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Am J Physiol Gastrointest Liver Physiol 295:1182-1189, 2008. First published Oct 2, 2008;
doi:10.1152/ajpgi.90294.2008

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Endothelin-3 stimulates survival of goblet cells in organotypic cultures of fetal human colonic epithelium

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Submitted 16 April 2008; accepted in final form 25 September 2008

Kalabis J, Li G, Fukunaga-Kalabis M, Rustgi AK, Herlyn M. Endothelin-3 stimulates survival of goblet cells in organotypic cultures of fetal human colonic epithelium. *Am J Physiol Gastrointest Liver Physiol* 295: G1182–G1189, 2008. First published October 2, 2008; doi:10.1152/ajpgi.90294.2008.—Cells within the normal human colonic epithelium undergo a dynamic cycle of growth, differentiation, and death. The organotypic culture system of human fetal colonic epithelial cells seeded on top of collagen gels with embedded colonic fibroblasts allowed prolonged culture of the colonic epithelial cells (Kalabis J, Patterson MJ, Enders GM, Marian B, Iozzo RV, Rogler G, Gimotty PA, Herlyn M. *FASEB J* 17: 1115–1117, 2003). Herein, we have evaluated the role of endothelin-3 (ET3) and both cognate endothelin receptors (ETRA, ETRB) for human colonic epithelial cell growth and survival. ET3 was produced continuously by the fibroblasts as a result of adenovirus-mediated gene transfer. The presence and function of the endothelin receptors (ETRs) in epithelial cells was evaluated by [³H]thymidine incorporation using primary epithelial cells in monoculture and by immunohistochemistry on human fetal and adult paraffin-embedded tissues. In organotypic culture, ET3 increased the number of goblet cells but not of enteroendocrine cells. The increase in goblet cells was caused by prolonged cell survival and differentiation. The inhibition of both ETRA and ETRB significantly decreased the number of goblet cells and proliferation in epithelial cells, whereas the number of enteroendocrine cells remained unchanged. ET3 induced activation of I κ B and MAPK in the epithelial cells, suggesting that these signaling pathways mediate its proliferation and prosurvival activities. Our results demonstrate that ET3 is involved in regulating human colonic epithelial cell proliferation and survival, particularly for goblet cells, and may be an important component of colonic homeostasis.

endothelin; proliferation; survival

THE COLONIC EPITHELIUM COMPRISES three major cell types: absorptive enterocytes, goblet cells, and enteroendocrine cells, all of which differentiate from a common stem cell pool in crypts (3). Proliferation, differentiation, and migration of cells are regulated by a complex network of cell-cell and cell-matrix interactions and modulated by local growth factors, luminal factors, and hormones (20, 29). Goblet cells are the major mucus-producing cells and are believed to play an important role in mucosal cytoprotection. Goblet cells secrete mucins, trefoil proteins, and other factors that help protect the intestinal mucosa from injury and facilitate tissue repair (8, 14, 19). Mice deficient in the *Muc2* gene display aberrant intestinal crypt morphology and altered cell maturation and migration (36). They also frequently developed adenomas in the small intestine that progressed to invasive adenocarcinoma as

well as rectal tumors. The number and activity of goblet cells in the gut can be altered by inflammatory cytokines and bacterial products (26) during intestinal infection (1), inflammatory bowel disease (5, 34), and colon carcinogenesis (5). In the mouse colon, cells of goblet lineage form ~16% of the entire crypt cell population (6). The mucous granules in these cells are large and numerous and confer a characteristic gobletlike appearance. The overall turnover time of goblet cells is 4.6 days (6).

Endothelin (ET) 1, -2, and -3 comprise a family of three 21-amino acid peptides encoded by distinct genes and are synthesized as larger precursor molecules that have to be cleaved by proteases to produce biologically active peptides (31). Two receptors have been identified: the endothelin A receptor (ETRA) that binds ET1 and -2, and the endothelin B receptor (ETRB) that has affinities for ET1, -2, and -3 (31). All three ETs and their receptors are present in human small intestine and colon (32–33). ETs act predominantly locally, in a paracrine or autocrine fashion. ET1 is a potent vasoconstrictor that also stimulates degranulation of mast cells in the intestinal lamina propria and induces leukostasis of leukocytes within intestinal vessels. In the intestinal epithelium, ETs stimulate epithelial cell secretions of ions (22) and they are upregulated in acute and chronic intestinal inflammation as well as colon carcinoma to foster cell proliferation and survival (1, 24).

In this study we evaluated the physiological role of ET3 in a unique three-dimensional organotypic culture of the human colonic epithelium. This *in vitro* model consists of proliferative, polarized, and differentiated normal human fetal intestinal epithelial cells grown on top of collagen gels containing colonic fibroblasts (18). In this system, adenovirus-mediated ET3 gene transfer in the fibroblasts increased the number of goblet cells by stimulating the proliferation of epithelial cells and prolonging the survival of goblet cells. Only simultaneous inhibition of both ETRA and ETRB significantly decreased the presence of goblet cells and the proliferation and total number of epithelial cells, but the number of enteroendocrine cells remained unchanged. ET3 effects may be attributed to phosphorylation of I κ B and MAP kinase. Our results suggest that ET3 and its receptors are involved in the regulation of colonic epithelial cell proliferation, differentiation, and survival.

MATERIALS AND METHODS

Antibodies and reagents. Studies used Ki-67 (DAKO, Carpinteria, CA); α -smooth muscle actin and β -actin (Sigma-Aldrich, St. Louis, MO); chromogranin A and Bcl-2 (Novocastra, New Castle Upon Tyne, UK); ETRA and ETRB (Abcam, Cambridge, MA); phospho-

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I κ B, total I κ B, phospho-AKT, and total and phospho-MAPK (p42/44 MAPK; Cell Signaling, Beverly, MA). BQ123 and BQ788 (Sigma) were dissolved in 0.01% NaOH (1 mM) and used as 1 μ M. 0.01% NaOH dissolved in BSA (Sigma) was used as negative control.

Adenoviral vectors. The adenoviral vectors carrying genes for ET3 (4), hepatocyte growth factor (HGF) (25), and stem cell factor (SCF) (4) have been described. Plaque-purified virus propagated in 293 cells was purified by ultracentrifugation in cesium chloride gradients. Virus titer was assayed by plaque formation in permissive 293 cells. Control adenoviral vector containing green fluorescent protein (GFP) was obtained from the Cell Culture Core of the University of Pennsylvania, Philadelphia, PA. Subconfluent (80%) fetal colonic fibroblasts FC331 (18) were infected with replication-deficient adenoviruses at 20 pfu/cell for 4 h at 37°C in protein-free medium. The viral suspension was then replaced with regular medium. Cells were allowed to recover for at least 24 h before assays. Optimal transfection of fibroblasts by adenovectors was monitored in cells per area by detecting adenovector-introduced GFP (Ad/GFP) 24 h after transfection by flow cytometry. Ad/GFP (20 pfu) were able to transfect ~95% of fibroblasts without signs of toxicity.

Isolation of human colonic epithelial cells. Specimens of fetal tissue were received from Advanced Bioscience Resources (Alameda, CA), and normal adult human colon samples were obtained from the Cooperative Human Tissue Network after approval by the Institutional Review Board. The epithelial cells were isolated from fetal colon as described previously (18). Single cells and cell clusters were washed and resuspended in base medium (18) supplemented with 2 ng/ml human recombinant EGF (Sigma), 5 μ g/ml insulin (Sigma), or 26.4 ng/ml ET3 (Peninsula Laboratories, San Carlos, CA).

Fibroblasts. Human colonic fibroblasts were derived from colon explants as described (18). Fibroblasts from three specimens were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (CanSera International, Rexdale, Canada) and antibiotics. Cultures were used up to passage 10. The fibroblasts were infected with adenovector containing either GFP, SCF, HGF, or ET3.

Colon organotypic culture. Colon organotypic cultures were prepared as described (18) with modifications. Untransfected, control GFP-, HGF-, SCF-, or ET3-transfected fibroblasts at 8×10^5 /ml were embedded in collagen type I (Organogenesis, Canton, MA) at 0.9 to 1.1 mg/ml in DMEM supplemented with 50 μ M vitamin C (Sigma), 1.66 mM L-glutamine (Invitrogen), and 1% FCS. The suspension was neutralized to pH 7.2, using 7.5% sodium bicarbonate. Chambered slides, Lab-Tek 8 wells (Nunc International, Rochester, NY), were filled with 0.2 ml collagen containing suspended fibroblasts.

Colonic epithelial cells were seeded on top of collagen gels containing embedded fibroblasts in 0.4 ml of base growth medium. After seeding, samples were incubated at 37°C in 5% CO₂ for 60–90 min in a low volume of medium (50–100 μ l) to enhance attachment before adding excess growth medium. Medium was changed daily for 10 days. Proliferation of organotypic cultures was determined by adding bromodeoxyuridine (BrdU) to the medium according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ).

Thymidine incorporation. Thymidine incorporation was measured in cells by incubation with 1 μ Ci [³H]thymidine/well in 96-well plates. Duplicates of four samples were incubated in medium as described in RESULTS for 18 h before harvest, and radioactivity was determined on day 2.

ELISA. ET3 protein in the culture supernatants was measured with an ET3 ELISA kit (Peninsula Laboratories, Torrance, CA), according to the manufacturer's protocol. All experiments were repeated three times.

Western blotting. HT-29 colon carcinoma cells (ATCC, Manassas, VA) were washed with PBS and harvested in Nonidet P-40 (NP-40) buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris·HCl pH 7.4), supplemented with Complete Mini, EDTA-free protease inhibitors (Roche Diagnostics, Penzberg, Germany), 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 mM PMSF (Sigma). Fetal epithelial cells were isolated as described

previously, spun down, and lysed in NP-40 buffer. Normal adult colonic tissue was thawed and minced while submerged in NP-40 lysis buffer using a tissue homogenizer. Total protein concentrations were measured using the bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL). Samples were loaded at 20 μ g/lane, separated on 8–12% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with specific primary antibodies. To detect the signal, peroxidase-conjugated secondary antibody was added, followed by exposure using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Characterization of organotypic cultures. Harvested organotypic cultures were fixed in 1.5% paraformaldehyde and embedded in paraffin. For histology and immunohistochemistry, 5- μ m-thick sections were cut from the paraffin blocks and stained with hematoxylin and eosin (H&E) or Alcian blue by standard procedures. The presence of goblet cells in samples of organotypic cultures was confirmed previously by electron microscopy (18). Enteroendocrine cells were identified by chromogranin A staining. Immunohistochemical staining was performed as described (18) by standard techniques using specific antibodies.

Quantifications were carried out as described previously (18) with some modifications. The number of cells per high-power field (HPF; 0.11 mm wide \times 0.18 mm long) was determined using a \times 40 lens and a digital camera attached to a microscope. Quantification was carried out with respect to the shape of the surface and the constriction of the matrix. The length of the epithelium on the surface of the organotypic cultures was equalized by rotating the digital image such that the length of each HPF was parallel to the epithelial layer. Each HPF of epithelial lining thus measured 0.18 mm. Epithelial cells on the surface of the collagen matrix were identified from cross sections. Those within the length of 12 consecutive HPFs (surface units) were scored. The results were expressed as cells/surface unit. Total and specially stained epithelial cells (Ki-67, BrdU, Alcian blue, or chromogranin A) per surface unit of each sample were counted as means \pm SE of 20 cross sections. Results for different experimental conditions were normalized with the control value representing 100%. For quantifications, we used the results from duplicate cultures, derived from 8 different specimens of fetal human colon in 3 independent experiments. The relative number of positive cells per surface unit was expressed as mean \pm SE positive cells vs. total cells per surface unit.

Constriction of collagen gels was measured as the length of the longitudinal axis of collagen harvested on day 10. The mean \pm SE of 56 measurements was determined by counting seven samples in duplicate and using four random cross sections of each. The number of fibroblasts was determined per area unit of collagen matrix in 10 consecutive HPFs ($10 \times 0.18 \times 0.11 = 0.198$ mm² representing one area unit). BrdU-positive fibroblasts per area unit of collagen matrix were counted as means \pm SE of 16 area units of two random cross sections from 4 samples in duplicate. The relative number of positive cells per area unit of collagen matrix was expressed as mean \pm SE of positive per total cells per surface area.

Statistics. Student's *t*-test was used to test for the differences in means of positive cells per unit under various conditions. *P* < 0.05 was considered statistically significant.

RESULTS

Normal colonic epithelial cells express functional ETRs. ETRA and ETRB expression in epithelial cells of the human fetal and adult colon was detected by immunohistochemistry using specific antibodies (Fig. 1, *a–g*). Both ETRA and ETRB are expressed on the basolateral surface of the epithelial cells of fetal colon (gestation 17–21 wk; Fig. 1, *a* and *c*). The expression patterns of ETRA and ETRB in adult colon are similar (Fig. 1, *b* and *d*). Intestinal nervous ganglia cells (fetal intestine) do not express ETRA (Fig. 1*e*) but are positive for ETRB staining (Fig. 1*f*), whereas control sections showed no

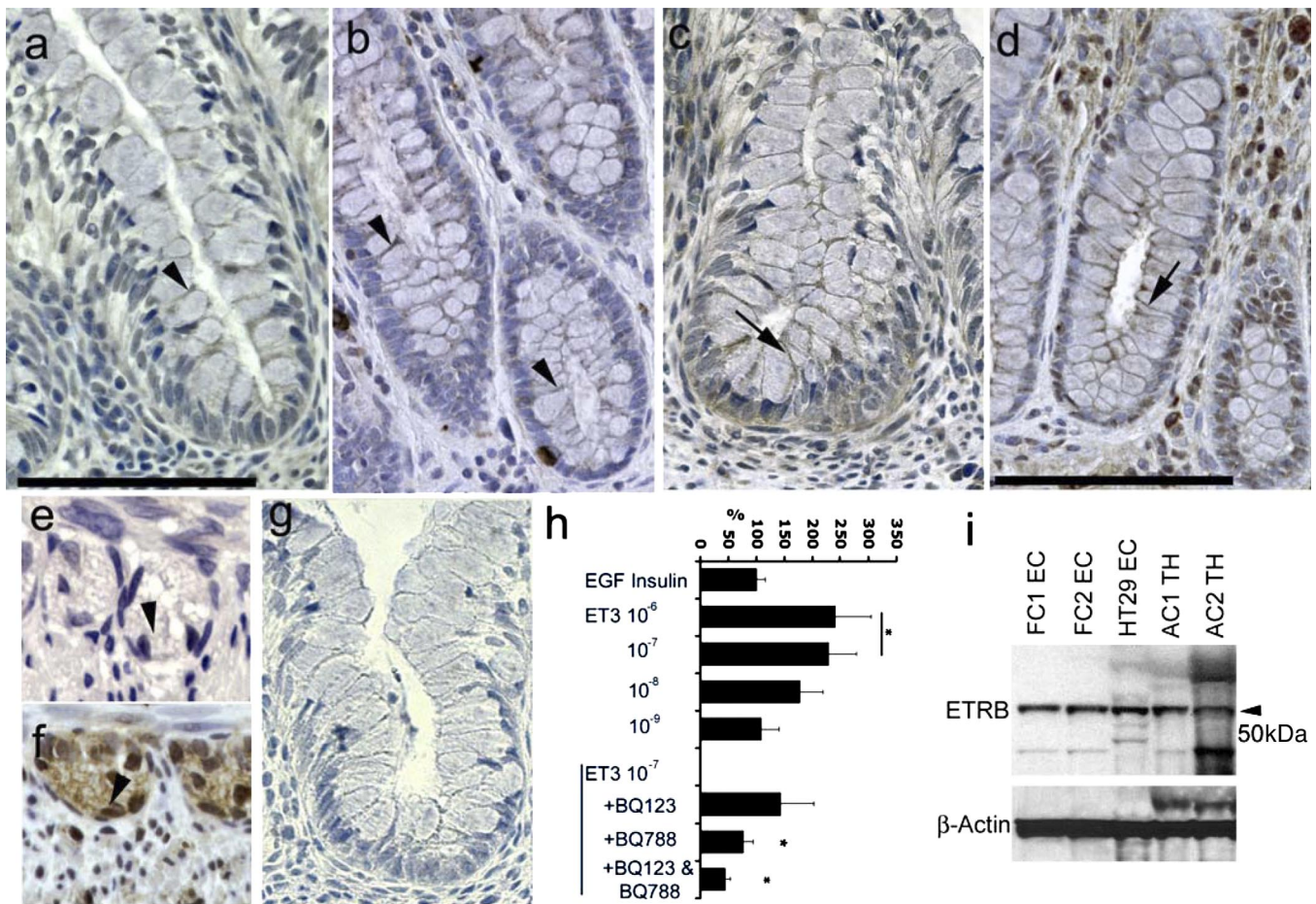


Fig. 1. Intestinal epithelial cells express functional endothelin receptors (ETRs). *a*: Normal fetal human colon (gestation 20 wk) epithelial cells express ETRA. Note the membrane-positive epithelial cells in the bottom of the crypt (arrowhead). Magnification $\times 400$ (bar 50 μm). *b*: Adult human colon stained for ETRA. Note the membrane-positive epithelial cells in the bottom of the crypt (arrowheads). *c*: Same sample as in *a*, stained for ETRB. Note membrane-positive epithelial cells (arrow). *d*: Adult human colon stained for ETRB (arrow). Magnification $\times 400$ (bar 50 μm). *e*: ETRA is not expressed in intestinal ganglion (arrowhead). *f*: ETRB is expressed by ganglionic cells (arrowhead). *g*: Isotype-matched control showed no staining. *h*: Concentration-dependent stimulation of [^3H]thymidine incorporation by endothelin-3 (ET3) in epithelial cells, when grown in monoculture (without fibroblasts) on *day 2*. *i*: Western blot of ETRB expression in fetal colonic epithelial cells: samples 1 and 2 (FC1, FC2), HT colon cancer cells, adult colon tissue homogenate samples 1 and 2 (AC1 TH, A2 TH). β -Actin served as loading control. * $P < 0.05$ relative to control cells (ET3 10^{-7} M stimulated sample was a control for ET3 10^{-7} M stimulated and inhibitor-treated samples).

staining (Fig. 1g). Other positive cell types included mesenchymal fibroblasts (scattered cells positive for both ETRs in fetal and adult tissue), endothelial cells, and cells of macrophage morphology in adult tissue only (ETRB positive).

The human fetal colonic epithelial cells seeded on plastic in complete growth medium (18) died within the first 4–6 days of the culture and were Ki-67 negative by *day 4* (Supplemental Fig. S1a) (the online version of this article contains supplemental data). The function of ETRs in normal colonic epithelial cells was tested by the ability of ET3 to stimulate [^3H]thymidine incorporation in short-term monocultures (*day 2*) of primary epithelial cells without fibroblasts *in vitro* (Fig. 1h). ET3 stimulated [^3H]thymidine incorporation of epithelial cells ($n = 4$) in a concentration-dependent manner ranging from 10^{-6} to 10^{-9} M. At 10^{-7} M, ET3-stimulated thymidine uptake was significantly blocked by the addition of 10^{-6} M BQ 788, a selective inhibitor of ETRB, but only decreased minimally by BQ 123, a selective blocker of ETRA, at the same concentration (Fig. 1h). Inhibition of both ETRA and ETRB receptors in epithelial cells further decreased [^3H]thymidine incorporation to a level significantly lower than that of control.

Effects of ET on organotypic cultures of human colonic epithelial cells. Because of the strong phenotype of the ET3-containing medium (18), we tested the effects of ET3 in organotypic culture. The adenovector-mediated gene transfer to colonic fibroblasts in organotypic culture was used to continuously deliver ET3 and examine its effect on the epithelial cell phenotype, growth, and differentiation. The improvement of organotypic culture technique, which shortened time needed to establish cultures, allowed prolonged cultivation of normal human intestinal epithelial cells in base medium and observation of the epithelial cell proliferation on *day 12* after seeding (Fig. 2).

ET3 production by adenovector-transfected fibroblasts (4.5×10^6 in 6-well plate) was measured by ELISA on *day 3* after transfection (24 h after medium change). There was 0.17 ± 0.15 and 0.11 ± 0.11 ng/ml of ET3 in supernatants of untransfected and GFP adenovector-transfected fibroblasts, respectively, whereas there was 1.07 ± 0.02 ng/ml in samples transduced with 20 pfu of ET3 adenovector (Fig. 2C). To test whether the adenovector-transfected fibroblast can maintain an elevated level of secreted growth factor, ET3 was measured by

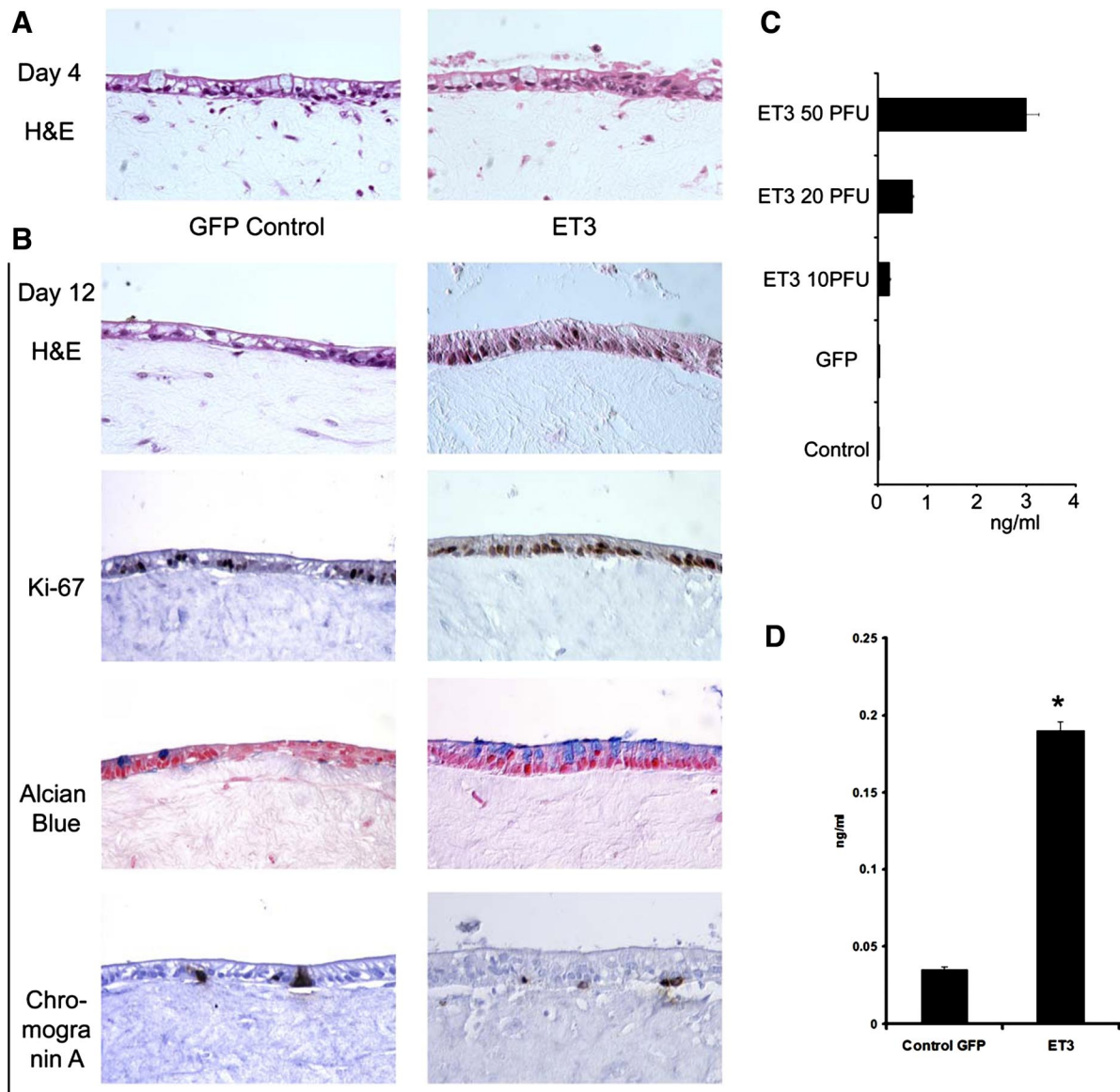


Fig. 2. Effects of ET3 on epithelial cells in the organotypic culture of human colonic epithelial cells. Duplicates of the human fetal intestine samples ($n = 9$) were used to establish organotypic cultures with ET3 adenovirus (ET3) or green fluorescent protein (GFP)-transfected colonic fibroblasts controls (Control GFP). **A:** organotypic culture *day 4* [hematoxylin and eosin (H&E)]; there was not a significant difference between GFP control and ET3 adenovector-transfected samples. **B:** organotypic culture *day 12*; there was a marked difference in morphology (H&E), number of Ki-67-positive cells (Ki-67), number of goblet cells (Alcian blue positive), and number of enteroendocrine cells per area unit (detected as chromogranin-A positive). Original magnification $\times 200$ in all panels. **C:** ELISA of ET3 in supernatants of untransfected (control), GFP adenovector-transduced (GFP), and different plaque-forming units (PFU) of ET3 adenovector-transfected colonic fibroblasts on *day 3* after transfection with medium change 24 h before measurement. **D:** ELISA of ET3 in the supernatant of organotypic cultures with ET3 adenovector-transduced fibroblasts on *day 12*, 24 h after medium change. All results are expressed as means \pm SE.

ELISA in the supernatant of organotypic cultures with ET3 adenovector-transduced fibroblast on *day 12*. The basal level in coculture was lower than that in monoculture; there was 0.20 ± 0.018 ng/ml in samples transduced with 20 pfu of ET3 adenovector, whereas there was 0.01 ± 0.013 ng/ml of ET3 in the supernatants of the organotypic cultures with the GFP adenovector-transfected fibroblasts (Fig. 2D).

There was no significant difference in the morphology or the number of goblet, enteroendocrine, and Ki-67-positive cells when samples were evaluated on *day 4* (Fig. 1a for H&E, other data not shown). Prolonged culture (12 days) permitted the determination of the long-term effect of ET3 on the morphology of epithelial cells (Fig. 2B, H&E), Ki-67-positive cells

(Fig. 2B, Ki-67), Alcian blue-positive goblet cells (Fig. 2B, Alcian blue), and chromogranin-A-positive enteroendocrine cells (Fig. 2B, chromogranin-A). Interestingly, there were clusters of 5–10 Ki-67-positive epithelial cells in control samples, whereas ~5–20 Ki-67-positive cells in ET3 adenovector-transfected samples.

Both ETRA and ETRB confer survival of goblet cells. To study further the functions of ET3 in colonic epithelial cells, inhibitors of the ETRs were added to organotypic cultures and cultures were grown in the presence of BrdU. All samples were constricted equally by *day 9*, allowing direct quantitation of cell numbers per area unit. To facilitate comparison between samples, the results were normalized against controls (Fig. 3E).

ET3 overexpression in fibroblasts increased significantly the total number of epithelial cells per surface unit ($P < 0.05$) and the number of BrdU-positive epithelial cells per surface unit ($P < 0.01$) in organotypic culture (Fig. 3E). The effect of ET3 on epithelial proliferation in organotypic culture (when ET3 overexpressed by adenovector, Fig. 3E) was not inhibited by BQ123 and only ~50% inhibited by BQ788. However, the presence of both ETR inhibitors decreased BrdU incorporation below the level of GFP-transfected controls ($P < 0.01$). In cultures with untransfected fibroblasts (Fig. 3F) only treatment with both ETR inhibitors decreased the number of BrdU-positive epithelial cells below the level of untreated control ($P < 0.01$).

Goblet cells were increased in ET3-transfected samples $355 \pm 92\%$ ($P < 0.001$, $n = 9$) compared with GFP-transfected controls $100 \pm 69\%$ (Fig. 3, A, B, E, and F). Alcian blue-stained goblet cells were BrdU negative in most samples. There were a few BrdU-positive goblet cells in ET3-treated samples (arrow, Fig. 3B); however, the number of positive cells was low compared with the total number of BrdU-negative goblet cells. ET3-mediated effect was reduced by BQ123 ($n = 0.08$) and significantly reduced by BQ788 ($P < 0.05$) and the combination of both inhibitors BQ 123 and 788 $34.6 \pm 20\%$ ($P < 0.01$). In samples with untransfected fibroblasts, there was a paradoxical trend with an increase in goblet cells when treated with BQ123 or BQ788 ($P = 0.17$ and 0.20 , respectively), whereas there was a sharp decrease in the goblet cells when combination of BQ123 and 788 was used $16.3 \pm 5.9\%$ ($P < 0.001$). To test whether ET3 or inhibitors would affect secretion of mucus by goblet cells, we measured the size of Alcian blue-positive goblet cells (Supplemental Fig. S3). There was no difference in size in GFP and BSA controls and ET3-treated samples. Interestingly, there was an increase in BQ788-treated samples (7.44 ± 0.56 mm compared with 5.93 ± 0.65 mm for BSA control $P < 0.05$).

The number of enteroendocrine cells (chromogranin A positive) was only increased slightly by ET3 $147 \pm 18.5\%$ ($P < 0.05$) vs. $100 \pm 14.6\%$ for control, and the number was not affected by treatment with combination of inhibitors ($78.2 \pm 15.3\%$; Fig. 3F). All enteroendocrine cells were negative for BrdU.

Effect of ET3 on fibroblasts. Similarly to the epithelial cells, ET3 stimulated the [3 H]thymidine incorporation in fibroblasts in a concentration-dependent fashion, when grown in monoculture on day 2 (Supplemental Fig. S2a). The maximum stimulation was with 10^{-7} to 10^{-9} M, which was less than maximum for epithelial cells (Fig. 1h). Similarly, ET3 increased BrdU incorporation in fibroblasts when quantified in the organotypic culture (Supplemental Fig. S2b). Interestingly, although ET3 significantly increased the number of BrdU-positive fibroblasts, the total number of fibroblasts remained the same (there were fewer BrdU-negative fibroblasts).

ET3 stimulated phosphorylation of I κ B α and MAPK in intestinal epithelial cells. The epithelial cells and fibroblasts grown for 9 days in base medium did not stain positive for phosphorylated I κ B α (p-I κ B α) (Fig. 4a) and stained weakly for phosphorylated p44/42 MAPK (Fig. 4c). After 15-min stimulation of organotypic cultures with 10^{-6} M ET3 protein, epithelial cells showed strong positive staining for both p-I κ B α (Fig. 4b) and p44/42 MAPK (Fig. 4d). A time course study of human epithelial cell line HT29 stimulated with 10^{-6} M ET3

demonstrated that, whereas total I κ B remained unchanged, p-I κ B α levels increased immediately after stimulation and remained elevated for at least 45 min before returning to the basal level (Fig. 4e). This increase was blocked by 15-min pretreatment with BQ788 (Fig. 4e). There were no changes in phospho-AKT or Bcl2 (data not shown). Similarly, stimulated HT29 cells also showed increased p44/42 MAPK 5 min after stimulation, which lasted for at least 45 min and was inhibited partially by BQ788 (Fig. 4f).

DISCUSSION

Previous studies have demonstrated pleiotropic effects of ETs in the human intestine, such as organogenesis of the intestinal neuronal plexus (9, 30), chloride secretion by intestinal crypts (23), modulation of ischemic injury (28), and regulating survival and proliferation in colon carcinoma cells (2). ETs and ETRs are expressed in the vasculature, ganglionic system, intestinal musculature, immune cells, myofibroblasts, and epithelial cells (11), thereby making a precise determination of ET function on epithelial cells difficult. For example, the vascular effects of ET blockage during reperfusion injury in the intestine may mask any effects on epithelial cells (28).

We have reported previously the effects of exogenously added growth factors, for example of leukemia inhibitory factor, on the growth of primary epithelial cells in vitro (18). We found that ET3, SCF, and HGF containing medium fostered increased proliferative capacity and an increased presence of goblet cells (18). We further modified the coculture system by introducing adenoviruses into fibroblasts that in turn mediate growth factor production. These viruses then served as a continuous source of growth factors at physiologically relevant levels. Although information on the physiological role of ETs came from studies of endothelin's circulatory effects, recent evidence suggests a more tissue-specific role for the ET system. Many growth factors are produced in vivo by intestinal myofibroblasts in direct proximity to the basolateral side of polarized epithelial cells (29).

Current knowledge of ET function in intestinal epithelial cells has been derived from cancer cell lines and animal models, with very little data available on the human colonic epithelium. Using a human colon organotypic culture system, we have demonstrated that ET3 increased proliferation of intestinal epithelial cells and survival of goblet cells, likely through stimulation of the I κ B and MAPK pathways, which could be inhibited by a combination of ETRA and ETRB inhibitors. Although the addition of BQ788 (ETRB inhibitor) was able to block the ET3-stimulated increase (relative to the unstimulated control level in [3 H]thymidine incorporation assays), combined inhibition of both receptors decreased [3 H]thymidine uptake below the levels of unstimulated controls in epithelial monoculture (without fibroblasts). Similarly, combined inhibition of both receptors was necessary to observe a similar effect in the organotypic cultures. The presence of ETRs in intestinal epithelial cells was demonstrated by 125 I-labeled ET1 binding studies (15) and at mRNA level (13), but it was not confirmed at the protein level. Here, we present evidence for both the expression and functionality of ETRB in human colonic epithelial cells. The simultaneous addition of ETRA and ETRB inhibitors further inhibited [3 H]thymidine uptake below basal levels, confirming that both ETRA and ETRB

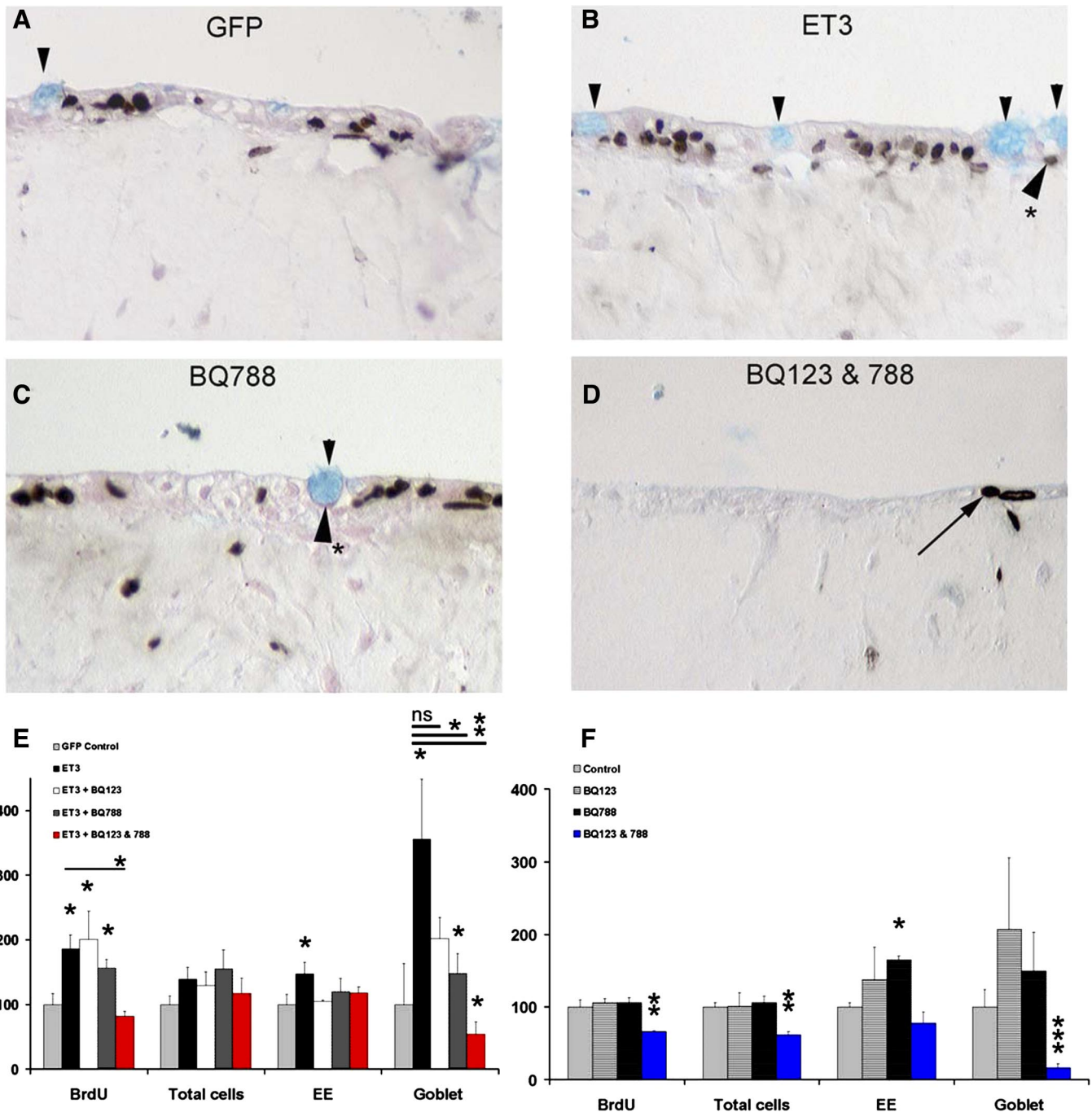


Fig. 3. Activation of the endothelin system promotes presents of goblet cells in organotypic cultures. *A–D*: *day 12* of the organotypic culture grown in the continuous presence of bromodeoxyuridine (BrdU) during the culture period, double staining of BrdU and Alcian blue. Magnification, $\times 400$. *A*: fibroblasts transfected with GFP. *B*: fibroblasts transfected with ET3. Goblet cells are indicated with arrowheads, note BrdU-positive nucleus in the proximity of goblet cell granule (arrowhead with *). *C*: untransfected fibroblasts treated daily with ETRB blocker BQ788 (1 μ M). *D*: untransfected fibroblasts treated daily with both ETRA and ETRB blockers BQ123 and BQ788 at 1 μ M each. Arrow indicates presence of the epithelial cell layer and arrowheads point to BrdU incorporation; Alcian blue-positive goblet cells were absent. *E*: quantification of the epithelial cells in the organotypic culture with control (GFP) or ET3-transfected fibroblasts treated with BSA ($n = 9$ in duplicate) or ET receptor antagonists ($n = 4$ in duplicate) on *day 12*; number of BrdU-positive cells (BrdU), total epithelial cells (Total cells), enteroendocrine cells based on the chromogranin-A-positive pattern (EE), and goblet cells (Goblet) per surface unit of organotypic cultures. * $P < 0.05$, ** $P < 0.01$. *F*: quantification of the epithelial cells in the organotypic culture with untransfected BSA (control) or ET receptor inhibitor-treated fibroblasts on *day 12*; number of BrdU-positive cells, total epithelial cells, enteroendocrine cells based on chromogranin-A positivity, and goblet cells (goblet) per surface unit of organotypic cultures ($n = 4$, in duplicate). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

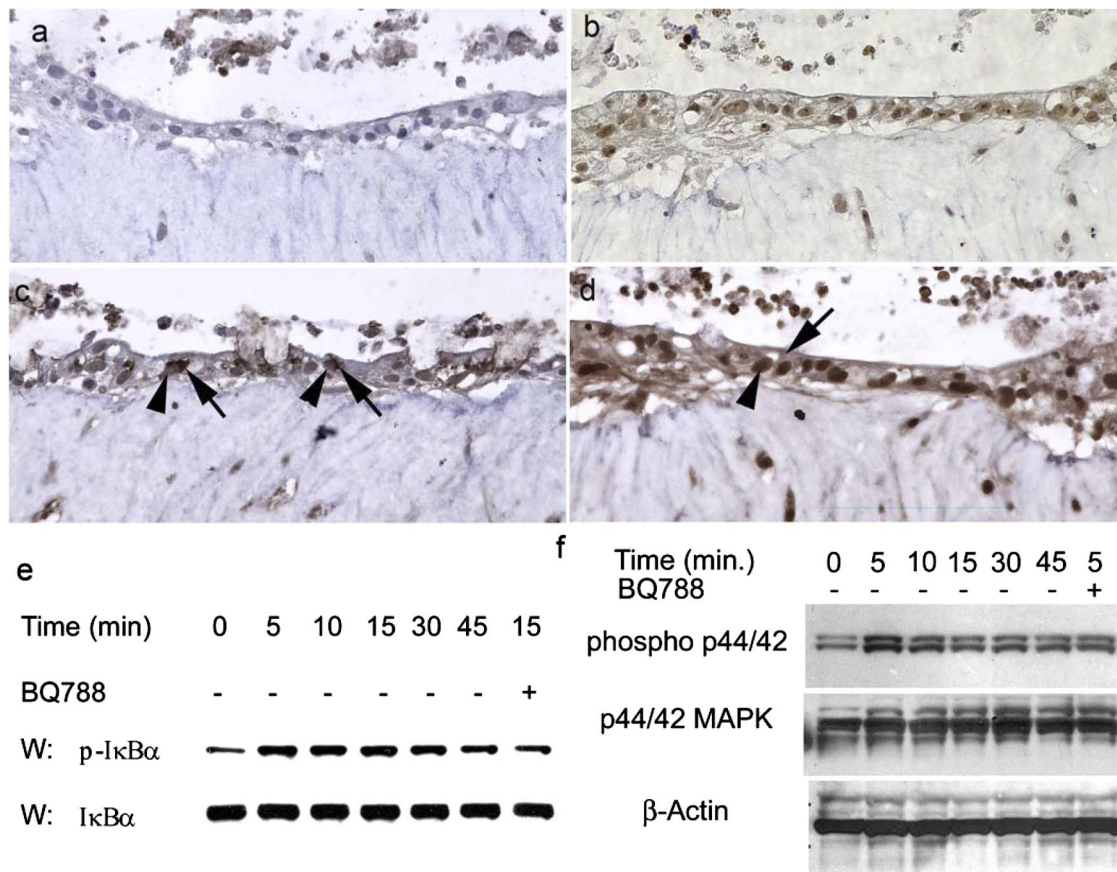


Fig. 4. ET3 stimulates phosphorylation of IκBα and MAPK in intestinal epithelial cells. Organotypic cultures grown for 9 days in base medium were stimulated with BSA (*a* and *c*) or ET3 (*b* and *d*) for 15 min and stained by immunohistochemistry for phospho-IκBα (*a* and *b*) and phospho-p44/42 MAPK (*c* and *d*). *e*: Time course study of human epithelial cell line HT29 stimulated with 10^{-6} M ET3 and probed with anti-phosphorylated IκBα (p-IκBα) and anti-IκBα antibodies by Western blot. *f*: Same as *e*; probed with phospho-p44/42 MAPK, total MAPK, and β-actin antibodies by Western blot.

mediated proliferation signals for primary colonic epithelial cells and that an autocrine loop might exist for both receptors in epithelial cells (33). Thus the ligands of both ET receptors are likely produced by intestinal epithelial cells (10, 32, 33). Interestingly, ET1 is upregulated by β-catenin activation, which occurs frequently in colon cancer (21).

Recently, several pathways have been implicated in the regulation of goblet cell differentiation. For example, *Klf4*^{-/-} mice lacked goblet cells (36), and Notch signaling may influence the lineage fate of intestinal epithelial stem cells (38). Higher numbers of goblet cells were achieved in mice treated with the γ-secretase inhibitor LY-411,575 (37). In addition, keratinocyte growth factor and its effector, intestinal trefoil factor, also modulated goblet cell differentiation in the gastrointestinal tract (12, 17). Less is known about factors that may influence goblet cell survival, for example, during the acute phase of intestinal injury. It has been shown that goblet cell function is preserved during acute colitis and increased during the recovery phase of colitis in a disease model. However, no factor is known to stimulate prolonged survival of already differentiated goblet cells during intestinal injury or colitis.

Recently, ET1 was shown to block secretion of mucin from bovine airways epithelial cells (7). By contrast, ETRB inhibition increased size of goblet cells, which may occur when secretion by goblet cells is inhibited. The inhibition of the secretion in airways epithelium was mediated by the ETRA

blocker BQ123 (7). Also, after goblet cell secretes, it resynthesizes mucus and remains Alcian blue positive. Our results seem to suggest that the inhibition of both receptors was necessary for significant inhibition of proliferation and goblet cells survival, whereas ETBR inhibition results in increase goblet cell size, suggesting a role of ETBR in mucus secretion.

In this study we demonstrated that ET3 increases the number of goblet cells in organotypic culture and this process involves both ETRA and ETRB. In addition, we have demonstrated that ET3 stimulates IκBα phosphorylation in intestinal epithelial cells, which is noteworthy because of evidence that IκB is involved in goblet cell survival. For example, IκBζ-knockout mice develop chronic inflammation in the ocular surface epithelium. During the onset of inflammation, there is a gradual loss of conjunctival goblet cells as inflammation progress (35).

Other growth factors such as HGF and SCF have been suggested to stimulate repair and proliferation of intestinal cells. They did not, however, promote goblet cell survival in our organotypic culture model (data not shown). HGF and SCF, which play a role in the migration and survival of colonic epithelial cells (16, 27), were not able to sustain or increase the number of goblet cells. Therefore, the effects of the ET system on goblet cell survival appear to be rather specific. In summary, our results suggest that endothelins contribute to the homeostasis of intestinal epithelium.

GRANTS

This work was funded by grants from the National Institutes of Health (CA-74294, CA-25874, DK-056645), the Commonwealth Universal Research Enhancement Program, Pennsylvania Department of Health, the Hansen Foundation and the NCCRA/EIF.

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