

Erythromycin and clarithromycin attenuate cytokine-induced endothelin-1 expression in human bronchial epithelial cells

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ABSTRACT: Erythromycin and its fourteen-member macrolide analogues have attracted attention for their efficacy in bronchial asthma. However, their mechanisms of action remain unclear. We evaluated the effects of the macrolide antibiotics on endothelin-1 (ET-1) expression in normal and transformed human bronchial epithelial cells, one of the sources of this potent bronchoconstrictor important in the pathogenesis of asthma.

Human bronchial epithelial cells were obtained from the resected bronchi, and the effect of several antimicrobial and antiasthmatic drugs on the production and messenger ribonucleic acid (mRNA) levels of ET-1 was evaluated. Bronchoepithelial cells were also isolated from the mucosa of asthmatic patients under fiberoptic bronchoscopy, and the modulating effects of the drugs were studied.

Erythromycin and clarithromycin uniquely suppressed mRNA levels as well as the release of ET-1 at therapeutic and non-cytotoxic concentrations (percentage inhibition of ET-1 protein release: $26.4 \pm 5.22\%$ and $31.2 \pm 7.45\%$, respectively, at 10^{-6} M). Furthermore, erythromycin and clarithromycin inhibited ET-1 expression in bronchoepithelial cells from patients with chronic, stable asthma. A glucocorticosteroid, dexamethasone, also inhibited ET-1 expression. In contrast, theophylline, salbutamol and FK506 had no effect on ET-1 production.

Our findings demonstrated that these fourteen-member macrolide antibiotics had an inhibitory effect on endothelin-1 expression in human bronchial epithelial cells. Moreover, this new mode of action may have some relevance to their clinical efficacy in bronchial asthma.

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Endothelin-1 (ET-1), a prototype of the endothelin family [1], is a potent bronchoconstrictive peptide that is believed to play an important role in the pathogenesis of bronchial asthma [2]. There is an increase of endothelin-1 in bronchoalveolar lavage fluid from patients with asthma [3], and its immunoreactivity is increased in the airway epithelium from patients with asthma [4]. It has also been shown that ET-1 levels decreased as the clinical disease severity declined by treatment [3, 4]. Recent experiments with animal models of asthma [5] showed that endothelin receptor antagonists significantly suppressed allergic airway inflammation as well as late-phase asthmatic responses characteristic of chronic, persistent asthma. Airway epithelial cells have been considered to be important sources of this potent bronchoconstrictive agent [6-8], and therefore, their modulation may lead to the clinical amelioration of asthma.

Erythromycin, a macrolide antibiotic, has been reported to be effective as one of the choices for asthma treatment, especially for severe, intractable, or steroid-dependent asthma [9, 10]. Erythromycin and its relative compound, roxithromycin, reduce the severity of bronchial hyperresponsiveness in asthmatic adults and children [11, 12].

However, its precise mechanisms remain unclear. Recently, KONNO *et al.* [13] showed that roxithromycin inhibited cytokine expression in peripheral mononuclear cells. We showed that erythromycin and clarithromycin suppress the expression of a pro-inflammatory cytokine interleukin (IL)-6 in human bronchial epithelial cells *in vitro* [14]. These effects may have some relevance to the beneficial effect seen in the treatment with macrolides.

The purpose of the present study was to determine whether or not erythromycin and its fourteen-member analogue, clarithromycin, suppress ET-1 expression and release in human airway epithelial cells, and to compare their potency with other antiasthma drugs including theophylline, β -agonist salbutamol, a glucocorticosteroid dexamethasone, and a potent macrolide immunosuppressant, FK506 [15].

Materials and methods

The study was planned according to the ethical guidelines following the declaration of Helsinki and given the institutional approval, and an informed consent was obtained from each patient.

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Preparation of normal human bronchoepithelial cells

Normal human bronchial epithelial cells were prepared by the method reported previously [8, 16, 17]. Briefly, a piece of macroscopically and microscopically normal human lobe or segmental bronchus (about 7 mm in width) was obtained either at the time of lung tumour resection or on autopsy. The bronchus was rinsed in sterile Hanks' balanced salt solution (HBSS) (GIBCO, Grand Island, NY) without calcium and magnesium and incubated in Ham's F12 medium (GIBCO) containing 0.1% protease (Sigma Chemical Co., St. Louis, MO) at 4°C overnight. The bronchus was rinsed with Ham's F12 medium supplemented with 10% foetal calf serum (FCS; heat inactivated, GIBCO), and the recovered cells were washed twice in HBSS. The number of cells was counted using a standard haemocytometer, and the cell viability was assessed by trypan blue dye exclusion technique.

Culture of bronchial epithelial cells

The cells were placed on to collagen-coated 24-well flat-bottom tissue culture plates (Koken, Tokyo, Japan) at a density of 5×10^4 cells-well⁻¹ in hormonally defined Ham's F12 medium (HD-F12) as reported previously [8, 16, 17]. HD-F12 contained 1% penicillin-streptomycin, 5 µg·mL⁻¹ insulin (GIBCO), 5 µg·mL⁻¹ transferrin (GIBCO), 25 ng·mL⁻¹ epidermal growth factor (Collaborative Research Corp., Lexington, MA), 15 µg·mL⁻¹ endothelial cell growth supplement (Collaborative Research Corp.), 2×10^{-10} M triiodothyronin (GIBCO), and 10^{-7} M hydrocortisone (GIBCO). The cells were incubated in a humidified atmosphere at 37°C and 5% CO₂. The medium was changed on day 1 and then every 2 days. Confluent monolayers of epithelial cells were stained with anti-keratin (KL-1, Immunotech, Marseille, France) or anti-vimentin (DAKO-Vimentin, DAKOPatts, Glostrup, Denmark), or with control immunoglobulin-G1 (IgG1) monoclonal antibodies using the avidin-biotin complex method [8, 18]. We used primary and secondary passaged cells for the experiments. In all preparations of primary and secondary passaged bronchial epithelial cells, no less than 98% of the cells were positive to keratin, but negative to vimentin, indicating that the cells were of epithelial cell origin as described [8, 18].

The human transformed bronchoepithelial BEAS-2B cell line [19] (a kind gift from J.F. Lechner and C.C. Harris, National Cancer Institute, Bethesda, MA) was cultured in HD-F12 as reported previously [17].

Isolation of airway epithelial cells from patients with chronic, stable bronchial asthma, and effect of the drugs on ET-1 expression and release from these cells

To assess the effect of macrolide antibiotics on ET-1 production by inflamed airway epithelium, bronchial epithelial cells were obtained from eight Japanese patients with chronic, stable bronchial asthma (table 1) in fibre-optic bronchoscopy as previously reported [20]. All the patients received neither inhaled nor oral corticosteroid treatment for at least 1 month. Eight nonsmoking control subjects with no clinical history of pulmonary disease also underwent bronchoscopy after informed consent was obtained. Briefly, under local anaesthesia, a fibreoptic bronchoscope (Olympus BF-20, Tokyo, Japan) was inserted transorally. A sheath-covered brush was introduced *via* the sampling channel, and the epithelial surface of bilateral main bronchi was brushed several times. The number of harvested cells was $1.05 \pm 0.65 \times 10^6$, and the cell viability was $65 \pm 12\%$ for the cells from the main bronchi. The cells were plated at a density of 5×10^4 ·mL⁻¹ in 48-well culture plates with hormonally supplemented Ham's F12 media and cultured until confluent as described above. Then, the cells were treated with different drugs, and the supernatants were harvested after 48 h and stored at -80°C until assayed. In some cases (n=5), the cell pellet was immediately processed for ribonucleic acid (RNA) isolation, as described below.

ET-1 assay

The specific immunoreactivity for ET-1 in culture supernatants was measured by sandwich enzyme-linked immunosorbent assay (ELISA) kits previously reported by SUZUKI *et al.* [8, 21]. Each sample was assayed in duplicate. This assay cross-reacted <2% with ET-3 and big-endothelin, but could not distinguish ET-2 from ET-1. Since studies with fast liquid chromatography revealed no detectable ET-2 in the samples as previously reported [8], we designated the immunoreactivity as for ET-1. The sensitivity was 0.2 pg·mL⁻¹, and intra- and interassay variations were <10%.

Northern blot analysis for ET-1 messenger RNA expression in human bronchial epithelial cells

Northern blot analysis was performed to study the effect of the drugs on ET-1 messenger (m)RNA expression in human bronchial epithelial cells by the method described previously [8]. Briefly, total cellular RNA was extracted by the method of CHOMCZYNSKI and SACCHI [22], and

Table 1. – Clinical characteristics of patients with chronic asthma

Patient no.	Age/sex	Smoking	Therapy	Blood eosinophil count·µL ⁻¹	%PEFR	%FVC	%FEV ₁
1	40/M	–	Theophylline, ambroxol	545	78.3	98.3	76.8
2	32/M	–	Theophylline, ambroxol	876	65.7	101.9	65.8
3	49/F	–	Ambroxol	123	68.6	87.7	78.9
4	67/M	–	Carbocisteine	890	89.5	76.2	68.7
5	64/F	–	Theophylline, carbocisteine	254	55.7	76.0	70.8
6	28/M	–	None	660	49.7	77.9	67.6
7	33/F	–	Bromhexine hydrochloride	465	45.7	67.8	80.9
8	45/M	exsmoker	None	776	67.5	89.9	70.5

–: never smoked. PEFR: peak expiratory flow rate; FVC: forced vital capacity; FEV₁: forced expiratory volume in one second; M: male; F: female.

electrophoresed on formaldehyde denatured agarose gel (10 µg·lane⁻¹) followed by capillary transfer onto Biodyne nylon membrane. The RNA integrity and equivalency of loading were routinely evaluated by ethidium bromide fluorescence. The *EcoRI* fragment of human preendothelin-1 complementary deoxyribonucleic acid (cDNA) clone (pHET91, the vector is pUC118) [23] was labelled with digoxigenin by random priming (DNA Labelling Kit, Boehringer Mannheim GmbH, Mannheim, Germany) and hybridized with blots followed by enzymatic reaction (DIG Luminescent Detection Kit, Boehringer). Briefly, blots were baked, prehybridized, and hybridized with digoxigenin-labelled cDNA at 42°C overnight and then washed at appropriate stringency. After incubation in blocking solution for 1 h, the membrane was incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody for 30 min.

After washing twice, 3-(2'-spiradamantane)-4-methoxy-4-(3'-phosphorylaxyl)phenyl-1,2-dioxetane disodium salt (AM-PPD) was added as a substrate for alkaline phosphatase to detect signals by chemiluminescence on Kodak X-ray film. As an internal control, β-actin mRNA transcripts were evaluated with a β-actin cDNA probe (pHFβ A-1) [8, 24].

Preparation of antimicrobial and antiasthmatic drugs

Erythromycin, clarithromycin, josamycin, tetracycline (generous gifts from S. Ohmura, Kitasato Institute, Tokyo, Japan) and dexamethasone (Banyu Pharmaceutical Ltd, Tokyo, Japan) were solubilized in ethanol as stock solutions and diluted in saline for experiments. Preliminary experiments showed that the final concentrations of ethanol used in the experiments had no significant effect on cell viability, as assessed by the trypan blue dye exclusion technique and on ET-1 release (data not shown). Aminobenzyl penicillin, cefazolin, theophylline, salbutamol and FK506 (a kind gift from Fujisawa Pharmaceuticals, Tokyo) were solubilized in saline and diluted for further experiments.

Statistics

The results were analysed by nonparametric equivalents of analysis of variance (ANOVA) as reported previously [8]. For evaluating the correlation between ET-1 release and pulmonary function data, a Pearson correlation analysis was used.

Table 2. – Production of immunoreactive endothelin (ET)-1 by human bronchial epithelial cells

	Normal primary passage bronchial epithelial cells	BEAS-2B
Baseline	74.0±6.87	80.4±10.2
+cycloheximide 10 µg·mL ⁻¹	12.0±12.0 ⁺	9.0±3.90 ⁺
+IL-1α 1 ng·mL ⁻¹	89.9±2.00 ⁺	120.0±12.0 ⁺
10 ng·mL ⁻¹	129.0±31.0 ⁺	145.0±23.7 ⁺
+IL-1β 1 ng·mL ⁻¹	79.6±12.9	109.0±23.9
10 ng·mL ⁻¹	130.0±12.5 ⁺	178.0±34.9 ⁺
+TNF-α 1 U·mL ⁻¹	100.0±14.1 ⁺	98.0±16.3
10 U·mL ⁻¹	189.0±11.0 ⁺	134.0±7.98 ⁺
+IL-1α 10 ng·mL ⁻¹		
+cycloheximide 10 ng·mL ⁻¹	34.9±12.1**	20.9±9.99**

The data are shown as means±SEM from three experiments. +: p<0.01 as compared with baseline release in each cell type (analysis of variance (ANOVA)). **: p<0.01 as compared with IL-1α (10 ng·mL⁻¹)-stimulated group in each cell type (ANOVA). IL-1: interleukin-1; TNF-α: tumour necrosis factor-α.

Results

Suppressive effect of erythromycin and clarithromycin on ET-1 production by normal and transfer bronchial epithelial cells

As previously reported [8], normal bronchial epithelial cells from surgical specimens obtained as well as transformed human bronchoepithelial cells BEAS-2B cell line constitutively released immunoreactive ET-1, and this process appeared to require protein synthesis, as assessed by the effect of cycloheximide (10 µg·mL⁻¹) (table 2). Pro-inflammatory cytokines such as IL-1α, IL-1β and tumour necrosis factor (TNF)-α stimulated ET-1 release in a dose-dependent fashion in both types of cells as reported previously [8] (table 2). Northern blot analysis showed that the epithelial cells expressed constitutive ET-1 mRNA, which

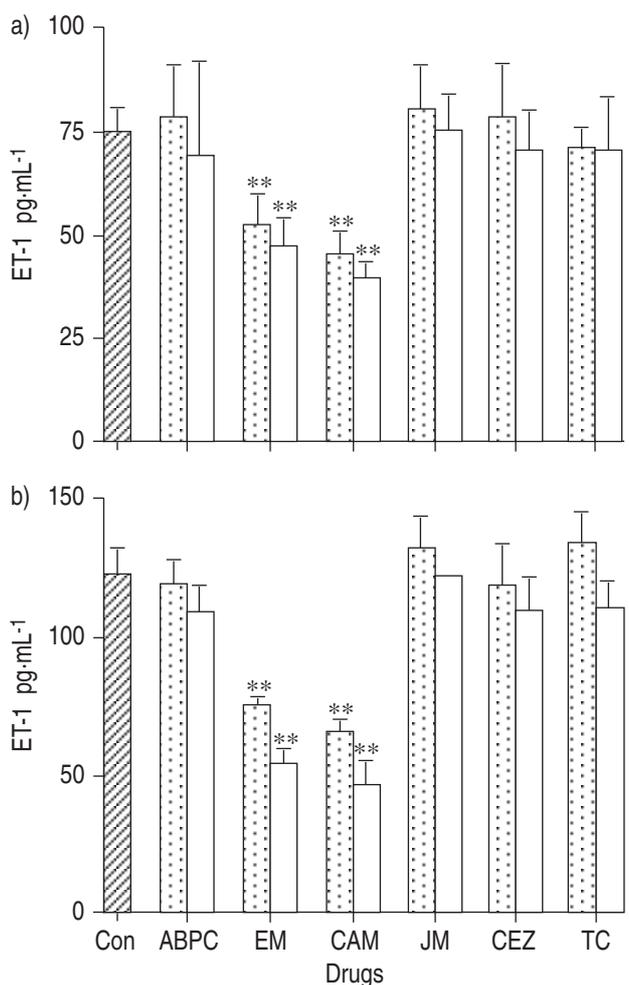


Fig. 1. – Effect of a variety of antimicrobial agents on endothelin (ET)-1 production by transformed human bronchial epithelial cells BEAS-2B. Erythromycin (EM) and clarithromycin (CAM), but not the other antibiotics, significantly inhibited ET-1 release from human bronchial epithelial cells in culture. a) Effect of antibiotics (▨: 10⁻⁶ and □: 10⁻⁵ M) on baseline release of ET-1 after 48 h. b) Effect of antibiotics on ET-1 release from interleukin (IL)-1α (10 ng·mL⁻¹)-stimulated bronchial epithelial cells in culture. The antibiotics (▨: 10⁻⁶ and □: 10⁻⁵ M) were added simultaneously with IL-1α, and the supernatants were harvested after 48 h. The data (mean±SEM) from three representative experiments, **: p<0.01 as compared with the controls (analysis of variance (ANOVA)). Con: control; ABPC: aminobenzyl penicillin; JM: josamycin; CEZ: cefazolin; TC: tetracycline.

was significantly upregulated by the cytokines listed above [8].

Among the antimicrobial drugs tested, only the fourteen-member macrolides erythromycin and clarithromycin showed any inhibitory action on ET-1 release by unstimulated and stimulated human bronchial epithelial cells BEAS-2B (fig. 1). This was also the case when the cells were pretreated with erythromycin or clarithromycin 12 h before the treatment of IL-1 stimulation (percentage inhibition at 10^{-6} M, erythromycin: $22.5 \pm 5.30\%$, clarithromycin: $28.5 \pm 2.98\%$). Lactate dehydrogenase (LDH) release assay, trypan blue dye exclusion test as well as a colorimetric 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay [25] revealed that this effect was not due to cytotoxicity (data not shown). Erythromycin and clarithromycin had a dose-dependent inhibitory effect on ET-1 release by primary, second-passaged bronchoepithelial cells as well as BEAS-2B cells (table 3). Northern blot analysis showed that both drugs, but not aminobenzyl penicillin, cefazolin, or a sixteen-member macrolide josamycin, inhibited the steady-state levels of ET-1 mRNA in IL-1 α ($10 \text{ ng}\cdot\text{mL}^{-1}$)-stimulated BEAS-2B cells (fig. 2). Erythromycin and clarithromycin had a dose-dependent inhibitory action on ET-1 mRNA in BEAS-2B cells (fig. 3).

Effect of antiasthma drugs on ET-1 production

Another potent anti-inflammatory agent, dexamethasone, clearly suppressed ET-1 production in normal cultured bronchial epithelial cells and in BEAS-2B cells (table 3 and fig. 4). In contrast, neither theophylline nor salbutamol showed any significant effect (fig. 4). A potent macrolide immuno-suppressant, FK506, showed no effect, either (fig. 4).

FK506 or motilin was given to BEAS-2B cells 18 h before the addition of erythromycin, and the influence of such agents on erythromycin effect was studied ($n=3$). Neither FK-506 nor motilin revealed any effect (percentage inhibition of ET-1 release: erythromycin (10^{-6} M) alone, $25.6 \pm 10.5\%$; with FK-506, $27.8 \pm 7.9\%$; with motilin, $24.9 \pm 11.3\%$, $p>0.05$). In addition, theophylline (10^{-4} M) and erythromycin (10^{-6} M) were simultaneously added to BEAS-2B cells, and the effect on ET-1 release was studied. Theophylline did not show any augmenting effect on erythromycin-induced suppression of ET-1 release (per-

centage inhibition: erythromycin alone, $25.9 \pm 3.13\%$, theophylline+erythromycin, $27.9 \pm 4.503\%$, $p>0.05$).

Northern blot analysis was performed to evaluate the effect of these drugs on ET-1 mRNA expression in BEAS-2B. Again, dexamethasone alone had a significant inhibitory effect on the IL-1-stimulated steady-state levels of

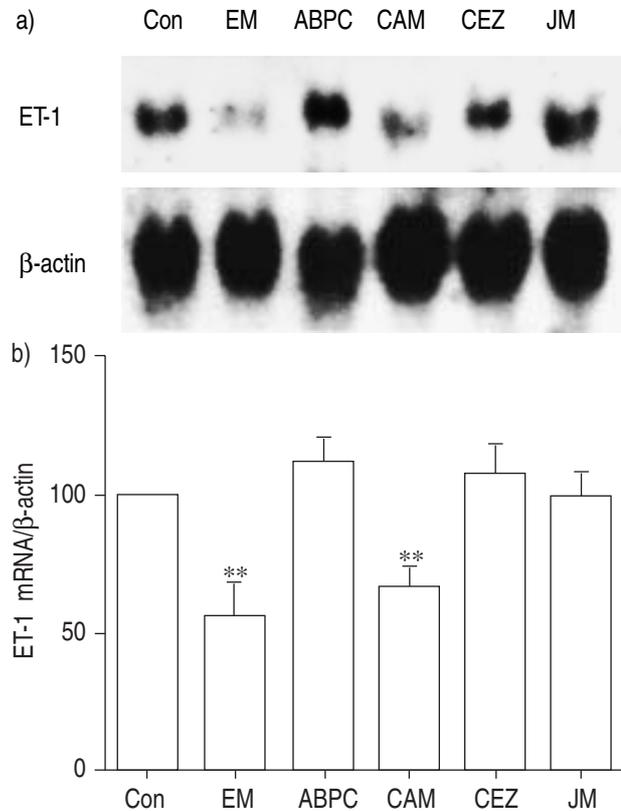


Fig. 2. – a) Northern blot analysis showing effect on erythromycin (EM), clarithromycin (CAM) and the other antibiotics on endothelin (ET)-1 messenger ribonucleic acid (RNA) expression by interleukin-1 α ($10 \text{ ng}\cdot\text{mL}^{-1}$)-stimulated BEAS-2B cells in culture. The total cellular RNA was extracted 6 h after treatment and electrophoresed as described in the methods section. EM and CAM, but not aminobenzyl penicillin (ABPC), cefazolin (CEZ) or josamycin (JM) showed an inhibition at 10^{-5} M. b) Measurement of densitometric signals of ET-1 corrected by actin transcripts showed a significant inhibition by EM and CAM (10^{-5} M). **: $p<0.01$ compared with controls (analysis of variance (ANOVA), $n=5$). Con: control.

Table 3. – Inhibitory effect (% inhibition) of erythromycin (EM), clarithromycin (CAM), FK506 and dexamethasone on endothelin-1 release by human bronchial epithelial cells

	Concentration M	Bronchial epithelial cells		
		Normal primary	Normal second passage	BEAS-2B
EM	10^{-7}	21.7 ± 3.22	24.0 ± 8.85	$24.1 \pm 4.70^{**}$
	10^{-6}	$26.4 \pm 5.22^{**}$	$30.9 \pm 5.02^{**}$	$30.4 \pm 9.02^{**}$
	10^{-5}	$48.4 \pm 4.80^{**}$	$38.1 \pm 4.90^{**}$	$35.4 \pm 4.42^{**}$
CAM	10^{-7}	21.2 ± 5.67	20.8 ± 8.52	$24.0 \pm 4.78^{**}$
	10^{-6}	$31.2 \pm 7.45^{**}$	$40.4 \pm 8.90^{**}$	$33.9 \pm 8.87^{**}$
	10^{-5}	$49.0 \pm 5.50^{**}$	$37.1 \pm 8.90^{**}$	$41.1 \pm 3.98^{**}$
FK506	10^{-7}	2.04 ± 3.24	Not tested	Not tested
	10^{-6}	-1.89 ± 7.05	Not tested	Not tested
	10^{-5}	3.00 ± 3.90	Not tested	Not tested
Dexamethasone	10^{-7}	$46.6 \pm 0.75^{**}$	Not tested	$45.0 \pm 2.90^{**}$
	10^{-6}	$66.3 \pm 10.3^{**}$	Not tested	$65.4 \pm 8.90^{**}$
	10^{-5}	$75.8 \pm 6.34^{**}$	Not tested	Not tested

Data are shown as mean \pm SEM. **: $p<0.01$ as compared with interleukin (IL)-1 α -stimulated control release in each cell type (analysis of variance (ANOVA)).

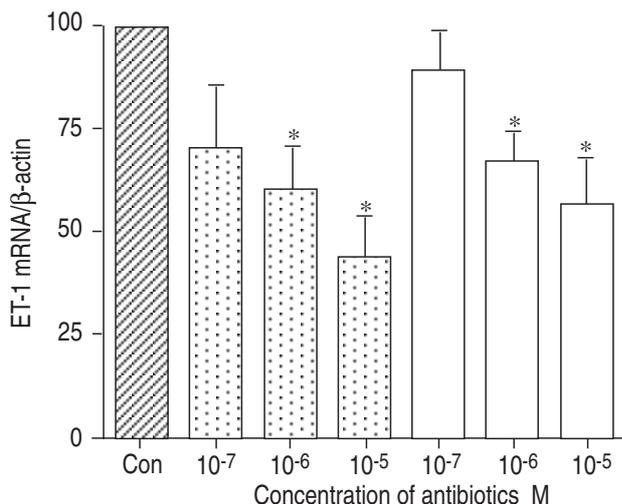


Fig. 3. – Erythromycin (EM; ▨) and clarithromycin (CAM; □) showed a dose-dependent inhibitory effect on endothelin (ET)-1 messenger ribonucleic acid (mRNA) levels as assessed by Northern blot analysis. Different concentrations of EM and CAM were added with interleukin-1α (10 ng·mL⁻¹) to BEAS-2B cells, and the total RNA was extracted as described previously. The data were from four representative experiments. *: p<0.05 compared with control (analysis of variance (ANOVA)). Con: control.

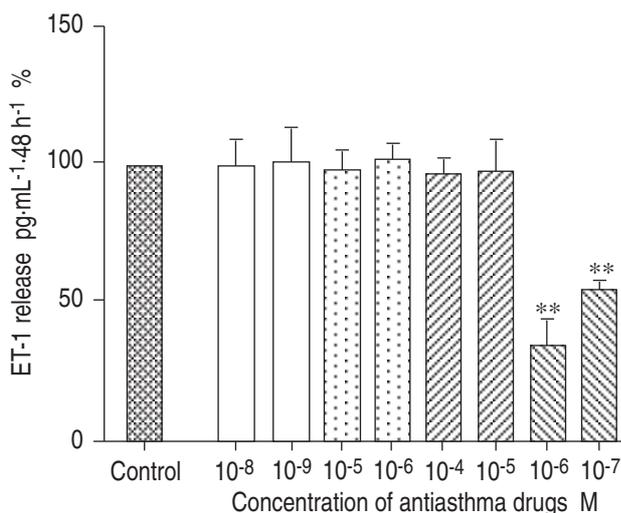


Fig. 4. – Effect of antiasthma drugs on endothelin (ET)-1 release from human bronchial epithelial cell line BEAS-2B. Dexamethasone (▨), theophylline (▧), salbutamol (▩) or FK506 (□) were added to the cells simultaneously with interleukin-1α (10 ng·mL⁻¹), and after 48 h, the supernatants were harvested for ET-1 assay. Dexamethasone, but not theophylline, salbutamol or FK506, showed a significant effect at the therapeutic concentrations. The data are shown as means±SEM from three representative experiments. **: p<0.01 (analysis of variance (ANOVA)).

ET-1 gene expression (percentage inhibition, dexamethasone at 10⁻⁶ M, 45.5±8.95%, p<0.01, ANOVA). Neither theophylline (10⁻⁴ M), salbutamol (10⁻⁵ M), nor FK506 (10⁻⁸ M) showed any effect (percentage inhibition, theophylline, 0.05±5.45%, salbutamol, -3.25±6.86%, FK506, 2.15±3.54%, p>0.05, ANOVA).

Spontaneous release of ET-1 by bronchial epithelial cells from patients with asthma and its relationship to lung functions

Human bronchoepithelial cells were obtained by fiberoptic bronchoscopy from eight patients with mild, stable asthma and eight nonasthmatic control subjects to evaluate ET-1 production. The cells were cultured until confluency, and the spontaneous release of ET-1 was measured. The amount of ET-1 spontaneously released from the primary bronchial epithelial cells of asthma patients was significantly greater than that from those of nonasthmatic subjects (ET-1 from asthmatic epithelial cells: 93.8±12.2 pg·48 h⁻¹ in a 48-well culture plate (n=8), ET-1 from nonasthmatic epithelial cells: 50.2±7.1 pg·48 h⁻¹ in 48-well plates (n=8), p<0.05, Student's t-test). As shown in figure 5, the amount of ET-1 spontaneously released for 48 h showed a significant negative correlation with the most recent value of the percentage peak expiratory flow rate (%PEFR) in each patient (n=8). ET-1 release did not correlate with the percentage forced expiratory volume in one second (%FEV₁), percentage forced vital capacity (%FVC), FEV₁/FVC or peripheral blood eosinophil count (r=0.321, 0.389, 0.330 and 0.332, p>0.05, respectively).

Suppressive effect of erythromycin and clarithromycin on ET-1 expression and release by bronchial epithelial cells obtained from patients with asthma

The effect of each of the drugs was evaluated after adding the drugs for 48 h. ET-1 protein levels in the supernatants of airway epithelial cells were significantly suppressed with the addition of erythromycin and clarithromycin as well as dexamethasone (percentage inhibition at 10⁻⁶ M, 25.2±11.2%, 32.6±12.3% and 34.2±5.20%, respectively), but not with theophylline (percentage inhibition at 10⁻⁴ M, 0.05±7.50%, p>0.05) or FK506 (percentage inhibition at 10⁻⁸ M, -3.25±6.78%, p>0.05). Northern blot analysis for

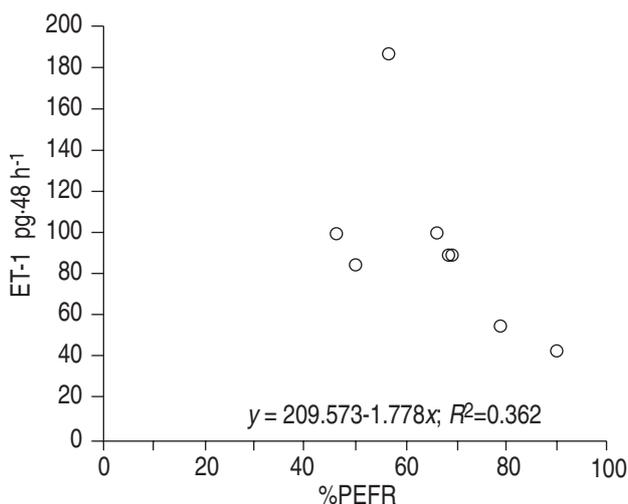


Fig. 5. – Relationship between percentage peak expiratory flow rate (PEFR) and spontaneous release of endothelin (ET)-1 by airway epithelial cells from patients with asthma. The percentage of PEFR was obtained from each patient within a week before bronchoscopic examination. There was a significant negative correlation between the extent of airflow limitation and airway production of ET-1 (Pearson correlation analysis).

the detection of ET-1 mRNA was performed in five cases, and erythromycin, clarithromycin and dexamethasone showed a suppressive effect on ET-1 mRNA levels (percentage inhibition of ET-1/ β -actin ratio at 10^{-6} M, $30.9 \pm 5.30\%$, $32.1 \pm 7.90\%$ and $51.9 \pm 13.5\%$, respectively, $p < 0.01$, ANOVA).

Discussion

In the present report, we found that fourteen-member macrolide antibiotics uniquely suppressed ET-1 expression and release in human bronchoepithelial cells. A glucocorticosteroid, dexamethasone, also showed a potent inhibitory effect, whereas neither theophylline, salbutamol, nor FK506 had any effect.

ET-1 is one of the important bronchoconstrictors [3]. It causes a strong, persistent constriction *in vitro* and *in vivo*. Clinical and experimental studies [3–5] have suggested the importance of this polypeptide in allergic airway inflammation. This polypeptide induces bronchoepithelial cells to release lipoxygenase products [26] and inhibits the proliferation of rat airway epithelial cells [27]. Therefore, ET-1 is one of the modulators of epithelial functions. Appropriate modulation of overproduced ET-1 may, therefore, be one mechanism that leads to the attenuation of airway hyperresponsiveness.

Erythromycin has been shown to be effective for the treatment of asthma in adults [9] and children [10]. *In vitro* studies showed that these macrolide antibiotics modulate neutrophil migration [28], lymphocyte proliferation [29], and monocyte differentiation [30]. Erythromycin has also been shown to inhibit cytokine release from monocytes [31]. We previously reported that erythromycin and clarithromycin as well as dexamethasone uniquely inhibited IL-6 expression by normal bronchial epithelial cells [14]. In the present study, erythromycin at the range of therapeutic concentration (10^{-6} M) reduced ET-1 expression at mRNA as well as at protein levels in human bronchoepithelial cells. This action appeared to be unique because other antibiotics, including sixteen-member macrolide josamycin did not show any effect. We further obtained airway epithelial cells from patients with asthma and studied ET-1 production by these cells. The spontaneous release of ET-1 from airway epithelial cells had a negative correlation with the magnitude of bronchial obstruction (%PEFR), highlighting a role of ET-1 in the pathogenesis of asthma. It was not clear why the percentage FVC or FEV₁ showed no significant correlation with ET-1 release. This might be partially because the abnormalities of study groups were relatively mild (table 1). Erythromycin and clarithromycin as well as dexamethasone, but not theophylline or salbutamol, inhibited ET-1 expression and release from such an inflamed epithelium.

We also studied the effect of antiasthma drugs on ET-1 production. A glucocorticosteroid dexamethasone [32], but not theophylline or salbutamol, inhibited ET-1 release from bronchial epithelial cells. Since theophylline has recently attracted attention for its anti-inflammatory action [33], it would be important to study the combined effect of theophylline and other drugs. In BEAS-2B cells, theophylline did not show any augmenting effect on erythromycin-induced ET-1 suppression. FK506, a macrolide immunosuppressant, has been reported to stimulate ET-1 expression

in human endothelial cells *in vitro* [34]. However, this drug showed no effect in the present experiment.

Another important issue would be to elucidate the molecular mechanisms of how these fourteen-member ring macrolides suppressed endothelin-1 expression. KEICHO *et al.* [29] showed that erythromycin affected the proliferation of peripheral blood lymphocytes at the later stages of signals as compared with FK506, another macrolide agent. Pretreatment with erythromycin did not affect the action of FK506, and, thus, this antibiotic showed its effect *via* a pathway different from that of FK506. Erythromycin also has a motilin-like stimulating activity on gastrointestinal smooth muscles [35], but neither the gastrointestinal hormone motilin itself nor FK506 had any effect on endothelin-1 release by bronchial epithelial cells in our studies. Recent studies have demonstrated the important roles of transcription factors in endothelin-1 expression [34, 36]. Further studies will be necessary to elucidate the mechanism of erythromycin and clarithromycin effects on transcription rate and degradation of endothelin-1 messenger ribonucleic acid. Characterization of the chemical structure responsible for its potential would also be important to pursue for a possible new type of anti-inflammatory agent in the treatment of asthma.

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