The localization of repair proteins for alternative non-homologous end joining and single strand annealing after stepwise fractionation

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DNA double-strand breaks (DSBs) are the most severe forms of DNA damage caused by ionizing radiation. In mammalian cells, DSBs are repaired primarily by non-homologous end joining (NHEJ) or homologous recombination (HR), whereas alternative non-homologous end joining (alt-NHEJ) and single strand annealing (SSA) are considered to function only when both NHEJ and HR are impaired. Accelerated heavy ions with high LET (linear energy transfer) induce clustered DNA damage. The complexity of clustered damage depends on LET and affects the chosen repair pathway. However, the repair mechanism of DSBs is not fully understood.

Previous studies have shown that DSBs caused by high LET radiation promote end-resection, which could lead to resection-dependent HR, alt-NHEJ, and SSA.[1)] Our previous study has also demonstrated that HR is favored in G2-phase after heavy-ion irradiation.[2)] Additionally, the chromatin recruitment of Rad51 increases up to 5 Gy in a dose-dependent manner but is suppressed by high dose (> 15 Gy) irradiation.[3)] These results suggest that the irradiation of heavy-ions at high doses suppresses HR and promotes SSA and/or alt-NHEJ. Since alt-NHEJ and SSA are error-prone pathways, it is important to know whether they are involved in repair to estimate the risk of secondary carcinogenesis during radiotherapy. In this report, we examined the localization of DNA polymerase theta (pol θ) and Rad52, which are essential components of alt-NHEJ and SSA, respectively.

The chromatin-bound Rad51 was localized in the Triton X-100-insoluble fraction.[4)] However, estimating the amount of chromatin-bound pol θ and Rad52 by Triton extraction was difficult because substantial amounts of these proteins seemed to bind to insoluble structures including nucleoli or nuclear bodies (data not shown). Therefore, we used the stepwise fractionation kit from Thermo Scientific (cat. no. 78840), which enables the stepwise extraction and preparation of cytoplasmic, membrane, nuclear soluble, chromatin-bound, and cytoskeletal fractions from mammalian cells.

HeLa cells were irradiated with 10 Gy of X-ray. Subsequently, cell extracts were prepared 1 hour after irradiation and subjected to immunoblotting (Fig. 1). Lamin b1, histone H3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were localized in cytoskeletal, chromatin-bound, and cytosol fractions, respectively. These results indicate the validity of fractionation. However, this method must be improved, as discussed below.

The major fraction (90%) of pol θ was localized in nuclear soluble fraction whereas the minor fraction (10%) was found in chromatin-bound fraction. The distribution of pol θ did not change after 1 hour of X-ray irradiation. Since alt-NHEJ is slower process, its longterm distribution must be investigated.

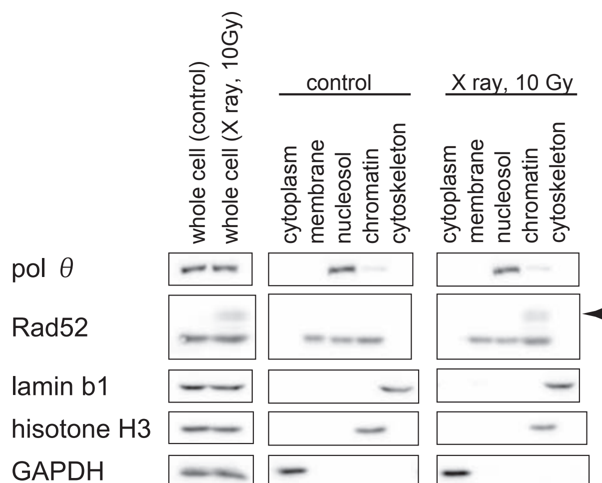

Fig. 1. Immunoblot analysis of repair proteins after irradiation. HeLa cells were irradiated with X ray (10 Gy) and fractionated after 1 hour of irradiation. Whole cell extracts and fractionated extracts were subjected to immunoblotting. Pol θ and Rad52 were detected as markers for alt-NHEJ and SSA. Arrowhead indicates the modified Rad52. Lamin b1, histone H3, and GAPDH were detected to confirm the validity of fractionation.

In contrast, Rad52 was found in membrane, nuclear, and chromatin-bound fractions, suggesting that the second reagent solubilized not only the mitochondria and ER/golgi membranes but also the nuclear membranes partially. The major fraction (40%) of Rad52 was found in the chromatin-bound fraction without irradiation, which may be because Rad52 contributes to multiple pathways, including break-induced replication, alternative lengthening of telomeres, and mitotic DNA synthesis in the normal cell cycle.[5)] The amount of Rad52 in the chromatin fraction increased after 1 hour of X-ray irradiation by 30%. Additionally, modified Rad52 was detected (Fig. 1, arrowhead). Rad52 sumoylation is important for DSB repair in yeast. The modified Rad52 was found only in chromatin fraction, suggesting that Rad52 was sumoylated through DNA damage responses in mammalian cells as well.

Currently, we are optimizing the fractionation and investigating the time course of chromatin-bound pol θ

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and Rad52 after heavy-ion irradiation at various doses. Rad52 is involved in HR as well as SSA. Therefore, we are also investigating the modification of Rad52 in quiescent cells where HR does not occur.

References

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