Radio-sensitivity of the Cells from Amyotrophic Lateral Sclerosis Model Mice Transfected with Human Mutant SOD1

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In order to clarify the possible involvement of oxidative damage induced by ionizing radiation in the onset and/or progression of familial amyotrophic lateral sclerosis (ALS), we studied radio-sensitivity in primary cells derived from ALS model mice expressing human mutant SOD1. The primary mouse cells expressed both mouse and the mutant human SOD1. The cell survival of the transgenic mice (with mutant SOD1), determined by counting cell numbers at a scheduled time after X-irradiation, is very similar to that of cells from wild type animals. The induction and repair of DNA damage in the transgenic cells, measured by single cell gel electrophoresis and pulsed field gel electrophoresis, are also similar to those of wild type cells. These results indicate that the human mutant SOD1 gene does not seem to contribute to the alteration of radio-sensitivity, at least in the fibroblastic cells used here. Although it is necessary to consider the difference in cell types between fibroblastic and neuronal cells, the present results may suggest that ionizing radiation is not primarily responsible for the onset of familial ALS with the SOD1 mutation, and that the excess risks are probably not a concern for radiation diagnosis and therapy in familial ALS patients.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder in adults, and is characterized by a gradual loss of motor neurons in the spinal cord and progressive muscle wasting and weakness. The disease occurs in both sporadic and familial forms. Approximately 20% of familial cases of ALS are caused by single-site mutations in the Cu/Zn superoxide dismutase (SOD1) gene.¹,²) However, the actual relationship between the SOD1 mutation and the progression of the disease has not been clarified. The SOD1 protein catalyzes the formation of hydrogen peroxide through the dismutation of superoxide free radicals, and plays an important role in mitigating oxidative damage.³,⁴) Some reports indicate that oxidative damage induced by mal- or dysfunction of SOD1 may be involved in the progression of familial ALS.⁵-⁷)

Ionizing radiation induces oxidative damage in cells mainly by hydroxyl radicals, and it has been demonstrated that the manipulation of SOD activity can influence cellular radiation responses. In general, the increase in SOD activity produces additional protective effect, as reviewed by Petkau.⁸) It is of interest whether or not the SOD1 mutation, which may be a cause of familial ALS, could change the response of cells to an oxidative stress such as ionizing radiation. Several lines of transgenic mice have been established that express a mutant SOD1 gene and provide valuable models for human ALS.⁹-¹¹) However, there has been no report on the radio-sensitivity of the cells derived from these transgenic mice. In the present paper, the cell survival and the induction of DNA damage after irradiation were determined in primary fibroblastic cells expressing the same SOD1 mutation as human familial ALS. In addition, we discuss the involvement of oxidative stress induced by ionizing radiation in the pathogenesis of ALS, and the possible risks of the use of ionizing radiation for diagnosis and therapeutics in familial ALS patients.

MATERIALS AND METHODS

Transgenic mice

Wild-type (WT) and mutant SOD1 transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). WT and mutant SOD1-expressing mice are strains B6
and B6SJL-TgN8SOD1-G93A1Gur, respectively. Since a sexual difference in the onset of the disease has been reported, only females were used here. The transgenic mice showed no motor abnormalities in the pre-symptomatic period (12 weeks of age), a high-frequency tremor of one hindlimb at the early-symptomatic age (13 weeks of age), and paralysis of the hind- and fore-limbs at the late-symptomatic age (19 weeks of age). Wild type littermates of G93A SOD1 transgenic mice were used as control animals. All animal breeding and experiments were carried out with the permission of and under the regulation of the Institutional Committee for Animal Safety and Welfare of the Kansai Medical University, and in accordance with the Regulations on Appropriate Animal Breeding and Treatment, Ministry Office of Japan.

**Cell preparation, culture and X-ray irradiation**

At 8 and 20 weeks, the transgenic animals and age-matched wild-type animals were anesthetized with ether and their kidneys were dissected. Primary cultures of fibroblastic cells were established using a procedure described elsewhere. In brief, cell suspensions were prepared by mincing the kidneys and incubating fragments in α–MEM medium containing collagenase (Type III; 200units/ml) at 37°C for 4–5 h. The digested cells were washed extensively in cold PBS and resuspended in α–MEM medium containing 10% fetal bovine serum, antibiotics and fungizone for cell attachment and growth. After 3–4 days of incubation with one medium change, cells were ready for use and split into culture dishes. Some of the dishes were used for the following experiments, and others were used for determining the doubling time by counting the number of cells at scheduled times.

Approximately 2 × 10⁴ cells were incubated in a 60 mm culture dish, and irradiated by X-rays at doses of 4 to 16 Gy. The irradiation conditions of X-rays were 200keV, 20 mA, 1mm Al and 1mm Cu filter, and the dose rate was 0.8–1.5 Gy/min. To determine cell survival, culturing of the cells was continued for approximately 4 days after irradiation, and the number of cells was counted using a hemocytometer.

**DNA damage and repair measured by single cell gel electrophoresis (comet assay)**

The cells were irradiated with a ¹³⁷Cs γ source at a dose rate of approximately 10 Gy/min and used for comet assays immediately or after incubation for an indicated time to allow DNA damage repair to occur. The cells were washed several times with chilled phosphate buffered saline (PBS) and then treated with 10% Zapo-globin-II (Coulter Electronics, Hialeah, FL, USA) in PBS on ice. This treatment dissolved plasma membranes while leaving the nuclei intact and made it possible to recover the samples for comet assay in a chilled condition without trypsin-EDTA treatment. The nuclei recovered by gentle pipetting were centrifuged at 400g for 5 min at 0°C, and the nuclear pellets were re-suspended in chilled PBS. The comet assay was performed following the method reported by Sasaki et al. with a slight modification. In brief, 75 µl of agarose GP-42 (Nakarai Co. Ltd., Tokyo, Japan) was quickly layered on a fully frosted slide (Matsumani Glass Industries, Ltd., Osaka, Japan) and covered with another slide. The sandwiched slides were placed on ice to allow the agarose to gel. After removal of the covering slide, the agarose was air-dried. Next, the cell nuclei suspension was mixed at 1:1 (v/v) with 1%, 45°C SeaPlaque agarose (BMA, Rockland, ME, USA) and the mixture was quickly layered on the slide. The slides were placed immediately in a chilled lysing solution (pH10) of 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Trizma, 1% sarkosyl, 10% DMSO, and 1% Triton X-100 and kept at 0°C in the dark for 60 min. The slides were placed on a horizontal gel electrophoresis platform and covered with an ice-chilled alkaline solution made up of 0.3 M NaOH and 1 mM Na₂EDTA. The slides were left in the solution in the dark at 0°C for 10 min. Then electrophoresis was conducted at approximately 1 V/cm for 60 min. After the electrophoresis was finished, the slides were rinsed gently 2 times with 400 mM Tris solution (pH7.5) to neutralize the excess alkali. Each slide was stained with 50 µl of 20 µg/ml ethidium bromide, covered with a cover slip and observed under a fluorescent microscope. Fluorescent images of individual comets were digitalized using a digital camera system (Fuji Digital Camera HC-2500 system, Fuji Photo Film Co. Ltd., Tokyo, Japan) and displayed on a monitor. The tail length, which is the distance of DNA migration, was used as a parameter of comet analysis. In order to quantify the tail length, the total length of the comet and the head diameter perpendicular to the tail direction were manually measured on the monitor using a millimeter grid and the head diameter was subtracted from the total length. Thus the tail length is expressed in arbitrary units, corresponding to millimeters on the monitor screen of the microscope image. At least 30 individual comets were measured for each dose or time point in an assay and three independent assays were made.

**Measurement of DNA double strand breaks (DSB) and repair**

Approximately 1.5 × 10⁵ cells were inoculated in a 35 mm tissue culture dish in 2 ml of the medium, and labeled with 0.74kBq/ml [¹³⁷C]-thymidine plus 5 µM cold thymidine added at the time of culture preparation. The labeled cells were collected and used for the experiment 48–52 h after incubation.

The DNA DSB assay was carried out with pulsed field gel electrophoresis as described elsewhere. In brief, the recovered cells were mixed with melted 0.5% agarose solution, pipetted into plastic molds, and cooled down on ice until solidification. These agarose samples were immersed in an
ice cold lysis solution containing 0.5M EDTA, 0.01M Tris, 2% Sarcosyl, and 0.2 mg/ml proteinase-K for 1 h, then incubated overnight at 50°C. Following lysis, samples were washed and treated with 0.1 mg/ml ribonuclease-A for 1 h. Sample plugs were electrophoresed in 0.5X TBE buffer (45 mM Tris, 45 mM Boric Acid, 1.5 mM EDTA, pH 8.2) in a clamped homogenous electric field gel box (BioRad, Tokyo, Japan), on 0.8% agarose gel (CHEF-grade, BioRad) at 14°C. The applied voltage was 200V with a 60 second pulse time for the first 9 h followed by a 120 second pulse time for the last 14 h (total run time, 23 h). After electrophoresis, the gels were stained with ethidium bromide, photographed under UV light, and cut to separate the plug from the lane of each sample. The 14C activity of each piece was measured in a scintillation counter and the fraction of activity released (FAR) was calculated as the disintegration per minute (DPM) of a lane divided by the total DPM (lane + plug) per sample. Background FAR values were typically between 2 and 5% and were subtracted from the FAR values of treated samples.

**Confirmation of SOD1 with Western blotting**

The expression of the mouse SOD1 gene and human mutant SOD1 gene were confirmed with Western blotting. The following methods were used: Cells were lysed in 50 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Igepal CA-630 supplemented with complete protein inhibitor cocktail tablets (Roche Diagnostics GmbH, Germany) on ice for 15 min. Insoluble material was removed by microcentrifugation at 6,000 x g for 10 min at 4°C. Protein concentration for all samples was determined with a BCA Protein Assay Kit (Pierce). Cell extracts (35 µg protein per sample) were applied to 16% SDS-polyacrylamide gel. Proteins were separated on an SDS-PAGE and transferred to nitrocellulose membrane. After blocking the membrane with 1 x TBST (25 mM Tris (pH 8.0), 125 mM NaCl, and 0.025% Tween 20) containing 5% non-fat dry milk, the membrane was incubated with rabbit anti-Cu/Zn SOD polyclonal antibody (Stress-Gen Biotechnologies Corp. Canada) for 2 h and then washed with 1 x TBST. The membrane was incubated with goat anti-rabbit IgG (H+L) coupled to alkaline phosphatase. Human mutant SOD1 and mouse SOD1 protein were detected with NBT/BCIP ready-to-use tablets (Roche Diagnostics GmbH, Germany).

**RESULTS**

**Effect of X-ray irradiation on the cell proliferation**

The cell growth rates at an exponential stage, measured as doubling time, were similar for cells from the control (WT) and cells from the transgenic mice (Table 1). There also was no difference between the cells from 8- and 20-week-old animals. Cell growth was affected by X-irradiation, and was totally inhibited by a dose of 16 Gy. From the cell-growth curve in Fig. 1, it seems that the cells from WT and transgenic mice had similar radio-sensitivity with respect to cell proliferation.

**DNA damage and repair measured by single cell gel electrophoresis (comet assay)**

The cells were irradiated at various doses and the DNA damage was quantified using the comet assay. Fig. 2 shows representative fluorescent images of comets. Fig. 3a and 3b show the relationship between tail length and radiation dose. In these experiments, the comet assay was performed immediately after irradiation without allowing DNA damage repair. The tail length increased with the irradiation dose, but the rate of increase decreased at doses of more than 5 Gy. The tail length did not significantly differ between the wild-type and the SOD transgenic mice. The dose-response of the increase in tail length was also similar between the cells from 8- and 20-week-old animals. In order to evaluate DNA damage repair, the cells were incubated at 37°C for various periods after irradiation at a dose of 20Gy, and then prepared for the comet assay. A large part of the radiation-induced DNA damage was repaired rapidly within 15 min and the remaining DNA damage was repaired gradually at a decreased rate (Fig. 4a and 4b). There was no significant difference in the repair of DNA damage between the wild type and the SOD transgenic mice for both 8 and 20-week-old samples.

![Fig. 1. The effect of X-irradiation on cell survival (proliferation).](https://academic.oup.com/jrr/article-abstract/46/1/67/927119)

![Table 1. Mean doubling time of the cells from wild and transgenic mice kidney](https://academic.oup.com/jrr/article-abstract/46/1/67/927119)

| Age of donor animals | Wild Type | Transgenic |
|----------------------|-----------|------------|
| 20 weeks             | 30.9 ± 4.7 (n=5) | 28.0 ± 4.3 (n=5) |
| 8 weeks              | 29.9 ± 2.7 (n=6)  | 31.5 ± 5.7 (n=6)  |

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Fig. 2. Typical image of single cell gel electrophoresis (comet assay). The tail length, which is the distance of DNA migration, was used as a measure of DNA damage. Fig. 2a shows cells irradiated at 20Gy and Fig. 2b shows control cells.

Fig. 3. DNA damage, measured by the tail length in comet assays, immediately after γ-ray irradiation. Fig. 3a and 3b are the data for 8- and 20-week-old mice, respectively.

Fig. 4. The repair of DNA damage after γ-ray irradiation. The tail length in the comet assay became shorter with the time after irradiation. Fig. 4a and 4b are the data for 8- and 20-week-old mice, respectively.
Measurement of DNA double strand breaks and repair

Fig. 5 shows FAR values at 0 min (non-repair) and 30 min (repair) after 40 Gy gamma irradiation. The FAR values, indicating the number of DNA double strand breaks, were 3–4% in the non-irradiated cells from the wild and transgenic mice. The gamma irradiation of 40 Gy induced a substantial number of DNA double strand breaks, which increased the FAR values to 13–18%. There was no difference in the FAR values after 40 Gy irradiation between the wild and transgenic cells. Although not statistically significant, the FAR values in the cells from 20-week-old mice were a bit lower than those from 8-week-old mice both in wild and transgenic animals. The post-irradiation culture allowed the damaged DNA to be repaired, and the FAR values decreased to 6–8%. The amount of repaired DNA, which is the difference of the FAR values at 0 and 30 min post-irradiation, was similar in wild and transgenic cells. The age of the animals did not seem to affect DNA repair efficiency.

Confirmation of SOD1 with Western blotting

The expression levels of human mutant SOD1 and mouse SOD1 in cell extracts derived from primary cells of WT and human mutant SOD1 transgenic mice were examined with Western blotting. The blotting patterns are shown in Fig. 6. In the extracts from WT primary cells (lanes 2, 4, and 6), only mouse SOD1 protein was detected. In the cell extracts from mutant SOD1 cells (lanes 3, 5 and 7), the expression of the human mutant SOD1 gene was identified clearly in addition to mouse SOD1. In a few trials, the degree of expression seemed to be similar regardless of the age (8 and 20 weeks) of the mice.

DISCUSSION

Few reports have been published on the radiosensitivity of cells derived from sporadic ALS patients. Chamberline and Lewis noted normal clonal growth and recovery after induction of potentially lethal damage by X-rays in fibroblastic cells from sporadic ALS patients. Other researchers also found that survival, induction of DNA damage, and free radical production were normal in X-irradiated sporadic ALS lymphoblastoid cell lines. In those studies, however, the cells used were collected from the ALS patients, and thus the cytogenetic characteristics were unclear. Recently, a mutation of the SOD1 gene was discovered in familial ALS patients, and using gene technology, several lines of transgenic mice have been established that express a mutant SOD1 gene. These mice provide valuable models for human ALS, because the transgenic mice expressing the mutant SOD1 develop progressive weakness with a pathology that resembles what is observed in human ALS. It is already known that oxidative damage increases in the mitochondria of these model mice. However, it is not known whether the expression of mutant SOD1 changes the cellular response to external oxidative agents such as ionizing radiation. This is of particular interest in relation to the involvement of such external oxidative stresses in the onset and progression of familial ALS.

In the present study, we examined cell survival and DNA damage and repair after gamma or X-ray exposure in fibroblastic cells from ALS model mice and WT mice. Our cell survival studies, though not as sensitive as the colony forming assay, showed no significantly reduced survival in ALS fibroblasts compared to WT fibroblast following exposure to X-rays. Interference in cell proliferation seemed to begin at 4 Gy of X-irradiation, and proliferation was completely stopped at 16 Gy. These dose ranges are similar to...
those interfering with primary cultured fibroblasts isolated from other strains of mouse.\(^{13}\)

The induction and repair of DNA damages were assayed by two methods, single cell gel electrophoresis (comet assay) and pulsed field gel electrophoresis. The former assay was used for examining the induction and repair of relatively small (not severe) DNA damages such as single strand breaks, and the latter for DNA double strand breaks that are a typical severe DNA damage. In both assays, the degree of DNA damage and rejoining after incubation was similar between wild and transgenic cells (Fig. 3 and 4). Therefore, we can speculate that the mutant SOD1 did not affect the induction and repair of a variety of types of DNA damage.

Several pieces of evidence suggest that mutant SOD1 newly acquires a toxic function and works as a cytotoxicant through some mechanisms. One possible mechanism is endoplasmic reticulum (ER) stress induced by the mutant SOD1 protein. The mutant SOD1 protein is aggregated in cells, induces ER stress, and finally leads to cell death.\(^{21,22}\) Another explanation is increased oxidative stress induced by the mutant SOD1. There is more evidence indicating that oxidative stress increases in cells with the mutant SOD1. The increased oxidative damage leads to the formation of peroxynitrite and subsequent tyrosine nitration of proteins that is highly toxic to the cell.\(^{23,24}\) According to the results of the present study, the mutant SOD1 does not alter the cellular defense potential against external oxidative stress due to ionizing radiation, at least in the fibroblastic cells used here. Although a direct demonstration using neuronal cells may be necessary, it seems that the oxidative damage induced by ionizing radiation may not be directly responsible for neuronal cell death in familial ALS with mutant SOD1.

Cell survival and DNA damage and repair were used as biological endpoints to predict radio-sensitivity in the present study. Although an additional investigation using other endpoints may be necessary to draw a concrete conclusion, the present results indicate that the expression of mutant human SOD1 in fibroblastic cells may not change their sensitivity to ionizing radiation under the acute exposure conditions used here. Some diseases associated with neuronal degeneration, ataxia-telangiectasia for example, are caused by or are related to a deficiency in DNA damage repair function.\(^{25}\) In such cases, not only neuronal cells but other types of cells are sensitive to ionizing radiation. Therefore, the potential risk of ionizing radiation is much higher in these patients, and special caution is necessary in the application of diagnostic and therapeutic radiation. In the case of ALS, however, the sensitivity to radiation seems to be normal, at least in fibroblastic cells. This may allow us to speculate that the potential risk of medical radiation may not be so much higher as with other types of neuronal disease which are caused or accompanied by a deficiency of DNA damage repair.

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