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Detection of DNA strand breaks in mammalian cells using the radioresistant bacterium PprA protein

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Abstract

We have previously found that the PprA protein from *Deinococcus radiodurans* possesses ability to recognize DNA carrying strand breaks. In the present study, we attempted to visualize radiation-induced DNA strand breaks with PprA protein using immunofluorescence technique to elucidate the DNA damage response mechanism in mammalian cultured cells. As a result, colocalization of Cy2 and DAPI fluorescent signals was observed. This observation suggests that DNA strand breaks in the nucleus of CHO-K1 cells were effectively detected using the PprA protein. The amount of DNA strand breaks (integrated density of Cy2 fluorescent signals) was increased with the increase in the radiation dose.

Keywords; *Deinococcus radiodurans*, PprA protein, DNA strand breaks, mammalian cell, radiation risk

1. Introduction

For living organisms, it is essential to stably maintain and inherit DNA that carries genetic information. However, they are always facing risk of DNA damage during life in various environments. The damage is caused by both intrinsic and extrinsic factors; respiration-generating active oxygen, DNA replication error, desiccation, ionizing radiation and genotoxic chemicals etc. Failure in DNA damage repair can lead to the loss of genetic information by mutation, chromosome loss, or rearrangement, and thereby in some instances, the failure results in the death of cell. DNA damage is classified into two types; base damage (pyrimidine dimer, oxidative damage, base loss, and cross-link etc) and DNA strand break (single and double strand break). Among them, DNA double strand break is the most lethal damage. Organisms had acquired DNA repair mechanism to cope such genetic lesions. However, radiosensitivities of organisms vary

extensively with species. In mammalian cultured cells that are comparatively sensitive to ionizing radiation, it is important to assess intracellular distribution and generative frequency of lethal DNA strand breaks induced by irradiation in order to get insight into cell's damage response and repair potential. Therefore, the development of effective technique is required to analyze DNA damage response mechanism in mammalian cells. However, such development had been hampered by the lack of appropriate method to evaluate the number of DNA strand breaks in cells immediately after irradiation.

The radioresistant bacterium *Deinococcus radiodurans* is characterized by its extraordinary resistance to the lethal and mutagenic effects of ionizing and UV radiation and to many other DNA damaging agents (1-3). This resistance is considered to be due to a highly proficient DNA repair capacity. The most noteworthy characteristic is a remarkable capacity for repairing DNA double-strand breaks (4, 5). Through the analysis of a DNA repair-deficient mutant strain that exhibits considerably extreme sensitivities to gamma ray, UV, and mitomycin-C. We have previously demonstrated that the *D. radiodurans* genome encodes a novel DNA repair protein (designated PprA for promoting prominent repair) responsible for its DNA repair proficiency. We have found that the PprA protein could bind to double-stranded DNA (dsDNA) with open circular form and linear dsDNA, but not bind to either dsDNA with closed circular form or single-stranded DNA. This result suggested that PprA protein possesses ability to recognize DNA carrying strand breaks (6).

In the present study, we attempted to visualize radiation-induced DNA strand breaks with PprA protein using immunofluorescence technique to elucidate the DNA damage response mechanism in mammalian cultured cells.

2. Methods

2.1 Antibody

The *D. radiodurans* PprA rabbit antiserum was raised against purified *D. radiodurans* PprA protein. Preparation of the antiserum was carried out by Immuno-Biological Laboratories (Gunma, Japan). The antiserum was precipitated with ammonium sulfate (50% saturation). After dialysis, anti-PprA IgG was purified by several chromatographic separations using HiTrap rProtein A and Mono S columns (Amersham Biosciences) (Fig. 1). The fluorescent dye Cy2 was directly conjugated to the purified anti-PprA IgG using an Ab Cy2 labeling kit (Amersham Biosciences).

2.2 Cell preparation

Chinese hamster ovary (CHO-K1) cells were cultivated in Ham's F-12 medium supplemented with 10% fetal bovine serum (FCS) at 37°C in an atmosphere of 5% CO_2 and 95% air. Exponentially growing cells were washed twice with phosphate-buffered saline (PBS). The cells were trypsinized, collected, and resuspended in the same medium. The resulting cell suspension was applied onto a cover glass in a culture dish, and incubated for 2 h at 37°C in an atmosphere of 5% CO_2 and 95% air. The dish was filled with the same medium and incubated for 18 h to homogeneously adhere cells onto a cover glass.

2.3 Irradiation

CHO-K1 cells adhered onto a cover glass was irradiated at 4°C by ^{60}Co gamma rays (0, 0.5, 1, 5, 30, 50, 75, and 100 Gy). These doses were regulated by changing the distance of sample from the gamma ray source.

2.4 Immunofluorescent assay

After irradiation, the cells were immediately fixed with a fixation buffer (10 mM Tris-HCl [pH 7.6], 50 mM EDTA, and 4% paraformaldehyde) for 30 min at 4°C. Following fixation, the cells were washed in buffer 1 (10 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, and 1 mM DTT) for 5 min (twice). The cells were then permeabilized with buffer 1 containing 1 μg/ml protenase K and 1% SDS for 30 s and washed in buffer 1 for 5 min (three times). The cells were incubated with buffer 1 containing 1% Nonidet P40 (Roche) for 90 min at 37°C and then incubated with buffer 1 containing 1% bovine serum albumin (BSA) for 60 min at 37°C. The cells were incubated with buffer 2 (10 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 1 mM DTT, and 0.1% BSA) containing 250 ng/ml PprA protein for 60 min at 37°C, followed by the incubation with Cy2-conjugated anti-PprA IgG (2 μg/ml in buffer 2) for 60 min at 37°C. The cells were counterstained with 0.1 μg/ml DAPI (4',6-diamino-2-phenylindole) for 1 min, mounted on a slide glass with an antifade solution (0.5% *p*-phenylendiamine in glycerol) and subjected to fluorescence microscopy observation (Fig. 2). Images were captured by using a cooled charged-coupled device (CCD) camera (Penguin 600CL, Pixera). The integrated density of Cy2 fluorescent signals in nuclei was measured by using Komet 4.0 (Kinetic Imaging).

3. Results & Discussion

3.1 Colocalization of Cy2 and DAPI fluorescent signals

Immunofluorescence analysis of irradiated CHO-K1 cells was performed with *D. radiodurans* PprA protein that possesses ability to recognize DNA carrying strand breaks. Incubation with Cy2-conjugated anti-PprA IgG enables to detect PprA protein bound with DNA strand break. Images of nuclei were captured with a DAPI (nucleus) filter (Fig. 3A), and images of the same field of view were then captured with a Cy2 filter (DNA strand breaks) (Fig. 3B). Colocalization of DAPI and Cy2 fluorescent signals was observed in the merged image (Fig. 3C). This observation suggests that DNA strand breaks in the nucleus of CHO-K1 cells are effectively detected with PprA protein.

3.2 Dose dependency of DNA strand breaks in CHO-K1 cells

To investigate whether the amount of DNA strand breaks is dependent on radiation dose, we examined the cells irradiated by ^{60}Co gamma rays with different doses (0, 0.5, 1, 5, 30, 50, and 100 Gy). The integrated density of Cy2 fluorescent signals of nuclei was scored for each dose. The integrated density corresponding to the amount of DNA strand breaks was increased with the increase in the radiation dose (Fig. 4, 5). The Cy2 fluorescent signals even were observed in non-irradiated cells (Fig. 4A), suggesting DNA strand breaks induced by respiration-generating active oxygen or during DNA replication. In previous study, some DNA strand breaks detection methods using DNA binding protein and its specific antibody were developed (7-9). However, it is difficult to detect an initial damage immediately after irradiation by these methods, because of necessity of post-irradiation incubation. In contrast, our method can apply for the effective detection of initial damage immediately after irradiation.

4. Conclusions

We have been successful in development of an effective detection method to evaluate DNA strand breaks in mammalian cells using PprA protein. Thus, this method can be useful in establishing the radiation risk assessment in molecular and cellular levels. By improving the sensitivity for the detection of DNA strand breaks, this method can be available as a biological dosimeter on the evaluation of low-dose radiation risk. This method is technically possible to apply to the genetic toxicity test that is used for the detection of genotoxic chemicals.

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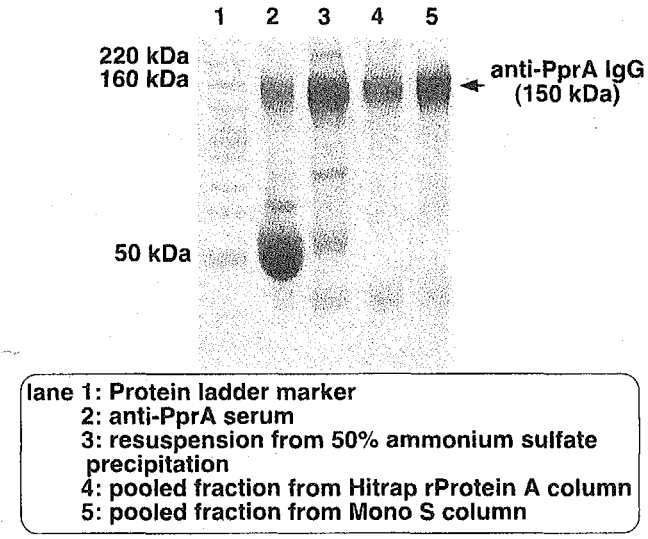

Fig. 1. Purification of anti-PprA IgG. The purified anti-PprA IgG migrated on a polyacrylamide gel with an apparent molecular mass of 150 kDa. The fluorescent dye Cy2 was directly conjugated to purified anti-PprA IgG.

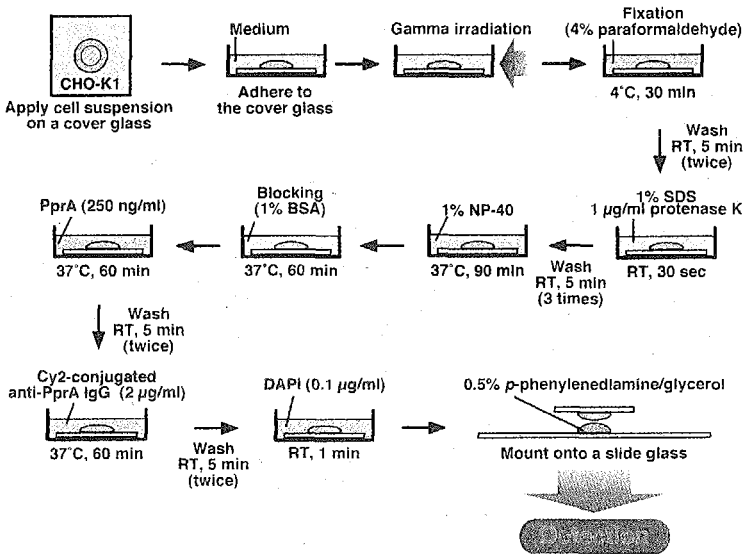

Fig. 2. Procedure for detection method of radiation-induced DNA strand breaks.

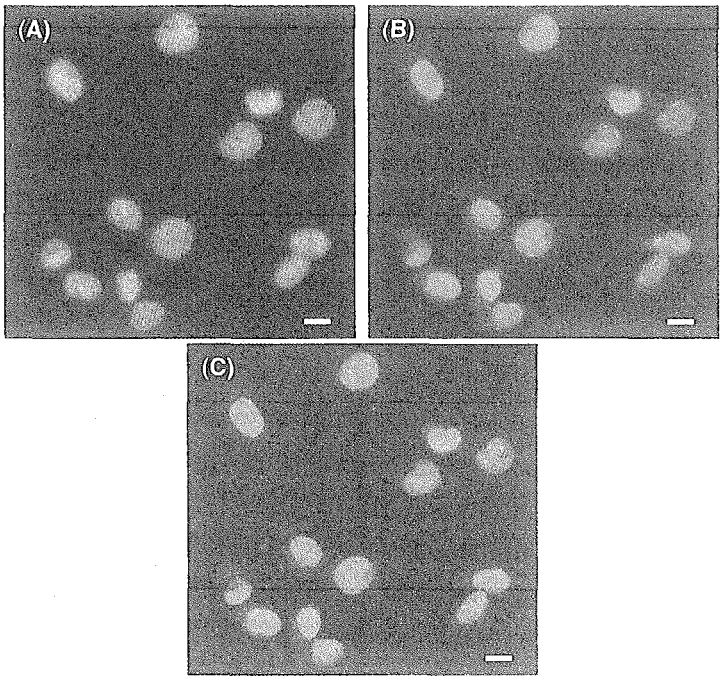

Fig. 3. Colocalization of Cy2 and DAPI fluorescent signals. Cells were stained with Cy2-conjugated anti-PprA IgG and DAPI. Images of the same field of view were captured under DAPI (nucleus) (A), Cy2 (DNA strand breaks) (B) filters, and merged (C). Bars indicate 10 μm.

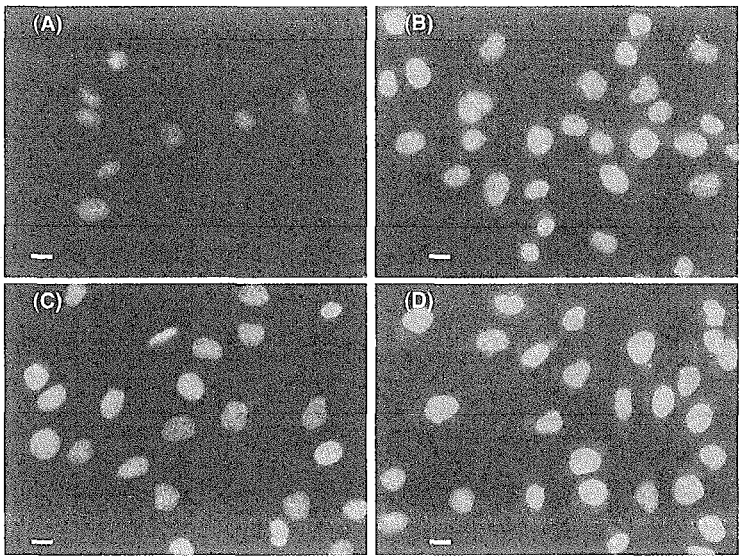

Fig. 4. Increase of DNA strand breaks in CHO-K1 cells following gamma irradiation. The cells were irradiated by ^{60}Co gamma rays; non-irradiation (A), 30 Gy (B), 50 Gy (C), and 100 Gy (D), and stained Cy2-conjugated anti-PprA IgG. Bars indicate 10 μm.

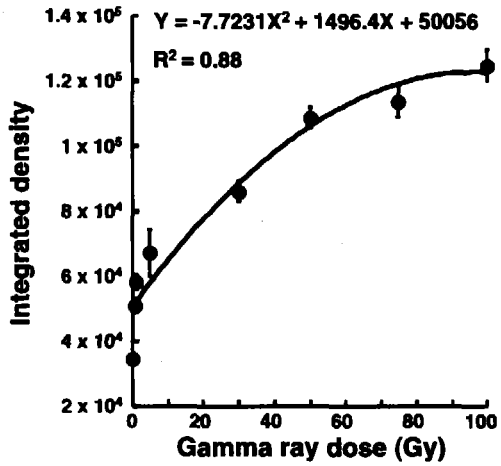

Fig. 5. Dose response curve of DNA strand breaks in CHO-K1 cells. The cells were irradiated by ^{60}Co gamma rays (0, 0.5, 1, 5, 30, 50, and 100 Gy) and treated with Cy2-conjugated anti-PprA IgG. Dots indicate the integrated density of Cy2 fluorescent signals in nuclei at each dose. The integrated density is corresponding to the amount of DNA strand breaks.