Lysozyme Is Up-regulated in Columnar-lined Barrett’s Mucosa: A Possible Natural Defence Mechanism Against Barrett’s Esophagus-associated Pathogenic Bacteria

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Abstract. Background: Lysozyme is a natural antimicrobial enzyme that is up-regulated in inflammatory diseases of the gastrointestinal tract. Pathogenic microbes have recently been identified in the esophageal mucosa in patients with Barrett’s esophagus (BE). Lysozyme expression was evaluated in biopsies from patients with BE. Materials and Methods: Ninety-seven consecutive esophageal biopsies with columnar-lined Barrett’s mucosa (BM) were investigated: 16 had oxyntic gland-only BM, 19 pyloric gland-only BM and 62, intestinal metaplasia BM. Twenty normal gastric biopsies and 20 normal duodenal biopsies were included as controls. Sections were stained with human lysozyme antiserum. Results: Lysozyme was up-regulated in the neck glands in 94% of the biopsies with oxyntic gland-only BM, in the pyloric gland in 79% of the biopsies with pyloric gland-only BM, and in goblet cells in 65% of the biopsies with intestinal metaplasia BM. Goblet cells with faint lysozyme expression were often found in glands overexpressing lysozyme in mucous secretions in the lumen. When compared to controls, lysozyme was up-regulated in all three BM phenotypes (p<0.05). Conclusion: Lysozyme is up-regulated in BM. It is therefore, believed that lysozyme’s up-regulation might mirror a molecular mechanism of self-defence aimed to safeguard the BM against the hostile pathogenic microbiota present in the esophageal microenvironment in patients with BE.

Following protracted gastric reflux, the normal esophageal squamous cell epithelium may undergo columnar-lined metaplastic transformation, with accessory glands of oxyntic type and/or pyloric type with or without intercalated goblet cells (GC) (1). The phenotype carrying GC, known as specialized epithelium or intestinal metaplasia (IM) (2), is regarded by the American College of Gastroenterology (3) as a prerequisite for the histological diagnosis of Barrett’s esophagus (BE). More recently the British Society of Gastroenterology (BSG) (4) defined BE as a columnar-lined esophagus on biopsies taken from endoscopical areas, suggestive of BE. Thus, the presence of areas of GC is not a sine qua non requirement for the diagnosis of Barrett’s mucosa (BM) for the BSG (4). This new definition has gained acceptance both in Europe (5) and Asia (6).

Patients with gastroesophageal reflux (GER) often receive medication with proton pump inhibitors. The reduction of gastric acid secretion by such inhibitors encourages bacterial growth in BE and hence, increased production of nitrosamines, with secondary epithelial damage. In fact, esophageal biopsies with BM often show signs of ongoing or past mucosal inflammation (7). Recently, it was demonstrated in esophageal biopsies that pathogenic bacteria often proliferate with BE (8-10). Yang et al. (8) classified esophageal microbiomes into two types: type I microbiome is dominated by the genus Streptococcus and is concentrated in the phenotypically-normal esophagus, and type II microbiome contains a greater proportion of Gram-negative anaerobes/microaerophiles, being primarily correlated with esophagitis and BE. Pei et al. (9) reported that residential bacterial populations in GER contained 21 distinct bacterial species. Members of four bacterial phyla were found, including Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria. In a more recent study of esophageal biopsies and aspirates, Mc Farlane et al. (10) found that the mucosa of BE often contain colonies of 46 bacterial species belonging to 16 genera with unique levels of Campylobacter consuis and Campylobacter rectus. Taken together, these microbiological findings indicate a close association between the proliferation of abnormal bacterial flora in the esophageal microenvironment and the presence of columnar-lined esophageal mucosa.
Many years ago, during a deliberate search for medical antibiotics, Alexander Fleming discovered one of the natural defense substances against infection, that he named lysozyme (11). Lysozyme, also known as muramidase or N-acetylmuramidase glycanhydrolase, is part of a family of enzymes (EC 3.2.1.17) which damage bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycans, and between N-acetyl-D-glucosamine residues in chitodextrins (12). Lysozyme, encoded by the LYZ gene (13) is regarded as an innate enzyme with potent non-immunological antibacterial properties. Lysozyme is up-regulated in many organs of the gastrointestinal tract (GI) undergoing chronic inflammation, such as chronic gastritis with or without intestinal metaplasia (14), celiac disease (15), ulcerative colitis, Crohn’s colitis and microscopic colitis (16), strongly suggesting that the associated bacterial flora plays an important role in the expression of this antimicrobial enzyme in the GI tract.

Unaware of the occurrence of particular bacteria in patients with BE, we reported preliminary results on lysozyme expression in BM (17). In view of the current knowledge regarding the existence of an alien microflora in patients with BE (8-10), it was considered of interest to investigate the expression of the natural antibacterial enzyme lysozyme in a larger cohort of esophageal biopsies exhibiting BE.

Materials and Methods

Ninety-seven consecutive esophageal biopsy cases with BM (4) were explored: 16 had oxyntic gland-only BM, 19 had pyloric gland-only BM and 62 had intestinal metaplastic BM (with or without pyloric glands). In addition, 20 normal gastric and 20 normal duodenal biopsies were included as controls. Sections were stained with hematoxylin-eosin (H&E) and with human lysozyme antiserum (Dako A 0099; DAKO, Glostrup, Denmark), dilution 1:1600, and incubation time of 5 min on a Leica Bond XT (Leica Microsystems, Wetzlar, Germany).

Definitions. BM: The BSG define BM as a columnar-lined esophagus with at least one of the following structures: esophageal glands proper, squamous islands and/or double muscularis mucosa (4). The columnar mucus-secreting cells are usually taller than the columnar cells that line the gastric surface epithelium; their cytoplasm exhibits apical mucin, resembling surface gastric cells, fine vacuolated cytoplasm or pseudoabsorptive cells with microvilli on their free border but not true enterocytes (18).

Esophageal glands in BM: Esophageal glands in BM were classified according to their metaplastic phenotype into oxyntic (type I), pyloric type II), and intestinal type III). i) Oxyntic phenotype: BM lined with columnar cells having subjacent accessory racemose glands of oxyntic type. ii) Pyloric phenotype: For some time the cardia mucosa has been a matter of much dispute in the literature (4-6, 18-21). The cardia is, in fact, an anatomical term defining the most proximal gastric region juxtaposing the esophagus. This term was subsequently extrapolated to describe a particular histological phenotype of columnar-lined mucosa with mucus-producing glands. Some authors regard the cardia mucosa as a millimetre-long structure found at birth (18-20), whereas others consider this mucosa to be a post-natal metaplastic transformation resulting from protracted GER (21, 22). Our own studies in baboons, animals in which gastric regurgitation with rumination is a natural process of digestion, favor the latter view (22, 23), since the columnar-lined mucosa with accessory mucus-producing glands was not found at birth, but evolved following post-natal, daily GER. Against this background, accessory mucus-producing glands present in esophageal biopsies lined by columnar epithelium will be referred to here as being of pyloric phenotype. The term cardio-pyloric glands was not used in this communication. iii) Intestinal phenotype: A columnar-lined mucosa with crypts and glands exhibiting scattered, intercalated barrel-shaped mucus-producing GCs. Sections with BM exhibiting GCs, may coexist with accessory glands of intestinal phenotype. In some cases, occasional Paneth cells may be found at the bottom of the crypts. Islands of squamous cell epithelium can be present in all three mucosal phenotypes.

Immuno histochemical Evaluation. Intensity of lysozyme expression was classified as negative (0)/slight (+), and moderate (++)/marked (+++). The highest degree of lysozyme expression in each biopsy was classified as negative (0)/slight (+), and moderate (++)/marked (+++). The highest degree of lysozyme expression in each biopsy was compared to that in 20 gastric biopsies with normal histology. Lysozyme expression in GCs in BM of IM phenotype was compared to that in 20 gastric biopsies with normal histology.

Table I. Lysozyme expression in consecutive esophageal biopsies in 97 cases with columnar-lined epithelium (Barrett’s mucosa): 16 had accessory glands of oxyntic phenotype-only, 19 accessory glands of pyloric phenotype-only and 62 accessory glands of intestinal phenotype.

<table>
<thead>
<tr>
<th>Degree of lysozyme expression</th>
<th>Oxyntic (n=16)</th>
<th>Pyloric (n=19)</th>
<th>Intestinal (n=62)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface columnar epithelium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/+</td>
<td>11 (69%)</td>
<td>17 (89%)</td>
<td>30 (48%)</td>
</tr>
<tr>
<td>++/+++</td>
<td>5 (31%)</td>
<td>2 (11%)</td>
<td>32 (52%)</td>
</tr>
<tr>
<td>Foveolar epithelium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/+</td>
<td>8 (50%)</td>
<td>15 (79%)</td>
<td>39 (63%)</td>
</tr>
<tr>
<td>++/+++</td>
<td>8 (50%)</td>
<td>4 (21%)</td>
<td>23 (37%)</td>
</tr>
<tr>
<td>Neck epithelium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/+</td>
<td>1 (6%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>++/+++</td>
<td>15 (94%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pyloric glands</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/+</td>
<td>None</td>
<td>4 (21%)</td>
<td>15 (24%)</td>
</tr>
<tr>
<td>++/+++</td>
<td>None</td>
<td>15 (79%)</td>
<td>47 (76%)</td>
</tr>
<tr>
<td>Oxyntic glands</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/+</td>
<td>16 (100%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>++/+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Goblet cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/+</td>
<td>None</td>
<td>22 (35%)</td>
<td></td>
</tr>
<tr>
<td>++/+++</td>
<td>None</td>
<td>40 (65%)</td>
<td></td>
</tr>
<tr>
<td>Paneth cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>++</td>
<td>None</td>
<td></td>
<td>7 (11%)</td>
</tr>
</tbody>
</table>
Statistical analysis. The Mann-Whitney test was used to compare difference between groups. Statistical significance was defined as \( p < 0.05 \).

The Regional Ethical Committee approved the study.

Results

Esophageal biopsies with BM. i) Oxyntic phenotype: Table I shows that lysozyme overexpression (moderate/marked) was found in the surface epithelium in 31% (5/16) of the biopsies, and in the foveolar epithelium in 50% (8/16). Moderate/marked lysozyme immunoreactivity was also found in 94% (15/16) of the biopsies with oxyntic phenotype BM. Oxyntic and chief cells were non-reactive (Figure 1). ii) Pyloric phenotype: Lysozyme was found to be overexpressed in the surface epithelium in 11% (2/19), in the foveolar epithelium in 21% (4/19) and in the pyloric glands (Figure 2), in 79% (15/19) of the biopsies with pyloric type BM. iii) Intestinal phenotype: The Table shows that lysozyme overexpression was recorded in the surface epithelium in 52% (32/62), in the foveolar epithelium in 37% (23/62), in coexisting pyloric glands in 76% (47/62), and in GCs in 65% (40/62) (Figure 3) of the biopsies with IM phenotype BM.

When immunoreactivity was compared in the various epithelia in all three phenotypes, it was found that lysozyme was more frequently overexpressed in the surface epithelium of the biopsies with IM phenotype than in those of the other

Rubio: Lysozyme Expression in Barrett’s Mucosa

Figure 1. Barrett’s mucosa, oxyntic phenotype, showing marked lysozyme expression in neck glands but negative expression in oxyntic glands (lysozyme immunostain, ×10).

Figure 2. Barrett’s mucosa, pyloric phenotype, showing moderate lysozyme expression in surface epithelium, foveolar epithelium and pyloric glands (lysozyme immunostain, ×10).

Figure 3. Barrett’s mucosa, intestinal phenotype, showing marked lysozyme expression in goblet cells (lysozyme immunostain, ×20).

Figure 4. Close-up view of Barrett’s mucosa of intestinal phenotype, showing faintly-stained goblet cells and marked lysozyme expression in the secreted mucus, in the lumen of the gland (lysozyme immunostain, ×40).
two phenotypes \( p < 0.05 \). Lysozyme overexpression was more frequently recorded in the foveolar epithelium of biopsies with oxyntic BM phenotype than in biopsies with pyloric BM phenotype \( p < 0.05 \). The highest percentage of biopsies exhibiting lysozyme overexpression was recorded in the neck epithelium, in the oxyntic BM phenotype. No difference in immunoreactivity was observed between the pyloric glands in biopsies with pyloric BM phenotype and IM BM phenotype. 

**Control gastric biopsies.** i) Pyloric mucosa: In the surface IM BM phenotype. Pyloric glands in biopsies with pyloric BM phenotype and difference in immunoreactivity was observed between the neck epithelium, in the oxyntic BM phenotype. No biopsies exhibiting lysozyme overexpression was recorded in the 20 normal biopsies. lysozyme was slightly \((+)\) or not expressed, whereas it was moderately expressed \((++)\) in pyloric glands. ii) Oxyntic mucosa: In the 20 normal biopsies, lysozyme was slightly expressed \((+)\) in the surface and foveolar epithelium, and moderately expressed \((++)\) in the mucus neck cells. Lysozyme was not expressed in fundic glands.

**Duodenal biopsies.** In the duodenal mucosa of 20 normal biopsies, GCs were lysozyme-negative, with the exception of occasional GC with slight \((+)\) lysozyme immunoreactivity. All duodenal biopsies contained Paneth cells at the bottom of the crypts. The presence of Paneth cells was assessed under fluorescence microscopy (24).

**Discussion**

Protracted gastric reflux elicits columnar-lined metaplastic transformation of the distal esophagus both in humans (1, 2) and in non-human primates (22, 23). On the other hand, the provenance of the accessory oxyntic and pyloric glands and of the intestinal glands in columnar-lined mucosa is less known. Accessory glands of oxyntic and pyloric phenotypes in BM might be the result of mucosal adaptation to an alien microenvironment. Recent studies in baboons suggested that BM (pyloric phenotype) might be an integrated part of the natural phenomenon of mucosal adaptation to daily regurgitation of gastric acid into the distal esophagus (natural GER), whereas BM (pyloric phenotype) in humans might reflect an evolutionary atavism triggered by a non-physiological disorder (pathologic GER) (25).

Recent knowledge strongly suggests that the intestinal phenotype, is neither a mucosal relocation nor a mucosal restoration of intestinal cells, but a phenomenon of reconstruction by migrant stem cells of bone marrow origin (26, 27) that adapts to hostile chemical microenvironmental conditions. Under this hypothesis, circulating bone marrow cells would appear to engraft into ulcerated mucosal areas affected by ongoing chronic inflammation. In this context, Sarosi et al. (28, in a murine model, demonstrated that multipotential progenitor cells of bone marrow origin contributed to the IM epithelium found in the esophagus of these animals.

When compared to controls, lysozyme was overexpressed in all three BM phenotypes. Although the causes for this phenomenon are not fully understood, it is tempting to speculate that the pathogenic bacteria flora present in the luminal microenvironment in BM might have stimulated stem cells (29) to produce more antibacterial enzyme. This is assumed, since it is most unlikely that the signaling responsible for the increased production of lysozyme is directed to already-committed, fully differentiated cells (8).

Why stem cells of bone marrow origin (26, 27) are more prone to produce the antibacterial enzyme lysozyme than are oxyntic and pyloric metaplastic glands, remains elusive. Another question is why only about 10% of the cases with intestinal phenotype displayed Paneth cells, a cell phenotype present in all crypts in the normal duodenal mucosa. In this respect, Scarbati et al. (30) postulated that “despite the wide use of the term intestinal metaplasia in medical literature, experimental data clearly failed to detect enterocytes in the columnar-lined esophagus, and ultrastructural data do not support the concept of intestinal metaplasia”. The present survey substantiates the notion of Scarbati et al. (30), inasmuch as only a small number of cases with intestinal phenotype displayed Paneth cells, at variance with the duodenal mucosa.

Lysozyme was slightly expressed in some GCs. This phenomenon might be due to a prior release of lysozyme-rich intracellular mucin; in fact, GCs with faint lysozyme expression were often found in glands exhibiting lysozyme-rich mucous discharge into the lumen (Figure 4). Lysozyme was not expressed in parietal and chief cells, neither in BM nor in controls, indicating that the up-regulation of this enzyme exclusively takes place in mucus-secreting cells.

In sum, lysozyme is up-regulated in the BM of patients with BE. The pathogenic bacterial flora known to be present in the BM microenvironment might have re-programmed stem cells (29) to encourage differentiated cells to produce more antimicrobial enzyme. Further studies are necessary to unveil the molecular signals responsible for the purported cross-talk between the microbiota in patients with BE and putative stem cells in BM with IM.

Today, bacterial antibiotic resistance continues to exhaust our supply of effective antibiotics. The future challenge is how to solve the conundrum of bacterial resistance to antibacterial drugs. It seems therefore pertinent to recall what Alexander Fleming said in his Presidential address 80 years ago: “I choose lysozyme as the subject for this address for two reasons, firstly because I have a fatherly interest in the name and, secondly, because its importance in connection with natural immunity does not seem to be generally appreciated” (31).

**Acknowledgements**

Dedicated to Sir Alexander Fleming (1881-1955).
References

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