Concentration-Dependent Modes of Cell Death in Chinese Hamster V79 Cells after Treatments with H$_2$O$_2$

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Deterioration in the clonogenic ability of Chinese hamster V79 cells was observed after treatments with low concentrations of H$_2$O$_2$ (0.06–0.2 mM) for 1 h and subsequent incubation for 6–8 days, whereas no loss of ability of the cells to exclude trypan blue after treatments with H$_2$O$_2$ for 1 h and subsequent incubation for 6 h was observed in this concentration range. The loss of the dye-exclusion ability became observable when the cells were treated with concentrations greater than 0.9 mM H$_2$O$_2$ for 1 h and subsequently incubated for 6 h. Agarose gel electrophoresis of DNA extracted from cells treated with 1–5 mM H$_2$O$_2$ showed specific regular fragmentation of DNA, suggesting that apoptotic cell death was induced. The induction of apoptotic cell death was further confirmed by observing the protective effects of several modifiers, a protein synthesis inhibitor (cycloheximide), a Ca$^{2+}$-chelator (BAPTA-AM) and an antioxidative compound (PBN), against cell survival and DNA fragmentation. From these results, it was concluded that apoptotic cell death was induced by H$_2$O$_2$ in Chinese hamster cells at high concentrations, where their clonogenic ability completely disappeared.

INTRODUCTION

In most cases in which cultured mammalian cells are exposed to ionizing radiation, the mode of cell death defined as the loss of reproductive integrity (i.e., loss of clonogenic survival) cannot be accounted for by apoptosis occurring either before or shortly after the cells divide following irradiation except in apoptotic sensitive-cell lines. For example, when Chinese hamster ovary cells are irradiated with 10–15 Gy, only 3% of the population is susceptible to apoptosis$^{1,2}$. On the other hand, human B lymphoblast cell line TK 6 is classified as an apoptotic-sensitive one$^{1,2}$ since 90% of the population is susceptible to apoptosis induced by high irradiation doses (10–15 Gy). However, the fraction of apoptosis observed for this cell line with low radiation doses is not considered to be the main fraction of the loss of the reproductive integrity, because after 1.5 Gy, 90% of the cells survive apoptotic cell death, compared with only 10% of the cells that survive reproductive death$^{1,3}$. These results indicate that the mode of cell death is dependent on the irradiation dose.
when the cultured mammalian cells are exposed to ionizing radiation. Reactive oxygen intermediates (ROI) and ionizing radiation are known to cause oxidative stress. Since ionizing radiation produces OH radicals as one ROI and leads to cell death, mechanisms of oxidative stress induced by ionizing radiation in cultured cells are considered to overlap those induced by usual ROIs like \( \text{H}_2\text{O}_2 \) or \( \text{O}_2^- \). It is, therefore, of interest to find the common processes leading to cell death due to ionizing radiation and \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \), which is steadily generated in vivo under both physiological and pathological conditions in several organella, induces damage leading to apoptosis\(^{4-7} \). The present study was carried out to examine whether the mode of cell death induced in mammalian cells after treatments with \( \text{H}_2\text{O}_2 \) is similar to that induced by ionizing radiation. For this purpose, Chinese hamster V79 cells were chosen because data concerning responses of this cell line to ionizing radiation have been accumulated in our previous studies\(^{8-10} \) and they are recognized as being extremely resistant to radiation-induced apoptosis, as are Chinese hamster ovary cells\(^{11} \). Then, concentration-dependent modes of cell death were examined in \( \text{H}_2\text{O}_2 \)-exposed cells by both clonogenic and trypan blue-exclusion activities.

**MATERIALS AND METHODS**

*Chemicals*

\( \text{O,O'}\)-Bis(2-aminophenyl)ethyleneglycol-\( N,N,N',N' \)-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) was supplied by Dojindo Laboratories (Kumamoto, Japan). \( \alpha \)-Phenyl \( N \)-tert-butylnitrone (PBN) was purchased from Aldrich, Chemical Company, Inc. (Milwaukee, WI). Cycloheximide was supplied by Sigma Chemical Company (St. Louis, MO).

*Cell Culture*

Chinese hamster cells, subline V79-B310H, were a kind gift of Dr. H. Utsumi, Research Reactor Institute, Kyoto University. The cells were maintained in log-phase culture with \( \alpha \)-minimum essential medium (GIBCO)\(^{11} \), supplemented with 10% fetal bovine serum (GIBCO). Under these conditions, the doubling time was 9–10 h.

*\( \text{H}_2\text{O}_2 \)-treatments and survivals of Chinese hamster V79 cells*

The cells were inoculated in plastic dishes 10 h before \( \text{H}_2\text{O}_2 \)-treatment. \( \text{H}_2\text{O}_2 \) was diluted with ice-cold PBS to the desired concentrations. The log-phase cells in a plastic dish were twice washed with PBS, and then \( \text{H}_2\text{O}_2 \) was added to the cells. After incubation for 1 h, the medium containing \( \text{H}_2\text{O}_2 \) was aspirated and fresh medium was added to the cells. Viabilities of cells were examined for the clonogenic ability by counting the colonies formed after subsequent incubation for 6–8 days and for 0.2% trypan blue-exclusion ability by counting cells which excluded the dye after subsequent incubation for 6 h.

*Treatments of cells with metabolic inhibitors*

Cycloheximide was added to the cell cultures at the concentration of 15 mM and incubated for 1 h before \( \text{H}_2\text{O}_2 \)-treatment and for 2 h during and after \( \text{H}_2\text{O}_2 \)-treatment. In the case of BAPTA-AM and PBN, cells were treated by incubating them with 5 \( \mu \)M BAPTA-AM for 1 h and with 10 mM PBN for 3 h before \( \text{H}_2\text{O}_2 \)-treatment. Viabilities of the treated cells were determined as above.
**Agarose Gel Electrophoresis of DNA**

DNA fragmentation was assayed as described by Ramakrishnan and Catravas. The cells were inoculated in 10-cm plastic dishes 10 h before H$_2$O$_2$-treatment. Log-phase cells containing 5x10$^6$ were harvested with rubber policeman. The cells were washed twice with ice-cold PBS and lysed with 0.5 mL of hypotonic buffer (10 mM Tris-HCl, pH 7.5 containing 0.2% Triton X-100 and 1 mM EDTA) at 0°C for 20 min. The homogenate was centrifuged at 13,500g for 30 min, and the supernatant was recovered as a solution containing fragmented DNA. The solution was then incubated for 1 h at 37°C with 50 µg/mL RNase followed by treatment with 0.5 mg/mL Protease K for 1 h at 37°C. The DNA was extracted with an equal volume of phenol followed by washing with chloroform/isoamyl alcohol. The DNA was then precipitated by twice the volume of 99.5% ethanol with 0.3 M sodium acetate. The precipitate was dissolved in 0.3 mL of TE buffer (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA). DNA was electrophoresed at 100 V for 1 h with 1.5% agarose gel in Tris-acetate buffer. DNA bands were visualized under UV light after staining with ethidium bromide.

**RESULTS AND DISCUSSION**

Figure 1 shows the dose-response curves of H$_2$O$_2$-treated cells assayed for both clonogenic and dye-exclusion abilities. The curves showed quite different dose-dependencies. The clonogenic ability of cells began to decrease with a concentration of H$_2$O$_2$ of above 0.06 mM, while dye-exclusion ability began to decrease above 0.9 mM H$_2$O$_2$. The incorporation of trypan blue occurred at about 1 h after the H$_2$O$_2$ treatment and the number of stained cells became approximately constant at 6 h (data not shown). These results suggested that two types of cell death were present in H$_2$O$_2$-treated cells, depending on the H$_2$O$_2$ concentrations.

![Figure 1](image-url)  
**Figure 1.** Viabilities of H$_2$O$_2$-treated Chinese hamster V79 cells. •: Assayed by clonogenic ability. ○: Assayed by 0.2% trypan blue exclusion at 6 h after the H$_2$O$_2$ treatment.
The exposure of Chinese hamster V79 cells to high concentrations of $\text{H}_2\text{O}_2$ (> 0.9 mM) induced the loss of ability to exclude trypan blue, which occurred before the cells divided following the $\text{H}_2\text{O}_2$ treatment (at 6 h after the treatment). To characterize this type of cell death, DNA was extracted from cells treated with various concentrations of $\text{H}_2\text{O}_2$ and assayed by gel electrophoresis. Figure 2 shows the electrophoretic patterns of DNA from cells incubated for 6 h after the $\text{H}_2\text{O}_2$ treatment. For comparison, DNA obtained from the cells treated with a low concentration of $\text{H}_2\text{O}_2$ (0.2 mM) was also analyzed under the same conditions. DNA obtained from the cells treated with high concentrations of $\text{H}_2\text{O}_2$ (1 and 5 mM) showed electrophoretic patterns with a ladder structure. Since fragmentation of DNA at the nucleosome level is characteristic of apoptosis\textsuperscript{13}, the cell death which occurred before the cells divided following the $\text{H}_2\text{O}_2$ treatment was regarded as apoptotic. When surviving cells were examined for clonogenic ability, its loss was observed at low concentrations of $\text{H}_2\text{O}_2$ (0.06–0.2 mM). DNA from the cells treated with low concentrations of $\text{H}_2\text{O}_2$ remained intact with no evidence for the induction of apoptotic cell death.

To further characterize these types of cell death, the effects of various metabolic inhibitors on cell viability and DNA fragmentation were examined. Figure 3 shows the effects of the metabolic inhibitors cycloheximide, BAPTA-AM and PBN on cell viability. Cycloheximide is known as an inhibitor of protein synthesis\textsuperscript{17}. When 15 mM cycloheximide was added to the cell cultures at 1 h before the $\text{H}_2\text{O}_2$ treatment and incubated for 2 h during and after the $\text{H}_2\text{O}_2$ treatment, it largely protected against cytotoxicity of $\text{H}_2\text{O}_2$ as well as DNA fragmentation (Figs. 3 and 4), suggesting that the rapid cell death caused by the high concentration of $\text{H}_2\text{O}_2$ required protein synthesis. Since several endonucleases are known as enzymes responsible for the nucleosomal fragmentation of DNA\textsuperscript{16}, this result may be explained by the fact that these enzymes were newly synthesized after treatment with a high concentration of $\text{H}_2\text{O}_2$. When a selective intracellular $\text{Ca}^{2+}$ chelator, BAPTA-AM\textsuperscript{16,17}, was added to cells, which were then incubated for 1 h before treatment with $\text{H}_2\text{O}_2$, marked protection against both cell death and DNA fragmentation was observed (Figs.

![Figure 2](image-url)  
**Figure 2.** Agarose gel electrophoresis of DNA extracted from $\text{H}_2\text{O}_2$-treated cells. Cells were incubated for 6 h after the $\text{H}_2\text{O}_2$ treatment.
Figure 3. Viabilities of cells treated with a high concentration of \( \text{H}_2\text{O}_2 \) in the presence of various inhibitors as determined by their dye-exclusion ability. □: \( \text{H}_2\text{O}_2 \)-treated cells. ▲: Cells treated with \( \text{H}_2\text{O}_2 \) in the presence of 15 mM cycloheximide. The cells containing cycloheximide were pretreated for 1 h before \( \text{H}_2\text{O}_2 \)-treatment and further posttreated for 2 h. ◆: Cells treated with \( \text{H}_2\text{O}_2 \) and 5 μM BAPTA-AM. The cells were preincubated for 1 h together with BAPTA-AM before the \( \text{H}_2\text{O}_2 \) treatment. ○: Cells treated with \( \text{H}_2\text{O}_2 \) and 10 mM PBN. The cells were preincubated for 3 h together with PBN before the \( \text{H}_2\text{O}_2 \) treatment.

Figure 4. Agarose gel electrophoresis of DNA extracted from cells treated with both \( \text{H}_2\text{O}_2 \) and various inhibitors. The cells were incubated for 6 h after the \( \text{H}_2\text{O}_2 \) treatment until DNA extraction. The concentrations and incubation times in the presence of inhibitors were the same as those of Fig. 3.
When the cells were pretreated with 10 mM PBN for 3 h before incubation with H₂O₂, the surviving fractions of the cells increased more than those of the cells treated with H₂O₂ alone (Fig. 3). Furthermore, PBN protected against H₂O₂-induced fragmentation of DNA (Fig. 4). Since the lipophilic spin-trapping agent PBN has been reported to serve as an antioxidant, it is probable that PBN inhibited Ca²⁺ release from intracellular Ca²⁺ stores by protecting against lipid peroxidation in the organelle membrane. From these results, it was inferred that Ca²⁺, which was released from Ca²⁺ stores due to H₂O₂-induced membrane denaturation, triggered the signal-transduction pathways leading to the induction of endonuclease synthesis.

However, since a typical endonuclease inhibitor, the zinc ion, showed no significant effect on cell viability (data not shown), endonucleases responsible for the DNA fragmentation could not be precisely identified. A spot of DNA smaller than 50 bp was observed at the concentrations of H₂O₂ higher than 5 mM (Figs. 2 and 4). This could not be explained by the direct action of H₂O₂ on DNA, because metabolic inhibitors suppressed it (Fig. 4). It is not clear at present whether apoptotic fragmentation is associated with the appearance of this spot.

The effects of these modifiers on cell viability at low concentrations of H₂O₂ were also examined. As shown in Figure 5, BAPTA-AM and PBN showed protective effects against the cytotoxicity of H₂O₂, while cycloheximide and zinc ions showed no protective effects. In particular, intracellular Ca²⁺-chelation by BAPTA-AM completely inhibited the cytotoxicity of H₂O₂. It is noted that BAPTA, which does not permeate into cells and chelates extracellular Ca²⁺, had no effect on the viabilities of H₂O₂-treated cells. These results
meant that H$_2$O$_2$ could serve as a cytotoxic agent in the presence of intracellular Ca$^{2+}$. The protective effect of PBN was explained by its antioxidative property inhibiting Ca$^{2+}$ release caused by lipid peroxidation in the Ca$^{2+}$ store membrane. The lack of protective effects of cycloheximide and zinc ions against the cytotoxicity of H$_2$O$_2$ suggested that protein synthesis and endonuclease ability were not required for the cytotoxicity of the low concentration of H$_2$O$_2$.

The present study showed that though the loss of clonogenic ability was observed in Chinese hamster V79 cells exposed to low concentrations of H$_2$O$_2$ (0.06 ~ 0.2 mM), apoptotic cell death became observable only in the cells exposed to high concentrations of H$_2$O$_2$ (> 0.9 mM). Kondo has also reported that the mode of apoptosis is dose-dependent and that high concentrations of cytotoxic chemicals induce apoptosis in cells arrested at the G2 phase$^{22}$. It is noted that the apoptosis observed in this experiment was that before cell division and not a delayed type after several cell divisions$^{129}$. Quite different dose dependence in the mode of cell death was reported in leukemia cells$^{24}$. Low-level H$_2$O$_2$ exposure allows them to activate the apoptotic process, whereas higher levels of H$_2$O$_2$ induce the loss of clonogenic ability. From the fact that Chinese hamster fibroblasts were resistant to the induction of apoptosis by ionizing radiation, Warters suggested that radiation-induced apoptosis might be limited to cells previously programmed to respond to certain stimuli$^{25}$. Chinese hamster V79 cells were apoptotic resistant not only for ionizing radiation but also H$_2$O$_2$. However, the treatment of cells with a high concentration of H$_2$O$_2$ could induce apoptotic cell death. This is a difference between ionizing radiation and H$_2$O$_2$. When H$_2$O$_2$ is added to cultured cells, it is inferred that it reacts mainly with surface-membrane and cytoplasmic components and only a small fraction of H$_2$O$_2$ can react with nucleosomal DNA, while ionizing radiation produces equal amounts of OH radicals in both the cytoplasm and nucleus. Thus, a high dose of ionizing radiation may induce chromosomal damage leading to the loss of reproductive integrity with no apoptotic processes. The identification of particular DNA damage involved in the loss of reproductive integrity, as has been performed by spin-trapping damage in γ-irradiated DNA, could provide further insights into the mechanisms involved$^{26,27}$. The treatment of cells with a high concentration of H$_2$O$_2$ preferentially induces damage in cytoplasm. Accumulation of damage in the cytoplasm may prime apoptotic processes even in apoptotic-resistant cell lines.

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