Research Article

Hydrogen peroxide and hydroxyl radical involvement in the activation of caspase-3 in chemically induced apoptosis of HL-60 cells


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Abstract. Apoptosis of HL-60 cells induced by actinomycin D, H7, or daunorubicin was shown to involve the activation of caspase-3-like protease, 2 h after the addition of these drugs, based on microassay of enzyme activity by high-performance liquid chromatography. Catalase and a spin trap, N-t-butyl-α-phenylnitrone, which effectively inhibited the apoptosis induced by these drugs, also inhibited the activation of caspase-3-like protease. These results suggest that hydrogen peroxide and the hydroxyl radical are common mediators of caspase-3 activation caused by these chemicals, with apparently different functional mechanisms. Based on mitochondrial activity determined by oxygen consumption, complexes I, II, and IV were inhibited by actinomycin D. H7 inhibited complexes I and IV, 1 and 1.5 h respectively, after the addition of the drug to HL-60 cells. Daunorubicin inhibited complex IV, 1.5 h after the addition of the drug to HL-60 cells. Inhibition of complex IV by actinomycin D, H7, and daunorubicin were almost fully restored by the addition of cytochrome c. The release to the cytosol of cytochrome c by these drugs was also demonstrated by Western blot analysis. Addition of catalase inhibited the depression of complex IV activity induced by actinomycin D and H7. These observations indicate a direct relationship between hydrogen peroxide and the release of cytochrome c during apoptosis caused by actinomycin D, H7, and daunorubicin.

Key words. Apoptosis; caspase-3; hydroxyl radical; HL-60; actinomycin D; hydrogen peroxide.

Since the first appearance of the term ‘apoptosis’ [1], great advances have been achieved in understanding the signalling systems of apoptosis [2], and a large body of evidence has accumulated to suggest that reactive oxygen species (ROS) play a key role as common mediators of apoptosis [3–5]. However, many problems remain to be clarified, for example, identifying the kind of inducers that use ROS as intracellular messengers of apoptosis, where, when, and how they are generated, and the mechanism by which ROS cause apoptosis. ROS have been suggested [6–7] to cause a mitochondrial permeability transition resulting in the release of caspase activators such as apoptosis-inducing factor [8] and cytochrome c [9]. However, Simizu et al. [10] recently reported that caspase-3 mediated the production of hydrogen peroxide in the apoptosis of human lung carcinoma Ms-1 cells induced by tyrosine kinase inhibitor and anticancer drugs [11]. The relationship between ROS and the activation of caspase is not well elucidated. Recently, we reported [12] that apoptosis of HL-60 cells caused by actinomycin D, H7, or daunorubicin was significantly inhibited by catalase and a spin trap, N-t-butyl-α-
phenylnitroline (PBN). These results suggest that hydrogen peroxide and the hydroxyl radical are common mediators of the apoptosis caused by these chemicals with apparently different functional mechanisms. In this report, we examined whether apoptosis caused by these drugs involves the activation of caspase-3 [13], and the involvement of ROS in the activation of the protease.

**Materials and methods**

RPMI 1640 medium (No. 22400), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from GIBCO BRL, Life Science Technologies. Actinomycin D, H7, daunorubicin, and the spin trap PBN were supplied by Sigma. Acetyl-Asp-Glu-Val-Asp-CHO (DEVD-CHO) and acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide) (DEVD-MCA) were purchased from the Peptide Institute. All other reagents were purchased from Nacalai Tesque. Monoclonal anti-human cytochrome c antibody (clone 7H8.2C12) was purchased from Genzyme/Techno and goat anti-mouse IgG-HRP conjugate was from Santa Cruz Biotechnology.

**Cell culture conditions**

The human promyelocytic HL-60 leukemic cells were purchased from the Japan Health Sciences Foundation. Cells were cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated FBS, penicillin (50 U/ml) and streptomycin (50 μg/ml) as described elsewhere [12]. The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

**Drug-induced apoptosis**

Cells were pelleted by centrifugation at 600 × g for 2 min and resuspended at 1.0 × 10⁶ cells/ml in RPMI 1640 medium without FBS. After the addition of actinomycin D dissolved in a mixture of ethylene glycol and ethanol (9:1) to a final concentration of 10 μg/ml [12], an aqueous solution of H7 (50 μM) [12], or daunorubicin (5 μM) [12], the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ for 2, 4, and 6 h.

**Microassay of caspase-3-like protease activity**

Zero, 2, 4, and 6 h after the addition of an inducer, a 2-ml aliquot (2.0 × 10⁶ cells) was taken and mixed with 2 ml of 100 mM Tris-HCl buffer at pH 7.5 containing 0.4% Triton X-100. After keeping on an ice bath for 30 min, the mixture was centrifuged at 15,000 × g for 5 min. To 1 ml of the supernatant were added 790 μl of a solution at pH 7.5 containing 100 mM of Tris-HCl, 2 mM of EDTA, 20 mM of EGTA, 200 μl of 10 mM dithiothreitol, and 10 μl of 10 mM DEVD-MCA solution. The resulting mixture was incubated at 37°C. After 0 and 30 min, a 190-μl aliquot was taken, and the reaction was terminated by the addition of 10 μl of 5% HClO₄. After centrifugation, the supernatant (10 μl) was directly applied for high-performance liquid chromatography (HPLC) analysis to determine the release 7-amino-4-methylcoumarin (AMC). A reversed-phase column (μ-Bondasphere 5-μm C₁₈, 100A, 3.9 × 150 mm; Waters) was eluted with a 1:4 mixture of acetonitrile and water containing 0.1% H₃PO₄ at a flow rate of 1.0 ml/min. Detection was made with a fluorescence detector (type RF-10AXL; Shimadzu) using excitation at 380 nm and emission at 460 nm. The caspase-3 reaction of the cells was confirmed as being linear at least for 1 h in that condition. Calibration was made by HPLC analysis using AMC solutions of a series of concentrations. The concentration range of AMC was chosen to give a linear relationship between the peak area and the AMC concentration. One unit (U) of enzyme activity was defined as 1 pmol AMC liberated/10⁴ cells per minute.

**Determination of apoptotic rate**

Apoptotic cells were counted using a Nikon TMD300 fluorescence microscope after fixation and staining with Hoechst 33258 as described previously [12].

**Inhibition experiments**

Catalase (175 U/ml) was dissolved in RPMI 1640 medium, and cells were suspended in the resulting solution. The apoptosis inducer was added to the suspension and incubated at 37°C in a humidified atmosphere of 5% CO₂ for 6 h. After the addition of inducers, a cell bottle was swirled gently for 1 min every 10 min during the first h and every 30 min thereafter [12]. PBN was dissolved in ethanol and added to the cell suspension at a final concentration of 20 mM. The final concentration of ethanol was 0.25%. The addition of PBN was followed by a 30-min preincubation at 37°C for 30 min in a humidified atmosphere of 5% CO₂, and then inducers were added.

**Determination of mitochondrial activities**

Oxygen consumption was measured using a Clark-type oxygen electrode and a Rank Brothers polarograph with a thermostatted chamber at 37°C under constant stirring. The determination was essentially made as described previously [14]. Apoptosis was induced as described above. At the times indicated, a 30-ml aliquot was withdrawn, centrifuged, and washed twice with respiration buffer (0.25 M sucrose, 0.1% bovine serum albumin, 10 mM MgCl₂, 10 mM K⁺Hepes, 5 mM KH₂PO₄, pH 7.2). Cells were suspended in 2 ml of the respiration buffer at a final concentration of 1.5 × 10⁷ cells/ml and injected into a chamber. Oxygen consumption was measured with the sequential addition of substrates and inhibitors in the following order and final concentrations: ADP-K, 1 mM; malate, 50 mM; digitonin, 0.005%;