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Induction of Apoptosis in HL-60 Cells by Photochemically Generated Hydroxyl Radicals

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Abstract—Reactive oxygen species (ROS), especially hydroxyl radicals are postulated to mediate apoptosis of the cell. Here we demonstrate that hydroxyl radicals generated selectively by photolysis of a photo-Fenton reagent, *N*,*N*′-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthaldiimide (NP-III), induce apoptosis in HL-60 (human promyelocytic leukemia) cells involving the activation of caspase-3. © 2002 Elsevier Science Ltd. All rights reserved.

Recently a great advancement has been achieved in signaling systems of apoptosis. Among cellular processes, a large body of evidence has accumulated to suggest that reactive oxygen species (ROS) play a key role as a common mediator of apoptosis.[1,2] It is well documented that hydrogen peroxide serves as a mediator of apoptosis[1–4] and that hydrogen peroxide itself induces apoptosis in many types of cells, including HL-60 cells.[5] Hydroxyl radicals, assumed to be easily generated in the cell by Fenton and/or Haber–Weiss reactions, have been suggested as a cause of apoptosis in many studies.[1–4] However, no direct experimental evidence has been presented to indicate that the hydroxyl radical itself causes apoptosis. To clarify this point, a specific method to generate hydroxyl radicals is necessary. Recently it has been established that *N*,*N*′-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthaldiimide (NP-III)[6] selectively generates hydroxyl radicals on UVA irradiation in the quantum yield of 0.03.[7] NP-III oxidizes DNA[6] and protein[8,9] and also causes DNA damage[10] in V79 cells and growth inhibition[11] of *Acanthamoeba* by photochemical generation of hydroxyl radical. In this study, we demonstrate that photochemically generated hydroxyl radicals using NP-III cause apoptosis in HL-60 (human promyelocytic leukemia) cells involving the activation of caspase-3, which is specifically activated during apoptosis.[12]

HL-60 cells were suspended in a dish at 1.0×10^6 cells/mL in RPMI 1640 medium without fetal bovine serum. After the addition of NP-III (0.1 mM) dissolved in 25% acetonitrile to a final concentration of 1 μM (therefore, final concentration of acetonitrile was 0.25%), the cells in the dish were photoirradiated (366 nm) with a trans-illuminator (Model UVGL-58, UVP Inc., San Gabriel, CA, USA) at a distance of 5 cm for 15 min. The photoirradiated cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 5 h. A typical apoptosis was induced (Fig. 1 A). The DNA ladder was clearly observed in these cells (Fig. 1C, lane 2). The percentage of apoptotic cells was $60.8 \pm 3.0\%$ ($n = 11$), which was significantly ($p < 0.0001$) larger than that in the control experiment described below and approximately corresponded to that of HL-60 cells treated with 100 μM of hydrogen peroxide.[5] In this study, data were expressed as means ±SE and analyzed by ANOVA using StatView software (Abacus Concepts, Berkeley, CA, USA). Differences between group means were analyzed using Bonferroni/Dunn (Dunn's procedure as a multiple comparison procedure) generated by this program. Differences were considered significant at $p < 0.05$.

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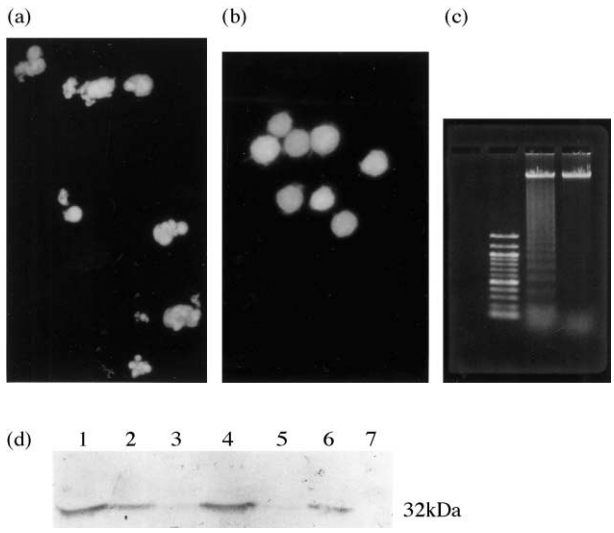

Figure 1. Changes in HL-60 cells by photochemical treatment with NP-III. The cells were pelleted by centrifugation at 600*g* for 2 min and re-suspended at 1.0×10^6 cells/mL in RPMI 1640 medium without FBS. After the addition of NP-III (0.1 mM) dissolved in 25% acetonitrile to a final concentration of 1 μM, the cells in the dish were photoirradiated with a transilluminator (366 nm) at a distance of 5 cm for 15 min. Then cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 5 h. After fixation and staining with Hoechst 33258, a photograph was taken using a Nikon TMD300 fluorescence microscope. (B) Photograph of the control cells (NP-III added but not irradiated). (C) DNA ladder by agarose gel electrophoresis (lane 1: DNA marker, lane 2: cells irradiated with NP-III, lane 3: the control cells). (D) Immunoblotting with anti-human procaspase-3 (lane 1: normal HL-60 cells, lane 2: control cells after 2 h, lane 3: 2 h after the irradiation with NP-III, lane 4: control cells after 3 h, lane 5: 3 h after irradiation with NP-III, lane 6: control cells after 4 h, lane 7: 4 h after irradiation with NP-III.

Control experiment was carried out by covering a dish with aluminium foil to interrupt UV light in the presence of NP-III. The rate of apoptosis in this control experiment after a similar treatment was $24.5 \pm 2.6\%$ ($n = 11$) (Fig. 1B), and much slighter DNA ladder was observed compared to the photoirradiated cells at the same cell number (Fig. 1C, lane 3). The apoptotic rate of cells irradiated without NP-III but in the presence of acetonitrile which was used to dissolve NP-III was [$25.7 \pm 2.5\%$ ($n = 9$)], which was not significantly different from that of the control run. The rate of apoptosis in cells which were not irradiated in the presence of acetonitrile was [$15.3 \pm 2.6\%$ ($n = 7$)], which was also not significantly different from that of the control run. These results indicate that a relatively high apoptotic rate in the control experiment was due to acetonitrile. Based on these results, it is concluded that hydroxyl radicals photochemically generated from NP-III cause apoptosis in HL-60 cells, because it is well established that NP-III selectively generates hydroxyl radicals on UVA irradiation.[6,7]

The activity of caspase-3 was determined as previously described[4] using a specific peptide substrate acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide) (DEVD-MCA). After 4 h of UV irradiation in the presence of NP-III, the activity of caspase-3 increased significantly to 0.79 ± 0.25 U (1 U of the enzyme activity was defined as 1 pmol 7-amino-4-methylcoumarin liberated/10^4, cells/min) while that for the control cells after 4 h was 0.33 ± 0.11 U.

Western blot analysis showed that procaspase-3 (32 kDa) was activated and had decreased at 2 h after irradiation (Fig. 1D, lane 3) compared to HL-60 cells before irradiation (Fig. 1D, lane 1), while procaspase-3 in the control cells in the same reaction conditions remained (Fig. 1D, lane 2). Similar results were obtained at 3 and 4 h after photoirradiation (Fig. 1D, lanes 4–7). The procaspase-3 remained in the control reaction even after 4 h (Fig. 1D, lane 6). These results indicate that hydroxyl radicals cause apoptosis in HL-60 cells involving the activation of caspase-3 more effectively than hydrogen peroxide,[5] by at least two orders of magnitude.

In conclusion, we presented the first confirmed evidence that hydroxyl radicals generated selectively by photolysis of a photo-Fenton reagent, *N*,*N*′-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthaldiimide (NP-III), induce apoptosis in HL-60 cells involving the activation of caspase-3.

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