

# Inhalation Anesthetics Induce Apoptosis in Normal Peripheral Lymphocytes In Vitro

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**Background:** The authors hypothesized that perioperative lymphocytopenia is partially caused by apoptosis of lymphocytes induced by inhalation anesthetics. Therefore, they evaluated whether sevoflurane and isoflurane induce apoptosis of normal peripheral lymphocytes.

**Methods:** Normal peripheral blood mononuclear cells were exposed to sevoflurane and isoflurane, and the percentages of apoptotic lymphocytes was measured by Annexin V-fluorescein isothiocyanate-7-amino actinomycin D flow cytometry after 24 h of exposure (0.5, 1.0, and 1.5 mM) and after 6, 12, and 24 h of exposure (1.5 mM). The percentages of lymphocytes with caspase 3-like activity were also measured after 24 h of exposure (1.5 mM).

**Results:** The percentages of apoptotic lymphocytes were increased in a dose-dependent manner (controls:  $5.1 \pm 1.4\%$ ; sevoflurane:  $7.3 \pm 1.3\%$  [0.5 mM],  $9.1 \pm 1.5\%$  [1.0 mM],  $12.6 \pm 2.1\%$  [1.5 mM]; isoflurane:  $7.5 \pm 1.6\%$  [0.5 mM],  $10.5 \pm 1.5\%$  [1.0 mM],  $16.3 \pm 2.7\%$  [1.5 mM]) after 24 h of exposure and in a time-dependent manner (controls:  $1.2 \pm 0.4\%$  [6 h],  $3.4 \pm 0.7\%$  [12 h],  $5.6 \pm 1.2\%$  [24 h]; sevoflurane:  $1.8 \pm 0.4\%$  [6 h],  $6.4 \pm 1.2\%$  [12 h],  $11.3 \pm 2.2\%$  [24 h]; isoflurane:  $2.6 \pm 0.5\%$  [6 h],  $8.8 \pm 1.5\%$  [12 h],  $16.0 \pm 1.9\%$  [24 h]) at the concentration of 1.5 mM. The percentages of lymphocytes with caspase 3-like activity were increased (controls:  $10.0 \pm 1.1\%$ ; sevoflurane:  $13.8 \pm 1.2\%$ ; isoflurane:  $17.0 \pm 1.3\%$ ).

**Conclusions:** Both sevoflurane and isoflurane induced apoptosis in peripheral lymphocytes in dose-dependent and time-dependent manners *in vitro*.

INHALATION anesthetics are known to affect the immune system, and some studies have described peripheral lymphocytopenia after inhalation anesthesia.<sup>1,2</sup> Oka *et al.*<sup>2</sup> reported that the number of lymphocytes was reduced to 60–70% of baseline on days 1 and 4 after isoflurane and nitrous oxide anesthesia, and lymphocytic apoptosis was induced in cultured peripheral blood mononuclear cells (PBMCs) obtained from the same patients 2 or 24 h after anesthesia. Delogu *et al.*<sup>3</sup> found that the incidences of apoptosis increased in cultured CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes obtained from patients 24 h after isoflurane and fentanyl anesthesia. However, it is difficult to determine whether inhalation anesthetics or surgical stress mainly induced this lymphocytic apoptosis.

We hypothesized that perioperative peripheral lymphocytopenia is partially caused by lymphocytic apoptosis

induced by inhalation anesthetics. Recently, we reported that sevoflurane and isoflurane induce apoptosis in murine thymocytes and splenic T cells.<sup>4</sup> Therefore, using flow cytometry, we evaluated whether sevoflurane and isoflurane induce apoptosis in peripheral lymphocytes obtained from healthy volunteers *in vitro*.

In apoptosis, caspases play important roles and work as a cascade. In particular, caspase 3 and caspase 7 (Mch3) are effector caspases that cleave substrates responsible for producing the morphologic changes associated with apoptosis.<sup>5,6</sup> However, apoptosis that is not dependent on caspases was reported.<sup>7</sup> Therefore, we measured intracellular caspase 3-like activity in lymphocytes using flow cytometry to investigate whether caspase 3 or caspase 7 is involved in apoptosis induced by inhalation anesthetics.

## Materials and Methods

### Collection of PBMCs

This study was approved by the Ethical Committee of the Medical Department of Tohoku University (Sendai, Miyagi, Japan). The purpose of this study was sufficiently explained, and written informed consent was obtained from 12 healthy volunteers (6 men and 6 women between 25 and 35 yr of age). RPMI-1640 medium (GIBCO RBL, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (GIBCO RBL) was used for the cell culture. Twenty milliliters of blood was taken from volunteers two times on different days. Heparinized blood was diluted two times with the medium and then poured over 20 ml Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). PBMCs were separated by gradient centrifugation at 400g for 30 min at 20°C. Isolated PBMCs were washed three times with the medium, and greater than 95% cell viability was confirmed by trypan blue staining. Subsequently,  $5 \times 10^5$ /ml PBMCs suspended in the medium were used in all the experiments. PBMCs from the first blood collection were used in all experiments except for the time course study of Annexin V-7-amino actinomycin D (7-AAD) staining, and that from the second blood collection was used in the time course study of Annexin V-7-AAD staining.

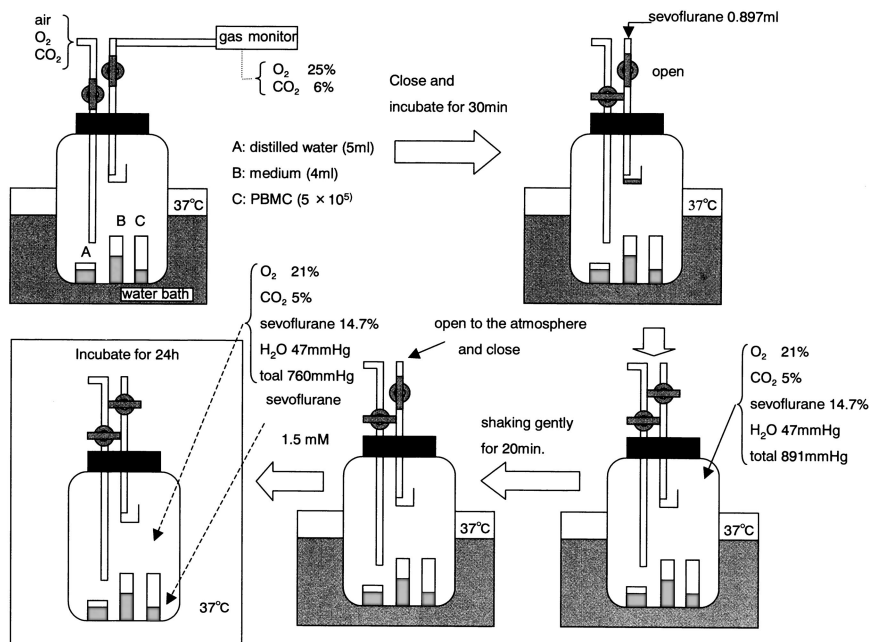
### Measurement of Medium-Gas Partition Coefficients of Inhalation Anesthetics

A culture tube (No. 336-335; INA-OPTIKA, Tokyo, Japan) containing 4 ml medium was placed in a 1-l container equipped with two sealing cocks. In addition, a

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**Fig. 1.** Experimental setup to incubate peripheral blood mononuclear cells (PBMCs) with 1.5 mM sevoflurane in medium. A culture tube containing  $5 \times 10^5$  PBMCs suspended in 1 ml medium was placed in a 1-l container. Then, the concentrations of gases in the container were adjusted to 5% for carbon dioxide (CO<sub>2</sub>), 21% for oxygen (O<sub>2</sub>), and 14.7% for sevoflurane to obtain 1.5 mM sevoflurane in the medium, and the inside pressure was adjusted to 1 atmosphere. The concentration of sevoflurane in the gas phase was calculated based on the previously obtained medium–gas partition coefficients. After incubation at 37°C, several experiments were performed.

culture tube containing 5 ml distilled water was placed in the container for humidification. Subsequently, using a gas analyzer (5250RGM; Datex-Ohmeda, Helsinki, Finland), the container was filled with carbon dioxide and oxygen to adjust the final concentrations after the injection of the inhalation anesthetic to 5% and 21%. Liquid sevoflurane (Maruishi Pharmaceutical, Osaka, Japan) or isoflurane (Dinabot, Tokyo, Japan) was injected into the container to adjust the final concentration in the gas phase to 3, 6, or 9 minimum alveolar concentration (MAC). We used MAC values of 1.71% for sevoflurane and 1.15% for isoflurane. After shaking gently, one of the two sealing cocks was opened to the atmosphere to adjust the pressure inside the container to 1 atmosphere. The sealing cock was then closed. The container was placed in an incubator for 5 h at 37°C. The experimental setup was the same as in figure 1, but without the culture tube with PBMCs.

Thereafter, the concentrations of the inhalation anesthetics dissolved in the medium were measured by the static head space method using a gas chromatograph equipped with a flame ionization detector (GC390B; GL Sciences, Tokyo, Japan) and a head space autosampler (7000HT; Tekmar, Mason, OH) to obtain the medium–gas partition coefficients as follows.

After 5 h of incubation, the culture tube containing medium was taken out of the container. We injected the 4 ml of medium in which the inhalation anesthetic was dissolved into a 9-ml glass bottle using a gas-tight syringe and sealed the bottle completely. The bottle was shaken for 10 min and equilibrated for 5 min at 37°C using the 7000HT. Subsequently, a part of the gases of the bottle was concentrated by cooling to  $-130^{\circ}\text{C}$ . The concentrated gases were applied to a capillary column (60 m; ID, 0.25 mm; film thickness, 1.0  $\mu\text{m}$ ; liquid phase, 25%

phenyl-75% dimethylpolysiloxane; GL Sciences, No. 1010-29165) and then to a flame ionization detector. The temperature of the flame ionization detector was established at 200°C. Helium was used as a carrier gas at a flow rate of 1.5 ml/min. Using an external standard method, we made calibration curves for sevoflurane and isoflurane between 0.3 and 1.8 mM on every experiment day. The concentrations of inhalation anesthetic dissolved in the medium were measured in twelve samples under the respective concentrations of the inhalation anesthetics, and the medium–gas partition coefficients were calculated.

#### Cell Culture

Using the same method described, we set up the experimental condition based on the previously obtained medium–gas partition coefficients to adjust the concentrations of the inhalation anesthetics in the medium to 0.5, 1.0, and 1.5 mM. Figure 1 shows the culture procedure in the 1.5-mM sevoflurane group. During the following experiments,  $5 \times 10^5$  PBMCs suspended in 1 ml medium were incubated in culture tubes under the respective concentrations of the inhalation anesthetics. Simultaneously, a culture tube containing 4 ml medium was allowed to stand to measure the concentration of the inhalation anesthetics. At the end of the experiment, the concentrations of inhalation anesthetics in the medium were measured as described and were confirmed to be within the expected ranges (data not shown).

#### Dose-Response Study of Annexin V-FITC-7-AAD Staining

In the early stage of apoptosis, phosphatidylserine is translocated from the inner side of the cell membrane to

the outer layer. Because Annexin V has a high affinity for phosphatidylserine in the presence of  $\text{Ca}^{2+}$ , fluorescent Annexin V can be used as a sensitive probe for phosphatidylserine exposed on the cell surface. The translocation of phosphatidylserine occurs also in necrotic cells. Necrotic and late apoptotic cells are stained with 7-AAD, a DNA-binding fluorescent dye, because of the increased permeability after the loss of the cell membrane integrity. Using this property, necrotic and late apoptotic cells can be differentiated from other cells.<sup>8,9</sup> Therefore, Annexin V(+)-7-AAD(-) cells are regarded as early apoptotic cells. Late apoptotic cells cannot be distinguished from necrotic cells with this method. Using this method, we evaluated whether the inhalation anesthetics induce lymphocytic apoptosis.

In seven experimental groups (control group: 0.5, 1.0, and 1.5 mm; sevoflurane groups: 0.5, 1.0, and 1.5 mm; isoflurane groups),  $5 \times 10^5$  PBMCs were cultured for 24 h. Subsequently, the cells were washed two times with phosphate-buffered saline and then suspended in 85  $\mu\text{l}$  binding buffer (BV-1035-3; MBL, Nagoya, Japan) containing  $\text{Ca}^{2+}$ . The PBMC suspension supplemented with 10  $\mu\text{l}$  FITC-conjugated Annexin V (MBL, BV-1001-5) and 1  $\mu\text{g}$  7-AAD (PNIM3422; Beckman Coulter, Fullerton, CA) was incubated at room temperature for 15 min in darkness. Subsequently, 400  $\mu\text{l}$  binding buffer was added, and the percentage of early apoptotic lymphocytes was measured using a flow cytometer (FCM, Epics XL System II, Beckman Coulter). Lymphocytes were gated using forward scatter and side scatter, and fluorescence intensity was measured in  $1 \times 10^4$  lymphocytes. The fluorescence intensity of Annexin V-FITC was measured at the fluorescence 1 (FL1) channel (band pass filter,  $525 \pm 15$  nm), and the fluorescence intensity of 7-AAD was measured at the FL4 channel (band pass filter;  $675 \pm 15$  nm).

Because the fluorescence intensity of Annexin V-FITC and that of 7-AAD show a biphasic distribution, we defined the threshold between the cells with dark and bright fluorescence. All samples were analyzed based on the same threshold.

#### *Time Course Study of Annexin V-FITC-7-AAD Staining*

A time course study of Annexin V-7-AAD staining was performed in the control, 1.5-mm sevoflurane, and 1.5-mm isoflurane groups. Using  $5 \times 10^5$  PBMCs, Annexin V-7-AAD flow cytometric analysis was performed as described, after culturing for 6, 12, and 24 h under the respective conditions.

#### *Intracellular Caspase 3-like Activity*

We measured intracellular caspase 3-like activity using the PhiPhiLuxG<sub>1</sub>D<sub>2</sub> (OncoImmunin, Gaithersburg, MD). This cell-permeable fluorescent substrate containing the amino acid sequence Asp-Glu-Val-Asp (DEVD) increases

green fluorescence when it is cleaved by caspase 3-like proteases, such as caspase 3 or caspase 7.<sup>10,11</sup>

Intracellular caspase 3-like activity was measured in the control, 1.5-mm sevoflurane, and 1.5-mm isoflurane groups. After culturing for 24 h under the respective conditions,  $5 \times 10^5$  PBMCs were suspended in the 75  $\mu\text{l}$  of 10  $\mu\text{M}$  PhiPhiLuxG<sub>1</sub>D<sub>2</sub> substrate. PBMCs were then cultured for 1 h at 37°C under the condition of 95% air-5% CO<sub>2</sub>. After washing, caspase 3-like activity was measured by FCM. Lymphocytes were gated as described, and the fluorescence intensity in  $1 \times 10^4$  lymphocytes was measured at the FL1 channel. Cells that were not stained were used to set the threshold point.

#### *Statistical Analysis*

The medium-gas partition coefficients were compared among the concentrations in the gas phase of each inhalation anesthetic using one-way analysis of variance. The percentages of early apoptotic lymphocytes obtained in the dose-response study of Annexin V-FITC-7-AAD staining and the percentages of lymphocytes with caspase 3-like activity were compared among the groups using one-way analysis of variance followed by Tukey-Kramer test. The percentages of early apoptotic lymphocytes obtained in the time course study of Annexin V-FITC-7-AAD staining were compared among the groups using 2-way analysis of variance followed by Tukey-Kramer test. All values were expressed as mean  $\pm$  SD, and a statistically significant difference was assumed if the *P* value was less than 0.05.

## **Results**

The medium-gas partition coefficient of sevoflurane and that of isoflurane were  $0.26 \pm 0.01$  and  $0.43 \pm 0.02$  (mean  $\pm$  SD), respectively. There was no significant difference in the mean medium-gas partition coefficient among the concentrations in the gas phase of each inhalation anesthetic.

In the dose-response study of Annexin V-FITC-7-AAD staining, significantly higher percentages of early apoptotic lymphocytes were detected in all the anesthetic groups except the 0.5-mm sevoflurane group than in the control group (figs. 2 and 3). The percentages of early apoptotic lymphocytes were significantly higher in the 1.0- and 1.5-mm isoflurane groups than in the sevoflurane groups at the same concentrations.

In the time course study of Annexin V-FITC-7-AAD staining, the percentages of early apoptotic lymphocytes were significantly increased among the control groups, the sevoflurane groups, and the isoflurane groups in a time-dependent manner and significantly higher in the isoflurane group, followed by the sevoflurane group and control group in descending order at the 12- and 24-h time points. There were no significant differences among the groups at the 6-h time point (fig. 4).

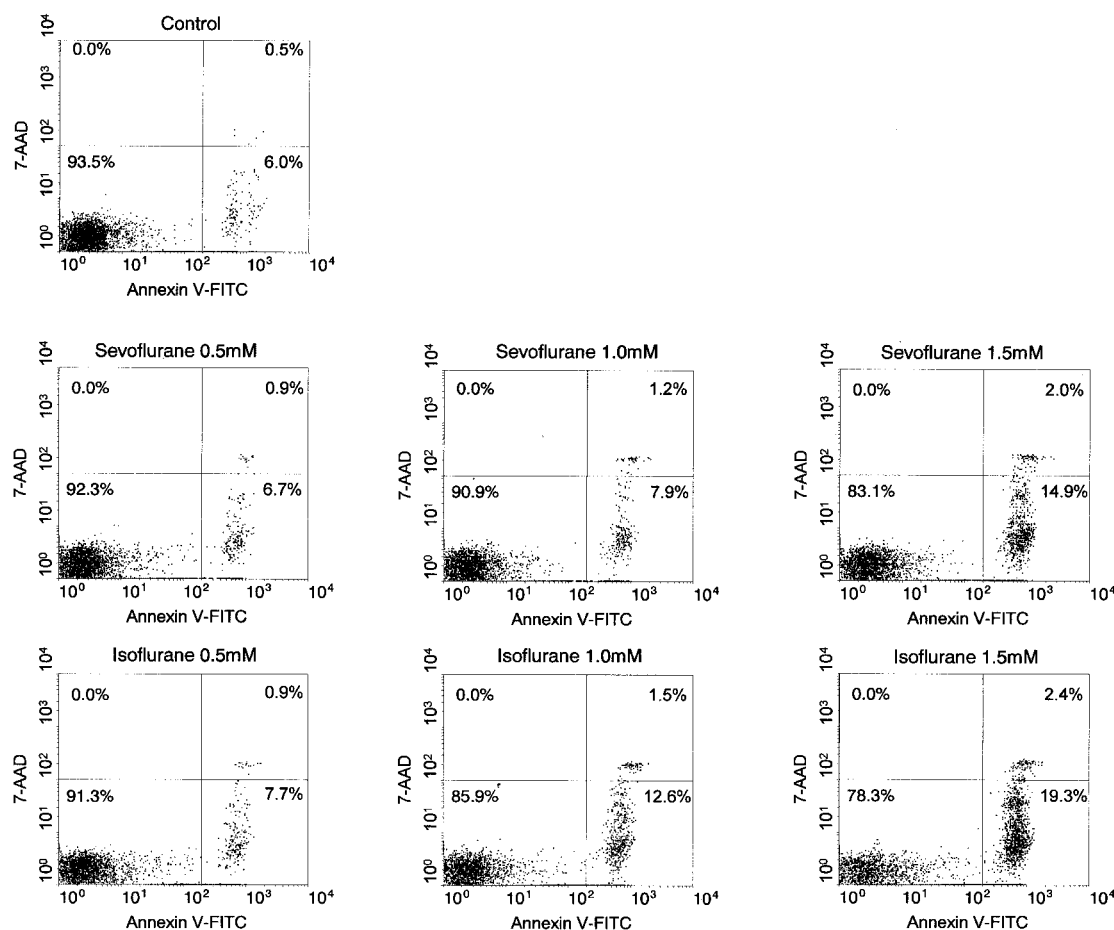


Fig. 2. Data from a representative flow cytometric analysis of lymphocytes stained with Annexin V-FITC-7-AAD after 24 h of incubation with inhalation anesthetics. The relative fluorescence intensity of Annexin V-FITC (FL1/x-axis) and 7-AAD (FL4/y-axis) is given in the dot plots.

The percentages of lymphocytes with caspase 3-like activity were significantly higher in the 1.5-mM isoflurane group ( $17.0 \pm 1.3\%$ ), followed by the 1.5-mM sevoflurane group ( $13.8 \pm 1.2\%$ ) and the control group ( $10.0 \pm 1.1\%$ ) in descending order (fig. 5).

## Discussion

The results of this study using Annexin V-FITC-7-AAD staining show that both sevoflurane and isoflurane induce apoptosis in human peripheral lymphocytes in dose-dependent and time-dependent manners *in vitro*. Based on the medium-gas partition coefficients of inhalation anesthetics obtained in this study and the previously reported values of blood-gas partition coefficients (0.63 for sevoflurane and 1.5 for isoflurane), the concentrations of inhalation anesthetics in the medium used in this study (0.5–1.5 mM) were calculated to be equivalent to 2–6% sevoflurane and 0.85–2.5% isoflurane in the gas phase of the blood-gas system, which would be high as the clinical dose. Although the results of this *in vitro* study cannot be extrapolated to the clinical situation

because of the higher concentrations and long duration used, our data suggest the possibility that lymphocytic apoptosis induced by inhalation anesthetics may cause perioperative lymphocytopenia.

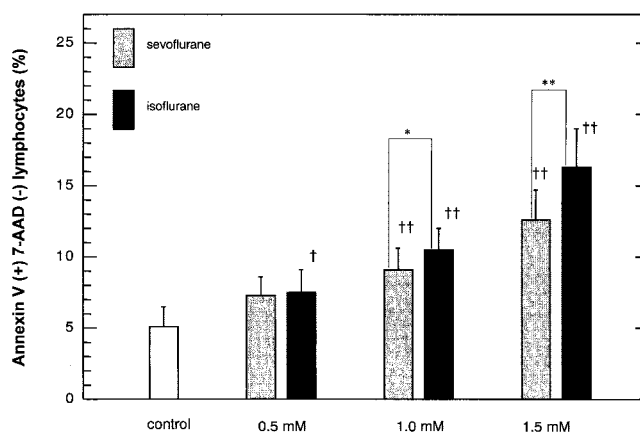


Fig. 3. The dose-dependent histogram of the percentages of early apoptotic lymphocytes after 24 h of exposure to the inhalation anesthetics. Data are expressed as mean  $\pm$  SD.  $\dagger\dagger P < 0.01$  versus control values.  $\dagger P < 0.05$  versus control values.  $** P < 0.01$  between groups.  $* P < 0.05$  between groups.  $n = 12$  per group.



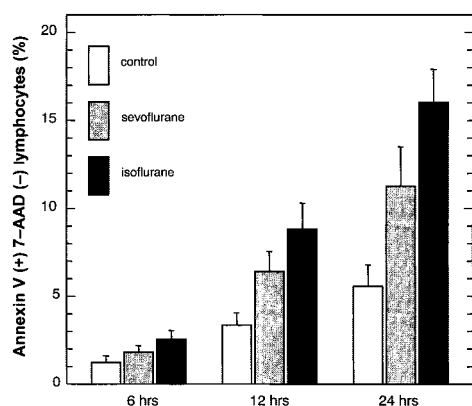


Fig. 4. The time course histogram of the percentages of early apoptotic lymphocytes after 6, 12, and 24 h of exposure to 1.5-mm inhalation anesthetics. Data are expressed as mean  $\pm$  SD. The percentages of early apoptotic lymphocytes in all the groups were significantly higher than that at one previous time point ( $P < 0.01$ ) and significantly higher in the isoflurane group, followed by the sevoflurane group and the control group in descending order at the 12- and the 24-h time points ( $P < 0.01$ ). There were no significant differences among the groups at the 6-h time point.  $n = 12$  per group.

The percentage of lymphocytes with caspase 3-like activity increased after 24 h of exposure to sevoflurane and isoflurane at a concentration of 1.5 mM. This result suggests that caspase 3 or 7 might play a role in the apoptosis of lymphocytes at high concentrations of anesthetics. Further study at lower concentrations is needed.

At the concentrations of 1.0 and 1.5 mM after 24 h of exposure and at the concentration of 1.5 mM after 12 h of exposure, the percentages of apoptotic lymphocytes were significantly higher in the isoflurane groups than in the sevoflurane groups. These data show that the potency of lymphocytotoxicity of isoflurane is higher than

that of sevoflurane at equimolar aqueous concentrations. Based on the medium-gas partition coefficients of inhalation anesthetics obtained in this study and the previously reported values of oil-gas partition coefficients (47.8 for sevoflurane and 94.5 for isoflurane), the oil-medium partition coefficients were calculated as 186 for sevoflurane and 218 for isoflurane. Because the calculated oil-medium partition coefficients are almost equal, the concentrations of both anesthetics in cell membrane phospholipids are almost equal in this study. However, the potency of lymphocytotoxicity of two anesthetics is different. Therefore, regarding lymphocytotoxicity, the site of action of inhalation anesthetics might be different from cell membrane.

The medium-gas partition coefficient of sevoflurane and that of isoflurane determined in this study were lower than the previously reported values of the water-gas partition coefficient (0.36 for sevoflurane and 0.6 for isoflurane). The medium that we used in this study contained many electrolytes, little protein, and no lipids. It is well-known that electrolytes in the fluid decrease the solubility of hydrophobic solutes by a salting-out effect. The possible reason for the low medium-gas partition coefficient obtained in this study might be that the salting-out effect outweighed the increased solubility associated with the protein.

The mechanism of lymphocytic apoptosis induced by inhalation anesthetics is unclear. As courses of apoptosis, cytokines, such as TNF- $\alpha$ , FAS-FAS ligand interaction, and damages of DNA, are well-known. It has been reported that inhalation anesthetics inhibit cytokine release from PBMCs *in vitro*.<sup>12</sup> There are no reports that inhalation anesthetics directly affect FAS-FAS ligand system. Conversely, some *in vitro* studies<sup>13,14</sup> and clinical

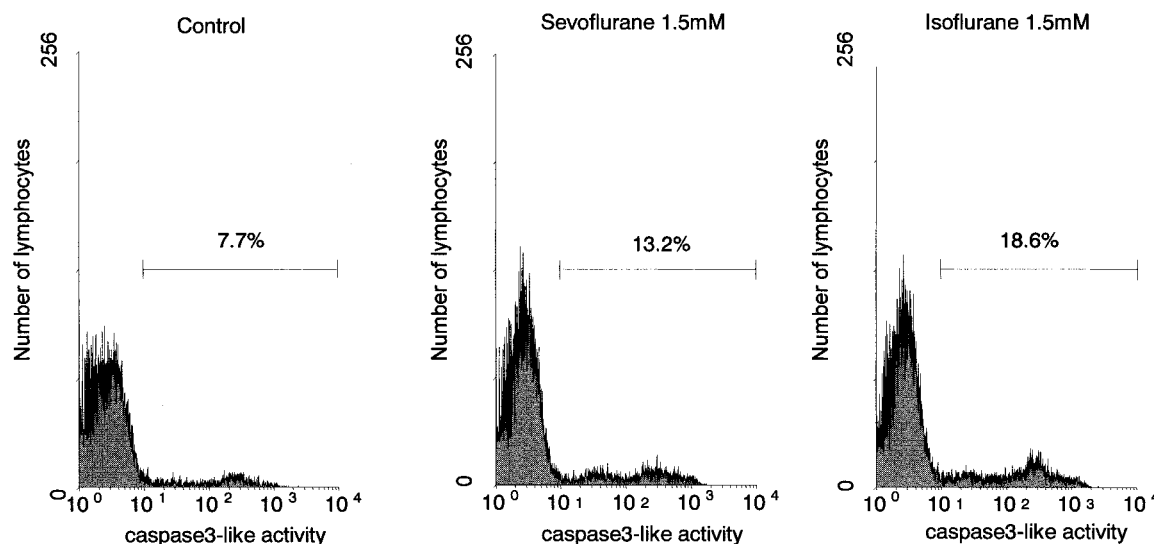


Fig. 5. Data from a representative flow cytometric analysis of intracellular caspase 3-like activity in lymphocytes after 24 h of incubation with inhalation anesthetics. The relative fluorescence intensity of cleaved PhiPhiLuxG<sub>1</sub>D<sub>2</sub> substrate (FL1/x-axis) is given in the histograms.

studies<sup>15-17</sup> that showed genotoxicity of isoflurane have been reported. Therefore, we speculate that the genotoxicity of inhalation anesthetics might be the cause of the apoptosis observed in the current study.

In conclusion, both isoflurane and sevoflurane induced apoptosis in human peripheral lymphocytes in dose-dependent and time-dependent manners *in vitro*. Further studies are warranted to elucidate the roles of lymphocytic apoptosis induced by inhalation anesthetics in lymphocytopenia after anesthesia.

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