Expression of trefoil peptides pS2 and human spasmolytic polypeptide in gastric metaplasia at the margin of duodenal ulcers

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Abstract
Duodenal ulcers are associated with gastric metaplasia in the duodenum, both at the ulcer margin and at more distant sites in the duodenal bulb. pS2 and human spasmolytic polypeptide (hSP) are secretory peptides expressed in gastric epithelial cells and in gastric metaplasia. As these peptides may be important in ulcer healing, this study investigated the possibility that the expression of pS2 and hSP is increased in gastric metaplasia at the margin of duodenal ulcers. Duodenal bulb biopsy specimens from 12 duodenal ulcer patients were assessed. Sections were immunostained with monoclonal antibodies for pS2 and hSP. Cytoplasmic stain intensities were measured by an image analysis system and expressed as integrated optical density (IOD) units. In situ hybridisation for pS2 and hSP mRNA was carried out on parallel sections. Duodenal sections were also stained with diastase periodic acid Schiff/Falcian blue to localise areas of gastric metaplasia. pS2 antigen staining in the duodenum was restricted to surface epithelial cells, and hSP to acinar and ductular components of Brunner’s gland. mRNA localisation corresponded to immunostaining cells. In gastric metaplasia, pS2 expression was greater at the ulcer margin than away from the ulcer, as judged by the intensity of antibody staining (mean IOD units (SEM), 20.6 ± 9.5 (3.0); p<0.001). There was a trend towards greater hSP staining at the ulcer margin but this did not achieve statistical significance. These findings support the putative role of pS2 and possible hSP in mucosal healing and provide further evidence for an autocrine ‘ulcer-healing’, ‘metaplasia repair’ loop involving these trefoil peptides.

Keywords: trefoil peptides, gastric metaplasia.

Trefoil peptides are a group of polypeptides considered to have a distinctive three loop or clover leaf shape secondary structure. They are widely expressed in cells of the gastrointestinal tract. The gene for pS2, the first trefoil peptide to be discovered, was found by differential screening of a cDNA library from the human breast carcinoma cell line MCF-7. The gene product was identified as a secreted polypeptide, comprised of a 60 amino acid mature protein and a 24 residue signal peptide. Studies using anti-pS2 antibodies and RNA blotting analysis initially localised pS2 to breast cancer cells, and subsequently to normal gastric epithelium but have failed to detect its expression in a variety of other normal tissues. Human spasmolytic polypeptide (hSP), a trefoil peptide with a double trefoil structure identified from a human stomach cDNA library shares amino acid sequence homology with pS2 and is coexpressed with it in gastric foveolar cells.

The functions of the various trefoil peptides have yet to be fully defined. hSP has recently been shown to stimulate migration of colonic carcinoma cells, while porcine spasmolytic polypeptide, the porcine equivalent of hSP is mitogenic for colorectal carcinoma cells and MCF-7 cells in culture. pS2 expression is associated with oestrogen dependent status and a good prognosis in mammary tumours, but in the gastrointestinal tract pS2 synthesis and secretion by gastric foveolar cells is independent of oestrogen status. pS2 gene expression may instead be constitutive or regulated by growth factors such as epidermal growth factor/urogastrone. Although the function of pS2 and hSP remains unclear, it has been suggested that they could be growth factors and their strong expression at the sites of intestinal injury suggest that they play a part in mucosal repair.

Gastric metaplasia of the duodenum, defined as the replacement of part of the normal duodenal epithelium by patches of gastric foveolar cells, is almost a constant feature in patients with duodenal ulceration. Animal studies have shown that gastric metaplasia is rapidly induced by mucosal damage in the duodenum and resolves after ulcer healing, indicating a potential role in mucosal regeneration. The cells of origin of gastric metaplasia are believed to arise from the crypts of Lieberkuhn or alternatively from Brunner’s gland ducts. Brunner’s gland ducts secrete epidermal growth factor/urogastrone, which has been shown to be mitogenic and to promote ulcer healing. In addition pS2 and hSP are coexpressed in cells of Brunner’s gland and duct, and may also be important in the putative role of these cells in mucosal regeneration.

The aim of this study was to measure the expression of pS2 and hSP in gastric metaplasia at the margin of duodenal ulcer and in areas of gastric metaplasia more distant from...
the ulcer, to provide evidence for an autocrine 'ulcer-gastric metaplasia-repair' loop involving these trefoil peptides.

**Methods**

Twelve duodenal ulcer patients were studied, and informed consent was obtained from all patients. The study was approved by the St George's Hospital ethics committee. Multiple biopsy specimens were obtained from the ulcer edge and from distant sites in the duodenal bulb. All material was fixed in formal saline and embedded in paraffin wax within 12 hours. Sections were cut and stained with haematoxylin and eosin, and also diastase periodic acid Schiff/alcian blue (dPAS/Ab). Parallel sections were processed for both immunohistochemistry and in situ hybridisation.

**Immunohistochemistry**

Sections of duodenal biopsy specimens were immunostained for pS2 using a monoclonal IgG raised against the 28 C-terminal amino acids of the molecule; and for hSP using an IgM monoclonal antibody raised against the 15 C-terminal amino acids of hSP. Both antibodies were in the form of a supernatant and were used neat in conjunction with a streptavidin-biotin technique as previously described. A brown reaction product was obtained using a peroxidase substrate comprising of diaminobenzidine in phosphate buffered saline with 0.3% hydrogen peroxide. All the immunostaining for each peptide was performed at the same time with the same batch of antibody. A computerised digital imaging system comprising: a Hitachi KP116 video camera (Hitachi Denshi), Leitz Ergolux Orthoplan microscope (Leitz Wetzlar, Germany), and an Elonex PC 3865-200 visual image processor and densitometer card (Free Lance Sight System, Cambridge, UK) was used to measure the intensity of immunostaining in 15–20 randomly selected cells in areas of gastric metaplasia from specimens adjacent to the ulcer and from a similar number of gastric metaplasia cells in specimens from sites 2–3 cm away from the ulcer margin. The intensity of staining, with background intensity subtracted, was expressed as integrated optical density (IOD) units.

**In situ hybridisation**

The distribution of pS2 and hSP mRNAs were determined by hybridisation in situ with 35S antisense riboprobes (specific activity 0–8–1.7×10^9 dpm/mg RNA transcribed with T7 RNA polymerase) followed by autoradiography. The method used was as previously described.

**Controls**

Sections of normal stomach, small and large intestine acted as both positive and negative controls for the immunohistochemical and in situ hybridisation techniques. The localisation of both pS2 and hSP peptides and their mRNAs in these tissues has been extensively described.

** Statistical methods**

For each patient, the mean IOD of cells from immediately adjacent to the ulcer and from more distant sites was compared using the Student's t test. All IOD measurements were carried out in duplicate (coefficient of variation 5–9%).

**Results**

**Controls**

pS2 and hSP peptides and mRNA were appropriately localised to sites described previously. There was coexpression of pS2 and hSP in gastric foveolar epithelium, whereas hSP alone was seen in the pyloric glands. Enterocytes, inflammatory and neural cells were appropriately negative for both hSP and pS2 and their mRNAs.

**Histochemical staining**

Gastric metaplasia was shown on haematoxylin and eosin staining as columnar epithelium similar in morphology to gastric foveolar epithelium with cells containing large amounts of clear to weakly eosinophilic cytoplasm and basal nuclei. Epithelial cells of the terminal Brunner's gland duct were very similar in appearance. The same cells stained magenta with dPAS/Ab indicating the presence of neutral mucin. Staining pattern in all areas of gastric type epithelium irrespective of distance from the ulcer was similar (Figure (A)).

**Immunohistochemistry**

pS2 staining in the duodenum was largely restricted to gastric metaplasia cells and the distal ductular parts of Brunner's gland, which showed a diffuse cytoplasmic distribution with anti-pS2 antibody, although no staining could be detected in the acinar components of Brunner's gland. Rarely, pS2 staining was detected more remotely in goblet cells but other non-metaplastic enterocytes did not stain with anti-pS2 antibody (Figure (B)). To compare the comparative amounts of antigen in different cells, the intensity of staining was measured densitometrically. This was shown to be greater in the cytoplasm of those metaplastic cells immediately adjacent to the ulcer than those in more distant sites (mean IOD (SEM), 20.6 (3.3) v 9.5 (3.0); p<0.001).

hSP staining was restricted to the acinar and ductular components of Brunner's gland, which showed similar staining patterns. There was a trend towards greater stain intensity in components of Brunner's ducts close to the ulcer than in similar duct cells away from the ulcer. The difference in mean IOD, however, did not achieve statistical significance (4.4 (0.7) adjacent to the ulcer v 3.9 (0.6) away...
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In situ hybridisation
pS2 mRNA was shown in the distal Brunner's gland duct and in patches of gastric metaplasia matching the distribution of the peptide (Figure (C)). No pS2 mRNA was seen in either the proximal Brunner's gland duct or their acini. hSP mRNA colocalised with pS2 mRNA to areas of gastric metaplasia (Figure (D)), confirming that pS2 and hSP peptides were synthesised in the immunostaining cells. In addition, hSP mRNA was localised to areas of gastric metaplasia where its peptide was only weakly shown and occasionally not shown at all. pS2 and hSP mRNA were seen in only a few goblet cells, otherwise most non-metaplastic enterocytes did not contain pS2 or hSP mRNA.

Discussion
This study shows that the expression of pS2 in gastric metaplasia at the margin of duodenal ulcer is significantly greater than in metaplastic epithelium more distant from the ulcer, supporting the concept that up regulation of this peptide and its secretion into the micro-environment around an ulcer may play a part in regeneration and healing. Higher hSP expression is also present in Brunner's gland ducts close to the ulcer margin, although this trend does not achieve statistical significance, suggesting that up regulation of hSP expression may be in part dependent on the presence of a mucosal defect but that other regulating factors are also important.

An alternative explanation is that trefoil peptides are structural proteins. Jeffrey et al.25
have shown that spasmyotic polypeptide is secreted into rat gastric mucus, and suggest that its resistance to proteolysis stabilises mucus structure and protects against acid/peptic digestion. Playford et al. 11 have recently shown, however, that hSP induces cell migration and probably plays a part in epithelial restitution after mucosal injury. Furthermore, a structural role does not explain the greater quantities of pS2 present at the margin of duodenal ulcers.

This study also confirms the findings of Hanby et al. that pS2 mRNA and peptide are expressed by the surface and upper duct cells of Brunner’s gland, in an identical manner to areas of gastric metaplasia. 4 This distribution of trefoil peptides, as well as morphological and histochemical similarities, provide further evidence that gastric metaplasia in the duodenum, including that at the margin of ulcers, may arise from Brunner’s gland ducts.

Wyatt et al. showed that the extent of gastric metaplasia in the duodenal bulb is related to gastric acid output, 19 and a number of studies 20–26 including our own 27 have shown a close relation between the extent of gastric metaplasia and severity of duodenal inflammation. It is possible that areas of gastric metaplasia distant from an ulcer and those close to the ulcer edge originate from the same stem cells derived from Brunner’s glands. Brunner’s glands are present in the duodenal bulb from early fetal development and it has been suggested that this permanent anatomical cell lineage provides an ‘on site’ repair mechanism for this area of the bowel, which is prone to repeated damage. Brunner’s gland ducts may provide a reserve of cells that can be stimulated under adverse conditions, such as increased duodenal acidity or duodenal inflammation as well as frank ulceration, and extend across the surface of the duodenum providing increased protection for the surface mucosa.

Metaplastic epithelium at the margins of chronic intestinal ulcers, such as those in Crohn’s disease, is believed to originate from a novel cell line, the ulcerated associated cell lineage. 28 The ulceration associated cell lineage shares many of the attributes of Brunner’s glands both in terms of morphology and peptide secretion 4 and has previously been regarded as Brunner’s gland metaplasia. This cell line arises only after mucosal damage, by extension from the base of a crypt with the formation of a coiled ‘acinar’ component and ductular components, which ramify through the lamina propria and emerge at the surface close to the ulcer. 28

Cells of Brunner’s gland and those of the ulcerated associated cell lineage synthesise and secrete epidermal growth factor/urogastrone, which stimulates cell proliferation in the intestine, 21 and prevents as well as heals artificially induced ulcers in the rat. 22,29 Epidermal growth factor/urogastrone has been shown to stimulate pS2 gene expression 13 and it can be postulated that up regulation of pS2 may be mediated by epidermal growth factor/urogastrone, produced in response to mucosal ulceration. Immunostaining has identified no epidermal growth factor/urogastrone receptors on the surface cells of the ulceration associated cell lineage, indicating that pS2 peptide expression at least in the ulceration associated cell lineage is not under epidermal growth factor/urogastrone regulation. 24

The serum concentration of pS2 peptide has been shown to be significantly increased in active Crohn’s disease 30 supporting the concept that increased secretion as well as synthesis of this peptide occurs during active ulceration.

Similar cell lines to the ulceration associated cell lineage have been shown in the pancreas in chronic pancreatitis as well as in a number of other inflamed tissues such as gall bladder, fallopian tube, urinary bladder, and nasal polyps. 2 These cell lines express abundant pS2 and hSP and have been suggested to play a part in preserving mucosal integrity.

pS2 peptide and mRNA have been shown in this study to be present in some goblet cells of duodenal epithelium. A similar finding has been made in goblet and in neuroendocrine cells of intestinal epithelium bordering the ulceration associated cell lineage. 24 The Golgi apparatus extends from goblet cells of both cell types. It remains unclear why such diverse cell types around an ulcer express pS2 and whether epidermal growth factor/urogastrone secreted by Brunner’s gland ducts or the ulceration associated cell lineage have any influence on regulating their level of expression.

In this study we have provided evidence that pS2 is raised in gastric metaplasia close to an ulcer compared with gastric metaplasia at sites more remote from the ulcer. This finding supports the view that there is an autocrine ‘ulcer-gastric metaplasia-repair’ loop and that pS2 and possibly hSP play a part in the repair process. This study was funded by the Imperial Cancer Research Fund.

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