Effects of X-ray Irradiation on Genomic DNA Methylation Levels in Mouse Tissues

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Effects of ionizing radiation on the level of genomic DNA methylation in liver, brain and spleen of mouse as well as in two kinds of cultured cells were examined by high-performance liquid chromatography. Ten Gy of whole body X-radiation reduced the 5-methyldeoxycytidine contents by about 40% within 8 hours after irradiation in liver. Similar effects were observed at 4 or 7 Gy of X-ray irradiation. However, no such change was detected in brain, spleen and cultured cells. The data indicate that radiation-induced alteration in genomic DNA methylation is not ubiquitous among different tissues and cells.

INTRODUCTION

Methylated deoxycytidine (5mdC) is widely observed in vertebrate DNA and many lines of evidence suggest that the methylation is one of the factors involved in regulation of gene expression through modification of DNA-protein interaction. Levels of 5mdC in genomic DNA are known to change in developmental process resulting in tissue-specific levels and in tissue-specific patterns. They also change in senescent period and tumor development process. A variety of DNA damaging agents are known to alter the DNA methylation. Ionizing radiation is one of such agents. In 1989, Kalinich et al. showed that several Gy of γ-ray reduced 5mdC
levels by about 50% within a few days after irradiation in four established cell lines\(^\text{17}\).

We have thought that these lines of evidence could suggest a possible involvement of DNA methylation alteration in radiation-induced late effects such as tumor induction and life shortening, because a change in DNA methylation could remain for a long period of time and could contribute to cellular changes associated with carcinogenesis or aging. In fact, alteration of DNA methylation in some genes are found in radiation-induced tumors\(^\text{18}\) and transformed cells\(^\text{19}\). In order to examine further the possible correlation, we have studied the effects of X-ray irradiation on 5mdC levels in animal tissues. A significant reduction has been observed in liver but not in spleen and brain. It suggests that the effects do not simply depend on DNA damage but also on the other factor(s).

**MATERIALS AND METHODS**

**Mice**

Adult C57BL/6NJcl mice were purchased from Nihon Clea Co. Ltd. (Tokyo) at 8–9 weeks of age and kept in our animal facility. They were irradiated with 4–10 Gy of X-rays as a whole body at 10 weeks of age and sacrificed by cervical dislocation at various times after irradiation. Tissues were excised, frozen on dry ice and kept at \(-70^\circ\text{C}\) till use.

**Cells**

Murine cell line m5S/1M and Chinese hamster CHO/K-1 cells were used. m5S/1M cells were derived from a mouse embryo and became immortal during passages but not transformed\(^\text{20–22}\). The cells showed contact-inhibition and their karyotype was near diploid. Cells were cultured in \(\alpha\)-modified minimum essential medium supplemented with 10% heat-inactivated fetal calf serum\(^\text{22}\). The cells in log phase or stationary phase were irradiated with 10 Gy and incubated for various times in fresh medium. CHO/K-1 cells were provided through Japan Health Science Foundation (Tokyo), cultured in F-12 medium with 10% heat-inactivated fetal calf serum, and irradiated at log phase.

**Irradiation**

Mice were confined in a plastic box where they could move freely. X-ray was 230kVp, 20mA from Shimazu Therapy Machine (SHT 250M-3, Kyoto) with a filter of 1mm Al, 1mm Cu. The dose rate in the center of the body was estimated to be 0.27 Gy/min by the use of mouse phantom and TLD system\(^\text{23}\). The cells in culture were irradiated similarly.

**HPLC analysis of 5mdC**

DNA was extracted with phenol and contaminating RNA was removed by RNases A and T1 treatment\(^\text{24}\). Approximately 5\(\mu\)g of DNA was digested with P1 nuclease (Penicillium citrinum) and alkaline phosphates (calf intestine) to obtain nucleosides, and run through high-performance liquid chromatography (HPLC)\(^\text{7}\). The amounts of 5mdC, dC, dT, dA and dG were measured at 273 nm at a sensitivity of 0.005 absorbance-unit full scale (AUFS) following separation on Nucleosil C18 (particle size 5 \(\mu\)m; Macherey and Nagel, Düren, Germany) using 0.1% (v/v)
phosphoric acid as a mobile phase. The level of 5mdC was calculated as a percentage in total nucleosides, that was the sum of 5mdC, dC, dT, dA, and dG.

RESULTS

A typical profile of HPLC analysis is shown in Fig. 1. The amounts of nucleosides including 5mdC were estimated from similar profiles of standard compounds. The effects of 10 Gy whole body irradiation on the levels of 5mdC in liver, brain, and spleen are shown in Fig. 2. The levels in non-irradiated mice differed among tissues as reported before. Immediately after irradiation,
Figure 2. Radiation effects on 5mdC levels in liver (A), brain (B) and spleen (C) in mice. Irradiation was done to a whole body with 10 Gy and the levels of 5mdC in DNA were measured at 0, 8, 24, 48 and 72 hours after irradiation. The data were means and standard deviations of 3 to 5 individuals. Open circles indicate the levels in unirradiated tissues.

Figure 3. Dose-response of radiation-induced reduction of 5mdC level in liver. Mice were irradiated with 4, 7 and 10 Gy and the 5mdC levels at 72 hours after irradiation were determined. Means and standard deviations were determined from the data of 4 individuals.

Figure 4. Radiation effects on 5mdC levels in cultured m5S/1M cells. Cells in exponentially growing phase (triangles) and stationary phase (circles) were irradiated with 10 Gy and the 5mdC levels at various times after irradiation were determined. Open symbols indicate the levels in non-irradiated cells. The data were averages of two determinations.
no appreciable alteration was observed in all of the tissues. It indicates that irradiation itself does not affect 5mdC level. At 8 hours after irradiation, the level decreased from 0.97% to 0.56% in liver and this lower level stayed for 3 days after irradiation. In brain and spleen, on the other hand, no such change was observed.

Concerning the effect on liver DNA, dose-dependency was examined. As is shown in Fig. 3, irradiation with 4, 7 and 10 Gy resulted in similar reduction of 5mdC level.

Since we observed tissue-dependency for radiation effects on the 5mdC level, we wondered why the previous paper observed radiation-induced reduction in all of the 4 different lines of cultured cells, neuroblastoma C-1300/NIH-115, CHO/K-1, V79/AO3 and HeLa/S-3\(^\text{[17]}\). As one approach, we examined another cell line, m5S/1m cells. In this cell line, the 5mdC level was not affected by 10 Gy of radiation (Fig. 4). The effects were studied at both log phase and stationary phase of growth. We further examined the effect on CHO/K-1, a cell line examined by Kalinick et al.\(^\text{[17]}\), but did not see an alteration (data not shown).

**DISCUSSION**

DNA methylation has been shown to be altered by many kinds of DNA damaging agents\(^\text{[10–16]}\) and by chemicals which inhibit DNA replication\(^\text{[25]}\). In 1989, ionizing radiation has been demonstrated to reduce 5mdC levels in four different cell lines, neuroblastoma C-1300/NIH-115, CHO/K-1, V79/AO3 and HeLa/S-3\(^\text{[17]}\). Present study in vivo shows that the radiation effect on the 5mdC level is not ubiquitous but depends on tissues. Liver DNA revealed a similar radiation response to those of cells examined previously by Kalinich et al.\(^\text{[17]}\). However, little effects were observed in brain and spleen.

The two cultured cell lines we examined revealed no alteration after irradiation. Inconsistency among cell lines in the effect of DNA damaging agent on DNA methylation has been reported before for the effects of benzo (a) pyrene on two cell lines, 10T1/2 and 3T3 cells\(^\text{[11]}\). The drug reduced the 5mdC level in BALB/3T3 cells but not in C3H/10T1/2. Therefore, cell line-dependency in the reduction of DNA methylation level by DNA damaging agents could be a common phenomenon. However, one of the two cell lines we examined was CHO/K-1, which was the same cell line as that used by Kalinick et al.\(^\text{[17]}\). We do not understand why the results are not reproducible. We speculate that there was an unidentified difference in experimental procedures conducted by Kalinick et al. and us.

The effects of radiation on DNA methylation could be considered at two levels. One is the change of methylation in a small number of nucleotides inserted by repair synthesis at damaged sites\(^\text{[10]}\). The other is the alteration taking place in wide range of genomic DNA, which could results from reduced DNA methyltransferase activity or from activated DNA demethylating activity. The number of DNA damage induced by 10 Gy of radiation is in the order of \(10^4/\text{cell}\) irrespective of cell types\(^\text{[26–28]}\), and the number of nucleotides which could be replaced by repair is estimated to be in the order of \(10^1–10^2\) for each damaged site\(^\text{[29]}\). Thus the total number of nucleotides incorporated into DNA as repair synthesis after 10 Gy should be in the order of \(10^5–10^6\) per cell. It consists a very small fraction of total genomic DNA (about \(10^{10}\) nucleotides/cell). The
present method cannot detect such a small change. Instead, the alterations observed in liver and four cultured cell lines must be widespread in the genome. At present, we are trying to elucidate which part of the genome is affected by radiation in liver. A search of DNA methylation in the c-myc gene and endogenous viruses (Env2 and Mtv) by their sensitivity to a restriction enzyme HpaII followed by Southern blot analysis using probes reported before did not show any difference. Therefore, it seems that the alteration is not taking place at random throughout the genome. Further studies are needed on this point.

A mechanism for the radiation-induced reduction of 5mdC level in liver is only speculative. Kalinich et al. have suggested redistribution of DNA methyltransferase from nucleus to cytoplasm. However, demethylation caused by a reduced level of DNA methyltransferase is assumed to take place only through DNA replication and it is not likely that DNA synthesis occurs within 8 hours after 4 Gy of irradiation in liver, the time when reduction of 5mdC was observed (Fig. 1). Therefore, the reduction of DNA methyltransferase in nucleus does not seem to be a likely explanation. Another possibility would be an enzymatic replacement of 5-methylcytosine with cytosine, which has been suggested to take place in a process of differentiation of Friend erythroleukemia cells. Razin et al. have observed a reduction of 30–50% of DNA methylation in 6–12 hours after differentiation started and showed evidence to support the replacement of 5-methylcytosine with cytosine. More detailed analyses of the DNA demethylation activity have been reported more lately.

Whatever the mechanism is, the tissue-specific reduction of DNA methylation would be an interesting effect of ionizing radiation in considering biological consequences of radiation.

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REFERENCES


