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Inhibition of E-selectin-mediated leukocyte adhesion by volatile anesthetics in a static condition

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Abstract

Purpose. Leukocyte recruitment from blood vessels to inflamed tissues is the central step in the process of inflammation. This may cause damage of the inflamed tissues in the case of severe inflammatory conditions such as ischemia reperfusion or graft rejection. Adhesion molecules, such as E-selectin, are induced on activated endothelium and play an important role in this process. Volatile anesthetics protect tissues or organs in such conditions, and inhibition of leukocyte adhesion by anesthetics has been implicated. However, little is known about how the anesthetics act on individual adhesion molecules. We examined the effects of volatile anesthetics on E-selectin mediated leukocyte adhesion in a static condition using HL-60 cells, a granulocyte cell line, and E-selectin-coated plates as well as cytokine-activated human umbilical vein endothelial cells (HUVEC).

Methods. The adhesion assay was carried out by overlaying fluorescence-labeled HL-60 cells on E-selectin-coated plates or cytokine-activated HUVEC. E-selectin in the coated plates or activated HUVEC were quantified by enzyme-linked immunosorbent assay. E-selectin in the activated HUVEC was analyzed by immunoblot.

Results. Isoflurane and sevoflurane concentration-dependently suppressed adhesion of HL-60 cells to E-selectin-coated plates. Although isoflurane did not change the amount of expression, or the molecular weight of E-selectin in the activated HUVEC, it significantly suppressed HL-60 cell adhesion to activated HUVEC.

Conclusion. Volatile anesthetics suppress E-selectin-mediated cell adhesion in a static condition without changing the expression of E-selectin. A role for E-selectin in the organ protection by volatile anesthetics is suggested.

Key words Anesthetic · HL-60 · HUVEC · Inflammation · Cytokine

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Introduction

Leukocyte recruitment plays an important role in various pathological inflammatory conditions such as postischemic reperfusion injury, rejection of graft tissue, and septic shock [1]. In these conditions, proinflammatory cytokines are released and activate vascular endothelium, leading to expression of various adhesion molecules such as selectins and integrins. Leukocytes adhere to these molecules, migrate out of vessels, and damage the tissues or organs [1, 2]. Among the expressed adhesion molecules, E-selectin is prominently induced on endothelial cells and interacts with its ligands, such as P-selectin glycoprotein ligand-1 (PSGL-1) and E-selectin ligand-1 (ESL-1), which are constitutively expressed on granulocytes [3]. A central role for E-selectin in tissue damage has been postulated based on reports in which blockade or inhibition of E-selectin was shown to have beneficial effects [4–6].

Volatile general anesthetics such as isoflurane and sevoflurane inhibit adhesion of leukocytes to H_2O_2-activated endothelium [7]. Isoflurane inhibits leukocyte adhesion to postischemic coronary vessels and reduces reperfusion injury of myocardium [8]. However, how anesthetics influence individual adhesion molecules is not well known. To clarify the influence of anesthetics on pathological inflammation, characterization of anesthetic action on the individual adhesion molecule is essential.

As a starting point, we investigated anesthetic action on E-selectin-mediated leukocyte adhesion in a static condition, using the HL-60 cell, which is a granulocyte cell line that expresses E-selectin ligand such as PSGL-1 [9]. This cell line has been regarded as a model system to characterize E-selectin-dependent cell adhesion [4, 9–13]. We found that isoflurane and sevoflurane suppressed HL-60 cell adhesion to an E-selectin-coated plate in a static condition. Moreover, isoflurane suppressed adhesion of HL-60 cells to cytokine-activated

human umbilical vein endothelial cells (HUVEC). These results suggest a possible mechanism in that volatile anesthetics attenuate tissue injury by inhibiting E-selectin.

Materials and methods

Materials

CS-C complete medium for HUVEC culture was obtained from Cell Systems (Kirkland, MA, USA). RPMI 1640, Dulbecco's phosphate-buffered saline (PBS), recombinant human tumor necrosis factor-alpha (TNF-α) and 2,2-azino-di-(3-ethyl-benzthiazol-6-sulphonic acid) (ABTS) were purchased from Sigma Chemical (St. Louis, Mo, USA). Monoclonal mouse antihuman E-selectin antibody (HAE-1f), which recognizes the lectin domain of E-selectin, was purchased from Genzyme Diagnostics (Cambridge, MA, USA). Horseradish peroxidase (HRP) and indocyanin (Cy3) conjugated antimouse IgG immunogloblin was purchased from Amersham Biosciences (Uppsala, Sweden). Human E-selectin/Fc chimera was from R&D Systems (Minneapolis, MN, USA) Biscarboxyethylcarboxyfluorescein acetoxymethyl ester (BCECF-AM) was from Molecular Probes (Eugene, OR, USA). Isoflurane and sevoflurane were purchased from Dinabot (Osaka, Japan).

Cell culture

According to the guideline of the Ethics Committee of Kyoto University Hospital, human umbilical cords of healthy donors were used after obtaining informed consent. HUVEC were isolated by 0.05% trypsin digestion of the umbilical cords and plated on 0.5% gelatin-coated culture dishes as described [14] and cultured in CS-C complete medium at 37°C in 5% CO_2. HUVEC were characterized by a "cobblestone" appearance and used between second and fifth passages. HL-60 cells, a human leukocyte cell line, were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (ICN Biomedicals, Aurora, OH, USA) at 37°C in 5% CO_2.

Application of volatile anesthetics

Isoflurane or sevoflurane in humidified 5% CO_2/air was delivered through appropriate vaporizers (Forawick: Muraco, Tokyo, Japan; PPV_{Σ}: Penlon, Abingdon, UK, respectively) into an air-tight chamber containing culture plates for at least 30 min at 37°C. The anesthetic concentration was monitored with a gas analyzer (Atom, Tokyo, Japan) to maintain a fixed anesthetic concentration in the gas phase throughout the experiment. The corresponding anesthetic concentrations in the culture media were measured by gas chromatography (GC-17A; Shimazu, Kyoto, Japan) configured with a headspace sampler (HS40; PerkinElmer, Wellesley, MA, USA) (Fig. 1). The concentrations of both anesthetics reached a plateau at 30 min and were stable thereafter throughout the experiments (data not shown).

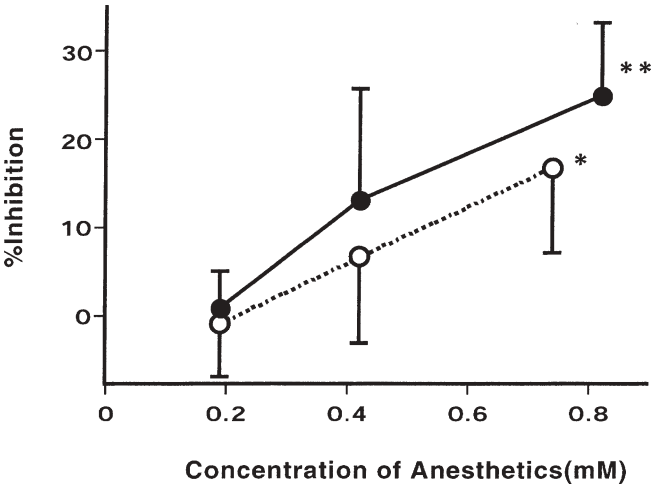

Fig. 1. Inhibition (%) of HL-60 cell adhesion to E-selectin by isoflurane and sevoflurane. Fluorescence-labeled HL-60 cells were overlaid on E-selectin-coated plates with or without isoflurane or sevoflurane. Nonadherent HL-60 cells were removed by inverting the plate. The number of remaining cells was measured by a fluorescent plate reader. Data are presented as mean ± SD of three separate experiments using five to eight wells per experiment. ●, isoflurane; ○, sevoflurane; $*P < 0.05$, $**P < 0.01$ compared to control wells without anesthetic

E-selectin plate coating

E-selectin/Fc chimera consists of the extracellular domain of human E-selectin fused with the Fc region of human IgG_1 and supports binding to E-selectin ligands (manufacturer's brochure and Walz et al. [15]). The chimera was coated on a 96-well flat-bottom plate (Falcon, Lincoln Park, NJ, USA) as described previously [15]. Briefly, the 96-well plate was incubated with rabbit antihuman IgG antibody (Biogenesis, London, UK) in 50 mM Tris HCl, pH 9.0, overnight, and blocked by 2% bovine serum albumin (BSA) in PBS for 2 h. Then, the plate was incubated for 2 h with the E-selectin chimera. Unbound E-selectin chimera was washed away with PBS. Bound E-selectin on the plate was quantified by enzyme-linked immunosorbent assay (ELISA), the method of which is described below. It remained stable throughout the experiment: 99.8% ± 2.0% and 99.7%

± 14.8% of the E-selectin was detected after 70 min exposure of 0.82 mM isoflurane and 0.74 mM sevoflurane, respectively (mean ± SD of three independent experiments).

HUVEC activation

When activated, HUVEC support dramatically increased HL-60 cell adhesion. This adhesion peaks at 4 h after the stimulation and declines gradually toward basal level by 24 h [12], and is mediated mainly through E-selectin [10, 16, 17]. HUVEC were plated at 5×10^4 cells per well in a 96-well gelatin-coated flat-bottom microplate and grown to confluence. Cells were activated by incubating with 1.0 ng ml^{-1} TNF-α for 4 h. When indicated, an anesthetic was applied to the microplate from 30 min before the addition of cytokine until the end of activation.

HL-60 cell adhesion assay

HL-60 cells were labeled with 1 μM BCECF-AM, a fluorescent dye, for 30 min [18], washed, and resuspended in RPMI containing 1% FBS. The adhesion assay was initiated by the addition of 1×10^5 labeled HL-60 cells to each well of the microplate. After incubating for 30 min at 37°C, nonadherent cells were removed by inverting the plates for 5 min. Fluorescent signals of adhered HL-60 cells were measured by a microplate fluorescent reader (Spectra Fluora; Wako, Osaka, Japan), using 485-nm excitation and 515-nm emission. The quantity of adhered HL-60 cells was calculated based on the fluorescence signal of a known number of HL-60 cells on the same microplate. Three to eight wells were used for each experimental condition. In the E-selectin-coated plate adhesion experiments, the plate and cells were treated with the anesthetics 30 min before adhesion, and anesthetics application was continued throughout the adhesion experiments. In the HUVEC adhesion experiments, application of isoflurane was initiated 30 min before addition of TNF-α to HUVEC. For HL-60 cells, anesthetic treatment began 30 min before adhesion. For the washout experiments shown in Table 1, HL-60 cells were treated with the anesthetics for 90 min, washed with RPMI 1640 with 1% FBS, and used for the adhesion assay without anesthetics.

Table 1. HL-60 cell adhesion after washout of anesthetics

Anesthetic	Inhibition (%)[a]	N[b]
Isoflurane 0.82 mM	1.4 ± 17.4	3
Sevoflurane 0.74 mM	0.31 ± 6.9	3

[a] Values are expressed as mean ± SD
[b] Number of independent experiments

E-selectin quantitation by ELISA

After blocking with 2% BSA containing 0.05% Tween 20, the wells of the E-selectin-coated plates were stained with monoclonal mouse antihuman E-selectin antibody HAE-1f (1 μg ml^{-1}) followed by antimouse IgG conjugated with HRP and developed using ABTS as a HRP substrate. The optical density (OD) was read using a Thermo max microplate reader (Molecular Devices, Sunnyvale, CA, USA) with a 405-nm filter. Four to eight wells were used for each experimental condition. To quantify E-selectin on activated HUVEC plates, cells were fixed with 1% paraformaldehyde in PBS for 20 min before the assay.

Immunoblot analysis

Cytokine-activated HUVEC were lysed in a sample buffer, separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis [19] under non-reducing condition, and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was incubated with HAE-1f (1 μg ml^{-1}) followed by HRP-conjugated antimouse IgG and developed using an ECL-plus developing kit (Amersham Biosciences, Uppsala, Sweden).

Analytical procedures

Values of percent (%) loss and percent (%) inhibition by anesthetics were calculated as $\{1 - (a - b)\,(c - d)^{-1}\} \times 100$ where a is the value of E-selectin-coated (or activated HUVEC) well incubated with anesthetic, b is the value of control Ig-coated (or control HUVEC) well incubated with anesthetic, c is the value of E-selectin-coated (or activated HUVEC) well incubated without anesthetic, and d is the value of control Ig-coated (or control HUVEC) well incubated without anesthetic. Data are presented as mean ± SD of separate experiments. The differences between two values were tested by Student's t test. Significant difference was assumed when $P < 0.05$.

Results

Suppression of HL-60 cell adhesion to E-selectin-coated plate by anesthetics

We first examined the effect on HL-60 cell adhesion to an E-selectin-coated plate to evaluate anesthetic action on E-selectin. As shown in Fig. 1, isoflurane inhibited the adhesion concentration-dependently, and at the

Table 2. Effect of 0.71 mM isoflurane on tumor necrosis factor-alpha (TNF-α)-activated human umbilical vein endothelial cells (HUVEC)

Effect	Inhibition (%)[a]	N[b]
HL-60 cell adhesion	22.3 ± 19.7*	7
E-selectin expression	−6.6 ± 39.0	4

[a] Values are expressed as mean ± SD
[b] Number of independent experiments
* $P < 0.05$

highest concentrations (0.82 mM) inhibited the adhesion by 24.9% ± 8.3% ($P < 0.01$ compared to control wells without anesthetics). To evaluate the effect of the anesthetic on HL-60 cells, the cells were treated with the highest concentration of the anesthetic for 90 min and the adhesion assay was performed after washing the cells. As shown in Table 1, the adhesion was maintained after washing out the anesthetic, indicating the anesthetic did not have irreversible effects that may inhibit E-selectin-mediated adhesion of HL-60 cells. We then tested whether other inhaled anesthetics could exert a similar effect. In fact, sevoflurane suppressed adhesion at 0.74 mM by 16.7% ± 9.6% ($P < 0.05$), and had a reversible effect on HL-60 cells as isoflurane did (see Fig. 1, Table 1). These results suggest that volatile anesthetics inhibit E-selectin-dependent adhesion.

Inhibition of HL-60 cell adhesion to cytokine-activated HUVEC by isoflurane

As shown in Table 2, 0.71 mM isoflurane inhibited HL-60 adhesion to TNF-α-activated HUVEC ($P < 0.05$). To exclude the possibility that the anesthetic modified E-selectin induction in HUVEC, we examined the isoflurane effect on activation. Isoflurane did not attenuate the E-selectin expression measured by ELISA (Table 2).

We further examined the influence of isoflurane on HUVEC by immunoblot analysis. E-selectin is modified by N-glycosylation, and in some conditions immunoblot analysis revealed bands of immaturely glycosylated E-selectin ranging from 78 to 110 kDa [10, 20]. Among them, the partially glycosylated form of E-selectin is not transported to the cell surface and thus does not mediate adhesion [21]. Immunoblot analysis of the anesthetic-treated HUVEC showed a single band at about 100 kDa as well as the control cells (Fig. 2), indicating that the anesthetic does not modify E-selectin expression and glycosylation during activation. These results suggest that induction of E-selectin is not altered by isoflurane and that fully glycosylated E-selectin is expressed on the cell surface.

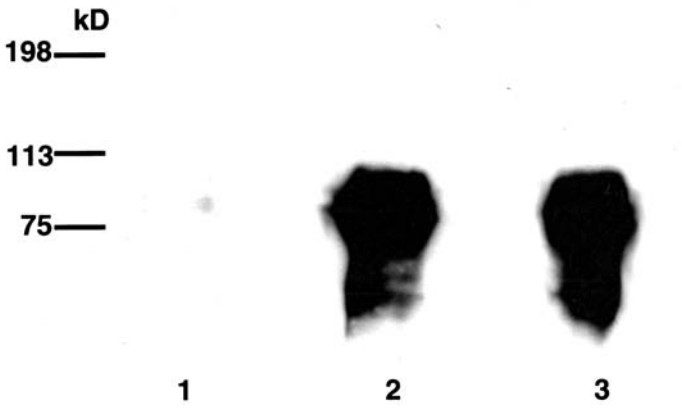

Fig. 2. Influence of isoflurane on E-selectin expression in activated human umbilical vein endothelial cells (HUVEC). Cell lysates of control or activated HUVEC were subjected to immunoblot analysis of E-selectin. *Lane 1*, control HUVEC; *lane 2*, activated HUVEC with 1 ng ml^{-1} tumor necrosis factor-alpha (TNF-α); *lane 3*, activated HUVEC with 1 ng ml^{-1} TNF-α and 0.71 mM isoflurane

Discussion

We describe here an inhibitory effect of volatile anesthetics on HL-60 cell adhesion to both E-selectin-coated plates and cytokine-activated HUVEC. In the E-selectin-coated plate experiments, we observed a statistically significant inhibition only at concentrations of 0.82 and 0.74 mM for isoflurane and sevoflurane, respectively. These concentrations in blood correspond to 3.0 and 2.5 minimum alveolar concentration (MAC) of isoflurane and sevoflurane clinically inhaled by humans, respectively [22], and 0.71 mM isoflurane in the HUVEC adhesion experiment corresponds to 2.6 MAC. These values are within clinically relevant concentrations.

Reversibility of the isoflurane effect suggests that isoflurane does not modify the E-selectin ligand in HL-60 cells. Although the ligand(s) for E-selectin are yet to be determined, a number of reports suggest PSGL-1 is one of the most probable candidates [23–26]. Indeed, in the membrane fractions of HL-60 cells, PSGL-1 has been identified as the only and major E-selectin binding molecule [9]. Isoflurane does not alter PSGL-1 expression in neutrophils up to 1 MAC concentration [27]. Considering that anesthetic was also unable to modify expression of E-selectin on activated HUVEC (see Table 2, Fig. 2), we speculate that the affinity between E-selectin and its ligands is altered. For example, the extracellular part of E-selectin contains a C-type lectin domain that binds both Ca^{2+} and the selectin ligands [28]. Anesthetics may inhibit adhesion activity of E-selectin by preventing Ca^{2+} from binding to the lectin domain.

The adhesion between HL-60 cells and TNF-α-activated HUVEC has been well described and consistently reported to be E-selectin dependent. For example, flow cytometric analysis of HL-60 cells revealed that the ligand of E-selectin is expressed ten times more than those of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 [16]. Anti-E-selectin antibody inhibited HL-60 cell adhesion to TNF-α-activated HUVEC more than 60% [10]. Antisense E-selectin oligonu-cleotide, which inhibits cell surface expression of E-selectin selectively, reduced HL-60 cell adhesion to TNF-α-activated HUVEC by 79% [17]. However, contribution of other adhesion molecules such as ICAM-1 and VCAM-1 is still possible. Anesthetic effects on other adhesion molecules remain to be investigated.

Volatile anesthetics protect organs or tissues including the heart, brain, and liver against ischemia and reperfusion injury [29–31]. A possible mechanism of this protective effect includes stimulation of the adenosine receptor and K_{ATP} channel [32, 33], suppression of metabolism [34], reduction of the frequency of transient ischemic depolarizations [35], and activation of protein kinase C [36]. Recently, reduction of leukocyte adhesion has also been proposed for the protection mechanism [8, 37]. Although actions of anesthetics on other adhesion molecules are to be examined, our results suggest the possibility that E-selectin inhibition plays a role in reducing leukocyte adhesion.

Among selectins, P-selectin is also modulated by volatile anesthetics such as halothane and sevoflurane. P-selectin is upregulated and adhesion of leukocytes is increased by the anesthetics in the rat mesenteric artery in vivo [38], contrary to E-selectin inhibition as described in this report. However, this is inconsistent with the several reports in which anesthetics inhibit leukocyte adhesion in ischemia-reperfusion of guinea pig heart models in vitro [8, 37], where P-selectin mediates the adhesion [39]. Moreover, anesthetics including isoflurane do not inhibit H_2O_2-mediated cell-surface expression of P-selectin in HUVEC [7]. This difference may be because Morisaki et al. used an in vivo model and the other reports concerned in vitro models. The P-selectin upregulation of an in vivo model may not be the direct action of anesthetics on microcirculation itself. It is possible that modulation of the hormonal or autonomic environment by volatile anesthetics induces P-selectin. Another difference in these reports is that Morisaki et al. examined the effect on normal and noninflamed tissue whereas the others used cytokines to induce adhesion molecules. Because P-selectin is already highly upregulated in inflamed tissues [1–3], it is possible that anesthetics do not induce P-selectin further. Therefore, it is likely that their result cannot be used to elucidate the anesthetic effect on inflammation-induced tissue damage.

We acknowledge that this study was performed only in a static condition without taking into account the effects of blood flow and shear stress. To explore anesthetic effects on E-selectin function fully, it is necessary to examine the anesthetic effects on E-selectin-mediated rolling, activation, and firm adhesion under shear stress, as well as E-selectin-mediated signal transduction. Nevertheless, this study is the first to demonstrate the anesthetic effect on E-selectin-mediated leukocyte adhesion, providing a new insight into the mechanism of organ protection by anesthetics. Examination of the other aspects of E-selectin function is indispensable to clarify the relevance of our present finding to clinical practice.

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