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Effect of Endothelin-1 on Proliferation and Differentiation of Rat Tracheal Epithelial Cells

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A number of substances involved in the proliferation and differentiation of the tracheobronchial epithelium have been identified. The defects in the control of the proliferation and differentiation of tracheobronchial epithelial cells appear to constitute crucial steps in the transition of normal cells to neoplastic ones. Endothelin-1 is produced by tracheal epithelial cells, and its receptors are present in tracheal epithelial cells. However, the effect of endothelin-1 on the proliferation and differentiation of tracheal epithelial cells has not been clearly elucidated. This study was undertaken to investigate these actions of endothelin-1 in primary cultured cells of rat tracheal epithelia. Endothelin-1 stimulated proliferation of tracheal epithelial cells 1.5-fold when compared with that of control cells. Endothelin-1 increased mitogen-activated protein kinase (MAPK) activity. Herbimycin A, a tyrosine kinase inhibitor, inhibited endothelin-1-induced proliferation of epithelial cells. The treatment of endothelin-1 during the primary culture of tracheal epithelial cells increased AB-PAS-stained cell population and ciliated cell population 6.5 fold and 1.5 fold, respectively, when compared with those in control cells. The responsiveness to carbachol and forskolin in the Cl^- secretion was increased 1.7 and 1.9 fold, respectively, in the endothelin-treated epithelial cells. These results indicated that endothelin-1 increases proliferation via MAPK pathway and stimulates differentiation to secretory and ciliated cells in rat tracheal epithelial cells.

Key Words: Endothelin-1, Rat, Tracheal epithelial cells, Proliferation, Differentiation

INTRODUCTION

The normal tracheobronchial epithelium is a pseudostratified epithelium consisting of three major cell types: basal cells, secretory cells, and ciliated cells (Jing et al, 1994). Like other tissues, the tracheobronchial epithelium turns over continuously: cells slough off into the tracheobronchial lumen and are replaced via the proliferation and differentiation of tracheobronchial stem or progenitor cells. In normal epithelium, the rates of proliferation, differentiation, and cell loss must be equal to create a balance. This delicate balance is maintained via an equilibrium between regulatory factors that have either a positive or negative effect on the growth and differentiation of these cells. The defects in the control of proliferation and differentiation appear to constitute crucial steps in the transition of normal to neoplastic cells (Anton et al, 1990; Hubbs et al, 1989).

Endothelin-1 (ET-1) was originally characterized from cultured porcine endothelial cells (Yanagisawa et al, 1988). Subsequent cDNA cloning revealed the presence of three isopeptides, termed ET-1, ET-2, and ET-3 (Inoue et al, 1989) in human. Synthesis of ET-1 has been identified in a wide variety of tissues including vascular endothelium, neurons in the central nervous system, and airway epithelium, suggesting its diverse physiological functions (Rozengurt et al,

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1990). Its role as a growth factor has been seen in vascular smooth muscle cells (Komuro et al, 1988), fibroblasts (Takuwa et al, 1989) and glomerular mesangial cells (Simonson et al, 1989). In these cells endothelin-1 increases cell proliferation and the expression of protooncogenes such as c-myc and c-fos. Endothelin-1 is produced by tracheobroncial epithelial cells, and its specific binding sites are also present in airway epithelial cells (Power et al, 1989; Mattoli et al, 1990; Rozengurt et al, 1990). It has been shown that endothelin-1 stimulates proliferation of airway epithelial cell growth (Murlas et al, 1995; Ninomiya et al, 1998). However, the signaling pathway of endothelin-induced proliferation has not been examined, and the effect of endothelin on differentiation of tracheobronchial epithelial cells has not been reported.

To investigate the mechanism of endothelin-induced proliferation and its effect on differentiation of airway epithelial cells, primary culture of rat tracheal epithelial cells were used. The results indicated that endothelin-1 increased proliferation via activation of MAPK pathway and stimulated differentiation of tracheal epithelial cells to secretory and ciliated cells.

METHODS

Primary cell culture

Sprague Dawley rats (200~300 g) were anesthetized with intraperitoneal injection of pentobarbital sodium (120 mg/100 g b.w.). Primary cell culture was done by the minor modification of the procedures as described prevously (Jung et al, 1997). Briefly, freshly excised tracheae were incubated at 4°C for 18~24 h in a Ca^{2+}- and Mg^{2+}-free, serum-free modified Eagle's minimum essential medium (MEM) containing 0.01% protease XIV (Sigma, St. Louis, MO). The epithelial cells were removed from the airways by scraping the epithelial surface of the tracheae, and the cells were then washed with fresh MEM medium containing fetal bovine serum (FBS) to neutralize the protease. After the final wash, the cells (-10^6) were resuspended in DMEM/Ham's F-12 mixture containing insulin, transferrin, hydrocortisone, triiodothyronine, prostaglandin E, epidermal growth factor (EGF), 500 units/ml penicillin and 500 pg/ml streptomycin (K1-10 medium). 0.5 ml of final cell suspension was plated to culture plate, or a permeable filter support for electrophysiological studies (12 mm TRANS WELL; Costar, Cambridge, MA). The cells were incubated in an atmosphere of 5% CO_2-95% air at 37°C.

Clonal growth assay

The total number of cells were counted 1, 3, 5 days after seeding 10^4 tracheal epithelial cells in the 6 well culture plate (Willey et al, 1984). For clonal growth assay, the medium was replaced with the basal medium (K1-10 medium without EGF and FBS), which removed the effect of EGF and FBS.

Measurement of mitogen activated protein kinase (MAPK) activity

Epithelial cells were lysed with ice-cold lysis buffer (20 mM Tris-HCl pH 8, 1% Triton X-100, 10% glycerol, 131 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 1 mM PMSF, 20 μM leupeptin and 10 μg/ml aprotinin) for 10 min with occcasional vortexing. Lysates were cleared off nuclei and detergent insoluble material when centrifuged for 10 min at 14,000 rpm. 100 μg of cell lysate were immunoprecipitated with the anti-MAPK antibody (Zymed Laboratories. Inc.) and protein A-Sepharose beads (Sigma Chemical Co.) overnight. Immune precipitates were collected by centrifugation for 10 sec at 14,000 rpm and were washed three times with 1 ml of kinase buffer (30 mM Tris pH 8, 10 mM $MgCl_2$) before being resuspended in 30 μl of kinase assay cocktail containing kinase buffer, 7 μg of myelin basic protein (MBP, Sigma Chemical Co.), 2 μM cold ATP, and 1 μCi of [γ-^{32}P] ATP (Amersham Co.) per sample. Incubation was carried out for 30 min at 30°C and were terminated by the addition of 2x SDS-PAGE sample buffer, followed by boiling for 5 min at 95°C. Samples were resolved on a 12% SDS-PAGE. The gel was dried and autoradiographed (Kang et al, 1996).

Alcian blue-periodic acid schiff (AB-PAS) staining

After fixation by 10% neutral buffered formalin (NBF), the tracheal epithelial cells were incubated in the following solutions: 30 min in 1% alcian blue in 3% acetic acid, 30 min in periodic acid, 5 min in Schiff's reagent (Sigma), and 3 min in 0.5% sodium metabisulfite. Slides were counterstained with 1%

methyl green. For each experimental group, the number of AB-PAS positive cells in a total of 500 cells per slide was determined, with a total of 1,500 cells scored per experimental group (Elizabeth & Paul, 1996).

Electrophysiology

Transepithelial electrophysiological measurements are performed in a modified Ussing chamber constructed to accept TRANSWELL filter (World Precision Instrument). Short-circuit current (I_{SC}) was measured with a DVC-1000 voltage-current clamp (World Precision Instrument) with the voltage clamp mode. The filters above 1,000 $\Omega \times cm^2$ in transepithelial resistance measured at the EVOM epithelial ohmmeter were used for all experiments. The bath solution was Kreb's bicarbonate Ringer's solution (KBR) which was composed of (in mM) 140 NaCl, 2.3 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, and 10 glucose (pH 7.4). Both mucosal and serosal bath solutions were subjected to constant recirculation, maintained at 37°C, and oxygenated gently with 95%/5% O_2/CO_2. (Jung et al, 1997).

Data presentation

The I_{SC} reported in this study was the maximum achieved, independent of the phase of the response. Paired or unpaired Student's t-test was utilized to analyze differences between means.

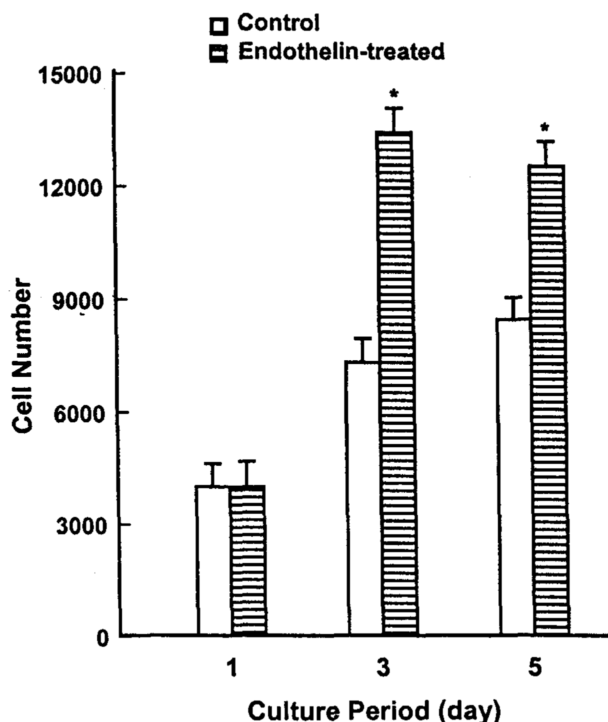

Fig. 1. Effect of endothelin-1 on proliferation of tracheal epithelial cells. Tracheal epithelial cells were protease-dissociated and plated in the K1-10 medium. After 1 day, the culture medium was changed to the basal medium containing hormone mixture (insulin, transferrin, hydrocortisone, triiodothyronine, prostaglandin E) with(▤) or without(□) 0.1 μM endothelin-1. The total numbers of cells per plate were determined on the 1st, 3rd and 5th day after seeding as described in "Materials and Method". Data are mean±S.E. of four experiments. Asterisk(*) indicates value where $p < 0.001$ compared to the control value.

RESULTS

Effect of endothelin-1 on proliferation of tracheal epithelial cells

To investigate the effect of endothelin-1 on proliferation of tracheal epithelial cells, we performed clonal growth assay. The treatment of 0.1 μM endothelin-1 increased the proliferation of tracheal

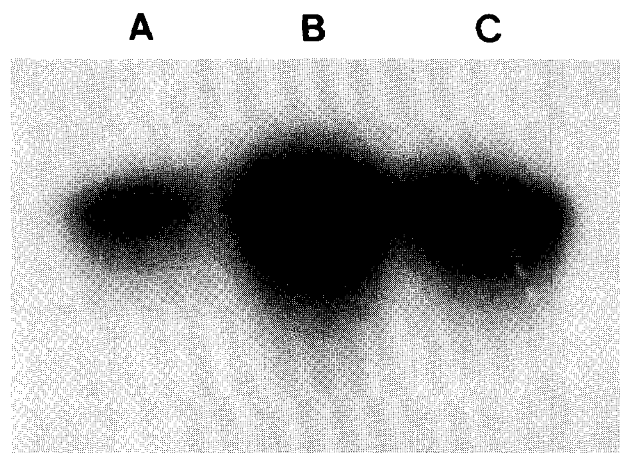

Fig. 2. Activation of MAPK by endothelin-1 in tracheal epithelia cells. Tracheal epithelial cells were incubated in serum-free DMEM/Ham's F12 medium for 6 h and treated with 25 ng/ml EGF or 0.1 μM endothelin-1 for 5 min. Then epithelial cells were scraped and MAPK activity was determined as described in "Materials and Method". A: Control, B: EGF-treated, C: Endothelin-treated.

epithelial cells. On the third day after treatment the number of tracheal epithelial cells in 0.1 μM endothelin-1-treated group was about 1.5 times more than in the control (Fig. 1).

To determine the mechanism for the endothelin-induced proliferation of tracheal cells, we measured mitogen-activated protein kinase (MAPK) activity of tracheal epithelial cells. The addition of EGF (25 ng/ml), a well-known stimulator of MAPK, strongly activated MAPK activity in tracheal epithelial cells grown in serum-free medium. The treatment of 0.1 μM endothelin-1 increased MAPK activity with respect to the control (Fig. 2). To identify whether activation of tyrosine kinase is involved in the endothelin-induced proliferation of tracheal cells, the effect of herbimycin A, an inhibitor of tyrosine kinase, was examined. Herbimycin A (1 μM) totally blocked the endothelin-induced increase of cell proliferation (Fig. 3).

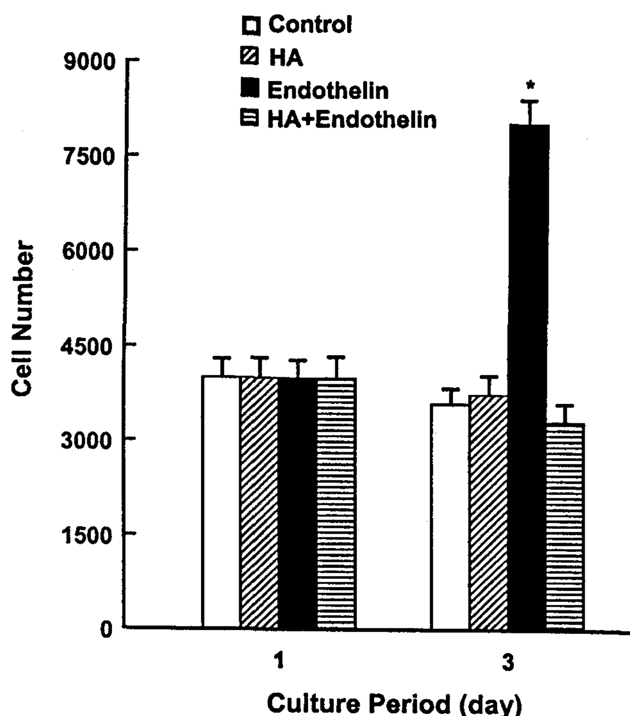

Fig. 3. Effect of herbimycin A, a tyrosine kinase inhibitor, on endothelin-1-stimulated proliferation of epithelial cells. Tracheal epithelial cells were protease-dissociated and plated in the K1-10 medium. After 1 day, culture medium were changed to the basal medium(□); basal medium containing 1 μM herbimycin A (▨); 0.1 μM endothelin-1 (■); 0.1 μM endothelin-1 and 1 μM herbimycin A(▤). The total number of cells per plate were determined on the 1st and 3rd day after seeding as described in "Materials and Method". Data are mean± S.E. of five experiments. Asterisk(*) indicates value where p<0.001 compared to the control value. HA: herbimycin A.

Effect of endothelin-1 on differentiation of tracheal epithelial cells

To determine whether endothelin-1 stimulates differentiation of tracheal epithelial cells to secretory or ciliated cells, change in the number of secretory and ciliated cells was investigated. Secretory cells were identified by AB-PAS staining and the number of ciliated cells was directly counted under inverted microscopy. The proportion of secretory and ciliated cells was increased 4.3 times and 1.5 times more by the treatment of 0.1 μM endothelin-1 than to the control (Table 1, Fig. 4). We also observed the morphology of tracheal epithelial cells cultured in transwell filters. In endothelin-treated group the shape of tracheal epithelial cells was cuboid, and epithelial cells formed two layers partly. However, in the control group, the shape of tracheal epithelial cells was flat, and epithelial cells formed a monolayer (Fig. 5). These results mean that endothelin-1 stimulates differentiation of tracheal epithelial cells to secretory and ciliated cells.

Effect of endothelin-1 on electrophysiological characteristics of tracheal epithelial cells

In this experiment, endothelin-1 stimulated diffe-

Table 1. Quantitation of secretory cells and cilliated cells in tracheal epithelial cultures. Primary tracheal epithelial cells were protease-dissociated and plated in the K1-10 medium. After 1 day, culture medium were changed to the basal medium or the basal medium containing 0.1 μM endothelin. Tracheal epithelial cells were stained with AB-PAS on the 4th day after seeding. The number of stained cells was determined as described in "Materials and Method". Data are mean±S.E. of five experiments. Asterisk(*) indicates value where p<0.001 compared to the control value.

	Control	Endothelin-treated
% AB-PAS positive cells	2.0±0.5	8.0±1.5*
% Cilliated cells	12.3±2	17.5±2.5*

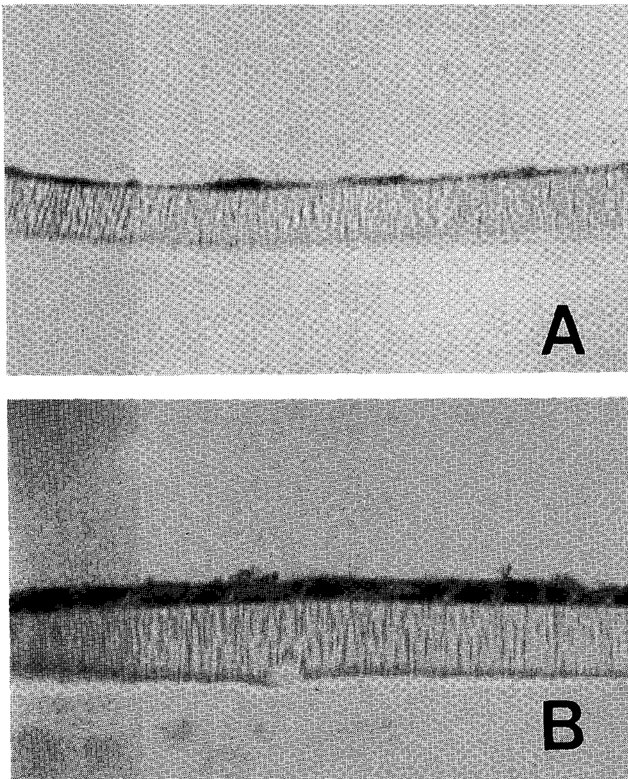

Fig. 4. Effect of endothelin-1 on morphology of tracheal epithelial cells cultured in SNAPWELL filter. Tracheal epithelial cells were cultured in the K1-10 medium with(B) or without(A) 0.1 μM endothelin-1. Tracheal epithelial cells were fixated and stained with Hamatoxylin-Eosin on the 6th day after seeding (×450).

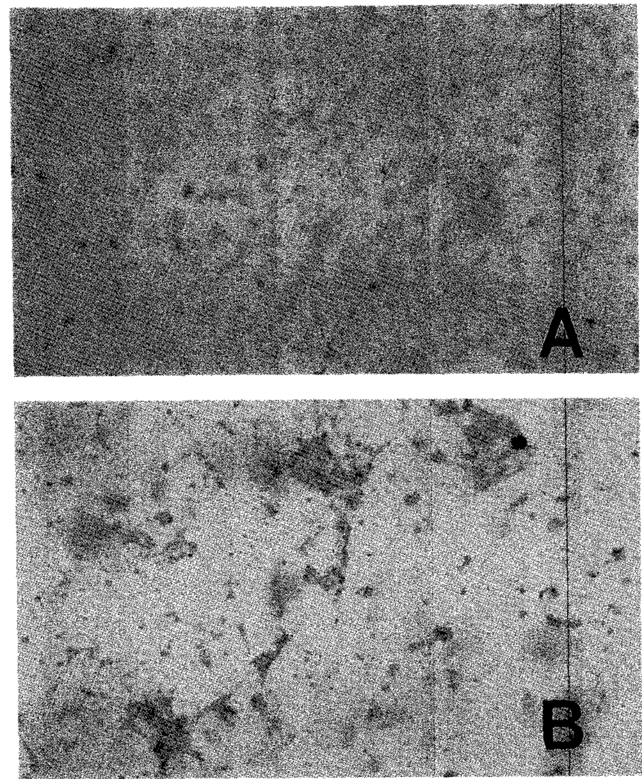

Fig. 5. AB-PAS staining of tracheal epithelial cells. Tracheal epithelial cells were protease-dissociated and plated in the K1-10 medium. After 1 day, culture medium was changed to the basal medium (A); basal medium containing 0.1 μM endothelin (B). Epithelial cells were fixated and stained with AB-PAS on the 4th day after seeding as described in "Materials and Method" (×100).

Table 2. Change of transepithelial potential difference (PD) and transepithelial resistance by endothelin-1 in tracheal epithelial cells. Tracheal epithelial cells were cultured in the K1-10 medium with or without 0.1 μM endothelin-1. PD was measured by DVC-1000 voltage-current clamp (World Precision Instrument, Sarasota, FL) and transepithelial resistance was measured by EVOM epithelial ohmmeter (World Precision Instrument, Sarasota, FL). Data are mean±S.E. of ten experiments. Asterisk(*) indicates value where $p < 0.01$ compared to the control value.

	Control	Endothelin-treated
Resistance (Ω)	1310±60	1160±54
Transepithelial potential difference (mV)	10.5±1.8	29.6±2.3*

rentiation of treacheal epithelial cells to secretory and ciliated cells, suggesting that endothelin-1 might induce functional differentiation of tracheal epithelial cells. Putting this possibility to the test, we examined the change of electrophysiological characteristics in tracheal epithelial cells maintained in endothelin-1 -treated media.

In the tracheal epithelium, transepithelial potential difference (PD) is generated from basal Cl^- secretion and Na^+ absorption. PD was higher in the endothelin-treated group than in the control group. However, transepithelial resistance was similar in both groups (Table 2).

We tested the effects of secretagogues on I_{SC} to define the change of responsiveness to secretogogues in endothelin-1-treated tracheal epithelial cells. In tracheal epithelial cells, major signaling pathways of Cl^- secretion are Ca^{2+} and cAMP pathways

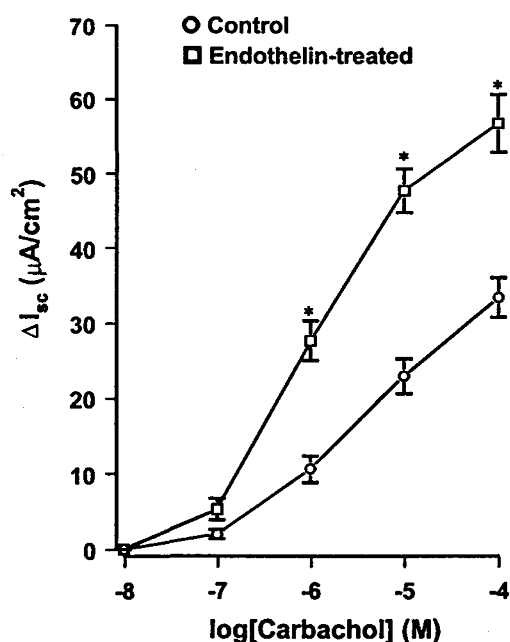

Fig. 6. The increase in Cl^- secretion induced by carbachol in endothelin-1-treated cells. Isc was measured by voltage-current clamp. Tracheal epithelial cells were cultured in the K1-10 medium with (□) or without (○) 0.1 μM endothelin-1. Data are mean±S.E. of four experiments. Asterisk (*) indicates values where $p<0.001$ compared to the control value.

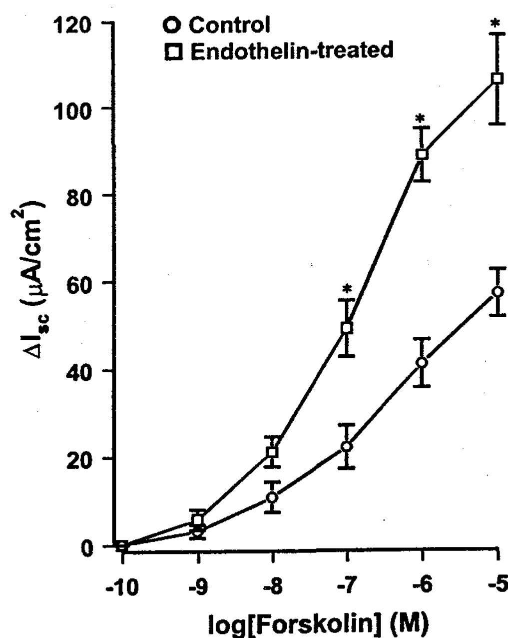

Fig. 7. The increase in Cl^- secretion induced by forskolin in endothelin-1-treated cells. Isc was measured by voltage-current clamp. Epithelial cells were cultured in the K1-10 medium with (□) or without (○) 0.1 μM endothelin-1. Data are mean±S.E. of four experiments. Asterisk (*) indicates values where $p<0.001$ compared to the control value.

(Anderson et al, 1991). Therefore, we examined effects of carbachol and forskolin, which increase intracellular Ca^{2+} and cAMP, respectively, on Isc. In endothelin-1-treated group, the responses of Isc to carbachol and forskolin were 1.7 and 1.9 times higher, respectively, than those in the control group (Fig. 6 and Fig. 7).

DISCUSSION

The normal turnover of the tracheal epithelium depends on a delicate balance between the cell loss and the cell renewal. The role of endothelin-1 in the control of proliferation and differentiation of airway epithelial cells was investigated in this study. Endothelin-1 was found to increase proliferation via MAPK pathway and stimulate differentiation to secretory and ciliated cells in rat tracheal epithelial cells, indicating that endothelin-1 may have substantial importance in autocrine and paracrine control of airway epithelial cell growth.

In this study, endothelin-1 increased proliferation of tracheal epithelial cells. Endothelin-induced proliferation has been reported in tracheal epithelial cells (Murlas et al, 1995; Ninomiya et al, 1998) as well as vascular smooth muscle cells (Komuro et al, 1988), fibroblasts (Takuwa et al, 1989), glomerular mesangial cells (Simonson et al, 1989). Most growth factors induce cell proliferation via activation of receptor tyrosine kinase and Ras-MAPK kinase pathway (Bruce et al, 1994). The data in the present study also supported that the effect of endothelin-1 in tracheal epithelial cells was mediated by increase in tyrosine phosphorylation and activation of MAPK pathway. The effect of endothelin-1 is mediated by the activation of G protein-coupled receptor (Rubanyi & Polokoff, 1994). Although we did not examined mechanisms of MAPK activation by endothelin-1, recent studies demonstrated that activation of G-protein coupled receptors by various agonists could induce MAPK activation by transactivation of EGFR (Dikic et al, 1996; Henrik et al, 1996) or by tyrosine phosphorylation of Pyk2 (Dikic et al, 1996), or Lyn and Syk protein (Wan et al, 1996).

A number of substances including retinoic acid,

hormones and growth factors, are involved in the differentiation of tracheal epithelial cells (van Scott et al, 1988). Retinoic acid, transferrin and insulin are known to stimulate differentiation of airway epithelial cells to secretory and ciliated cells, although their mechanisms have not been clearly elucidated (Anton, 1991; van Scott et al, 1988). In the present study, endothelin-1 increased proportion of PAS-positive cells and ciliated cells, indicating differentiation of tracheal epithelial cells. Stimulation of differentiation by endothelin-1 has been reported in neural crest cells and osteoblastic cells (Kasperk et al, 1997).

The induction of differentiation of tracheal cells by endothelin-1 was also supported by data obtained by electrophysiological measurement. Endothelin-treated monolayers developed higher potential difference without significantly affecting transepithelial resistance than that in control monolayers, and also increased secretagogues-induced I_{SC}. These results can be explained by an increase of chloride secretion in endothelin-treated tracheal epithelial cells, which is resulted from increase in the number of secretory cells in tracheal monolayers. Effect of endothelin-1 on cellular differentiation was also reported in adipocytes (Hauner et al, 1994) and airway fibroblast (Sun et al, 1997).

In summary, this study shows that endothelin-1 increases proliferation via activation of MAPK pathway and stimulates differentiation of tracheal epithelial cells to secretory and ciliated cells.

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