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Analysis of Common Deletion (CD) and a novel deletion of mitochondrial DNA induced by ionizing radiation

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Abstract

Purpose: In order to identify supportive evidence of radiation exposure to cells, we analyzed the relationship between exposure to ionizing radiation and the induction of deletions in mitochondrial DNA (mtDNA).

Materials and methods: Using human hepatoblastoma cell line, HepG2 and its derivatives, HepG2-A, -89 and -400, established after long term exposure to X-ray, mtDNA deletions were analyzed by polymerase chain reaction (PCR) and real-time PCR after cells were subjected to radiation and genotoxic treatments.

Results: Common Deletion (CD), the most extensively studied deletion of mtDNA, was induced within 24 h after exposure to 5 Gray (Gy) of X-rays and was associated with replication of mtDNA. CD became undetectable several days after the exposure due to the death of cells containing mitochondria within which CD had been induced. Furthermore, we found a novel mtDNA deletion that consisted of a 4934 base-pair deletion (4934del) between nucleotide position 8435 and 13,368. A lower dose of ionizing radiation was required to induce the 4934del than for CD and this was independent of the quality of radiation used and was not induced by treatments with hydrogen peroxide (H_2O_2) and other genotoxic reagents including bleomycin.

Conclusion: CD is induced by ionizing radiation, however, the amount of CD detected at a certain point in time after radiation exposure is dependent on the initial frequency of CD induced and the death rate of cells with mtDNA containing CD. The novel mtDNA deletion found in this study, therefore, will be used to determine whether cells were exposed to ionizing radiation.

Keywords: *Mitochondrial DNA, ionizing radiation, deletion, cell death*

Introduction

DNA is known to be one of the major targets for radiation DNA damage due to radiation exposure result in cell death, mutation and carcinogenesis. Radiation induces various types of genetic changes such as point mutations, small and large deletions and complex rearrangements. We have studied molecular genetic mechanisms of radiation carcinogenesis by analyzing Thorotrast-induced liver tumors which are caused by chronic exposure to ionizing radiation (Fukumoto et al. 2006). However, a characteristic spectrum of associated DNA mutations has not been determined for radiation-induced human cancers. In order to estimate the genetic risk resulting from radiation exposure, we need to know the differences between spontaneous and radiation-induced mutations in terms of their natures and mechanisms of induction. Although the most frequently induced mutations reported are multiloci deletions, the full spectrum of genetic changes induced by radiation remains unknown (Sankaranarayanan 1991, Brooks 2005). This may be partially due to the fact that most studies have focused on the effects of radiation on nuclear DNA.

In order to more accurately identify biological changes specifically induced by radiation, markers that are more specific than changes in nuclear DNA are required. Mitochondria are essential for respiration and oxidative energy production in aerobic cells

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and are involved in apoptosis and probably also in tumorigenesis (Petros et al. 2005). Human mitochondrial DNA (mtDNA) is a 16,569 base-pair (bp) double-strand circular DNA molecule which contains 37 genes, encoding 13 polypeptides for the mitochondrial electron transport chain, 2 ribosomal RNA and 22 transfer RNA for mitochondrial protein synthesis (Anderson et al. 1981). Somatic cells have an average of 100–500 mitochondria with 1–15 mtDNA molecules per mitochondrion (Satoh & Kuroiwa 1991). Mitochondrial DNA is more susceptible than nuclear DNA to structural damage due to the lack of protective histones and a limited capacity for DNA repair. Therefore, mtDNA has a 10-fold higher mutation rate than nuclear DNA (Brown et al. 1979). These factors suggest that mtDNA may be a more sensitive indicator of exposure to ionizing radiation than nuclear DNA. The most abundant large-scale deletion reported in mtDNA is called a 'Common Deletion' (CD). CD is a 4977-bp deletion specifically occurring from nucleotide position (nt) 8470 to nt13,446 in the mtDNA sequence and can be used as a marker of oxidative damage to mtDNA. CD is a very sensitive marker of mtDNA damage because the lesion is amplified during mtDNA replication and is easily detected and quantified by polymerase chain reaction (PCR) using primers adjacent to the CD site. It is assumed that mtDNA may be particularly susceptible to radiation damage, resulting in CD formation and may therefore be a good indicator for total DNA damage. Induction of CD has been reported 72 h after the exposure to X-ray. However, the number of deletions induced was not dose dependent. In addition, the relationship between the radiation doses required to induce CD and radiosensitivity of the cell was inconsistent (Kubota et al. 1997, Prithivirajsingh et al. 2004).

Ionizing radiation is known to induce genomic instability that is transmitted through many generations after irradiation via the progeny of surviving cells. Radiation-induced genomic instability, including chromosome aberrations, is commonly observed in various types of mammalian cells (Suzuki et al. 2003). These imply that the biological effects and cellular alterations induced by chronic exposure to ionizing radiation could be different from those merely resulting from an accumulation of acute exposures. Therefore, in order to understand the biological effects of radiation, we need to examine the temporal fluctuation of deleted mtDNA after exposure to radiation. We also thought that the high copy number of mtDNA per cell and the high mutation rate of mtDNA might be advantageously exploited for the detection of radiation-specific DNA alterations. In this study, using cells chronically exposed to X-ray, we performed time course observations to measure the quantity of mtDNA present and the number of CD induced after radiation exposure, and sought to identify novel mtDNA deletions specifically induced by radiation.

Materials and methods

Cell lines

Human hepatoblastoma cell line, HepG2 was obtained from the Cell Resource Center for Biomedical Research in our institute and established 3 sub-cell lines by exposing HepG2 cells to 0.5 Gy of X-rays twice a day, everyday, for more than 4 years: this comprised a total dose of more than 1600 Gy of X-rays. HepG2-A was treated only by X-ray, HepG2-400 was treated by 400 nM N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG; Fluka Chemika Biochemika, Buchs, Switzerland) 24 h once before starting X-ray exposure and HepG2-89 was exposed once to 2 Gy of alpha particles by boron-neutron capture before continuous X-ray treatment. HepG2-89 was the most radioresistant followed by HepG2-A. HepG2-400 was found to have the same sensitivity as HepG2. All the cells were maintained in Roswell Park Memorial Institute (RPMI1640) medium (Sigma-Aldrich Inc., St Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS, Gibco Invitrogen Corp., Carlsbad, CA, USA). Establishment and characteristics of the cell lines will be published elsewhere (Kuwahara et al. manuscript in preparation). Maintenance radiation was applied to long-term irradiated cells at a level of 0.5 Gy twice a day. This procedure was used for the present study and exposure experiments were performed 12 h after the last maintenance irradiation. For X-ray irradiation, we used a 150-KVp X-ray generator in our institute (Model MBR-1520R, Hitachi, Tokyo, Japan) with a total filtration of 0.5 mm aluminum plus 0.1 mm copper filter. The X-ray dose rate measured by a thimble ionization chamber (IC 17A, Far West Technology, Goleta, CA, USA) at the same position as the samples was about 1 Gy/min. HepG2 cells were maintained in culture without any treatment for the same period as other 3 sub-cell lines.

Irradiation, treatments with hydrogen peroxide (H_2O_2) and anti-cancer drugs

HepG2, HepG2-A, -89 and -400 cells were irradiated by X-ray at the doses indicated. Cells were harvested at 24, 48, 72 h, and 10, 20, 30 and 45 days after exposure to 5 Gy of X-rays for time course studies of mtDNA deletions. H_2O_2 treatment was performed according to the studies by Prithivirajsingh et al. (2004) and Wang et al.

(2002). Briefly, a suitable aliquot of 30% H_2O_2 (Wako Chemical, Tokyo, Japan) solution was freshly diluted into phosphate-buffered saline (PBS) immediately before the experiment. After treatment with 200 μM H_2O_2 for 1 h in RPMI1640 without FBS, cells were washed once with PBS. Cells were harvested at 24, 48, 72 h, and 10, 20, 30 and 45 days after treatments.

For the treatment with anticancer reagents, HepG2 cells were washed once with PBS and incubated in RPMI1640 without serum for 1 h with one of following chemicals:

- 5 μg/ml bleomycin (Nihon Kayaku Co., Ltd., Tokyo, Japan)
- 20 μg/ml etoposide (Nihon Kayaku Co., Ltd., Tokyo, Japan)
- 200 μg/ml fluorouracil (5-Fu, Kyowa Hakko, Co., Ltd., Tokyo, Japan)
- 10 μM vincristine (Shionogi Pharmaceutical Co., Ltd., Tokyo, Japan)

Bleomycin treatment was carried out according to the report of Paul et al. (2001). Before bleomycin treatment, cells were washed with PBS containing 1 mM Calcium Chloride ($CaCl_2$) and permiabilized with 12.5 μg/ml L-α-lysophosphatidylcholine (Sigma-Aldrich, St Louis, MO, USA) in PBS-1 mM $CaCl_2$ for 1 min on ice. After treatment with anticancer drugs, the medium was removed and cells were washed once with PBS. Cells were harvested at 24, 48 and 72 h after treatment. From treatment to cell harvest, cells were cultured in RPMI1640 without irradiation. All the post-treatment conditions were the same for both radiation and chemical treatments, unless otherwise specified. The medium was changed 24 h before cell harvest. As a control for all treatments, cells without any treatment were used. The control was subjected to a change of medium 24 h before cell harvest, as was used for the treatments. In order to examine mtDNA deletion after exposure to different qualities of radiation, cells were irradiated by alpha particles, neutrons and gamma rays, respectively. Boron neutron capture (BNC) was used for the exposure to alpha particles. A ^{10}B compound, Sodium mercaptoundeca-Hydrodo-decaborate (BSH; Katchem Ltd., Prague, Czech Republic) was suspended in physiological saline at a concentration of 50 parts per million (ppm) in the culture medium, the cells were exposed to neutron radiation at the Research Reactor Institute, Kyoto University (KURR). The average fluency of the thermal neutron source was 2.1×10^{12} n/cm^2 and the average flux was 2.3×10^9 n/cm^2/s at 5 MW. We determined the exposure period at the calculated dose of 5 Gy of alpha particles. For the neutron-exposed group, the cells were exposed to the neutron source for the same period as the BNC group corresponding to 0.35 Gy. For the gamma ray exposed group, the cells were irradiated at a dose of 5 Gy (0.34 Gy/min) with a ^{60}Co gamma ray source at KURR. Twenty-four hours after exposure cells were analyzed for mtDNA deletions. All the exposure experiments were carried out using 25 cm^2 flasks (Nunc, Roskilde, Denmark) in triplicate.

Mitochondrial staining

Mitochondria of live cells were stained by incubating with 500 nM MitoTracker Green FM (Molecular Probes Inc., Eugene, OR, USA) for 30 min at 37°C. Cells were fixed with 3.7% formaldehyde (Wako chemical, Tokyo, Japan) for 30 min at room temperature and washed three times with PBS. Images were captured by fluorescence microscopy (Keyence BZ-8000, Keyence Co., Osaka, Japan) and fluorescence intensity was calculated using Photoshop software 7.0.1.1 (Adobe Systems Inc., San Jose, CA, USA).

Flow cytometry

Twenty four hours after exposure to 5 Gy of X-rays, HepG2 cells were trypsinized to make a single cell suspension. Pre-apoptotic and dying cells were stained by an Annexin V-fluoresceinisothiocyanate (FITC) apoptosis detection kit (BD Biosciences Japan, Tokyo, Japan) according to the manufacture's protocol. Annexin V positive pre-apoptotic cells were combined with propidium iodide positive and dying cells were separated from surviving/viable cells in a BD Fluorescence Activated Cell Sorting (FACS) Vantage (Becton, Dickinson Co., Franklin Lakes, NJ, USA).

Primers and PCR

Primers were synthesized by Gene Design Inc., Osaka, Japan. To detect CD, primer F1 and R1 sites were chosen at the region flanking the 4977-bp CD region and PCR was carried out under conditions that allowed a product to form only if the deletion had occurred. For the assessment of mtDNA copy number, a primer set to amplify the mitochondrial cytochrome *b* gene, where mutations rarely exist, was designed (F: 5′-tatccgccatcccatacatt-3′, R: 5′-ggtgattcctaggggttgt-3′). Primers for amplifying the nuclear β-actin gene (forward primer (F): 5′-ttctacaatgagctgcgtgtgg-3′, reverse primer (R): 5′-tcctacggaaaacggcagaaga-3′) were used to normalize the copy number of PCR products from mtDNA in each sample relative to the copy number of nuclear DNA. We designed the primers Fnd and Rnd to amplify the novel deletion that we found in this

study. The sequence of mtDNA and the annealing site for the primers, that is, F1, R1, Fnd and Rnd are shown in Figure 1.

Total DNA was extracted from cells using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Real-time PCR analysis was performed in a BIO-RAD icycler iQ, SA-THK Real-time PCR analysis system (BIO-RAD, Hercules, CA, USA). Amplification was conducted in a final volume of 15 μl containing 10 ng DNA, 0.15 μM of each primer, and AB solute syber green ROX mix (ABgene, Epsom, Surrey, UK). The PCR program included initial denaturation at 95°C for 15 min followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, annealing at a suitable temperature (61°C for CD, 66°C for β-actin and 58°C for cytchrome *b*) for 30 sec, and extension at 72°C for 30 sec. Ordinary PCR was performed in a GeneAmp PCR system 9600 (Perkin-Elmer Corp., Norwalk, CT, USA). The reactions were carried out in a 10 μl reaction volume containing 10 ng DNA, 0.25 U platinum™ *Taq* DNA polymerase High Fidelity (Invitrogen Corp., Carlsbad, CA, USA), 1x High Fidelity PCR Buffer (Invitrogen Corp., Carlsbad, CA, USA), 2 mM $MgSO_4$, 0.2 mM of each dNTP (Roche Applied Science, Penzberg, Germany) and 0.2 μM primers. Amplification was performed under the same conditions as for real-time PCR, but with 35 amplification cycles. PCR products were electrophoresed on a 10% polyacrylamide gel in Tris borate ethylenediaminetetraacetic acid (EDTA) (Wako chemical, Tokyo, Japan) buffer, and stained with ethidium bromide (Wako chemical, Tokyo, Japan) or SYBR Green Nucleic Acid Gel Stain Starter Kit (Molecular Probes Inc., Eugene, OR, USA). All PCR analyses were performed in triplicate.

Cloning and sequencing

After PCR products were electrophoresed in a 1% agarose gel, strips of agarose corresponding to DNA bands were cut out and purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, USA). Sequencing was performed in an ABI Prism 310 (Perkin-Elmer, Foster City, CA, USA) according to the manufacture's protocol after subcloning into TOPO vectors (TOPO TA Cloning Kit, Invitrogen Corp., Carlsbad CA, USA).

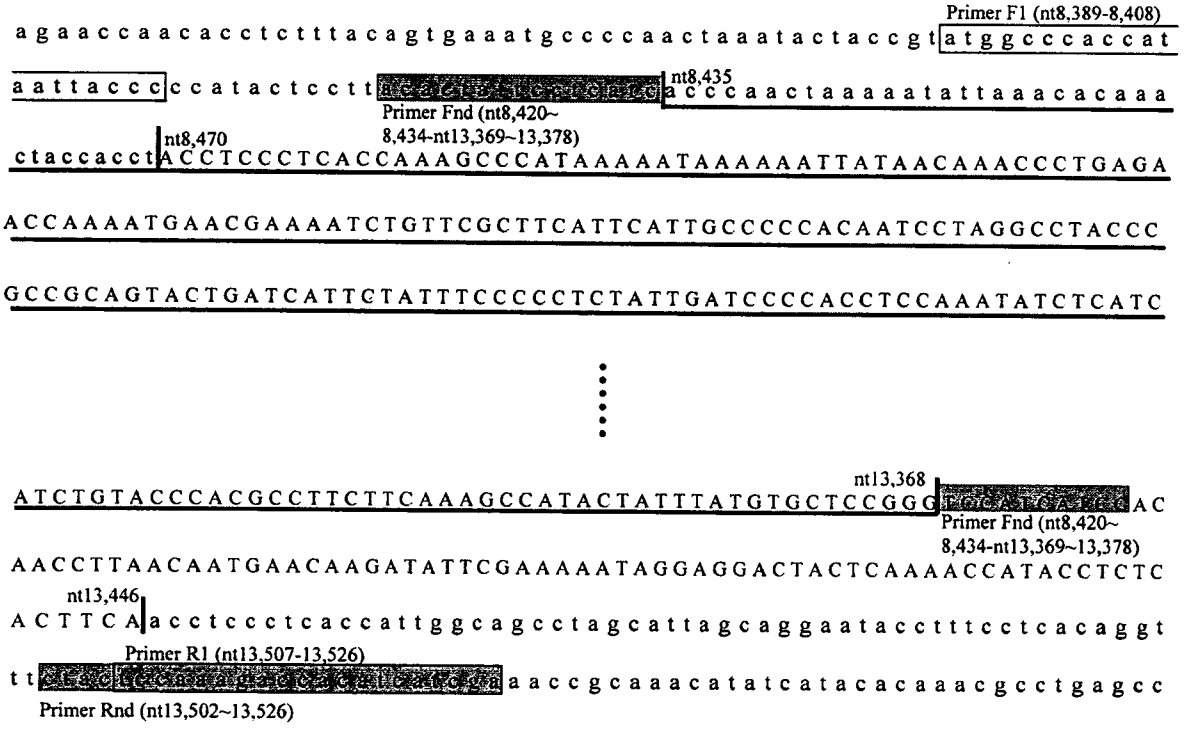

Figure 1. Mitochondrial DNA (mtDNA) sequence around common deletion (CD). Capital letters indicate nucleotides within CD. Solid underlines (__) indicate a novel deletion (4934del) specific to the radiation exposure found in this study. Vertical lines (|) indicate the boundaries of deletions. DNA sequences where PCR primers anneal are enclosed with squares (□) or hatched (■). Primer set F1-R1 was used to detect CD. Primer set Fnd-Rnd was used for detection of the novel deletion found in this study. The 'nt' number indicates nucleotide position of mtDNA. CD associated 13-nucleotide direct repeats are shown in bold letters.

Results

HepG2 cells were exposed to 2, 5 and 10 Gy of X-rays to determine whether deletions of mtDNA were dose dependent. Sequencing confirmed that a 161-bp fragment amplified using the F1-R1 primer set was derived from CD in this study. As shown in Figure 2, CD was detected 72 h after irradiation by X-rays at 5 and 10 Gy but not at 2 Gy by PCR using the F1-R1 primer set. Since the amount of CD formed was not dependent on the dose, thereafter we fixed the irradiation at 5 Gy for further experiments with X-rays in the current study.

To confirm whether PCR products using the F1-R1 primer set were from target sequences or not, real-time PCR products were electorphoresed in a polyacrylamide gel (Figure 3). The band from CD was induced by irradiation, but 10 days after irradiation it became undetectable.

In order to investigate the temporal pattern of CD occurrence in total mtDNA and nuclear DNA after exposure to 5 Gy of X-rays, we performed real-time quantitative PCR to measure the copy number of CD and total mtDNA per cell, normalized by PCR fragments from the nuclear β-actin gene. As shown in Figure 4, the copy number of CD reached a maximum from 24–48 h after exposure to 5 Gy of X-rays, and then decreased sharply. Assuming that the number of CD at the peak mostly reflects the induction of CD, maximal relative copy number of CD was between 1 out of 7000 (HepG2-A at 24 h) and 25,000 (HepG2 at 24 h) total mitochondrial DNA. CD was nearly undetectable 10 days after the exposure in all the cell lines examined. The copy number of total mtDNA per cell also increased 24 h after irradiation and recovered to basal levels after 45 days. During this period the copy number of mtDNA was not constant but fluctuated with different patterns amongst the cell lines studied.

The amount of mitochondria in a HepG2 cell was determined by the intensity of fluorescence after staining with Mitotracker. Exposure to X-rays increased the fluorescence intensity evenly as a whole (Figure 5). Total mitochondrial mass was calculated by 'mean intensity × area'. Compared to non-irradiated cells, mitochondrial mass increased by 3.2-fold 24 h after X-ray irradiation. The value of this increase was close to the increase of mtDNA copy number (4.4-fold). In order to determine why CD disappeared 72 h after exposure, we separated viable/surviving cells from dying/dead cells using a cell sorter 24 h after exposure to X-rays and stained with Annexin V and propidium iodide. We found that CD was only detected in the dying/dead cells but not in viable/surviving cells (Figure 6).

Because the frequency of homologous sequences in mtDNA is high, at first we had tried several pairs of primers for PCR, including nested PCR to amplify only CD specific sequences. Using the primer set reported by Kubota et al. (1997), we noticed that one of the PCR products appeared only after irradiation and sequencing revealed it to be a novel 4934-bp

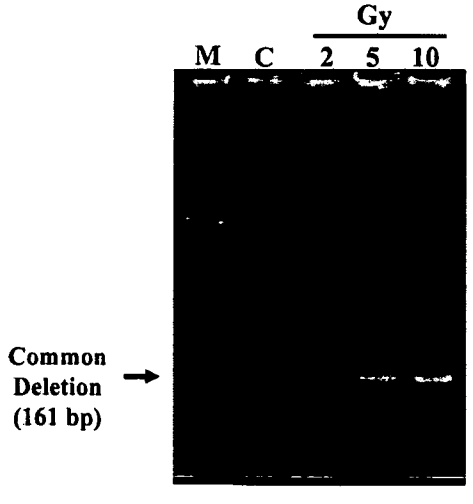

Figure 2. Profile of PCR products using primer set F1-R1 (Figure 1). Common deletion was detected 72 h after exposure of HepG2 cells to 5 and 10 Gy of X-rays but not detectable at an exposure of 2 Gy. C: without irradiation. M: 100-bp marker.

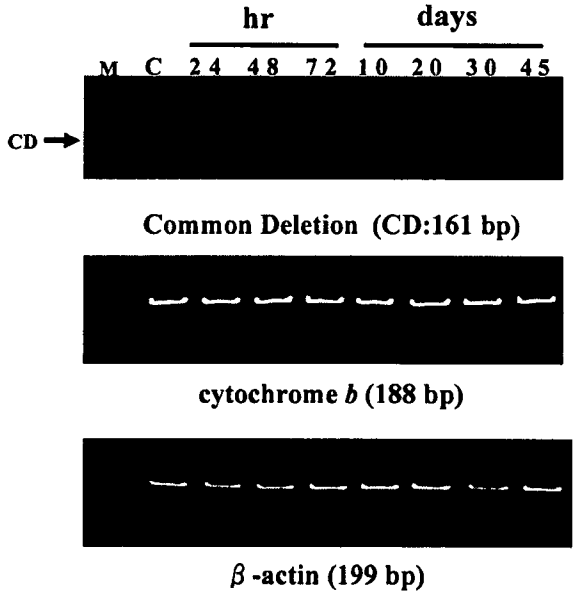

Figure 3. Electrophoretic pattern of real-time PCR products after HepG2 cells were exposed to 5 Gy of X-rays. A pair of primer set, F1-R1 in Figure 1, was used to detect common deletion (CD). CD is apparent at 72 h but not detectable 10 days after exposure. The band from cytochrome *b* represents the copy number of total mitochondrial DNA and the β-actin band that of nuclear DNA. C: without irradiation, M: 100-bp marker.

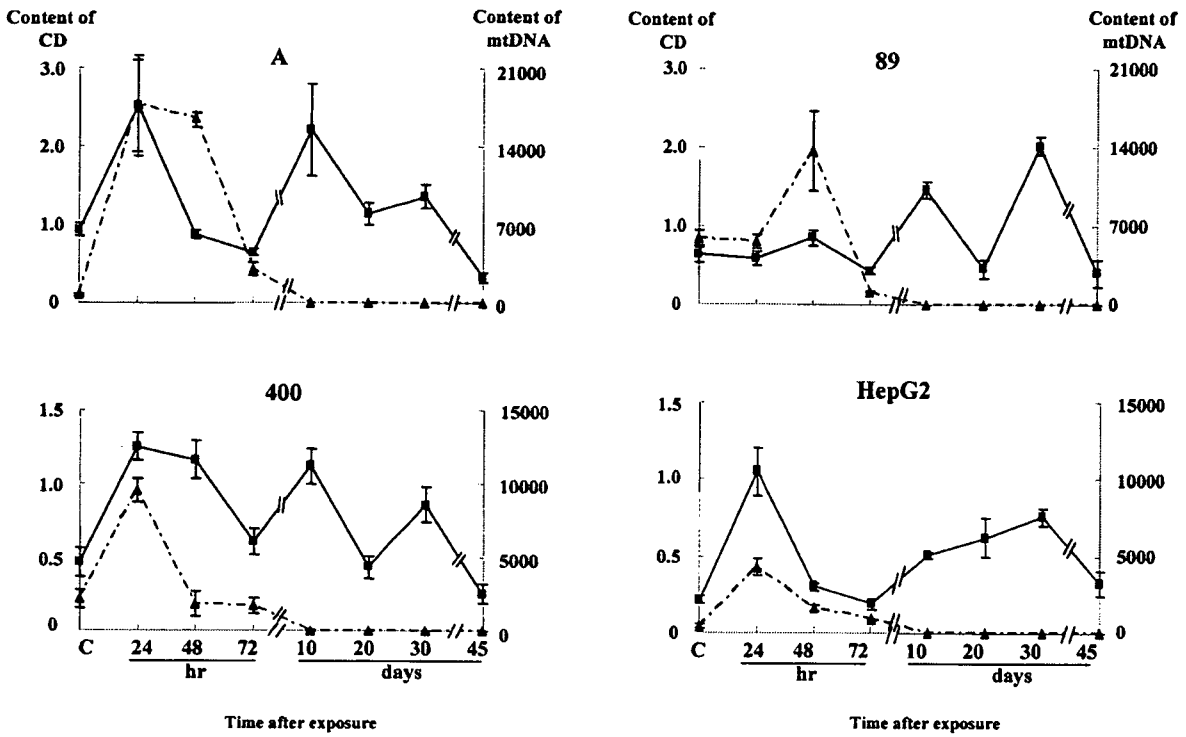

Figure 4. Temporal fluctuation profile of common deletion (▲) and total mitochondrial DNA (■). Copy number of common deletion (CD, left scale) and total mitochondrial DNA (mtDNA, right scale) was respectively normalized to that of the nuclear β-actin gene after real-time PCR. C: without irradiation. Data are means ± SD of triplicate in each experimental sample.

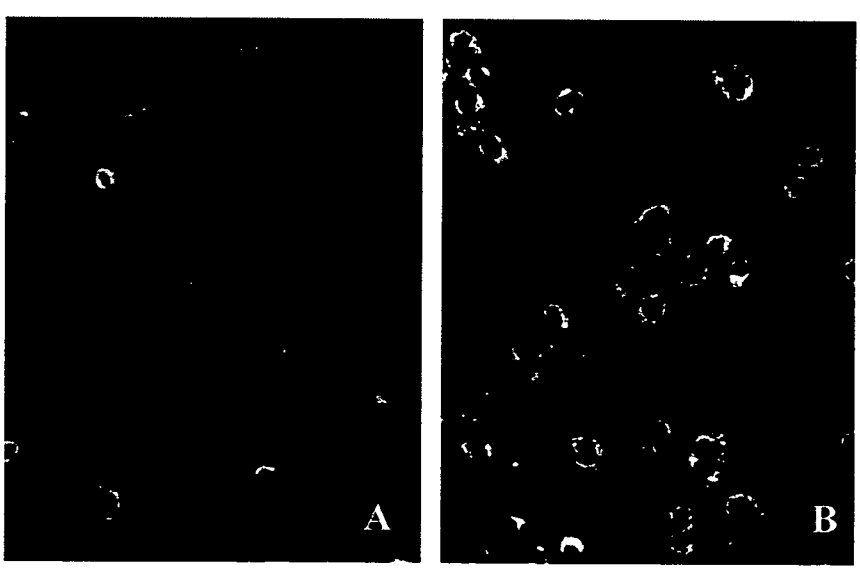

Figure 5. Mitochondria of HepG2 cells stained with Mitotracker. Compared with non-irradiated cells (A), the intensity as a whole increased 24 h after exposure to 5 Gy of X-rays (B). Original magnification: 20×.

deletion between nt8435 and nt13,368 (4934del) of mtDNA, and the 3 first deleted sequences were 'ACC' (Figure 1). This 4934del, to our knowledge, has not been previously reported. To amplify this novel 4934del effectively, we designed a new primer set, Fnd and Rnd. Primer Fnd overlays the breakpoint of 4934del but outside this novel deletion (Figure 1). Using the Fnd-Rnd primer set, 4934del

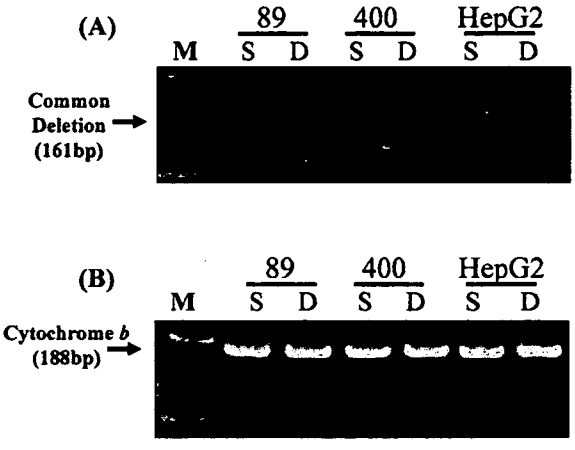

Figure 6. Detection of common deletion (CD) in dying/dead cells (D) but not in viable/surviving cells (S). Viable/Surviving cells (S) and dying/dead cells (D) were separated by a cell sorter 24 h after the cells were exposed to 5 Gy of X-rays. A primer set, F1-R1, was used to detect common deletion (CD) and cytochrome *b* to show total mitochondrial DNA. M: 100-bp marker.

was observed from 24 h after irradiation of 5 Gy X-rays to HepG2 cells and maintenance irradiation at 0.5 Gy to long-term exposed cell lines (Figure 7A). We performed real-time PCR to measure the copy number of 4934del per cell in HepG2 cells after exposure to X-rays. The copy number of 4934del before irradiation was negligible and increased up to 72 h after exposure. The copy number decreased thereafter but was detectable at a constant level (Figure 7B). This 4934del also appeared in HepG2 cells 24 h after exposure to gamma rays, alpha particles and neutrons (Figure 8A). The 4934del was not induced in HepG2 cells from 24 h to 45 days after treatment with 200 μM H_2O_2, 24–72 h after treatment with 5 μg/ml bleomycin (Figure 8B) or other anti-cancer drugs examined (data not shown).

Discussion

We could not detect CD 72 h after HepG2 cells were exposed to 2 Gy of X-rays and no apparent difference in the amount of CD induced was observed between 5 and 10 Gy-exposures. These observations suggest that a threshold dose exists for the induction of CD, but the amount of CD induced is not dependent on dose.

We found the dramatic increase in copy number for both total mtDNA and CD 24–72 h after exposure to X-rays. This indicates that CD formation is associated with the mtDNA replication induced by the preceding genetic insults by radiation, and that radiation induces over-replication of mtDNA. Basal levels of mtDNA were 2- to 3-fold higher in long-term irradiated cells compared with their parental HepG2 cells. These results suggest that a higher amount of mtDNA than that contained by HepG2 cells is needed to tolerate twice daily exposure to X-rays at a level of 0.5 Gy and that this is not enough to act as an inducible stimulus for CD. In this study, our calculation revealed that the maximum occurrence of CD corresponds to 1 out of 7000 to 25,000 copies of mitochondrial DNA. Although this occurrence of CD was insufficient to cause mitochondrial dysfunction, all traces of CD disappeared 10 days after irradiation. Therefore, we thought that mitochondria with CD were segregated into certain fractions of the cells.

In some instances, the mutated mtDNA coexists with wild-type mtDNA in a living cell (heteroplasmy) because mtDNA is present in multiple copies within a cell and wild type mtDNA can compensate for the mutated mtDNA (Morares et al. 2003). In the present study the maximum occurrence of CD was 5 copies per cell, CD was detectable only in dying cells but not at all in surviving cells. Brief treatment of cybrids containing CD mtDNA with H_2O_2 initiates a vicious cycle of mitochondrial reactive oxygen species (ROS) production resulting in apoptosis (Jou et al. 2005, Schoeler et al. 2005). Therefore, we thought that mitochondria with CD were segregated into certain fractions of the cells, and X-ray do not induce CD in all cells but only in a class of X-ray sensitive cells that are promptly eliminated by apoptosis. Characterization of cells that are prone to CD accumulation will help individualized radioprotection.

CD is reportedly induced by receiving genetic insults including ionizing radiation. Kubota et al. (1997) reported that minimal doses required for detecting CD range from 1–10 Gy of X-rays, dependent on the radiosensitivity of the cells exposed. Prithivirajsingh et al. (2004) reported that significant levels of CD accumulate 72 h after irradiation of all the human cells studied *in vitro* but they could not find a dose-response relationship or any clear relationship between sensitivity to radiation-induced deletion and sensitivity to cell death caused by radiation. In the present study, we determined the fluctuation profile of the copy number and deletions of mtDNA and showed that the total amount of CD detected in a cell population is the sum of CD induced and eliminated by cell death. Although cumulative doses for long-term irradiated cells were extraordinary high, the maintenance dose (0.5 Gy/exposure) sustained a low but variable CD copy number. Considering that cells with CD were eliminated by cell death, it would be interesting to investigate the contribution of mtDNA deletions to sub-lethal damage repair and the adaptive response.

It has been demonstrated that one of the mechanisms that produces mtDNA deletions is the pairing

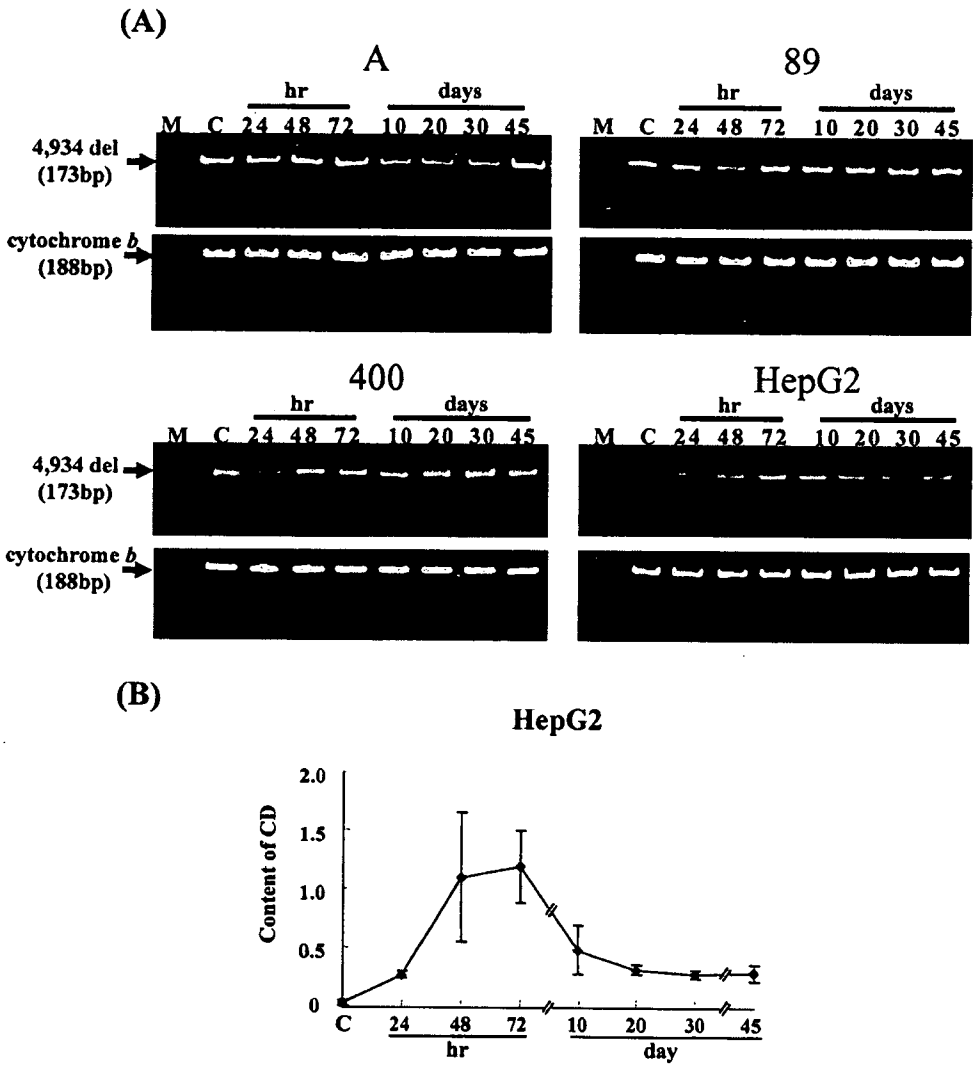

Figure 7. (A) Temporal fluctuation profile of a novel deletion (4934del) found in this study. A pair of primer set, Fnd-Rnd in Figure 1, was used to detect 4934del. 4934del was clearly induced shortly after HepG2 cells were exposed to 5 Gy of X-rays and was sustained for at least 45 days after irradiation including other sub-cell lines. (B) The change in 4934del copy number compared with that for a nuclear gene fragment in HepG2 cells exposed to X-rays at a dose of 5 Gy. Data are means ± SD of triplicate in each experimental sample. C: non-irradiated HepG2 cells and other long-term irradiated cells only, with maintenance exposure to 0.5 Gy of X-rays. M: 100-bp marker.

between two direct repeats separated by an intervening DNA fragment. Slipped-strand mispairing (SSM) is performed by non-homologous end-joining molecules which are isolated in the mitochondrial fraction of the cell (Coffey et al. 1999, Lakshmipathy & Campbell 1999). CD may arise from recombination or slippage between 13-nucleotide sequence repeats (at nt8470–8482 and nt13,447–13,459) during replication (Holt et al. 1989, Schon et al. 1989). The ends of 4934del were not related to a direct repeat but resided close to the break points of CD and the first 3-nucleotide sequence of the deleted part was ACC, which is common in CD. Both CD and 4934del were formed after irradiation, indicating that direct repeats play only a minor role in generating mtDNA deletions. This is consistent with the previous report that the vast majority of break points of different mtDNA deletions are close to the break points of CD, suggesting a common mechanism related to mtDNA replication (Samuels et al. 2004). We also found a mtDNA deletion whose 3′ end was adjacent to the sequence 'ACC' (nt13,304) after exposure to X-rays (data not shown). These results suggest the triplet sequence 'ACC' is crucial to form mtDNA deletions upon exposure to ionizing radiation. At 10 days after X-ray irradiation, CD disappeared due to the death of cells containing CD and thereafter several other PCR

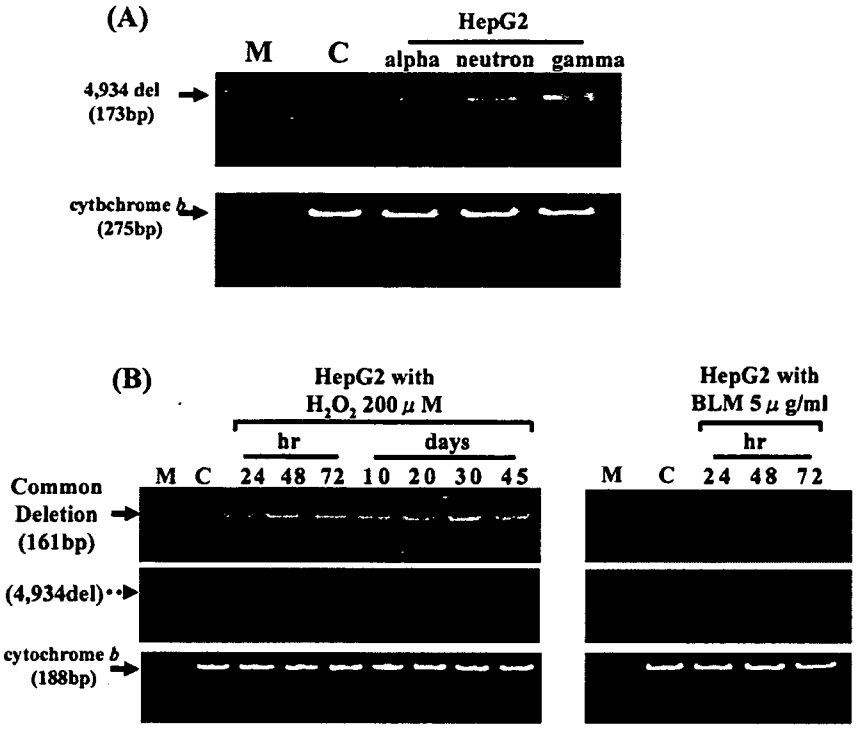

Figure 8. Association of a novel deletion, 4934del with radiation exposure. A novel deletion, 4934del that study showed was detectable 24 h after exposure to different qualities of ionizing radiation (A). 4934del was not induced in HepG2 cells by H_2O_2 treatment whereas common deletion (CD) was. Bleomycin did not induce either 4934del or CD (B). C: without irradiation. M: 100-bp marker.

products were observed using primers to detect CD partly because of abundant homologous sequences in mtDNA.

Since CD induced by UV is diminished by scavengers of ROS, this indicates that CD formation is closely linked to ROS (Berneburg et al. 1999). Bleomycin induces DNA double strand breaks via free radicals. Etoposide inhibits DNA synthesis by blocking re-ligation of DNA cleaved by topoisomerase II. 5-FU acts as a pyrimidine antagonist. Vincristine acts as an antimicrotubule agent that blocks mitosis by arresting cells in the metaphase (Ratain 2001). All the anticancer agents except vincristine used in this study are genotoxic. This is especially true for bleomycin, which is generally regarded as a radiomimetic agent because it efficiently induces DNA double strand breaks and chromatid breaks (Adema et al. 2003). In this study, CD was induced by H_2O_2 treatment and irradiation, whereas 4934del was induced by irradiation but not by H_2O_2 or other anti-cancer reagents. In addition, we did not find 4934del either in brain tissues diagnosed with Alzheimer's disease or in normal brain tissues from elder persons (data not shown). Furthermore, 4934del was clearly observed in all the cell lines 24 h after exposure either to alpha particles, neutrons, gamma rays, or X-rays. These results suggest that genetic insults are caused by ionizing radiation via not only ROS but also by other non-random mechanisms irrespective of the quality of radiation. We are now undertaking a study of 4934del in tissues from patients injected with Thorotrast to confirm its relevance to long-term radiation exposure. Exposure to alpha particles and low doses of neutrons induced 4934del, suggesting that high Linear Energy Transfer (LET) radiation is more effective than low LET radiation in inducing mtDNA deletions. We need further study to confirm whether this assumption is correct or not, and understand the similarity in mechanism between different qualities of radiation.

In conclusion, CD is induced after exposure to radiation irrespective of the quality of radiation. Cells with CD selectively died within 10 days of exposure. The novel mtDNA deletion found in this study could be useful as a marker of radiation damage irrespective of the quality of radiation.

Acknowledgements

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Regular Paper

Oxidative Stress Induced Lipocalin 2 Gene Expression: Addressing its Expression under the Harmful Conditions

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Lipocalin 2/H_2O_2/oxidative stress/radiation/liver.

Lipocalin 2 (Lcn2, NGAL) is a member of the lipocalin superfamily with diverse functions such as the transport of fatty acids and the induction of apoptosis. Previous reports indicated that expression of *Lcn2* is induced under harmful conditions. However, the mechanisms of the induction of *Lcn2* expression remain to be elucidated. In this report, we intended to identify the factor or factors that induce *Lcn2* expression. Up-regulation of *Lcn2* expression after X-ray exposure was detected in the heart, the kidney and especially in the liver. Primary culture of liver component cells revealed that this up-regulation in the liver was induced in hepatocytes. Up-regulation of *Lcn2* expression was also detected in HepG2 cells after the administration of X-rays or H_2O_2. Interestingly, up-regulation of *Lcn2* expression after H_2O_2 treatment was canceled by the addition of the anti-oxidants, dimethylsulfoxide or cysteamine. These results strongly suggest that *Lcn2* expression is induced by reactive oxygen species. Therefore, Lcn2 could be a useful biomarker to identify oxidative stress both *in vitro* and *in vivo*.

INTRODUCTION

The lipocalins constitute a broad but evolutionally conserved family of small proteins. Although the primary function of the lipocalins is thought to be involved in the transport of small ligands such as fatty acids and pheromones, they have also been implicated in a variety of different functions such as retinol transport, cryptic coloration, olfaction, prostaglandin synthesis, regulation of the immune response and cell homeostatic mediation.[1)] Neutrophil gelatinase-associated lipocalin (NGAL, lipocalin 2, Lcn2) is a 25 kDa glycoprotein that was initially purified from neutrophil granules.[2)] The Lcn2 protein exists as a 25 kDa monomer, as a 46 kDa homodimer, and in a covalent complex with neutrophil gelatinase that is known as matrix metalloproteinase 9.[2)] A variety of functions of the Lcn2 protein have been reported, such as the transport of fatty acids and iron,[3–4)] the induction of apoptosis,[5)] the suppression of bacterial growth,[6)] and the modulation of inflammatory responses.[7)] The expression of the *Lcn2* gene is detected in mouse fibroblasts stimulated by a number of growth factors such as serum, basic fibroblast growth factor and phorbol esters. *Lcn2* expression is also observed in the mouse liver after treatment with some carcinogens or reactive oxygen species (ROS) producing agents such as diethylnitrosamine.[8)] These findings suggest that the Lcn2 protein may play a role in regulating cellular growth. This hypothesis is further supported by the expression of the Lcn2 protein in various malignant tumors.[9–11)] Contrary to previous reports, most functions of the Lcn2 protein, with the exception of its role in innate immunity, are not verified in *Lcn2* deficient mice.[12)] Another member of the lipocalin family, human tear lipocalin (Lcn1), acts as an oxidative-stress induced scavenger of potentially harmful products.[13)] In spite of the fact that there is little similarity between the Lcn1 and the Lcn2 proteins, they commonly exhibit antimicrobial activity.[6,14)] Several reports indicated that *Lcn2* expression is induced in various cells under harmful conditions such as cancer, intoxication, infection, inflammation, kidney injury, heart injury and burn injury where production of free radicals has been reported.[9–11,15–19)] Our previous study revealed that *Lcn2* expression was induced in the mouse liver when exposed to alpha particles (Roudkenar *et al.*, manuscript submitted). It has also been

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reported that *Lcn2* expression was up-regulated in some radio-resistant cell lines established by continuous fractionated exposure to X-rays.[20] From the above results we hypothesized that *Lcn2* expression is induced by ROS. In this study we examined *Lcn2* expression in mouse tissues and HepG2 cells after irradiation. We also examined *Lcn2* expression after the administration of H_2O_2 and its scavengers.

MATERIALS AND METHODS

Mice

Seven-week old male C3H/Hex mice were used. Animal protocols were approved by the ethical committee of the Institute of Development, Aging and Cancer (IDAC), Tohoku University, and were performed according to the institutional guidelines.

Cell culture

HepG2 cells derived from human hepatoblastoma were obtained from the Cell Resource Center for Biomedical Research in IDAC, Tohoku University. Cells were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Cell cultures were raised at 37°C in an atmosphere of 5% CO_2.

H_2O_2 treatment

Before H_2O_2 treatment, HepG2 cells were cultured in serum-free medium for 16 hrs. After washing with phosphate buffered saline (PBS), cells were cultured in serum-free medium containing 0.1 mM H_2O_2 for 45 min. For scavenger treatments we also added 1 mM cysteamin or 5% dimethyl sulfoxide (DMSO; Sigma) to the H_2O_2 containing medium.

Irradiation

Mice were exposed to 2, 5 or 8.5 Gy of ^{60}Co γ-rays (0.34 Gy/min) at the Research Reactor Institute, Kyoto University. Liver, lungs, heart, spleen, testis and kidneys were dissected out from mice after sacrificed by cervical dislocation. All tissues were immediately frozen and kept at –80°C until use. HepG2 cells and mice for the isolation of parenchymal cells (hepatocytes, PC) and non-parenchymal cells (NPC) were exposed to 8.5 Gy X-rays (1.0 Gy/min.) using a 150-KVp X-rays generator (Model MBR-1520R, Hitachi, Tokyo, Japan) with a total filtration of 0.5 mm aluminum plus 0.1 mm copper filter. Long-term irradiated HepG2 cells were exposed to 0.5 Gy X-rays every 12 hrs for more than 4 years, and the total exposure dose was over 1,600 Gy.

Assessment of Lcn2 *gene expression*

Total RNA from mouse tissues and cells were extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription (RT) was performed by SuperScript III reverse transcriptase (Invitrogen) with 500 ng of *DNaseI* (Invitrogen) treated total RNA. Polymerase chain reaction (PCR) was performed using Animal *Taq* DNA polymerase (ABgene, Surrey, UK) in a GeneAmp PCR system 9600 (PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA, USA). After initial denaturation (5 min at 94°C), complementary DNA was subjected to 30 cycles of PCR. Primers for the amplification of mouse *Lcn2* were; forward 5'-CCA GTT CGC CAT GGT ATT TTT C-

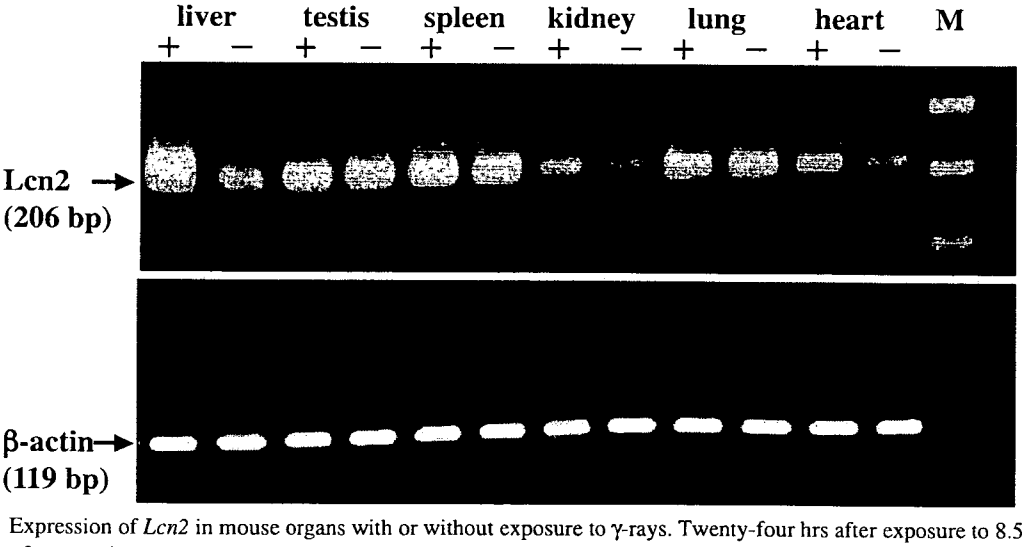

Fig. 1. Expression of *Lcn2* in mouse organs with or without exposure to γ-rays. Twenty-four hrs after exposure to 8.5 Gy of γ-rays *Lcn2* expression was determined by semi-quantitative RT- PCR. *Lcn2* expression was detected in all the organs examined before irradiation. After γ-ray irradiation *Lcn2* expression was up-regulated in the liver, the kidney and the heart. M; 100 bp marker. +; with γ-rays. –; without γ-rays.

3' and reverse 5'- CAC ACT CAC CAC CCA TTC AGT T-3'. Primers for human *Lcn2* were; forward 5'-TCA CCT CCG TCC TGT TTA GG-3' and reverse 5'-CGA AGT CAG CTC CTT GGT TC-3'. Gene expressions were normalized by *β-actin* expression and primers were; forward 5'-TTC TAC AAT GAG CTG CGT GTG G -3' and reverse 5'-GTG TTG AAG GTC TCA AAC ATG AT-3'. PCR annealing temperature was 60°C for human and mouse *Lcn2* and 59°C for *β-actin*. PCR products were separated in a 2% agarose gel. In order to determine gene expression, real-time PCR was performed in a BIO-RAD icycler iQ, SA-THK Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Amplification was conducted using AB solute SYBR green ROX mix (ABgene) according to the manufacture's instructions. The PCR conditions were; initial denaturation at 96°C for 15 min followed by 40 amplification cycles consisting of denaturation at 96°C for 30 sec, annealing at a suitable temperature for 30 sec and extension at 72°C for 30 sec. Threshold cycle values were normalized by *β-actin* expression.

Isolation of parenchymal cells (PC) and non-parenchymal cells (NPC)

PC and NPC were isolated from mouse liver tissue as described elsewhere.[21] Briefly, 24 hrs after exposure to 8.5 Gy of X-rays, mice were anaesthetized with pentobarbital (Dainippon Sumitomo Pharma, Co., Ltd., Osaka, Japan) followed by cannulation of the portal vein with a 24-gauge needle. Then, the liver was perfused with prewarmed calcium-free Hank's Balanced Salt Solution (HBSS, Sigma), at a flow rate of 3 ml/min. Subsequently, the liver was perfused with collagenase solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan; 0.5 mg/ml HBSS containing 5 mM calcium) for 7 min. Liver component cells were gently isolated and were maintained in HBSS. Differential centrifugations were used to separate PC from NPC. The PC fraction was separated from total cells by centrifugation for 2 min at 50*g* and the pellet was washed three times with HBSS. Viability of isolated cells was assessed by the trypan blue dye exclusion test immediately after isolation and was found to be more than 70%. For separation of the NPC fraction, the cell suspension was centrifuged twice for 5 min at 50*g* to eliminate PC. Subsequently, the NPC fraction was sedimented by centrifugation of supernatant for 10 min at 150*g*.

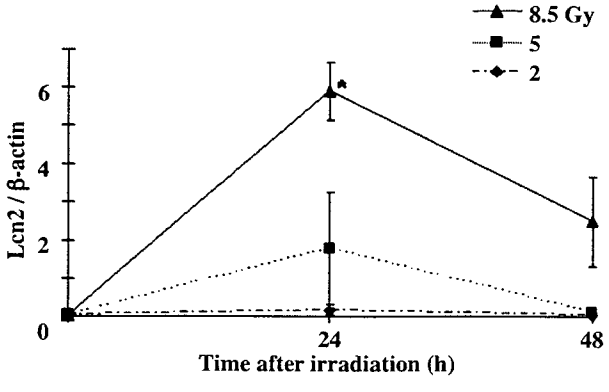

Fig. 2. Real-time PCR analysis of *Lcn2* expression in the mouse liver after exposure to γ-rays. Dose dependent up-regulation of *Lcn2* expression was observed 24 hrs after irradiation. (Mean ± SD, *; $p < 0.001$)

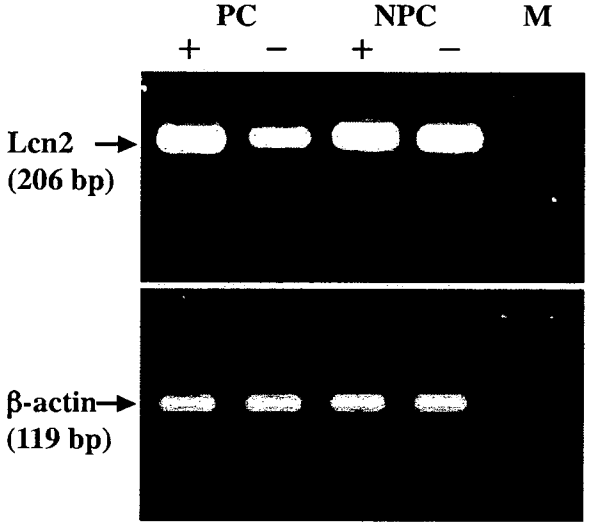

Fig. 3. Up-regulation of *Lcn2* expression in PC after exposure to 8.5 Gy of X-rays. PC and NPC were separately obtained from the irradiated mouse liver by perfusion. PC; parenchymal cells. NPC; non-parenchymal cells. M; 100 bp marker. +; with X-rays. −; without X-rays.

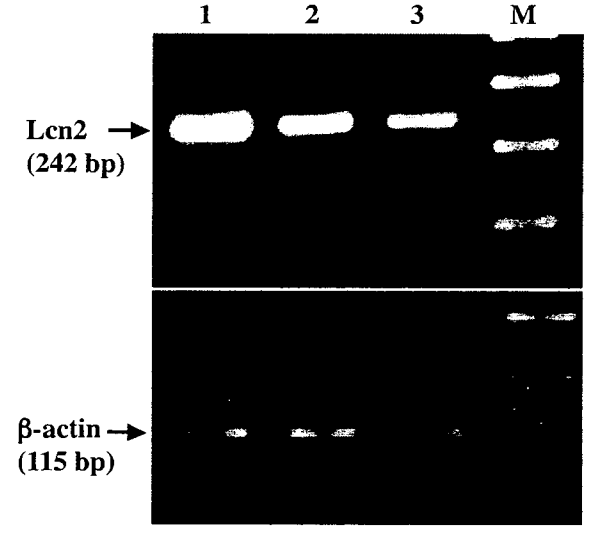

Fig. 4. Up-regulation of *Lcn2* expression after acute or long-term X-ray irradiation. Total RNA was extracted from HepG2 cells and *Lcn2* expression was examined by semi-quantitative RT-PCR. Lane 1; Long-term X-rays. Lane 2; 8.5 Gy of X-rays. Lane 3; without X-rays. M; 100 bp marker.

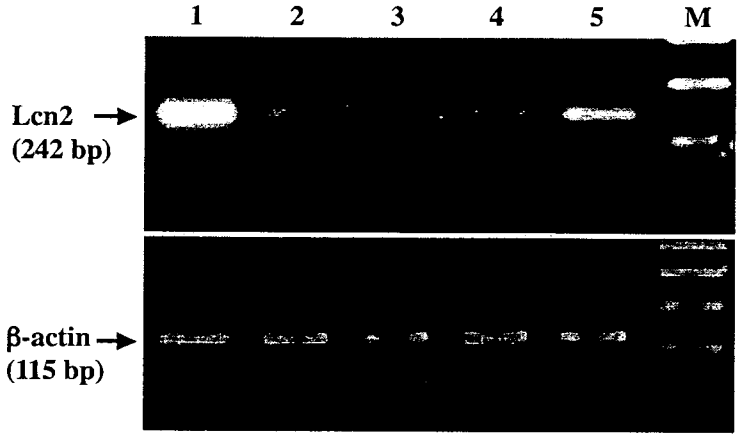

Fig. 5. Up-regulation of *Lcn2* expression after treatment with H_2O_2. Up-regulation of *Lcn2* expression was induced by the administration of H_2O_2. This up-regulation was suppressed by the addition of 5% DMSO or 1 mM cysteamine in the medium containing H_2O_2. Lane 1; 0.1 mM H_2O_2. Lane 2; 0.1 mM H_2O_2 and 1 mM cysteamine. Lane 3; 0.1 mM H_2O_2 and 5% DMSO. Lane 4; serum-free medium. Lane 5; No treatment. M; 100 bp marker.

Statistical analysis

The results were expressed as the mean ± SD of three independent experiments. Differences between groups were compared using the Student's *t*-test.

RESULTS

Lcn2 *expressions in mouse tissues after exposure to γ-rays*

We examined the effect of γ-rays on *Lcn2* expression by semi-quantitative RT-PCR. Before irradiation, *Lcn2* expression was detected in all the organs examined (Fig. 1). Twenty-four hrs after exposure to 8.5 Gy of γ-rays, *Lcn2* expression was up-regulated in the kidney, the heart and especially in the liver (Fig. 1). We then quantified *Lcn2* expression in the irradiated liver by real-time PCR (Fig. 2). Up-regulation of *Lcn2* expression was dose dependent and statistical significance was detected in the 8.5 Gy exposed group compared to non-irradiated mice ($p < 0.001$). In order to identify which compartment of the liver expressed *Lcn2*, we separated mouse liver cells into PC and NPC by the perfusion sedimentation technique. PC and NPC fraction mainly contained hepatocytes and Kupffer cells, respectively. Basal level of *Lcn2* expression in NPC was higher than that in PC (Fig. 3). After 8.5 Gy X-rays administration, up-regulation of *Lcn2* expression was detected in PC but not in NPC (Fig. 3).

Induction of Lcn2 expression in HepG2 cells after acute and long-term X-rays exposure

Since the liver was the most sensitive organ for the induction of *Lcn2* after irradiation (Fig. 1), we examined *Lcn2* expression *in vitro* using HepG2 cells. Up-regulation of *Lcn2* expression was detected 12 hrs after exposure to 8.5 Gy of X-rays (Fig. 4). *Lcn2* expression level in cells with long-term fractionated irradiation was higher than in cells after an acute exposure (Fig. 4).

Induction of Lcn2 *expression after H_2O_2 treatment*

The effect of ROS on *Lcn2* expression was examined by treating HepG2 cells with H_2O_2. Before H_2O_2 treatment, HepG2 cells were cultured in serum-free RPMI-1640 medium to avoid any influence of external free radicals such as lipid peroxidation products or antioxidants present in the serum. Without serum, *Lcn2* expression decreased, however, the administration of H_2O_2 drastically up-regulated *Lcn2* expression (Fig. 5). With the presence of 5% DMSO or 0.1 mM cysteamine in the H_2O_2 containing medium, up-regulation of *Lcn2* expression was canceled (Fig. 5).

DISCUSSION

Several studies indicate that *Lcn2* expression is induced under harmful conditions such as intoxication, infection, inflammation or other forms of cellular stresses,[8–11,15–19] however, the reason for the induction remains to be elucidated. Previous reports indicated that expression of *Lcn1*, a member of the lipocalin superfamily, was induced by harmful products such as ROS under conditions such as kidney injury, heart failure and burn injury.[15–19] Therefore, we hypothesized that harmful products such as ROS also induce *Lcn2* expression. To confirm our assumption, we examined *Lcn2* expression after the administration of H_2O_2 and also after Ionizing Radiation (IR), that is also a potent inducer of ROS.[22–23]

Without irradiation, *Lcn2* expression was detected in all the mouse organs examined. The Lcn2 protein in the testis

is known to be involved in protection of spermatogenic cells from genotoxic stresses such as ROS, and high expression of the *Lcn2* protein in the lung might be necessary to cope with the high level of ROS produced in this organ.[24] In this study, drastic up-regulation of *Lcn2* expression induced by IR was detected in the liver. Previous reports indicated that hepatocyte growth factor (HGF) induced by IR subsequently induces *Lcn2* expression.[25] HGF expression is reportedly induced under the condition of renal tubular injury, heart and liver diseases[26–28] accompanied with the production of ROS and induction of the *Lcn2* gene expression.[15–19] Therefore, high level of *Lcn2* expression in the irradiated liver might be due to the HGF induced by ROS. However, there is no report which indicates that ROS induces HGF expression. The up-regulation of *Lcn2* expression in the irradiated mouse liver may be attributed to both HGF and ROS. A previous report indicated that HGF is produced in Kupffer cells and endothelial cells.[29] Therefore, we further studied *Lcn2* expression after separating liver cells into PC and NPC. Basal level of *Lcn2* expression in NPC was higher than that in PC presumably because ROS are endogenously produced in Kupffer cells for the mediators in antigen presentation.[30] In this study, IR induced *Lcn2* upregulation in PC but not in NPC. It is noted that ROS are prominently produced in hepatocytes, but not in Kupffer cells after the administration of toxic injury.[31] These suggest that *Lcn2* expression in the irradiated liver is attribute to ROS produced in PC.

After acute and long-term exposure to X-rays, *Lcn2* expression was found to be upregulated in HepG2 cells. The level of up-regulation after long-term exposure to X-rays was higher than that after single acute exposure. This is consistent with the previous result using esophageal cancer cell lines.[20] In the present study, H_2O_2 induced up-regulated *Lcn2* expression in HepG2 cells and this up-regulation was canceled by the administration of ROS scavengers. Thus, we confirmed that *Lcn2* up-regulation in the liver is induced via ROS produced by IR. It is suggested that the Lcn2 protein might have a function as a scavenger and protect cells from ROS. The study to confirm these assumptions is now underway in our laboratory. This study also suggested that Lcn2 could be a useful biomarker for the detection of oxidative stress *in vitro* and *in vivo*.

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Regular Paper

Gene Expression Profiles in Mouse Liver Cells after Exposure to Different Types of Radiation

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Gene expression/Microarray/Mouse liver/Radiation quality/Endothelial cell/Kupffer cell.

The liver is one of the target organs of radiation-induced cancers by internal exposures. In order to elucidate radiation-induced liver cancers including Thorotrast, we present a new approach to investigate *in vivo* effects of internal exposure to α-particles. Adopting boron neutron capture, we separately irradiated Kupffer cells and endothelial cells in mouse liver *in vivo* and analyzed the changes in gene transcriptions by an oligonucleotide microarray. Differential expression was defined as more than 3-fold for up-regulation and less than 1/3 for under-regulation, compared with non-irradiated controls. Of 6,050 genes examined, 68 showed differential expression compared with non-irradiated mice. Real-time polymerase chain reaction validated the results of the microarray analysis. Exposure to α-particles and γ-rays produced different patterns of altered gene expression. Gene expression profiles revealed that the liver was in an inflammatory state characterized by up-regulation of positive acute phase protein genes, irrespective of the target cells exposed to radiation. In comparison with chemical and biological hepatotoxicants, inductions of Metallothionein 1 and Hemopexin, and suppressions of cytochrome P450s are characteristic of radiation exposure. Anti-inflammatory treatment could be helpful for the prevention and protection of radiation-induced hepatic injury.

INTRODUCTION

The biological effects of exposure to high linear energy transfer (LET) radiation have a particular relevance to radiation protection and risk assessment. Although internal exposure to high LET radiation is of a major concern, it is characterized by the existence of target organs and the difficulty of dose estimation. Thorotrast, a colloidal suspension of radioactive $^{232}ThO_2$ that naturally emits α-particles, was used as a radiographic contrast agent in the 1930s–1950s. More than half of intravasculary injected Thorotrast deposited in the liver caused liver cancers decades after the injection because of its life-long deposition and exposure to α-particles. Our histological examination of the liver from 144 cases of Thorotrast patients revealed intrahepatic cholangiocellular carcinoma (ICC, 25.7%), angiosarcoma (AS, 20.8%), hepatocellular carcinoma (HCC, 14.6%) and combined tumors (2.1%). Considering that Japan is an endemic area of hepatitis virus B and C, and that HCC comprises more than 80% of liver cancers, ICC and AS may be considered to be characteristic of Thorotrast-induced liver tumors. Our previous study showed that injected Thorotrast is phagocytosed by macrophages and radioactive Thorium is always migrating within the affected livers via Thorotrast-laden macrophages. These suggest that the liver is evenly exposed to α-particles at the organ level despite the short range of α-particles.[1)] Internal deposition of plutonium also causes chronic exposure to high levels of α-particles with increased risk of liver cancers including AS.[2)] Neither the deposited amount of Thorium nor the incubation period from injection to tumor induction is significantly different between cases with ICC and AS (manuscript in preparation). Consequently we thought that cell-to-cell interaction between irradiated macrophages and/or epithelial cells and parenchymal cells of the liver is involved in the development of ICC while direct irradiation of endothelial cells of the

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sinusoid is the principal contributor to the development of AS. A few studies also have been shown that exposure to α-particles induces liver tumors in mouse and other rodents.[3,4]

Thermal neutrons cause the boron atom to split into an α-particle and a lithium nucleus via the boron neutron capture reaction (BNC). Both of these particles have a very short range (about one cellular diameter) and cause significant damage to the cell in which boron atoms are located. BNC therapy (BNCT) adopts this cytotoxic effect by selective delivery of boron-10 (^{10}B) to tumor cells: the short range nature of the effects of BNC minimizes the damage to adjacent normal cells. A large amount of ^{10}B compound can be administered in a liposome-incorporated form, which is then phagocytosed by macrophages. Conjugation of the liposome with polyethylene glycol (PEG) is known to increase blood levels of ^{10}B compounds and reduced uptake by macrophages.[5] Recent radiological studies focus on the molecular mechanisms underlying transcriptional responses of mammalian cells to ionizing radiation. It is now apparent that the cellular reactions to ionizing radiation are complex and involve the activation of secondary messenger pathways and increased transcription of immediate early response genes.[6]

These observations prompted us to adopt BNC to investigate *in vivo* effects of internal radiation exposure to α-particles. In this study, we prepared the ^{10}B-liposome treatment and the ^{10}B PEG-liposome treatment to expose Kupffer cells and endothelial cells to α-particles respectively, and analyzed the changes of gene expression using a microarray containing probes for 6,050 genes. As well as elucidation of the biological relevance of radiation, the present study also

Table 1. Primer sets for RT-PCR

Symbol	GenBank		Sequence	Size(bp)
AK3	AK005194	forward reverse	5'-GTG TGT TGG CCA AGA CTT TC-3' 5'-ATG TAT CCA GCG AGC AGT AAG-3'	236
Atp5b	AK010314	forward reverse	5'-GCA CAA TGC AGG AAA GGA TCA C-3' 5'-ACG TCA TAA TGC TCA TTG CCA AC-3'	241
ATP5c1	AK007063	forward reverse	5'-CGC CCC ATG GCA ACT CTG AAA G-3' 5'-GCC AAA GAA CCT GTC CCA TAC A-3'	160
Brap	AK013885	forward reverse	5'-AAA GGG CTG AAG TGC TGA ATC-3' 5'-TCT GGC GTT TGA CAG TAT CGG C-3'	211
Car3	AK003671	forward reverse	5'-CTT GAT GCC CTG GAC AAA AT-3' 5'-AGC TCA CAG TCA TGG GCT CT-3'	180
Egfr	AK004944	forward reverse	5'-TGA GCA ACA TGT CAA TGG ACT TAC-3' 5'-GCA TGT GGC CTC ATC TTG GAA C-3'	263
Galnt3	AK019995	forward reverse	5'-CAC TAT TTA CCC GGA AGC GTA TG-3' 5'-GTG GCA CGT GTA CAG AAT CAA TG-3'	139
Gsta3	AK014076	forward reverse	5'-TGA CCT GGC AAG GTT ACG AAG TG-3' 5'-CAT TAT CTC CAG ATC CGC CAC TC-3'	199
Hpxn	BB610094	forward reverse	5'-ATC TCA GCG AG GTG GAA GAA TC-3' 5'-CCT TCA CTC TGG CAC TCT CCA C-3'	215
Lcn2	AK002932	forward reverse	5'-CCA GTT CGC CAT GGT ATT TTT C-3' 5'-CAC ACT CAC CAC CCA TTC AGT T-3'	206
Mt1	AK018727	forward reverse	5'-ACC TCC TTG CAA GAA GAG CTG CT-3' 5'-GCT GGG TTG GTC CGA TAC TAT T-3'	160
Mt2	AK002567	forward reverse	5'-GGG TCC CCA CAT CTG TG TAA-3' 5'-CAA CGG CTT TTA TTG TCA GTT AC-3'	115
β-actin		forward reverse	5'-TTC TAC AAT GAG CTG CGT GTG G-3' 5'-GTG TTG GAA GGT CTC AAA CAT GAT-3'	110

contributes to the understanding of general idea of potential target molecules for cancer therapy.

MATERIALS AND METHODS

Mice and radiation

Male C3H/Hex mice (6 weeks old) were exposed to whole-body irradiation. For irradiation of mice by α-particles, specifically to macrophages and endothelial cells, ^{10}B-liposomes and polyethylene glycol (PEG)-^{10}B-liposomes were respectively administered. There were two mice analyzed by microarray, independently treated, and five mice by real time PCR. Mice used for these 2 assays were from 2 different courses of experiments. The ^{10}B compound sodium mercaptoundecahydrododecaborate (BSH) was used.[7] Each compound was suspended in physiological saline at a concentration of 4,000 ppm and 100 μl of ^{10}B-liposome solution and 300 μl of PEG-^{10}B-liposome solution were injected via the tail vein. Four hours (hrs) after the administration, the mice were exposed to neutron radiation at the Research Reactor Institute, Kyoto University (RRIKU). Before the irradiation experiments for gene expression, the neutron fluence was monitored by radioactivation of gold foils in the front and back of the mouse container. The average fluence of the thermal neutron source was 2.1×10^{12} n/cm^2 and the average flux was 2.3×10^9 n/cm^2/s at 5 MW. The boron concentration of the liver was measured by γ-ray spectrometry using a thermal neutron guide. We determined the exposure period at the calculated dose of 8.5 Gy at an organ level. For control irradiation, the mice were exposed to the neutron source for the same period as the BNC group. The contribution of neutrons and γ-rays to the total exposure was 4.2 cGy and 33 cGy, respectively. As a control for the quality of radiation, the mice were exposed to γ-rays at a dose of 8.5 Gy (0.34 Gy/min) with a ^{60}Co γ-ray source. Twenty hrs after irradiation, the mice were sacrificed by cervical dislocation. The dissected liver was immediately frozen and stored at −80°C until use. Animal experiments were approved by the Ethical Committee of the Institute of Development, Aging and Cancer, Tohoku University and were performed in accordance with institutional guidelines.

Oligonucletide microarrays

In accordance with 'Functional Annotation of Mouse' for the RIKEN full-length cDNA clone (http://fantom2.gsc.riken.go.jp/) and GenBank (http://www.ncbi.nih.gov/Genbank), 6,050 mouse genes were chosen for microarray analysis. These consisted of genes associated with signal transduction (766), cancer (506), autoimmune/inflammatory disease (455), cytokine/inflammatory response (267), stem cell

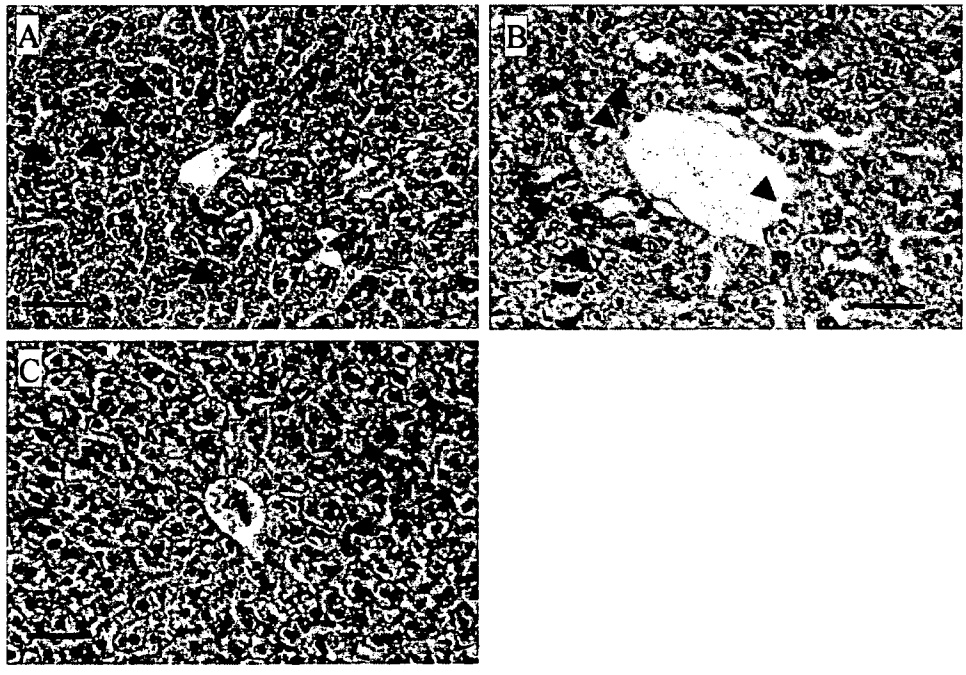

Fig. 1. Histological findings of the mouse liver following irradiation of Kupffer or endothelial cells. A: Compared with non-irradiated control, mice injected with ^{10}B-liposome solution showed a slight increase of the number and the size of Kupffer cells (arrows), indicating Kupffer cells were mainly irradiated (Kupffer exposure). B: Endothelial cells (arrow heads) were swollen in irradiated liver. The dilatation of sinusoids was noticed in mice injected with PEG-^{10}B-liposome, indicating sinusoidal endothelial cells were mainly insulted (Endothelial exposure). C: Non-irradiated control. Scale Bar: 50 μm.