

Trefoil Factor Family–Peptides Promote Migration of Human Bronchial Epithelial Cells

Synergistic Effect with Epidermal Growth Factor

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A process termed “restitution” enables rapid repair of the respiratory epithelium by migration of neighbouring cells. Mucin-associated TFF-peptides (formerly P-domain peptides or trefoil factors) are typical motogens enhancing migration of cells in various *in vitro* models mimicking restitution of the intestine. The human bronchial epithelial cell line BEAS-2B was used as a model system of airway restitution. The motogenic activities of recombinant human TFF2 as well as porcine TFF2 were demonstrated by *in vitro* wound healing assays of BEAS-2B cells. TFF2 did not induce phosphorylation of the epidermal growth factor (EGF) receptor. EGF was capable of enhancing the motogenic effect of human TFF2 at a concentration of 3×10^{-10} M whereas EGF itself (i.e., in the absence of TFF2) did not stimulate migration at this low concentration. Furthermore, TFF2 as well as monomeric and dimeric forms of TFF3 enhanced migration of BEAS-2B cells in Boyden chambers. Motogenic activity of TFF2 was also shown for normal human bronchial epithelial (NHBE) cells in Boyden chambers. These results suggest that TFF-peptides act as motogens in the human respiratory epithelium triggering rapid repair of damaged mucosa in the course of airway diseases such as asthma.

The respiratory epithelium is frequently injured by inhaled toxic agents, by micro-organisms, or during inflammatory diseases such as asthma. This often leads to desquamation of cells from the epithelium and denudation of the basement membrane. Migration of neighboring cells is an important component for the rapid repair of damaged airway epithelium. Rapid re-epithelialization is promoted by microcirculation-derived factors together with an intact basement membrane; this repair starts within minutes after damage, long before cell proliferation can be turned on (1, 2). This process was termed “restitution” (3). Various model systems of restitution in the airway epithelium include primary cultures of human (4), bovine (5), or guinea-pig (6)

bronchial epithelial cells; cultured alveolar epithelial cells (7); and *in vivo* epithelial shedding of guinea-pig trachea (1). Migration is induced by several peptides (“motogens”) including insulin, insulin-like growth factors, calcitonin gene-related peptide, and epidermal growth factor (EGF). Restitution was also extensively studied in the gastrointestinal tract (8), where the family of trefoil factor family (TFF)-peptides (formerly P-domain peptides, trefoil factors; [9]) was shown to promote migration *in vitro* (reviews: 10–12).

The family of TFF-peptides consists of the members TFF1, TFF2, and TFF3 in human beings (13). These peptides are lumenally secreted from a variety of mucous epithelia on one side of the membrane and represent—together with mucins—characteristic constituents of mucus gels. For example, TFF3 is the predominant TFF-peptide of the human respiratory tract, where it is released mainly from submucosal glands and, to a lesser extent, from goblet cells (14, 15). TFF3 is also a secretory product of the hypothalamo-neurohypophyseal system released into the bloodstream together with oxytocin on the other side (16, 17).

All three TFF-peptides act as motogens during *in vitro* wound healing assays; this has been shown only for various gastrointestinal cell lines (18–21). Further, an antiapoptotic effect could be demonstrated for TFF3 in certain cell lines requiring transactivation of the EGF receptor in certain cell lines (22–24). All three TFF-peptides show protective or healing effects *in vivo* for the gastrointestinal tract, and it stands to reason that this will also apply to other mucous epithelia. For example, transgenic animals lacking TFF1 or TFF3 show abnormalities of the gastrointestinal mucosae (25, 26). Systemic application of TFF-peptides to the basolateral side of the mucosae seems to be more effective than oral application in preventing experimental damage of the gastrointestinal tract (reviews: 11, 12). However, it is still a question of debate how TFF-peptides exert their molecular function, and a putative TFF2 receptor was characterized only recently (27).

The aim of the present study was to investigate for the first time whether TFF-peptides are capable of promoting cell migration of human bronchial epithelial cells. The virally transformed cell line BEAS-2B (28) or normal human bronchial epithelial (NHBE) cells were used as *in vitro* models of airway epithelium. The peptides TFF2, consisting of two TFF-domains (29–31); TFF3, containing only a single TFF-domain (32, 33); and EGF were tested for their motogenic activity either after *in vitro* wounding of monolayers or in Boyden chambers.

(Received in original form February 21, 2001 and in revised form May 14, 2001)

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Abbreviations: epidermal growth factor, EGF; extracellular regulated kinase, ERK; fetal calf serum, FCS; recombinant nonglycosylated human TFF2, hTFF2; recombinant glycosylated human TFF2, hTFF2/glyc; recombinant human TFF3/dimer, hTFF3/di; recombinant human TFF3/monomer, hTFF3/mono; relative molecular mass, M_r ; normal human bronchial epithelial, NHBE; TFF2 purified from porcine pancreas, pTFF2; synthetic rat TFF3/monomer blocked at Cys-57 by an acetamidomethyl group, rTFF3/mono; standard error of the mean, SEM; trefoil factor family, TFF.

Materials and Methods

Culture of BEAS-2B Cells

The BEAS-2B cell line is from NHBE cells immortalized using a simian virus-40/adenovirus-12 hybrid virus (28).

Three cell-culture media were used. Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (Fischer Scientific, Schwerte, Germany), 1% 100× nonessential amino acids, 1 mM sodium pyruvate, and 2 mM L-glutamine (all from Biochrom, Berlin, Germany) served as the base for two different growth media. This basal medium was supplemented either with 10% fetal calf serum (FCS) (Biochrom) or 5% AC2 (Cell Concept, Umkirch, Germany). Starvation medium was DMEM/Ham's F12 without any supplements. No antibiotics were used.

Stocks of the cells were maintained in TPP tissue culture flasks (growth area: 25 or 75 cm²) with filter caps (Renner, Dannstadt, Germany) in a humidified 37°C incubator (NU4500; NuAire, Plymouth, MN) in an atmosphere of 93.5% air and 6.5% CO₂, fed every 2 to 3 d, and passaged once or twice a week. The cells, originally propagated in the presence of 10% FCS, were sequentially adapted to serum-free media by replacing FCS in the medium with AC2 in the following manner. Cells grown in 10% FCS were passaged into medium containing 5% FCS and 2.5% AC2. The percentage of FCS was halved at each feeding whereas the percentage of AC2 was raised so that 1 wk later, when the cells were split, they were passaged into a medium containing 0.63% FCS and 4.69% AC2. The cells were fully adapted and maintained in 5% AC2 without FCS in the third week after the start of adaptation. Stocks of the adapted cells were frozen in liquid nitrogen and defined as passage 3.

Culture of NHBE Cells

Primary cultures of NHBE cells (NHBE 4683; Clonetics, Walkersville, MD) were grown in bronchial epithelial growth medium (Clonetics) to a confluence of 60 to 80% (37°C, 5% CO₂). Only cells between passages 4 and 6 were used. The culture medium was removed 1 d before starting the analyses and the cells were incubated in Ham's F12 nutrient mix without serum (GIBCO Life Technologies, Karlsruhe, Germany).

TFF-Peptides, EGF

The following TFF-peptides were used: TFF2 purified from porcine pancreas (pTFF2; relative molecular mass [M_r]: 11797 [29, 34]) as described previously (35), recombinant glycosylated human TFF2 (hTFF2/glyc; average M_r: 14465 [31]), recombinant nonglycosylated human TFF2 (hTFF2; M_r: 11961.5 [31]), recombinant human TFF3/monomer (hTFF3/mono; M_r: 6694 [33]), recombinant human TFF3/dimer (hTFF3/di; M_r: 13147 [33]), synthetic rat TFF3/monomer blocked at Cys-57 by an acetamidomethyl group (rTFF3/mono; M_r: 6608 [36]), or recombinant human EGF (M_r: 6222; Sigma, Deisenhofen, Germany; stock solution at 50 µg/ml in 10 mM acetic acid and 0.1% bovine serum albumin [BSA]).

Cell Migration Assays after *In Vitro* Wounding

BEAS-2B cells (passages 4 to 18) adapted to 5% AC2 were plated on TPP 58 × 15-mm petri dishes (Renner). The cells attached within 15 to 30 min, flattened widely, started to proliferate, and formed a confluent sheet 3 d later. The confluent cultures were washed with Hanks' balanced salt solution (HBSS; GIBCO Life Technologies), and then starved for about 18 h by replacing AC2-containing culture medium with the starvation medium. The cells were scratched under sterile conditions in 20 to 35 areas per petri dish with ~ 5-mm fragments of a double-edge stainless-steel razor blade (American Safety Razor Co., Staunton, VA) fixed in a Barraquer razor blade holder (Deutschmann,

Zittau, Germany). The scratches extended over areas ~ 5-mm long. Each scratch site was marked at the bottom of the petri dish with a felt pen. The cells were washed twice with HBSS to remove the dislodged cells and residual cell debris. These "wounded" cultures were subsequently incubated with 5 ml of fresh starvation medium supplemented with TFF-peptides or EGF at the stated concentrations. Untreated controls received no supplements to the starvation medium.

The cells in the same scratched area were photographed (Axiovert [Zeiss, Oberkochen, Germany] and TMX100 [Kodak, Rochester, NY]) approximately 24 and 72 h after the start of the experiment. The scratches to be photographed fulfilled three requirements at 24 h: first, the scar in the plastic had to be clearly visible; second, the inner scratched area had to be free of cells; and third, a large number of cells had to have already crossed the scratch boundary. The first four areas of each petri dish fulfilling these requirements were photographed. The scratches photographed were labeled with a pen on the base of the petri dish so that they could easily be found again at 72 h. The photograph always included the part of the boundary where the cells had apparently traveled farthest. The micrographs were digitized, revealing an area ~ 750 µm wide (Sprint Scan 35plus; Polaroid). The digitized micrographs were analyzed with the graphical analysis software Digitrace v. 2.10a (Imatec, Munich, Germany). Fully automatic analysis was not possible due to the low contrast of the micrographs; instead, the scratch mark was drawn in manually as an overlay and the center of about 50 cells farthest away from the scratch mark was also marked manually with an asterisk. The Digitrace software then automatically measured the perpendicular distance from the line to the asterisk, and the mean distance of the 30 cells in an area ~ 750 µm wide farthest away from the start scratch was finally stored in a data file. The accuracy of the absolute length measurements was ± ≤ 5%.

The mean distance traveled by the 30 fastest cells after 24 h, subtracted from the mean distance traveled by the 30 fastest cells from the same scratch after 72 h, was used to calculate the mean distance traveled in 48 h. There was a variability in velocity from passage to passage; the mean velocity of the untreated controls of the fastest passage was 10.66 µm h⁻¹ whereas the slowest passage had a mean velocity of 5.57 µm h⁻¹. The variation in proportional stimulation from one passage to another was, however, lower. For this reason the migration distances of the 24-to-72-h time bracket are expressed as percentages: the distance traveled, divided by the mean distance traveled by the untreated controls of the same experiment, and multiplied by 100. Statistical analysis was performed on these percentages from four scratched areas from two different petri dishes from at least three different experiments so that *n* was ≥ 24, with the exception of the value for TFF2 at 5 × 10⁻⁶ M and EGF at 3 × 10⁻⁷ M, both with *n* = 8, and hTFF2 at 5 × 10⁻⁶ M with *n* = 11. Error bars in the figures show the standard error of the mean (SEM). High significance from Student's *t* test, *P* ≤ 0.01, is indicated by double asterisks and significance of *P* ≤ 0.05 by a single asterisk.

Time Course of Migratory Activity after *In Vitro* Wounding

Wounded areas were produced exactly as for the normal assay. If fewer than 30 cells had crossed the wound boundary in particular at early time points after wounding, asterisks were placed on the scratch mark (distance = 0 µm) until 30 marks were present. Photographs were made approximately every 12 h starting at 10 h after wounding until 72 h had passed.

Cell Migration Assays Using Boyden Chambers

Cell migration assays were performed as previously described (37). Modified Boyden chambers (tissue culture-treated, 6.5-mm diameter, 10-µm thickness, 8-µm pores) (Transwell; Costar Corp.,

Cambridge, MA) containing polycarbonate membranes were used. The underside of the membrane of the upper chamber was coated with 10 $\mu\text{g/ml}$ collagen type 1 from rat tail (Upstate Biotechnology, Lake Placid, NY) for 2 h at 37°C, rinsed once with phosphate-buffered saline (PBS), and then placed into the lower chamber containing 500 μl migration buffer (starvation medium supplemented with 0.25% BSA) and one of the different TFF-peptides tested. Serum-starved cells were removed from culture dishes with diluted trypsin/EDTA (0.01% trypsin and 5 mM ethylenediaminetetraacetic acid), washed twice with migration buffer, and then resuspended in migration buffer (10^6 cells/ml). A total of 100,000 cells was then added to the top of each migration chamber and allowed to migrate to the underside of the top chamber for various times. Nonmigratory cells on the upper membrane surface were removed with a cotton swab. The migratory cells attached to the bottom surface of the membrane were stained with 0.1% crystal violet in 0.1 M borate (pH 9.0) and 2% ethanol for 30 min at room temperature. The stained cells were eluted with 10% acetic acid and the absorbance was determined at 600 nm.

Error bars in Figures 7 and 8 show the SEM. Significance from Student's *t* test ($P \leq 0.05$ compared with the control) is shown by one asterisk, high significance ($P \leq 0.01$) by two asterisks, and extremely high significance ($P \leq 0.001$) by three asterisks.

Immunoblotting for EGF Receptor Phosphorylation

Just-confluent AC2 adapted BEAS-2B cells were starved in supplement-free DMEM/Ham's F12 overnight and then stimulated with hTFF2 or EGF; washed in cold PBS; and lysed at 4°C in a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid (pH 7.5), 10 mM ethyleneglycol-*bis*-(β -aminoethyl ether-*N,N'*-tetraacetic acid, 40 mM β -glycerophosphate, 1% Triton X-100, 25 mM MgCl_2 , 2 mM orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg aprotinin, and 20 $\mu\text{g/ml}$ leupeptin. The quantity of 30 μg of total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8% gel) and analyzed by Western blotting using a polyclonal anti-phosphotyrosine antibody (PY99; Santa Cruz Biotechnology, CA.). Immunoreactivity was detected with the ECL Western blotting analysis system (Amersham Pharmacia Biotech, Europe GmbH, Freiburg, Germany) using a Biomax MR film (Kodak).

Results

hTFF2/glyc and pTFF2 Enhance Migration of BEAS-2B Cells after *In Vitro* Wounding

Figure 1 shows typical micrographs used for analysis in the scratch wound assay. Recombinant hTFF2/glyc evidently enhanced migration of BEAS-2B cells. The enhancement remained roughly proportional over the 48-h span analyzed, 24 to 72 h after adding the peptide (Figure 2). Figure 3 summarizes the dose-dependent migration of BEAS-2B cells. Significant enhancement of migration ($P \leq 0.05$) was observed starting at a concentration as low as 5×10^{-9} M. The motogenic effect of hTFF2/glyc reached a peak at 5×10^{-7} M with about a 155% increase of the relative speed or distance traveled compared with control cells not treated with hTFF2/glyc.

pTFF2 (purified from porcine pancreas) also acted as a motogen; however, it needed a 10-fold higher minimal concentration of 5×10^{-8} M when compared with hTFF2 (Figure 4). The maximal effect (about 135% over control cells) was observed at a concentration comparable to hTFF2 of 5×10^{-7} M.

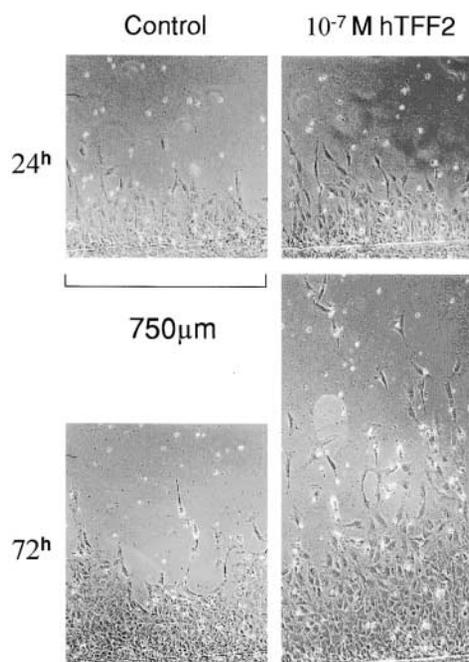


Figure 1. Cell migration assay. Typical micrographs of untreated BEAS-2B control cells and cells treated with 10^{-7} M recombinant glycosylated hTFF2 photographed after 24 and 72 h. The width of the micrographs is $\sim 750 \mu\text{m}$. The Digitrace software calculated a mean distance traveled for the 30 fastest cells of 187 μm at 24 h and 524 μm at 72 h ($\Delta = 337 \mu\text{m}$) for the control, and 265 μm at 24 h and 925 μm at 72 h ($\Delta = 660 \mu\text{m}$) for the hTFF2-treated cells.

EGF Enhances Migration of BEAS-2B Cells after *In Vitro* Wounding

The dose-dependent motogenic effect of EGF is presented in Figure 5. Significant promotion of migration of BEAS-2B cells was observed at concentrations at or above 3×10^{-9} M with a 55% increase at 3×10^{-7} M compared with controls. This enhancement is also visible in the time course illustrated in Figure 2.

hTFF2/glyc and EGF Synergistically Enhance Migration of BEAS-2B Cells after *In Vitro* Wounding

The motogenic effects of various combinations of recombinant hTFF2/glyc and EGF were investigated (Figure 6). The presence of 3×10^{-10} M EGF, which on its own has no effect, enhanced migration of BEAS-2B cells (by about 10 to 20%) whenever they were costimulated by various concentrations of hTFF2/glyc. This is an indication for a synergistic effect of TFF2/glyc and EGF. This synergistic effect is masked at higher EGF concentrations (3×10^{-9} M), where it appeared to be an additive effect.

TFF-Peptides Enhance Migration of Bronchial Epithelial Cells Cultured in Boyden Chambers

The motogenic activity of TFF2 and also that of TFF3 was tested in a complete different assay using migration of BEAS-2B cells in Boyden chambers (Figure 7). Nonglycosylated hTFF2 was by far the most active peptide in this assay (about 300% increase when compared with the con-

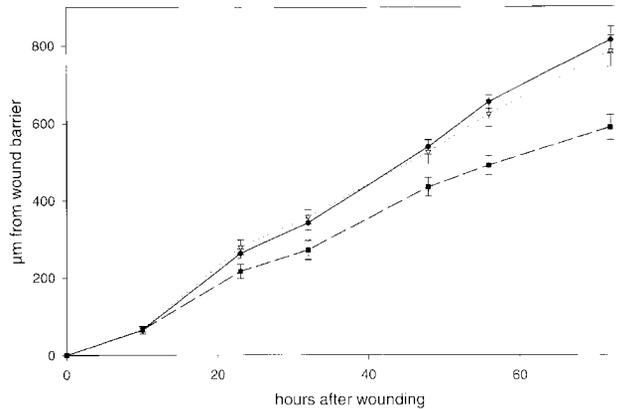


Figure 2. Time course of a typical wound-healing assay. Comparison of the migration distances (absolute means originating from one series so that they did not have to be compared with other passages) under three different conditions (eight scratches for each condition). Treatment with 5×10^{-7} M hTFF2: solid line with filled circles; treatment with 5×10^{-8} M EGF: dotted line with open triangles; untreated control: dashed line with filled squares. Error bars show the \pm SEM at the various time points.

ontrol), followed by hTFF2/glyc and hTFF3/di (about 150% increase); hTFF3/mono, rTFF3/mono, and pTFF2 showed smaller increases (between 60 and 120%).

The Boyden chamber assay was also used as a test for bronchial epithelial cells other than BEAS-2B. The primary NHBE cells showed an enhanced migratory activity after treatment with hTFF2 (Figure 8). Again, the nonglycosylated hTFF2 was more active than hTFF2/glyc (125% increase versus 45% increase).

hTFF2/glyc Does Not Activate the EGF Receptor in BEAS-2B Cells

The synergistic motogenic effect of hTFF2/glyc and EGF did not originate from transactivation of the EGF receptor by hTFF2/glyc. Figure 9 shows the constantly low phosphorylation state of the EGF receptor after treatment with

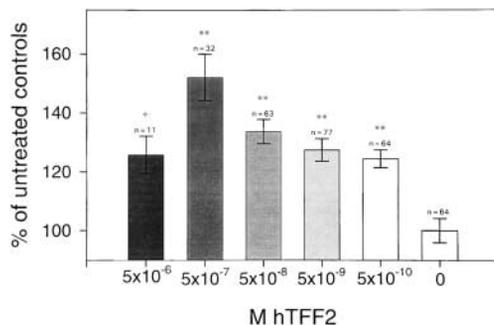


Figure 3. Motogenic effects of recombinant glycosylated hTFF2 at various concentrations. The plot shows the mean relative distance or speed of BEAS-2B cells measured in the time bracket between 24 and 72 h after application of hTFF2 compared with the control cells. Error bars show the \pm SEM; *n* represents the number of scratches analyzed at the stated concentration. *Significant difference from the untreated controls ($P \leq 0.05$); **high significance ($P \leq 0.01$).

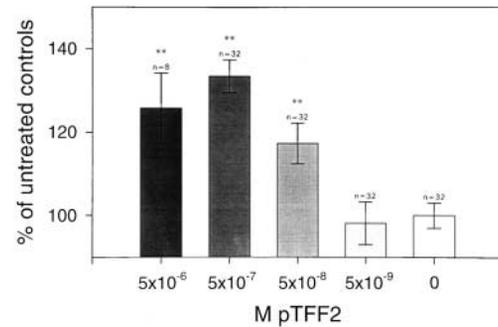


Figure 4. Motogenic effects of pTFF2 at various concentrations. The plot shows the mean relative distance or speed of BEAS-2B cells measured in the time bracket between 24 and 72 h after application of pTFF2 compared with the control cells. Error bars show the \pm SEM; *n* represents the number of scratches analyzed at the stated concentration. *Significant difference from the untreated controls ($P \leq 0.05$); **high significance ($P \leq 0.01$).

8×10^{-7} M hTFF2/glyc for up to 40 min. hTFF2/glyc was incapable of triggering tyrosine phosphorylation of the EGF receptor, whereas EGF treatment resulted in a strong but transient activation of the EGF receptor within 2 min.

Discussion

BEAS-2B cells did not migrate in an orderly fashion after *in vitro* wounding (see Figure 1). This is probably the reason that no data on the migration of BEAS-2B cells appears to be published and a time-consuming two-point assay had to be developed. Single cells stretched out into the cell-free area, often losing immediate contact with the neighboring cells but evidently leaving a film of extracellular matrix on the plate so that other cells often followed the same path, producing a string of cells protracting into the scratched area. The result was a migratory front with many fjordlike indentations, which are larger the faster the cells are. In contrast, IEC-6 cells, a rat colon cell line established as a classic *in vitro* model of epithelial restitution (38), behave differently in a similar scratch assay in con-

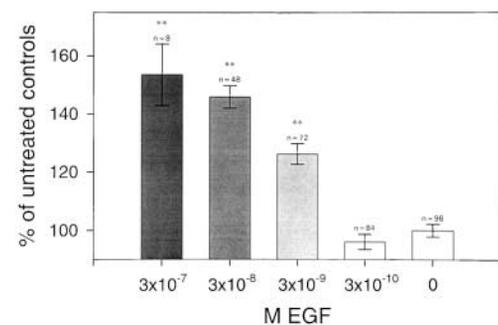


Figure 5. Motogenic effects of EGF at various concentrations. The plot shows the mean relative distance or speed of BEAS-2B cells measured in the time bracket between 24 and 72 h after application of EGF compared with the control cells. Error bars show the \pm SEM; *n* represents the number of scratches analyzed at the stated concentration. *Significant difference from the untreated controls ($P \leq 0.05$); **high significance ($P \leq 0.01$).

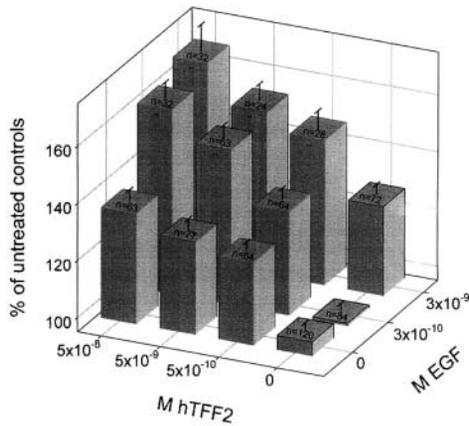


Figure 6. Motogenic effects of recombinant glycosylated hTFF2, EGF, or combinations of both. The 3-D plot shows the mean relative distance or speed of BEAS-2B cells measured in the time bracket between 24 and 72 h after treatment with hTFF2 glyc, EGF, or combinations of both compared with the control cells. The unidirectional error bars show only the \pm SEM; *n* represents the number of scratches analyzed at the stated conditions. *Significant difference from the controls (lacking EGF) with the same hTFF2 concentration ($P \leq 0.05$); **high significance ($P \leq 0.01$).

text with TFF-peptides (18). The cells move forward so that a lightly undulating migration front results that is almost parallel to the scratch mark. Thus, the distance between the migratory front and the scratch can simply be measured in IEC-6 cells. Where, however, should this parallel line be made through this ragged indented migratory front of the BEAS-2B cells? Our answer was simply to measure the distance of the 30 fastest cells and to define the ensuing mean as the migration front. BEAS-2B cells are also problematic in that they have difficulties negotiating the start of the scratch wound, especially for the inexperienced experimenter. Some wound barriers are overcome within hours; other scratches need more than 12 h,

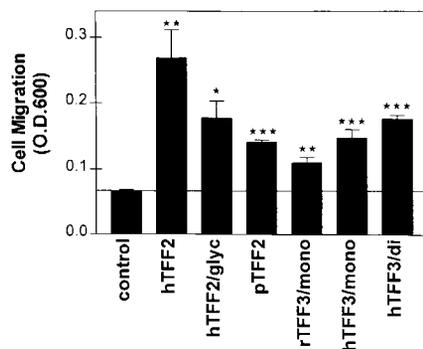


Figure 7. TFF-peptides promote migration of BEAS-2B cells in Boyden chambers. Serum-starved BEAS-2B cells were allowed to migrate for 14 h in the presence or absence of different TFF-peptides (1.6 μ M each) using Transwell migration chambers coated with collagen type I and quantified as described in MATERIALS AND METHODS. Each determination represents the average of three individual wells. *Significance ($P \leq 0.05$); **high significance ($P \leq 0.01$); ***extremely high significance ($P \leq 0.001$).

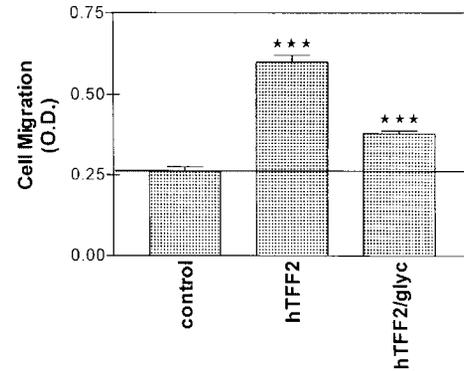


Figure 8. Glycosylated and nonglycosylated forms of hTFF2 promote migration of NHBE cells in Boyden chambers. Serum-starved NHBE cells were allowed to migrate for 5.5 h in the presence or absence of hTFF2 or hTFF2/glyc (1.6 μ M each) using Transwell migration chambers coated with collagen type I and quantified as described in MATERIALS AND METHODS. Each determination represents the average of four individual wells. ***Extremely high significance ($P \leq 0.001$).

so an end-point assay would have resulted in large error bars. The cells migrated proportionally to the treatment after succeeding in surpassing the scratch barrier in the time bracket between 24 and 72 h (Figure 3), so a two-point assay could be implemented with statistically significant results.

Using the *in vitro* wounding assay, hTFF2/glyc showed basal but significant motogenic activity already at a concentration of 5×10^{-9} M, whereas pTFF2 was still inactive at that concentration. This species difference could be explained by the fact that hTFF2 and BEAS-2B cells represent a homologous human system whereas pTFF2 is heterologous to BEAS-2B cells. However, both peptides showed a dose-dependent increase in their activity and were maximally active at about 5×10^{-7} M. This concentration is considerably (about two magnitudes) lower when compared with previous reports on the motogenic activity of hTFF2 on various intestinal epithelial cell lines, i.e., IEC-6 (8×10^{-5} M [18]), HT-29 (1×10^{-6} M [19]), and LIM1215 (4×10^{-5} M [20]).

The *in vitro* motogenic response for EGF (3×10^{-8} M) after *in vitro* wounding of BEAS-2B cells is in the same range as that of hTFF2/glyc. This situation is comparable with that of various intestinal epithelial cell lines, i.e., IEC-6 (39), HT-29 (40), and LIM1215 (20), where EGF and TFF2 act about equally as motogens.

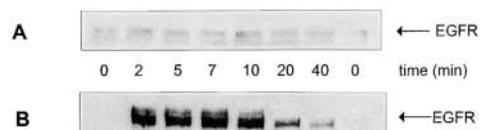


Figure 9. Time course of EGF receptor phosphorylation. Starved BEAS-2B cells were incubated with either 8×10^{-7} M recombinant glycosylated hTFF2 (A) or with 1.5×10^{-8} M EGF (B) for the times indicated. Tyrosine phosphorylation of the EGF receptor was detected by Western blot analysis.

The signaling cascades triggered by hTFF2/glyc, promoting migration of BEAS-2B cells, are currently not known and are the subject of detailed investigations. However, activation of extracellular regulated kinase (ERK) 1/ERK2 was shown to be essential for migration of various cell lines leading to phosphorylation of myosin light-chain kinase (37, 41–43). Activation of ERK1/ERK2 was also demonstrated in IEC-6 cells after treatment with TFF3 (23, 44), with ERK activation shown to be parallel to the mitogenic activity independent of phosphorylation of the EGF receptor (23). Further, EGF, which is mitogenic for IEC-6 cells (39), is also capable of activating ERK1/ERK2 in these cells (45). Thus, an additive synergistic mitogenic effect of TFF3 and EGF can be expected for IEC-6 cells through activation of nitrogen-activated protein kinases ERK1/2. However, there is only a single report published previously demonstrating such a synergistic mitogenic effect, and that only for HT-29 cells (40). Interestingly, TFF3 and EGF also cooperate in regulating epithelial chloride transport (46). The synergistic mitogenic effect of TFF2 and EGF observed for BEAS-2B cells could be explained by a joint activation of ERK leading to phosphorylation of myosin light-chain kinase. However, TFF-peptides do not bind the EGF receptor (47, 48) and hTFF2/glyc did not trigger phosphorylation of the EGF receptor in BEAS-2B cells (Figure 9). There is probably a threshold of ERK activation that must be reached to significantly promote the migration of BEAS-2B cells. An EGF concentration of 3×10^{-10} M is obviously not enough to generate this threshold activation (Figure 5) but is obviously physiologically more feasible than high concentrations.

The mitogenic activity of hTFF2/glyc was also demonstrated by means of a second migration assay using the Boyden chamber. This assay is certainly more convenient for most future studies because of the easier evaluation compared with the *in vitro* wounding assay. Migratory activity of hTFF2/glyc was evident in the Boyden chamber testing two different human bronchial epithelial cells, i.e., the transformed BEAS-2B cell line and the primary NHBE cells. Further, a series of different TFF-peptides was tested in the Boyden chamber assay, all of them being active mitogens. Surprisingly, the nonglycosylated hTFF2 was the most active peptide for both cells tested (Figures 7 and 8). The reason why glycosylation diminished migratory activity of hTFF2 is not currently known. Perhaps the sugar moiety represents a steric hindrance for ligand binding of TFF2 to a putative TFF-receptor. Interestingly, hTFF2/glyc was reported to be more active in preventing indomethacin-induced gastric damage *in vivo* when compared with the nonglycosylated form (19). Further, hTFF3/di is somewhat more active than hTFF3/mono or rTFF3/mono (Figure 7). However, the monomeric form of TFF3 is still capable of acting significantly as a mitogen and dimerization is not required for activity. This result agrees with a previous study which showed that dimerization of TFF3 is not necessary to promote migration of IEC-6 cells or to activate ERK1/ERK2 (23). In contrast, the mitogenic activity of TFF1 seems to be dependent on dimerization (21).

TFF-peptides can be expected to play an important role in promoting restitution of the respiratory epithelium on the basis of the *in vitro* mitogenic activities of TFF2 and

TFF3. TFF3—and not TFF2—is clearly expected to act as the typical mitogen for the respiratory epithelium *in vivo*. This peptide could act luminally as a major constituent of the airway mucus after its release, particularly from submucosal glands, together with the mucin MUC5B (14). TFF3 could also act via specific receptors from the basolateral side of the airway epithelium supplied by its systemic release into the bloodstream from oxytocinergic neurons of the posterior pituitary (16, 17). This model is in line with the view that plasma-derived proteins contribute essentially to the airway re-epithelialization *in vivo* (2). Further, a basolateral receptor-mediated action of TFF3 is comparable with EGF, whose receptor is found predominantly on basolateral surfaces of cells (49, 50). Thus, a common beneficial effect of TFF-peptides and EGF on cell migration might be of significance during various pathologic conditions of the tracheobronchial tract, such as asthma.

Acknowledgments: The authors thank I. Schmid-Kunz for her excellent technical assistance, and C. Chwieralski and Dr. R. L. Klemke (La Jolla, CA) for many helpful discussions. This work was generously supported by the Land Sachsen/Anhalt (1918A/0025H and 1918A/2587B to one author [W.H.]) and the Fonds der Chemischen Industrie (0163615 and 0500058 to one author [W.H.]).

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