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 (3.3) vs 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” role of these peptides as a 24 residue signal peptide.

Keywords: trefoil peptides, gastric metaplasia.

Trefoil peptides are a group of polypeptides considered to have a distinctive three loop or clover leaf shaped 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. P2 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. P2 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 and resolves. 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,
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
eedge 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/alcan blue (dPAS/Ab).
Parallel sections were processed for both
immunohistochemistry and in situ hybridisa-
tion.

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 anti-
bodies 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 compris-
ing of diaminobenzidine in phosphate buffered
saline with 0.3% hydrogen peroxide. All the
immunostaining for each peptide was per-
formed 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 Elionex PC 3865-200 visual
image processor and densitometer card (Free
Lance Sight System, Cambridge, UK) was
used to measure the intensity of immunostain-
ing 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 autoradi-
ography. 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 extensiv-
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
9–5%).

Results
Controls
pS2 and hSP peptides and mRNA were
appropriately localised to sites described pre-
viously. 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 stain-
ing 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) vs 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 vs 3.9 (0.6) away

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Gastric metaplasia within the duodenum close to an area of ulceration. Staining with dPAS/Ab shows the uniform positivity of the metaplastic epithelium (GM) in contrast with the discrete goblet cell (GC) staining in the small amount of native small intestinal epithelium (SIE) included in the photomicrograph (A). Immunoreactive pS2 localises to the gastric metaplasia, and to nearby goblet cells marked by arrows (B). mRNA to pS2 colocalises to these sites (C). Although hSP immunoreactivity is not demonstrable in the metaplastic areas, hSP mRNA is present (D).

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 microenvironment 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

from the ulcer margin). Immunostaining hSP was weakly or not detected in surface gastric metaplasia.

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.
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.\(^\text{10}\) 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.\(^\text{9}\) 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.\(^\text{4}\) 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.\(^\text{9}\) showed that the extent of gastric metaplasia in the duodenal bulb is related to gastric acid output,\(^\text{19}\) and a number of studies\(^\text{26,28}\) including our own\(^\text{37}\) 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.\(^\text{28}\) The ulceration associated cell lineage shares many of the attributes of Brunner's glands both in terms of morphology and peptide secretion\(^\text{4}\) 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.\(^\text{28}\)

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,\(^\text{21}\) and prevents as well as heals artificially induced ulcers in the rat.\(^\text{22,29}\) Epidermal growth factor/urogastrone has been shown to stimulate pS2 gene expression\(^\text{13}\) 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.\(^\text{24}\)

The serum concentration of pS2 peptide has been shown to be significantly increased in active Crohn's disease\(^\text{30}\) 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.\(^\text{2}\) 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.\(^\text{24}\) The Golgi apparatus and rough endoplasmic reticulum are abundant in both cell types. It remains unclear why such diverse cell types expresses 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.

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