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Molecular Aspects of Restitution: Functions of Trefoil Peptides

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Healing of mucosal damage takes place in two phases: restitution of mucosal integrity and remodeling towards recreating the original glandular arrangements. These processes can be observed in several experimental rodent models: e.g., cryoprobe or NSAID-generated ulcers in the gastric or duodenal mucosa and following surgical resection of the small or large bowel. In some studies, it has been possible to detect changes in the expression of peptides, either in the reparative epithelium or adjacent to the damage, that may contribute to the healing processes. Trefoil peptides are expressed constitutively by epithelial cells in specific regions of the gastrointestinal tract, in association with mucins. Several studies have shown that trefoil peptide expression is enhanced at sites of damage in man and rat, and experimental evidence supports their active participation in the healing process. Recombinant trefoil peptides are able to enhance the rate of epithelial cell migration *in vitro* and are able to protect against indomethacin-induced damage *in vivo*, yet they do not depend upon TGF-β for enhancing cell migration and do not appear to affect acid secretion. The mode of action of trefoil peptides appears to be receptor-mediated but is not simple. There is good evidence that there are interactions between members of the trefoil family and the EGF family that are beneficial for mucosal defense and repair. This raises the possibility that combining trefoil peptides with other growth factors or small molecules may be advantageous for treatment of ulceration.

INTRODUCTION

Rapid restoration of mucosal integrity in the gastrointestinal tract is an important step in preventing the progression of small foci of damage into significant ulcers. Movement of epithelial cells to seal small breaches occurs swiftly and without the need for cell division [1].

A variety of gut peptides are able to increase the motility of epithelial cells, including epidermal growth factor (EGF)[c], transforming growth factor-α (TGF-α), interleukin-1-β (IL-1-β) and interferon-γ (IFN-γ). These appear to have their "motogenic" effects via increased expression of TGF-β [2, 3, 4]. Exactly how these peptides enhance cell migration is not known; migration depends upon the balance of spatial and temporal signals that regulate cell adhesion to substrate and to other cells and is dependent upon the ordered formation and breakdown of adhesive complexes that attach the cytoskeleton to integrin-matrix anchors [5].

Other peptides affect the remodeling following more extensive damage. Basic fibroblast growth factor (bFGF) is a potent angiogenic agent that facilitates healing in experimental models and perhaps also in man [6, 7, 8]. Trefoil peptides are the most recent additions to the list of gut repair peptides [9, 10]. In this article, we review evidence that trefoil

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[c]*Abbreviations:* EGF, epidermal growth factor; TGF-α, transforming growth factor-α; IL-1-β, interleukin- 1-β; IFN-γ, interferon-γ ; bFGF, basic fibroblast growth factor; SP, spasmolytic polypeptide; ITF, intestinal trefoil factor, UACL, ulcer-associated cell lineage.

peptides are involved directly in processes relevant to gastric ulcer healing and present new data indicating that trefoil peptide expression is altered during the healing of surgical anastomoses in the rat.

TREFOIL PEPTIDES

The trefoil peptide family was named originally after the three-looped (three-leaf) structures proposed to be formed by intramolecular disulphide bonding in porcine spasmolytic polypeptide (SP), pS2 and *Xenopus* spasmolysin [11]. Subsequently, detailed structural information obtained by X-ray crystallography [12] and nmr spectroscopy [13] confirmed the cysteine bridging pattern of porcine SP and established the trefoil motif as a distinct structural unit with a compact structure.

In man, rat and mouse, three trefoil peptides are known: pS2, a single-trefoil peptide expressed at high levels in the stomach [14, 15]; SP, a double-trefoil peptide also expressed at high levels in the stomach [15, 16, 17], particularly by antral glands [18, 19], and additionally by Brunner's glands in the duodenum [15, 19, 20, 21]; and ITF (intestinal trefoil factor) a single-trefoil peptide abundant in goblet cells throughout the small and large bowel [22, 23, 24, 25, 26]. In each case, the trefoil peptide is co-expressed with one or more mucin genes, although in some circumstances, pS2 expression can occur in neuroendocrine cells [27, 28]. In *Xenopus*, additional trefoil peptides are known, some of which are expressed contiguous with mucin core peptide sequences so that combined trefoil-mucin molecules can be made [29].

TREFOIL PEPTIDE EXPRESSION AND ULCERATION

Several studies have used immunohistochemical and *in situ* hybridization methods to determine the patterns of expression of trefoil peptides in normal and ulcerated endodermal tissues. These have indicated that there is enhanced, and sometimes ectopic, expression of trefoil peptides adjacent to ulcers [17, 30, 31, 32] within the sheet of epithelial cells presumed to be advancing over the ulcer base, in regenerative glands at the ulcer margins, and occasionally in nearby goblet or neuroendocrine cells. In chronic ulceration, local production of trefoil peptides together with EGF and TGF-α is augmented by the development of glands of an ulcer-associated cell lineage (UACL), and this is the subject of the next section of these proceedings.

One problem with studying archival clinical materials is that the chronology of alterations in gene expression cannot be established. Potentially, and if acceptable on ethical grounds, serial biopsies might be taken endoscopically from a lesion, but with the complication that sampling itself could trigger a further response. One way to circumvent these problems and learn about the sequence of events following ulceration is to use animal models.

Trefoil peptide expression during healing of cryoprobe ulcers in rat stomach

Application of a liquid nitrogen chilled rod to the exterior wall of the stomach of an anaesthetized rat produces a "freeze burn" that is reproducible in size and rate of healing. This model was used to establish [33] that increased levels of rSP mRNA (relative to that encoding the cytoskeletal protein β-actin) were produced rapidly in response to lesioning gastric body; the ratio was almost doubled between 30 min and two hr after freezing and then normalized. Normal body mucosa showed rSP expression in mucous neck cells. This persisted after ulceration, together with very strong signals for rSP and β-actin mRNAs in the regenerative glands. There was no detectable rITF mRNA in normal rat stomach (consistent with earlier observations) or in the first 24 hr of ulcer formation; levels increased from day two to three then decreased over the next week, by which time occasional ITF-expressing mucous cells in regenerative glands were detectable by *in situ* hybridization.

The relative levels of mRNAs encoding EGF and TGF-α did not increase as quickly as that of rSP or to such an extent as that of rITF. Analysis of control trephines of gastric body mucosa showed that there were no field changes increasing the expression of any of these mRNAs.

Immunohistochemical studies [33] indicated that SP synthesized by regenerative glands was able to leak through into the submucosa, where it might be predicted to exert the spasmolytic properties after which it was named [34].

Subsequently, it was possible to examine the expression of rpS2 by *in situ* hybridization using the same histology blocks. The mRNA for rpS2 is normally found in the superficial epithelial cells [15]. Figure 1 shows the distribution of rpS2 mRNA in sections through rat stomach at six hr (Figure 1a) and 24 hr (Figures 1b and 1c) after application of the cryoprobe. From such images, it seems there is accentuation of rpS2 signals in the glands closest to the damage at these early times. Unfortunately, it was not possible to quantify these changes in the same way as the other four peptide mRNAs because all of the RNA extracted had been used.

Trefoil peptide expression in response to NSAIDs

In contrast to the deep focal lesions produced by cryoprobe or topical application of acetic acid, indomethacin or ASA treatments produce erosions whose boundaries can be more difficult to detect. Immunohistochemically, the expression of rSP in the gastric body mucosa is altered within a few hours of indomethacin (s.c.); the zone of expression broadens and extends much more basaly beneath superficial erosions [35]. ASA also disrupts the pattern of rSP, assessed immunohistochemically and by *in situ* hybridization, but it has been very difficult to relate these changes to the histology (Yeomans and Poulsom, unpublished observations).

Trefoil peptide expression during healing of surgical anastomoses

We have set out to study the healing process following surgical resection of either the small or large bowel in rats to see if the tongue of epithelium that extends to cover the ulcer base expresses all three trefoil peptides as it can do in ulcerated regions of the bowel in patients with Crohn's disease. Also, resection of the bowel is a common surgical procedure in which the restoration of mucosal integrity might potentially be impaired by chemotherapy and or radiation, and we wondered if it may be possible to use this model to evaluate peptides as therapeutic agents to improve healing rates.

We found that the rate of mucosal healing (simply to restore gross mucosal integrity) was extremely variable between subjects. Nevertheless, a number of observations were made at times between four and 21 days where there were still breaks in the mucosa: 1) enhanced expression of rITF mRNA near the ulcer in loose glandular structures (Figures 1a and 1b) and in many cells of the extending epithelial sheet (Figure 1c) (including those not resembling goblet cells) (see also Figure 2); 2) ectopic expression of rSP mRNA and immunoreactivity in crypts near the resection (data not shown).

BIOLOGICAL PROPERTIES OF TREFOIL PEPTIDES

The only trefoil peptide to be purified in more than mg quantity from natural sources is porcine pancreatic spasmolytic polypeptide, which was discovered in a side fraction produced during the preparation of porcine insulin; it had a notable resistance to proteolysis and thermal denaturation [36]. This peptide corresponds to the SPs known in man, mouse and rat, although expression in the human pancreas seems to occur only in response to chronic inflammation [17]. Early characterization suggested that PSP was able to inhibit gastric acid secretion and muscle contraction [34], stimulate the proliferation of some epithelial cell lines in culture [37] and bind to intestinal epithelial cell membrane

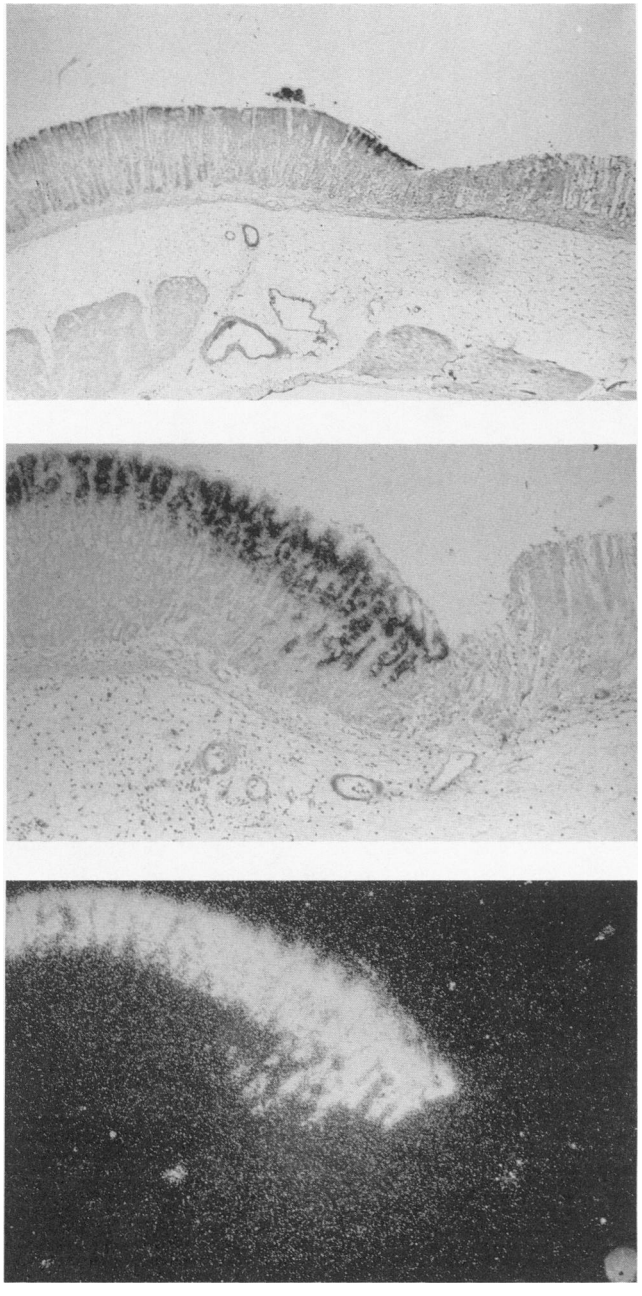

Figure 1. Expression of rpS2 mRNA in rat stomach following application of a cryoprobe. At six hr, pS2 mRNA (black autoradiographic silver grains) is localized to the superficial mucosa and appears more abundant in the glands nearest the damage, which is present on the right side of the picture (a, top). After 24 hr, the glands nearest to the damage are expressing pS2 mRNA in a broader zone than normal ([b, center], bright field illumination, and [c, bottom], reflected-light dark-field view to show autoradiographic grains in white).

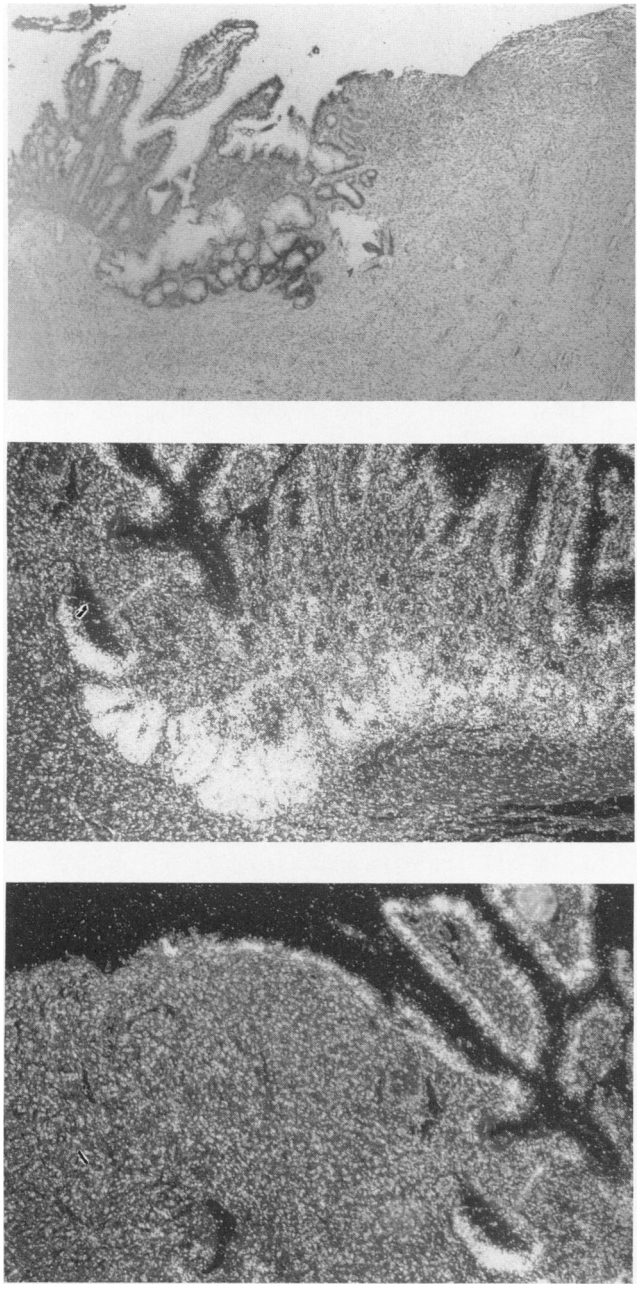

Figure 2. Expression of rITF mRNA in rat small bowel 14 days after surgical resection. (a, top) normal crypt/villus architecture is just visible on the left margin. There is a high level of rITF mRNA (black autoradiographic silver grains) in the regenerative glands at the margin of an extensive ulcer. (b, center) higher power reflected light dark-field view indicating that the regenerative glands show strong expression of rITF mRNA as do goblet cells in the rest of the intestine (the original section was counterstained by Giemsa's method which results in a gold-coloured reflection easily discriminated from silver grains in colour but not monochrome figures). (c, bottom) a region contiguous with that in (b) showing that the tongue of cells extending part way over the base of the ulcer (top left) expresses rITF mRNA.

receptors [38, 39]. Such properties would be highly desirable in a peptide designed to assist ulcer healing but have been difficult to reproduce. For example, the ability of PSP to promote proliferation *in vitro* appears dependent upon the presence of glutathione in the culture medium [40, 41], and *in vivo,* no effects of PSP could be detected [41]. Significant progress in learning the functions of trefoil peptides has become possible only recently, as they have been produced using recombinant DNA technology.

Rat ITF was first made as a fusion protein in *E. coli* and used to probe for trefoil binding sites [42]; subsequently, the mature rITF peptide was made using *E. coli* [43], yeast [44, 45] and baculovirus systems [2, 46], and a mutated form [43], in which the unpaired 7th cysteine is replaced with serine, was made to test the hypothesis that ITF can exist as a dimer, mimicking the two-trefoil structure of PSP.

Two forms of human SP, one unavoidably glycosylated, have been purified in gram quantities following expression in yeast [44]. Like PSP, hSP exhibits resistance to proteases, even in human gastric and intestinal juice [35], and is able to exert mild proliferative effects in cell culture [40].

Human pS2 has been made in *E. coli* [47] and *B. subtilis* [48] and as the natural mature peptide and as a variant in which the 7th cysteine has been replaced by serine [49]. A transgenic mouse strain was produced that secreted human pS2 in milk [50] but without effects upon breast morphology or nursing pups.

Motogenic effects of trefoil peptides

Variations of the "wounded cell monolayer" experiment have been used to establish that rITF, hITF and hSP are able either to increase the numbers of epithelial cells migrating into the "wound" from the edge or to increase the rate at which a wound gap closes [2, 3, 4, 35, 51]. Many studies assess the effects of test compounds after 24 hr, which may mean that any effect is not strictly modeling restitution; nevertheless, it is clear from time-course studies that hSP and rITF do improve the advance of the wound edge in the first few hours, with the rate then slowing to that of control wounds, possibly as the peptides become inactivated [35, 51]. The motogenic effects of ITF and hSP differ from that of EGF and TGF-α in that the trefoil effects are not mediated via TGF-β [2, 35].

Mitogenic effects of trefoil peptides

There was much interest in the possibility that pS2 was able to cause cell proliferation because it is expressed in approximately 50 percent of breast cancers. However, no such effects have been found [52]. Both PSP and hSP have weak proliferative activity *in vitro* depending upon the cell line and the glutathione status of the medium and cells (see above). There are no claims that ITF is proliferative, in fact some data support ITF being anti-proliferative [2].

Ulcer prevention by trefoil peptides

Pretreatment of rats with subcutaneous ITF or hSP is able to prevent, in a dose-dependent manner, much of the macroscopic hemorrhagic damage produced in response to a single subcutaneous dose of indomethacin [35, 51]. Oral doses of trefoil peptides appear to be less effective [46], possibly because the trefoil receptor [53] is located basolaterally on the epithelial cell membranes.

Synergy between rITF and EGF

Doses of EGF and rITF given subcutaneously that are themselves ineffective in reducing indomethacin-induced gastric damage are very effective when given together [51]. This synergy occurs in an assay that is performed over three to four hr, so it is possible that the benefit is due in part to complex interactions involving altered expression of

genes; the presence of an EGF-response element in the pS2 gene [54] shows that there is some functional relationship between the trefoils and EGFR-ligands, and there is evidence that bFGF can increase pS2 expression [55, 56]. Yet much more direct interactions occur. Electrophysiological studies of isolated gut mucosa and cell lines in Ussing chambers have showed that both EGF and rITF (but not hSP or PSP) rapidly increase electrogenic chloride transport when applied basolaterally [57]. The response to a maximally effective concentration of EGF (1nM) is increased further by adding rITF; the enhancing effect of rITF had a bell-shaped dose response below 10^{-7} M [58].

Other effects of trefoil peptides

Early observations reported that PSP could inhibit pentagastrin-stimulated gastric acid secretion [34] although recombinant hSP does not affect gastric pH [35]. There is speculation that trefoil peptides interact with mucus, e.g. [59], although there is as yet limited evidence that they do [60].

CONCLUSION

A growing body of evidence supports the view that trefoil peptides are important in the maintenance of mucosal integrity and may be actively involved in the healing of damage. It is not certain whether the properties of trefoil peptides could be useful therapeutically in peptic ulcer disease, but their potential for reducing NSAID-damage and chronic ulceration in Crohn's disease and ulcerative colitis may merit further study.

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