Susceptibility of Mouse Splenic Cells to Oxidative DNA Damage by X-Ray Irradiation

Shoichi TAHARA and Takao KANEKO*

Redox Regulation Research Group, Tokyo Metropolitan Institute of Gerontology; 35–2 Sakaecho, Itabashi-ku, Tokyo 173–0015, Japan. Received August 29, 2003; accepted October 29, 2003; published online October 31, 2003

Susceptibility to oxidative stress by X-ray irradiation was examined in splenic cells of BDF1 mouse and fetal human lung fibroblasts, TIG-7. Survival rates of splenic cells irradiated with X-rays were lower than those of TIG-7 cells irradiated similarly. The content of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) immediately after X-ray irradiation in the DNA of splenic cells increased until 2 Gy irradiation, but remained constant above 2 Gy. The 8-oxodG contents rose in proportion to the dose of X-rays in TIG-7 cells. Although the survival rate of splenic cells exposed to 1 Gy irradiation decreased with time, the survival rate of TIG-7 cells remained unchanged. The 8-oxodG content in splenic cells irradiated with X-rays did not decrease even 48 h after irradiation, while that in TIG-7 cells decreased with time, and recovered to the pre-irradiation level after 48 h. A DNA ladder was observed in splenic cells 2 h after X-ray irradiation, but the ladder was not found in fibroblasts. Furthermore, caspase-3 activity increased after X-ray irradiation of splenic cells. These results indicate that splenic cells are sensitive to oxidative stress induced by X-ray irradiation and that splenic cells damaged by even low doses of X-rays are removed through apoptosis rather than by a repair pathway.

Key words: splenic cell; 8-oxo-7,8-dihydro-2′-deoxyguanosine; apoptosis; X-ray; TIG-7

Reactive oxygen species (ROS) formed in cells by mitochondrial respiration, phagocytosis, ionizing radiation and chemical oxidizing agents have been shown to be a major cause of oxidative damage to lipids, proteins, DNA, etc. Hydroxyl radicals are suggested to play an important role in the damage caused by ionizing radiation. Many kinds of oxidative damage are induced in the body by X-ray irradiation. DNA lesions, such as modified bases, abasic sites, and DNA strand breaks, are formed by X-ray irradiation. For example, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is formed in DNA by ROS, such as hydroxyl radicals and singlet oxygen, and is recognized as a biomarker of oxidative DNA damage. If 8-oxodG residues are not removed before DNA replication, they cause G-C→T-A transversions.

A variety of antioxidant mechanisms in the body protect tissues against damage caused by ROS. In general, susceptibility of mitotic cells to oxidative stress is higher than that of postmitotic cells. If antioxidant defenses are insufficient, ROS could cause damage, including DNA lesions, to cells.

Two patterns of cell death, necrosis and apoptosis, are known. Necrosis results from metabolic collapse in cells, when cells can no longer maintain their homeostasis, while apoptosis is known as programmed cell death, and plays a central role in cell death that occurs during normal development in animals. In this study, the difference in susceptibility of splenic cells as undifferentiated cells and fibroblasts as differentiated cells to X-ray irradiation was examined based on changes in survival rates and 8-oxodG contents. In addition, in order to confirm the process of cell death, we examined whether apoptosis occurs in splenic cells and fibroblasts after X-ray irradiation.

MATERIALS AND METHODS

Animals Male BDF1 mice were obtained at 9 or 10 weeks of age from SLC Japan Inc. (Shizuoka, Japan) and housed 4—5 mice per cage under specific-pathogen-free conditions. The animals were fed a commercial laboratory diet, CRF-1 (Oriental Yeast Inc., Tokyo, Japan), and water ad libitum. The animals were maintained at 21±2 °C in a photoperiod-controlled (12h/d) room.

Cell Culture Mice were killed by cervical dislocation under anesthesia and their spleens were quickly isolated and put in phosphate-buffered saline (PBS, pH 7.2). Splenic cells were obtained by leaching the spleens on a #100 gauge wire mesh in α-medium (ICN Biomedicals Inc., Aurora, OH, U.S.A.), and suspended in α-medium supplemented with 30% fetal bovine serum (FBS; Moresgate, Melbourne, Australia), 1% deionized bovine serum albumin, 100 μM mercaptoethanol, 2 units of human urine erythropoietin (Sigma, St. Louis, MO, U.S.A.) and 10% (v/v) pokeweed-mitogen-stimulated murine spleen cell conditioned medium (PWM-SCCM; Stemcell Technologies Inc., Vancouver, BC, Canada). The cell suspension was mixed by pipetting and passed through another mesh. The suspensions (3 ml) containing 5×10⁶ splenic cells were incubated at 37 °C in 35 mm plastic dishes under a humidified atmosphere of 5% CO₂ and 95% air. Human embryonic fibroblasts, TIG-7, established from embryonic lungs in the Tokyo Metropolitan Institute of Gerontology, were incubated at 37 °C in Eagle’s MEM supplemented with 10% FBS in 10 cm plastic dishes under a humidified atmosphere of 5% CO₂ and 95% air.

X-Ray Irradiation Cells were irradiated with X-rays (1—10 Gy) from an X-ray generator (MTC-22CS, Mitsubishi Electric Corporation, Tokyo, Japan) at a dose rate of 1.9 Gy/min (10 MeV). Cell survival was determined by the trypan blue exclusion method. Cells were collected at various times (0—48 h) after X-ray irradiation by centrifugation at 1000 rpm for 5 min, and the cell pellets obtained were washed with PBS (pH 7.2) and stored at −20 °C until use.

Isolation of DNA from Cells and Quantification of 8-oxodG DNA was isolated using NaI as described previously. DNA was hydrolyzed with nuclease P1 and alkaline phosphatase, and the amounts of 8-oxodG were measured by HPLC with electrochemical detection (ESA Coulochem II 5200, Bedford, MA, U.S.A.).
Detection of DNA Ladders in Splenic Cells Irradiated with X-Rays

Pellets from ca. $1\times10^7$ cells were suspended in 100 μl of lysis buffer consisting of 10 mm Tris–HCl buffer (pH 7.4), 10 mm EDTA (pH 8.0) and 0.5% Triton X-100, and allowed to stand for 20 min at 4°C. After centrifugation at 15000 rpm for 5 min, the supernatant was transferred to new tube and treated 2 μl of a solution of ribonucleases T1 (40 units/ml, Sigma, St. Louis, MO, U.S.A.) and A (80 mg/ml, Sigma). After 60 min incubation at 37°C, 2 μl of a solution of proteinase K (25 mg/ml, Boehringer Mannheim, Indianapolis, IN, U.S.A.) was added and the extract incubated for an additional 30 min at 50°C. After incubation, 6× loading buffer (0.03% bromophenol blue, 0.03% xylene cyanol, 30% glycerol, and 30 mM EDTA; Takara Shuzo Co., LTD, Shiga, Japan) was added to the resulting solution. DNA was analyzed by horizontal 1% agarose gel electrophoresis (Agarose ME; Iwai Chemicals CO., Tokyo, Japan) with 5 μg/ml ethidium bromide.

Assay of Caspase-3 Activity

Caspase-3 activity was assayed using an Apoalert Caspase Colorimetric Activity Assay Kit (Clontech Laboratories Inc., Palo Alto, CA, U.S.A.). The cell pellet was suspended in cooled lysis buffer and left on ice for 10 min. The protein content in the supernatant was measured by the method of Bradford. Aliquots of the supernatant were added to 2× reaction buffer/dithiothreitol (DTT) mixture (313 mM HEPES (pH 7.5), 31.3% (w/v) sucrose, 0.313% (w/v) 3-[3-cholamidopropyl]-dimethylammonio]-1-propane-sulfonate and 10 mM DTT, and for control samples, the amino acid sequence Acetyl-Asp-Glu-Val-Asp-Aldehyde (DEVD-CHO, a caspase-3 inhibitor), and the mixtures were incubated for 30 min at 37°C. The amino acid sequence Asp-Glu-Val-Asp (DEVD, 1 mM), labeled with the chromophore p-nitroaniline (100 mM) solution was added to both the reaction and control solutions. The absorbance at 405 nm was measured after incubation for 1 h at 37°C. One unit of caspase-3 cleaves 1.0 nmol of DEVD under these conditions.

Statistical Analysis

Data are expressed as mean±S.D. The Fisher’s PLSD test was used for statistical analysis. Probability (p) values less than 0.05 were considered statistically significant.

RESULTS

Splenic cells and TIG-7 cells were irradiated with X-rays at dose of 1, 2, 5 or 10 Gy. The survival rates of both cells immediately after X-ray irradiation decreased in a dose-dependent manner as shown in Fig. 1A. The survival rate of splenic cells decreased significantly to 82.5% at 1 Gy and 24.4% at 10 Gy, while the survival rates of T7 cells were 94.3% at 1 Gy and 49.6% at 10 Gy. The 8-oxodG content in the DNA of T7 cells increased with X-rays irradiation in a dose-dependent manner as shown in Fig. 1B. The 8-oxodG content of splenic cells increased significantly from 0.05 8-oxodG/10⁵ dG immediately after 1 Gy X-ray irradiation, but the change was not significant. The 8-oxodG content of T7 cells increased immediately after irradiation with 1 Gy of X-rays. The survival rate of T7 cells was not influenced by 1 Gy of X-rays and remained nearly unchanged up to 48 h after irradiation as shown in Fig. 2A. The survival rate of splenic cells did not change up to 6 h after irradiation, but thereafter decreased in a time-dependent fashion. Furthermore, the 8-oxodG contents in splenic cells and T7 cells without irradiation are shown by the dotted line (-----) and the broken line (-----), respectively. They did not return to the pre-irradiation level even after 48 h. On the other hand, the 8-oxodG content of T7 cells increased immediately after X-ray irradiation, but the change was not significant. We investigated whether splenic cells undergo apoptosis

Fig. 1. Survival Rates (A) and 8-oxodG Contents (B) in Splenic Cells (●) and TIG-7 cells (○) Immediately after X-Ray Irradiation

Each value represents the mean±S.D. of three separate experiments. The contents of 8-oxodG in splenic cells and TIG-7 cells without irradiation are shown by the dotted line (-----) and the broken line (-----), respectively. *Significantly different at p<0.05 vs. 0 Gy of X-rays. **Significantly different at p<0.01 vs. 0 Gy of X-rays.

Fig. 2. Time Courses of Survival Rates (A) and 8-oxodG Contents (B) of Splenic Cells (●) and TIG-7 Cells (○) after Irradiation with 1 Gy of X-Rays

Each value represents the mean±S.D. of three separate experiments. The contents of 8-oxodG in splenic cells and TIG-7 cells without irradiation are shown by the dotted line (-----) and the broken line (-----), respectively. *Significantly different at p<0.05 vs. 0 h. **Significantly different at p<0.01 vs. 0 h.
following irradiation with 1 Gy of X-rays. The DNA of splenic cells and TIG-7 cells irradiated with X-rays was analyzed by agarose gel electrophoresis. No DNA laddering was observed in splenic cells 1 h after incubation. DNA ladders appeared 3 h after X-ray irradiation, and the density of the DNA ladders increased with time (Fig. 3A). On the other hand, no DNA ladders were found in TIG-7 cells irradiated with 1 Gy of X-rays even after 24 h (data not shown).

Caspase-3 is activated by damage that occurs in cells due to oxidative stress and induces cells to undergo apoptosis. Therefore, caspase-3 activity was examined in cells exposed to 1 Gy X-ray irradiation. The caspase-3 activity prior to X-ray irradiation was 17.8 ± 1.3 units/mg protein. Two hours after X-ray irradiation, the activity had risen to 27.4 ± 2.6 units/mg protein, and the level remained high even after 3 h (Fig. 3B). After 6 h, the activity decreased to 22.6 ± 2.5 units/mg protein. The caspase-3 activity (39.2 ± 5.0 units/mg protein) 2 h after irradiation with 2 Gy of X-rays was significantly higher than that induced by a dose of 1 Gy.

**DISCUSSION**

It has been generally accepted that hydroxyl radicals produced by radiolysis of water in cells play an important role in oxidative DNA damage and DNA strand breaks. A variety of antioxidant enzymes and repair enzymes in the body protect tissues against damage caused by ROS. The susceptibility to irradiation differs among cell species and strains. Furthermore, the susceptibility is dependent on the rate and phase of the cell cycle. Late S phase is the most resistant to irradiation, while the G1, G2, and M phases are more sensitive. It is known that rapidly dividing and undifferentiated cells are especially sensitive to irradiation. For example, bone marrow cells are more sensitive to irradiation than nerve cells, which are postmitotic. Splenic cells are predicted to be more sensitive to irradiation than TIG-7 cells, because splenic cells comprise undifferentiated and dividing hematopoietic cells. In contrast, TIG-7 cells are completely differentiated and are divided only when needed. Up to 2 Gy of X-rays, dose-dependent changes in the survival rate and 8-oxodG content are greater in splenic cells than in TIG-7 cells (Fig. 1). However, the 8-oxodG content in splenic cells did not change at the doses of 5 or 10 Gy. This strange result may be attributed to the fact that the majority of splenic cells die by X-ray irradiation at these doses. These results may support the assumption that splenic cells are more sensitive than TIG-7 cells to oxidative stress proposed by X-ray irradiation. We observed the increase in the 8-oxodG content by low doses of X-ray irradiation. Low doses of irradiation such as 1 to 5 Gy have been reported to increase the 8-oxodG contents in liver DNA of rodents and the mutations in mice, while no production of 8-oxodG has been reported at low doses of γ-irradiation in some studies. Further investigation would be required in order to resolve this confusion.

Time-dependent changes in the survival rates and 8-oxodG contents of cells exposed to oxidative stress are thought to be reflected in the ability of antioxidants and repair functions to protect them against oxidative damage. We examined changes in the survival rates and 8-oxodG contents of splenic cells and TIG-7 cells after irradiation with 1 Gy of X-rays. The survival rate of splenic cells decreased in a time-dependent manner after irradiation, whereas TIG-7 cells were scarcely influenced by irradiation with 1 Gy of X-rays (Fig. 2A). These results suggest that antioxidant and/or repair mechanisms are not sufficient to prevent damage to splenic cells caused by oxidative stress. In fact, the 8-oxodG content in splenic cells did not recover to the pre-irradiation level before irradiation even after 48 h (Fig. 2B).

Necrosis and apoptosis are widely recognized as two types of cell death. Apoptosis involves programmed signal cascades triggered by differential stimuli depending on a variety of physiological situations, and DNA damage induced by irradiation can also trigger apoptosis. The apoptotic process is generally characterized by extensive fragmentation of the genome. The cellular DNA is degraded at the nucleosomal linker sites yielding DNA fragments in multiples of 180 base pairs (bp). Lymphocytes are extremely sensitive to irradiation, and undergo apoptosis in response to irradiation. When comparing the response to irradiation of lymphocytes and fibroblasts, most of fibroblasts undergo necrosis, while lymphocytes show evidence consistent with apoptotic cell death. In this study, agarose gel electrophoresis showed DNA fragmentation in splenic cells to be present 3 h after X-ray irradiation at a dose of 1 Gy. However, DNA fragmentation was not observed in TIG-7 cells under similar conditions. These results indicate that splenic cells are highly sensitive to X-ray irradiation and tend to undergo apoptosis following small doses of X-rays.

Caspase-3 is a member of the cysteine aspartic acid-specific protease family, whose members have been shown to play key roles in apoptosis in mammalian cells. The activation of caspases occurs as a result of exposure to stresses such as irradiation. Active caspases participate in a cascade of cleavage events that disable repair enzymes, and bring about the systematic disassembly of dead cells. In this study, the caspase-3 activity of splenic cells increased significantly 2 h after X-ray irradiation at a dose of 1 Gy to a level 1.5-fold higher than that before irradiation. In addition, when splenic cells were exposed to 2 Gy of X-rays, the caspase-3 activity was strongly induced compared to that following irradiation with 1 Gy. These results also indicate that apoptosis is induced in splenic cells about 2—3 h after X-ray irradiation. Irradiation produces hydroxyl radical and other radical species by interaction with cellular water. Hydroxyl radicals...
modify DNA to form base and sugar lesions, single-strand breaks, double-strand breaks, abasic sites and DNA-protein cross-links.\textsuperscript{25,26}) It has been reported that DNA double-strand breaks are significantly increased in Chinese hamster fibroblasts by X-ray irradiation at the dose of 1 Gy.\textsuperscript{27}) In this study, we observed the increase in the 8-oxodG content by low doses of irradiation, although there are some reports that 8-oxodG does not increase at low doses of irradiation described above. At present, it is not clear which type of DNA damage induces apoptosis in splenic cells. Further studies are necessary to elucidate the mechanism of apoptosis caused with low doses of irradiation.

In conclusion, splenic cells are more sensitive to X-ray irradiation than TIG-7 cells, and they receive lethal damage by X-ray irradiation at a dose of 1 Gy. We observed that the increase in 8-oxodG contents in splenic cells by X-ray irradiation is hard to return the steady-state levels. This phenomenon has never been reported in other cell strains. The DNA repair system appears not to act functionally in splenic cells irradiated with X-rays and the apoptosis pathway progresses in the damaged cells.

REFERENCES