Original

**Lidocaine Induces Apoptosis in Peripheral CD4⁺ T-cells
of Patients with Bronchial Asthma**

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Abstract : Lidocaine inhibits the proliferative response and cytokine synthesis of peripheral blood mononuclear cells after allergen- and non-allergen-specific activation. In the present study, we investigated the role of apoptosis in the inhibitory effect of lidocaine on CD4⁺ T-cells. CD4⁺ T-cells from 8 patients with asthma and house dust mite allergy were cultured with lidocaine. Staining for Annexin V and propidium iodide was monitored by flow cytometry. The active forms of caspase-3 and caspase-9 were examined by flow cytometry and the expression of Bcl-2 was analyzed by immunoblotting. The inhibitory effects of lidocaine on CD4⁺ T-cell proliferation and interleukin-5 and interferon γ production after stimulation with phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore were also investigated. The extent of apoptosis in CD4⁺ T-cells stimulated with PMA plus calcium ionophore was examined. Addition of lidocaine (1 mM) to resting CD4⁺ T-cells increased the percentage of Annexin V⁺ PI⁻ T-cells in the culture ($P<0.01$); expression of the active forms of caspase-3 and caspase-9 was increased ($P<0.05$), while expression of Bcl-2 was decreased. The proliferative response ($P<0.01$) and the production of interleukin-5 and interferon- γ ($P<0.01$) by CD4⁺ T-cells after stimulation with PMA plus calcium ionophore were inhibited upon incubation with 1 mM lidocaine. Furthermore, the percentage of Annexin V⁺ PI⁻ T-cells in activated CD4⁺ T-cells treated with 1 mM lidocaine was also increased. Thus, lidocaine induces apoptosis in CD4⁺ T-cells by activation of caspase-3 and caspase-9 and down-regulation of Bcl-2.

Key words : lidocaine, T-cell, apoptosis, caspase, Bcl-2

Introduction

Bronchial asthma is a chronic inflammatory disease characterized by infiltration of activated T-cells and eosinophils in the airway mucosa leading to airflow limitation, airway hyper-responsiveness and mucus hypersecretion¹⁻³⁾. Presently, inhaled corticosteroids (ICS) are the first-line of treatment in bronchial asthma⁴⁾. The use of ICS has improved the quality of life of asthmatic patients; however, there are still a number of patients whose asthma can-

not be sufficiently controlled even with high doses of ICS and who require systemic steroids.

Lidocaine, a widely used local anesthetic and anti-ventricular arrhythmic agent, has a steroid-sparing effect in patients with steroid-dependent severe asthma^{5,6}. It was also demonstrated to induce apoptosis in eosinophils, suppress histamine release from basophils and mast cells, attenuate bronchoconstriction of airway smooth muscle, and inhibit the effect of granulocyte colony-stimulating factor on neutrophils⁷⁻¹¹. We previously demonstrated that although 100 μ M lidocaine did not have an inhibitory effect on the viability of CD4⁺ T-cells, it inhibited the proliferative response and cytokine production by peripheral blood mononuclear cells¹².

Apoptosis is a mechanism of cell death during which a complex set of proteins, which includes the caspase family of proteases and the Bcl-2 family, is involved^{13,14}. The induction of apoptosis in inflammatory cells is considered to be an important mechanism of drugs used in the treatment of patients with bronchial asthma. Both corticosteroid and theophylline induce apoptosis in eosinophils^{15,16}. In the present study, we examined the inhibitory effect of lidocaine on CD4⁺ T-cells and found that lidocaine induced apoptosis in CD4⁺ T-cells of patients with allergic asthma.

Materials and Methods

Reagents

The following reagents were used: hydrochloride lidocaine, hydrochloride tetracaine, hydrochloride dibucaine, phorbol 12-myristate 13-acetate (PMA), calcium ionophore, tetrodotoxin (Sigma, St. Louis, MO, USA), and anti Bcl-2 antibody (Upstate Biotechnology, Lake Placid, NY, USA).

Subjects

Eight non-smoking patients with allergic asthma (3 females and 5 males, 28 to 41 years old, mean age 34.4 years) were selected on the basis of (a) positive cutaneous reactions to *Dermatophagoides farinae* (Der f) extract, (b) high levels of house dust- and Der f-specific IgE in sera, (c) baseline FEV₁ more than 70% of the predicted value, (d) increased bronchial responsiveness to histamine, and (e) no treatment other than rescue β_2 -agonists for at least 3 months. None of the subjects had suffered from respiratory infections or other diseases in the previous 4 weeks. Written informed consent was obtained from the patients before the study.

Purification of CD4⁺ T-cells

CD4⁺ T-cells were purified by negative selection (MACS, Miltenyi Biotec, Auburn, CA, USA) from peripheral blood mononuclear cells isolated from heparinized venous blood on Ficoll-Hypaque gradients (Nacalai Tesque Co., Kyoto, Japan). In all experiments, cells were cultured in RPMI-1640 supplemented with 10% v/v human AB serum, 100 U penicillin, and 100 μ g/ml streptomycin in an atmosphere containing 5% CO₂ at 37°C. Lidocaine (10 μ M, 100 μ M, 1 mM), tetracaine (1 mM) or dibucaine (100 μ M) were added to the culture medium.

Staining with Annexin V and propidium iodide

CD4⁺ T-cells were activated or not activated with PMA (10 ng/ml) plus calcium iono-

phore ($1\text{ }\mu\text{g/ml}$) and cultured in the presence or absence of lidocaine for 3 days; they were stained with FITC-conjugated Annexin V antibody and red-fluorescent propidium iodide (PI) using the Vybrant Apoptosis kit (Molecular Probes, Eugene, OR, USA). The cells were analyzed using the FACS Calibur (BD Biosciences Immunocytometry Systems, San Diego, CA, USA). CD4^+ T-cells incubated for 3 days with tetracaine or dibucaine were also analyzed as described above. In some experiments, CD4^+ T-cells were treated with tetrodotoxin ($2\text{ }\mu\text{M}$) in the presence or absence of lidocaine.

Measurement of caspase-3, caspase-8, and caspase-9 activities

Intracellular expression of the active forms of caspase-3 and caspase-9 was analyzed in CD4^+ T-cells incubated with or without lidocaine for 3 days. Peptide inhibitors of caspase-3 (DEVD-FMK) and caspase-9 (LEHD-FMK) were added to the cell culture and coupled with the FMK carboxyfluorescein dye for 1 h at 4°C in PBS (CaspaTag kit, Intergen, Purchase, NY, USA). The cells were washed and fixed according to the manufacturer's recommendation. Stained cells were immediately examined using the FACS Calibur, and data were analyzed with the Cell Quest software (BD Biosciences Immunocytometry Systems). Caspase-8 activity was assayed using BD ApoAlert™ Caspase assay plates (Clontech Laboratories, Inc., Palo Alto, CA). Briefly, CD4^+ T-cells (2×10^6 cells) were incubated in the presence or absence of lidocaine for 3 days and lysed for 10 min on ice. After centrifugation, reaction buffer containing DTT and the respective substrates was added to the cell lysates. The lysates were then incubated at 37°C for 2 h, and the caspase activities were determined by measuring the absorbance at 405 nm in a microtiter plate reader. The results were calculated as percentage of caspase-8 activity compared to control.

Immunoblotting analysis

Cell lysates from CD4^+ T-cells incubated with or without lidocaine for 48 h were obtained using the Cellytic M (Sigma, St. Louis, MO, USA). Equal amounts ($10\text{ }\mu\text{g}$) of cell lysate protein were separated by SDS-PAGE through a 15% polyacrylamide gel and transferred onto polyvinyl difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk in TBST (1 M Tris Hcl (pH 8.0), NaCl 15 mM, Tween 20 0.05%) for 1 hour at room temperature and incubated with anti-Bcl-2 antibodies in TBST with 5% skim milk overnight at 4°C . After washing, the membranes were incubated for 1 h with the appropriate horseradish peroxidase (HRP)-conjugated secondary reagent at room temperature. Antigen-antibody complexes formed were detected with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Proliferation and cytokine assays

To assay proliferation, CD4^+ T-cells (10^5 cells/well) were stimulated with PMA (10 ng/ml) plus calcium ionophore ($1\text{ }\mu\text{g/ml}$) in the presence or absence of lidocaine for 72 h, and pulsed with $1\text{ }\mu\text{Ci}$ of ^3H -methylthymidine (ARC Inc., St. Louis, MO, USA) for the last 8 h of culture. The stimulation index (SI) was calculated by dividing the cpm of stimulated cultures by that of un-stimulated cultures. To assay cytokine production, CD4^+ T-cells (2×10^6 cells) were activated or not with PMA (10 ng/ml) plus calcium ionophore ($1\text{ }\mu\text{g/ml}$) in the presence or absence of lidocaine for 24 h. Production of interleukin-5 (IL-5) and interferon- γ (IFN- γ) in the culture supernatants was measured with ELISA kits obtained

Fig. 1. Flow cytometry analysis of CD4⁺ T-cells stained for Annexin V and PI. (a) Percentages of Annexin V⁺ PI⁻ CD4⁺ T-cells after incubation with different concentrations of lidocaine. In some experiments, CD4⁺ T-cells were incubated with lidocaine in the presence or absence of tetrodotoxin (2 μM). (b) CD4⁺ T-cells were treated with tetracaine (1 mM) or dibucaine (100 μM) for 72 h. Results are presented as mean ± SEM for 8 different patients.

from Pharmingen (San Diego, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively. The ELISA kits could detect IL-5 and IFN- γ at concentrations as low as 10 pg/ml.

Statistical analysis

The statistical significance of the differences between groups was calculated using the Wilcoxon rank test. Bonferroni correction was applied for multiple comparisons. Data were expressed as mean ± standard error of mean (SEM) and a probability less than 0.05 was considered to indicate significance.

Results

Lidocaine and its analogues induce apoptosis in resting CD4⁺ T-cells

Annexin V-positive, PI-negative cells were identified as apoptotic cells. In freshly isolated CD4⁺ T-cells, 98.2 ± 3.5% were live cells, negative for both Annexin V and PI staining (data not shown). In non-activated CD4⁺ T-cells, incubation for 3 days with 1 mM lidocaine increased the percentage of Annexin V⁺ PI⁻ cells; 100 μM lidocaine had no such effect (6.3 ± 2.6% without lidocaine, 8.8 ± 3.7% with 100 μM lidocaine, and 18.9 ± 5.5% with 1 mM lidocaine, $P < 0.01$; Fig. 1a). These results suggest that lidocaine induces apoptosis in CD4⁺ T-cells. Treatment with tetrodotoxin, a selective voltage-gated sodium channel blocker, failed to inhibit the lidocaine-induced apoptosis in CD4⁺ T-cells (18.9 ± 5.5% Annexin V⁺ PI⁻ cells in the presence of 1 mM lidocaine vs 19.1 ± 5.0% Annexin V⁺ PI⁻ cells in the presence of 1 mM lidocaine and 2 μM tetrodotoxin; Fig. 1a).

To examine whether the derivatives of lidocaine can induce apoptosis in CD4⁺ T-cells, tetracaine and dibucaine were used in some experiments. Apoptotic CD4⁺ T-cells were in-

(b) *Caspase-9*

Fig. 2. The active forms of intracellular caspase-3 and caspase-9 in CD4⁺ T-cells after incubation with lidocaine for 72 h. Active forms of caspase-3 (a) and caspase-9 (b) in CD4⁺ T-cells treated with 1 mM lidocaine are expressed as percentages of total cells. Results are presented as mean $\pm$ SEM for 8 different patients.

Fig. 3. Effect of lidocaine on Bcl-2 expression in CD4⁺ T-cells. CD4⁺ T-cells were incubated with lidocaine for 48 h. Bcl-2 was detected by immunoblotting. Representative data for 1 patient out of 8 tested are shown.

creased after incubation with 1 mM tetracaine ($6.3 \pm 2.6\%$ without tetracaine vs $36.2 \pm 3.8\%$ with 1 mM tetracaine, $P < 0.01$; Fig. 1b) or 100 μM of dibucaine ($6.3 \pm 2.6\%$ without dibucaine vs $50.8 \pm 4.6\%$ with 100 μM dibucaine, $P < 0.01$; Fig. 1b).

Lidocaine increases caspase-3 and caspase-9 activities and down-regulates Bcl-2

To further investigate the mechanism involved in lidocaine-induced apoptosis in CD4⁺ T-cells, we evaluated caspase-3 and caspase-9 activities and the expression of Bcl-2. Although 1 mM lidocaine increased the expression of the intracellular active form of caspase-3 ($4.8 \pm 2.3\%$ without lidocaine vs $16.0 \pm 4.9\%$ with 1 mM lidocaine, $P < 0.05$; Fig. 2a) and caspase-9 ($5.1 \pm 2.5\%$ without lidocaine vs $19.3 \pm 2.7\%$ with 1 mM lidocaine, $P < 0.05$; Fig. 2b), caspase-8 activity was not significantly increased ($100 \pm 0\%$ without lidocaine vs

Fig. 4. Inhibitory effects of lidocaine on CD4⁺ T-cell proliferation (a) and IL-5 and IFN- γ production (b) after stimulation with PMA (10 ng/ml) plus calcium ionophore (1 μ g/ml). Proliferative response was measured on day 3 of incubation and the stimulation index (SI) was calculated. Culture supernatants were harvested after 24 h and cytokines (IL-5 and IFN- γ) were measured with ELISA. Results are presented as mean $\pm$ SEM for 8 different patients.

Fig. 5. Flow cytometry analysis of activated CD4⁺ T-cells stained for Annexin V and PI. Percentage of Annexin V⁺ PI⁻ CD4⁺ T-cells after stimulation with PMA plus calcium ionophore in the presence or absence of different concentrations of lidocaine. Results are presented as mean $\pm$ SEM for 8 different patients.

115.3 $\pm$ 7.6% with 1 mM lidocaine, $P=0.07$). In addition, treatment with lidocaine decreased Bcl-2 expression in a dose-dependent manner (Fig. 3).

Lidocaine inhibits proliferative response and cytokine production by CD4⁺ T-cells

CD4⁺ T-cell proliferation and cytokine production were analyzed to determine the inhibitory effects of lidocaine on CD4⁺ T-cells activated by PMA plus calcium ionophore.

The proliferative response of activated CD4⁺ T-cells incubated in the presence of 1 mM lidocaine was significantly inhibited (SI: 14.9 ± 3.3 without lidocaine vs 7.0 ± 2.3 with 1 mM lidocaine, $P < 0.01$; Fig. 4a). Lidocaine (1 mM) also significantly inhibited the production of IL-5 (347.2 ± 108.7 pg/ml without lidocaine vs 184.8 ± 91.7 pg/ml with 1 mM lidocaine, $P < 0.01$) and IFN- γ (5298.0 ± 1788.4 pg/ml without lidocaine vs 2554.2 ± 1173.4 pg/ml with 1 mM lidocaine, $P < 0.01$) by CD4⁺ T-cells after stimulation (Fig. 4b).

Lidocaine induces apoptosis in activated CD4⁺ T-cells

Because lidocaine inhibited the proliferation and cytokine production of activated CD4⁺ T-cells, we investigated apoptosis in these cells. As seen with resting CD4⁺ T-cells, the percentage of apoptotic cells (Annexin V⁺ PI⁻) increased in activated CD4⁺ T-cells treated with 1 mM lidocaine ($15.1 \pm 8.1\%$ without lidocaine vs $20.1 \pm 7.9\%$ with 1 mM lidocaine, $P < 0.05$; Fig. 5).

Discussion

Our major findings in this study are that the local anesthetic lidocaine induced apoptosis in CD4⁺ T-cells by activating caspase-3 and caspase-9 and down-regulating Bcl-2. Moreover, lidocaine inhibited the proliferative response and production of Th1 (IFN- γ) and Th2 (IL-5) cytokines by CD4⁺ T-cells stimulated with PMA plus calcium ionophore, by increasing apoptosis.

Apoptosis has gained relevance as one of the specific aims of therapeutic approach in the treatment of bronchial asthma. Together with glucocorticosteroid and theophylline, the local anesthetic lidocaine was reported to induce apoptosis in eosinophils⁷⁾. Inhaled anesthetics, isoflurane and sevoflurane, were found to induce apoptosis in lymphocytes; however, whether local anesthetics can also have such an effect has not been fully established¹⁷⁻²²⁾. In the present study, we observed induction of apoptosis in CD4⁺ T-cells incubated with 1 mM lidocaine, which was the same concentration used by Okada et al. to induce apoptosis in eosinophils, suggesting that the inhibitory effects of lidocaine may be attributed to apoptosis⁷⁾.

Local anesthetics are divided into two major types depending on their binding property, the amide type, exemplified by lidocaine and dibucaine, and the ester type, represented by tetracaine. The specific actions of these local anesthetics may differ depending on this binding property; however, apoptosis was induced in CD4⁺ T-cells regardless of the type of the anesthetics used. Local anesthetics are known to directly interact with voltage-gated Na⁺ channels preventing Na⁺ ion from entering the cell during depolarization²³⁾. Although the blockade of sodium channels is the proposed mode of action of lidocaine, induction of apoptosis by lidocaine is independent of sodium channel inhibition because tetrodotoxin, a selective voltage-gated sodium channel blocker, failed to inhibit apoptosis. Therefore, the mechanism of apoptosis induced by lidocaine may not be affected by its binding property or mediated through its action on the sodium channels, but rather, lidocaine-induced apoptosis may occur through other mechanisms.

The caspase family of proteases is known to be the central operator of apoptosis²⁴⁾. There are two signal pathways in apoptosis that both result in activation of caspase-3, the mitochondria-independent and the mitochondria-dependent pathways. The former pathway involves a signal from the death receptor (Fas/CD95, tumor necrosis factor receptor) and

triggers caspase-8, which in turn activates caspase-3. The latter pathway involves a death stimulus, such as stress and ischemia, which increases the permeability of the mitochondrial membrane and results in the release of cytochrome c. The cytochrome c released from the mitochondria promotes caspase-9 activation leading to activation of caspase-3. Local anesthetics including lidocaine induce human renal cell apoptosis by the activation of the caspase family²⁵⁾. In this study also, we have demonstrated the activation of both caspase-3 and -9 in CD4⁺ T-cells after incubation with lidocaine. Because lidocaine did not activate caspase-8, the mitochondria-independent pathway is not involved in lidocaine-induced apoptosis. Bcl-2, which promotes cell survival, is one of the several endogenous molecules involved in the regulation of mitochondrial permeability and is part of the mitochondria-dependent pathway²⁶⁾. We observed that treatment of CD4⁺ T-cells with lidocaine resulted in decrease of Bcl-2. Therefore, our results suggest that the mitochondria-dependent pathway may be one of the mechanisms involved in lidocaine-induced apoptosis of CD4⁺ T-cells.

The stimulation of T-cells by PMA plus calcium ionophore increases FasL expression and activates caspase-8, leading to apoptosis; this phenomenon is known as activation-induced cell death²⁷⁾. In parallel with the occurrence of activation-induced cell death in activated CD4⁺ T-cells, 1 mM lidocaine further increased the number of apoptotic cells; thus, 1 mM lidocaine induced apoptosis in both resting and activated CD4⁺ T-cells. Consequently, inhibition of CD4⁺ T-cell proliferation and cytokine production by 1 mM lidocaine may be due in part to apoptosis.

Lidocaine is frequently used as a local anesthetic and anti-ventricular arrhythmic agent. The blood concentration of lidocaine after treatment of ventricular arrhythmia was reported to be 7.5–18.5 μ M, while the concentration of lidocaine in bronchoalveolar lavage fluid used as an anesthetic for bronchofiberscopy was detected to be 1.7–10.7 mM²⁸⁾. Because the concentration of lidocaine in the airway after inhalation therapy may be less than that used as an anesthetic for bronchofiberscopy, we used lidocaine at concentrations up to 1 mM in the present study. Although no clinical studies have measured the local concentration of lidocaine in the airway after nebulization, a recent randomized placebo-controlled study showed that 4% (148 mM) of nebulized lidocaine is safe and effective in patients with mild to moderate asthma²⁹⁾. Therefore, 1 mM lidocaine could be delivered in the airway after nebulization.

In conclusion, the local anesthetic lidocaine induces apoptosis in CD4⁺ T-cells of patients with bronchial asthma by activating caspase-3 and caspase-9 and down-regulating Bcl-2. Therefore, lidocaine may be useful in the treatment of steroid-dependent asthma by inducing apoptosis in CD4⁺ T-cells.

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