SPANDIDOS PUBLICATIONS

Regulation of amphiregulin and epiregulin expression in human colonic subepithelial myofibroblasts

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Abstract. Amphiregulin and epiregulin belong to the epidermal growth factor (EGF) family, and act as mitogenic stimulators via binding to EGF receptors (EGFRs). Amphiregulin and epiregulin are thought to play a role in regenerative responses in the gastrointestinal tract. In this study, we investigated secretion of amphiregulin and epiregulin in human colonic subepithelial myofibroblasts (SEMFs). The mRNA expression and protein secretion of amphiregulin and epiregulin were evaluated by Northern blotting and Western blotting, respectively. The trophic effects of amphiregulin and epiregulin on SEMFs were analyzed by MTT assays. Amphiregulin and epiregulin mRNAs were not detected in unstimulated SEMFs. Among the various cytokines and growth factors, interleukin-1ß, tumor necrosis factor-α, and EGF strongly induced amphiregulin and epiregulin mRNA expression. These responses were markedly reduced by AG1478, a specific inhibitor of EGF receptor tyrosine kinases. Amphiregulin and epiregulin secretion were also detected at the protein level. MTT assays demonstrated that amphiregulin and epiregulin stimulate the proliferation of SEMFs. We demonstrated expression of amphiregulin and epiregulin in SEMFs. Amphiregulin and epiregulin may play an important role in the mechanism underlying wound healing in damaged colonic mucosa.

Introduction

Amphiregulin and epiregulin belong to the epidermal growth factor (EGF) family which includes EGF, transforming growth factor (TGF)-α, heparin-binding (HB)-EGF, betacellulin, and various heregulins. These factors mediate biological functions of epithelial and mesenchymal cells through the EGF receptors (EGFRs) (1). Several studies have demonstrated that the EGF family and the EGFR signaling pathway play a crucial role in the regenerative response of mucosal damage in the gastrointestinal tracts (2-4).

Amphiregulin is a 252 amino-acid transmembrane glycoprotein, and was originally isolated from human breast carcinoma cell line MCF-7 (5). The mRNA expression for amphiregulin can be detected in a variety of carcinoma cell lines, and in non-transformed epithelial and mesenchymal cells from the colon, stomach, lung, breast, ovary and kidney (6). Amphiregulin stimulates the proliferation of keratinocytes, fibroblasts and epithelial cells (7-9). In some carcinoma cell lines, amphiregulin stimulates their proliferation through autocrine mechanisms (10,11). Beales *et al* demonstrated that amphiregulin stimulates proliferation of the gastric mucosa (12).

Epiregulin is a 46 amino-acid active protein, and was initially purified from the conditioned medium of the fibroblast-like cell line NIH3T3/T7 (13). Epiregulin stimulates the proliferation of non-transformed fibroblasts, hepatocytes, smooth muscle cells, and keratinocytes, but inhibits the growth of several tumor-derived epithelial cell lines (14-16). Lee *et al* reported that epiregulin null mice are highly susceptible to intestinal damage caused by oral administration of dextran sulfate sodium (17).

In this study, we evaluated secretion of amphiregulin and epiregulin from human colonic subepithelial myofibroblasts (SEMFs). SEMFs play a critical role in the pathophysiological processes involved in inflammation and wound healing in the intestine (18-22). We found that proinflammatory cytokines such as interleukin (IL)-1ß, tumor necrosis factor (TNF)-α, and EGF were strong inducers of amphiregulin and epiregulin in colonic SEMFs. Furthermore, amphiregulin and epiregulin act as autocrine growth factors for colonic SEMFs.

Materials and methods

Reagents. Recombinant human IL-1ß, TNF-α, amphiregulin and epiregulin were obtained from R&D Systems (Minneapolis, MN). All other cytokines and growth factors were purchased from PeproTech (Rocky Hill, NJ). The EGF receptor tyrosine kinase inhibitor (AG1478) was purchased from Calbiochem (San Diego, CA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO).

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Culture of human colonic myofibroblasts. Primary cultures of intestinal SEMFs were prepared according to the method reported by Mahida *et al* (18). The characteristics of these cells have been described in our previous report (23-25). The cells were cultured in DMEM containing 10% FBS. The studies were performed on passages 2-6 of myofibroblasts isolated from 3 resection specimens.

Immunoprecipitation and Western blotting. Conditioned medium (5 ml) was incubated with 2 μg/ml of anti-amphiregulin rabbit IgG (Chemicon, Temecula, CA) or epiregulin goat IgG (R&D Systems, Minneapolis, MN) for 1 h, and then 20 μl of Protein G-Sepharose beads (Amersham, Arlington Heights, IL) was added to the reaction mixture. After a 16-h incubation, beads were suspended in 20 μl of loading buffer and separated by 16% of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. SDS PAGE gels were then electrophoretically transferred onto nitrocellulose membranes. Membranes were soaked with 200 ng/ml of anti-amphiregulin or epiregulin goat IgG (R&D Systems) for 1 h, followed by incubation with peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The detection was carried out by means of ECL kit (Amersham).

Reverse-transcription polymerase chain reactions. The mRNA expression for EGFR, ERBB2, ERBB3 and ERBB4 was assessed by reverse-transcription polymerase chain reaction (RT-PCR) analyses (26). PCR products were ligated into TA cloning vectors (Promega, Madison, WI) and sequenced. Primers specific for human EGFR were as follows: 5':TGAT GGCCAGCGTGGACAACC [corresponding to nucleotides 2416-2436 (27)] and 3':CATGGTATTCTTTCTCTTCCGCA (nucleotides 2711-2733). Primers for ERBB2 were as follows: 5':GGCTGCTGGACATTGACGAG [nucleotides 2777-2796 (28)] and 3':GGGGCTGGGGCAGCCGCTC (nucleotides 2989-3007). Primers for ERBB3 were as follows: 5':GGAGT ACAAATTGCCAAGGGAA [nucleotides 2636-2657 (29)] and 3':CAGGTCTGGCAAGTATGGAT (nucleotides 2942-2962). Primers for ERBB4 were as follows: 5':CTCTGATCAT GGCAAGTATGGAT [nucleotides 2339-2361 (30)] and 3':CA TTGTATTCTTTTTCATCTCCTTC (nucleotides 2638-2662).

Northern blot analyses. Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (31). Total RNA (20 μg) was separated by 1.0% agarose/formaldehyde/MOPS gel electrophoresis, and transferred to a Hybond nylon membrane (Amersham). The cDNA probes for human amphiregulin and epiregulin were also prepared by RT-PCR using the following primers: amphiregulin 5':AGTGAGATTTCCCCTGTGAG [corresponding to nucleotides 391-410 (6)] and 3':CTCCTTCATATTTCCTG ACG (890-910), and epiregulin 5':GACAGTTCCCTATGC CAACT [1291-1310 (32)] and 3':GCTCCTTATGCAATAG CCCA (1992-2011). The hybridization was performed with ^{32}P-labeled probes generated by a random primed DNA-labeling kit (Amersham), and evaluated by autoradiography.

Cell proliferation assays. The number of cells was determined by a modification of the tetrazolium assay using MTT as a substrate (33). Colonic SEMFs were seeded at a density of $3x10^4$ cells per well in 96-well cell culture plates. Cells were exposed to amphiregulin, epiregulin or EGF. Cells were incubated for predetermined times, and then 50 μl of PBS containing 0.5 mg/ml MTT was added to each well. After a 4-h incubation, 100 μl of 0.04 N HCl in isopropanol was added and mixed thoroughly. The absorbance in each well was read at 550 nm by a microplatereader. The cell number was expressed as a percentage relative to the cell number of controls incubated with medium alone.

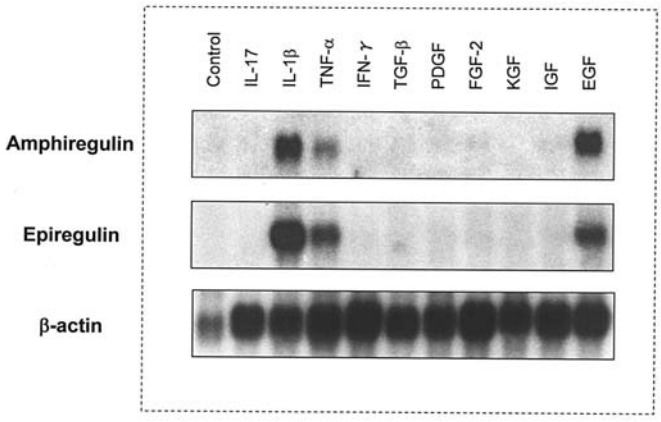

Figure 1. Effects of various cytokines and growth factors on mRNA expression of amphiregulin and epiregulin mRNA in human colonic SEMFs. Cells were incubated with each factor (100 ng/ml) for 12 h, and total RNA was extracted. The mRNA expression for amphiregulin and epiregulin was assessed by Northern blotting.

Statistical analyses. Data are expressed as means ± SD. Statistical significance of changes were determined with unpaired Student's t-test. Differences resulting in P-values <0.01 were considered significant.

Results

Effects of cytokines and growth factors on amphiregulin and epiregulin mRNA expression. Cells were stimulated with each factor (100 ng/ml) for 12 h, and the mRNA expression for amphiregulin and epiregulin was evaluated by Northern blotting. As shown in Fig. 1, mRNA expression of amphiregulin and epiregulin was not detected in unstimulated SEMFs. IL-1ß, TNF-α, and EGF induced a marked increase in this mRNA expression.

Effects of IL-1ß, TNF-α, and EGF on amphiregulin and epiregulin mRNA expression. Colonic SEMFs were incubated for 12 h with increasing concentrations of IL-1ß, TNF-α and EGF, and amphiregulin and epiregulin mRNA expression patterns were analyzed by Northern blotting. As shown in Fig. 2, IL-1ß dose-dependently induced amphiregulin and epiregulin mRNA expression. The effect of IL-1ß was detected at as low as 0.1 ng/ml, and reached a maximum at 10 ng/ml. TNF-α and EGF also induced a dose-dependent increase in amphiregulin and epiregulin mRNA expression (Fig. 2). The effects of TNF-α and EGF were detected at as low as 0.1 ng/ml, and reached a maximum at 100 ng/ml.

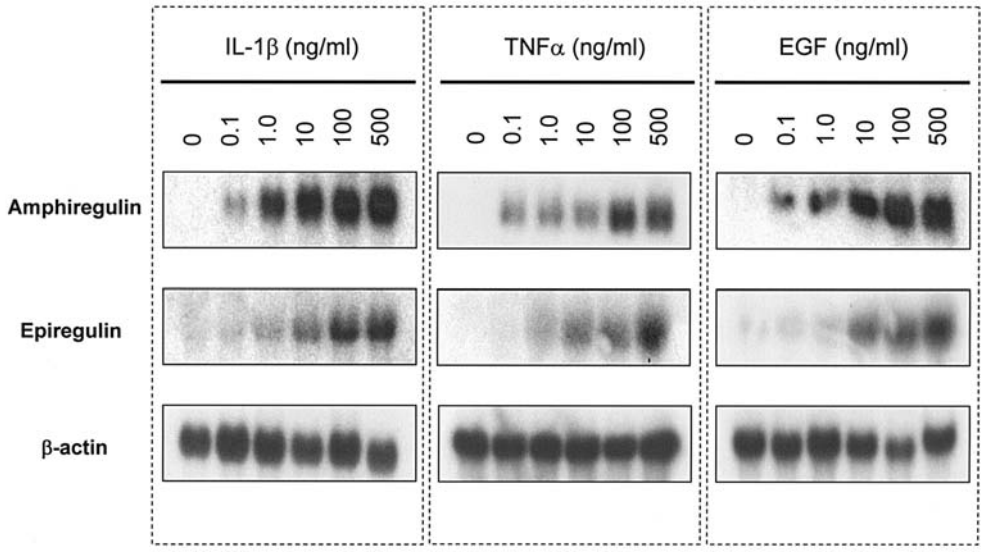

Figure 2. Induction of amphiregulin and epiregulin mRNAs in colonic SEMFs. Cells were stimulated for 12 h with various concentrations of IL-1ß, TNF-α, and EGF. The mRNA expression for amphiregulin and epiregulin was analyzed by Northern blotting.

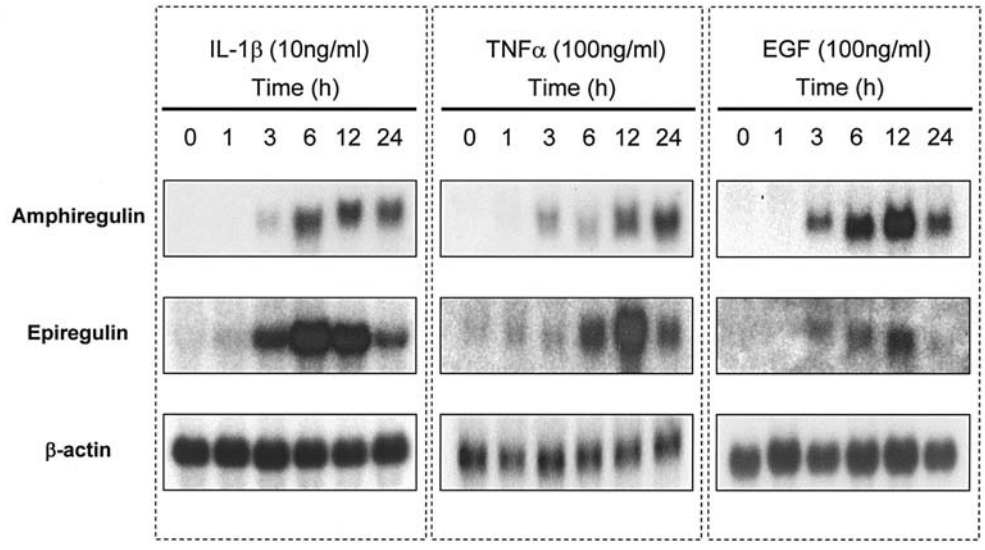

Figure 3. Kinetics of amphiregulin and epiregulin mRNA expression in colonic SEMFs. Cells were stimulated with IL-1ß (10 ng/ml), TNF-α (100 ng/ml), and EGF (100 ng/ml), and expression of amphiregulin and epiregulin mRNAs was sequentially determined by Northern blotting.

Kinetics of amphiregulin and epiregulin mRNA expression were evaluated (Fig. 3). Cells were stimulated with IL-1ß (10 ng/ml), TNF-α (100 ng/ml) or EGF (100 ng/ml), and the sequential changes in mRNA expression were determined by Northern blotting. IL-1ß induced a rapid increase in the accumulation of amphiregulin and epiregulin mRNA, which reached a maximum at 6 h after stimulation. Thereafter, the induced amphiregulin mRNA level decreased gradually. Kinetics revealed that TNF-α and EGF-induced amphiregulin and epiregulin mRNA expression reached 12 h after stimulation (Fig. 3), which also gradually decreased.

Amphiregulin and epiregulin protein secretion. Colonic SEMFs were stimulated for 48 h, and amphiregulin and epiregulin secretion was analyzed by immunoprecipitation and Western blotting. As shown in Fig. 4, the addition of IL-1ß, TNF-α, and EGF induced amphiregulin or epiregulin protein secretion. In previous reports, various soluble forms of amphiregulin (9.5-10, 28-30, 35, and 55-60 kDa) have been identified in several cell types (5,6). In this study, amphiregulin was detected as one band at 9.5-10 kDa. Epiregulin secretion was also induced by the addition of IL-1ß, TNF-α, and EGF. Epiregulin was detected as a band at 5 kD, which is compatible with previous reports (15).

Trophic effects of amphiregulin and epiregulin on SEMF proliferation. Since amphiregulin and epiregulin have been reported to stimulate proliferation of mesenchymal cells, we tested how these growth factors modulate colonic SEMF proliferation. Colonic SEMFs were cultured with various

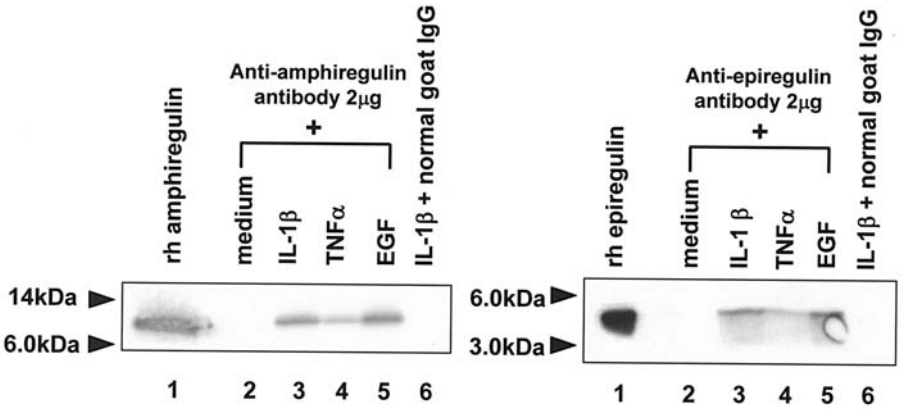

Figure 4. Amphiregulin and epiregulin secretion in colonic SEMFs. Cells were incubated for 48 h with IL-1ß (10 ng/ml), TNF-α (100 ng/ml), or EGF (100 ng/ml), and then amphiregulin and/or epiregulin proteins in supernatants were immunoprecipitated and analyzed by Western blotting. (A) Lane 1, recombinant amphiregulin (10 ng); lane 2, medium alone; lane 3, IL-1ß; lane 4, TNF-α; lane 5, EGF; and lane 6, precipitated with normal rabbit IgG. (B) Lane 1, recombinant epiregulin (10 ng); lane 2, medium alone; lane 3, IL-1ß; lane 4, TNF-α; lane 5, EGF; and lane 6, precipitated with normal rabbit IgG.

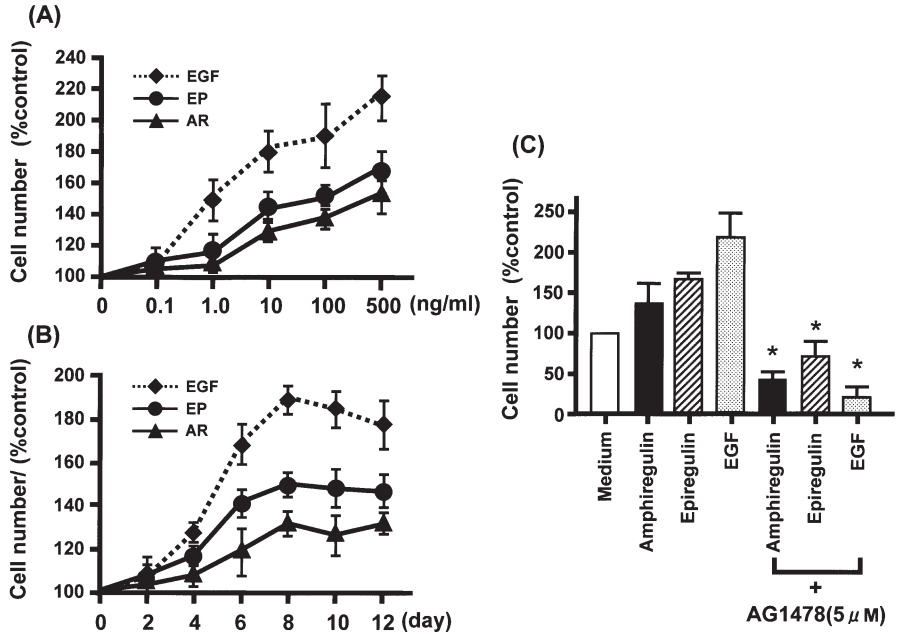

Figure 5. Effects of amphiregulin, epiregulin and EGF on proliferation of intestinal SEMFs. (A) Cells were incubated with increasing concentrations of recombinant amphiregulin, epiregulin, EGF for 8 days and total viable cell numbers were assessed by MTT assays. Data represent means ± SD (n=3). (B) Cells were incubated with amphiregulin (100 ng/ml), epiregulin (100 ng/ml) or EGF (100 ng/ml), and total viable cell numbers were sequentially assessed by MTT assay. Data represent means ± SD (n=3). (C) Cells were treated with amphiregulin (100 ng/ml), epiregulin (100 ng/ml) or EGF (100 ng/ml) in the presence or absence of AG1478 (5 μM) for 8 days. Total viable cell numbers were assessed by MTT assays. Each column represents the mean of three independent experiments. Data represent means ± SD (n=4). $^{*}P<0.01$.

concentrations of amphiregulin, epiregulin, and EGF for 8 days, and cell proliferation was analyzed by MTT assays (Fig. 5). These growth factors stimulated an increase in the cell number in a dose-dependent manner (Fig. 5A). Amphiregulin, epiregulin and EGF promoted the proliferation of colonic SEMFs up to day 8 (Fig. 5B). As shown in Fig. 5C, these effects were significantly inhibited by the addition of AG1478, a specific inhibitor of EGFR tyrosine kinase (34), suggesting that trophic effects of amphiregulin and epiregulin as well as EGF are mediated through EGFR-mediated signalling pathways.

It has been reported that amphiregulin and epiregulin exert their biological effects through the ERBB receptor family including EGFR, ERBB2, ERBB3 and ERBB4 (1,35,36). To confirm the expression of these receptor subunits in colonic SEMFs, we performed RT-PCR analyses. As shown in Fig. 6, mRNA expression for EGFR, ERBB2, ERBB3, and ERBB4 was detected in colonic SEMFs.

Autocrine induction of amphiregulin and epiregulin mRNA expression. Previously, amphiregulin stimulation has been reported to induce amphiregulin expression in bronchial epithelial cells (13). Based on this report, we tested similar responses in colonic SEMFs. As shown in Fig. 7, amphiregulin induced a rapid increase in the accumulation of amphiregulin mRNA within 3 h after stimulation. Similarly, epiregulin

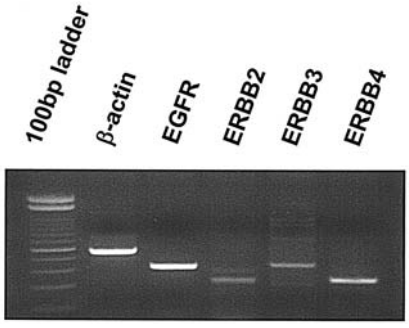

Figure 6. Reverse transcription-polymerase chain reaction (RT-PCR) analyses for mRNA expression of the EGF receptor family. Total cellular RNA was extracted from unstimulated cells, and the mRNA expression was analyzed by means of RT-PCR.

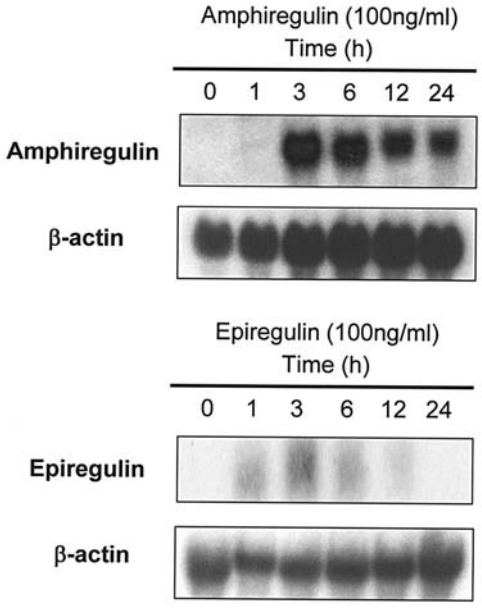

Figure 7. Effects of amphiregulin on amphiregulin mRNA expression (upper) and those of epiregulin on epiregulin mRNA expression (lower). Cells were incubated with amphiregulin (100 ng/ml) or epiregulin (100 ng/ml), and mRNA expression for each growth factor was analyzed by Northern blotting.

induced a rapid accumulation of epiregulin mRNA. The aforementioned observations suggest that amphiregulin and epiregulin exert biological effects on SEMFs through an autocrine fashion.

To confirm this possibility of autocrine regulation, we analyzed how AG1478 modulates the effects of IL-1ß and TNF-α as well as those of EGF. As shown in Fig. 8, AG1478 completely blocked EGF- and amphiregulin-induced amphiregulin mRNA expression. IL-1ß- and/or TNF-α-induced amphiregulin mRNA expression was partially blocked by AG1478. Similarly, the effects of IL-1ß and TNF-α on epiregulin mRNA expression were partially blocked by AG1478, suggesting that EGFR-mediated responses might be involved in the responses induced by IL-1ß and TNF-α.

Discussion

This study shows that among various cytokines and growth factors, IL-1ß, TNF-α and EGF specifically induce amphiregulin and epiregulin expression in human colonic SEMFs. To our knowledge, EGF-induced amphiregulin and epiregulin expression has not been reported in other cell types. Furthermore, amphiregulin and epiregulin exert their biological actions through an autocrine fashion. These observations suggest that colonic SEMFs may be a local source of amphiregulin and epiregulin, and that these phenomena may be important mechanisms controlling mucosal proliferation in healthy and diseased colons.

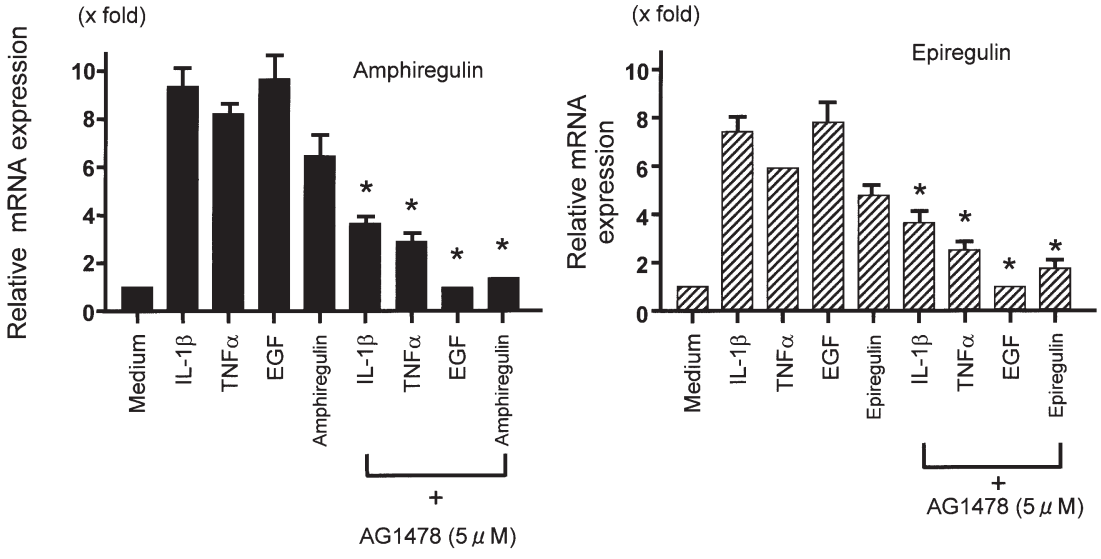

Figure 8. Effects of the EGFR tyrosine kinase inhibitor, AG1478, on mRNA expression for amphiregulin (A) and epiregulin (B). Cells were stimulated with IL-1ß (10 ng/ml), TNF-α (100 ng/ml), EGF (100 ng/ml), amphiregulin (100 ng/ml) or epiregulin (100 ng/ml) in the presence or absence of AG1478. The total RNA was then extracted, and Northern blotting was performed. The radioactivity of each band was determined from an Instant Imager™ Electronic Autoradiography system model (Packerd). Each amphiregulin or epiregulin mRNA value was standardized by an internal control for ß-actin mRNA. Data were converted to relative expression to the value of medium alone. Data represent means ± SD (n=4). *P<0.01.

The proinflammatory cytokines, IL-1ß and TNF-α, are representative proinflammatory cytokines that share several biological functions. Both cytokines promote inflammatory responses, regulate aspects of cellular immunity and are important in host defense against infection. IL-1ß and TNF-α are produced by monocytes/macrophages and epithelial cells, and expression of these cytokines has been reported to be elevated in inflamed mucosa of inflammatory bowel disease (37). Based on these notions, the findings in this study suggest that some parts of proliferative responses in inflamed colonic mucosa are mediated by IL-1ß- and TNF-α-induced amphiregulin and/or epiregulin. Furthermore, since amphiregulin (epiregulin) stimulated amphiregulin (epiregulin) expression, responses to IL-1ß and TNF-α might be amplified in the inflamed mucosa through an autocrine fashion. It was previously reported that IL-1ß and TNF-α stimulated the proliferation of intestinal SEMFs with a relatively longer incubation of 72 h (38,39). The proliferation of intestinal SEMFs may be explained by indirect effects of amphiregulin and epiregulin induced by IL-1ß and TNF-α. Amphiregulin and epiregulin are stimulators of proliferation of epithelial cells as well as mesenchymal cells, it is likely that proinflammatory cytokines such as IL-1ß and TNF-α may play an important role in the process of tissue remodeling and wound healing through the induction of amphiregulin and epiregulin in intestinal mucosa.

EGF is a 6,000-Da polypeptide hormone, and exists in external secretions in the gastrointestinal tract (40). Previous studies demonstrated that EGF is a potent stimulator of proliferation of epithelial and mesenchymal cells, and suggested that EGF promotes repair processes in the inflamed mucosa of the intestine. For example, EGF treatments enhance resolutions of tri-nitrobenzenesulfonic acid-induced colitis in rats (41). Clinically, EGF enemas are effective for active distal ulcerative colitis (42). In this study, we found that among several growth factors EGF specifically induced amphiregulin and epiregulin expression in colonic SEMFs, suggesting that trophic effects of EGF in the colonic mucosa may be partially mediated by SEMF-derived amphiregulin and epiregulin induced by EGF. It is likely that EGF coordinates with proinflammatory cytokines to promote tissue repair processes via induction of amphiregulin and epiregulin.

EGF has been known to exert its trophic effects via the tyrosine kinase pathway through phosphorylation of EGF receptors (EGFRs) (1). In this study, we identified the four EGFRs in human colonic SEMFs, and demonstrated that AG-1478, a specific inhibitor of EGFR tyrosine kinases, markedly blocked EGF-induced amphiregulin and epiregulin mRNA expression in colonic SEMFs. The aforementioned result indicates that in colonic SEMFs phosphorylation of EGFR tyrosine kinase is actually involved in mechanisms underlying EGF-induced amphiregulin and epiregulin induction.

AG1478 partially blocked IL-1ß- and TNF-α-induced mRNA expression for amphiregulin and epiregulin in colonic SEMFs. Since amphiregulin (epiregulin) is a potent inducer of amphiregulin (epiregulin) expression (Fig. 7), some parts of the effects of IL-1ß and/or TNF-α on amphiregulin (epiregulin) expression may be indirectly mediated by EGFR phosphorylation in response to induced-amphiregulin and/or epiregulin. Furthermore, it has previously been reported that IL-1ß and TNF-α induces phosphorylation of EGFR in CaCo-2 intestinal epithelial cells (43), suggesting a possibility that IL-1ß and/or TNF-α directly induce phosphorylation of EGFRs in colonic SEMFs.

In various cells, amphiregulin and epiregulin are bifunctional growth factors. To access the biological activity of amphiregulin and epiregulin in our system, we tested how these growth factors modulate proliferation of colonic SEMFs by means of MTT assays. Recombinant amphiregulin and epiregulin actually stimulated the proliferation of colonic SEMFs in a dose- and time-dependent manner. Combined with findings in previous studies that amphiregulin and epiregulin stimulate the proliferation of colonic epithelial cells (10,11,17,44), amphiregulin and epiregulin play a crucial role in the process of wound healing through their action on both epithelial and mesenchymal cells in the intestine. The hypothesis may be supported by the previous reports which indicate that mice deficient in EGFRs showed an increased susceptibility to DSS-induced colitis (17,45).

In conclusion, this study demonstrates that amphiregulin and epiregulin are expressed by human colonic SEMFs. Colonic SEMFs may play an important role in promoting repair processes following mucosal injury via amphiregulin and epiregulin expression.

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