodeficiency (*Scid*) mouse (C.B-17 *scid*/*scid*) and isogenic wild-type mouse (C.B-17 +/+) embryonic fibroblasts were cultured in DMEM supplemented with 10% FBS [28]. Embryonic fibroblasts from C3H/He wild-type mouse and $ATM^{-/-}$ knockout mouse were cultured in DMEM supplemented with 10% FBS [29]. The human KU86 gene was introduced into xrs5 cells by electroporation, and the cells were cultured in αMEM containing 200 μg/ml of G418 and 10% fetal bovine serum (TRACE Bioscience PTY Ltd., Australia). Exponentially growing cells were irradiated with X-rays from an X-ray generator at 150 kVp and 5 mA with a 0.1-mm copper (SOFTEX M-150WE, Softex, Osaka). The dose rate was 0.44 Gy/min. Dose rates were determined with an ionization chamber.

2.2. Cell survival

Cell survival was determined by colony formation assay. Cells cultured in T25 flasks were irradiated with various doses of X-rays, collected by trypsinization, counted the cell number, and appropriate numbers of cells, which give 10^2 surviving cells, were seeded into at least three 100-mm dishes. The cultures were incubated in a CO_2 incubator for 10 days before fixation with ethanol. The colonies were stained with Giemsa's solution, and those consisting more than 50 cells were counted.

2.3. Analysis of delayed effects

Cells cultured in T25 flasks were irradiated with various doses of X-rays. CHO cells were irradiated with 8 and 10 Gy of X-rays, while xrs5 and xrs6 cells were irradiated with 2 and 4 Gy. EM-9 cells were irradiated with 6 and 8 Gy of X-rays. After irradiation, the irradiated cells and the control cells were collected by trypsinization, and appropriate numbers of cells, which give 10^2 surviving cells, were seeded into ten 100-mm dishes. Ten days after irradiation, the primary colonies formed in ten independent dishes were collected by trypsinization. The number of cells was counted, and total population doubling levels (PDLs) were calculated by using the total numbers of cells in ten dishes divided by the number of colonies formed in ten dishes. They were used as the cells at 15–20 population doublings. Rest of the cells was used for the secondary colony formation, and 10^2 cells were re-inoculated into another ten 100-mm dishes. After 10 days, all the colonies formed in ten dishes were collected by trypsinization, counted the number of cells, and total population doubling levels were calculated. They were used as the cells at 30–35 population doublings after irradiation.

2.4. Detection of giant cells and delayed chromosomal bridge formation

Exponentially growing cells were plated onto 22 mm × 22 mm coverslips, and incubated for 24 h before fixation with methanol. Then, cells were stained with 5% Giemsa's solution. The cells, which occupied an area in the colony several times greater than the rest of the cells, were considered to be giant cells as described previously. Chromosomal bridges, which were detected between two dividing daughter nuclei in the anaphase cells, were counted.

2.5. Detection of delayed chromosomal aberrations

Exponentially growing cells were treated with 0.033 μg/ml colcemid (GIBCO, Grand Island, NY) for 1 h, and mitotic cells were collected. They were treated with 0.075 M potassium chloride for 20 min, fixed in ice-cold Carnoy's fixative (methanol:acetic acid, 3:1) for 30 min, and spread on slide glasses using an air-drying method. After staining with 3% Giemsa's solution, chromosome aberrations were classified as previously described [14]. Three independent experiments were performed, and total 200 metaphases were counted per each sample.

2.6. Detection of delayed DNA damage

Delayed induction of DNA double strand breaks was determined by 53BP1 foci. The cells cultured on coverslips were fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, and then were washed extensively with phosphate buffered saline (PBS). The primary antibody, anti-53BP1 antibody (Bethyl laboratories Inc., TX), were diluted in 100 μl of TBS-DT (20 mM Tris–HCl, 137 mM NaCl, pH7.6, containing 50 mg/ml skim milk and 0.1% Tween-20), and applied on the cover slips. The samples were incubated for 2 h in a humidified CO_2 incubator at 37 °C. The primary antibody was washed with PBS, and Alexa594-labelled anti-rabbit IgG antibodies (Molecular Probes, Inc., OR) were added. The cover slips were incubated for 1 h in a humidified CO_2 incubator at 37 °C. They were then washed with PBS and counterstained with 0.1 mg/ml of DAPI. The samples were examined with an Olympus fluorescence microscope AX80 (Olympus, Tokyo). Digital images were captured by a Quantix 1400 camera (Photometrics, AZ), and the images were analyzed by IPLab Spectrum analysis software (Signal Analytics Corporation, VA).

2.7. Immunoblotting and detection

Exponentially growing cells were lysed in lysis buffer (50 mM Tris–HCl (pH7.2), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing 1 mM 4-(2-aminoethyl)-benzensulfonyl fluoride hydrochloride. The cell lysate was sheared through 28 G needle 5 times and cleared by centrifugation at 15,000 rpm for 10 min at 4 °C, and then supernatant was used as total cellular protein. Total protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). Protein samples (16 μg) were electrophoresed on SDS-polyacrylamide gel and were electrophoretically transferred to a polyvinyl difluoride membrane in a transfer buffer (100 mM Tris, 192 mM glycine). After overnight incubation with blocking solution (10% skim milk), the membrane was incubated with anti-Ku80 monoclonal antibody (clone 111, KAMIYA Biomedical Co.) or anti-XRCC1 polyclonal antibody (NOVUS Biologicals), a biotinylated anti-mouse IgG antibody, and streptavidine–alkaline phosphatase. The bands were visualized after addition of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as a substrate.

2.8. Data analysis

A Student's *t*-test was used to evaluate significant difference between the control and irradiated cells. *P* values of less than 0.05 were considered significant difference.

3. Results

Exponentially growing CHO, XRCC1-defective EM-9 cells, and Ku80-deficient xrs5 and xrs6 cells were exposed to various doses of X-irradiation. Both xrs5 and xrs6 cells show significant reduction of cell survival as compared to CHO cells (Fig. 1). In order to compare delayed induction of genomic instability at the same survival levels, 8 and 10 Gy of X-rays were irradiated to CHO, while 2 and 4 Gy