Expression of cholecystokinin in the duodenum of patients with coeliac disease: respective role of atrophy and lymphocytic infiltration

Pierre H. DEPREZ*, Christine SEMPOUX†, Christine DE SAEGER*, Jacques RAHIER†, Paul MAINGUET*, Stanislas PAUWELS‡ and André GEUBEL*

*Department of Gastroenterology, Cliniques Universitaires St-Luc, Catholic University of Louvain, Ave Hippocrate 10, B-1200 Brussels, Belgium, †Department of Pathology, Cliniques Universitaires St-Luc, Catholic University of Louvain, Ave Hippocrate 10, B-1200 Brussels, Belgium, and ‡Department of Nuclear Medicine, Cliniques Universitaires St-Luc, Catholic University of Louvain, Ave Hippocrate 10, B-1200 Brussels, Belgium

ABSTRACT

Cholecystokinin (CCK) release after a standard test meal is decreased in coeliac patients. The aim of the study was to determine the origin of the CCK deficiency and the relationship between the distinctive types of mucosal lesions observed in coeliac disease and the number of duodenal CCK cells and their peptide and mRNA content. Duodenal biopsies were obtained in ten controls and nineteen coeliac patients [seven with atrophic mucosa, six with increased numbers of intraepithelial lymphocytes (IELs) and six with fully normalized mucosa]. Immunocytochemistry was performed with a CCK C-terminal-specific polyclonal antiserum. The CCK cells were counted and related to the epithelial area using a semi-automated image analyser. CCK content in mucosal extracts was determined by radioimmunoassay. mRNA was measured with a semi-quantitative reverse transcriptase–PCR method using specific CCK and ribosomal protein L19 (RPL19) primers. CCK tissue concentration and CCK mRNA were significantly reduced in patients with atrophic mucosa [12.2 (range 6.9–17.5) pmol/g; CCK/RPL19 ratio 0.64 (0.30–0.99)] compared with patients with normal mucosa [40.5 (30.4–50.7) pmol/g; CCK/RPL19 ratio 1.40 (0.41–2.40)] or controls [42.7 (18.2–67.2) pmol/g; CCK/RPL19 ratio 1.35 (1.09–1.62)]. A similar decrease was observed in patients with an excess of IELs, 13.9 (3.8–31.8) pmol/g and 0.86 (0.57–1.15) pmol/g respectively. The number of CCK cells was, however, similar in all groups. Duodenal CCK concentration and mRNA are decreased not only in the mucosa presenting atrophic changes but also when disease activity is limited to infiltration by IELs. Reduced expression of the CCK gene could therefore be related to suppressive factors induced by the inflammatory infiltrate.

INTRODUCTION

Coeliac disease is associated with a reduced circulating concentration of cholecystokinin (CCK), which accounts for the diminished gallbladder contraction and pancreatic function observed in patients with a flat mucosa [1–8]. Appropriate CCK extraction methods and radioimmunoassays confirmed that this was related to a defective release rather than to a lack of end-organ responsiveness to cholecystokinin [9–12]. A gluten-free diet was also demonstrated to reverse the defective CCK release [13]. The mechanism by which CCK release is...
impaired in coeliac disease is, however, still poorly understood. Lower intestinal concentrations of CCK have been reported in coeliac atrophic mucosa by means of CCK extraction and radioimmunoassay [6,14]. These observations are contrary to immunocytochemistry reports showing an increased number of CCK cells in the coeliac mucosa [15,16]. Impaired release could also be explained by decreased stimulation of the mucosal CCK cells due to impaired intraduodenal hydrolysis of nutrients observed with mucosal atrophy [17].

Coeliac sprue was defined more than 30 years ago in terms of the flat mucosal lesion, usually associated with a malabsorption syndrome that responds to a gluten-free diet [2,3]. Recently, more refined descriptions of gluten-induced mucosal changes have been given that might be termed the spectrum of gluten sensitivity. At least five distinct types of mucosal lesions have been recognized and have been called pre-infiltrative, infiltrative, hyperplastic, destructive and hypoplastic (flat) [18]. Even with histological changes to a lesser degree, some functional abnormalities have been reported, e.g. increased permeability [19,20].

The aim of our work was to determine the origin of CCK deficiency and to study the relationship between the distinctive mucosal patterns observed in coeliac disease and the mucosal CCK content, and specifically to determine if CCK deficiency was due to a decrease in the number of CCK cells, a decrease in peptide concentration or a decrease in CCK mRNA content.

**METHODS**

**Patients**

Biopsies (n = 6) were obtained from the distal duodenum, just proximal to the angle of Treitz, during upper gastrointestinal endoscopy in ten controls and 19 coeliac patients (five men, 14 women). Coeliac patients were divided into three groups: untreated patients presenting with atrophic mucosa (group A, n = 7), patients on a gluten-free diet with infiltration by IELs but no architectural changes of the mucosa (group B, n = 6), and patients on a gluten-free diet with a fully normalized mucosa (group C, n = 6). Diagnosis of coeliac disease was made using duodenoscopy and duodenal biopsies. Endoscopic signs of coeliac disease included loss or reduced number of duodenal folds, scalloped duodenal folds and mosaic pattern [21,22]. Histological criteria suggestive of coeliac disease were: flat mucosa with injured surface epithelium, elongated crypts with mitosis, increased number of intraepithelial lymphocytes (IELs) and chronic inflammation in lamina propria [18]. IEL count was performed on a Leitz microscope at ×40 magnification on haematoxylin/eosin stained slides. The number of IELs was evaluated on 100 villous epithelial cells and expressed as a percentage. A value below 30 IELs/100 villous epithelial cells was considered normal [23–25]. We also performed morphometric studies, including a count of villous-crypt units, measurement of the respective length of the villi and the crypts with the determination of a villous/crypt length ratio, and measurement of the epithelial surface per villous-crypt unit.

Control subjects (three men, seven women) underwent upper gastrointestinal endoscopy for evaluation of dyspeptic complaints; their gastric or duodenal mucosa showed a normal endoscopic appearance and no histological changes. They were matched for age (41 ± 4 years) with coeliac patients. The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and was approved by the Ethics Committee of the Faculty of Medicine. Informed written consent was obtained for each subject or patient.

**Immunocytochemistry**

Double immunocytochemical staining was performed to specifically recognize the CCK cells. A polyclonal antibody raised against the N-terminal gastrin 34 (LGW21) was used at a 1:4000 dilution, and revealed using a streptavidin-biotin-peroxidase system and 3,3'-diaminobenzidine staining (brown stain) [26]. After elution, the same slides were incubated with a polyclonal antibody raised against the C-terminal CCK-10 (Dino) at a 1:10000 dilution and revealed by an alkaline phosphate anti-alkaline phosphate system (red stain) [27,28]. This antibody, although highly specific for CCK in radioimmunoassays, had a low affinity for gastrin cells in immunocytochemistry. After exclusion of the brown stained cells, the red stained cells were considered to be specifically CCK cells and were counted. The number of CCK-positive cells was determined per villous-crypt unit. Their number was also related to the epithelial area of each biopsy. This epithelial area was measured on adjacent tissue sections immunostained brown with an anti-human cytokeratin monoclonal antibody (Clone CAM 5-2; 1:25; Becton Dickinson, Erembodegem, Belgium) using a semi-automatic image analyser (IBAS 2000-Kontron, Munich, Germany).

**Tissue extraction and radioimmunoassay**

CCK was extracted from intestinal biopsies in acid [trifluoroacetic acid (TFA) 2% (w/v)] and boiling water (one biopsy in 2 ml) for 5 min. After homogenization and centrifugation [10 min, 2000 g (10000 rev./min)], the supernatant was decanted. Further extraction and concentration of CCK were immediately performed by adsorption on Sep-Pak C18 cartridges (Waters Millipore, Harrow, Middx., U.K.) previously washed with 10 ml of acetonitrile, 10 ml of water and 10 ml of 0.1% TFA [28]. CCK was eluted with 3 ml of acetonitrile in 0.1% TFA.
Semiquantitative reverse transcriptase (RT)–PCR

Total RNA was prepared from duodenal biopsies (47–195 mg) using the guanidine thiocyanate and cesium chloride method [29]. Duodenal RNAs (5 μg) were preincubated with random hexamer and water for 10 min at 70 °C. Moloney murine leukaemia virus RT (400 units; Gibco BRL, Merelbeke, Belgium) was added together with diithothreitol and dNTPs, and the reaction was continued for further 60 min at 37 °C. PCR was carried out with primers specific for human CCK and ribosomal protein L19 (RPL19) as an internal standard [30]. PCR was performed in two steps to ensure that both CCK (5 cycles) and RPL19 (25 cycles) were in the linear range for amplification. The first 5-cycle PCR step (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) was performed using only the CCK primers in 30 μl of a solution containing 3 μl of 1× PCR buffer, 30 pmol each of 5′- and 3′-CCK primers, 6.4 μl of dNTPs, 0.4 μl of deoxy[32P]CTP (1.25 μCi; Amersham) and 1.25 units of Taq polymerase (Boehringer Mannheim, Brussels, Belgium). The reaction was kept at 25 °C and was followed by a second 25-cycle PCR (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) after the addition of 50 μl of a solution containing 1× PCR buffer, 30 pmol each of 5′- and 3′-RPL19 primers, 1.1 mM of MgCl₂, and 1.25 units of Taq polymerase. DNA contamination of RNA was excluded by the absence of product in the reaction performed without RT. The following primers were used: CCK sense, GTATCGCAGAGAACGGAT; antisense, TACTCATACTCCTCGGCACT (size of product 203 bp); RPL19 sense, AGTATGGCTAGGGCTTCA-GAA; antisense, TTCTCTGTGCTTAGACCCGTG (size of the product 501 bp). PCR products were separated by electrophoresis using a 1.5 % (w/v) agarose gel. The gels were vacuum-dried (LKB 2030, Slab gel dryer; LKB-Pharmacia, Uppsala, Sweden) and exposed to X-ray film (Kodak Biomax MS, Boston, MA, U.S.A.) for 1 h at room temperature (25 °C). The PCR bands were quantified by computer-assisted densitometric scanning (Ultrascan XL LKB Bromma and Gelscan PC; LKB Pharmacia). Individual PCR reactions were calculated as the ratio of CCK to RPL19 and the ratio was averaged across four dilutions (1:1, 1:2.5, 1:5 and 1:10) from each sample [30]. Experiments were performed in duplicate.

Statistical analysis

Data were analysed on a logarithmic scale and reported as geometric means and 95% confidence intervals. The number of CCK cells was expressed as the ratio of cells/mm² of epithelial area; duodenal extract CCK concentrations as CCK immunoreactivity in pmol/g of tissue, and CCK mRNA as the ratio of CCK to RPL19. Groups were compared using ANOVA with F-tests, and post-hoc tests were performed when significant, using the Bonferroni method. A value of P < 0.05 was considered statistically significant.

RESULTS

Patients

Patients’ age, body mass index and biological findings are shown in Table 1. Typical endoscopic features of disease, atrophic mucosa and endomysium antibodies were characteristic of the untreated group of patients (group A). The villous/crypt length ratio was 0.7 (range 0.4–1.2) compared with 2.9 (2.2–3.4) for the control group, confirming the significantly altered duodenal mucosal morphology, with loss of villi and elongation of crypts (P < 0.01). The epithelial surface per villous-crypt unit [0.029 mm² (0.021–0.038)] was significantly decreased compared with controls [0.048 mm² (0.034–0.058), P < 0.05]. These patients had a significantly lower body mass index (P < 0.05) and lower serum concentration of β-carotene, iron and vitamin D, compared with patients from groups B and C, which reflected malabsorption. Patients from group B (coeliac patients on a gluten-free diet with a fully normal architecture but an inflammatory infiltrate) and group C (coeliac patients on a gluten-free diet with a fully normal duodenal mucosa) had similar general characteristics. The morphometric studies confirmed that the mucosal architecture had returned to normal; the villous/crypt ratio was comparable with controls as well as the epithelial surface per villous-crypt unit (Table 1). The following differences were observed between group B and group C: a significantly higher number of IELs (P < 0.05) in group B, a low serum iron concentration and a more frequent presence of endomysium antibodies in group B.

Immunocytochemistry

The results of cell quantification are shown in Table 2. The CCK-positive cell count was higher than the...
Table 1  Coeliac patient characteristics

Group A refers to untreated patients with atrophic mucosa, group B to patients on a gluten-free diet with an infiltrative pattern, and group C to patients on a gluten-free diet without infiltrative or destructive histological lesions. Results are expressed as means and 95% confidence intervals (in parentheses). Groups were compared using one-way ANOVA. Post-hoc tests were performed when significant ($P < 0.05$), using the Bonferroni method. Comparison between groups: **, group C versus group B; †, group A versus group C. ND, not determined.

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n)</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>39 (33–44)</td>
<td>42 (36–48)</td>
<td>45 (35–55)</td>
<td>0.181</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>19 (18–20)</td>
<td>24 (21–27)**</td>
<td>23 (20–26)†</td>
<td>0.005</td>
</tr>
<tr>
<td>Flat destructive mucosa</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>ND</td>
</tr>
<tr>
<td>Villous/crypt length ratio</td>
<td>0.7 (0.4–1.2)</td>
<td>1.9 (1.5–2.3)</td>
<td>2.5 (1.8–3.1)†</td>
<td>0.01</td>
</tr>
<tr>
<td>Epithelial surface area (mm²)/villous-crypt unit</td>
<td>0.029 (0.021–0.038)</td>
<td>0.040 (0.029–0.051)</td>
<td>0.037 (0.032–0.043)†</td>
<td>0.2</td>
</tr>
<tr>
<td>IEL count (IELs/100 enterocytes)</td>
<td>43 (33–55)</td>
<td>35 (34–36)</td>
<td>21 (18–25)**†</td>
<td>0.0001</td>
</tr>
<tr>
<td>β-Carotene (µmol/l)</td>
<td>0.7 (0.3–1.1)</td>
<td>1.7 (0.7–3.7)*</td>
<td>3.1 (2.3–4.2)†</td>
<td>0.001</td>
</tr>
<tr>
<td>Vitamin D (µmol/l)</td>
<td>43 (25–75)</td>
<td>82 (63–106)</td>
<td>129 (74–223)†</td>
<td>0.004</td>
</tr>
<tr>
<td>Iron (µmol/l)</td>
<td>6.3 (3.4–16.7)</td>
<td>13.2 (12.4–14)</td>
<td>20.9 (17.8–24.6)†</td>
<td>0.02</td>
</tr>
<tr>
<td>Endomysium antibodies</td>
<td>Positive 6/7</td>
<td>Positive 3/6</td>
<td>Positive 0/6</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 2  CCK cell quantification

Group A refers to untreated patients with atrophy, group B to patients on a gluten-free diet with an infiltrative pattern, and group C to treated patients with a normalized mucosa without histological lesions. Results are expressed as means and 95% confidence intervals.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>F-test P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n)</td>
<td>10</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>CCK-positive cells/epithelial surface ratio</td>
<td>12.1 (2.6–21.4)</td>
<td>8.7 (2.1–19.4)</td>
<td>9.3 (1.6–17.1)</td>
<td>10.8 (5.2–16.4)</td>
<td>0.96</td>
</tr>
<tr>
<td>CCK-positive cells/villous-crypt unit</td>
<td>0.49 (0.19–0.63)</td>
<td>0.39 (0.28–0.47)</td>
<td>0.45 (0.32–0.56)</td>
<td>0.46 (0.33–0.58)</td>
<td>0.21</td>
</tr>
<tr>
<td>[CCK] neutral extracts (µmol/g)</td>
<td>42.7 (18.2–67.2)</td>
<td>12.2 (6.9–17.5)</td>
<td>13.9 (8.8–31.8)</td>
<td>40.5 (30.4–50.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>[CCK] acid extracts (µmol/g)</td>
<td>12.3 (6.4–19.5)</td>
<td>6.4 (1.9–12.1)</td>
<td>7.8 (2.2–14.5)</td>
<td>11.3 (5.7–22.3)</td>
<td>0.04</td>
</tr>
<tr>
<td>CCK mRNA (CCK/RPL19 ratio)</td>
<td>1.35 (1.09–1.62)</td>
<td>0.64 (0.30–0.99)</td>
<td>0.86 (0.57–1.15)</td>
<td>1.40 (0.41–2.40)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Figure 1  Immunocytochemical staining for CCK cells (alkaline-phosphatase stained red) in a duodenal biopsy from a coeliac patient with atrophic mucosa (magnification × 520).

A non-significant decrease in the number of CCK cells, in the epithelial surface and in the ratio of CCK cells to epithelial surface was observed in group A (patients with atrophic mucosa). When CCK-positive cells were expressed per villous-crypt unit, a trend towards a decreased number of CCK-positive cells was observed without reaching statistical significance. When the individual patient’s characteristics were analysed, we observed some distinctive patterns: two patients (group A) showed an absence of CCK cells and two other patients (group A) showed a very high CCK cell count (40 and 67) and higher ratios of CCK cells to epithelial surface (15.6 and 39.9). No relationship or correlation could be seen between these results and those obtained from tissue extraction or RT–PCR. These discrepancies were not observed in the other groups.

Tissue extraction and radioimmunoassay

The results are shown in Table 2. Between-group comparison showed a significant decrease in CCK concentration in patients from group A with atrophic changes ($P = 0.006$), but surprisingly also in patients from group B with an infiltrative pattern ($P = 0.01$).
DISCUSSION

The present studies provide evidence that the defective release of CCK, observed in patients with coeliac disease, is not related to a decrease in the number of CCK cells present in the proximal part of the small intestine, but rather to a decrease in CCK synthesis mediated by a decrease in mRNA content. Previous results in the literature are at variance with this finding with reports of increased CCK cell counts in coeliac mucosa [15,16,31] and decreased amounts of CCK in duodenal extracts [6,14]. These discrepancies could be related to the site where the pathological specimen was taken (duodenum, Treitz angle or proximal jejunum), to methodological difficulties in counting endocrine cells in atrophic or non-atrophic mucosa, to the method used to relate the CCK-positive cells to the epithelial surface area, to the various antisera used (recognition of smaller and larger CCK forms), to differences in the degree of mucosal lesions, or to the type of coeliac patients studied (on a normal diet or a gluten-free diet). The size of the cells could also account for some discrepancy, since CCK cells were shown to be more numerous but smaller in one report [31] and larger in another study [15]. We studied 19 patients, divided into three groups according to the severity of their mucosal lesion, and we could not show any significant differences in the number of CCK cells in specimens taken from the distal duodenum. A nonsignificant trend towards fewer CCK-expressing cells was observed in our group of patients with mucosal atrophy. This trend was observed when CCK-positive cells were expressed per surface ratio and per villous-crypt unit. A similar trend was not observed, however, in the two other groups of coeliac patients (fully recovered mucosa and mucosa with raised IELs). Some individual differences could be seen, particularly in the group of patients with atrophic mucosa, either with an absence of CCK cells or with a larger number of cells. These results were not related to the degree of atrophy and could be explained by sampling differences, since coeliac disease is said to be patchy.

Decreased amounts of CCK in the duodenal mucosa of coeliac patients were observed both in neutral and acidic extracts. Our CCK antiserum was able to recognize all molecular forms of CCK with a comparable affinity [27]. The similar decrease in CCK content in neutral and acidic extracts would confirm that all molecular forms are affected in coeliac disease, as published previously [6]. The decreased level of CCK in the mucosa of patients on a gluten-free diet (for a mean duration of 3.6 years) showing persistent infiltration of IELs was more surprising. This persistent infiltrate is not uncommon in our patients (> 65%; P. H. Deprez and P. Mainguet, unpublished work) and has been reported previously [32]. It could reflect some low but persistent intake of gluten, since gluten is known to evoke a dose-responsive infiltration of IELs into the epithelium [18,33]. In recent years, it became apparent that gluten sensitivity is associated with a spectrum of mucosal lesions, arbitrarily termed pre-infiltrative, infiltrative-hyperplastic, flat destructive and atrophic-hypoplastic [18,34]. These lesions comprise a dynamically interrelated series of events, culminating in the severe flat-destructive lesion. IEL counts in our group of patients with the infiltrative type of mucosal lesion ranged from 31 to 37 IELs/100 enterocytes (Table 1). Little is known about the functions of IELs and their impact on enterocytes and mucosal endocrine cells. Their localization in coeliac disease matches that of the CCK mucosal cells found in highest numbers in the duodenum and proximal jejunum and scattered evenly in the villi and crypts. Our results could therefore support some inhibitory effect of this epithelial infiltration on CCK secretion. It is tempting to speculate about the activity and interaction of duodenal cytokines on CCK cells. Although there is clear evidence of communication between the immune and neuroendo-
crine systems, very few direct interactions have been studied in the digestive area. Raised pro-inflammatory cytokines detected by immunohistochemistry and molecular biology could provide interesting clues on such a link in coeliac disease [35–38].

A decreased CCK content in the duodenal mucosa could also be explained by paracrine inhibition (somatostatin) or defective stimulation by intraduodenal nutrients. Somatostatin is located in D cells throughout the gastrointestinal tract and is believed to act primarily as a paracrine factor [39]. Coeliac patients have been shown to have higher somatostatin levels than controls and this increase was proposed to cause the higher gallbladder volumes observed in fasting coeliac patients [8]. Immuno-cytological studies have, however, reported normal or decreased somatostatin levels in coeliac patients with a flat mucosa [14,31]. These data do not support a role for somatostatin in impairing CCK secretion.

Defective stimulation of CCK release has been demonstrated in coeliac patients with a destructive pattern [17]. In the presence of an atrophic mucosa, crypts are elongated and most CCK cells reside in the bottom of the crypts where there would be little contact with food [31]. Moreover, administration of pre-digested fat corrected the defective CCK secretion in those patients. However, a similar mechanism has never been shown in patients with mucosal lesions limited to an inflammatory infiltrate. Those patients do not show any clinical or biological sign of malabsorption and their mucosa has no architectural modification that could lead to impaired contact between nutrients and the CCK cells. Our studies therefore suggest a more complex interaction between food, CCK release, CCK duodenal cells and the various patterns of intestinal lesions observed in coeliac disease, including IEL infiltration.

The decrease in duodenal CCK concentrations was associated with decreased mRNA abundance, showing evidence of a coupling between hormonal secretion and gene expression. Although short-term experiments failed to demonstrate an association between CCK secretion and corresponding increases in mRNA [40], dietary stimulation of CCK secretion was reported to be associated with an increase in CCK mRNA levels that was due, at least in part, to an increase in CCK gene transcription [40–42]. A similar coupling between duodenal CCK content and mRNA levels could therefore have been expected in our results, since the patients studied were either untreated coeliac patients or treated patients following a gluten-free diet for a mean of 3.6 years.

In conclusion, our results confirm an impaired upper intestinal endocrine function in coeliac disease. We demonstrated a decrease in duodenal CCK concentrations and a reduction in CCK mRNA transcripts without significant changes in the number of CCK expressing cells, especially in patients with high IEL counts. Reduced expression of the CCK gene could, therefore, be related to suppressive factors induced by the inflammatory infiltrate.

REFERENCES


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