

Induction and mechanism of apoptotic cell death by propofol in HL-60 cells

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Background: Apoptosis (programmed cell death) occurs in various physiological and pathological conditions, exhibits a characteristic mechanism of intracellular sequential reaction and may be involved in determining clinical outcome. The antioxidant activity of propofol (2,6-diisopropylphenol) together with the stimulating effect of protein kinase C suggests that propofol might have the potential to modulate apoptosis. Thus, it is of both clinical interest and biomedical importance to investigate and clarify the effect and mechanism of propofol upon the intracellular reactions underlying apoptotic cell death.

Methods: The effect of propofol on apoptosis was investigated using cultured human promyelocytic leukemia HL-60 cells. This well-characterized cell line is useful for the study of apoptosis because the various biochemical steps occurring during apoptosis have been well documented.

Results: Treatment of HL-60 cells with propofol resulted in growth inhibition with the formation of apoptotic bodies in a concentration-dependent manner. DNA fragmentation and ladder formation was also observed in a concentration-dependent

manner. Propofol treatment resulted in activation of caspase-3, -6, -8 and -9, thereby suggesting that cell surface death receptor activation of the caspase cascade mediates propofol-induced apoptosis with consequent formation of the cleaved product of Bid (a pro-apoptotic Bcl-2 family member protein) and activation of the mitochondrial pathway with cytosolic release of cytochrome c.

Conclusion: Propofol may induce apoptosis, which is dependent on the mechanism that activates both the cell surface death receptor pathway and the mitochondrial pathway.

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IN recent years, apoptosis and necrosis, two fundamentally different forms of cell death, have been defined in terms of their incidence, morphology and biochemistry (1). Apoptosis (programmed cell death) is an active form of cell death observed in various physiological and pathological scenarios and may play a role in determining clinical outcome (1). Therefore clinicians are interested in the results of on-going efforts to clarify the mechanism and modulation of this mode of cell death. Recent studies have indicated that apoptosis is characterized by the initiation of an intracellular cascade of biochemical reactions that culminate in the activation of caspase-3 and other caspases, resulting in the proteolytic cleavage of myriad substrates including lamins, α -fodrin, poly(ADP-ribose)-polymerase and the inhibitor of the caspase-activated DNase, ICAD (2–5). Based on these findings, we have found that the potency of drugs to induce apoptosis is closely related to their ability to modulate the activity of these intracellular events. For example, dibu-

caine activates caspase-3, -6, -8 and -9 and thereby accelerates the apoptotic process (6).

Propofol (2,6-diisopropylphenol) is an anesthetic agent and possesses potent antioxidant activity similar to tocopherols (7,8) as well as stimulating protein kinase C (9). As the anesthetic potency of propofol is not strong, it is used at a relatively higher range of concentration. These pharmacological properties, which are uniquely different from those of inhalational anesthetic agents, may exert significant effects upon various intracellular apoptotic death processes (10–13). Recently, it has been reported that propofol might trigger neurodegeneration in neurons during development (14) and it has also been reported that its derivative may affect neuronal injury, including apoptotic cell death (15). Therefore, it is conceivable that propofol may modulate apoptosis.

Propofol is usually used for surgery, in which the human body receives serious invasive stress. These life-threatening stresses may be processed to further biologi-

cal response through the modification of apoptosis of cells participating in host defence such as neutrophils, monocytes, lymphocytes, etc. (16–18). Thus, the effect of propofol on the apoptosis of these cells may well be clinically important. However, Delogu *et al.* reported that apoptosis of neutrophils was accelerated after elective surgery (19), while Fanning *et al.* reported inhibition of neutrophil apoptosis (20). The discrepancy of these reports suggests that many factors, such as host conditions and cell types, concerned might interact in a complex manner in the apoptosis process. Investigating propofol under such a complicated *in vivo* condition might confound data interpretation. Thus, in order to better understand the basic property of propofol regarding apoptosis, we performed investigations under controlled and *in vitro* conditions. According to this investigative strategy, we aimed to study the effect of propofol on apoptosis using cultured promyelocytic leukemia HL-60 cells. The cell line is one of the characterized and standardized cell lines for apoptosis study in which the various biochemical steps of apoptosis have been well elucidated and also maintains some properties of neutrophils and monocytes (21,22). Furthermore, the simpler conditions facilitated the detailed investigation of intracellular apoptotic molecular reactions induced by propofol, which was another important aim of the present study.

Materials and methods

Chemicals

Ribonuclease (RNase) A, proteinase K and cytochrome c were obtained from Sigma Chemical Co. (St. Louis, MO). Various fluorogenic tetrapeptide substrates including Ac-DEVD-MCA (acetyl-Asp-Glu-Val-Asp-MCA) for caspase-3, Ac-YVAD-MCA for caspase-1 and -4, Ac-VEID-MCA for caspase-6, Ac-IETD-MCA for caspase-8 and Ac-LEHD-MCA for caspase-9 were obtained from the Peptide Institute (Osaka, Japan). Anti-Bid antibody and anticypochrome c antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and PharMingen (San Diego, CA, USA), respectively. Diprivan™ (medical product of propofol) and 2,6-diisopropylphenol (propofol) were obtained from Astra-Zeneca (Osaka, Japan) and Aldrich Chem. Co. (Milwaukee, WI), respectively. All other chemicals were of analytical grade and obtained from Nacalai Tesque (Kyoto, Japan).

Cell line

Dr Saito (Jichi Medical University) kindly donated the human promyelocytic leukemia cell line HL-60. The cells were maintained at $0.2\text{--}1 \times 10^6$ cells/ml in RPMI

(Roswell Park Memorial Institute) 1640 medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Co. Carlsbad, CA, USA), 100 U/ml of penicillin and 100 µg/ml of streptomycin. The cells were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO₂/95% air. The cells were used in experiments during the exponential phase of growth. They were routinely counted to maintain a low population density and assayed for viability using the trypan blue exclusion method. The HL-60 cells (3×10^5 cells) were generally treated in 1.5 ml of RPMI medium containing 10% fetal bovine serum and various reagents and then incubated in an O₂/CO₂ culture incubator (BNP-110, SanRel Co., Tokyo, Japan). Preincubation was normally performed for at least 1 h before adding the reagents.

Experiments were repeated at least five times and data are expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed using the analysis of variance (ANOVA) test with repeated measurements. A *P*-value of less than 0.05 was considered statistically significant.

Analysis of DNA fragmentation

The extent of DNA fragmentation was determined spectrophotometrically using diphenylamine (6). The cells were lysed in 150 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA and 0.5% Triton X-100) on ice for 20 min. The lysate was centrifuged at 13 000 *g* at 4°C for 20 min to separate the intact and fragmented chromatin. Both the pellet and the supernatant were precipitated at 4°C for 30 min with 6% perchloric acid, after which the precipitates were sedimented at 13 000 *g* at 4°C for 20 min. Next, the DNA precipitates were heated at 70°C for 20 min in 50 µl of 6% perchloric acid followed by mixing with 100 µl of diphenylamine solution (1.5% w/v diphenylamine, 1.5% sulfuric acid and 0.01% acetaldehyde in glacial acetic acid). After overnight incubation at 30°C in the dark, both optical densities were measured at 600 nm and the percentage of DNA fragmentation was calculated as the ratio of DNA in the supernatant to total DNA.

The laddering pattern of the DNA fragmentation was detected by agarose gel electrophoresis. The lysate was first treated with 400 µg/ml RNase A at 37°C for 1 h and then 400 µg/ml proteinase K at 37°C for 1 h. The DNA was precipitated with an equal volume of isopropanol, and then electrophoresed at 100 V through a 2% agarose gel containing 0.1 µg/ml ethidium bromide in TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.3). DNA bands were visualized under ultraviolet illumination and photographed on Polaroid type 667 film 38 (3000 ASA).

Assay for caspase-like activity

Caspase-like activities were determined as described previously. Total HL-60 cell extract was treated with 250 μ M 2,6-diisopropylphenol and then incubated with 10 μ M of Ac-YVAD-MCA for caspase-1, Ac-DEVD-MCA for caspase-3, Ac-VEID-MCA for caspase-6, Ac-IETD-MCA for caspase-8 or Ac-LEHD-MCA for caspase-9. These various caspase substrates were in 20 mM of Hepes buffer (pH 7.5) containing 0.1 M NaCl and 5 mM dithiothreitol, and the mixture was incubated for 1 h at 37°C following the addition of cell lysate. The fluorescence of released 7-amino-4-methylcoumarin was then measured with an excitation wavelength of 335 nm and an emission wavelength of 460 nm, and reflected caspase-like activity.

Western blot analysis

Cell lysates were prepared as described elsewhere (6). The lysate (10^7) was dissolved in a SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% β -mercaptoethanol, 20% glycerol and 0.002% bromophenol blue) and boiled at 100°C for 5 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis. The proteins in the gel were transferred to an Immobilon filter (Millipore Co., Tokyo, Japan) and the filter was incubated with the relevant primary antibodies (1:1000 dilution for cytochrome c and 1:500 dilution for Bid) followed by horseradish peroxidase-linked secondary antibody (1:2000 dilution for cytochrome c and 1:50000 dilution for Bid). The filters were analyzed using an ECL plus kit (Amersham Biosciences Co. Piscataway, NJ, USA). Protein concentrations were determined by the method of Lowry *et al.* with bovine serum albumin as the standard (23).

Results

Effect of propofol on HL-60 cell growth and resultant morphological changes

Growth of HL-60 cells was significantly inhibited by 2,6-diisopropylphenol (propofol) in a concentration-dependent manner (Fig. 1). This inhibitory effect was observed at the lower concentration of 56 μ M and with concentrations of 2,6-diisopropylphenol greater than 280 μ M, with a reduction in cell numbers being evident during the period of exposure to 2,6-diisopropylphenol. We observed no morphological changes related to cell differentiation but apoptotic bodies were evident after incubation for 16 h with 280 μ M 2,6-diisopropylphenol (data not shown). DiprivanTM, the medical product of 2,6-diisopropylphenol dissolved in lipid emulsion, exhibited the same effects as 2,6-diisopropylphenol (data not shown).

Effect of propofol on DNA fragmentation and laddering of HL-60 cells

Incubation of HL-60 cells with 2,6-diisopropylphenol induced cellular DNA fragmentation in a time- and concentration-dependent manner (Figs 2A and 3). DNA fragmentation was significantly evident with 150 μ M 2,6-diisopropylphenol. The fragmentation was

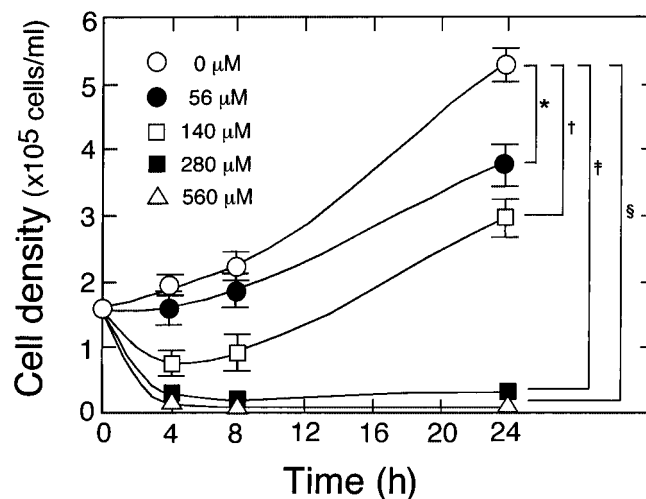


Fig. 1. Effect of 2,6-diisopropylphenol on HL-60 cell growth. HL-60 cells (2.25×10^5 cells) at the exponential phase of growth were incubated in 1.0 ml of RPMI medium containing 10% fetal bovine serum and various concentrations of 2,6-diisopropylphenol (0, 56, 140, 280, 560 μ M) and then incubated in an O_2/CO_2 culture incubator for the indicated periods. Cell number was counted using a hemocytometer and cell viability was evaluated by trypan blue exclusion. Data are expressed as mean \pm SEM, and *, †, ‡ and § indicate that the indicated curve is significantly different from the control curve (obtained in the absence of 2,6-diisopropylphenol) ($P < 0.05$).

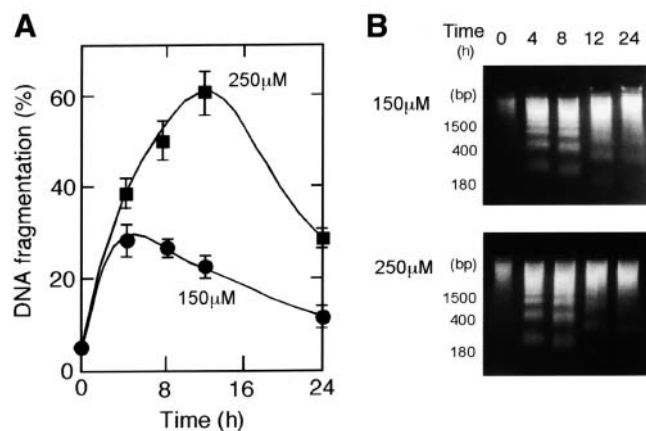


Fig. 2. Effect of 2,6-diisopropylphenol on DNA fragmentation in HL-60 cells. (A) Time-dependent changes in the percentage of fragmented DNA as determined by the diphenylamine method. Data are expressed as mean \pm SEM. (B) Gel electrophoresis pattern of fragmented DNA corresponding to each incubation time. Cells (1.5×10^5 cells/ml) were treated with 150 μ M or 250 μ M of 2,6-diisopropylphenol.

confirmed by gel electrophoresis, which exhibited a typical DNA ladder pattern (Fig. 2B). Diprivan™ also induced DNA fragmentation and a ladder pattern in the same manner (Fig. 4).

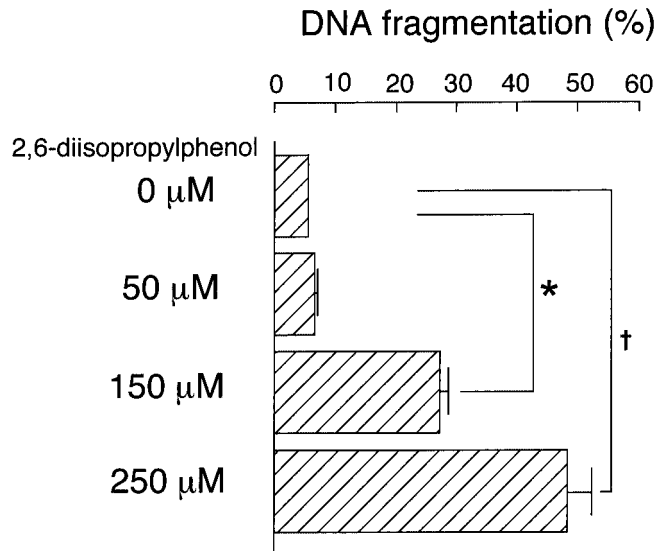


Fig. 3. Dependency of HL-60 cell DNA fragmentation on 2,6-diisopropylphenol concentrations. After 8 h of incubation (1.5×10^5 cells/ml) with 0, 50, 150 and 250 μ M of 2,6-diisopropylphenol, the percentages of fragmented DNA were determined by the diphenylamine method. Data are expressed as mean \pm SEM, and * and † indicate that the shown value is significantly different from the control value (obtained in the absence of 2,6-diisopropylphenol) ($P < 0.05$).

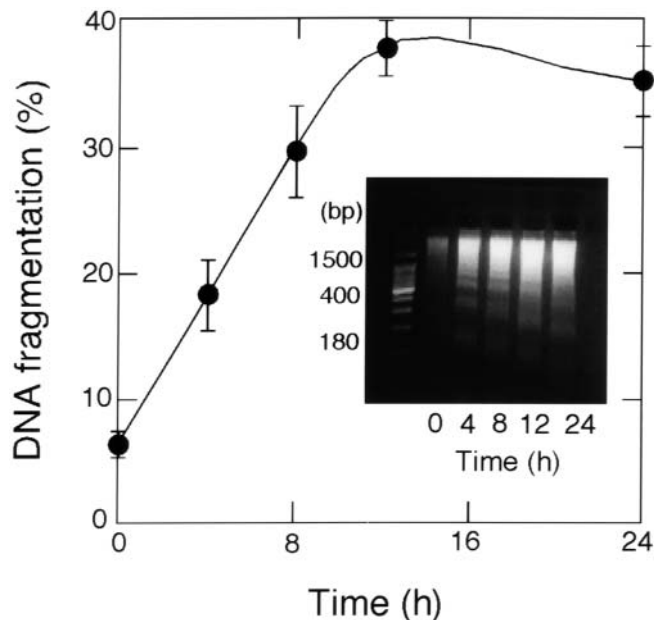


Fig. 4. Effect of Diprivan™ (the medical product of 2,6-diisopropylphenol, dissolved in lipid emulsion) on DNA fragmentation in HL-60 cells as determined by the diphenylamine method. The inset shows the gel electrophoresis pattern of fragmented DNA. Cells (1.5×10^5 cells/ml) were treated with 560 μ M Diprivan™. Data are expressed as mean \pm SEM.

Effect of propofol on activities of various caspases in HL-60 cells

The effect of 2,6-diisopropylphenol on the induction of various caspase activities was examined to analyze the mechanism of 2,6-diisopropylphenol-induced apoptosis in HL-60 cells using specific substrates. Cas-

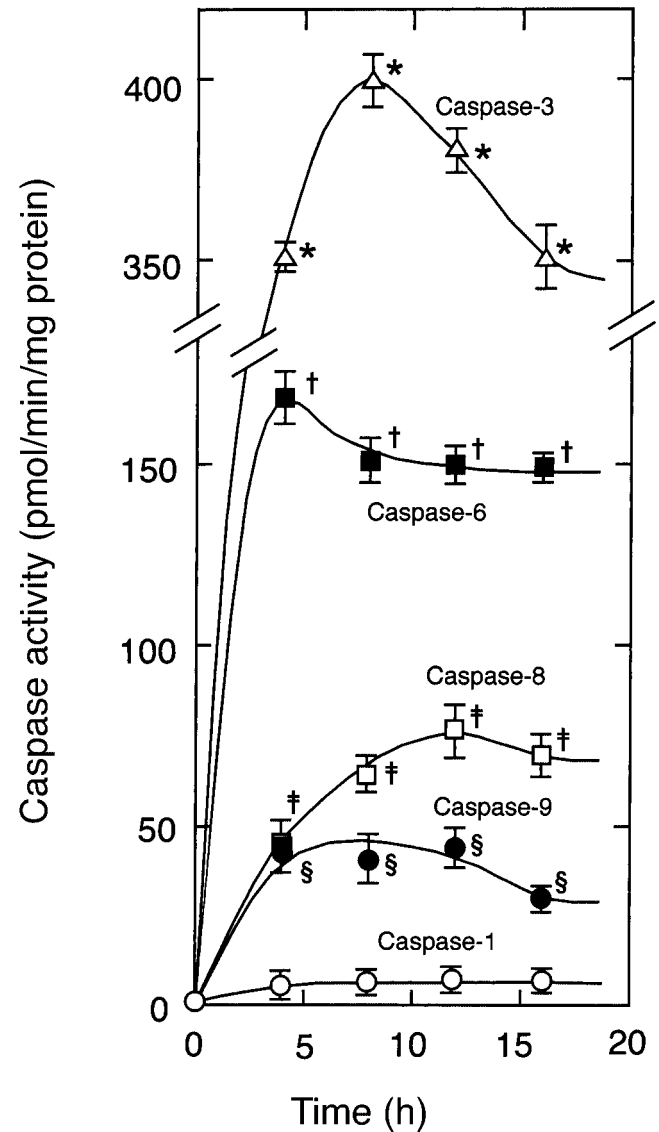


Fig. 5. Effect of 2,6-diisopropylphenol on caspase-like activities in HL-60 cells. Cells were treated with 250 μ M 2,6-diisopropylphenol for the indicated periods. Total cell extracts were incubated in 0.1M NaCl and 5 mM dithiothreitol (DTT) with 10 μ M fluorogenic peptide substrates for each caspase (Ac-YVAD-MCA for caspase-1, Ac-DEVD-MCA for caspase-3, Ac-VEID-MCA for caspase-6, Ac-IETD-MCA for caspase-8 and Ac-LEHD-MCA for caspase-9) at 37°C for 1 h. The fluorescence of released 7-amino-4-methyl-coumarin was measured as caspase activity with an excitation wavelength of 335 nm and an emission wavelength of 460 nm. Data are expressed as mean \pm SEM, and *, †, ‡ and § indicate that each indicated value is significantly different from the control value (no treatment with 2,6-diisopropylphenol) ($P < 0.05$).

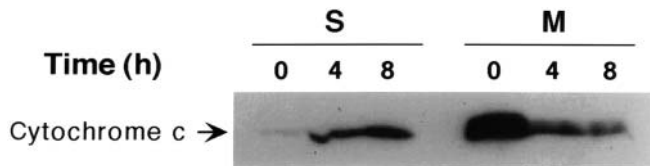


Fig. 6. Effect of 2,6-diisopropylphenol on the release of cytochrome *c* from mitochondria in HL-60 cells. Cells were incubated with 250 μ M 2,6-diisopropylphenol for the indicated periods. Total cell lysates (20 μ g protein) were analyzed by Western blotting using anticytochrome *c* antibody. Depending on the incubation time, cytochrome *c* in the cytosolic (S) fractions increased while cytochrome *c* in the mitochondrial (M) fractions decreased.

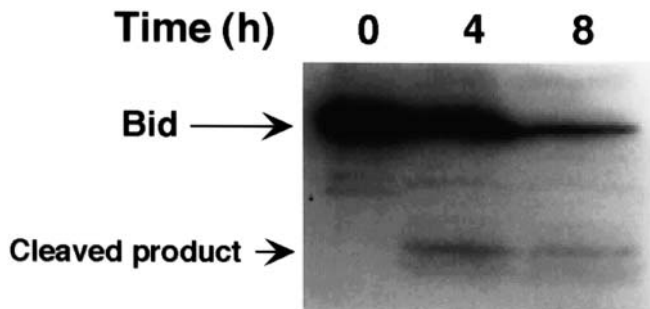


Fig. 7. Effect of 2,6-diisopropylphenol on the cleavage of Bid in HL-60 cells. Cells were incubated with 250 μ M 2,6-diisopropylphenol for the indicated periods. Total cell lysates (30 μ g protein) were analyzed by Western blotting using anti-Bid antibody.

pase-3 and -6 were activated by 2,6-diisopropylphenol in a time-dependent manner (Fig. 5). Caspase-8 and -9 were also activated, though the effect of 2,6-diisopropylphenol was slightly weaker. In contrast, 2,6-diisopropylphenol did not activate caspase-1.

Effect of propofol on the release of cytochrome c from mitochondria to cytosol

As 2,6-diisopropylphenol activated caspase-9, we speculated that cytochrome *c* would be released from mitochondria into the cytosol. As expected, 2,6-diisopropylphenol-treated HL-60 cells exhibited cytochrome *c* release from mitochondria into the cytosol in a time-dependent manner with a resultant decrease in the mitochondrial fraction (Fig. 6).

Effect of propofol on Bid cleavage

Recent studies have demonstrated that caspase-8-mediated cleavage of Bid directly triggers the release of cytochrome *c* from mitochondria via a Bcl-2-inhibitable mechanism, thus resulting in an amplification of the apoptotic signal from the cell surface. Therefore, we used Western blots with an anti-Bid antibody to study the effect of 2,6-diisopropylphenol on the cellular level of cleaved Bid. Interestingly, we

found that incubation of HL-60 cells with 2,6-diisopropylphenol resulted in the appearance of cleaved Bid (Fig. 7).

Discussion

We found that treatment of HL-60 cells with propofol resulted in growth inhibition and formation of apoptotic bodies. Indeed, the concomitant DNA ladder formation and fragmentation indicated that this growth inhibition was mainly the result of the induction of apoptosis in HL-60 cells (6,22). It is known that apoptosis of HL-60 cells may develop subsequent to cell differentiation (24). However, as demonstrated with dibucaine in our previous work (6), some agents can directly induce apoptosis in the absence of differentiation. We found no evidence of morphological changes associated with differentiation before apoptosis in this study and therefore consider that propofol directly induces apoptosis of HL-60 cells in the absence of cell differentiation. In order to elucidate the mechanism underlying the pro-apoptotic effects of propofol in further detail, we studied the effects of propofol on the activities of various caspases. These cysteine proteases form a proteolytic cascade, which can be initiated by ligation of the cell surface Fas death receptor (4,25). The present findings that propofol activated caspase-3, -6, -8 and -9 thereby indicate that this typical death protease cascade mediates the propofol-induced apoptosis of HL-60 cells.

Recently, we and others have demonstrated that the mitochondrial release of cytochrome *c* plays an important role in the amplification of the caspase cascade (6,22,26,27). Released cytosolic cytochrome *c* forms a complex with Apaf-1, resulting in activation of caspase-9 and consequent activation of downstream caspases. Cytochrome *c* release from mitochondria is a consequence of the proteolytic processing of Bid secondary to the activation of caspase-8. Bid is a pro-apoptotic member of the Bcl-2 family (28). The Bcl-2 gene was discovered at a chromosomal breakpoint in human B-cell lymphomas. The Bcl-2 family of proteins has expanded significantly and now includes both pro- and anti-apoptotic molecules. Family members possess up to four conserved Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4, with the Bid protein only bearing the BH3 domain. Proteolytic generation of the cleaved product of Bid results in translocation of Bid to mitochondria and insertion into the mitochondrial membrane where it inhibits the anti-apoptotic action of Bcl-2 and results in the release of cytochrome *c*. In the present study, the generation of the cleaved product of Bid and the

release of cytochrome c from mitochondria by propofol treatment indicates that propofol activates the mitochondrial pathway of apoptosis.

A characteristic of propofol is its hindered phenolic structure (7,8), which is typical of tocopherols (29). The structure of this amphipathic small molecular compound is considered to provide antioxidant activity as well as to determine the molecular behavior of the compound (7,8). Because of this characteristic feature, propofol is considered to easily interact with various membranous and protein fractions, thereby enabling the molecule to modulate the function of membrane-bound and/or cytosolic enzymes and subsequently initiate a sequence of events leading to apoptosis. This possibility should be studied in the future.

The relevance of the concentrations of propofol used in the present study may be of importance. HL-60 cells have properties in common with both monocytes and neutrophils (21,22), which are considered to be possible cellular targets of propofol *in vivo*. The peripheral blood count of these cells ranges from approximately $2\text{--}40 \times 10^5$ cells/ml whilst the concentration of propofol in whole blood is maintained at approximately $25\text{--}130 \mu\text{M}$ during anesthesia (7,8,30). In the present study, the pro-apoptotic effect of propofol upon HL-60 cells was observed in the micromolar concentration range with cell densities between 1.5 and 2.3×10^5 cells/ml. Although the cell number and propofol concentration used in our *in vitro* cell culture system are not identical to those found *in vivo*, this does not invalidate the results and it is not unreasonable to speculate that propofol may result in the apoptosis of monocytes and neutrophils *in vivo*. Clinically, a pathological delay of apoptosis of these leukocytes has been reported in severely injured and septic patients, which may result in the exacerbation of tissue injury and facilitate the development of multiple organ failure (31,32). Thus, the possible acceleration of leukocyte apoptosis by propofol might be favorable in such patients. In contrast, in some immunodeficient conditions such as severe neutropenia or immune deficiency syndrome (33,34), it may be prudent to avoid propofol.

Fas is expressed on various human cells, particularly on leukocytes and macrophages and in the thymus, liver, spleen, lung, testis, brain, intestines, heart and ovaries (1). Furthermore, 11 caspases have been cloned in these cells (35). Therefore, the observed molecular mechanism of the pro-apoptotic effects of propofol on HL-60 cells could be extended to many human cell types, as well as monocytes and neutrophils (36). If propofol does augment apoptosis of various

human cells then this might relate to the recently reported adverse effects of propofol (37–39). However, it should be noted that different cell types exhibit different and diverse levels of sensitivity to apoptosis despite the fact that apoptotic mechanisms may be common to such cells (1,6,40). This diversity in the vulnerability to apoptosis is considered to be partly a result of the diverse activity of anti-apoptotic mechanisms (1). HL-60 cells are rather sensitive to pro-apoptotic stresses (1,6,22) and this may indicate that the pro-apoptotic effect of propofol may be limited to cells that are closely related to HL-60 cells with propofol functioning as a weaker modulator of apoptosis in many other cell types *in vivo*.

In conclusion, we have demonstrated that propofol can induce apoptosis in HL-60 cells via the activation of both the cell surface death receptor pathway and the mitochondrial pathway.

References

- Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell death. *Pharmacol Ther* 2001; **92**: 57–70.
- Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extract: requirement for dATP and cytochrome c. *Cell* 1996; **86**: 147–157.
- Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis and its inhibitor ICAD. *Nature* 1998; **391**: 43–50.
- Nagata S. Apoptosis by death factor. *Cell* 1997; **88**: 355–365.
- Vaux DL, Strasser A. The molecular biology of apoptosis. *Proc Natl Acad Sci* 1996; **93**: 2239–2244.
- Arita K, Utsumi T, Kato A, Kanno T, Kabuchi H, Ihoue B et al. Mechanism of dibucaine-induced apoptosis in promyelocytic leukemia cells (HL-60). *Biochem Pharmacol* 2000; **60**: 905–915.
- Tsuchiya M, Asada A, Maeda K, Ueda Y, Sato EF, Shindo M et al. Propofol versus midazolam regarding their antioxidant activities. *Am J Respir Crit Care Med* 2001; **163**: 26–31.
- Tsuchiya M, Asada A, Kasahara E, Sato EF, Shindo M, Inoue M. Antioxidant protection of propofol and its recycling in erythrocyte membranes. *Am J Respir Crit Care Med* 2002; **165**: 54–60.
- Hemmings Jr. HC, Adamo AIB. Effects of halothane and propofol on purified brain protein kinase C activation. *Anesthesiology* 1994; **81**: 147–155.
- Satoh K, Ida Y, Hosaka M, Arakawa H, Kaeda M, Ishihara M et al. Induction of apoptosis by cooperative action of vitamins C and E. *Anticancer Res* 1998; **18**: 4371–4376.
- Yu W, Simmons-Menchaca M, Gapor A, Sanders BG, Kline K. Induction of apoptosis in human breast cancer cells by tocopherols and tocotrienols. *Nutr Cancer* 1999; **33**: 26–32.
- Palomba L, Sestili P, Cantoni O. The antioxidant butylated hydroxytoluene induces apoptosis in human U937 cells: the role of hydrogen peroxide and altered redox state. *Free Rad Res* 1999; **31**: 93–101.
- Franklin RA, McCubrey JA. Kinases. positive and negative regulators of apoptosis. *Leukemia* 2000; **14**: 2019–2034.
- Olney JW, Farber NB, Wozniak DF, Jevtovic-Todorovic V, Ikonomidou C. Environmental agents that have the poten-

- tial to trigger massive apoptotic neurodegeneration in the developing brain. *Environ Health Perspect* 2000; **108** (Suppl. 3): 383–388.
15. Sagara Y, Hendler S, Khoh-Reiter S, Gillenwater G, Carlo D, Schubert D et al. Propofol hemisuccinate protects neuronal cells from oxidative injury. *J Neurochem* 1999; **73**: 2524–2530.
 16. Sendo F, Tsuchida H, Takeda Y, Gon S, Takei H, Kato T et al. Regulation of neutrophil apoptosis – its biological significance in inflammation and the immune response. *Hum Cell* 1996; **9**: 215–222.
 17. Schuurman HJ, Van Loveren H, Rozing J, Vos JG. Chemicals trophic for the thymus: risk for immunodeficiency and autoimmunity. *Int J Immunopharmacol* 1992; **14**: 369–375.
 18. Oberholzer A, Oberholzer C, Moldawer LL. Sepsis syndromes: understanding the role of innate and acquired immunity. *Shock* 2001; **16**: 83–96.
 19. Delogu G, Moretti S, Famularo G, Antonucci C, Signore L, Marcellini S et al. Circulating neutrophils exhibit enhanced apoptosis associated with mitochondrial dysfunctions after surgery under general anaesthesia. *Acta Anaesthesiol Scand* 2001; **45**: 87–94.
 20. Fanning NF, Porter J, Shorten GD, Kirwan WO, Bouchier-Hayes D, Gotter TG et al. Inhibition of neutrophil apoptosis after elective surgery. *Surgery* 1999; **126**: 527–534.
 21. Ishii Y, Hori Y, Sakai S, Honma Y. Control of differentiation and apoptosis of human myeloid leukemia cells by cytokinins and cytokinin nucleosides, plant redifferentiation-inducing hormones. *Cell Growth Differ* 2002; **13**: 19–26.
 22. Yabuki M, Tsutsui K, Horton AA, Yoshioka T, Utsumi K. Caspase activation and cytochrome c release during HL-60 cell apoptosis induced by a nitric oxide donor. *Free Radic Res* 2000; **32**: 507–514.
 23. Lowry O, Rosebrough N, Farr A, Randall R. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
 24. Charrad RS, Gadhoom Z, Qi J, Glachant A, Allouche M, Jocmin C et al. Effects of anti-CD44 monoclonal antibodies on differentiation and apoptosis of human myeloid leukemia cell lines. *Blood* 2002; **99**: 290–299.
 25. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Ann Rev Cell Dev Biol* 1999; **15**: 269–290.
 26. Nishikawa M, Sato EF, Kuroki T, Utsumi K, Inoue M. Macrophage-derived nitric oxide induces apoptosis of rat hepatoma cells in vivo. *Hepatology* 1998; **28**: 1474–1480.
 27. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281**: 1309–1312.
 28. Kaufmann SH, Hengartner MO. Programmed cell death: alive and well in the new millennium. *Trends Cell Biol* 2001; **11**: 526–534.
 29. Tsuchiya M, Kagan VE, Freisleben HJ, Manabe M, Packer L. Antioxidant activity of alpha-tocopherol, beta-carotene, and ubiquinol in membranes: cis-parinaric acid-incorporated liposomes. *Meth Enzymol* 1994; **234**: 371–383.
 30. Riu PL, Riu G, Testa C, Mulas M, Caria MA, Mameli O et al. Disposition of propofol between red blood cells, plasma, brain and cerebrospinal fluid in rabbits. *Eur J Anaesthesiol* 2000; **17**: 18–22.
 31. Fanning NF, Kell MR, Shorten GD, Kirwan WO, Bouchier-Hayes D, Gotter TG et al. Circulating granulocyte macrophage colony-stimulating factor in plasma of patients with the systemic inflammatory response syndrome delays neutrophil apoptosis through inhibition of spontaneous reactive oxygen species generation. *Shock* 1999; **11**: 167–174.
 32. Ertel W, Keel M, Infanger M, Ungethüm U, Steckholzer U, Trentz O. Circulating mediators in serum of injured patients with septic complications inhibit neutrophil apoptosis through up-regulation of protein-tyrosine phosphorylation. *J Trauma* 1998; **44**: 767–776.
 33. Palmblad J, Papadaki HA, Eliopoulos G. Acute and chronic neutropenias. What is new? *J Intern Med* 2001; **250**: 476–491.
 34. Pitrak DL. Apoptosis and its role in neutrophil dysfunction in AIDS. *Oncologist* 1997; **2**: 121–124.
 35. Grutter MG. Caspases: key players in programmed cell death. *Curr Opin Struct Biol* 2000; **10**: 649–655.
 36. Chang HY, Yang X. Proteases for cell suicide: functions and regulation of caspases. *Microbiol Mol Biol Rev* 2000; **64**: 821–846.
 37. Blakey SA, Hixson-Wallace JA. Clinical significance of rare and benign side effects. propofol and green urine. *Pharmacotherapy* 2000; **20**: 1120–1122.
 38. Cray SH, Robinson BH, Cox PN. Lactic acidemia and bradyarrhythmia in a child sedated with propofol. *Crit Care Med* 1998; **26**: 2087–2092.
 39. Islander G, Vinge E. Severe neuroexcitatory symptoms after anaesthesia – with focus on propofol anaesthesia. *Acta Anaesthesiol Scand* 2000; **44**: 144–149.
 40. Morishita S, Sato EF, Takahashi K, Manabe M, Inoue M. Insulin-induced hypoglycemia elicits thymocyte apoptosis in the rat. *Diabetes Res Clin Pract* 1998; **40**: 1–7.

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