Role of trefoil peptides and IL-6 cytokine family signaling in gastric homeostasis.

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Abstract

Gastric trefoil peptides mediate mucosal repair by stimulating cell migration, inhibiting apoptosis and inflammation, and likely augmenting the barrier function of mucus. One of these, \textit{tff1}, is a gastric-specific tumor suppressor gene, which when repressed is associated with gastric cancer progression. IL-6 family cytokines play an important role in maintaining gastric homeostasis, by regulating \textit{tff1} and other mediators of mucosal proliferation, inflammation, angiogenesis and apoptosis. In this review the signaling cascades downstream of the common IL-6 cytokine family co-receptor gp130 which contribute to control of this homeostasis are described, as are the pathological outcomes of imbalancing these pathways.

Key words: trefoil peptides, IL-6, gp130, gastric cancer, gastric homeostasis
What are trefoil peptides and what do they do?

Three members of the mammalian trefoil peptide family are currently known: trefoil factor 1 (TFF1 formerly pS2) is expressed by surface and pit epithelial cells of both the fundic (body) and especially the antral (distal) stomach; trefoil factor 2 (TFF2 formerly spasmolytic polypeptide or SP) is produced by antral gland and fundic mucus neck as well as Brunners gland cells; trefoil factor 3 (TFF3 formerly intestinal trefoil factor or ITF) is a product of intestinal and colonic goblet cells and is expressed in intestinal metaplasia of the esophagus and stomach. A common feature of these peptides is that their synthesis is closely associated with that of gastrointestinal mucus (TFF1 and MUC5AC; TFF2 and MUC6; TFF3 and MUC2), and that their genes are clustered together on chromosome 21q22.3 in humans. All are synthesized ectopically throughout the gut in response to chronic inflammatory or acute ulcerative damage. Their common documented functions in normal gastric homeostasis include inhibition of apoptosis, promotion of cell migration in response to day-to-day injury, inhibition of inflammation, and augmentation of the barrier function of mucus (22, 23), although the mechanism by which the latter is accomplished physiologically is unknown. Only the homeostatic stomach-expressed trefoils TFF1 and 2 will be reviewed here.

Gastric trefoil peptides are also likely to play a critical role in disease processes including chronic inflammation, ulceration and in cancer progression (18). TFF1 is secreted from surface/pit mucus cells of the stomach, the cell type with which \textit{H. pylori} most closely associates. \textit{Tff1} gene expression is strongly inhibited in about 50% of distal cancers of the
human stomach (18, 23), and this observation coupled with the discrete phenotype of \textit{tff1} null mice, which develop antral tumours and subsequently adenocarcinomas, suggests that this trefoil functions as a stomach-specific tumor suppressor gene (23). Support for this idea comes from numerous studies including: (i) an investigation of a large series of human gastric tumors which show reduced TFF1 expression, loss of heterozygosity, and numerous somatic mutations particularly in the region encoding the trefoil motif and which is essential for biological activity (17); (ii) the observation that TFF1, given exogenously or when the gene is transfected, effectively inhibits cancer cell proliferation by delaying G1-S phase transition (23); (iii) the biological activity of trefoil factor loop 1 proteins, engineered to express known human mutations, which lose growth inhibitory ability and show enhanced cell invasion (25); (iv) the observation that depletion of TFF1 inhibits epithelial cell maturation pathways resulting in an accumulation of undifferentiated cells and endoplasmic reticulum stress (23), both of which may promote the initiation of carcinogenesis.

Under normal conditions, TFF1 is secreted apically into the overlying mucus layer (and subsequently into gastric juice), where it interacts with mucins and promotes repair after mucosal damage. Although classical receptors have not been identified for any of the trefoil peptides, 2 hybrid studies have shown a specific association between TFF1 and the vonWillebrand c-domains (vWD-c) of Muc 5AC (23), the main secreted mucin of stomach surface and pit epithelial cells. However the functional mechanism for this association is not well understood. This is important because mucins are generally accepted as being integral in protecting the underlying epithelium from acid and
proteases as well as bacterial antigens, and evidence exists that trefoil peptides can regulate mucin gel formation (22). Coincidentally, *H. pylori* which resides beneath the luminal mucus layer also binds Muc 5AC in the stomach (19), raising the possibility that infection with the bacterium may influence the barrier function of mucus. In addition *H. pylori* can bind, but not signal via secreted TFF1 (4), supporting the idea that *H. pylori* may traffic to the adherent mucus/antral apical membrane junction using TFF1 as a chemotactic signal, and anchor there by binding MUC 5AC. It is also possible that *H. pylori* may influence TFF1 expression and function, since *H. pylori* infection correlates with a decrease in the number of TFF1-positive cells (24) in humans. When, how and to what extent this occurs in the path to cancer has not yet been addressed, however these data suggest that one mechanism by which *H. pylori* might subvert gastric defence is by inhibiting proteins important in maintaining barrier function, repair and proliferation such as TFF1.

**Reciprocal regulation of trefoil peptide expression by cytokines**

We previously showed that TFF2 is expressed not only by gastrointestinal epithelial mucus cells, but also by several lymphoid organs including the spleen, lymph nodes and thymus, and is upregulated by the bacterial antigen lipopolysaccharide (reviewed in 2). Subsequently the TFF2 knockout mouse was demonstrated to have a compromised immune system (2), marked by constitutively active IL-1 receptor signaling in macrophages (Jones et al. in Proceedings of the 4th International Trefoil Conference, Strasbourg, 2004), and suggesting a role for TFF2 in negatively regulating the inflammatory response in the gut. Likewise, TFF1 knockout mice develop antropyloric
tumors and show increased lymphoid infiltrate in the small intestinal mucosa (23), again
commensurate with an anti-inflammatory role for this peptide.

An inspection of the promoter region of the stomach trefoils TFF1 and TFF2 shows the
presence of consensus motifs for a number of transcription factors including AP-1, NF-
κB, STAT 6, C/EBPβ, PPARγ and USF (1), commonly utilized by both pro- and anti-
inflammatory cytokines and growth factors to regulate target gene transcription. In
support of this, IL-1β and IL-6 inhibit tff1 promoter activity in gut cancer cell lines via
NF-κB and C/EBPβ respectively (6), and C/EBPβ expression is inversely related to that
of TFF1 in gastric cancer (20). The pleiotrophic nature of IL-6 signaling is demonstrated
by its potent positive control of tff1 gene expression in the stomach after gp130/SHP-
2/ras//Erk/AP-1 pathway activation, and the loss of TFF1-mediated tumour suppressor
activity when this pathway is blocked (21). Thus IL-6 is a key regulatory cytokine in the
stomach, acting by several different pathways under homeostatic conditions to both
positively and negatively regulate TFF1 gene expression.

2. IL-6 family cytokine signaling

The cytokines of the IL-6 family, including IL-6, IL-11, IL-27, leukemia inhibitory
factor, oncostatin M, ciliary neurotrophic factor, cardiotropin-like related cytokine, and
cardiotropin-1, all commonly utilise the trans-membrane receptor glycoprotein gp130 for
signal transduction. IL-6 and IL-11 are the most prevalent and physiologically relevant
family cytokines expressed in the gastrointestinal tract. These cytokines initiate signaling
by binding their specific α receptors (IL-6Rα and IL-11Rα respectively) which in turn
associate with, and lead to recruitment and homodimerization of the common signal transducing receptor subunit gp130, ultimately resulting in the activation of the JAK/STAT and SHP-2/ras/MAPK/ERK1-2/AP-1 signaling cascades (9, 14), which under normal conditions are in homeostatic balance (Fig. 1A). *In vitro* studies using recombinant cytokines and cancer cell lineages or primary cultures have illustrated that gp130 signals in numerous organ systems including the gut, and regulates diverse biological functions. In these tissues, gp130 signaling has been found to play roles in the regulation of cell differentiation, proliferation, survival, as well as hormone secretion and inflammatory responses (9, 14).

Following ligand binding, intracellular activation of the JAK/STAT pathway occurs by autophosphorylation of the Janus Kinases (JAKs), consisting of JAK1,2 and Tyk2. The JAKs subsequently phosphorylate multiple specific tyrosine residues on the intracellular arm of gp130 which act as docking sites for molecules which encompass a Src-homology-2 (SH-2) domain, in particular members 1 and 3 of the signal transducer and activator of transcription (STAT) family. Once docked at the receptor complex, STAT proteins are tyrosine phosphorylated by JAK1/2, dimerise, then undergo translocation to the nucleus, where they promote transcription of target genes. Likewise, the SHP-2 tyrosine phosphatase also contains an SH-2 domain, and is recruited to tyrosine residue 757 (mouse) or 759 (human) of gp130, where it undergoes activation after phosphorylation by JAK1/2, leading to binding of the adapter molecules Grb2 and son-of-sevenless (SOS) and recruitment of Ras, then activation of a series of MAP kinases culminating in gene regulation by AP-1 transcription factors (9, 14). Crucially, the
endogenous negative regulator of STAT3 activation, suppressor of cytokine signaling (SOCS3) also binds the same tyrosine residue on gp130 as SHP-2, and it is this pivotal binding site which when mutated results in a profound gastric tumor phenotype.

**How does dysregulated IL-6 family cytokine signaling impact on gastric homeostasis and trefoil gene expression?**

We have recently characterised a novel and informative mouse model of gastric cancer, based on dysregulated gp130 signaling in response to IL-6 family cytokines, that displays many of the temporal phenotypic changes characteristic of the intestinal form of human gastric cancer including gastritis, atrophy, intestinal metaplasia, dysplasia and submucosal invasion (13, 21).

The gastric tumor or gp130<sup>757F/F</sup> mouse was generated after mutation of the common SHP2 and SOCS3 binding site at tyrosine 757 (Phe for Tyr) on the intracellular arm of gp130 (21; Fig.1B). Mutation at this site prevents SHP2 docking after ligand binding with the receptor complex, thereby blocking signal transduction via the SHP-2/ras/ERK/AP-1 signaling cascade (21). Inhibition of this cascade not only prevents activation of downstream targets by AP-1 transcription factors, but also serves to augment STAT3 signaling, due in part to the loss of feedback inhibition of STAT3 activation by the SHP-2/ras/erk signaling cascade, and also because of the inability of SOCS3 to dock and inhibit STAT3 signalling. The phenotypic outcome is rapid, fully penetrant gastric tumorigenesis and accompanying chronic inflammation, with genes normally regulated by IL-6 family cytokine activation of Erk/AP-1 being down-
regulated, while those regulated by STAT3 being strongly up-regulated in a constitutive manner.

How does imbalancing of IL-6 family cytokine signaling pathways result in loss of homeostasis and selective gastric tumorigenesis? We showed both in vivo and in vitro that loss of SHP-2/ras/ERK/AP-1 signaling in response to IL-6, results in inhibition of tff1 gene expression (13, 21). Moreover we demonstrated that activated STAT3 accompanies tumour progression in the gp130$^{757F/F}$ mouse, and that allelic loss in compound STAT3$^{+/-}$ x gp130$^{757F/F}$ mutant mice resulted in much smaller tumors than in age-matched gp130$^{757F/F}$ littermates, due to reduced epithelial cell proliferation and inflammation, and loss of cytostatic TGF-β activity (12 and our own work in press). Precedents for independent loss of TFF1, and activation of STAT3, culminating in oncogenic transformation are already established. As described previously, the TFF1 null mouse develops gastric tumors and loss of TFF1 expression is associated with human gastric adenocarcinoma development. On the other hand, STAT3 can be oncogenic in the absence of other transforming factors (3), and constitutive activation occurs in many epithelial cancers including the stomach (15). Since STAT3 is a target of intracellular signaling by both growth factors and cytokines, and atypical control of signaling has increasingly been implicated in disease development and progression, it is likely that dysregulated STAT3 activation may be an early event in cancer development in many organs. The discrete development of gastric tumors in gp130$^{757F/F}$ mice despite global mutation of gp130, may reflect the fact that TFF1 is expressed primarily in the stomach.
where it has specific tumour suppressor activity, and that IL-6 family cytokines are particularly important determinants of TFF1 synthesis.

**Which IL-6 family cytokines regulate gastric mucosal pathology?**

IL-6 is generally considered to be a pro-inflammatory cytokine, the expression of which is associated with *H. pylori*-induced inflammation, and several stomach and colon-derived cancer cell lines synthesise IL-6. Despite this, definitive evidence for a causal role for IL-6 in gastric cancer progression is lacking. In fact genetic polymorphisms in IL-6 have been shown not to contribute to *H. pylori* induced gastric cancer in susceptible humans (11), suggesting that this cytokine may play a protective rather than pathogenic role in the stomach. In order to investigate this possibility further, we compared the tumour phenotype of gp130<sup>757FF</sup> and compound gp130<sup>757FF</sup> x IL-6<sup>KO</sup> mice (10). Surprisingly, in the absence of IL-6, gastric tumour growth occurred with the same incidence, and at the same age of onset and rate, as in gp130<sup>FF</sup> mice. In addition STAT3 activation was undiminished and correlated with tumour progression. Significantly, we found that although IL-6 appeared to be dispensable for tumour development, in its absence the incidence and extent of submucosal invasion, the first step in tumour metastasis, was increased 10 fold. This suggests that a cytokine signalling through gp130 other than IL-6 is responsible for tumour progression, and further that IL-6, at least in this model, inhibits tumour submucosal invasion in the stomach. Subsequent analysis showed that IL-6 is likely to inhibit submucosal invasion in part by limiting the expression of the metalloproteinases (MMPs) 9 and 13 (10). These MMP’s have already been implicated in human gastric cancer progression and metastasis, however their regulation by IL-6
family cytokines has not been well investigated and may offer new avenues for therapeutic intervention especially in targeted disruption of critical MMP function.

A clue to the identity of the mitogenic cytokine involved in stomach tumourigenesis comes from other mouse models of gastric pathology, in which we have shown that the proximal gastric hyperproliferation accompanying murine autoimmune gastritis, and the fundic hyperplasia in mice lacking the H⁺K⁺ ATPase-β subunit of the parietal cell acid pump, are both strongly associated with increased IL-11 expression (7). gp130 is ubiquitously expressed, both IL-11 and the IL-11Rα are synthesized in the stomach (5), and IL-11 expression parallels the proliferative phase of gastric repair, together lending support for the idea of a permissive role for enhanced IL-11 signalling in both normal and pathological gastric proliferation.

An interesting corollary to the gp130<sup>757F/F</sup> model is the perturbation of gastric homeostasis brought about by <i>H. pylori</i>. A consequence of <i>H. pylori</i> infection of the stomach is the constitutive activation of Erk kinases. This occurs after the <i>H. pylori</i>-derived cytotoxin CagA, which has been shown to be injected into gastric epithelial cells via the bacterial type IV injection system encoded by the Cag pathogenicity island, interacts with the phosphatase SHP-2, thereby activating Erk signaling pathways (8). We hypothesised that since activated SHP-2 negatively regulates STAT3 signaling, then sequestration or preferential binding by <i>H. pylori</i>-derived proteins like CagA might reduce the intracellular pool of SHP-2, thereby removing the brake on STAT3 activation, resulting in ongoing STAT3-induced transcriptional activation of target genes, many of
which promote proliferation, inflammation, angiogenesis and inhibition of apoptosis. Additionally, CagA might promote STAT3 binding or recruitment to gp130 thereby promoting hyperactivation directly. In unpublished studies we and others have shown that STAT3 phosphorylation occurs in human gastritis in response to *H. pylori* infection, and is more marked in CagA positive strains. Activated STAT3 has also been shown in human gastric cancers (15). Together these data support the idea that *H. pylori* activation of both SHP-2/ras/erk and STAT3 pathways may promote initiation and progression of gastric neoplasia.

**Summary of the link between cytokine signaling and trefoil peptide expression in gastric neoplasia**

Genetic dysregulation of IL-6 family cytokine signaling in the absence of *H. pylori* infection has been shown to have pathological sequelae in the mouse gastric mucosa. Loss of Erk/AP-1 signalling downstream of gp130 results in down-regulation of TFF1 tumour suppressor activity concomitant with constitutive and oncogenic STAT3 activation, which promotes proliferation and inflammation and inhibits apoptosis. The result is mucosal atrophy, mucus metaplasia and the TFF2-associated SPEM lineage, cellular proliferation, and subsequently dysplasia and submucosal invasion. Human intestinal-type gastric cancer development is associated with *H. pylori*-induced inflammation, and is also frequently marked by loss of *tff1* gene expression and the occurrence of the TFF2-associated SPEM lineage, as well as over-expression of IL-6 family cytokines. The pathological outcome of the association of *H. pylori* cytotoxins such as CagA with elements of the cell signaling machinery downstream of gp130 such
as SHP-2 has been linked to an increase in activated Erk-mediated transcription, but the occurrence and mechanism leading to STAT3 activation is unknown. Further investigation of these pathways is likely to be instructive in understanding the way in which chronic *H. pylori* infection and inflammatory cytokine production promote gastric epithelial cell transformation.
References


Figure legend

Figure 1.

(A) Balanced IL-6 family cytokine signalling via SHP2/ras/Erk and STAT3 leads to gastric mucosal homeostasis.

(B) Mutation of tyrosine 757 on the intracellular arm of mouse gp130, results in the loss of IL-6 family cytokine-mediated SHP2/ras/Erk/AP-1 regulation of the tumor suppressor gene *tff1*, and hyperactivation of STAT3 after loss of negative regulation by SHP-2 and SOCS3.