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TFF (trefoil factor family) peptide-triggered signals promoting mucosal restitution

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Abstract. Rapid repair of mucous epithelia is essential for preventing inflammation which is a critical component of cancer progression. 'Restitution' is an early repair process which can begin within minutes and is achieved via the migration of neighbouring cells into the wounded area. Mucosal restitution is a multistep process which requires continuous blood flow and includes at least (i) the reduction of cell-cell contacts and a shift in the cell shape towards a migratory phenotype (characteristics of the epithelial-mesenchymal transition), (ii) migration of cells, (iii) repolarization and formation of tight junctions (morphological restitution) and (iv) restoration of barrier function (transmucosal epithelial resistance, functional restitution). Secretory TFF (trefoil factor family) peptides TFF1, TFF2 and TFF3 are well known for their potent protective and healing effects after mucosal damage (function as 'luminal surveillance peptides'). Here, the contributions of the TFFs during the different steps of mucosal restitution are discussed, i. e. the modulation of cell-cell contacts, their motogenic activity and synergy with epidermal growth factor, their anti-apoptotic and pro-angiogenic effects. Special emphasis has been given to discussion of the various signal transduction networks triggered by TFFs. It is becoming increasingly clear that these pathways differ depending on the respective TFF.

Key words. TFF peptides; trefoil peptides; restitution; epithelial repair; cell migration; ERK; mucous epithelia; cell-cell contacts; apoptosis.

Introduction

The three mammalian TFF (trefoil factor family) peptides TFF1, TFF2 and TFF3 [1] (in short: TFFs), are major secretory products of mucous epithelia. They are also expressed in minute amounts within the brain (for reviews, see [2–5]). Mucin-producing cells lining these epithelia or specific glands represent the predominant sites for TFF synthesis. Each of these specialized mucosae secretes its specific cocktail of TFFs and secretory mucins, and TFFs represent integral constituents of the mucus (for compilation, see [2, 3, 5]). Furthermore, pathological expression is observed in response to mucosal damage as well as during chronic inflammatory diseases, various types of metaplasia and many tumours (for reviews, see [2, 3, 6, 7]. Of interest, the expression of TFFs, as well as other growth factors and their receptors, follows a strict time scale after mucosal injury [7, 8]. A unique glandular structure known as the ulcer-associated cell lineage (UACL) is a hallmark of various chronic inflammatory conditions and a prominent site of synthesis for all TFFs as well as for epidermal growth factor (EGF) (for review, see [9]). This points to a natural synergy of TFFs and EGF for mucosal repair because the UACL is thought to play an important role for ulcer healing. This complex process of re-epithelialization and reconstruction of glandular structures is triggered by numerous growth factors (for review, see [8, 10, 11]).

The potent protective and healing effects of all three TFFs after various types of induced mucosal damage have been documented by numerous in vivo studies [12–17] (for review and older references, see [2, 3]). Also, a synergistic protective effect with EGF was shown [15]. Recent detailed comparative studies documented

that luminal application is superior over systemic delivery [13, 17]. It has even been shown that systemic TFFs (in particular TFF3/monomer) can aggravate the mucosal insults [17]. A further strong indication for the superior potential of the luminal route is the effective prevention of induced colitis after intragastric administration of TFFs by genetically modified *Lactococcus lactis* [16]. Taken together, secretory TFFs are thought to act as typical 'luminal surveillance peptides', a concept proposed by Playford [18].

Significant progress has been made within the last years in understanding the molecular function of TFFs. Besides their function as neuropeptides, there is a body of evidence that TFFs support a variety of different mucosal defence and repair mechanisms, synergistically enhancing the surface integrity of the gastrointestinal (GI) mucosa (for reviews, see [4, 5]). On the one hand, intracellular TFFs are probably involved during their secretory pathway in the complex oligomerization and packaging of secretory mucins, e.g. via the unusual TFF1-TFIZ1 heterodimer [19], and extracellular TFFs after their exocytosis bind to mucins, influencing their rheological properties [20, 21]. On the other hand, extracellular TFFs are also thought to act via hypothetical receptors postulated to be localized on the basolateral membrane of mucous epithelia. Possible candidates are gastric DMBT1 (porcine CRP-ductin) and a β-subunit of the fibronectin receptor (i.e., a β-integrin) [22]. However, in humans only soluble forms of DMBT1 have been detected thus far which are implicated in mucosal defence and epithelial differentiation [23].

The multiple protective functions of TFFs include (i) formation and stabilization of the mucus barrier, (ii) enhancement of rapid mucosal repair ('restitution'), (iii) modulation of mucosal differentiation processes and (iv) modulation of the mucosal immune response (for review, see [5]). This article will focus only on restitution (for other points, see further articles in this issue).

Mucosal restitution

Mucous epithelia cover the delicate internal surfaces of the body. These interfaces to the external environment are constantly exposed to a broad spectrum of potentially injurious factors that can induce damage from the luminal (apical) side. Rapid repair is essential for preventing mucosal inflammation, which is a critical component of cancer progression [24]. For example, some 90% of fatal malignancies in adult humans arise from epithelia. Generally, regeneration by proliferation and differentiation processes is simply too slow in order to protect the mucosa efficiently. Thus, rapid re-epithelialization is achieved in a first phase via the migration of neighbouring cells into the wounded area which, in a second phase, re-establish a tight mucosal barrier (fig. 1). This fundamental early repair process can begin within minutes, well before extensive inflammatory processes occur, and has been termed 'restitution' [25]. It is typical of the GI mucosa (for reviews, see [25–30], the respiratory tract [31–34], the urothelium [35], the gall bladder epithelium [36], the oral epithelium [37] and the cornea [38]. For example, surface mucous cells migrate in the stomach (starting within 3 min after injury), whereas enterocytes (not goblet cells) participate in duodenal restitution with a first sign of repair after 3 h [26].

In vivo restitution is dependent upon uninterrupted mucosal blood flow [29]. However, the initial phases of this multi-step process do not require cell proliferation or protein biosynthesis. Furthermore, the extracellular mucus plays an essential role [29, 30], and restitution is also strictly energy dependent. Ongoing glycolysis is essential for the early migratory phase, including re-polarization and formation of tight junctions (morphological restitution), whereas full recovery of the mucosal barrier function also relies on ATP generated by mitochondrial respiration (functional restitution) [39]. Of interest, migrating cells and cells in a tight mucosal barrier have dif-

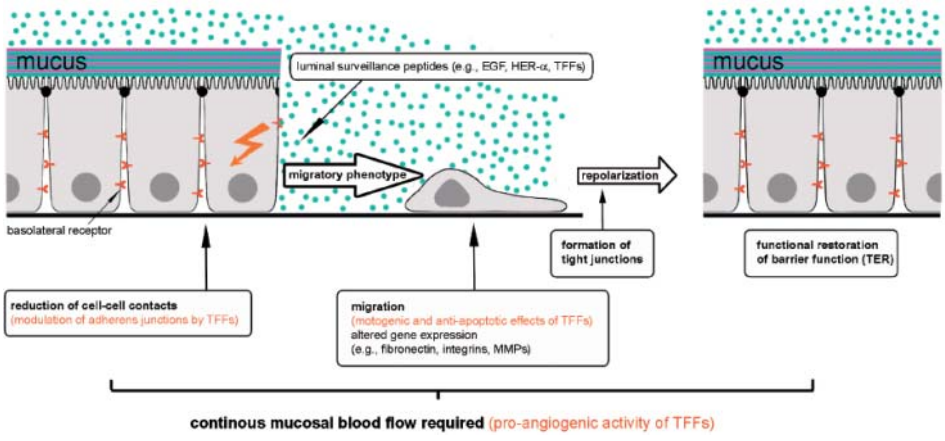

Figure 1. The different steps of mucosal restitution. After local mucosal damage, luminal surveillance peptides activate only those cells which directly neighbour the wounded area. Morphological restitution is complete after formation of tight junctions, whereas functional restitution also requires full restoration of the transmucosal epithelial resistance (TER). The contribution of TFF peptides, which are also integral mucus constituents, after binding to their hypothetical basolateral receptors is shown in red.

ferent energy requirements (reminiscent of the Warburg effect in cancer cells).

There are a number of regulatory peptides known which stimulate restitution including EGF, TGF-α, TGF-β, bFGF, HGF, IGF-I, IGF-II, IL-1β, and IL-8 (for reviews, see [10, 11, 18, 30, 40]). These peptides differentially regulate the regeneration of mucous epithelia through specific modulation of the various restitution steps [41]. A central role has been reported for transforming growth factor (TGF)-β at least in vitro [42]. However, the question arises why at least some of these peptides act only when needed. Particularly for the EGF family of receptor ligands, it has been shown that they act from the luminal side, whereas the corresponding receptors are localized at the basolateral side. Thus, these 'luminal surveillance peptides' [18] can reach their receptors only after exposure of the basolateral membrane, i.e., after local mucosal damage (fig. 1). This simple but highly effective mechanism has been demonstrated in airway epithelial repair by the EGF receptor ligand heregulin [43], enabling activation of only those cells which directly neighbour the wounded area.

One of the first steps in mucosal restitution is a shift in the cell shape towards a specialized migratory phenotype (fig .1), i.e., the cells abandon their columnar shape (loss of polarity) and assume a flattened squamoid appearance (cell spreading) [44]. Particularly TGF-α has been reported to exert strong effects (even more than EGF) [41]. This phenotypic shift shows characteristics of the epithelial-mesenchymal transition (EMT) [33, 45] and points to a close connection to cancer progression [46] (see also Machado and Wright, this issue). Adapting the migratory phenotype requires changes at least in cell-cell contacts and reorganization of the cytoskeleton [47]. TGF-β was recently established as the key regulator of EMT by dissolution of tight junctions [48].

The next step is migration of the cells (fig. 1) in order to cover the denuded basal lamina (e.g., in the gastric mucosa cells migrate approximately 1–2 μm/min [44]). This is supported by various regulatory peptides ('motogens') [41], cytosolic Ca^{2+} [49] and polyamines [50]. Cell migration is also dependent upon extracellular matrix (ECM) proteins [27]), and migratory cells in the wounded area change their expression pattern, e.g. concerning fibronectin, integrins and metalloproteases [33, 51]. This is in line with the fact that cell migration requires both ligation of growth factor receptors as well as integrins [52]. The complex spatial and temporal organization of the signalling mechanisms regulating the formation of pseudopodia is on its way to being understood (for review, see [53]).

In the final step, the monolayer of flattened cells reestablishes tight junction structure (and cell polarity: morphological restitution; fig. 1) that acts to restore barrier function (transmucosal epithelial resistance, TER: functional restitution). The latter is thought to require at least tyrosine phosphorylation of occludin and ZO-1 [39]. Polyamines are necessary for the synthesis of tight junction proteins, such as occludin [54]. There are also clear indications that intercellular communication via gap junctions plays an important role in restitution [55].

When mucosal damage extends deeper than the superficial epithelium, the mucosa is able to undergo additional repair steps, including proliferation (1–2 days) and angiogenesis [8, 11]. The final stage, which is often overlooked and can take months, is remodelling, where an essentially normal-looking mucosa is re-established.

TFFs enhance restitution in vitro: a multistep process

All three TFFs have been shown in a variety of in vitro models to be involved in the different steps of restitution, particularly by modulating cell-cell contacts, cell migration, apoptosis and angiogenesis (fig. 1). These exocrine products of mucous epithelia typically act from the luminal side. This view is in total agreement with recent in vivo protection studies.

TFFs modulate cell-cell contacts

There have been numerous reports in the past that TFFs reduce cell-cell and cell-matrix interactions and enhance cell scattering (for review, see [3]). For example, the overexpression of TFF1 or TFF3 induced dispersed growth patterns of cells in collagen gels [56, 57], and TFF2 induced a change in the growth pattern from compact spheres to complex branching tubular structures [58]. TFFs also induced scattering of Src- and RhoA-transformed cells and caused them to invade collagen gels [59]. Here, activation of Ras and Src pathways could easily be capable of reducing adherens junctions, as similarly described for the EMT [60, 61].

TFFs have indeed repeatedly been reported to modulate adherens junctions, e.g. by reducing E-cadherin, α- and β-catenin levels [62–64], and E-cadherin is necessary for the motogenic activity of TFF2 [65]. Tyrosine phosphorylation of components of the E-cadherin/catenin adhesion complex has been postulated [64] based upon the single report on TFF3-triggered phosphorylation of β-catenin (at an unusually high concentration, i.e. 10^{-2} M [62]) and the reduced E-cadherin half-life of TFF3-transfected cells. However, the TFF3-triggered signalling pathways are not known thus far. There is emerging evidence that TFFs also influence tight junctions.

Motogenic signaling of TFFs, synergy with EGF

TFFs have been shown to speed up migration of epithelial cells and also immune cells in different in vitro model

systems (motogenic effect; for compilation, see [3]). TFF1 dimer was much more potent than TFF1 monomer [66], whereas dimerization did not play a key role for TFF3 [67, 68]. There were also differences in the motogenic activity between the glycosylated and the non-glycosylated forms of TFF2 [68]. All three TFFs have mainly chemotactic, but almost no chemokinetic activity [66, 69]. Some of the signalling cascades involved have only been identified within the last years.

Originally, there were contradictory reports on phosphorylation of extracellular signal-related kinase (ERK)1/2 by TFF3 in IEC-6 cells, claiming either inactivation [70] or activation [71]. However, the latter has been confirmed, and the motogenic activity of TFF3 also depends upon this signal transduction pathway [67]. TFF3 dimerization was not required for either the motogenic effect or ERK1/2 activation in IEC-6 cells [67]. TFF3-triggered phosphorylation of ERK1/2 has also been demonstrated for gastric KATO-III cells, and activation of the TFF1 promoter by TFF3 strictly relies upon functional Ras and ERK1/2 stimulation [72].

Further studies with bronchial BEAS-2B cells demonstrated that TFF2 also is capable of inducing moderate sustained activation of ERK1/2 as well as phosphorylation of c-Jun N-terminal kinase (JNK) [73]. The motogenic effect of TFF2 was critically dependent upon ERK1/2, protein-kinase C (PKC)-α, and the Src family of tyrosine kinases, but not on p38, cyclic AMP (cAMP)-dependent protein kinase or phospatidylinositol 3-kinase (PI3K) [73]. The key role of the Ras/MEK/ERK pathway for the motogenic activity of TFFs during restitution is in total agreement with the fact that sustained ERK activation enhances cell migration processes via phosphorylation of myosin light-chain kinase, which is independent of gene expression [74].

TFF2-induced migration of BEAS-2B cells in Boyden chambers was also enhanced by haptotactic substrates, particularly collagen I or fibronectin [69]. This might point to the importance of both growth factor and integrin ligation for TFF-induced cell migration. Cas/Crk coupling provides the adhesion-dependent component of this signalling cascade and serves as a molecular switch promoting cell migration on the ECM [52].

Furthermore, all three TFFs (10^{-7} M) induced kidney epithelial cells MDCK transformed by a temperature-sensitive mutant of v-src (MDCKts.src) to invade collagen gels at the non-permissive temperature 40 °C, whereas non-transformed cells did not respond to TFFs [59]. TFF3-induced invasion was dependent upon PLC/PKC, RhoA, COX-2 and the PI3K/Akt/mTOR/$p70^{S6K}$ pathway [59, 75]. TFF1- and TFF3-induced invasion was also abolished by an agonist for the thrombin PAR-1 receptor and the constitutively activated form of the G-protein subunit Gαi3 [76], as well as a thromboxane A2 receptor antagonist [75]. The precise mechanism as to how TFFs trigger cell invasion in this artificial system has not been elucidated thus far (for review, see [77]). However, Src appears to play a key role which is in agreement with previous studies [73]. Src is well known for its role in cell migration and invasion. v-Src particularly induced activation of STAT transcription factors [61]. Of major interest, TFF1 and TFF3 were recently shown to differ completely in their ability to induce activation of STAT3 in kidney epithelial HEK-293T cells [78]. TFF3 (10^{-7} M) caused transient tyrosine phosphorylation of STAT3α (maximal levels at 5–10 min) and sustained activation of the splice variant STAT3β, whereas TFF1 did not activate STAT3 at all. This points to different TFF receptors specific for TFF1 and TFF3, respectively. Furthermore, this indicates that TFF3 can act via autocrine and paracrine activation loops, triggering its own expression via STAT3 signalling.

TFFs and EGF act synergistically in several wound healing models in vitro [15, 68, 69, 79], and there is also a single publication on the modulatory effect of TFF3 on EGF-induced ion transport [80]. Furthermore, phosphorylation of the EGF receptor (EGFR) and erbB-2 has been reported in various cell lines after treatment with TFF2 or TFF3 [62, 67, 72]. However, some of these effects require careful evaluation due to the exceedingly high TFF concentrations employed (10^{-2} M). Also, the motogenic effect of TFF2 in LIM1215 cells has been reported to require EGFR activation [81]. In contrast, TFF3-triggered restitution of IEC-6 cells did not require EGFR phosphorylation [67], and the motogenic activity of TFF2 in bronchial BEAS-2B cells is neither accompanied by EGFR phosphorylation [68] nor does it depend upon EGFR activation [69]. It has rather been demonstrated that the synergistic motogenic effect of TFF2 and EGF in BEAS-2B cells depends upon different signalling cascades, i.e. Ras/ERK versus PI3K/p38 [69]. However, repeated attempts have clearly failed to demonstrate direct binding of TFFs to the EGFR [72, 77, 82]. This argues for a potential indirect transactivation of the EGFR by certain TFFs which could only occur in specific cells. This view is congruent with the observation that the pro-invasive activity of TFF1 and TFF2 in MDCKts.src cells relies upon EGFR activation whereas TFF3-induced invasion is EGFR-independent in this artificial system [83]. This is in line with the different STAT3 responses triggered by TFF1 and TFF3 [78] and points again to the existence of different TFF receptors.

Anti-apoptotic effect of TFFs

Efficient repair by cell migration can be accomplished only if the cells do not die during this process. Thus, an intimate relationship between cell migration and cell survival developed whose biochemical pathways were only recognized within the last years [84, 85]. Major players

are IAP and Rac1, which probably form a physical complex with profilin. IAP is a known inhibitor of caspase-9, thus reducing apoptosis, and the BIR domain of IAP is important for cell migration. Recently, C1-TEN was also established as a molecule regulating both cell migration and apoptosis [86].

Consequently, numerous studies on the anti-apoptotic effect of TFFs [58, 67, 87–91] are in agreement with their motogenic function during restitution. TFF1 was found to protect cells from three different types of induced apoptosis by partially or completely blocking caspase-3, -6, -8 and -9 activities [89]. The anti-apoptotic effect of TFF3 has been reported to require intact TFF3 dimer, EGFR activation and the PI3K pathway leading, to phosphorylation of Akt [67, 87]. Another group described inhibition of anchorage-related apoptosis (anoikis) by TFF3 via a PI3K/Akt/nuclear factor κB (NF-κB) pathway, and they also showed activation of NF-κB-regulated genes such as NOS-2 and COX-2 [88]. Also, TFF3-induced expression of decay-accelerating factor (DAF) via NF-κB has been reported [92]. However, all attempts failed to repeat these results with highly purified, biologically active TFF3 [I. Schnurra, L. Thim and W. Hoffmann, unpublished results]. Activation of the extremely sensitive NF-κB pathway is easily subject to artifacts due to impurities. Thus, the signalling pathway mediating the anti-apoptotic effect of TFFs still requires careful evaluation.

Pro-angiogenic activity of TFFs

Restitution in vivo is dependent upon continous mucosal blood flow and angiogenesis is a typical process observed when mucosal damage extends deeper than the superficial epithelium. Consequently, the pro-angiogenic activity of TFFs [93] would perfectly support restitution, particularly during the later stages of remodelling. Here, expression of vascular endothelial growth factor (VEGF) could be induced by TFF3 via Src and activation of STAT3 [61, 78].

Future perspectives

Thus far, there are no molecular data published unambiguously describing TFF receptors in spite of circumstantial evidence for their existence on the basolateral side of mucous epithelia (for reviews, see [2, 5]). Integrins would be particularly interesting candidates because this would be in agreement with binding studies [22], and integrins easily could account for the complex signalling mechanisms observed, including the interaction with the EGFR system. There are even clear indications that different TFFs trigger distinct signalling pathways. Molecular characterization of such binding sites will be of invaluable assistance for the further characterization of the complex signalling network involved. This will eventually not only help to better understand the physiological function of TFFs during restitution, but also their pathological role in tumour progression and metastasis.

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