Trefoil peptides: what are they and what do they do?

ABSTRACT—The trefoil peptides help to protect the lining of the gastrointestinal tract. They have two beneficial roles in the gastrointestinal tract:
1. In basal circumstances, they may play a role in mucus stabilisation.
2. When an acute injury occurs, their rapid upregulation is important in stimulating the repair process, particularly that of epithelial restitution.

The trefoil peptide motif consists of a unique 'three-loop' structure formed by intrachain disulphide bonds. Three members of this family have been identified in humans; psmalolytic polypeptide, intestinal trefoil factor and pS2. The trefoil peptides are expressed by mucus-producing cells throughout the normal gastrointestinal tract in a site-specific manner. In addition, the production of all three trefoil peptides is ectopically expressed in cells surrounding areas of damage in conditions such as peptic ulceration and inflammatory bowel disease.

The trefoil peptide family in mammals consists of a group of small proteins, each containing one or two copies of the trefoil motif. This motif comprises a three-loop (trefoil) configuration with six highly conserved cysteine residues allowing three intrachain disulphide bridges (Fig 1). Trefoil peptides have been identified in many species, including humans, mice and toads, and are normally found in mucus-producing epithelia. This review will focus on the three trefoil peptides isolated from humans (together with their mouse and rat homologues):
- pS2 (named after the plasmid from which it was originally derived),
- psmalolytic polypeptide (SP), and
- intestinal trefoil factor (ITF).

A historical perspective

The first trefoil peptide to be identified was pS2. It has a single trefoil domain, and was originally isolated from the human breast cell line MCF7 [1]. Interest in its role in the tumour process was heightened when it was realised that it had (weak) structural similarities to epidermal growth factor (EGF) and that its production could be upregulated by oestrogens, EGF and the oncoproteins fos and jun [2]. Although not enough of the purified peptide was available to perform in vivo physiological studies, a transgenic mouse strain was produced in which expression of (human) pS2 was markedly upregulated in the mouse breast by linking it to an acid-whey protein promoter [3]. Disappointingly (for the researchers), these animals showed no abnormalities of breast architecture, and their offspring, fed on this pS2-containing milk, were morphologically normal.

Porcine psmalolytic polypeptide (PSP) was the second member of the family to be identified. It contains two trefoil domains, and was isolated from a side-fraction produced during the commercial purification of (pig) insulin. In contrast to pS2, enough peptide was available to perform various physiological and pharmacological studies. PSP was found to be remarkably resistant to luminal pancreatic proteases (of particular relevance to peptides being secreted into the gut lumen), to reduce gastric acid secretion, increase proliferation of MCF7 cells and, as the name suggests, to relax in vitro smooth muscle preparations [4]. However, these apparent properties of PSP have recently been disputed (see later).

The realisation by Thim [5] that these peptides can be considered to be a structurally distinct family of molecules, well conserved across evolution, has renewed interest in their physiological role. The sequences of human, rat and mouse homologues of SP, ITF and pS2 have now been established, and sufficient recombinant trefoil peptides are being produced to begin to examine their effects both in vitro and in vivo.

Where are the trefoil peptides found?

All three members of the trefoil peptide family identified in humans are expressed in the normal gastrointestinal tract in a site-specific distribution. pS2 and SP are produced by mucus-producing cells of the stomach (pS2 by the superficial epithelial cells throughout the stomach, and SP by the deeper glandular elements within the gastric antrum), whereas ITF is predominantly produced by goblet cells of the small and large intestine.

In addition to their production by mucus-producing cells, all these trefoil peptides are produced in the epithelial cells around areas of damage in conditions such as peptic ulceration (Fig 2) and inflammatory bowel disease [6]. The mechanism(s) by which this upregulation occurs is unclear, but a working hypothesis suggests that luminal EGF may be important in this response. This idea derives from the observations...
that pS2 has an upstream EGF response element in its DNA sequence, and that the EGF receptor in the cells of the normal gastrointestinal tract is present on the basolateral, but not the apical, membranes. When an injury occurs, luminal EGF may be able to reach its receptor on the basolateral surface of the damaged mucosa, resulting in the upregulation of trefoil peptide production [7]. The trefoil peptides are also produced by a specific glandular system, the ulcer-associated cell lineage (UACL), which develops at sites of chronic ulceration. It produces a host of ‘healing factors’, including EGF, trefoil peptides and pancreatic secretory trypsin inhibitor, which are secreted on to the wound edge [8]. This has led to the suggestion that the UACL should be considered as the ‘first-aid kit’ of the gastrointestinal tract.

The distribution of expression, together with mucus in the normal gastrointestinal tract, and upregulation in epithelial cells around sites of injury, suggest that these peptides may have two related but distinct roles:

- ‘stabilising’ mucus, and
- acting directly on the epithelium to stimulate repair at sites of injury.

Experimental evidence in support of both these ideas is beginning to accumulate (see later).

**What do the trefoil peptides do (and not do)?**

**Effects on growth**

pS2 was originally identified in breast carcinoma cells. Because it was likened to EGF, it was suspected that pS2 might be important in stimulating growth—an idea initially supported by the report that PSP stimulates growth of the breast and colonic carcinoma cell lines MCF7 and HCT116, respectively [9]. However, more recent studies suggest that (recombinant) trefoil peptides have virtually no trophic effect when tested against a relatively wide panel of cell lines [10,11]. It is also relevant that the transgenic mouse strain which overexpressed pS2 within the breast showed no abnormalities of growth [3]. The general (majority) consensus is therefore that trefoil peptides have little or no physiologically relevant activity on cell growth.

**Effects on acid secretion and smooth muscle contractility**

The isolation of ‘purified’ PSP allowed physiological and pharmacological studies to be performed. PSP caused smooth muscle relaxation of guinea pig ileum (hence the term spasmolytic polypeptide) and also reduced gastric acid secretion. However, although structurally similar to the porcine peptide, human PSP did not affect gastric acid secretion [10]. We therefore tested the original PSP product, and found that PSP did not affect smooth muscle activity or acid secretion. The reasons for these different results are unclear. However, if the SP family is confirmed not to be spasmolytic, the nomenclature will need to be reconsidered! It is also important to note that the human pancreas (unlike that of the pig) does not produce SP.

**Effects on mucus**

Mucus is present in the stomach and colon as a continuous viscoelastic layer. In the stomach, the mucus layer (in combination with bicarbonate secretion) maintains the surface of the gastric cells at neutral pH, despite the gastric juice being at about pH 2. In the colon, the
mucus layer acts as a lubricant for the passage of faeces—and probably also acts as a barrier to reduce the translocation of bacteria.

Trefoil peptides are present within mucus-producing cells and are secreted together with the mucus. This has led to the suggestion that they may be intimately involved in mucus ‘function’. Initial studies tend to support this idea: the addition of recombinant trefoil peptides to (porcine) gastric mucus increases mucus viscosity (D Podolsky; personal communication, manuscript submitted), which may be relevant to the diffusion of acid through the mucus layer. This is somewhat surprising as trefoil peptides are small (about 60-110 amino acids long), and the mechanisms by which this interaction occurs are therefore of great interest. The three-dimensional structure of PSP (using X-ray crystallography [12] and nuclear magnetic resonance) will help in understanding this interaction at the molecular level. In addition to these mucus stabilisation studies, there has recently been a report that mucus and trefoil peptides can also act together in stimulating epithelial cell migration (see later).

Effects on cells in vitro

One of the earliest responses to an acute injury (within the first hour) is migration of surviving cells from the edge of the wound over the denuded area to re-establish epithelial continuity. The physiological stimuli responsible for this response are unknown but trefoil peptides (as well as EGF) may well play an important role.

Evidence in favour of this idea is that trefoil peptides (particularly SP) are upregulated at the time that restitution is taking place [10,13], and that all the recombinant peptides tested to date stimulate the rate of cell migration of the human colonic cell line HT29 and the rat small intestinal-like cell line IEC6 in an in vitro wounding assay of cell migration [10,11]. These effects do not depend on increasing cell proliferation and, in contrast to other peptides such as EGF and transforming growth factor α (TGFα) which show a similar effect, the response to trefoil peptides is not dependent on the cells producing TGFβ. The intracellular messengers and extracellular responses involved in this stimulation of cell migration are unclear but changes in cell-cell and cell-matrix adhesion are likely to be important.

In addition to stimulating lateral cell migration, we have found that human SP stimulates cell migration through collagen gel [10]. This may be important in the healing process in vivo as, following an acute injury, the denuded area is rapidly covered in a ‘slough’ of fibrin and necrotic tissue (the so-called ‘mucoid cap’). Trefoil peptide-mediated stimulation of cell migration through this matrix may therefore be important during the initial restitution process.

In vitro studies examining the effect of ITF on epithelial ion transport (using the human colonic carcinoma cell line Colony-29) suggest that trefoil peptides may also play a role in controlling the amount of chloride secretion produced by the intestinal epithelium. This effect might be relevant in controlling the local apical fluid environment and the viscosity of the overlying colonic mucus gel [14].

It is important to appreciate that the stimulation by trefoil peptides both of cell migration (an effect which can be blocked by adding a neutralising antibody) and also of chloride secretion strongly supports the idea that, in addition to any mucus stabilising effect, trefoil peptides act via specific receptors on the cells (see later).

Effects on in vivo models of injury

We have shown that systemic administration (subcutaneous infusions) of the trefoil peptides SP and ITF decreases the amount of gastric injury which occurs following a (subcutaneous) injection of indomethacin [10,15]. The dose of SP required to achieve this effect was 25 μg/kg/h, so each animal received a total of about 22 μg of recombinant SP [10]. We also showed that intragastric doses of up to 200 μg/rat failed to reduce the amount of gastric damage sustained. Similar results have been found using PSP (M Parsons; manuscript submitted). Much higher doses (ca 1,000-fold higher) of intragastric trefoil peptides reduce gastric injury caused by ethanol and indomethacin [16]. How trefoil peptides mediate these effects is unclear, but stimulation of epithelial restitution is one obvious mechanism. Further studies examining their effects on small intestinal and colonic models of damage are presently under way.

Transgenic models

We have recently produced a transgenic mouse model which specifically overexpresses the human trefoil peptide pS2 within the jejunum by linking it to the intestinal form of fatty acid binding protein. This animal model showed normal gastrointestinal morphology and proliferation under basal conditions but markedly increased resistance to non-steroidal anti-inflammatory drug-induced small intestinal injury [17]. Importantly, only those areas of the bowel that expressed pS2 had increased resistance, indicating that this effect is probably mediated by a local rather than a systemic mechanism. It is also of note that the degree of initial damage sustained in these transgenic animals was reduced, rather than their healing response increased. This is difficult to explain solely in terms of stimulation of epithelial restitution, and suggests that trefoil peptide expression may somehow ‘stabilise’ the cells against injury.

Preliminary studies using mice in which the ITF gene has been ‘knocked out’ have given complementary results: these mice do not show an abnormal
phenotype under basal conditions but have an increased sensitivity to injurious agents [18]. A pS2 knockout mouse model has recently been produced [19]. These animals do not produce gastric mucus giving further support for a close link between trefoil peptides and mucus. Somewhat surprisingly, these animals also develop gastric adenomas. This is a completely unexpected phenotype given that trefoil peptides do not seem to influence growth. Further experiments are required to examine whether the development of adenomas is due to a direct effect of the lack of pS2 or whether it is due to the absence of gastric mucus production. Other transgenic trefoil peptide overexpressed and ‘knockout’ models are presently being produced by various groups. It will be of interest to see if these result in similar phenotypes, and also if double ‘knockout’ animals (in which two of the trefoil peptides have been knocked out in the same animal) show an abnormal phenotype in the normal (ie not exposed to ulcerogens) state.

Is there a receptor for trefoil peptides?

The findings that trefoil peptides stimulate cell migration and that systemic administration of recombinant peptides reduces intestinal injury strongly support the idea that they are acting via a receptor. Immunoprecipitation and cross-linking experiments have recently identified a 45 kDa protein complex from rat intestinal membranes which binds 125Iodine-labelled ITF [20]. This binding of labelled ITF could be displaced by unlabelled ITF and by unlabelled human SP, suggesting that there may be a common receptor(s) for several of the trefoil peptides. Binding of the unlabelled ITF resulted in tyrosine phosphorylation within the complex. This suggests that tyrosine kinase activity is important in mediating trefoil peptide responses. The presence of specific trefoil peptide receptors is also supported by the preliminary studies of Rasmussen and colleagues [21] who showed that radiolabelled PSP binds to rat enterocytes when administered systemically.

Detailed experiments examining the distribution of this putative receptor have not been performed. However, our finding that systemic administration of trefoil peptides is much more potent than when they are given intragastrically suggests that these receptors are likely to be present on the basolateral membrane of the enterocytes. A similar distribution for the EGF receptor has recently been reported in rats and humans [22].

Isolation of the trefoil peptide receptor(s) should facilitate the development of receptor blockers and also of non-peptide ligands (which would be much cheaper to produce than the recombinant peptides). These agents should allow much greater insight to be gained into the role of these peptides in vivo, and the hunt for the trefoil peptide receptor(s) is one of the most active areas of research at present.

Clinical implications

There are now sufficient in vitro and animal data to consider using recombinant trefoil peptides to stimulate gastrointestinal repair. Large-scale production of recombinant peptides is (relatively) simple, and they are being increasingly used in clinical practice (eg erythropoietin for the treatment of renal failure-induced anaemia). It is unlikely that trefoil peptides will play a major role in the treatment of peptic ulceration (due to the great efficacy of acid suppressant and Helicobacter pylori eradication therapies). There may well be a place for them to treat conditions such as inflammatory bowel disease, necrotising enterocolitis and chemotherapy-induced mucositis where therapy is presently suboptimal. In addition, because of the marked synergistic action between EGF and the trefoil peptides in reducing injury in experimental models [15], combination peptide therapy (eg ITF and EGF given together) may prove particularly useful.

Conclusions

These studies suggest that the trefoil peptides have two roles:
1. In basal circumstances, they may help to stabilise mucus.
2. When an acute injury occurs, their rapid upregulation stimulates the repair process, particularly that of epithelial restitution.

The advent of recombinant peptide technology now allows us to explore the potential benefit of recombinant trefoil peptides in the clinical setting.

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