Augmented Intestinal Trefoil Factor (TFF3) and Loss of pS2 (TFF1) Expression Precedes Metaplastic Differentiation of Gastric Epithelium

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SUMMARY: The trefoil peptides spasmolytic polypeptide (SP), intestinal trefoil factor (ITF), and pS2 show lineage-specific expression in the normal gut and are strongly induced after mucosal injury. We assessed the relationship between this induction and the development of the regenerative epithelial lineage over time in the rat stomach and verified these observations in the metaplastic and dysplastic human stomach. Antral or colonic ulcers were induced in Wistar rats by application of serosal acetic acid and tissues harvested 2 hours to 125 days later. Human endoscopic biopsies or gastric resection specimens were also assessed. Tissues were examined by radioimmunoassay, immunoblotting, or immunohistochemistry for ITF, SP, and transforming growth factor α (rat) or ITF and pS2 (human) expression. ITF and SP mRNA in antral ulcer margins was localized by in situ hybridization. ITF and SP peptide expression rose steadily in ulcer margins after 4 days, with the rise in ITF being more pronounced. By 40 days, several hundred-fold elevations in ITF levels were present, with a field effect in uninvolved mucosa. Hyperproliferative, elongated glands of undifferentiated cells expressing abundant trefoil peptides and acid sulfomucins were present after day 12 and persisted after ulcer healing. ITF mRNA was aberrantly expressed in basal and mid-regions of these regenerative glands. In contrast, transforming growth factor α peptide expression rose promptly after injury then fell to baseline levels with healing. Seven months after injury, gastric atrophy, intestinal metaplasia, and severe dysplasia with conserved ITF expression were seen. ITF was also induced in human intestinal metaplasia and conserved in all gastric cancers, whereas expression of the gastric peptide pS2 was progressively reduced in the progression from metaplasia to dysplasia. Persistent, selective overexpression of ITF, possibly acting in an autocrine fashion, is a feature of regeneration after antral ulceration, and may provide insight into the nature of metaplastic phenotypes arising from chronic gastric injury. The loss of pS2 expression in metaplasia and cancer supports a role for this protein in gastric tumor suppression. (*Lab Invest* 2001, 81:397–408).

Gastric adenocarcinoma is a common malignancy with over 20,000 new cases each year in the United States alone (Antonioli, 1994). The identification of early curable lesions is necessary to reduce the grim outlook associated with the majority of cases, and depends on the recognition of precursor phenotypes and knowledge of the pathogenesis of these lesions (Miller, 1994; Weinstein and Goldstein, 1994). At present, however, the mechanisms underlying the formation of gastric cancer precursors such as intestinal metaplasia and gastric atrophy are virtually unknown.

The identification of regulatory molecules induced at early times during mucosal healing and marking the metaplastic phenotype may lead to a better understanding of the mechanisms. Trefoil peptide family

members are attractive candidates for this role. Comprising *spasmolytic polypeptide* (SP or TFF2), *pS2* (TFF1), and *intestinal trefoil factor* (ITF or TFF3), the trefoil gene family encodes small (7 to 12 kd), abundant, acid-stable secreted proteins of the gastrointestinal epithelium (Thim, 1989). These proteins seem to function locally by augmenting epithelial restitution (Babyatsky et al, 1996; Dignass et al, 1994; Kindon et al, 1995; Playford et al, 1995), and resealing epithelial breaches by migration of neighboring cells rather than by proliferation (Nusrat et al, 1992). Thus, mice lacking ITF have deficient colonic epithelial restitution after injury induced by dextran sodium sulfate (Mashimo et al, 1996). In addition to high constitutive levels of expression, and in keeping with a role in ensuring rapid epithelial repair, the induction of trefoil gene expression after experimentally induced injury is rapid (Alison et al, 1995).

Trefoil peptides normally exhibit lineage-specific expression within the gastrointestinal tract. SP is predominantly synthesized and secreted by the gastric mucous neck cell in humans and rodents (Jeffrey et al, 1994; Taupin et al, 1995; Tomasetto et al, 1990), pS2 by the surface (foveolar) cells of the gastric corpus and

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antrum (Lefebvre et al, 1993; Tomasetto et al, 1990). ITF peptide is expressed by human and murine intestinal goblet cells (Podolsky et al, 1993; Suemori et al, 1991). Conserved expression of human ITF is shown by colon cancer cell lines (Podolsky et al, 1993) and in colon cancer tissue (Taupin et al, 1996). Human pS2 is expressed and secreted by breast cancer tissue (Rio et al, 1987), the estrogen-responsive breast tumor line MCF-7 (Nunez et al, 1987), and other epithelial malignancies (Luqmani et al, 1992; Welter et al, 1992; Wysocki et al, 1990).

An epidermal growth factor (EGF) response element within the human pS2 promoter suggests that EGF receptor (EGFR) activation may regulate gastric pS2 transcription (Nunez et al, 1989). SP and pS2 peptide expression is upregulated in chronically ulcerated human gastrointestinal epithelium, compared with neighboring normal mucosa (Rio et al, 1991; Wright et al, 1990). An expanded EGFR-expressing region is described in these tissues and can also be induced after experimental gastrointestinal ulceration in the rat (Hansson et al, 1990; Tarnawski et al, 1992; Wright et al, 1990). These data suggest an interaction between the EGFR and the regulation of trefoil peptide expression in glandular regeneration. There is recent evidence that ITF treatment of cell lines causes EGFR phosphorylation, and EGFR activation is necessary for ITF-mediated upregulation of SP and pS2 expression (Liu et al, 1997; Taupin et al, 1999). These data raise the possibility that trefoil peptide expression may play a role in regeneration of these epithelia after injury, which would imply an involvement in growth and differentiation of new glands. However, it is not clear whether induction of trefoil peptide expression is an integral, reparative early response of injured mucosa or is a phenotypic display by newly formed glands. Whereas up-regulation of ITF and SP gene expression after acute injury (Alison et al, 1995), SP and pS2 in chronic gastrointestinal injury (Rio et al, 1991), and SP in established metaplasia (Hanby et al, 1993) have been reported, it has not been established whether these phenomena are governed by separate mechanisms or represent a continuum of gene induction running parallel to the morphogenic evolution of metaplasia.

In this study, an established model of discrete *in vivo* gastric injury was used to assess trefoil peptide induction over time, focusing on events in the late regenerative phase of repair. Rat SP and ITF expression were measured in ulcer margins and surrounding mucosa at intervals after injury. These levels were correlated at each time with peptide localized by immunohistochemistry and mRNA by hybridization *in situ*. Expression of the EGFR ligand transforming growth factor α (TGF α), an immediate-early gene product trophic to gastric epithelium (Beauchamp et al, 1989; Jhappan et al, 1990), was also quantified at relevant times after injury, as this gene is also activated during glandular repair (Russell et al, 1993). The phenotype of the regenerating gastric epithelium was further characterized by mucin histochemistry, helping to define the mucosa as gastric or intestinal in type,

and by an assessment of location and number of proliferating cells. To extend these observations to the human stomach, antral endoscopic biopsies were assessed, to determine the levels and pattern of expression of the trefoil peptides pS2 and ITF in normal, metaplastic, and dysplastic epithelium. Healing gastric glands in the rat had marked up-regulation of trefoil peptide production, which was localized to relatively undifferentiated and hyperproliferative cells. Prolongation of this gene induction heralded a subsequent metaplastic phenotype analogous to intestinal metaplasia and dysplasia of the human stomach.

Results

Ulcer Healing

The maximum ulcer area was observed 2 days after injury (mean: 24.4 mm², range: 17.3 to 32.6 mm²). Macroscopic ulcer healing, defined as complete coverage of the epithelial defect with a shiny pink mucosa, with no breaches seen at 7 $\times$ magnification under a dissecting microscope, was complete between days 12 and 24, although a shallow pit covered by mucosa was still apparent after 42 days after injury. This is consistent with healing rates previously observed with a similar model (Tarnawski et al, 1990a). There was no relationship between the area of the ulcer remaining at any time point and expression of SP in the ulcer margin. For ITF, only at 12 days was a correlation noted between expression in the residual ulcer margin (region 1, the area immediately around and containing the ulcer crater; Fig. 1B, pmol/mg of protein) and the area of that ulcer (in mm²; Pearson Product moment correlation, $r = 0.85$, $p = 0.008$, $n = 8$).

Sustained Induction of ITF Peptide Expression in Experimental Ulcer Margins Was Associated with Spread of ITF Expression to Neighboring Mucosa

In control rats, gastric samples had similar levels of immunoreactive ITF and SP at each time analyzed. There was no significant difference between peptide levels in either region 1 or 3 ($p > 0.15$, t test). In rats given ulcers, a rise in SP immunoreactivity was not seen in region 1 until after day 4 (Fig. 1A). The increase reached significance by day 12 after injury (median: 15.0 versus 3.9 pmol of SP/mg of protein; $n = 11$, $p < 0.001$, Mann-Whitney rank sum test). There was no further significant rise in levels after this time, although the elevated levels were sustained. The rise in SP immunoreactivity was approximately 4-fold. A "ripple effect" seemed to be present, with levels in region 3 (remote mucosa) elevated at week 6, but this elevation was not statistically significant (median: 14.5 versus 4.1 pmol of SP/mg of protein; $n = 6$, $p > 0.1$, Mann-Whitney rank sum test).

ITF expression was assessed. There was an initial (2 to 8 hours) fall in median ITF levels in injured animals in all three regions, although this finding was not statistically significant (Mann-Whitney rank sum test, $p > 0.1$) in any region. After 4 days, a steady rise in ITF was seen in region 1, and it reached significance at

day 12 (median: 8.8 versus 1.2 pmol of ITF/mg of protein; $n = 11$, $p = 0.007$, Mann-Whitney rank sum test), and levels continued to rise until after week 6 (Fig. 1B). A "ripple effect" was clearly apparent, with the rise in ITF levels in region 2 (adjacent to region 1) occurring by day 24, and a rise in region 3 evident at the latest time (median: 14.5 versus 1.5, $n = 11$, $p = 0.05$, Mann-Whitney rank sum test). The levels of ITF at week 6 in region 1 (230.5 ± 159 pmol of ITF/mg of protein) approximated levels previously obtained in normal rat intestine (Taupin et al, 1995).

Because of these findings, a further three animals received an acetic acid injury to the antro-fundic junction at day 30 of life (10 days after weaning) and had a laparotomy 32 to 33 weeks later. These animals seemed healthy and gained weight normally. However, at the laparotomy the mucosal aspect of the gastric antrum was grossly nodular and thickened, although other abdominal organs and the peritoneum were grossly normal. Values for ITF immunoreactivity were 150.5, 63.1, and 10.9 pmol/mg of protein in tissue from the affected areas (compared with the above median control value of 1.2 pmol of ITF/mg of protein).

Up-Regulation of TGF α in Gastric Ulcer Margins is Transient

In contrast to trefoil peptide induction, TGF α expression in the ulcer margin (both in region 1 and 2) was elevated by 2 hours after injury and reached a peak of 19 ± 3.0 pmol/mg from a baseline of 6.5 ± 0.1 pmol/mg at 8 hours. Immunoreactive TGF α concentrations declined slowly to near baseline by 12 days after injury (Fig. 1C). At day 24, tissue concentrations were similar in regions 1, 2, and 3.

Marked Trefoil Peptide Up-Regulation is Specific for Gastric Injury

To determine whether induction of trefoil synthesis was peculiar to the stomach, an analogous model of

Figure 1.

Trefoil peptide expression in gastric tissue surrounding induced ulcers. *Region 1*: ulcer and 5 mm of surrounding margins; *region 2*: 5 to 15 mm from the ulcer margin; *region 3*: mucosa from the opposite (posterior) aspect of the antrum. Results given are the mean of three radioimmunoassays (RIA), expressed as picomoles of immunoreactivity per milligram of protein, where $n = 5$ to 8 rats/group. *Zero*, control; *2h*, 2 hours after injury; *8h*, 8 hours after injury; *2d*, 2 days after injury; *4d*, 4 days after injury; *12d*, 12 days after injury; *24d*, 24 days after injury; *>6w*, > 42 days after injury. A, Spasmolytic polypeptide (SP) peptide concentration in ulcer tissue and within 5 mm of the ulcer margins (region 1, black bars) rose 4 days after injury, remaining significantly elevated 42 days later. Elevated levels were also seen in tissue adjacent to this area (region 2, white bars) and even in distant tissue at the latest time studied (region 3, hatched bars). Ordinate is linear scale. B, Intestinal trefoil factor (ITF) peptide levels in all three regions trended to fall in the first 8 hours after injury. Levels in region 1 rose after day 4, significantly by day 12, and markedly thereafter (to approximately 200-fold at day 42). A "ripple effect" is apparent, with tissue on the opposite gastric wall (region 3) being significantly elevated 6 weeks after injury. Ordinate is logarithmic scale. C, Transforming growth factor α (TGF α) peptide was measured by RIA in tissues corresponding to those in A and B. Peak immunoreactivity was present in region 1 (black bars) and region 2 (white bars) by 2 to 8 hours after injury with values gradually falling thereafter (2 and 8 hours values in region 1 and 2 compared with baseline; $n = 8$, $p < 0.05$, t test). Ordinate is linear scale.

colonic injury was used. A single-injury model was employed so that groups at each time after injury were comparable with respect to stage of repair. This model had reproducible injury and healing times, and produced a discrete lesion with a defined healing margin rather than a diffuse and patchy injury. After colonic ulceration, the observed healing rates were similar to those of gastric ulcers. Circular mucosal ulcers with 12 to 25 mm² areas were produced. Macroscopic healing occurred between days 12 and 24 after injury, although a nodule produced by submucosal thickening was frequently present after this time. There was no significant difference in immunoreactive ITF levels between tissue taken from ulcer margins and remote tissues. Although modest rises in ITF expression were evident in ulcer margins in day 24 animals compared with 24-hour animals and controls (Fig. 2), no statistically significant trend was seen ($p > 0.1$, Kruskal-Wallis one-way analysis of variance on ranks).

ITF and SP mRNA and Peptide Up-Regulation Marks Undifferentiated Columnar Cells of Regenerative Gastric Glands

Many of the structural changes of healing ulcers in this model have been previously described (Tarnawski et al, 1990a, 1990b). In the first 7 days after injury, the ulcer crater was well separated from the ulcer margin, with flattened cuboidal cells on the luminal surface of the crater. These cuboidal cells eventually provide epithelial continuity. Between 4 and 12 days after injury, the ulcer cleft immediately adjacent to the crater was lined by a single layer of columnar, relatively undifferentiated cells, with nuclei at different

Figure 2.

Trefoil peptide expression was assessed by RIA at various times in margins of cecal ulcers induced in Wistar rats ($n = 6$ per group) at laparotomy by topical serosal application of glacial acetic acid to the anterior cecum. Circular mucosal ulcers of area 12 to 25 mm² were produced by this treatment. Levels of ITF expression in ulcer margins (shaded bars) seemed to rise modestly and remain elevated, but this was not statistically significant compared with tissue from sham-operated animals at day 24 (open bar; $p > 0.1$, Kruskal-Wallis one-way ANOVA on ranks).

levels but predominantly at the basal pole, frequent mitotic figures, and occasional apoptotic bodies. Primary and secondary lumina were apparent within this layer of epithelium. In the first 4 days after injury, although the regenerating cuboidal cells showed faint cell surface SP and ITF immunoreactivity, the adjacent glands had diminished SP immunoreactivity and negligible ITF immunoreactivity, compared with control tissue (data not shown).

We focussed on the regenerative mucosa 12 days after injury, corresponding to the significant rise in tissue ITF and SP peptide concentrations, despite ostensible mucosal healing. In situ hybridization was performed on uninjured and injured antral specimens, using sense and antisense rSP riboprobes. In normal stomach, SP mRNA expression was restricted to the region of the gastric gland between the base and midgland, excluding the gland neck and surface (Fig. 3A). In contrast, in injured day 12 antral mucosa, SP mRNA hybridization signals were increased, and occurred at all levels of the regenerative gland (Fig. 3, B and C). No signal was seen after hybridization with a sense riboprobe (not shown). The increase in SP mRNA signal intensity and distribution was confined to the regenerative mucosa, with tissue on the opposite gastric wall displaying similar intensity and distribution of signal as control tissue.

SP peptide expression was also increased in intensity and distribution in the regenerative mucosa, with intense immunoreactivity in both elongated regenerative glands and in somewhat tortuous dilated glands (Fig. 3D). This mucosa was also characterized by a high proliferative rate, as assessed by nuclear proliferating cell nuclear antigen (PCNA) immunoreactivity, particularly at the bases of dilated glands (Fig. 3E). Mucin histochemistry demonstrated periodic acid-Schiff (PAS) positivity, corresponding to neutral mucins, and alcian blue staining, denoting acid sialomucins, localized to the bases of abnormal glands (Fig. 3F). Aberrant high-iron diamine staining, corresponding to intestinal-type acid sulfomucins (Fig. 4C), was also present in regenerative mucosa 12 days after injury.

The presence of this hyperproliferative regenerative mucosa, exhibiting increased SP mRNA and peptide expression, and intestinal-type mucins suggested that this was the mucosa responsible for increased levels of ITF peptide, which is normally present at low levels in the rat stomach (Taupin et al, 1995). This tissue was therefore hybridized with an antisense ITF riboprobe to assess the site of ITF mRNA expression. Twelve days after injury, ITF mRNA expression was restricted to the area encompassing the ulcer site (Fig. 4A). Immunohistochemistry showed that the regenerative epithelium expressed ITF peptide in the supranuclear region of cells throughout these abnormal glands, with evidence of secretion onto the luminal surface of cells (Fig. 4B).

This aberrant ITF expression, as predicted from radioimmunoassay (RIA) data, persisted after the time when macroscopic healing had taken place. ITF mRNA expression occurred at all levels of elongated

Figure 3.

Up-regulation of SP gene expression in regenerative mucosa after induced antral ulceration in the rat. A, In situ hybridization using antisense rat SP riboprobe, darkfield view, of normal rat gastric antrum. mRNA expression is restricted to the region of the gastric gland between the base and midgland. L, lumen; original magnification, $\times 100$. B, In situ hybridization to rat SP in regenerative (day 12) epithelium, darkfield view, showing increase in both signal intensity and distribution of SP mRNA, contrasting with tissue from the opposite antral wall. Original magnification, $\times 100$. C, Brightfield view of B, showing that the tissue with increased SP mRNA expression consists of elongated glands with SP mRNA expression along the length of these glands, in contrast to the glands of normal height on the opposite gastric wall. L, lumen; original magnification, $\times 100$. D, SP immunohistochemistry in regenerative (day 12) gastric epithelium. SP immunoreactivity is present along the length of the elongated gastric glands (*black arrow*). Tortuous and elongated glands with cystic dilation, also showing SP immunoreactivity, are seen to the right of the field (*white arrow*). Original magnification, $\times 200$. E, Proliferating cell nuclear antigen (PCNA) immunohistochemistry in a serial section corresponding to D. *Arrowheads* mark dilated glands with frequent PCNA-immunoreactive nuclei. F, Mucin histochemistry (periodic acid-Schiff (PAS)/alcian blue) in a serial section corresponding to D.

glands and was present 24 days after injury (Fig. 4D). ITF immunohistochemistry was performed on tissues obtained up to 7 months after injury. By this stage,

gross gastric mucosal and submucosal thickening, including the original site of injury, was present at laparotomy. No intraperitoneal or liver tumor was

Figure 4.

Morphologic assessment and localization of ITF expression in rat gastric antrum at 12 days (A to C), 24 days (D), and 120 days (E to F) after acetic acid-induced ulceration. A, In situ hybridization using antisense ITF riboprobe, darkfield view, demonstrating ITF mRNA expression localized to the area of regenerative epithelium. Original magnification, $\times 50$. B, ITF immunohistochemistry in tissue corresponding to A (original magnification, $\times 200$), showing discrete ITF immunoreactivity (*brown stain*) in columnar cells lining cystically dilated glands and the elongated cleft epithelium adjacent to the ulcer scar and lining the luminal aspect of this epithelium (*arrow heads*). C, High-iron diamine histochemistry, showing atypical acid sulfomucin staining of regenerative mucosa (section corresponds to Fig. 3, D to F). Nuclear-fast red counterstain. D, In situ hybridization, ITF riboprobe, brightfield view, in regenerative gland 24 days after acetic acid injury, showing persistence of ITF mRNA up-regulation (*arrowheads*). L, lumen. E and F, Gastric atrophy and intestinal metaplasia in ulcer region, 120 days after acetic acid injury. In E, PAS/alcian blue histochemistry shows predominant alcianophilia marking an area of gastric atrophy (*single arrow*). Adjacent glands continue to resemble regenerative epithelium (*double arrow*). Original magnification, $\times 50$. In F, dysplastic epithelium within the area of gastric atrophy displays persistent ITF expression (*arrows*).

apparent, and liver histology was normal. Two of three animals had evidence of gastric atrophy (Fig. 4E); many of the glands within areas of metaplasia were frankly dysplastic (Fig. 4F, *arrows*). Nevertheless, ITF immunoreactivity persisted (Fig. 4F). This atrophic tissue was alcianophilic but PAS-negative, and this, together with persistent aberrant ITF expression and the presence of goblet cells (not shown), suggested intestinalization of this mucosa. Nevertheless, no brush border was apparent at any time during the course of this experiment, nor were specialized gastric cells, such as parietal cells or endocrine cells, seen in the regenerative epithelium (not shown).

pS2 Expression in Normal, Inflamed, and Metaplastic Human Gastric Mucosa

An RIA for human SP was developed, based on a rabbit polyclonal antibody, but did not prove sufficiently sensitive to yield discriminative levels of SP immunoreactivity from endoscopic biopsies. Instead, RIA for pS2 was used to determine stomach-specific trefoil expression (see "Discussion"). Human gastric pS2 was characterized by gel filtration chromatography on G-50 of crude antral extracts from each of four patients, which revealed a single peak of pS2 immunoreactivity in each case, eluting at an approximate molecular weight of 14 kd and corresponding to a dimer of the mature protein (Jakowlew et al, 1984). These peaks were pooled, desalted, and further purified by reverse phase high-pressure liquid chromatography (HPLC). Separation on SDS/PAGE under reducing conditions gave a predominant band at 7 kd corresponding to the pS2 monomer with a minor band at 14 kd corresponding to the intact dimer.

pS2 concentrations in samples from patients with normal histology, active chronic gastritis, intestinal metaplasia, or gastric cancer were assessed. Normal antral pS2 levels (7290 ± 1842 pmol of pS2/mg of protein, $n = 6$) were more than 2.5-fold higher than in atrophic gastritis (2924 ± 493 pmol of pS2/mg of protein, $n = 8$, $p = 0.142$), 3.7-fold higher than in intestinal metaplasia (1957 ± 629 , $n = 5$, $p = 0.033$), and 54-fold higher than in gastric cancer (134 ± 57 , $n = 7$, $p = 0.001$) (Fig. 5). The normal gastric expression of pS2 had strong perinuclear staining of foveolar cells, leaving an apical clear region that was occupied by mucin. In Barrett's epithelium, where the epithelium was gastric in morphology, this expression pattern was repeated; where patches of intestinal metaplasia were present, goblet cells and endocrine cells were clearly positive, and other epithelial cells were negative.

ITF Peptide Expression is Highly Induced in Intestinal Metaplasia of the Stomach

Human ITF immunoreactivity was quantified by RIA. ITF was elevated in antral biopsies from patients exhibiting intestinal metaplasia (as assessed histologically in separate but contemporary antral biopsies) and compared with those having normal histology,

active chronic gastritis, and gastric cancer. All had comparable ITF peptide expression (Fig. 5) (intestinal metaplasia, median: 51.6, range: 31 to 109 pmol/mg of protein, $n = 7$; normal, median: 13.8, range: 11 to 18 pmol/mg of protein, $p = 0.006$, $n = 7$; active chronic gastritis, median: 19.9, range: 11.3 to 23.6 pmol/mg of protein, $p = 0.001$, $n = 7$; gastric cancer, median: 11.4, range: 5.6 to 41.9 pmol/mg of protein, $p = 0.039$, $n = 9$). The conservation of ITF expression in cancer progression suggests that the antral mucosa retains the ability to express this trefoil peptide independent of the gastric phenotype.

Figure 5.

Quantification of trefoil expression in endoscopic biopsies and gastric resection specimens. Two endoscopic biopsies from each patient were obtained for ITF and pS2 RIA, and a third was submitted for histology. A, Median ITF expression was significantly elevated in biopsies from patients exhibiting intestinal metaplasia (mean: 51.6, range: 31 to 109 pmol/mg of protein compared with those of the group with normal histology, mean: 13.8, range: 11 to 18 pmol/mg of protein, $p = 0.006$, and those with active chronic gastritis, mean: 19.9, range: 11.3 to 23.6 pmol/mg of protein, $p = 0.001$). Levels were in the same range as normal antrum in resection specimens demonstrating gastric cancer (mean: 11.4, range: 5.6 to 41.9 pmol/mg of protein, $p = 0.751$). Ordinate is logarithmic scale. B, Mean pS2 concentrations were substantially higher in the normal antrum (7290 ± 1842 pmol/mg of protein) than with gastritis (2924 ± 493 pmol/mg of protein, $p = 0.142$) and intestinal metaplasia (1957 ± 629 pmol/mg of protein, $p = 0.033$), and there was a highly significant difference in pS2 expression between these groups and those with gastric cancer (134 ± 57 pmol/mg of protein, all $p < 0.003$). ACG, active chronic gastritis; IM, intestinal metaplasia; GC, gastric cancer.

Positive control sections (five of five) of duodenum and colon demonstrated strong goblet cell ITF immunoreactivity. ITF immunoreactivity was weak and diffuse in normal human gastric antrum, but occasionally (two of five specimens), epithelial cell immunoreactivity in chronic active gastritis was seen. Where intestinal metaplasia was present in the antrum, goblet cells and occasional epithelial cells were positive (six of six specimens; Fig. 6A). Three of four specimens with intestinal metaplasia within Barrett's epithelium of the esophagus had a similar immunoreactivity pattern. There was diffuse cytoplasmic immunoreactivity within individual neoplastic cells in most cases of adenocarcinoma of the esophagus studied (seven of ten). Nine of nine cases of gastric carcinoma studied, including four of four cases of signet cell type, also showed supranuclear but not cell-surface immunoreactivity (Fig. 6B).

Discussion

In this study, experimental gastric injury in the Wistar rat produced an abnormal repair phenotype characterized by persistent induction of the trefoil peptides SP and ITF. Glands responsible for this expression were composed of morphologically undifferentiated cells. The single injury was subsequently found to produce true metaplastic changes in the gastric antrum, with histologic evidence of gastric atrophy, goblet cell metaplasia, and severe dysplasia. Evidence of ITF induction was obtained during each of these stages. Similarly, in the human stomach there was evidence of ITF induction in established intestinal metaplasia and expression of ITF was conserved in gastric cancers.

After the formation of gastric ulcers in rats, the induction of SP expression (appropriate to site) and ITF expression (inappropriate to site) denoted a sustained aberrant phenotype, initially recognized in the ulcer margins and subsequently in the ulcer scar. This abnormal phenotype consisted of elongated and branching glands lined by a columnar, acid sulfomucin-secreting epithelium. The lineage was hyperproliferative, with a shift in the proliferative region to the gland base. ITF and SP mRNA and peptide expression seemed to occur at all levels of the aberrant glands. The induction of ITF was not associated

with evidence of cellular specialization, such as goblet cells or a brush border.

In this model, the earliest morphologic evidence of increased trefoil synthesis occurred between 4 and 12 days after injury, corresponding to the rise in trefoil levels by RIA and the detection of SP and ITF mRNA transcripts in the epithelium. Increased immunoreactivity was seen in the emerging epithelium in the cleft between the ulcer and adjoining normal glands, also described as a "transitional region" (Tarnawski et al, 1990a), and comprised predominantly of intracellular, granular Golgi-region ITF and apical SP immunoreactivity. Some surface immunoreactivity was seen by day 4, consistent with secretion, and secretion into secondary lumina could also be seen. The presence of peptide in Golgi region and cell surface, rather than the apical compartment, suggests rapid secretion from these cells at this time. Secreted peptide within secondary lumina indicate the presence of basolateral secretion.

The magnitude of the rise in ITF immunoreactivity seen in this model of gastric injury was not observed in the colon. The modest (2-fold) rise in expression in the latter model may reflect increased, and possibly constitutive, expression by a newer population of cells in these regions. Cell proliferation was not directly assessed in the colonic injury model to confirm this. Substantial rises in ITF levels may have been difficult to detect in these tissue samples, because the major determinant is the number of goblet cells in the sample. Some of the architectural changes that accompany gastric injury were also present after colonic injury; namely, cystic dilation and secondary lumen formation. Nevertheless, the less exuberant regeneration of colonic mucosa suggests a fundamentally different response to injury.

Expression of SP and ITF mRNAs in the first 10 days after cryoprobe-induced gastric ulceration in rats has been reported (Alison et al, 1995). Healing rates were similar to those of the acetic acid model. A modest induction of SP mRNA, assessed by Northern blotting, occurred 30 minutes after injury, whereas substantial increases in ITF mRNA were detectable 2 days after ulcer induction and could be seen in discrete mucosal cells associated with regenerating glands at the ulcer margin. No changes in nearby body mucosa were

Figure 6.

ITF expression in intestinal metaplasia of the stomach and in gastric carcinoma. A, Cross-section of intestinal type gland, showing human ITF immunoreactivity in cytoplasm of goblet cells (white arrow). B, Gastric carcinoma, showing intense supranuclear ITF immunoreactivity (black arrow). Original magnification, $\times 200$.

seen. Our results do not show increased trefoil protein until at least 4 days after injury with a steady rise in SP and an exponential rise in ITF expression thereafter. Furthermore, we found evidence of a field effect, with tissue distant from the ulcer zone (region 3) expressing metaplastic ITF peptide and moderately increased SP peptide. However, this was only detectable at least 6 weeks after injury, whereas glands adjoining areas of ulceration (region 2) also underwent stereotyped morphologic changes of cystic dilation and gland branching and elongation, associated with de-differentiation of epithelial cells. We did not assess whether these histologic changes were present in remote (region 3) epithelium, although this would be of interest. A similar phenomenon was seen in expression of ITF after colonic injury. Dispersal of secreted peptide from the injury site cannot account for this effect, as peak levels in region 1 were apparent several weeks before those seen in region 3.

This field effect, therefore, suggests the influence of a factor expressed by the regenerating mucosa acting at a distance from the cell of origin. Trefoil peptides themselves may be responsible. Although there is some work supporting the existence of SP (Chinery and Cox, 1995; Frandsen, 1988; Frandsen et al, 1986) and ITF (Chinery et al, 1993) receptors, these entities have not been well characterized or cloned. Nevertheless, self- and cross-induction of trefoil expression has been shown in a model gastric cell line (Taupin et al, 1999), and is supported by a reduction in gastric SP expression both in mice lacking ITF (Taupin et al, 1999) and pS2 (Lefebvre et al, 1996). Classic growth factors should also be considered. Notably, EGFR activation induces pS2 expression in a breast cancer cell line (Nunez et al, 1989) and hSP expression in a colon cancer cell line (Giraud et al, 1994). An EGF receptor ligand, TGF α , is an epithelial mitogen and in the stomach is expressed in the superficial, nonproliferative compartment, consistent with a role also in epithelial differentiation (Beauchamp et al, 1989). Transgenic mice overexpressing human TGF α develop a range of hyperproliferative lesions (Jhappan et al, 1990). The stomachs of these mice show an increase in mucosal thickness caused by an expanded compartment of PAS-positive cells resembling mucous neck cells and expressing SP, but with loss of chief cells and parietal cells (Sharp et al, 1995). Similarly, TGF α treatment of cultured mouse keratinocytes blocks calcium-dependent terminal differentiation, an effect that mimics that of expression of oncogenic ras^Ha (Denning et al, 1996). The elongated, hyperproliferative glands composed of relatively undifferentiated cells seen after acetic acid injury to the rat antrum may reflect similar events.

The cells comprising this regenerative phenotype, although resembling mucous neck cells and overexpressing SP, show marked re-expression of the putative goblet cell marker ITF and express acid sulfomucins, and are capable of differentiation into an intestinal phenotype without further external initiation. We propose that the abnormal repair phenotype consists of multipotent undifferentiated cells rather than

cells committed to mucous neck cell differentiation. The mitogen(s) responsible for induction of this phenotype were not identified, although an early peak in ulcer-region TGF α expression together with the previous demonstration of EGFR upregulation in similar models (Hansson et al, 1990; Konturek et al, 1988; Tarnawski et al, 1992), as noted, suggests a role for TGF α . Although modest, the approximately 2-fold elevation in immunoreactive TGF α peptide 8 hours after acetic-acid injury to the gastric antrum is similar to that seen after orogastric administration of HCl (Polk et al, 1992), and in regenerating rat liver after partial hepatectomy (Russell et al, 1993). An inductive role for TGF α is supported by the partial abolition of gastric ITF re-expression after induced injury in TGF α null mice (Cook et al, 1997).

The model of gastric injury used here clearly represents an exaggerated response to injury, but one that is nevertheless applicable to human disease. The prominent histologic abnormalities that persist after experimental chronic gastric injury in the rat are well recognized (Seven et al, 1993; Tarnawski et al, 1990a; Taylor et al, 1991). For example, gastric carcinoma in the Wistar rat may be readily induced by gastroenterostomy (Mason, 1986). These adenocarcinomas are accompanied by adenocystic proliferation and increased sulfomucin production; less frequently, glandular atrophy and intestinal metaplasia are seen (Taylor et al, 1991). Rates of carcinoma induction vary greatly in methodologically similar studies, possibly because of strain differences (Seven et al, 1993; Tominaga et al, 1994). Ulcer induction similarly may give rise to varying responses. In one study, animals were observed for up to 300 days after acetic acid injury, with no apparent development of carcinoma (Tominaga et al, 1994). In mice, acetic acid injury led to a sustained rise in ITF expression without apparent metaplastic or neoplastic change (Cook et al, 1997).

It has been proposed that, because 'initial' gastric cancers occupy the neck region only, and the proliferative region of wholly intestinal glands lies at the gland base, malignant differentiation proceeds independently from intestinal differentiation (Hattori, 1986). This theory is supported by the current study. Gland morphology was suggestive of, but not diagnostic of, intestinalization as late as 90 days after injury; at the time typical intestinal metaplasia was recognized, severe dysplasia had also developed.

We studied the metaplastic and neoplastic human stomach to test the generalization that trefoil peptide induction is a form of re-expression of early rather than terminal differentiation markers. Because SP and ITF mark the same regenerative lineage in rats, we assessed human pS2 expression, which marks the foveolar cells in the normal human stomach. Human gastric pS2, predominating as a 14-kd dimeric form, was present at levels of approximately 20 μ g/mg of protein, compared with the approximately 11 ng/mg of protein found in breast cancer cytosols (Foekens et al, 1990). No induction of gastric pS2 expression was observed in metaplasia or cancer; rather, a loss of expression in metaplasia and a further reduction in

gastric cancer was observed despite the apparent ability of the neoplastic epithelium to express ITF. These observations are consistent with a recent report, which has shown that somatic mutations occur in the human pS2 gene in gastric cancer, and that pS2 gene expression is lost in 44% of cases examined (Park et al, 2000), suggesting a possible role for this gene in tumor suppression.

In contrast, ITF was elevated approximately 4-fold in biopsies exhibiting intestinal metaplasia; with levels always > 25 pmol/mg of protein. Biopsies obtained for histology were from adjacent rather than identical gastric sites, and because mild gastritis and intestinal metaplasia are patchy in distribution, sampling errors may account for the apparent wide range of ITF values in normal samples and those with gastritis. ITF immunoreactivity resided in goblet cells, endocrine cells, and occasional epithelial cells in intestinalized glands, and not in adjacent gastric epithelial cells. Intestinal metaplasia within Barrett's epithelium of the esophagus showed a similar staining distribution. Importantly, cytoplasmic immunoreactivity within individual neoplastic cells was seen in most cases of adenocarcinoma of the esophagus studied and in all cases of gastric carcinoma studied, including signet-cell type. In the human colon, ITF is also strongly expressed in neoplastic epithelial cells lacking goblet cell features (Taupin et al, 1996). It is therefore possible that ITF is expressed by gastrointestinal stem cells and then transcriptionally down-regulated in nongoblet cells in the normal adult gastrointestinal tract.

The mechanisms of development of metaplastic phenotypes after experimental and clinical gastric injury and subsequent tumor development are unclear at this time, but some of the factors responsible are being elucidated. Marked elevations of SP and particularly ITF seem to predict this behavior, as does the decrease in pS2 expression. The identification of elements regulating trefoil peptide transcription may therefore allow insight into the mechanisms of intestinal metaplasia and gastric tumorigenesis.

Materials and Methods

Plasmids and Peptides

Recombinant rat ITF was a gift from Dr. L. Thim (Novo Nordisk, Denmark). Recombinant rat TGF α and sheep anti-rat TGF α were gifts from Dr. R. Coffey (Vanderbilt University, Nashville, Tennessee). Rat ITF cDNA in pBluescript was a gift from Dr. D. K. Podolsky (Massachusetts General Hospital, Boston, Massachusetts). Rat SP cDNA in pBluescript was a gift from Dr. G. Jeffrey (University of Western Australia). The C-terminal human pS2 decapeptide was synthesized by Auspep (Melbourne, Australia).

Gastric and Colonic Ulcer Induction in Rats

Gastric ulcers were induced by acetic acid according to published methods (Okabe et al, 1971; Tarnawski et al, 1990b). Briefly, nonfasted, male Hooded Wistar rats ($n = 5$ to 8 per group, 250 to 300 g) were anesthetized

and 50 μ l of 100% acetic acid was applied through a 6-mm internal diameter plastic mold to the serosal surface of the junction of antrum and fundus for 25 seconds. At 2 hours, 4 hours, 8 hours, 2 days, 4 days, 12 days, 24 days, and over 6 weeks after injury, the animals were killed and the stomach removed, dissected along the greater curvature and gently rinsed with saline. Ulcer area was calculated by point counting. Tissue was either frozen in liquid nitrogen for RIA or fixed in neutral buffered formalin then embedded in paraffin. Tissue for RIA was obtained from three regions of the full thickness of the stomach. Region 1 comprised the ulcer center and margins and within 5 mm from the ulcer margins. Region 2 extended from 5 to 15 mm from the ulcer margins, but excluded gastric tissue within 10 mm of the pylorus or tissue proximal to the superior margin of region 1. Region 3 corresponded to region 1, but on the opposite (posterior, and uninvolved) antral wall. Control animals ($n = 12$), had laparotomy without acetic acid treatment. Tissues corresponding to regions 1 and 3 were dissected. After preliminary results were assessed, a further three animals had acetic acid injury to the antro-fundic junction at day 30 of life and were then housed as above for 32 to 33 weeks before being killed.

For colonic ulcer induction, nonfasted male Wistar rats ($n = 36$), 250 to 300g, were anesthetized, and 25 μ L of 100% acetic acid was applied for 15 seconds via a 6-mm internal diameter plastic mold to the anterior serosa of the cecum ($n = 30$). At 24 hours and 4, 12, 24, 100, and 120 days after injury, tissue was dissected from the ulcer margins and an area 3 cm cephalad as control. Additionally, a separate control group of rats ($n = 6$) had laparotomy only, and tissues were harvested 24 days later.

Animal experimentation and the procurement of animal tissues was conducted under guidelines of the National Health and Medical Research Council of Australia, and approved by the Animal Ethics Committee of the Royal Melbourne and Western Hospitals.

Human Gastric Biopsies

Endoscopic pinch biopsies from 20 patients undergoing diagnostic endoscopy for upper gastrointestinal symptoms were obtained and immediately frozen in liquid nitrogen, and 10 surgical specimens from 8 patients who underwent gastric and/or distal esophageal resection for gastric cancer were also obtained. Contemporary histology was obtained on all of these specimens for classification. Samples (25 to 50 μ g) were extracted for RIA or immunoblotting and assayed in duplicate. For immunohistochemistry, archived, endoscopic biopsies from 64 patients were retrieved, representing normal gastric antrum ($n = 20$), normal duodenum ($n = 5$) and colon ($n = 5$), active chronic antral gastritis ($n = 5$), intestinal metaplasia of the stomach ($n = 6$), gastric cancer ($n = 9$), and Barrett's epithelium of the esophagus, with ($n = 10$) or without ($n = 4$) carcinoma. The procurement of human tissues was approved by the Ethics Committee at Western Hospital.

Characterization of pS2 from Endoscopic Antral Biopsies

Antibody to pS2 was raised in rabbits against a synthetic peptide representing the C-terminal 10 amino acids of human pS2. Gel filtration chromatography (G-50 Sephadex) was performed on extracts of antral biopsies from four patients, representing 150 mg of biopsy material. Protein was eluted in 0.025 M phosphate buffer and fractions (1.0 ml) were assayed by RIA. The peaks obtained from each sample were pooled, lyophilized, and desalted on Sephadex PD-10. A single peak was obtained yielding approximately 31 pmol of immunoreactive pS2. This was separated by SDS/PAGE followed by electrophoretic transfer onto nitrocellulose or Coomassie R250 staining. Purity was also assessed by reverse-phase high-pressure liquid chromatography (HPLC, Brownlee C18, 4 × 750 mm) with 0% to 70% acetonitrile gradient.

Trefoil Peptide and TGF α Radioimmunoassay

Tissue extraction and ITF and SP RIA conditions were as previously described (Taupin et al, 1995). Samples were assayed in duplicate; results were the means of at least three assays. For human pS2 RIA, samples (50 to 100 μ L) were diluted in assay buffer (ammonium acetate 0.05 M, bovine serum albumin 0.1%, pH 6.8) containing polyclonal anti-human pS2 at 1:60,000 dilution and approximately 5000 cpm of 125 I-pS2 decapeptide. After overnight incubation at 4° C, 300 μ L of goat-anti rabbit IgG, diluted 1:60,000 in 3.3% polyethyleneglycol and 6% normal rabbit serum was added. Overnight incubation at 4° C was followed by precipitation of unbound antibody. Assays were counted against a standard curve of 1.9 to 10000 fmol of pS2 C-terminal decapeptide standard. The ED₅₀ (concentration of standard causing 50% inhibition of binding) for these assays was 700 fmol/tube. The cross-reactivity in assay against rITF C-terminal decapeptide or recombinant rat ITF was less than 0.5% in the pS2 radioimmunoassay.

For TGF α RIA, recombinant rat TGF α was iodinated and the tracer purified on Sephadex G-25. Samples were homogenized in 2 ml of assay buffer containing protease inhibitors (phenylmethylsulfonyl fluoride 1 mM, EDTA 1 mM, Leupeptin 10 μ g/ml, Pepstatin A 10 μ g/ml, Aprotinin 1 μ g/ml, Des-pro-bradykinin 10 μ g/ml, and DI-Thiorphan 10 μ g/ml in 0.05 M ammonium acetate, pH 6.8; all Sigma Chemical Company, St Louis, Missouri) at 4° C. Extracts (100 μ L) were diluted in 1.0 ml of assay buffer containing sheep anti-rat TGF α (1:500,000). This antibody recognizes both the mature 5.6 kd form and precursor forms (Russell et al, 1993). Approximately 5000 cpm 125 I-TGF α was added and samples were incubated for 24 hours at 4° C. PBS (300 μ L) containing normal sheep serum at 1:1800, donkey anti-sheep at 1:180 and 3.3% polyethylene glycol was added. After an additional 24 hours at 4° C, samples were centrifuged and precipitates were counted on a gamma-counter against a linear-logarithmic standard curve calibrated with 0.98 to 1000 fmol/tube recombinant rat TGF α as standard.

Immunohistochemistry and In Situ Hybridization

Mucin histochemistry was performed on deparaffinized sections according to standard protocols (Culling, 1974); PAS staining for neutral mucins, Alcian blue at pH 2.5 for acidic sialomucins, and high-iron diamine for acid sulfomucins. Immunohistochemistry of trefoil peptides was performed on rat and human samples as previously described (Taupin et al, 1995, 1996). For PCNA immunohistochemistry, deparaffinized 4 μ m sections were heated in a microwave oven in sodium citrate buffer, 0.1 M, pH 6.0, to retrieve antigen. Sections were blocked with normal goat serum (1:20) for 20 minutes and incubated with monoclonal anti-PCNA (Novocastra Laboratories, Newcastle-on-Tyne, United Kingdom) at 1:50 dilution overnight at 4° C, incubated with biotinylated horse anti-mouse, then avidin-biotin (Vectastain, Vector Laboratories, Peterborough, United Kingdom). Color was developed with diaminobenzidine tetrahydrochloride (Sigma), 0.5 mg/ml in 0.01% H₂O₂.

For ITF and SP hybridization in situ, deparaffinized 4 μ m sections of rat stomach were rehydrated, post-fixed in 4% paraformaldehyde in PBS, permeabilized with proteinase K, acetylated and then dehydrated in the presence of 0.4 M ammonium acetate. Rat ITF cDNA and rat SP cDNA were linearized, and antisense riboprobes were transcribed in the presence of 32 P using T7 polymerase. Sense control probes were transcribed with SP6 polymerase (both Promega, Madison Wisconsin). Hybridization was in 50% formamide buffer overnight at 55° C, followed by ribonuclease-A treatment. Final washes were in 0.1x to 0.5x SSC at 55° C. Slides were dehydrated and coated with NTB-2 emulsion (Kodak, Rochester, New York), with exposure for 10 to 14 days at 4° C.

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