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Involvement of Hydrogen Peroxide and Hydroxyl Radical in Chemically Induced Apoptosis of HL-60 Cells

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ABSTRACT. Effects of three kinds of antagonists against reactive oxygen species were evaluated at the same time in chemically induced apoptosis of human leukemic HL-60 cells. Apoptosis of HL-60 cells induced by actinomycin D, H7, 1-β-D-arabinofuranosylcytosine, and daunorubicin was inhibited significantly by radical scavengers (vitamin E, *N*-acetyl-L-cysteine, and mercaptoethanol), catalase, and a spin trap, *N*-*t*-butyl-α-phenylnitrone. These results suggest that hydrogen peroxide and hydroxyl radical are common mediators of apoptosis caused by these chemicals with apparently different functional mechanisms. The consumption of vitamin E to inhibit apoptosis induced by actinomycin D was undetectable, suggesting that the generation of reactive oxygen species during apoptosis was not very extensive. Radicals were suggested to be a mediator of apoptosis of HL-60 cells induced by cisplatin based on the observations that the above inhibitors, except catalase, effectively inhibited apoptosis by the drug. BIOCHEM PHARMACOL **57**;12:1361–1365, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. apoptosis; HL-60 cell; hydrogen peroxide; hydroxyl radical; actinomycin D; H7

Since the first appearance of the term "apoptosis" [1], a great advancement has been achieved in understanding the signaling systems of apoptosis [2]. Among cellular processes, a large body of evidence has accumulated to suggest that oxidative stress plays a key role as a common mediator of apoptosis [3–5]. However, many problems remain to be clarified, for example, what kinds of inducers use ROS‖ as an intracellular messenger of apoptosis, where, when, and how ROS are generated, the chemical nature of ROS, and the quantity of ROS.

To approach the role of ROS, we have evaluated the effect of ROS antagonists on apoptosis of a human promyelocytic leukemia cell line, HL-60, which is one of the most used cell lines in the study of apoptosis. Apoptosis was induced by drugs that had been established to cause apoptosis in HL-60 cells [6–8]. Thus far, fragmentary results have been reported on inhibition of apoptosis of HL-60 cells by antioxidants. However, in this study, we evaluated simultaneously the effects of ROS antagonists with different mechanisms, antioxidants, catalase, and a spin trap reagent, on chemically induced apoptosis of the HL-60 cell line. As a result, hydrogen peroxide and hydroxyl radical are suggested to be mediators of apoptosis.

MATERIALS AND METHODS

Materials

RPMI 1640 medium (No. 22400), FBS, and penicillin-streptomycin were purchased from GIBCO BRL, Life Science Technologies, Inc. Actinomycin D, H7, cisplatin, Ara-C, daunorubicin, 3-amino-1,2,4-triazole, and the spin trap PBN were supplied by the Sigma Chemical Co. All other reagents were purchased from the Nacalai Tesque Co.

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 [8]. Cells were maintained at 37° in a humidified 5% CO_2 atmosphere.

Determination of Apoptotic Rate

Apoptotic cells were counted using a Nikon TMD300 fluorescence microscope after fixation and staining with Hoechst 33258 as described previously [9].

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‖ *Abbreviations:* Ara-C, 1-β-D-arabinofuranosylcytosine; DMPO, 5,5-dimethy-1-pyrroline *N*-oxide; FBS, fetal bovine serum; NAC, *N*-acetyl-L-cysteine; PBN, *N*-*t*-butyl-α-phenylnitrone; and ROS, reactive oxygen species.

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TABLE 1. Effects of inhibitors on chemically induced apoptosis in HL-60 cells

	% Apoptotic cells					
Inhibitors	**Actinomycin D (10 μg/mL)**	**H7 (50 μM)**	**Ara-C (25 μM)**	**Daunorubicin (5 μM)**	**Cisplatin (50 μM)**	**None**
None	96.6 ± 6.8 (0)	74.0 ± 14.1 (0)	32.8 ± 12.5 (0)	88.4 ± 2.5 (0)	35.0 ± 7.8 (0)	9.1 ± 1.7
Vitamin E (1 mM)	14.5 ± 6.1** (88.2)	16.8 ± 3.5** (80.3)	8.4 ±2.4** (81.4)	25.9 ± 13.1** (72.4)	7.5 ± 2.9** (86.5)	4.0 ± 2.6
NAC (1 mM)	13.8 ± 3.9** (91.9)	14.5 ± 3.7** (87.8)	11.2 ± 0.6** (80.6)	38.9 ± 2.7** (59.3)	14.6 ± 4.5** (69.1)	6.6 ± 4.0
2-Mercaptoethanol (1 mM)	37.3 ± 11.6** (35.2)	6.8 ± 0.8** (98.6)	4.8 ± 1.8** (105)	17.0 ± 1.5** (82.2)	5.9 ± 1.4** (100)	5.9 ± 2.2
Catalase (175 U/mL)	30.5 ± 16.0** (68.0)	22.9 ± 3.9** (67.8)	9.6 ± 1.7** (67.9)	27.3 ± 3.3** (68.1)	26.6 ± 5.3 (5.0)	2.0 ± 0.2*
PBN (20 mM)	37.1 ± 13.9** (68.3)	14.5 ± 5.2** (91.2)	10.2 ± 3.0** (94.1)	8.7 ± 5.1** (100)	16.9 ± 5.4* (68.7)	8.8 ± 1.1

Cells were treated with inducers of apoptosis in the presence or absence of inhibitors in RPMI 1640 medium at 37° in a humidified atmosphere of 5% CO_2 for 6 hr. The percentage of apoptotic cells was determined as described in the text. Values are means ± SD of 3 or 4 independent runs. Asterisks indicate significant difference from the corresponding control run, which was made in the absence of inhibitors (ANOVA Bonferroni/Dunn procedure, $*P < 0.05$ and $**P < 0.01$). In parentheses is shown the inhibition percentage of apoptosis calculated as follows:

100 × [{(% apoptosis by the inducer)—(% apoptosis in the presence of the vehicle of the inducer)}—{(% apoptosis in the presence of both the inducer and the inhibitor)—(% apoptosis in the presence of the inhibitor)}]/{(% apoptosis by the inducer)—(% apoptosis in the presence of the vehicle of the inducer)}.

Drug-Induced Apoptosis

Cells were pelleted by centrifugation at 600 *g* for 2 min and re-suspended at 1.5×10^6 cells/mL in RPMI 1640 medium without FBS. After the addition of drug—actinomycin D, dissolved in a 9:1 mixture of ethylene glycol and ethanol, to a final concentration of 10 μg/mL [8], or aqueous solutions of H7 (50 μM) [6], cisplatin (50 μM) [6], Ara-C (25 μM) [6], or daunorubicin (5 μM) [7]—cells were treated at 37° in a humidified atmosphere of 5% CO_2 for 6 hr.

Inhibition Experiments

NAC (1 mM), 2-mercaptoethanol (1 mM), or catalase (175 U/mL) was dissolved in RPMI 1640 medium, and cells were suspended in the resulting solution. To the suspension, apoptosis inducer was added and incubated at 37° in a humidified atmosphere of 5% CO_2 for 6 hr. PBN was dissolved in ethanol and added to the cell suspension at the final concentration of 20 mM. The final concentration of ethanol was 0.25%. After the addition of PBN, preincubation was done at 37° for 30 min in a humidified atmosphere of 5% CO_2, and then inducers were added.

For the experiment with catalase, after the addition of inducers, a cell bottle was swirled gently for 1 min every 10 min during the first hour and every 30 min thereafter.

α-Tocopherol dissolved in ethanol at the final concentration of 1 mM was added to cultured cells. The concentration of ethanol was 0.5%, which did not affect the viability of the cells. After the addition, cells were preincubated in the presence of FBS for 48 hr. Cells were collected by centrifugation at 600 *g* for 2 min, washed twice with RPMI 1640 medium, and used for inhibition experiments. The concentration of α-tocopherol in the cells was determined by HPLC as described [10].

Data were expressed as means ± SD for at least three independent runs and analyzed by ANOVA using StatView software (Abacus Concepts). 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 $P < 0.05$.

RESULTS AND DISCUSSION

Apoptosis of HL-60 Cells Induced by Actinomycin D

It is well documented that actinomycin D causes apoptosis in HL-60 cells [8, 11, 12]. In the present experiment, vitamin E significantly inhibited apoptosis of HL-60 cells induced by this chemical (Table 1). The inhibition percentage by vitamin E was calculated as 100 × [{96.6 (% of apoptotic cells by the inducer) − 7.4 (% of apoptotic cells in the presence of the vehicle (a mixture of ethylene glycol and ethanol) of actinomycin D, i.e. spontaneous apoptosis)} − {14.5 (% of apoptotic cells in the presence of both the inducer and vitamin E) − 4.0 (% of apoptotic cells in the presence of the inhibitor)}]/{96.6 (% of apoptotic cells by the inducer) − 7.4 (% of apoptotic cells in the presence of the vehicle of the inducer)} = 88.2%. Other radical scavengers such as NAC and 2-mercaptoethanol significantly inhibited apoptosis induced by actinomycin D, and the inhibition percentage was calculated to be 91.9 and 35.2%, respectively (Table 1). These results suggest that radical reaction is involved in the apoptotic process caused by the drug. Cotter and coworkers [8] reported that an antioxidant, butylated hydroxyanisole, inhibited apoptosis of HL-60 cells induced by actinomycin D.

To shed more light on the nature of ROS, catalase was used as an inhibitor, and the enzyme was found to inhibit

apoptosis effectively (Table 1). In the presence of 20 mM 3-amino-1,2,4-triazole, a specific inhibitor of catalase, the inhibitory effect of catalase on apoptosis caused by actinomycin D was totally abolished. These observations support a view that hydrogen peroxide is involved in the process leading to apoptosis, because catalase is a specific enzyme to decompose hydrogen peroxide.

Catalase is reported to inhibit apoptosis of HL-60 cells induced by UV irradiation and low concentrations of camptothecin, etoposide, and melphalan [13]. It is reported that hydrogen peroxide is highly diffusible, and horizontal agitation is necessary to determine its steady-state concentration during thymocyte apoptosis [14]. Indeed, occasional swirlings of the bottle in the inhibition experiment using catalase were necessary to obtain reproducible results. It may be worthwhile to note that among the inhibitors, only catalase caused a significant reduction in spontaneous apoptosis, to 2.0 ± 0.2% compared with the control (9.1 ± 1.7%), which was made by the addition of RPMI 1640 medium, the vehicle of all inhibitors except PBN (Table 1).

A spin trap, PBN, at 20 mM significantly inhibited apoptosis caused by actinomycin D (Table 1). This result suggests that hydroxyl radicals are involved in the process leading to apoptosis, since hydrogen peroxide is suggested to be a mediator as described above, and it easily generates hydroxyl radical by a Fenton-type reaction. Effective inhibition of apoptosis of HL-60 cells induced by actinomycin D utilizing dithiocarbamates as a metal chelator [8] supports the involvement of metals causing a Fenton reaction. Cotter and colleagues [12] reported that a spin trap, DMPO, did not inhibit apoptosis of HL-60 cells caused by actinomycin D. This observation may be explained on the grounds that the concentration of DMPO used by those authors is 10 mM, which may not be sufficient to trap radicals, because a relatively high concentration of trapping reagents is necessary to trap radicals.

To evaluate the quantity of radicals produced, the consumption of vitamin E during apoptosis was determined. Vitamin E was enriched by treatment of the cells for 48 hr in medium containing 1 mM of the vitamin. The concentration of vitamin E increased from 0.57 ± 0.20 pmol/10^6 cells to 1.20 ± 0.38 nmol/10^6 cells. Six hours after the addition of actinomycin D, the concentration of vitamin E in the enriched cells was 1.43 ± 0.44 nmol/10^6 cells, which was not significantly different from either the initial value (1.20 ± 0.38 nmol/10^6 cells, as described above) or that of the control cells (1.52 ± 0.59 nmol/10^6 cells) treated for 6 hr in the absence of the inducer. These observations suggest that the generation of ROS and the resulting lipid peroxidation during apoptosis was not very extensive, and therefore the consumption of vitamin E was of an undetectable amount, considering that vitamin E scavenged almost all ROS to inhibit apoptosis effectively under the present conditions. This result is consistent with the report that low concentrations of hydrogen peroxide induce apoptosis, whereas necrosis occurs when millimolar amounts of the oxidant are present [15].

Apoptosis of HL-60 Cells Induced by H7

H7, an inhibitor of protein kinase C, is reported to cause apoptosis in HL-60 cells [6, 16], although the mechanism has not been clarified. To our knowledge, this is the first report that has examined effects of anti-ROS reagents on apoptosis of HL-60 cells caused by H7. The vehicle of H7 was RPMI 1640 medium, and the spontaneous apoptotic rate after 6 hr in the absence of H7 was 9.1 ± 1.7%. This value was common to Ara-C, daunorubicin, and cisplatin (Table 1). Apoptosis caused by H7 was inhibited significantly by radical scavengers (vitamin E, NAC, and mercaptoethanol), catalase, and PBN just as in the case of actinomycin D, and inhibition rates were 80.3, 87.8, 98.6, 67.8, and 91.2%, respectively (Table 1). These results suggest that hydrogen peroxide and hydroxyl radical also are involved in the apoptosis induced by H7, although the function of H7 apparently is different from that of actinomycin D.

Apoptosis of HL-60 Cells Induced by Ara-C

Ara-C is one of the most effective agents in the treatment of acute myelogenous leukemia, and incorporation of the drug into DNA is associated with its toxicity, including inhibition of DNA synthesis [17]. Ara-C is reported to cause apoptosis in HL-60 cells [18–21] by a protein kinase C-related mechanism [20, 21]. Present experiments demonstrate that apoptosis induced by Ara-C was inhibited significantly by radical scavengers (vitamin E, NAC, and mercaptoethanol), catalase, and PBN just as in the case of actinomycin D and H7 (Table 1). These results suggest that hydrogen peroxide and hydroxyl radical also were involved in apoptosis induced by Ara-C. The present report is also the first to examine systematically the effects of anti-ROS reagents on apoptosis of HL-60 cells caused by Ara-C. Previous reports describing the inhibition of apoptosis of leukemia cells induced by the drug using metal chelators [18] such as pyrrolidine dithiocarbamate, *o*-phenanthroline, and NAC [22] support the conclusion. ROS generation by Ara-C in OCI/AML-2 cells has been reported [23].

Apoptosis of HL-60 Cells Induced by Daunorubicin

The anthracycline daunorubicin is widely used in the treatment of acute myeloid leukemia and is reported to cause apoptosis in HL-60 cells [7, 24–27] through ceramide generation [7, 24] and activation of NFκB, which may involve ROS [25]. In the present experiment, apoptosis induced by daunorubicin was also inhibited significantly by radical scavengers (vitamin E, NAC, and mercaptoethanol), catalase, and PBN, just as in the case of actinomycin D, H7, and Ara-C (Table 1). Particularly, PBN completely inhibited apoptosis induced by the drug. This result is consistent with the reports that daunorubicin-induced apoptosis of HL-60 cells is inhibited by antioxidants such as *N*,*N*′-diphenyl-*p*-phenylene diamine [26] and NAC [27], as

well as metal chelators such as desferal [25, 26] and pyrrolidine dithiocarbamate [25]. Formations of superoxide anion, hydrogen peroxide, and hydroxyl radical by redox cycling of daunorubicin in the presence of rat tissue nuclei [28] and bovine heart mitochondria [29, 30] have been well documented. These results support a conclusion that hydrogen peroxide and hydroxyl radical are also mediators of apoptosis induced by daunorubicin.

Apoptosis of HL-60 Cells Induced by Cisplatin

The cytotoxicity of cisplatin is believed to be due to the formation of DNA adducts, which include DNA-protein cross-links, DNA monoadducts, and interstrand and intrastrand DNA cross-links [31]. Cisplatin is reported to induce apoptosis in HL-60 cells [6]. Apoptosis of HL-60 cells caused by cisplatin was inhibited significantly by vitamin E, NAC, mercaptoethanol, and PBN (Table 1), suggesting radicals as a mediator of apoptosis. This assumption is supported by the report by Kim *et al.* [32] demonstrating that ROS is involved in cell injury of rabbit renal cortical slices based on its inhibition by radical scavengers and iron chelator. They also reported [32] that catalase did not affect the cell damage induced by the drug, consistently with our present observation (Table 1). The ineffective inhibition by catalase may be explained on the grounds that cisplatin does not generate hydrogen peroxide, or that cisplatin directly interacts with DNA to form adducts, and therefore exogenous catalase cannot decompose effectively any hydrogen peroxide generated in the vicinity of DNA. It is reported that cisplatin generates superoxide anion with DNA in a cell-free system [33]. Recently Ishibashi and Lippard [34] reported that cisplatin caused telomere loss and triggered apoptosis in HeLa cells. This result also suggests that the action of cisplatin is localized in the vicinity of DNA.

In summary, hydrogen peroxide and hydroxyl radical are suggested to be common mediators in apoptosis of HL-60 cells caused by drugs with apparently different action mechanisms, such as actinomycin D, H7, Ara-C, and daunorubicin, based on inhibition experiments simultaneously using three kinds of anti-ROS reagents, antioxidants, catalase, and a spin trap. Radicals were suggested to be a mediator of apoptosis of HL-60 cells induced by cisplatin based on observations that the above inhibitors, except catalase, effectively inhibited apoptosis by the drug.

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