# Contribution of radiation-induced activation of NAD(P)H oxidase to the induction of apoptosis in human promyelocytic cell line HL 60

Keisuke Sakakibara

*Laboratory of Radiation Biology,*
*Department of Environmental Veterinary Sciences,*
*School of Veterinary Medicine,*
*Hokkaido University, Sapporo 060-0818, Japan*

Recently, the post-irradiation treatment with antioxidants is reported to have protective effects on radiation-induced apoptosis in leukemia cells, suggesting that reactive oxygen species (ROS) such as superoxide anions ($O_2^-$), hydrogen peroxides ($H_2O_2$), OH radical ($OH^\cdot$) and nitric oxide (NO), which can be scavenged by the antioxidants, are produced after irradiation and involved in apoptosis. However, there is no direct evidence for ROS production after irradiation. In this study, to clarify the production of ROS after irradiation and to identify enzymes responsible for this system, the production of ROS from X-irradiated human promyelocytic cell line HL 60 was measured and the effects of inhibitors on enzymes responsible for the production of ROS were evaluated. Flowcytometry using dihydrorhodamine 123 (DHR 123) was employed to detect the ROS production after X irradiation, and the following inhibitors were used to study the mechanisms of ROS production; NAD(P)H oxidase inhibitor (diphenyleneiodonium chloride [DPI]), NADH dehydrogenase inhibitor (rotenone), succinate dehydrogenase inhibitor (malonic acid), mitochondria uncoupler (carbamoyl cyanide m-chlorophenylhydrazone [CCCP]), nitric oxide synthase inhibitor ($N^G$-monomethyl-L-arginine [NMMA]), xanthine oxidase inhibitor (allopurinol), ribonucleotide reductase inhibitor (hydroxyurea), intracellular calcium ions chelater (1,2-bis (o-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid tetra (acetoxymethyl) ester [BAPTA-AM]) and calcineurin inhibitor (FK 506).

Flowcytometric profile obtained from cells at 15 min after irradiation with 15 Gy showed the rightward shift in comparison with that of unirradiated control, indicating the increase of ROS inside cells after irradiation. This increased-response of ROS after irradiation was significantly attenuated by a vitamin E analogue Trolox and a spin-trapping reagent N-*tert*-butyl-α-phenylnitrone (PBN) which were known to act as antioxidants. These antioxidants also inhibited a part of apoptotic cell-death and the activation of caspase 3 after X irradiation of HL 60 cells with 15 Gy. Since the caspase 3 inhibitor (Ac-DEVD-CHO) greatly decreased X-ray-induced apoptosis in HL 60 cells, it was considered that the ROS production after irradiation played as important roles in the induction of caspase 3-dependent apoptosis.

In experiments using various enzyme inhibitors, ROS production was found to be significantly attenuated by DPI but not rotenone, malonic acid, CCCP, NMMA, allopurinol, hydroxyurea and FK 506. These results indicated that NAD(P)H oxidase was a candidate to produce ROS after X irradiation. Furthermore, treatments with BAPTA-AM also partly attenuated not only the X-ray-induced ROS production but also apoptosis. This suggested that the increase of intracellular calcium ions

induced by X irradiation also participated in the ROS production.

In conclusion, the present study demonstrated that the ROS generated by NAD (P) H oxidase was partly responsible for the caspase 3-dependent apoptosis. Since this apoptosis was partly inhibited by antioxidants like PBN and trolox, the post-irradiation treatment with antioxidants may be used as a new remedy for radioprotection.

## Enhancement of radiation-induced apoptosis in HL 60 and MOLT-4 cells by oxygen and hypoxic radiosensitizer etanidazole

Kiyoshi Sugihara

*Laboratory of Radiation Biology,*
*Department of Environmental Veterinary Sciences,*
*School of Veterinary Medicine,*
*Hokkaido University, Sapporo 060-0818, Japan*

The distribution of hypoxic cells in the tumor is thought to be critical against a cure rate of radiation therapy, because ionizing radiation induces less efficiently cell death under the hypoxic conditions than under the aerobic condition. The killing effect of ionizing radiation on cultured mammalian cells was usually judged by the loss of clonogenic ability. Recent studies showed that two types of cell death, necrosis and apoptosis, were main components of the loss of clonogenic ability. However, there are little reports about oxygen effects on radiation-induced apoptotic cell death. The present study was carried out to examine how the hypoxia influenced the ionizing irradiation-induced apoptosis in cultured mammalian cells with the aid of a specially designed gas-exchangeable chamber. Furthermore, we examined whether the hypoxic cell radiosensitizer, etanidazole, sensitized the apoptotic cell death under the hypoxic condition.

Two cell-lines derived from human lymphocytes, HL 60 and MOLT-4, were employed and exposed to 15 Gy of X-rays under the aerobic and hypoxic conditions. In the case of experiments with etanidazole, both were treated with 10 mM etanidazole for 90 min before exposure to X-rays. The apoptotic morphological changes of nuclei and induction of ladder-like DNA fragmentation were accessed by fluorescence microscopy and agarose gel electrophoresis, respectively. In HL 60 cells, the results showed that the apototic cell death and the activation of caspase 8, 9 and 3 were less induced in the hypoxic cells than in the aerobic ones. Furthermore, treatments of the cells with etanidazole enhanced radiation-induced apoptosis as well as the activation of caspase family under the hypoxic condition. However, in MOLT-4 cells, hypoxia did not reduce radiation-induced apoptotic cells and the activation of caspase family. The treatment with etanidazole did not affect the induction of radiation-induced apoptosis under the hypoxic condition. To investigate the relationship between the radiation-induced DNA double-strand breaks (dsb) and the induction of apoptosis, the dsb were measured by pulsed-field gel electrophoresis immediately after X irradiation. In both cell lines, the radiation-induced dsb in the hypoxic condition was sig-