turned down a number of patients for LDLLT because of an unacceptable size mismatch. When grafts are too small, a limited amount of vascular bed might cause high pulmonary artery pressure, resulting in lung edema. Intrathoracic dead space can remain and cause complications, such as post-operative bleeding, persistent air leakage and empyema.[3] Moreover, hyperinflation of the grafted lungs may result in insufficient respiratory dynamics or hemodynamic collapse after LDLLT.[4]

Sugimoto et al demonstrated a technical feasibility of sparing native upper lobes using a canine lung transplant model.[5] Yamane et al reported a clinical case in which the right upper lobe with a relatively normal parenchyma was spared in LDLLT for severe pulmonary emphysema.[6] Our primary purpose for sparing the bilateral native upper lobes in the present case was not to preserve native lung function but to reduce intrathoracic dead space, and thus to provide adequate chest cavity for small grafts. As post-operative perfusion/ventilation scintigraphy demonstrated, native spared lungs showed marked air trapping and received very little blood flow. In contrast, the implanted small grafts received a majority of blood flow and provided sufficient lung function.

Candidates for this approach should have no infection in the spared lobes and minimum pleural adhesion with well-developed interlobar fissures. Considering these factors, a space-occupying, non-infectious disease, such as bronchiolitis obliterans, would be an ideal indication. Pulmonary fibrosis, pulmonary hypertension, emphysema and lymphangioleiomyomatosis may also be possible indications.

King et al reported that 25 (13.9%) of 180 patients who underwent single-lung transplantation developed significant native lung complications, such as pneumothoraces, malignancy, aspergilloma, pneumonia, bronchopleural fistulas and pulmonary embolism.[7] Therefore, the present technique of sparing bilateral native upper lobes is also potentially related to a risk of post-operative complications. Further careful observation is needed in our case. In terms of management during the operation, we were careful to continue ventilation of the spared upper lobes to avoid collapse of the lungs and subsequent shunt formation. Collapsed lungs with severe bronchiolar stenosis due to bronchiolitis obliterans would require a high airway pressure for reinflation and would disturb weaning from CPB.

In conclusion, we have reported the first case of bilateral native upper lobe–sparing LDLLT. Although the indications for this approach to LDLLT must be carefully selected, this new technique can provide an encouraging strategy for larger patients who have been contraindicated thus far because of size mismatches between graft lungs and the thoracic cavity.

Disclosure statement

The authors have no conflicts of interest to disclose.

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Immunopharmacology and Inflammation

Procaterol inhibits rhinovirus infection in primary cultures of human tracheal epithelial cells

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ABSTRACT

β_2 agonists reduce the frequency of exacerbations in patients with bronchial asthma and chronic obstructive pulmonary disease caused by respiratory virus infection. β_2 agonists reduce the production of pro-inflammatory cytokines. However, the inhibitory effects of β_2 agonists on the infection of rhinovirus, the major cause of exacerbations, have not been well studied. To examine the effects of a β_2 agonist, procaterol, on rhinovirus infection and rhinovirus infection-induced airway inflammation, human tracheal epithelial cells were infected with a major group rhinovirus, type 14 rhinovirus. Rhinovirus infection increased viral titers and the content of pro-inflammatory cytokines, including interleukin-1β and interlukin-6, in supernatant fluids and rhinovirus RNA in the cells. Procaterol reduced rhinovirus titers and RNA, cytokine concentrations, and susceptibility to rhinovirus infection. Procaterol reduced the expression of intercellular adhesion molecule-1 (ICAM-1), the receptor for type 14 rhinovirus, and the number of acidic endosomes in the cells from which rhinovirus RNA enters into the cytoplasm. Procaterol inhibited the activation of nuclear factor kappa-B (NF-κB) proteins including p50 and p65 in the nuclear extracts, while it increased the cytosolic amount of the inhibitory kappa B-α and intracellular cyclic AMP (cAMP) levels. A selective β_2-adrenergic receptor antagonist ICI 118551 [erythro-*dl*-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol] reversed the inhibitory effects of procaterol on rhinovirus titers and RNA, susceptibility to rhinovirus infection, pro-inflammatory cytokines production, ICAM-1 expression, acidic endosomes, and NF-κB. ICI 118551 also reversed the effects of procaterol on cAMP levels. Procaterol may inhibit rhinovirus infection by reducing ICAM-1 and acidic endosomes as well as modulate airway inflammation in rhinovirus infection.

1. Introduction

Rhinoviruses are the major cause of the common cold as well as of the most common acute infection illnesses in humans (Turner and Couch, 2006). They are also associated with exacerbations of inflammatory chronic pulmonary diseases such as bronchial asthma (Johnston et al., 1995) and chronic obstructive pulmonary disease (COPD) (Seemungal et al., 2000). Several mechanisms of rhinovirus-induced exacerbations of these diseases have been proposed, including virus-induced mucus hypersecretion, airway inflammation (Pizzichini et al., 1998; Seemungal et al., 2000), mast cell activation, and smooth muscle contraction.

Short-acting β_2 agonists as well as long-acting β_2 agonists improve the symptoms and lung function in patients with bronchial asthma and COPD. Furthermore, long-acting β_2 agonists themselves, or in combination with inhaled corticosteroids, reduce the frequency of exacerbations in patients with bronchial asthma (Barnes, 2007) and COPD (Calverley et al., 2007). It has been suggested that these clinical benefits of β_2 agonists are related to various effects of the agents, including the bronchodilating and anti-inflammatory effects (Johnson, 1991), improvement of mucociliary clearance and mucosal edema, and inhibition of mucus hypersecretion (Rogers and Barnes, 2006).

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Rhinovirus infection induces the production of cytokines and monokines including interleukin-1, interleukin-6, and interleukin-8 (Subauste et al., 1995; Zhu et al., 1996). These cytokines and monokines have pro-inflammatory effects (Akira et al., 1990) and may also be involved in the pathogenesis of rhinovirus infections. On the other hand, Edwards et al. (2006, 2007) demonstrated that a β_2 agonist, salmeterol, increased interleukin-6 production in bronchial epithelial cell line (BEAS-2B) cells and primary cultures of normal bronchial epithelial cells, and increased CXCL5 in primary cells after rhinovirus infection. By contrast, in primary cultures of normal bronchial epithelial cells, salmeterol alone reduced the rhinovirus-induced production of RANTES (regulated on activation, normal T cells expressed and secreted/CCL5) and interferon-γ-inducible protein 10 (IP-10/CXCL10), one of the chemokines. Furthermore, salmeterol had no effect on interleukin-8 production in BEAS-2B cells and in primary cells (Edwards et al., 2006).

The major group of rhinoviruses enters the cytoplasm of infected cells after binding to its receptor intercellular adhesion molecule (ICAM)-1 (Casasnovas and Springer, 1994; Greve et al., 1989). The entry of the RNA from this group into the cytoplasm of infected cells is suggested to be mediated by destabilization from receptor binding and by endosomal acidification (Casasnovas and Springer, 1994). Glucocorticoid (Suzuki et al., 2000), macrolide antibiotics bafilomycin (Pérez and Carrasco, 1993) and erythromycin (Suzuki et al., 2002), and a proton pump inhibitor lansoprazole (Sasaki et al., 2005) inhibit the infection of the major group of rhinoviruses through the reduction of ICAM-1 expression or an increased endosomal pH. β_2 agonists fenoterol and procaterol reduce ICAM-1 expression in the human bronchial epithelial cells and human lung fibroblasts, respectively (Oddera et al., 1998; Yoshida et al., 2009). However, the inhibitory effects of β_2 agonists on rhinovirus infection are still uncertain.

We studied the effects of a β_2 agonist, procaterol hydrochloride, on rhinovirus infection in the primary cultures of human airway epithelial cells. We also examined the effects of procaterol on the production of ICAM-1 and on the endosomal pH to clarify the mechanisms responsible for the inhibition of rhinovirus infection.

2. Materials and methods

2.1. Human tracheal epithelial cell culture

Human tracheal surface epithelial cells were isolated and cultured as described previously (Yamaya et al., 2007). The cells were plated at 5×10^5 viable cells/ml in plastic tubes with round bottoms (16 mm in diameter and 125 mm in length; Becton Dickinson, Franklin Lakes, NJ, USA) coated with human placental collagen. The plastic tubes were fixed in an inclined stainless-steel tube rack (30 cm wide, 10 cm high and 10 cm deep, TE-HER TUBE RACK INCLINABLE® RF-6; Hirasawa Works Co. Ltd., Tokyo, Japan), which was placed in a humid incubator. The long axis of the tubes was at a 5° angle with the flat bottom plate of the incubator. The tubes were kept stationary, and the cells were immersed in 1 ml of Dulbecco's modified Eagle's Medium (DMEM)-Ham's F-12 medium (50/50, vol/vol) containing 2% ultroser G (USG; Pall BioSepra, Cergy-Saint-Christophe, France) and cultured at 37 °C in 5% CO_2–95% air in the incubator.

In the preliminary experiments, we found that rhinovirus titers in the human tracheal epithelial cells cultured via rolling were much higher than those in the cells cultured in a stationary condition (data not shown). Therefore, as described below, to study the effects of procaterol on the release of rhinovirus and cytokines, rhinovirus RNA replication, NF-κB activation after rhinovirus infection, as well as susceptibility to virus infection, we cultured the epithelial cells in the tubes by rolling. By contrast, acidic endosomes and cellular cAMP levels could be observed in the cells living on coverslips in Petri dishes and in the cells cultured in 96-well plates, respectively, in a stationary condition. Therefore, cells used for measurement of acidic endosomes and cAMP were cultured in a stationary condition.

Tracheas for cell cultures were obtained after death from 41 patients (age; 73 ± 3 yr; 15 females, 26 males). All patients were not complicated with bronchial asthma, while three patients were complicated with chronic obstructive pulmonary disease (COPD). The causes of death were malignant tumors other than lung cancer (n = 21), acute myocardial infarction (n = 4), renal failure (n = 3), congestive heart failure (n = 4), malignant lymphoma (n = 3), cerebral bleeding (n = 2), rupture of an aortic aneurysm (n = 1), cerebral infarction (n = 1), sepsis (n = 1), and mitral stenosis (n = 1). Of 41 patients, 15 were ex-smokers and 26 had never smoked. This study was approved by the Tohoku University Ethics Committee.

2.2. Culture of human embryonic fibroblast cells

Human embryonic fibroblasts, HFL-III, were obtained from Riken Bio Resource Center Cell Bank (Cell No: RCB0523; Tsukuba, Japan). The cells, derived from human embryo lungs, are fibroblast-like in shape. Human embryonic fibroblast cells were cultured in flasks (25 cm^2 of surface area; Becton Dickinson) and then plated in plastic dishes (MICROTEST™ Tissue Culture Plate, 96 well; Becton Dickinson) or in plastic tubes with round bottoms (16 mm in diameter and 125 mm in length; Becton Dickinson) and cultured at 37 °C in 5% CO_2–95% air as previously described (Yamaya et al., 2007) with some modification.

2.3. Viral stocks

Type 14 rhinovirus stocks were prepared from a patient with a common cold by infecting human embryonic fibroblast cells as previously described (Numazaki et al., 1987).

2.4. Detection and titration of viruses

Type 14 rhinoviruses in supernatant fluids were detected and titrated using the endpoint methods (Condit, 2006) by infecting replicate confluent human embryonic fibroblast cells in plastic 96-well plates (Becton Dickinson) with serial 10-fold dilutions of virus-containing supernatant fluids as previously described, with some modification. Virus-containing supernatant fluids were added into the replicate human embryonic fibroblast cells in the wells (200 μl/well) of 96-well plates. Human embryonic fibroblast cells in the wells were then cultured at 33 °C in 5% CO_2–95% air, and the presence of the typical cytopathic effects of rhinovirus was observed in all replicate cells for 7 days (168 h) as described previously (Yamaya et al., 2007). Based on these data, $TCID_{50}$ (tissue culture infective dose) was calculated as previously described (Condit, 2006). Because the human tracheal epithelial cells were cultured in 1 ml of medium in the tubes, viral titers in supernatant fluids are expressed as $TCID_{50}$ units/ml (Yamaya et al., 2007). Furthermore, the rates were obtained by dividing the value of viral titer ($TCID_{50}$ units/ml) in supernatant fluids by incubation time and are expressed as $TCID_{50}$ units/ml/24 h (Yamaya et al., 2007).

2.5. Quantification of rhinovirus RNA

To quantify the rhinovirus RNA and ribosomal RNA (18S, rRNA) expression in the human tracheal epithelial cells after rhinovirus infection, two-step real-time quantitative reverse transcription (RT)-PCR using the Taqman technique (Roche Molecular Diagnostic Systems) was performed with a TaqMan® Gene Expression Master Mix (Applied Biosystems, Bedford, CA, USA) as previously described (Nolan et al., 2006), with some modification, using RNA extracted from the cells. Extracted RNA from the cells was divided into 10 ng of aliquots, and the amount of rhinovirus RNA or rRNA was measured by

using each 10 ng aliquot of extracted RNA from the same cells. To measure the amount of rhinovirus RNA and rRNA, RNA extracted from the cells was converted to cDNA, and the amount of cDNA for rhinovirus RNA and rRNA was measured. TaqMan technology exploits the 5′–3′ nucleolytic activity of AmpliTaq Gold® DNA Polymerase. In principle, the method uses a dual-labeled fluorogenic hybridization probe, a TaqMan probe that specifically anneals the template between the PCR primers.

In the first step in the quantification of rhinovirus RNA, cDNA from rhinovirus RNA was made using the QuantiTect Reverse Transcription Kit (Quiagen). In brief, an extracted aliquot containing RNA (10 ng/5 μl) was mixed with gDNA Wipeout Buffer (2 μl) and RNAase-free water (2 μl) and incubated at 42 °C for 2 min. The mixture (9 μl) was further mixed with Quantiscript Reverse Transcriptase (1 μl), Quantiscript RT Buffer (4 μl), RT Primer Mix (1 μl), and rhinovirus reverse primer (5′-CGGACACCCAAAGTAGTCGGT-3′; 5 μl, to make cDNA from rhinovirus RNA). This mixture was incubated at 42 °C for 15 min and then at 95 °C for 3 min using GeneAmp® PCR System 9700 (Applied Biosystems).

Likewise, in the second step in the quantification of rhinovirus RNA, real-time PCR was performed using cDNA from rhinovirus RNA with a TaqMan® Gene Expression Master Mix. The probe contains a fluorescent reporter (6-carboxyfluorescein [FAM]) at the 5′ end and a fluorescent quencher (6-carboxytetramethylrhodamine [TAMRA]) at the 3′ end. According to the progression of PCR, the TaqMan probe is degraded and releases the reporter, resulting in an increase in fluorescence emission. The use of a sequence detector (ABI PRISM 7700; Applied Biosystems) allows measurement of the amplified product in direct proportion to the continuous increase in fluorescence emission during PCR. Briefly, a cDNA sample (2 μl) made at the first step was mixed with TaqMan Gene Expression Master Mix (10 μl), forward primer (5′-GCACTTCTGTTTCCCAGGAGC-3′; 0.5 μl), reverse primer (5′-CGGACACCCAAAGTAGTCGGT-3′; 0.5 μl), Taqman probe type 14 rhinovirus (5′-[FAM] CCTTTAACCGTTATCCGCCA [TAMRA]-3′; 0.5 μl), and RNAase-free water (6.5 μl). cDNA made for rhinovirus RNA was amplified by PCR for 45 cycles (15 s at 95 °C and 1 min at 60 °C). We used the PrimerExpress program (version 3.0; Applied Biosystems) to design the probe and primers according to the guidelines for the best performance of the PCR. Taqman probe type 14 rhinovirus was designed for type 14 rhinovirus based on a previous report (Terajima et al., 1997). Whole reactions of the RT-PCR and detection of the fluorescence emission signal for every PCR cycle were performed at the same time in a single tube in a sequence detector (ABI 7700).

In the quantification of rRNA, conversion of rRNA to cDNA and real-time PCR were also performed using the same two-step process of the methods described above. To quantify rRNA expression, a forward primer (5′-GCACTTCTGTTTCCCAGGAGC-3′), reverse primer (5′-CGGACACCCAAAGTAG TCGGT-3′), and Taqman probe (5′-[FAM] CCTTTAACCGTTATCCGCCA [TAMRA]-3′] were designed for rRNA.

To obtain the quantitative data, the minimum PCR cycle to detect the fluorescent signal was defined as the cycle threshold (Cτ) in rhinovirus RNA and rRNA from the cells. To normalize the Taqman data using rRNA, the calculations for the quantification were performed by getting the difference (ΔCτ) between the Cτ values of the target (rhinovirus) and the reference (rRNA). The difference of ΔCτ value was also calculated between the cells treated with procaterol and the cells treated with vehicle. To calculate the absolute value, the difference of ΔCτ value was transformed according to the comparative (ΔΔCτ) method described in the manufacturer's instructions.

2.6. Viral infection of the epithelial cells

Infection of type 14 rhinovirus to human tracheal epithelial cells was performed with methods previously described (Yamaya et al., 2007). A stock solution of type 14 rhinovirus (100 μl in each tube, 1.0×10^{4} $TCID_{50}$ units/100 μl, 5.0×10^{-2} $TCID_{50}$ units/cell) was added to the human tracheal epithelial cells in the tubes. After a 1-h incubation at 33 °C in 5% CO_2–95% air, the viral solution was removed, and the epithelial cells were rinsed once with 1 ml of phosphate-buffered saline. The cells were then fed with 1 ml of fresh medium containing 2% USG. The opening of the tubes was sealed with rubber plugs and cells in the tubes were cultured at 33 °C by rolling in an incubator.

2.7. Treatment with procaterol

To examine the effects of procaterol, cultured human tracheal epithelial cells from the same donors were treated with either procaterol hydrochloride (0.1 μM, Sigma) (Koyama et al., 1999) or a vehicle (ethanol, 0.01%) from 3 days (72 h) before type 14 rhinovirus infection until the end of the experiments after type 14 rhinovirus infection (Yamaya et al., 2007), except where we describe other concentrations or treatment periods.

To examine the concentration-dependent effects of procaterol on type 14 rhinovirus infection and acidic endosomes, cells were treated with procaterol at concentrations ranging from 1 nM to 10 μM. Likewise, to examine the time-dependent effects of procaterol on acidic endosomes, cells were treated with procaterol (0.1 μM) for time periods ranging from 0 to 3 days (72 h).

To examine the effects of a selective β_2-adrenergic receptor antagonist ICI 118551 [erythro-*dl*-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol] (Bilski et al., 1983) on the inhibitory effects of procaterol on type 14 rhinovirus titers, cells were pretreated with ICI 118551 (1 μM, Sigma) for 10 min (Bilski et al., 1983). Then they were treated with ICI 118551 (1 μM) in the presence of procaterol (0.1 μM) from 3 days (72 h) before type 14 rhinovirus infection until the end of the experiments after infection.

To examine the effects of procaterol on ICAM-1 mRNA expression in the cells and concentration of a soluble form of ICAM-1 (sICAM-1) in supernatant fluids, cells were pretreated with procaterol (0.1 μM) from 3 days (72 h) before type 14 rhinovirus infection and supernatant fluids were collected and RNA in the cells were extracted just before type 14 rhinovirus infection.

2.8. Collection of supernatant fluids for measurements

We measured the time course of viral release with methods as previously described (Yamaya et al., 2007) with some modification. To measure type 14 rhinovirus release during the first 24 h, we used three separate cultures from the same trachea. We collected the supernatant fluids at either 1, 12, or 24 h after type 14 rhinovirus infection. We also collected supernatant fluids at 3 days (72 h), 5 days (120 h), and 7 days (168 h). At 1 day (24 h), 3 days (72 h) and 5 days (120 h) after infection, supernatant fluids were collected and tracheal epithelial cells were rinsed with PBS, 1 ml of fresh medium containing 2% USG was replaced, and the cell culture was continued. Supernatant fluids were also collected at 7 days (168 h) after infection to measure the release of rhinovirus.

Likewise, to examine the effects of procaterol on the secretion of interleukin-1β, interleukin-6, and interleukin-8, supernatant fluids were collected just before infection, and 1 day (24 h), 3 days (72 h) and 5 days (120 h) after type 14 rhinovirus infection.

2.9. Effects of procaterol on susceptibility to rhinovirus infection

The effects of procaterol on the susceptibility to type 14 rhinovirus infection were evaluated as previously described (Yamaya et al., 2007). Epithelial cells were pretreated with procaterol (0.1 μM) or vehicle (ethanol, 0.01%) from 3 days (72 h) before infection with type 14 rhinovirus until just finishing the infection. The epithelial cells

were exposed to serial 10-fold dilutions of type 14 rhinovirus at a dose ranging from 10^1 to $10^5 TCID_{50}$ units/ml, containing procaterol or vehicle for 1 h at 33 °C. After exposure to type 14 rhinovirus, cells were rinsed with PBS, and a fresh medium without the addition of procaterol was replaced. Cells in the tubes were then cultured at 33 °C by rolling in an incubator.

We collected the supernatant fluids at 1, 12, and 24 h after type 14 rhinovirus infection with the methods described above (Collection of supernatant fluids for measurements). Supernatant fluids were also collected at 3 days (72 h), 5 days (120 h) and 7 days (168 h) after type 14 rhinovirus infection.

Because we found in the preliminary experiments that the supernatant fluids collected at 3 days (72 h) after infection showed maximum viral titers, we measured the rhinovirus titers in the supernatant fluids collected at 3 days (72 h) after infection with the human embryonic fibroblast cell assay described above in order to assess whether infection occurred at each dose (10^1, 10^2, 10^3, 10^4 or $10^5 TCID_{50}$ units/ml) of type 14 rhinovirus used. After addition of virus-containing supernatant fluids to the fibroblast cells, the presence of the typical cytopathic effects of rhinovirus was observed for 7 days (168 h) (Yamaya et al., 2007).

2.10. Measurement of ICAM-1 expression

The mRNA of ICAM-1 was examined with the two-step real-time RT-PCR analysis with the methods described above (Quantification of rhinovirus RNA), by using a forward primer (5′-GCACTTCTGTTTCCCAG-GAGC-3′) and a reverse primer (5′-CGGACACCCAAAGTAG TCGGT-3′). Taqman probe (5′-[FAM] CCTTTAACCGTTATCCGCCA [TAMRA]-3′] was designed for ICAM-1. The expression of ICAM-1 mRNA was also normalized to the constitutive expression of rRNA. Concentrations of sICAM-1 in supernatant fluids were measured with the enzyme immunoassay (EIA) (Yamaya et al., 2007).

2.11. Measurement of changes in acidic endosomes

The distribution and the fluorescence intensity of acidic endosomes in the cells were measured as previously described with a dye, LysoSensor DND-189 (Molecular Probes, Eugene, OR, USA) (Suzuki et al., 2001). Live-cell imaging was performed. The cells on coverslips in Petri dishes were observed with a fluorescence microscope (OLYMPUS IX70; OLYMPUS Co. Ltd., Tokyo, Japan). The excitation wavelength was 443 nm, and the emitted light from the cells was detected through a 505-nm filter. The fluorescence intensity was calculated using a fluorescence image analyzer system (Lumina Vision®; Mitani Co. Ltd., Fukui, Japan) equipped with a fluorescence microscope. Fluorescence intensity of acidic endosomes was measured in 100 human tracheal epithelial cells, and the mean value of fluorescence intensity was expressed as a percentage of the control value compared with the fluorescence intensity of the cells before any treatment.

We studied the effects of a long period of treatment with procaterol (0.1 μM, 72 h) on acidic endosomes, because cells were pretreated with procaterol for 3 days (72 h) before type 14 rhinovirus infection. Furthermore, to examine the time-dependent effects of procaterol on acidic endosomes, cells were treated with procaterol (0.1 μM) for the time ranging from 0 h to 3 days (72 h).

To examine the concentration-dependent effects of procaterol on acidic endosomes, cells were treated with procaterol at concentrations ranging from 1 nM to 100 nM.

2.12. Measurement of cytokine production

We measured interleukin-1β, interleukin-6, and interleukin-8 of supernatant fluids by specific enzyme-linked immunosorbent assays (ELISAs) (Yamaya et al., 2007) in duplicate human tracheal epithelial cells in plastic tubes at all time points.

2.13. NF-kappa B assay

Nuclear extracts from human tracheal epithelial cells were prepared using a TransFactor extraction kit (BD Bioscience/CLONTECH, Mountain View, CA, USA) according to manufacturer's instructions. After centrifugation at 20,000×*g* for 5 min at 4 °C, nuclear extracts were assayed for p50, p65, and c-Rel content. An equal amount of nuclear lysate was added to incubation wells precoated with the DNA-binding consensus sequence. The presence of translocated p50, p65, and c-Rel subunit was assayed by using a TransFactor Family Colorimetric Kit-NFκB (BD Bioscience/CLONTECH) according to the manufacturer's instructions (Fiorucci et al., 2002). Plates were read at 655 nm and results are expressed as OD, which shows quantitative levels of NF-kB subunits (Fiorucci et al., 2002).

2.14. Western blot analysis

Western blot analysis for the degradation of inhibitory kappa B-α (IκB-α) and the analysis of the amount of phosphorylated IκB-α (p-IκB-α) and β-actin were performed with the methods described previously (Kim et al., 2008) with some modification. Total cellular proteins from human tracheal epithelial cells before and after type 14 rhinovirus infection in the presence or absence of procaterol (0.1 μM) and a selective β_2-adrenergic receptor antagonist ICI 118551 (1 μM) were harvested and lysed in modified radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 0.25% sodium deoxycholate, 150 mM NaC1, 20 mM Tris, pH 7.5, and 10 mM EDTA) supplemented with phosphatase and protease inhibitors.

Protein concentrations were determined using the Bio-Rad protein assay reagent, according to the manufacturer's instructions. Twenty micrograms of cellular proteins from treated or untreated cell extracts were separated on 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes that were incubated for 2 h with blocking solution (5% skim milk) at 4 °C and then with primary antibody overnight. Primary antibodies used in this study were a monoclonal rabbit anti-phospho-IκB-α (Ser32) antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), a polyclonal rabbit anti-IκB-α (C-21) antibody (1:200 dilution; Santa Cruz Biotechnonogy, Santa Cruz, CA, USA), and a monoclonal mouse anti-β-actin antibody (1:10,000 dilution; Sigma-Aldrich, St. Louis, MO, USA). Blots were then washed four times with Tween 20/Tris-buffered saline (TTBS), incubated with one of the following secondary antibodies for 1 h at room temperature: peroxidase-conjugated anti-rabbit IgG (1:1000 dilution; DakoCytomation, Glostrup, Denmark) for p-IκB-α and IκB-α or peroxidase-conjugated anti-mouse IgG (1:10,000 dilution; Jackson Immuno Research Laboratories, Avondale, PA, USA) for β-actin. Blots were rewashed three times with TTBS, and chemiluminescence was detected using an Amersham ECL Plus western blotting detection kit (GE Healthcare, Waukesha, WI, USA) and a LAS-1000 lumino image analyzer (Fujifilm, Tokyo, Japan). The results were quantified using ImageJ 1.42 software (http://rsb.info.nih.gov/ij/). The data were obtained by dividing the results in each culture condition by the results of β-actin.

2.15. Cyclic AMP assay

Intracellular cyclic AMP (cAMP) levels were measured as previously reported (Chiulli et al., 2000) with the cAMP-Screen™ System (Applied Biosystems). Human tracheal epithelial cells, cultured in a 96-well plate, were treated with either procaterol (0.1 μM), ICI 118551 (1 μM), procaterol (0.1 μM) plus ICI 118551 (1 μM), or vehicle (0.01% ethanol) for either 5 min, 10 min, 20 min, or 3 days (72 h). Intracellular cAMP was then extracted by adding Assay/Lysis buffer to the cells in the wells, and the extracted samples were incubated at 37 °C for 30 min. The samples containing cAMP were incubated with anti-cAMP antibody in the wells of the 96-well plates at room

temperature for 60 min. The combined cAMP with anti-cAMP antibody, which was adhered to the bottom of the wells, was then rinsed with wash buffer, and 100 μl/well of CSPD®/Sapphire-II™ RTU substrate/enhancer solution was added to the wells and incubated for 30 min. The signal was measured in a luminometer (Luminoscan acent; ThermoFisher Scientific Co., MA, USA) for 1 s/well. The standard curve was obtained by using 10-fold dilutions of cAMP at the concentrations ranging from 0.6×10^{-3} pmol to 6×10^{3} pmol in each well of 96-well plates. Results are shown as pmol/well according to the standard curve and expressed as pmol/mg protein after being normalized to the amount of protein in the cells in the wells.

2.16. Statistical analysis

Results are expressed as means ± S.E.M. Statistical analysis was performed using a one-way analysis of variance (ANOVA). Subsequent post-hoc analysis was made using Bonferroni's method. For all analyses, values of $P<0.05$ were assumed to be significant. n refers to the number of donors (tracheae) from which cultured epithelial cells were used.

3. Results

3.1. Effects of procaterol on rhinovirus infection in human tracheal epithelial cells

Exposing confluent human tracheal epithelial cell monolayers to type 14 rhinovirus (5.0×10^{-2} $TCID_{50}$ units/cell) consistently led to infection. No detectable virus was revealed at 1 h after infection, but type 14 rhinovirus was detected in culture medium at 12 h, and the viral content progressively increased between 1 and 12 h after infection (Fig. 1). Evidence of continuous viral production was obtained by demonstrating that each of the supernatant fluids collected during either 12 h to 24 h (1 day), 1 day (24 h) to 3 days (72 h), 3 days (72 h) to 5 days (120 h), or 5 days (120 h) to 7 days (168 h) after infection contained significant levels of type 14 rhinovirus (Fig. 1). The viral titer levels in supernatant fluids increased significantly with time for the first 3 days (72 h) ($P<0.05$ by ANOVA). Furthermore, in the tracheal cells from subjects whose cells were infected with rhinovirus, the supernatant fluids collected during 1 (24 h) to 3 days (72 h) after infection contained consistent levels of type 14 rhinovirus (4.53 ± 0.18 log $TCID_{50}$ units/ml/24 h, n = 38).

Treatment of the cells with procaterol (0.1 μM) significantly decreased the viral titers of type 14 rhinovirus in supernatant fluids from 12 h after infection (Fig. 1). Furthermore, a selective β_2-adrenergic receptor antagonist ICI 118551 (1 μM) reversed the inhibitory effects of procaterol on type 14 rhinovirus titer levels (Fig. 1), whereas ICI 11851 alone did not change the titer levels (data not shown).

Type 14 rhinovirus titer levels in supernatant fluids of the cells, collected from 13 ex-smokers over 1 day (24 h) to 3 days (72 h) after infection, did not differ from those of 25 patients who had never smoked (4.58 ± 0.32 log $TCID_{50}$ units/ml/24 h *vs* 4.50 ± 0.21 log $TCID_{50}$ units/ml/24 h, respectively, $P>0.02$). Likewise, type 14 rhinovirus titer levels in supernatant fluids of the cells from three patients complicated with COPD did not differ from those from 35 patients without COPD (data not shown). No virus was detected in supernatant fluids after infection of ultraviolet (UV)-inactivated type 14 rhinovirus (data not shown).

Treatment with procaterol (0.1 μM) for 3 days (72 h) did not change the viability (99 ± 1% in procaterol *vs* 98 ± 1% in vehicle, n = 5, $P>0.50$), as assessed by the exclusion of trypan blue. Furthermore, until 7 days (168 h) after the start of cell culture, cells made confluent sheets in the tubes in both the culture medium alone and the medium containing procaterol at the same time point. The cell number of confluent sheets cultured in the medium supplemented with procaterol (0.1 μM) did not differ from that in the medium alone ($2.1\pm0.3\times10^{6}$ of cells/tube in procaterol *vs* $2.2\pm0.3\times10^{6}$ of cells/tube in vehicle, n = 5, $P>0.50$). When LDH concentrations in supernatant fluids 3 days (72 h) after procaterol treatment (0.1 μM) were measured, treatment with procaterol (0.1 μM) for 3 days (72 h) did not change the LDH concentration (32 ± 3 IU/l/24 h in procaterol *vs* 33 ± 3 IU/l/24 h IU/ml in vehicle, n = 5, $P>0.50$).

Procaterol inhibited type 14 rhinovirus infection concentration-dependently. The maximum effect was obtained at 0.1 μM, 1.0 μM, and 10 μM, and the minimum effect was obtained at 3 nM (Fig. 2).

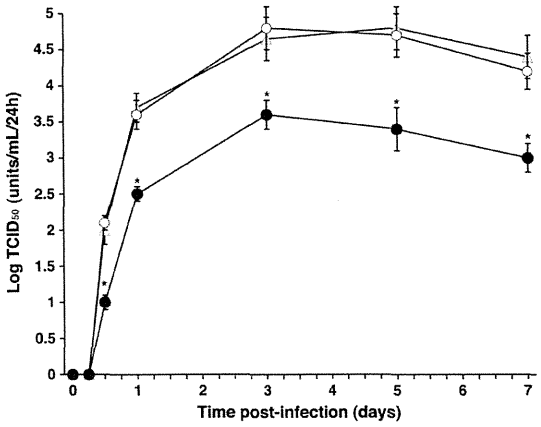


Fig. 1. The time course of viral release in supernatant fluids of human tracheal epithelial cells obtained at different times after exposure to 5.0×10^{-2} $TCID_{50}$ units/cell of type 14 rhinovirus in the presence of procaterol (0.1 μM) (closed circles), the vehicle of procaterol (ethanol, 0.01%) (open circles), or the presence of procaterol (0.1 μM) plus ICI 118551 (1 μM) (open triangles). The epithelial cells isolated from the same donors were treated with either procaterol, vehicle, or procaterol plus ICI 118551. To examine whether supernatant fluids contain significant amount of rhinovirus, cytopathic effects on human embryonic fibroblast cells were observed for 7 days (168 h) after placing the supernatant fluids on the fibroblast cells. The rates of change in type 14 rhinovirus concentration in the supernatant fluids are expressed as $TCID_{50}$ units/ml/24 h. Results are means ± S.E.M. from 6 different tracheae (2 ex-smokers and 4 non-smokers). Significant differences from viral infection alone are indicated by *$P<0.05$.

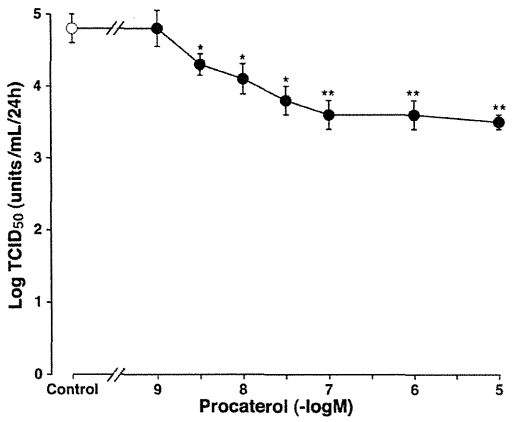


Fig. 2. Concentration-response effects of procaterol on the viral release in supernatant fluids collected over 1 day (24 h) to 3 days (72 h) after infection. The cells were treated with procaterol (closed circles) or vehicle (Control; ethanol, 0.01%, open circle) from 3 days (72 h) before type 14 rhinovirus infection until the end of the experiments after type 14 rhinovirus infection. The epithelial cells isolated from the same donors were treated with either procaterol or vehicle. In order to examine whether supernatant fluids contain significant amount of rhinovirus, cytopathic effects on human embryonic fibroblast cells were observed for 7 days (168 h) after placing the supernatant fluids on the fibroblast cells. The rates of change in type 14 rhinovirus concentration in the supernatant fluids are expressed as $TCID_{50}$ units/ml/24 h. Results are means ± S.E.M. from 10 different tracheae (4 ex-smokers and 6 non-smokers). Significant differences from vehicle alone (Control) are indicated by *$P<0.05$ and **$P<0.01$.

3.2. Effects of procaterol on viral RNA by real-time RT-PCR

Further evidence of the inhibitory effects of procaterol on type 14 rhinovirus RNA replication in human tracheal epithelial cells was provided by real-time quantitative RT-PCR analysis. The RNA extraction was performed at 1 day (24 h), 3 days (72 h) and 5 days (120 h) after type 14 rhinovirus infection. Type 14 rhinovirus RNA in the cells was consistently observed from 1 day (24 h) after infection, increased with time after infection (Fig. 3), and maximum rhinovirus RNA replication was observed at 3 days (72 h) after infection (data at 120 h not shown), whereas type 14 rhinovirus RNA in the cells was not observed before infection (data not shown). Procaterol (0.1 μM) decreased the type 14 rhinovirus RNA at 1 day (24 h) and at 3 days (72 h) after infection (Fig. 3).

On the other hand, ICI 118551 (1 μM) reversed the inhibitory effects of procaterol on the type 14 rhinovirus RNA replication, whereas ICI 118551 alone did not change it (Fig. 3). The amount of type 14 rhinovirus RNA in the cells treated with ICI 118551 (1 μM) alone did not differ from that in the cells treated with vehicle (ethanol, 0.01%) at 1 day (24 h) and at 3 days (72 h) after type 14 rhinovirus infection (Fig. 3). In contrast, the amount of type 14 rhinovirus RNA in the cells treated with procaterol (0.1 μM) plus ICI 118551 (1 μM) were significantly higher than that in the cells treated with procaterol alone and did not differ from that in the cells treated with vehicle at 1 day (24 h) and at 3 days (72 h) after type 14 rhinovirus infection (Fig. 3).

3.3. Effects of procaterol on susceptibility to rhinovirus infection

Treatment of the cells with procaterol (0.1 μM) decreased the susceptibility of the cells to infection by type 14 rhinovirus. When viral release was measured using supernatant fluids collected 3 days (72 h) after rhinovirus infection, the minimum dose of type 14 rhinovirus necessary to cause infection in the cells treated with procaterol (0.1 μM, 72 h) (3.2 ± 0.2 log $TCID_{50}$ units/ml, $n = 5$, $P<0.05$) was significantly higher than that in the cells treated with the vehicle of procaterol (ethanol, 0.01%) (2.2 ± 0.2 log $TCID_{50}$ units/ml, $n = 5$) (Fig. 4A). Likewise, when viral release was measured using supernatant fluids collected 5 days (120 h) and 7 days (168 h) after type 14 rhinovirus infection, the minimum dose of rhinovirus necessary to cause infection in the cells treated with procaterol and that in the cells treated with the vehicle of procaterol were the same values as those in supernatant fluids collected at 3 days (72 h) after rhinovirus infection (data not shown).

A selective β_2-adrenergic receptor antagonist ICI 118551 (1 μM) itself did not change the minimum dose of type 14 rhinovirus necessary to cause infection in the cells, and did not affect the susceptibility (data not shown). In contrast, ICI 118551 reversed the effects of procaterol on susceptibility to type 14 rhinovirus infection. Treatment of the cells with ICI 118551 (1 μM, 72 h) plus procaterol

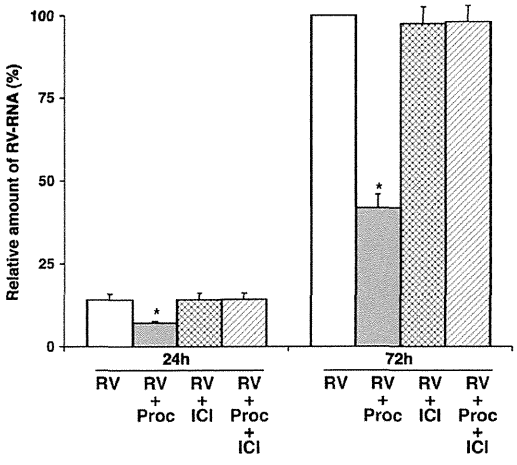


Fig. 3. Replication of viral RNA in human tracheal epithelial cells at 1 day (24 h) or 3 days (72 h) after infection with type 14 rhinovirus in the presence of procaterol (0.1 μM) (RV + Proc), vehicle (0.01% ethanol) (RV), ICI 118551 (1 μM) (RV + ICI), or the presence of procaterol (0.1 μM) plus ICI 118551 (1 μM) (RV + Proc + ICI) as detected by real-time quantitative RT-PCR. The epithelial cells isolated from the same donors were treated with either procaterol, vehicle, ICI 118551, or procaterol plus ICI 118551. Results are expressed as the relative amount of RNA expression (%) compared with that of maximal rhinovirus RNA at day 3 (72 h) in the cells treated with vehicle, and reported as means ± S.E.M. from five samples (2 ex-smokers and 3 non-smokers). Significant differences from treatment with a vehicle (RV) at each time are indicated by $^*P<0.05$.

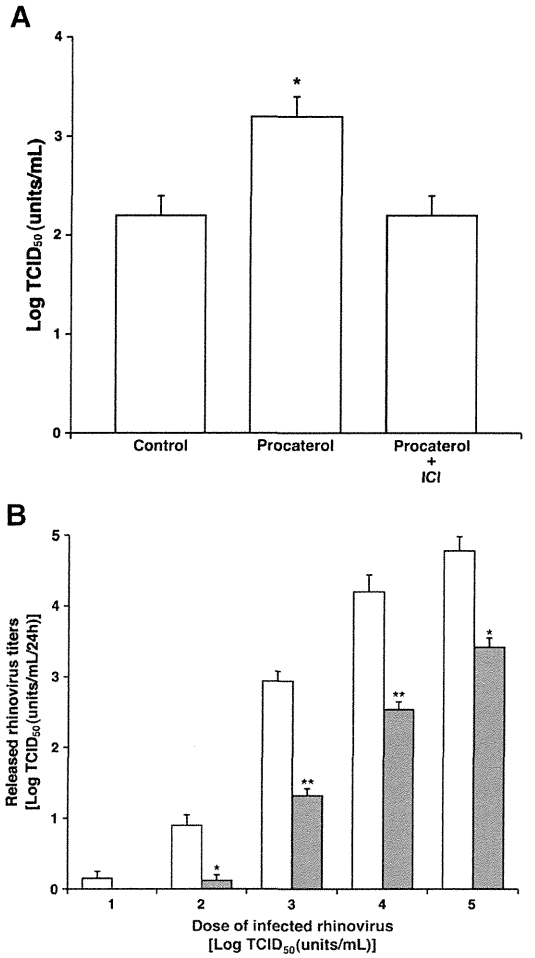


Fig. 4. (A) The minimum dose of type 14 rhinovirus necessary to cause infection in the human tracheal epithelial cells treated with either procaterol (0.1 μM, 72 h), vehicle (Control, 0.01% ethanol), or procaterol (0.1 μM) plus ICI 118551 (1 μM) (Procaterol + ICI). The epithelial cells isolated from the same donors were treated with either procaterol, vehicle, or procaterol plus ICI 118551. In order to examine whether supernatant fluids contain significant amount of rhinovirus, cytopathic effects on human embryonic fibroblast cells were observed for 7 days (168 h) after placing the supernatant fluids on the fibroblast cells. The minimum dose of type 14 rhinovirus necessary to cause infection is expressed as $TCID_{50}$ units/ml. Results are means ± S.E.M. from seven different tracheae (2 ex-smokers and 5 non-smokers). Significant differences from vehicle alone (Control) are indicated by $^*P<0.05$. (B) Viral release in supernatant fluids collected during 24 h (1 day) to 72 h (3 days) after infection with type 14 rhinovirus at a dose ranging from 10^1 to $10^5 TCID_{50}$ units/ml in the presence of procaterol (0.1 μM) (gray columns) or the vehicle of procaterol (ethanol, 0.01%) (open columns). The rates of change in type 14 rhinovirus concentration in the supernatant fluids are expressed as $TCID_{50}$ units/ml/24 h. Results are means ± S.E.M. from seven different tracheae (2 ex-smokers and 5 non-smokers). Significant differences from viral infection alone at each dose of type 14 rhinovirus are indicated by $^*P<0.05$ and $^{**}P<0.01$.

(0.1 μM, 72 h) decreased the minimum dose of type 14 rhinovirus necessary to cause infection in the cells (2.2 ± 0.2 log $TCID_{50}$ units/ml, $n = 5$) as compared with the dose in the cells treated with procaterol ($P<0.05$) (Fig. 4A), to the levels in the cells treated with the vehicle of procaterol (ethanol, 0.01%) (Fig. 4A).

Type 14 rhinovirus titer levels in supernatant fluids collected at 3 days (72 h) after rhinovirus infection increased with the dose of rhinovirus infected to epithelial cells ($P<0.05$ by ANOVA) (Fig. 4B) Furthermore, treatment with procaterol (0.1 μM, 72 h) reduced the type 14 rhinovirus titers in supernatant fluids at each dose of rhinovirus infection (Fig. 4B).

3.4. Effects of procaterol on the expression of ICAM-1

Procaterol (0.1 μM, 72 h) reduced the baseline ICAM-1 mRNA expression in the cells by about 40% compared with that of the cells treated with the vehicle of procaterol (ethanol, 0.01%) before type 14 rhinovirus infection (Fig. 5A). Furthermore, concentrations of sICAM-1 in supernatant fluids in the cells treated with procaterol (0.1 μM) were significantly lower than those in the cells treated with the vehicle of procaterol before type 14 rhinovirus infection (Fig. 5B).

ICI 118551 (1 μM) itself did not change ICAM-1 mRNA expression and sICAM-1 release in supernatant fluids (Fig. 5A and B). In contrast, ICI 118551 (1 μM) reversed the inhibitory effects of procaterol on the ICAM-1 mRNA expression in the cells and sICAM-1 release in supernatant fluids (Fig. 5A and B). The ICAM-1 mRNA expression and concentrations of sICAM-1 in supernatant fluids in the cells treated with procaterol (0.1 μM) plus ICI 118551 (1 μM) were significantly higher than those in the cells treated with procaterol (0.1 μM) alone and did not differ from those in the cells treated with the vehicle (ethanol, 0.01%) before type 14 rhinovirus infection (Fig. 5A and B).

3.5. Effects of procaterol on the acidification of endosomes

Acidic endosomes in human tracheal epithelial cells were stained green with LysoSensor DND-189 (Fig. 6A–C) as shown previously (Yamaya et al., 2007). Treatment with the vehicle (ethanol, 0.01%) for 3 days (72 h) did not change the number of acidic endosomes with green fluorescence in the cells (Fig. 6A and B) and the fluorescence intensity from acidic endosomes compared with that in the cells before any treatment (Fig. 6D). In contrast, treatment with procaterol (0.1 μM, 72 h) reduced the number of acidic endosomes with green fluorescence in the cells (Fig. 6C) and the fluorescence intensity from acidic endosomes in the cells compared with cells treated with vehicle of procaterol (ethanol, 0.01%) and before any treatment (Fig. 6D).

Furthermore, treatment of the cells with ICI 118551 (1 μM, 72 h) reversed the inhibitory effects of procaterol on the number of acidic endosomes with green fluorescence in the cells (data not shown) and the fluorescence intensity from acidic endosomes, while ICI 118551 alone did not change the fluorescence intensity (Fig. 6D).

The inhibitory effects of procaterol on the fluorescence intensity from acidic endosomes were time- and dose-dependent, and significant inhibitory effects were observed when cells were treated with procaterol (0.1 μM) for the time of 12 h or more (Fig. 6E). The maximum inhibitory effect was obtained when cells were treated with procaterol for 3 days (72 h) (Fig. 6E). The inhibitory effects of procaterol on the fluorescence intensity from acidic endosomes were also dose-dependent. Significant inhibitory effects were observed at 3 nM, and the maximum inhibitory effect was obtained at 100 nM (0.1 μM) (Fig. 6F).

3.6. Effects of procaterol on cytokine production

Procaterol (0.1 μM) reduced the baseline secretion of interleukin-1β, interleukin-6, and interleukin-8 for 24 h before type 14 rhinovirus infection compared with that in cells treated with the vehicle of procaterol (ethanol, 0.01%) (Fig. 7). Type 14 rhinovirus infection increased the secretion of interleukin-1β, interleukin-6, and interleukin-8. Maximum secretion was observed at 1 day (24 h) after type 14 rhinovirus infection in interleukin-6 and interleukin-8, and at 3 days (72 h) after the infection in interleukin-1β. Procaterol (0.1 μM) also reduced the type 14 rhinovirus infection-induced secretion of interleukin-1β, interleukin-6, and interleukin-8 compared with that in the cells treated with vehicle of procaterol (Fig. 7).

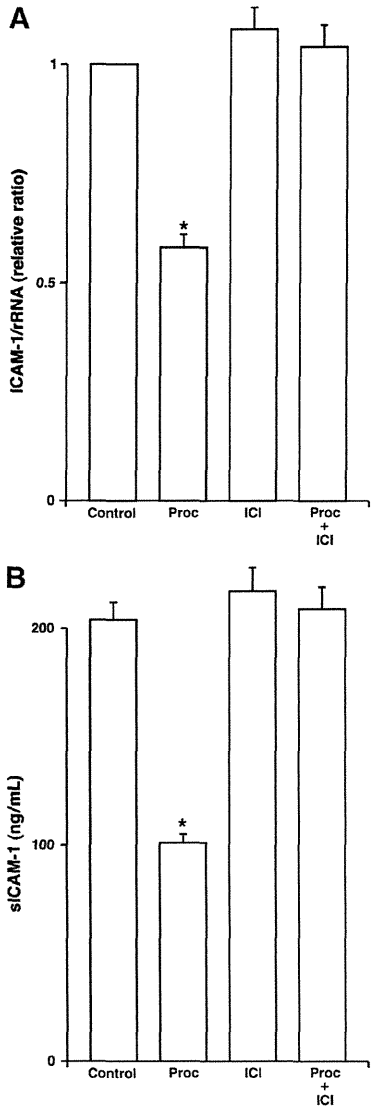


Fig. 5. (A) The expression of ICAM-1 mRNA before type 14 rhinovirus infection in human tracheal epithelial cells treated with procaterol (0.1 μM, 72 h, Proc), a vehicle of procaterol (0.01% ethanol, Control), ICI 118551 (1 μM, ICI), or procaterol plus ICI 118551 (Proc + ICI) detected by real-time quantitative RT-PCR. The epithelial cells isolated from the same donors were treated with either procaterol, vehicle, ICI 118551, or procaterol plus ICI 118551. ICAM-1 mRNA was normalized to the constitutive expression of ribosomal RNA (rRNA). The expression of ICAM-1 mRNA in the cells treated with vehicle (Control) was set to 1.0. Results are means ± S.E.M. from five different tracheae (2 ex-smokers and 3 non-smokers). Significant differences from control values are indicated by $^*P<0.05$. (B) The sICAM-1 concentrations in supernatant fluids before type 14 rhinovirus infection in human tracheal epithelial cells treated with procaterol (0.1 μM, 72 h Proc), the vehicle of procaterol (0.01% ethanol, Control), ICI 118551 (1 μM, ICI), or procaterol plus ICI 118551 (Proc + ICI) detected by enzyme immunoassay. The concentrations of sICAM-1 in the supernatant fluids are expressed as ng/ml. Results are means ± S.E.M. from five different tracheae (2 ex-smokers and 3 non-smokers). Significant differences from control values are indicated by $^*P<0.05$.

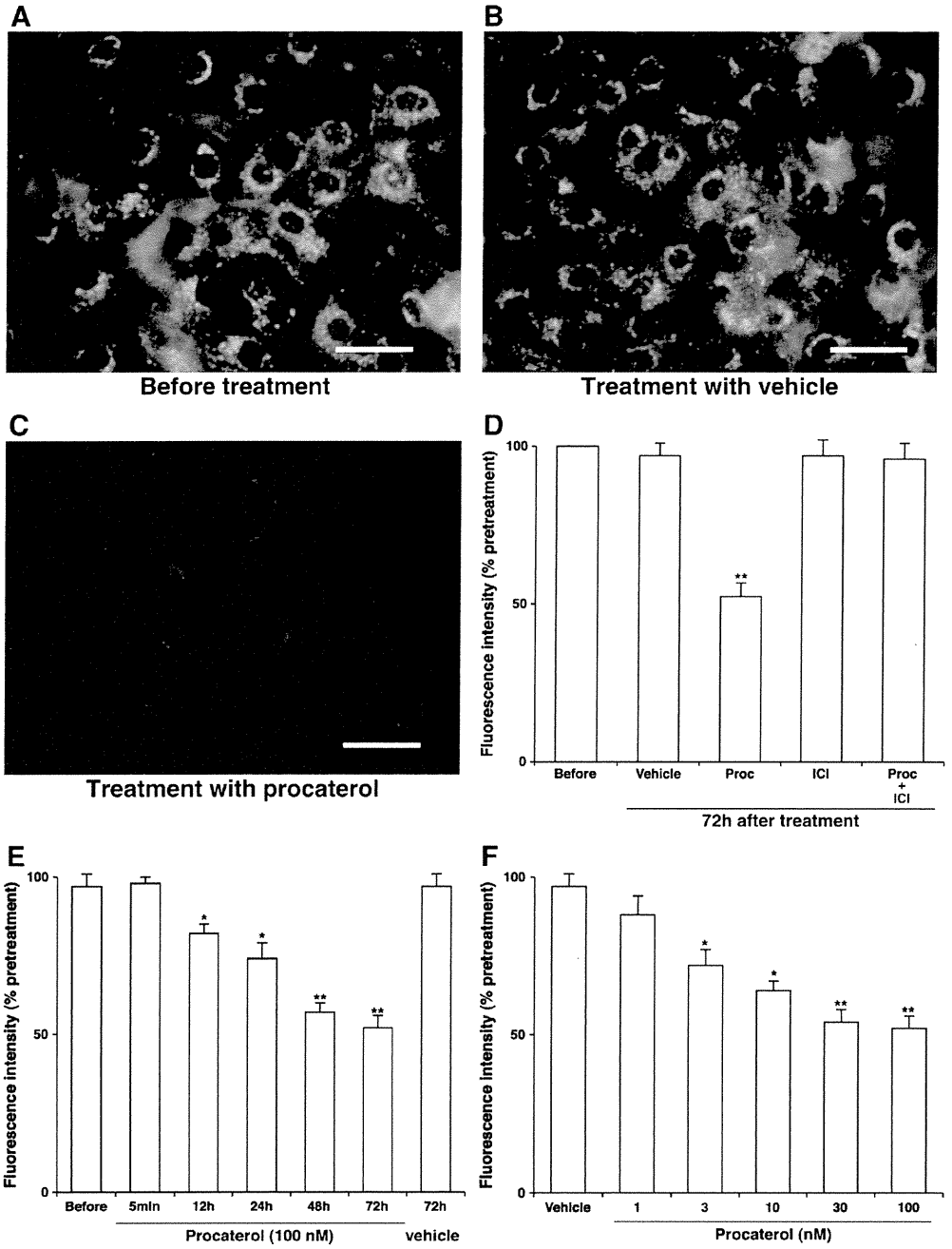


Fig. 6. (A–C) Changes in the distribution of acidic endosomes with green fluorescence in human tracheal epithelial cells before (A) and 3 days (72 h) after treatment with procaterol (0.1 μM) (C) or the vehicle of procaterol (0.01% ethanol) (B). Data are representative of five different experiments (2 ex-smokers and 3 non-smokers). (Bar = 100 μm). (D) The fluorescence intensity of acidic endosomes before and 3 days (72 h) after treatment with procaterol (0.1 μM, Proc), ICI 118551 (1 μM, ICI), or procaterol plus ICI 118551 (Proc + ICI), or the vehicle of procaterol (0.01% ethanol, Vehicle). Results are expressed as relative fluorescence intensity (%) compared with that before any treatment (Before) and reported as means ± S.E.M. from five samples (2 ex-smokers and 3 non-smokers). Significant differences from values before any treatment (Before) are indicated by $^{**}P<0.01$. (E) Time course of the effects of procaterol (0.1 μM) on the fluorescence intensity of acidic endosomes in the cells treated for times ranging from 0 (Before) to 3 days (72 h) and the fluorescence intensity in the cells treated with vehicle (0.01% ethanol, vehicle) for 3 days (72 h). Results are means ± S.E.M. from five different tracheae (2 ex-smokers and 3 non-smokers). Significant differences from before any treatment (Before) are indicated by $^{*}P<0.05$ and $^{**}P<0.01$. (F) Dose-response effects of procaterol on the fluorescence intensity of acidic endosomes 3 days (72 h) after treatment. The cells were treated with procaterol or the vehicle (0.01% ethanol, Vehicle) for 3 days (72 h). Results are means ± S.E.M. from five different tracheae (2 ex-smokers and 3 non-smokers). Significant differences from vehicle alone (Vehicle) are indicated by $^{*}P<0.05$ and $^{**}P<0.01$.

Treatment of the cells with ICI 118551 (1 μM, 72 h) reversed the inhibitory effects of procaterol on the baseline and rhinovirus infection-induced secretion of interleukin-1β, interleukin-6, and interleukin-8 (Fig. 7), whereas ICI 118551 alone did not change the secretion of these cytokines (data not shown).

In contrast, ultraviolet-irradiated type 14 rhinovirus did not increase interleukin-1β, interleukin-6, and interleukin-8 at any time after infection (Fig. 7). Secretion of interleukin-1β, interleukin-6, and interleukin-8 in supernatant fluids of the cells from three ex-smokers did not differ from those of three patients who had never smoked

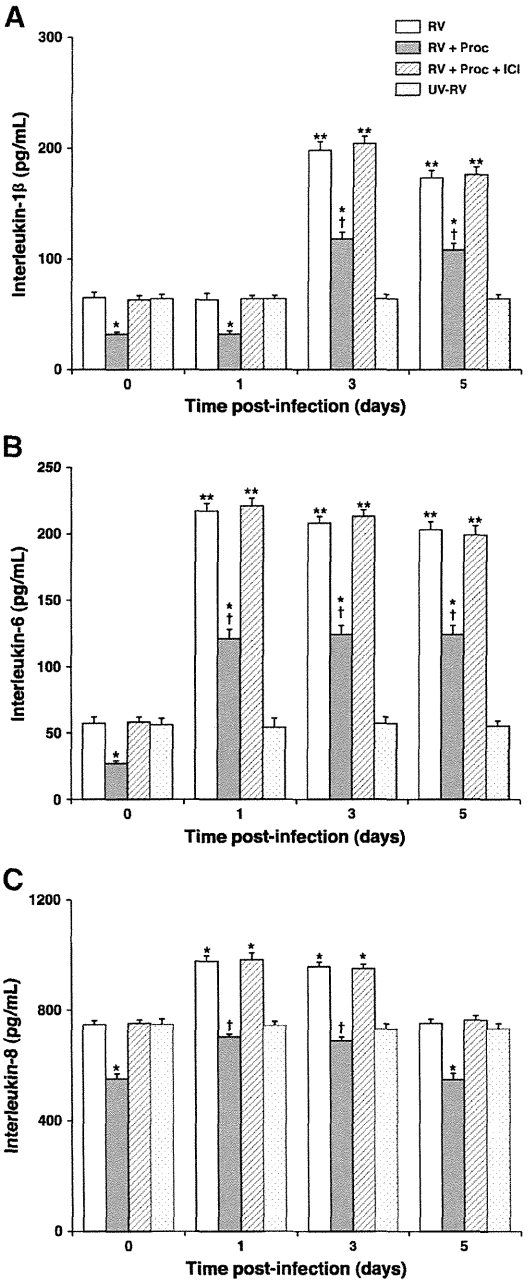


Fig. 7. (A–C) Time course changes in the release of cytokines into supernatant fluids of human tracheal epithelial cells before and after type 14 rhinovirus infection in the presence of procaterol (0.1 μM, RV + Proc), procaterol (0.1 μM) plus ICI 118551 (1 μM) (RV + Proc + ICI), or the vehicle of procaterol (0.01% ethanol, RV) and after UV-inactivated RV14 infection (UV-RV). The epithelial cells isolated from the same donors were treated with either procaterol, vehicle, or procaterol plus ICI 118551. The concentrations of cytokines in the supernatant fluids are expressed as pg/ml. Results are means ± S.E.M. from six different tracheae (3 ex-smokers and 3 non-smokers). Significant differences from values before type 14 rhinovirus infection (time 0) in the presence of vehicle of procaterol (0.01% ethanol) are indicated by $^{*}P<0.05$ and $^{**}P<0.01$. Significant differences from type 14 rhinovirus infection alone (RV) at each time after infection are indicated by $+P<0.05$.

(data not shown). Likewise, secretion of interleukin-1β, interleukin-6, and interleukin-8 in supernatant fluids of the cells from three patients complicated with COPD did not differ from those from 30 patients without COPD complications (data not shown).

3.7. Effects on NF-kappa B

Procaterol (0.1 μM, 72 h) significantly reduced the amount of p50, p65, and c-Rel of NF-κB in the nuclear extracts in the cells before type 14 rhinovirus infection (Fig. 8A–C). On the other hand, the amount of p50, p65, and c-Rel of NF-κB in the nuclear extracts increased at 2 h after type 14 rhinovirus infection (Fig. 8A–C), and procaterol (0.1 μM) also significantly reduced the amount of p50, p65, and c-Rel of NF-κB induced by type 14 rhinovirus infection (Fig. 8A–C). Furthermore, ICI 118551 (1 μM) reversed the inhibitory effects of procaterol on the NF-κB activation before (data not shown) and after type 14 rhinovirus infection (Fig. 8A–C), whereas ICI 118551 alone did not change the NF-κB activation before the infection (Fig. 8A–C).

Type 14 rhinovirus infection increased the cytosolic amount of p-IκB-α 2 h after infection, and procaterol (0.1 μM, 72 h) significantly decreased the amount of p-IκB-α induced by the infection (Fig. 9A and B). Furthermore, ICI 118551 (1 μM, 72 h) reversed the effects of procaterol on the p-IκB-α after type 14 rhinovirus infection (Fig. 9A and B). In contrast, before type 14 rhinovirus infection, neither procaterol (0.1 μM, 72 h), ICI 118551 (1 μM, 72 h), nor procaterol plus ICI 118551 had any effect on p-IκB-α in the cellular proteins.

On the other hand, type 14 rhinovirus infection decreased the cytosolic amount of IκB-α 2 h after infection, and procaterol (0.1 μM, 72 h) significantly increased the amount of IκB-α after the infection (Fig. 9A and C). Furthermore, ICI 118551 (1 μM) reversed the effects of procaterol on the IκB-α after the infection (Fig. 9A and C). In contrast, before type 14 rhinovirus infection, neither procaterol (0.1 μM, 72 h), ICI 118551 (1 μM, 72 h) nor procaterol plus ICI 118551 had any effect on the IκB-α in the cellular proteins (Fig. 9A and C).

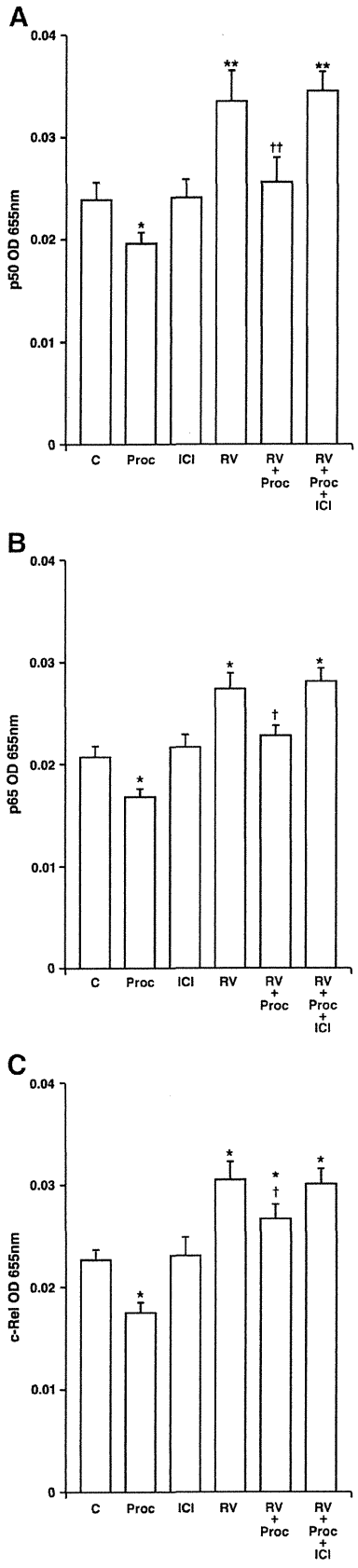
3.8. Effects on intracellular cAMP

Significant intracellular cAMP levels were detected in the cells before any treatment (44.0 ± 4.2 pmol/mg protein, n = 5) (Fig. 10A). Treatment of the cells with procaterol (0.1 μM) increased intracellular cAMP at 10 min and 20 min after the addition of procaterol, and increased levels of intracellular cAMP were still observed at 3 days (72 h) after the addition of procaterol (Fig. 10A).

Treatment with the vehicle alone (0.01% ethanol) for 20 min did not change intracellular cAMP levels (Fig. 10B). ICI 118551 (1 μM, 20 min) alone did not change the intracellular cAMP levels, while ICI 118551 reversed the effects of procaterol (0.1 μM, 20 min) on intracellular cAMP to the levels in the cells treated with vehicle (Fig. 10B). Intracellular cAMP levels in the cells treated with procaterol (0.1 μM) plus ICI 118551 for 20 min were significantly lower than those in the cells treated with procaterol alone (Fig. 10B). Treatment with ICI 118551 for 10 min or 3 days (72 h) also reversed the effects of procaterol (0.1 μM) on intracellular cAMP to the levels in the cells treated with the vehicle (data not shown).

4. Discussion

In the present study, we have shown that a β_2 agonist, procaterol hydrochloride, reduced the titers of a major group rhinovirus, type 14 rhinovirus, in supernatant fluids and RNA replication of the virus in the primary cultures of human tracheal epithelial cells. Pretreatment with procaterol reduced the expression of mRNA and protein of ICAM-1, the receptor for the major group of rhinoviruses (Greve et al., 1989) before rhinovirus infection. The minimum dose of type 14 rhinovirus necessary to cause infection in the cells treated with procaterol was significantly higher than that in the cells treated with the vehicle of procaterol. A selective β_2-adrenergic receptor antagonist ICI 118551 reversed the inhibitory effects of procaterol on type 14 rhinovirus titer levels, RNA replication of the virus, the expression of mRNA and protein of ICAM-1, and NF-κB. Treatment of the cells with ICI 118551 also reversed the inhibitory effects of procaterol on the susceptibility



of the cells to type 14 rhinovirus infection. These findings suggest that β_2-adrenoceptor-mediated effects of procaterol might inhibit type 14 rhinovirus infection partly through reducing the production of its receptor, ICAM-1.

Furthermore, treatment with procaterol reduced the number and fluorescence intensity of acidic endosomes from which rhinovirus RNA enters into the cytoplasm (Casasnovas and Springer, 1994; Turner and Couch, 2006), dose- and time-dependently, and ICI 118551 reversed the inhibitory effects of procaterol on the acidic endosomes. If only ICAM-1 expression is reduced on the cells, a similar amount of virus will be produced and released into supernatant fluids, and viral titers of procaterol-treated cells will reach to those of control cells after long periods of culture. On the other hand, in this study, procaterol also reduced the number of acidic endosomes where virus RNA enters into the cytoplasm. Therefore, by the reduction of acidic endosomes, inhibition of RNA entry into the cytoplasm might reduce the number of virus virions that enter into the cytoplasm, and viral titers of procaterol-treated cells did not reach control cells after long periods of culture. Procaterol might also inhibit type 14 rhinovirus infection partly through inhibiting rhinovirus RNA entry from acidic endosomes into the cells.

Treatment of the cells with procaterol significantly decreased the viral titers of type 14 rhinovirus in supernatant fluids from 12 h after infection. Procaterol also decreased the type 14 rhinovirus RNA at 1 day (24 h) and at 3 days (72 h) after infection. In contrast, there is no difference in the rate of increase in viral titers between rhinovirus infected control cells and procaterol-treated cells. These results suggest that procaterol may affect the ability of rhinovirus to infect cells and possibly does not affect the rate of rhinovirus replication once within the cell.

Likewise, to examine the susceptibility to type 14 rhinovirus infection, the epithelial cells were treated with procaterol from 3 days (72 h) before infection with type 14 rhinovirus until the end of the infection. The epithelial cells were exposed to serial 10-fold dilutions of type 14 rhinovirus at a dose ranging from 10^1 to $10^5 TCID_{50}$ units/ml of type 14 rhinovirus containing procaterol. After exposure to type 14 rhinovirus, cells were rinsed with PBS, and fresh medium without addition of procaterol was replaced. Cells in the tubes were then cultured. Therefore, in this experiment, we studied the effects of procaterol on the ability of type 14 rhinovirus to infect. In contrast, cells were treated with procaterol before and during type 14 rhinovirus infection but were not treated with procaterol after infection. Therefore, the minimum dose of type 14 rhinovirus necessary to cause infection might not explain the ability of type 14 rhinovirus to replicate within the cell.

Human embryonic fibroblast cells did not show any morphological change that demonstrates the presence of type 14 rhinovirus when supernatant fluids collected 1 h after infection were added to the fibroblast cells. In contrast, supernatant fluids 12 h after infection produced morphological change on the cells showing the presence of rhinovirus (Condit, 2006; Numazaki et al., 1987). These findings suggest that supernatant fluids 12 h after infection contained significant amounts of type 14 rhinovirus virions that were newly produced after infection.

Fig. 8. (A-C) Amount of p50 (A), p65 (B), and c-Rel (C) in nuclear extracts in human tracheal epithelial cells treated with procaterol (0.1 μM, Proc), vehicle (0.01% ethanol, C), or ICI 118551 (1 μM) (ICI) for 3 days (72 h) before type 14 rhinovirus infection, and the amount of p50, p65, and c-Rel in the cells 2 h after infection with type 14 rhinovirus in the presence of procaterol (RV + Proc), in the presence of the vehicle of procaterol (0.01% ethanol, RV), or the presence of procaterol plus ICI 118551 (RV + Proc + ICI) from 3 days (72 h) before rhinovirus infection until the end of the experiments after rhinovirus infection. Results are expressed as OD, and are means ± S.E.M. from five different tracheae (2 ex-smokers and 3 non-smokers). Significant differences from control values (C) before rhinovirus infection are indicated by $^*P<0.05$ and $^{**}P<0.01$. Significant differences from rhinovirus infection alone (RV) are indicated by $+P<0.05$ and $++P<0.01$.

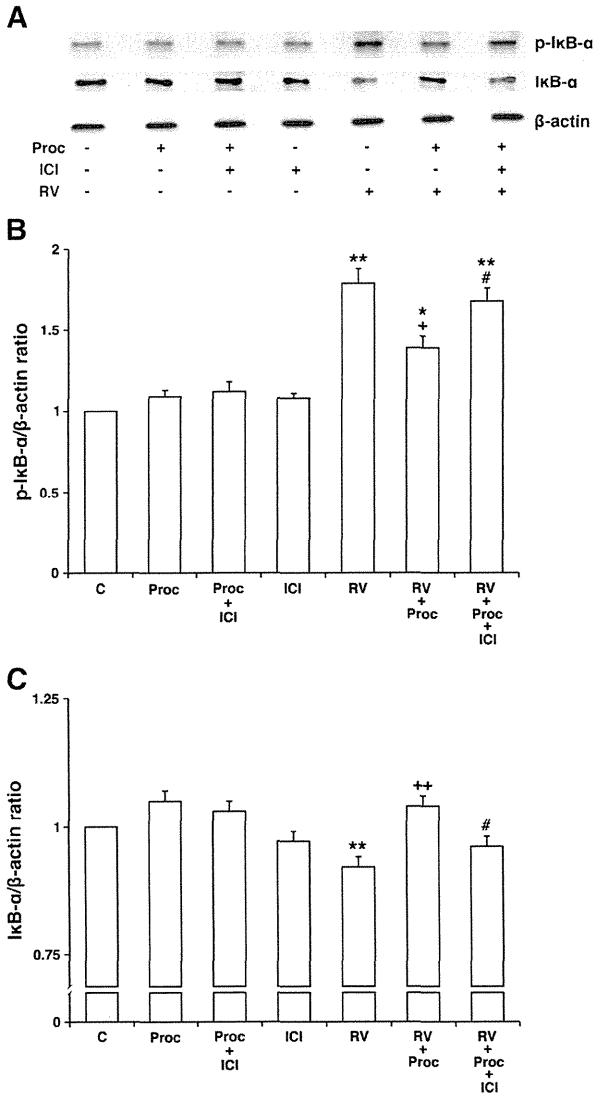


Fig. 9. (A) Cytosolic amount of p-IκB-α, IκB-α or β-actin in human tracheal epithelial cells before and 2 h after type 14 rhinovirus infection (RV) in the presence of procaterol (0.1 μM, Proc) or ICI 118551 (1 μM, ICI) for 3 days (72 h). The data are representative of three different experiments (2 ex-smokers and 1 non-smoker). (B and C) Cytosolic amount of p-IκB-α (B) and IκB-α (C) in the cells treated with procaterol (0.1 μM, Proc), procaterol plus ICI 118551 (1 μM) (Proc + ICI), ICI 118551 (ICI), or vehicle (0.01% ethanol, C) for 3 days (72 h) before type 14 rhinovirus infection and the amount in the cells 2 h after infection with type 14 rhinovirus in the presence of procaterol (RV + Proc), the presence of the vehicle of procaterol (0.01% ethanol, RV), or the presence of procaterol plus ICI 118551 (RV + Proc + ICI) from 3 days (72 h) before rhinovirus infection until the end of the experiments after rhinovirus infection. The data were obtained by dividing the results in each culture condition by the results of β-actin. The cytosolic amount of p-IκB-α and IκB-α in the cells treated with vehicle before rhinovirus infection (0.01% ethanol, C) was set to 1.0. Significant differences from control values (C) before rhinovirus infection are indicated by $^{*}P<0.05$ and $^{**}P<0.01$. Significant differences from rhinovirus infection alone (RV) are indicated by $+P<0.05$ and $++$ $P<0.01$. Significant differences from rhinovirus infection in the presence of procaterol (RV + Proc) are indicated by # $P<0.05$.

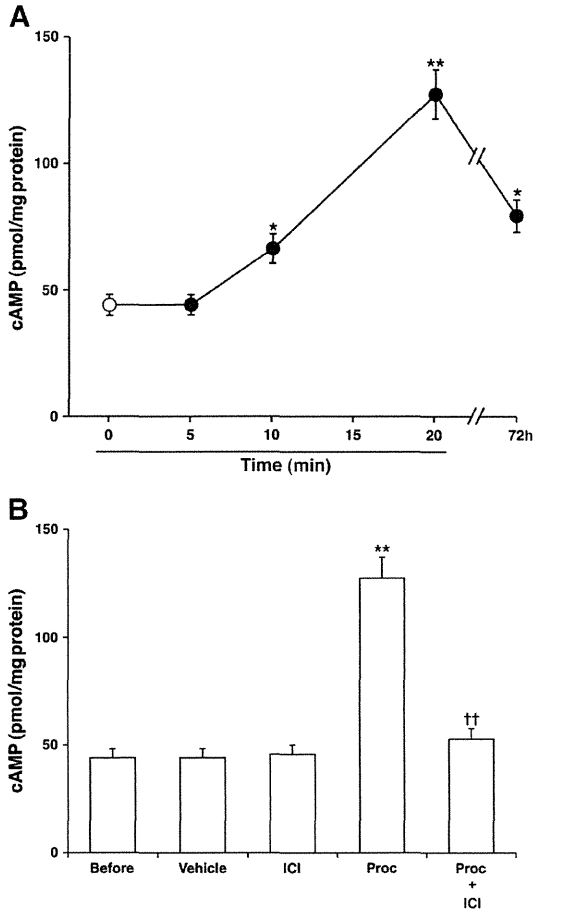


Fig. 10. (A) Intracellular concentrations of cAMP in human tracheal epithelial cells before (time 0, open circle), 5 min, 10 min, 20 min, and 3 days (72 h) after treatment with procaterol (0.1 μM, closed circles). Results are expressed as pmol/mg protein, and are means ± S.E.M. from five different tracheae (2 ex-smokers and 3 non-smokers). Significant differences from control values before treatment (time 0) are indicated by $^{*}P<0.05$ and $^{**}P<0.01$. (B) Intracellular concentrations of cAMP in human tracheal epithelial cells before (Before) and 20 min after treatment with either procaterol (0.1 μM, Proc), ICI 118551 (1 μM, ICI), procaterol (0.1 μM) plus ICI 118551 (1 μM) (Proc + ICI), or vehicle (0.01% ethanol, Vehicle). The epithelial cells isolated from the same donors were treated with either procaterol, vehicle, ICI 118551, or procaterol plus ICI 118551. Results are expressed as pmol/mg protein and are means ± S.E.M. from five different tracheae (2 ex-smokers and 3 non-smokers). Significant differences from control values before treatment (Before) are indicated by $^{**}P<0.01$. Significant differences from procaterol (Proc) are indicated by $++P<0.01$.

Furthermore, in the tracheal cells from all subjects which cells were infected with rhinovirus, the supernatant fluids collected during 1 day (24 h) to 3 days (72 h) after infection contained consistent levels of type 14 rhinovirus. These findings suggest that human tracheal epithelial cells from all subjects were constantly infected with type 14 rhinoviruses.

The major group of rhinoviruses enters the cytoplasm of infected cells after binding to its receptor ICAM-1 (Greve et al., 1989). In the present study, procaterol reduced ICAM-1 expression in the primary cultures of human tracheal epithelial cells. On the other hand, a β_2 agonist, fenoterol, reduces ICAM-1 expression in the human bronchial epithelial cells (Oddera et al., 1998), and salmeterol and procaterol reduce ICAM-1 expression in fibroblast cells (Silvestri et al., 2001; Yoshida et al., 2009). The results of reduced ICAM-1 expression observed in this study are consistent with the results of these reports. The inhibitory effects of procaterol on ICAM-1 expression in human tracheal epithelial cells might be associated with the inhibitory effects of procaterol on type 14 rhinovirus infection, as previously reported on the inhibitory effects of agents including dexamethasone, erythromycin, a proton pump inhibitor (lansoprazole), and a Japanese herbal medicine (Hochu-ekki-to) (Sasaki et al., 2005; Suzuki et al., 2000, 2002; Yamaya et al., 2007).

The endosomal pH is suggested to be regulated by vacuolar H^+-ATPase (Mellman et al., 1986) and by ion transport across the Na^+/H^+

exchangers (Marshansky and Vinay, 1996; Nass and Rao, 1998). A vacuolar H^+-ATPase inhibitor bafilomycin, an inhibitor of Na^+/H^+ exchangers 5-(N-ethyl-N-isopropyl) amiloride (EIPA) and N″-[3-(Hydroxymethyl)-5-(1 H-pyrrol-1-yl) benzoyl] guanidine methonesulfonate (FR168888) increase endosomal pH and inhibit type 14 rhinovirus infection in cultured human tracheal epithelial cells (Suzuki et al., 2001). In the present study, procaterol increased the endosomal pH, although we have no data with which to determine whether procaterol inhibits vacuolar H^+-ATPase or Na^+/H^+ exchangers. On the other hand, the addition of cyclic AMP (cAMP) increases endosomal pH in kidney epithelial cells through the inhibition of a Na^+/H^+ exchanger (Gekle et al., 2002), and procaterol increases cAMP levels in airway epithelial cells as shown in a previous report (Koyama et al., 1999) and in this study. In contrast, cAMP alone has no effect on vacuolar H^+-ATPase activities in Madin–Darby canine kidney cells, but has synergic action on vacuolar H^+-ATPase activation induced by arginine vasopressin (Oliveira-Souza et al., 2004). These findings suggest the possibility that procaterol has an inhibitory effect on Na^+/H^+ exchanger through the increased production of cAMP in airway epithelial cells.

ICAM-1 also plays a vital role in the recruitment and migration of immune effector cells to sites of local inflammation observed in patients with bronchial asthma and COPD (Grunberg and Sterk, 1999; Riise et al., 1994). The inhibitory effects of β_2 agonists on ICAM-1, as shown in the previous reports and in this study, may also be associated with the inhibition of airway inflammation and subsequently occurring exacerbations of bronchial asthma and COPD (Barnes, 2007; Calverley et al., 2007) after rhinoviruses infection.

Rhinoviruses are associated with exacerbations of bronchial asthma (Johnston et al., 1995) and COPD (Seemungal et al., 2000). Neutrophilic and eosinophilc inflammation in the exacerbations of bronchial asthma and COPD are suggested to be associated with a variety of mediators including interleukin-6 and interleukin-8 by rhinovirus infection (Pizzichini et al., 1998; Seemungal et al., 2000). Procaterol reduces the number of eosinophils in bronchoalveolar lavage fluids in mice after ovalbumin challenge (Tashimo et al., 2007) and inhibits interleukin-1β- and tumor necrosis factor (TNF)-α-mediated eosinophil chemotactic activity (Koyama et al., 1999). β_2 Agonists may also modulate eosinophil-related inflammation in bronchial asthma. Furthermore, in the present study, procaterol reduced type 14 rhinovirus infection-induced production of interleukin-1β, interleukin-6, and interleukin-8, and ICI 118551 reversed the inhibitory effects of procaterol on the release of these interleukins. These findings are consistent with previous findings showing the inhibitory effects of procaterol on plasma levels of cytokines including interleukin-1β in rats (Izeboud et al., 2004), and the inhibitory effects of salmeterol on the production of pro-inflammatory cytokines and monokines including RANTES (regulated on activation, normal T cells expressed and secreted/CCL5) in primary culture of normal bronchial epithelial cells after rhinovirus infection (Edwards et al., 2006). Similar to the inhibitory effects of a glucocorticoid (Suzuki et al., 2000), procaterol may also modulate airway inflammation induced by rhinovirus infections.

NF-κB increases the expression of genes for ICAM-1 and various pro-inflammatory cytokines (Papi and Johnston, 1999; Zhu et al., 1996). In the present study, procaterol reduced the expression of ICAM-1 before rhinovirus infection and the secretion of pro-inflammatory cytokines in supernatant fluids before and after rhinovirus infection. Rhinovirus infection increased activation of NF-κB as previously reported (Suzuki et al., 2002). Rhinovirus increased p50, p65, and c-Rel of NF-κB in the nuclear extracts, increased the cytosolic quantity of p-IκB-α, and decreased the cytosolic quantity of IκB-α. Procaterol reduced p50, p65, and c-Rel of NF-κB induced by rhinovirus infection as well as baseline NF-κB before rhinovirus infection. Procaterol also reduced p-IκB-α and increased IκB-α in the cellular proteins after rhinovirus infection, although procaterol had no effect on p-IκB-α and IκB-α in the cellular proteins before rhinovirus infection. The inhibitory effects of procaterol on NF-κB activation observed in this study are consistent with those of salmeterol in lung myofibroblasts (Baouz et al., 2005). Furthermore, a selective β_2-adrenergic receptor antagonist, ICI 118551, reversed the inhibitory effects of procaterol on the activation of NF-κB in the nuclear extracts before and after type 14 rhinovirus infection. ICI 118551 also reversed the effects of procaterol on p-IκB-α and IκB-α in the cellular proteins after rhinovirus infection. These findings suggest that procaterol might reduce the expression of ICAM-1 on the cells and secretion of pro-inflammatory cytokines partly through the reduction of NF-κB activation.

In contrast, Edwards et al. reported that salmeterol increases interleukin-6 production and enhances NF-kB pathway activation following rhinovirus infection in bronchial epithelial cell line (BEAS-2B) cells and primary cultures of normal bronchial epithelial cells (2007). Furthermore, another report demonstrated that β_2 agonists, treated for 30 min before stimulation with interleukin-1β do not affect NF-kB-induced activation of the interleukin-6 gene in airway smooth muscle cells (Kaur et al., 2008). On the other hand, production of interleukin-6 after rhinovirus infection through the activation of NF-κB has been reported in A549 alveolar epithelial type II-like cells (Zhu et al., 1996). Fragaki et al. demonstrated that salmeterol plus corticosteroid reduced interleukin-6 release in response to *Staphylococcus aureus* from transformed human tracheal gland cell line partly through the inhibition of NF-kB (2006). Inhibition of NF-κB and TNF-α-induced interleukin-6 production by salmeterol was also reported in lung myofibroblasts (Baouz et al., 2005). We previously reported that reduced production of interleukin-6 by a macrolide antibiotic erythromycin and a mucolytic agent L-carbocisteine is associated with the inhibition of NF-κB (Suzuki et al., 2002; Yasuda et al., 2006). Thus, these findings suggest that different effects of β_2 agonists on interleukin-6 and NF-κB after rhinovirus infection or after addition of stimulants may be partly associated with differences in cell type and with culture condition. However, the precise mechanisms are uncertain.

In this study, procaterol reduced viral titers and cytokine concentration in the supernatant fluids. It also decreased viral RNA replication, ICAM-1 expression, acidic endosomes, and NF-κB activation in the cells. Procaterol decreased susceptibility of the cells to virus infection and increased intracellular cAMP. The levels of intracellular cAMP induced by procaterol in this study were consistent with those reported previously (Koyama et al., 1999). On the other hand, a selective β_2-adrenergic receptor antagonist ICI 118551 reversed the inhibitory effects of procaterol on various cell functions and reduced cellular cAMP concentrations stimulated by procaterol. These findings suggest that the effects of procaterol observed in this study might be mediated by the β_2-adrenergic receptor.

Procaterol alone did not change cell viability, including cell number, assessed by the exclusion of trypan blue, and LDH concentrations in supernatant fluids. However, procaterol reduced NF-κB activation before and after RV infection. These findings suggest that reduced cytokine release might be partly associated with the inhibition of NF-κB activation but not with cell injury.

In the present study, type 14 rhinovirus titers in supernatant fluids in the cells from three patients with COPD did not differ from those in the cells from 35 patients without COPD complications. Epidermal growth factor receptor, which is up-regulated in COPD (O'Donnell et al., 2004), is associated with ICAM-1 and interleukin-8 production in airway epithelial cells, although we did not examine the production of epidermal growth factor receptor in the epithelial cells in this study. On the other hand, deficient induction of interferon-gamma (IFN-γ)-mediated high rhinovirus RNA replication was reported in the bronchial epithelial cells from patients with bronchial asthma (Contoli et al., 2006), but no patients were complicated with bronchial asthma in this study. We observed no significant mRNA expression of IFN-γ in the human tracheal epithelial cells from patients with or

without COPD complication (data not shown). Because we isolated the cells from human tracheae after death, the conditions before death, at the time of death, and the conditions from the time of death to cell isolation might also mask the characteristic features of cultured cell function. However, further studies are needed to clarify the difference of the magnitude of rhinovirus replication in the cells from COPD patients.

Likewise, the amount of cytokine release after rhinovirus infection did not differ between smokers and non-smokers. Conditions before death, at the time of death, and the conditions from the time of death to the cell isolation may also mask the cell condition including different response to beta agonists in smokers. The precise reason is uncertain.

Baseline levels for all cytokines observed in this study were higher than those in other studies including those by Edwards et al. (2007), while baseline levels in this study were consistent with those reported previously by us (Yamaya et al., 2007). Although the precise reason is uncertain, human bronchial epithelial cells in the study by Edwards were cultured in BEBM medium, and human tracheal epithelial cells in this study were cultured in Dulbecco's modified Eagle's Medium (DMEM)-Ham's F-12 medium (50/50, vol/vol) containing 2% USG. As previously reported (Yamaya et al., 1992), differences in the factors in culture medium may change cell functions including ion transport and protein production. Differences in culture medium may be partly associated with baseline levels of cytokines.

In summary, this is the first report that a β_2 agonist procaterol reduces type 14 rhinovirus titers in supernatant fluids, reduced rhinovirus RNA replication in cultured human tracheal epithelial cells, and decreases the susceptibility of the cells to rhinovirus infection. This may occur partly through the reduced expression of ICAM-1, the receptor for the major group of rhinoviruses, and reduction in the number of acidic endosomes from which rhinovirus RNA enters into the cytoplasm. Procaterol reduced baseline and rhinovirus infection-induced release of interleukin-1β, interleukin-6, and interleukin-8 in supernatant fluids. Procaterol may inhibit the infection of the major group of rhinoviruses and modulate the inflammatory responses in the airways after rhinovirus infection.

Conflict of interest

None.

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Prevalence of Uterine and Adnexal Involvement in Pulmonary Lymphangioleiomyomatosis: A Clinicopathologic Study of 10 Patients

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Abstract: Lymphangioleiomyomatosis (LAM), a systemic disorder affecting almost exclusively young women, is characterized by the abnormal proliferation of smooth muscle-like cells (LAM cells). LAM can occur either in association with the tuberous sclerosis complex (TSC) (TSC-LAM) or without TSC (sporadic LAM). Recent studies have demonstrated that LAM is a neoplasm arising from constitutive activation of the mammalian target of rapamycin signaling pathway dysregulated by a functional loss of *TSC* genes, but the primary organ of origin remains unclear. Therefore, we performed histologic and immunohistologic analyses of gynecologic organs in 20 patients, half with and the other half without pulmonary LAM, to determine how often LAM involves the uterus. The results showed that 9 of 10 (90%) patients with pulmonary LAM had uterine LAM lesions. In contrast, no patients without pulmonary LAM had so. All uterine LAM lesions were accompanied by LAM lesions in retroperitoneal or pelvic lymph nodes and LAM cell clusters, each enveloped by a monolayer of vascular endothelial growth factor receptor-3–positive lymphatic endothelial cells. Furthermore, when we compared uterine lesions of TSC-LAM with those of sporadic LAM, proliferation of HMB45-positive epithelioid-shaped LAM cells and infiltrates with a tongue-like growth pattern was more prominent in the former, whereas the extent of lymphangiogenesis within the myometrium was greater in the latter. These results indicate that uterine involvement is a common manifestation of LAM, and, possibly, that the uterus or an adjacent locale in the retroperitoneum or pelvic cavity is the primary site of origin of LAM.

Key Words: lymphangioleiomyomatosis, uterus, PEComa

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Lymphangioleiomyomatosis (LAM) is a rare, systemic disorder that affects almost exclusively young women and is characterized by a proliferation of abnormal smooth muscle-like cells (LAM cells) in the lungs and along the axial lymphatic system. LAM, which can occur either in association with the tuberous sclerosis complex (TSC-LAM) or without TSC (sporadic LAM), is usually recognized by its pulmonary manifestations because of cystic destruction of the lung parenchyma. The pathogenic mechanism of LAM has been attributed to a functional loss of *TSC* genes, causing neoplastic proliferation of cells through constitutive activation of the mammalian target of rapamycin signaling pathway, a crucial participant in mammalian cell growth, proliferation, and metabolism.[26] In addition, the metastatic potential of LAM cells was demonstrated by the clinical and genetic evidence that LAM recurred in transplanted lungs of recipients.[2,12,18] Accordingly, a metastatic mechanism is considered to be involved in the disease progression of LAM. However, LAM cells look benign in terms of morphologic appearance; further, neither the primary organ where LAM cells originate nor the normal counterpart of LAM cells is completely understood in the metastatic tumor model of LAM. Extrapulmonary LAM has 3 major locations: the mediastinum, the upper retroperitoneal areas close to the abdominal aorta, and the pelvic cavity.[17] All 3 are candidates for primary sites of LAM. We previously described an abundance of LAM-associated lesions in

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lymphatic vessels and a proliferation of LAM cells. Also, lymphangiogenesis-mediated fragmentation of LAM lesions and shedding of LAM cell clusters (LCCs) into the lymphatic stream likely play a role in the metastatic progression of LAM.[14] Furthermore, the frequency of LAM cell proliferation in lymph nodes was highest in the retroperitoneum and pelvic cavity along the axial lymphatics, indicating that LAM cells may originate in or distal to this area in a direction toward the lymphatic stream.

The World Health Organization defines "perivascular epithelioid cell tumors (PEComas)" as "mesenchymal tumors composed of histologically and immunohistochemically distinctive perivascular epithelioid cells."[8] At present, "PEComa" is a widely accepted concept of a group of tumors including angiomyolipoma, clear cell "sugar" tumor of the lung, and LAM. Perivascular epithelioid cells are consistently present in "PEComas," and no normal counterpart of the perivascular epithelioid cell has ever been identified in any organ. Recently, the uterus and retroperitoneum emerged as the most frequently reported anatomic sites where "PEComa" occurs.[7,9] Taken together with these findings, the striking gender predilection for female patients in the occurrence of LAM, and morphologic evidence that LAM cells are smooth muscle-like cells that express female hormone receptors, led us to speculate that the uterus could be a candidate for the primary site of LAM. However, still unclear is how often the uterus is also affected in patients with pulmonary LAM.

Here, we performed histologic and immunohistologic analyses of genital tracts from 20 women, 10 each with or without pulmonary LAM. We further characterized the histologic and immunohistologic features of uterine TSC-LAM compared with sporadic LAM.

MATERIALS AND METHODS

Study Population

The study population consisted of 10 women with pulmonary LAM whose genital organs were obtained at autopsy and/or after removal by surgical resection because of gynecologic disorders (Table 1). These tissues from 6 patients (patients 2, 3, 6 to 9) were acquired at autopsy. Of the surgical patients, patient 3 had received bilateral oophorectomy at the age of 37 years and before we obtained uterine blocks. Most of the patients died from respiratory failure because of the progression of pulmonary LAM; the exceptions were patients 8 and 9. Patient 8 had an endobronchial carcinoid tumor that led to massive hemoptysis, as previously reported.[20] In patient 9, an angiosarcoma of the uterine corpus had metastasized to multiple organs, including both lungs. Six patients (patients 1, 4, 5, 8, 9, and 10) received a total hysterectomy because of neoplasm of the uterus or ovary.

Patients' ages ranged from 25 to 83 years (median, 43 y) when blocks of female genital tracts were obtained. Two patients (patients 6 and 7) were menopausal. Seven patients (patients 1 to 7) had sporadic LAM, and 3 patients (patients 8, 9, and 10) had TSC (TSC-LAM). The diagnosis of LAM was confirmed histopathologically in all patients: in lung and lymph node tissue of 6 patients (patients 2, 3, 6, 7, 8, and 9), in lung tissue of 2 patients (patients 5 and 10), and in lymph node tissue of 2 patients (patients 1 and 4). The characteristic cystic appearance of the chest on computed tomography for all patients indicated the presence of pulmonary LAM. Two of 3 patients with TSC had renal angiomyolipomas. Four patients (patients 2, 3, 8, and 9) received antiestrogen therapy when their genital organs were removed.

Histopathologic and Immunohistochemical Examination of the Female Genital Tract

All genital organs were fixed in 10% buffered formalin, cut at 5-mm intervals (average 55 blocks per patient; ranging from 18 to 108 blocks), except for patient 7, and embedded in paraffin after routine processing. Each tissue block was sectioned at 4 μm and stained with hematoxylin-eosin and Elastica-Masson trichrome. Although 3 patients (patients 2, 3, and 8) were described previously,[13,19,20] their genital organs were reexamined (patients 2 and 8) or newly examined (patient 3) for this study. For patient 7, a retrospective study was conducted using several paraffin-embedded tissue blocks from the uterus, bilateral adnexa, and lymph nodes because of the absence of any other remnant tissues.

Immunohistochemical examinations were carried out using monoclonal antibodies against α-smooth muscle actin (α-SMA, dilution 1:200, Dako Cytomation, Carpinteria, CA), HMB45 (dilution 1:50, Dako Cytomation), vascular endothelial growth factor receptor (VEGFR)-3 (dilution 1:50, R&D Co. Ltd, Minneapolis, MN), and CD10 (dilution 1:200, Novocastra Laboratories Ltd., New Castle, UK). The EnVision kit (Dako Cytomation) was used for the immunostaining of α-SMA, HMB45, and CD10 to detect binding of the first antibodies according to the manufacturer's instructions, and 3, 3′-diaminobenzidine tetrahydrochloride was used as the chromogen. For the immunostaining of VEGFR-3, biotinylated anti-goat rabbit antibody (Dako Cytomation) and alkaline phosphatase-conjugated streptavidin (Dako Cytomation) were used to detect antibody binding. Fast red was used as the chromogen.

LAM lesions in the genital tissues were demonstrated by the existence of (1) smooth muscle-like, spindle-shaped cells or epithelioid-shaped cells with pale-to-eosinophilic cytoplasm, nuclei devoid of pleomorphism, mitotic activity, and proliferation sufficient to form foci; (2) positivity for α-SMA and HMB45; and (3) slit-like spaces positive for VEGFR-3. These criteria indicated the presence of LAM-associated lymphangiogenesis within or at the periphery of each foci. The following parameters were evaluated in the entire LAM lesion: (1) tongue-like growth pattern that was often seen in endometrial stromal sarcoma or uterine PEComa and (2) slit-like space positive for VEGFR-3 (lymphatic vessels). The extent of each was graded semiquantitatively as 0 (0%), 1+ (≤25%), 2+ (≤50%), or 3+ (> 50%). In addition, the number of LAM cells, spindle or epithelioid, immunoreactive for HMB45, α-SMA, or CD10, was

graded semiquantitatively as 0 (0% to 5% cells), 1+ (6% to 25% cells), 2+ (26% to 50% cells), or 3+ (51% to 100% cells).

Control Population

To clarify the frequency of LAM involving the female genital tract, we selected as controls surgical specimens of uteri from 10 patients of child-bearing age and without pulmonary LAM. These samples were selected from the archives of the Department of Human Pathology at Juntendo University Hospital and stored between 2009 and 2010. The patients' ages ranged from 31 to 43 years (median, 37.5 y), and their pathologic diagnosis included endometrioid adenocarcinoma of the uterine corpus (3 patients), squamous cell carcinoma of cervix uteri (1 patient), adenocarcinoma of cervix uteri (2 patients), squamous cell carcinoma arising from mature teratoma of the right ovary (1 patient), adenomyosis (2 patients), and leiomyoma (1 patient) of the uterine corpus. Each uterus was cut at 5-mm intervals (average 43 blocks per patient, ranging from 17 to 88 blocks).

RESULTS

Frequency and Histopathologic Features of LAM Lesions in Female Genital Tracts

LAM cells were readily identified in the genital tracts of 9 of 10 women with pulmonary LAM (90%; 3 TSC-LAM and 6 sporadic LAM); the single exception was patient 6 (Table 1). The frequency of these lesions was as follows: 9 of the 10 patients (90%) had LAM in the uteri, and 5 of 8 patients (63%) had it in the adnexa; 2 patients had LAM only in the broad ligaments, and 3 had LAM in both the ovaries and broad ligaments (Table 1). All these women had multiple LAM lesions in their genital organs with the distribution illustrated in Figure 1. Most of these LAM lesions in patients 1 and 2 were identified microscopically as multiple nodules; in addition, well-circumscribed yellowish masses (< 30 mm in size) with cleft-like spaces were detected macroscopically at the periphery of the uterine corpus or fundus in these 2 patients (Fig. 2A). In contrast, all LAM lesions in the adnexa were microscopic and smaller than 6 mm. In contrast, the 10 women without pulmonary LAM had no detectable LAM lesions in their genital organs.

Histologic and immunohistologic characterizations of uterine LAM lesions have been summarized in Table 2 and in Figures 2 to 4. In 6 patients with sporadic LAM (patients 1 to 5 and 7), LAM lesions were detected in all layers other than the uterine endometrium but primarily at the outer area of the myometrium or subserosa. Their margins were exceedingly well demarcated, although some microscopic LAM nodules in patient 1 had ill-defined margins with a focal tongue-like growth pattern. No such tongue-like growth pattern was seen in the other patients with sporadic LAM (Table 2). Each LAM nodule had abundant slit-like spaces that were irregularly dilated around some nodules. Each LAM nodule was exclusively composed of small-to-medium-sized spindle-shaped LAM cells with eosinophilic-to-clear cytoplasm and round-to-ovoid nuclei arranged in fascicles around a ramifying network of slit-like spaces. Only in patients 1 and 2 were small amounts of epithelioid-shaped LAM cells intermixed. In addition, hyalinized stroma was seen within LAM foci in patients 2 and 3. Spindle and epithelioid cells manifested neither nuclear pleomorphism nor mitotic activity. However, when present, both spindle-shaped and epithelioid-shaped LAM cells were immunopositive for α-SMA and HMB45, and the slit-like spaces were outlined by VEGFR-3–positive lymphatic endothelial

TABLE 1. Summary of the Clinicopathologic Findings of the 10 Patients With LAM

				LAM					
Patient	Age (y)*	TSC	Procedure	Uterus	Adnexa	Lung	LNs	AML	The Concomitant Diseases of Genital Organs
1	29	–	TAH-BSO	+	+ (BL)	+	+	–†	Endometrioid adenocarcinoma of the uterine corpus
2	33	–	Autopsy	+	+ (BL)	+	+	–	None
3	37	–	Oophorectomy		+ (ovary, BL)		+	–	Leiomyoma of the uterine corpus
	50		Autopsy	+		+			
4	45	–	TAH-BSO	+	–	+	+	–†	Serous borderline tumors of both ovaries Leiomyoma of the uterine corpus
5	48	–	TAH	+	NA	+	NA	–†	Leiomyoma of the uterine corpus Severe dysplasia of the cervix uteri
6	62	–	Autopsy	–	–	+	+	–	Leiomyoma of the uterine corpus
7	83	–	Autopsy	+	+ (ovary, BL)	+	+	–	Squamous cell carcinoma of the cervix
8	25	+	TAH-BSO	+	–				Adenomyosis and leiomyoma of the uterine corpus
	44		Autopsy			+	+	+	
9	41	+	TAH-BSO	+	+ (ovary, BL)				Angiosarcoma of the uterine corpus
	42		Autopsy			+	+	+	
10	50	+	TAH	+	NA	+	NA	–†	Adenomyosis and leiomyoma of the uterine corpus
Total (%)				9/10 (90)	5/8 (63)	10/10 (100)	8/8 (100)		

*The age of the patient when each examination was performed.
†Computed tomography showed no renal AML.
AML indicates renal angiomyolipoma; BL, broad ligament; LNs, lymph nodes in the retroperitoneum or pelvic cavity; NA, not available; TAH-BSO, a total abdominal hysterectomy and bilateral alpingo-oophorectomy.

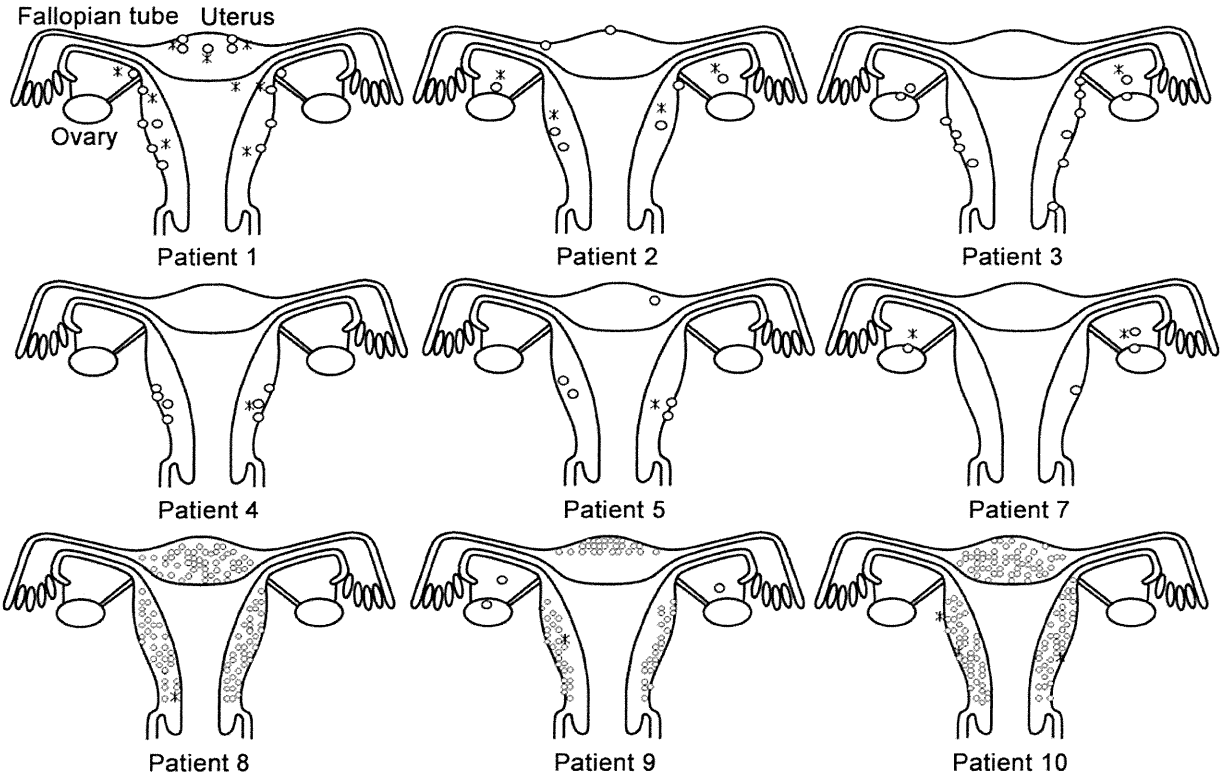

FIGURE 1. Schematic illustrating the distribution of LAM lesions in the female genital tract. The white circle indicates the site of a LAM lesion, and the asterisk indicates an LCC within the lymphatics. Note that the majority of LAM lesions in sporadic LAM (patients 1 to 7) are located along the outer area of the uterine myometrium, but those in TSC-LAM (patients 8 to 10) appear diffusely in the myometrium.

cells (Fig. 4). The constituent cells were negative for CD10 in all patients (Table 2).

In contrast, 3 patients with TSC-LAM (patients 8 to 10) had no macroscopically apparent nodules in the uteri. However, a mixed population of small-to-medium-sized spindle-shaped LAM cells and epithelioid-shaped LAM cells with clear-to-eosinophilic cytoplasm and round-to-ovoid nuclei infiltrated the entire myometrium in a tongue-like growth pattern (Fig. 3). Slit-like spaces were seen focally, and all patients had areas of spindle-shaped LAM cells with pale eosinophilic cytoplasm arranged in fascicles around slit-like spaces, similar to the uterine lesions of sporadic LAM patients. Transitional areas between spindle-shaped and epithelioid-shaped LAM cells were apparent in all patients. Some epithelioid-shaped LAM cells had mild nuclear atypia, but neither mitotic activity nor necrosis was present. Immunohistochemical findings resembled those of sporadic-LAM patients, but $>25\%$ of the cells were positive for HMB45, and a higher percentage of epithelioid-shaped LAM cells were immunoreactive for HMB45 compared with spindle-shaped LAM cells (Table 2 and Fig. 4).

Four patients with sporadic LAM and 1 with TSC-LAM had LAM lesions in the adnexa. Adnexal LAM lesions were detected as microscopic nodular lesions with slit-like spaces in both sporadic LAM and TSC-LAM patients. LAM cells proliferating in both ovaries and broad ligaments were predominantly spindle shaped with eosinophilic-to-clear cytoplasm and round-to-ovoid nuclei arranged in fascicles around a ramifying network of slit-like spaces. The tongue-like growth pattern was not seen in any adnexal LAM lesions.

Other LAM-Related Histopathologic Findings

A proliferation of LAM cells was identified in the retroperitoneal and pelvic lymph nodes of 8 patients with pulmonary LAM; the exceptions were patients 5 and 10 (Table 1). Close examination revealed that LAM cells proliferating in lymph nodes were predominantly spindle shaped. In patient 6, who lacked any LAM foci in her gynecologic organs, we observed the proliferation of LAM cells in retroperitoneal lymph nodes.

LCC (Fig. 5) composed of LAM cells enveloped by a monolayer of VEGFR-3–positive lymphatic endothelial cells were present in lymphatic vessels of all patients, except patient 6 (Table 2 and Fig. 1). LCCs were most frequently found in the uteri (7 patients), followed by detection in the broad ligaments of 3 (patients 2, 3, and 7) of 8 patients whose adnexa was available for this study; however, LCCs were not detected in the ovaries (Fig. 1).

Vascular walls, including the spiral arteries of the uterus and ovarian arteries, from 7 of 10 study patients

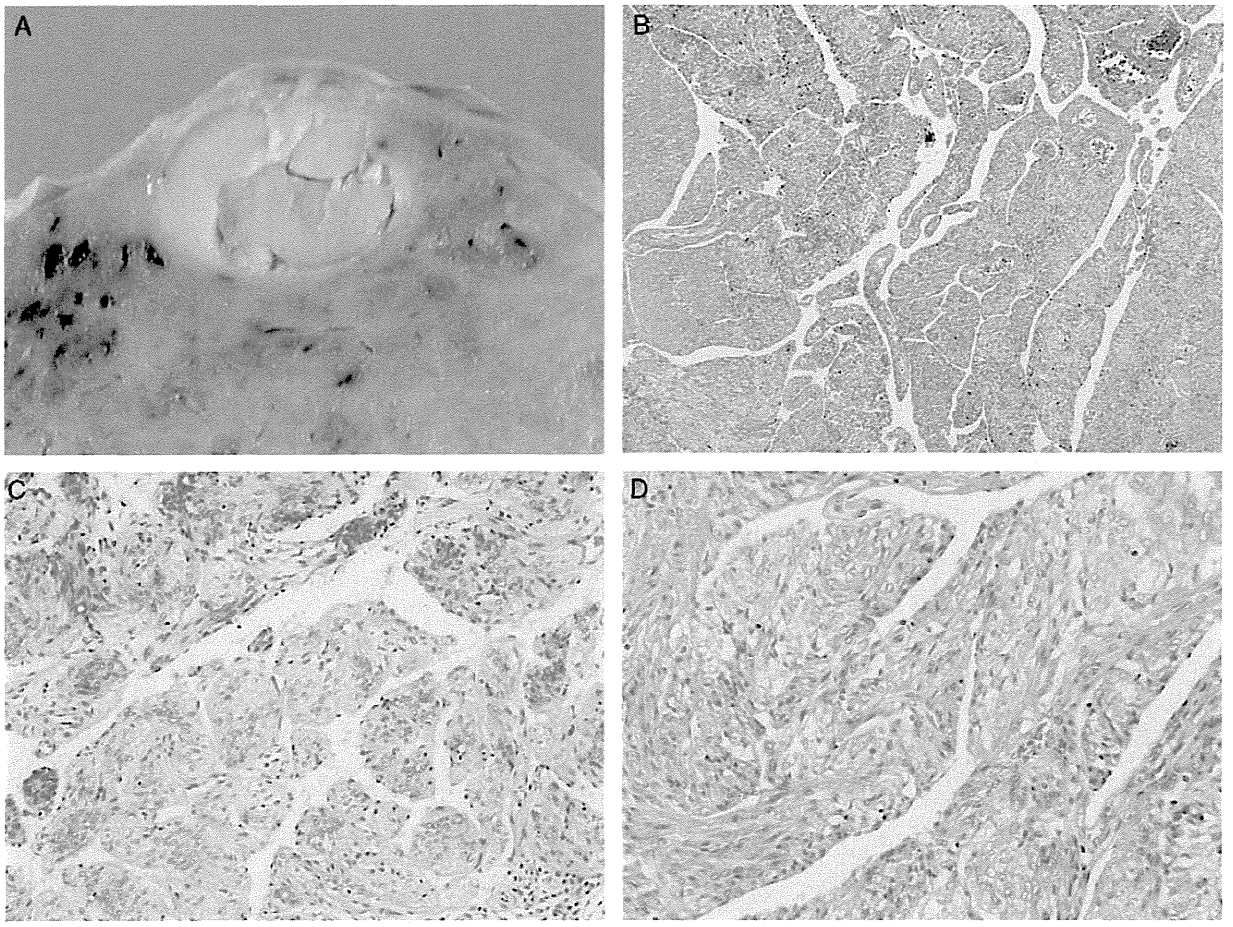

FIGURE 2. Representative pathologic findings of uterine LAM lesions in sporadic LAM patients. In patients with sporadic LAM, macroscopically identifiable, well-circumscribed yellowish masses with interior cleft-like spaces were demonstrated in the uterine corpus or fundus (A), but most uterine lesions in patients with sporadic LAM were microscopic, and each lesion had abundant slit-like spaces (B). Bundles of proliferating LAM cells with slit-like spaces produced a pericytoma-like appearance. C, LAM foci were exclusively composed of spindle-shaped LAM cells with pale-to-eosinophilic cytoplasm and nuclei devoid of pleomorphism or mitotic activity (D) (B–D, hematoxylin-eosin stain).

contained LAM cells (Table 2 and Fig. 6). Focal or entire destruction of elastic fibers of the tunica media was marked. However, endothelial cells were preserved, and no intravascular extension of LAM cells was detected. No LAM-related histopathologic findings of the type described above were present in genital organs of the 10 women without pulmonary LAM who have been designated here as controls.

TABLE 2. Histologic and Immunohistologic Characterization of Uterine Lesions in Patients With LAM

Patient	Tongue-Like Growth Pattern	Slit-Like Space	Cell Type		Immunohistochemistry			LCC	Involvement of the Vascular Wall
			Epithelioid	Spindle	HMB45	SMA	CD10		
1 (sporadic)	1+	3+	1+	3+	1+	3+	0	+	+
2 (sporadic)	0	3+	1+	3+	1+	3+	0	+	+
3 (sporadic)	0	3+	0	3+	1+	3+	0	+	+
4 (sporadic)	0	3+	0	3+	1+	3+	0	+	–
5 (sporadic)	0	3+	0	3+	1+	3+	0	+	+
7 (sporadic)	0	3+	0	3+	1+	3+	0	+	–
8 (TSC)	2+	1+	2+	3+	2+	3+	0	+	+
9 (TSC)	2+	1+	2+	3+	2+	3+	0	+	+
10 (TSC)	3+	1+	2+	3+	2+	3+	0	+	+

See Materials and methods for the meaning of semiquantitative evaluation.