**Inflammatory gradient in Barrett's oesophagus: implications for disease complications**

R C Fitzgerald, S Abdalla, B A Onwuegbusi, P Sirieix, I T Saeed, W R Burnham, M J G Farthing

**Introduction:** Barrett’s oesophageal epithelium (BE) is clinically important due to the associated inflammatory and malignant complications which are unevenly distributed throughout the BE segment. As the immunoregulatory environment may influence disease manifestations, we analysed the inflammatory and cytokine responses throughout the BE mucosa. We then investigated whether the inflammatory gradient is related to the distribution of metaplastic cell subtypes, epithelial exposure to the components of refluxate, or squamocolumnar cell interactions.

**Methods:** Fifty consecutive patients with long segment BE were recruited. The segmental degree of endoscopic and histopathological inflammation was graded, and expression of interleukin (IL)-1β, IL-8, IL-4, and IL-10 were determined by ELISA following organ culture with or without addition of acid or bile salts. Mucin staining and IL-10 immunohistochemistry were performed. The effect of squamocolumnar interactions on cytokine expression were analysed using cocultures of squamous (OE-21) and BE (TE7) carcinoma cell lines.

**Results:** There was a histopathological inflammatory gradient in BE. Inflammation was maximal at the new squamocolumnar junction with ≥2-fold increase in proinflammatory IL-8 and IL-1β expression. The proximal proinflammatory response could not be explained by the distribution of metaplastic subtypes. Pulsatile exposure of BE to acid and bile, as well as juxtaposition of BE to squamous epithelial cells in increased expression of IL-1β. In contrast, inflammation was minimal distally with a significant increase in anti-inflammatory IL-10 expression and 4/6 cancers occurred distally.

**Conclusions:** Specific cytokine responses may contribute to the localisation of inflammatory and malignant complications within BE.

Barrett’s oesophageal epithelium (BE) is important because of the potential for ulcers, strictures, and cancers to occur. Complications arising in BE are relatively uncommon but may result in substantial patient morbidity and mortality. Interestingly, the complications are not uniformly distributed throughout the columnar lined segment. For example, Barrett’s associated strictures are usually mid-oesophageal in contrast with the distal peptic strictures, and oesophageal adenocarcinomas tend to be located distally.

In our own retrospective series, cancers were located in the distal portion of a BE segment (mean length 6.5±0.41 cm) in 18/21 (86%) patients (unpublished data). Furthermore, tumours of the gastro-oesophageal junction (GOJ) are more frequent than in other segments of BE. More than 50% of patients with GOJ-derived cancers have moderate to severe BE inflammation, compared to 16% of patients with short BE segments or distal BE cancers. This study suggests that inflammation may contribute to the increased risk of cancer in patients with GOJ disease.

The relationship between inflammation and cancer risk in BE is complex. Inflammation has been shown to be a risk factor for the development of BE, and the presence of inflammation is associated with an increased risk of developing adenocarcinoma. However, the mechanism by which inflammation leads to cancer is not fully understood. It is thought that chronic inflammation may contribute to the genetic and epigenetic changes that occur in BE, leading to the development of cancer.

**Abbreviations:** GOJ, gastro-oesophageal junction; GORD, gastro-oesophageal reflux disease; BE, Barrett’s oesophageal epithelium; PPI, proton pump inhibitor; TLOS, transient lower oesophageal sphincter relaxation; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction.
we have examined whether any differences between the proximal-distal inflammatory profile could be explained by the predominant metaplastic subtype, the degree of exposure to refluxate, or the cellular interaction between squamous and columnar cells at the new squamocolumnar junction.

METHODS

Patient and tissue collection
Fifty patients with an endoscopic and histopathological diagnosis of BE (≥3 cm, containing intestinal metaplasia with goblet cells) were recruited from Havering Hospitals and St Barts and the London NHS Trusts. The study was approved by the research ethics committees of the Barking and Havering, and East London and City Health Authorities. Patients attending for endoscopy were recruited consecutively and included newly diagnosed patients as well as those undergoing cancer surveillance. The current severity of reflux symptoms and any acid suppressant medications were recorded. The degree of endoscopically visible oesophageal inflammation was graded according to the revised Savary-Miller classification grades 0–IV. Duodenal biopsies served as control intestinal epithelium. Quadrantic biopsies were taken from Barrett’s segment starting 2 cm above the GOJ to the new squamocolumnar junction. At least three low power fields on at least three sections per patient were measured by ELISA assays for IL-8, IL-10 (R&D Systems Europe Ltd, Abingdon, UK) was performed for 20 consecutive patients with non-dysplastic Barrett’s oesophagus and all six patients with high grade dysplasia/carcinoma according to a standard protocol. Immunohistochemistry for IL-10 (1:30 monoclonal antibody) (R&D Systems Europe Ltd, Abingdon, UK) was performed for 20 consecutive patients with non-dysplastic Barrett’s oesophagus and all six patients with high grade dysplasia/carcinoma according to a standard protocol. Organ culture and ELISA
Organ culture was performed for 20 consecutive BE patients in an oxygen enriched environment, as described previously. Culture was performed in Medium 199 supplemented with 10% heat inactivated fetal calf serum, 1 µg/ml of insulin, streptomycin (500 U/ml), and penicillin (250 U/ml), and tissue viability was confirmed following 24 hour culture. Culture was performed in Medium 199 supplemented with 10% heat inactivated fetal calf serum, 1 µg/ml of insulin, streptomycin (500 U/ml), and penicillin (250 U/ml), and tissue viability was confirmed following 24 hour culture. Culture was performed in Medium 199 supplemented with 10% heat inactivated fetal calf serum, 1 µg/ml of insulin, streptomycin (500 U/ml), and penicillin (250 U/ml), and tissue viability was confirmed following 24 hour culture.

Microscopic grading of inflammation
Formalin fixed paraffin embedded haematoxylin and eosin stained tissue was analysed by a single consultant histopathologist (ITS) who was unaware of the endoscopic degree of inflammation. Oesophagitis was diagnosed using the criteria of Ismail-Beigi as well as the presence of an inflammatory cell infiltrate. The updated Sydney system was employed to formally evaluate the histopathological degree of inflammation within glandular BE. Inflammation was graded using a visual analogue scale taking into account the degree of lymphocytic, neutrophilic, and eosinophilic infiltration (none 0, mild 1, moderate 2, and marked 3); this scale has been shown to have excellent interobserver agreement.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Endoscopic degree of inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0</td>
<td>Grade 1</td>
</tr>
<tr>
<td>No of patients</td>
<td>29</td>
</tr>
<tr>
<td>PPI</td>
<td>18</td>
</tr>
<tr>
<td>Segment length (cm)</td>
<td>6.6 (0.51)</td>
</tr>
</tbody>
</table>

The revised Savary-Miller classification was used to determine the degree of endoscopic inflammation. Neither a proton pump inhibitor (PPI) nor segment length (ANOVA) significantly affected the degree of endoscopic inflammation.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>High grade dysplasia and cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient No</td>
<td>Surveillance</td>
</tr>
<tr>
<td>1</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>No</td>
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<td>4</td>
<td>No</td>
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<td>5</td>
<td>No</td>
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<tr>
<td>6</td>
<td>No</td>
</tr>
</tbody>
</table>

Details of the patients with high grade dysplasia and cancer. The tumour stage for patient Nos 2–6 who were operated on is based on analysis of the resection specimen. The mid tumour point was assessed from the endoscopic and histopathological analyses.

BE, Barrett’s oesophageal epithelium; GOJ, gastro-oesophageal junction.
which are known to be expressed by these epithelial cells. ANOVA was used for all other multiple comparisons, and the
ences between metaplastic subtypes in the distal compared
grades. The Student’s paired
cmpare the effect of clinical variables on inflammation
software (Eastman Kodak Company, Rochester, New York,
phoresis Documentation and Analysis System 120 (EDAS)
bromide stained bands were quantified using Kodak Electro-
purchased from Life Technologies (Paisley, UK). The ethidium
were expressed as per biopsy weight which has been shown to
correlate well with tissue protein content.46
Cell culture
TE7 cells and OE-21 (subcloned from an oesophageal squamous carcinoma, gift of Dr Janusz Jankowski, University of
Birmingham, UK) were cultured in RPMI-1640 medium
supplemented with 10% fetal bovine serum, 100 U/ml penicil-
in, 100 µg/ml streptomycin, and 1 mM glutamine in 25 cm²
tissue culture dishes. For cocultures, OE-21 and TE7 cells were
seeded in equal density onto the culture dish which was veri-
ted by dual labelling for cytokeratins (CK 13 for TE7, CK19 for
OE-21). Cytokine analysis was restricted to IL-1β and IL-8
which are known to be expressed by these epithelial cells.37
Competitive RT-PCR for a panel of cytokines
mRNA expression of IL-1β and IL-8 in cell lines were quanti-
fied by competitive reverse transcription-polymerase chain
reaction (RT-PCR) using a standard RNA molecule encoded by
the plasmid pHCQ1 (gift of Dr M Kagnoff, La Jolla, California,
USA), as previously described.38 All reagents for RT-PCR were
purchased from Life Technologies (Paisley, UK). The ethidium
bromide stained bands were quantified using Kodak Electro-
phoresis Documentation and Analysis System 120 (EDAS)
software (Eastman Kodak Company, Rochester, New York,
USA).39
Statistical analysis
Data are expressed as mean (SEM). The χ² test was used to
cmpare the effect of clinical variables on inflammation
grades. The Student’s paired t test was used to identify differ-
ences between metaplastic subtypes in the distal compared
with the proximal BE segments. Analysis of variance
(ANOVA) was used for all other multiple comparisons, and the

Figure 1  (A) Degree of light microscopic inflammation (graded 0–3 using a visual analogue scale) in the proximal compared with
the distal Barrett’s oesophageal epithelium (BE) segment (n=50 patients) (p=0.001 proximal compared with distal segment). The
bars are subdivided to highlight the number of patients on acid suppressants (proton pump inhibitor [PPI]). (B) Mean inflammation
score progressing proximally from the gastro-oesophageal junction (GOJ) towards the teeth (n=16 patients with BE
=8 cm). **p=0.007 compared with the distal segment.

Figure 2  Following organ culture, expression of interleukin (IL)-8 (A), IL-1β (B), and IL-10 (C) were determined by ELISA. Biopsies from
squamous oesophagus, proximal Barrett’s oesophageal epithelium (BE), distal BE, and duodenum in the same patients are compared (at
least 10 patients per cytokine). Note that the units for IL-10 are pg/g
due to its short half-life. *p<0.05, **p<0.005 compared with distal
BE.

Kruskal Wallis test (in the case of inflammatory scores 0–3) or
the Mann-Whitney test (for cytokine expression) was applied
to identify specific differences. p<0.05 was required for
significance.

RESULTS
Characteristics of the patient cohort
The male to female ratio was 4:1 and mean age was 64 years
(range 34–78), with female patients being on average four
years older, as expected.47 The mean length of the segment was
6.5 (0.03) cm (median 6.0; range 3–15). Twenty eight of 50
(60%) patients were receiving a PPI which was effective at
relieving symptoms (p<0.005).

The presence of reflux symptoms (21/50 (42%) had at least
weekly heartburn) correlated with the endoscopic (p<0.05),
but not the light microscopic, grade of inflammation, similar
to other studies.47 There was minimal endoscopic evidence of
inflammation within or above Barrett’s segment in 70% of
patients (29/50 grade 0 and 6/50 grade 1) (table 1). Patients
with grade 4 inflammation included three ulcers (one malig-
nant) and one malignant stricture. There was no relationship
between the degree of endoscopic inflammation and the
length of Barrett’s segment (p>0.5) or the patient’s acid sup-
pressant medication (p>0.5) (table 1).
Histopathological and cytokine evidence for an inflammatory gradient

There was significant variation in the histopathological degree of inflammation throughout the segment (fig 1): 100% of patients had some degree of inflammation in the proximal BE and 60% of patients had oesophagitis above Barrett’s segment. In contrast, 66% of patients had minimal histopathological evidence of inflammation in their distal Barrett’s segment (12 patients grade 0, 22 patients grade 1; p=0.01) (fig 1A). The histopathological degree of inflammation was not related to the use of PPIs (p=0.06 proximal, p=0.18 distal) (fig 1A). Furthermore, there was a gradient of inflammation when multiple segments were analysed in long segments of Barrett’s oesophagus (≥8 cm; p=0.007) (fig 1B).

In keeping with the light microscopic findings and the prominent neutrophil component of the inflammatory cell infiltrate, IL-8 expression was increased twofold in the proximal (4297 (912) pg/mg) compared with the distal (2192 (634) pg/mg; p<0.005) segment. Similarly, expression of the pro-inflammatory cytokine II-1β was increased 3.8 times in the proximal (10 500 (3299) pg/mg) compared with the distal (2711 (520) pg/mg) BE segment (p=0.002). II-1β expression was also significantly increased in the inflamed squamous mucosa above the BE segment (7838 (1638) pg/mg) (p=0.01). In contrast, anti-inflammatory IL-10 expression was increased in the distal (4079 (605) pg/g) compared with the proximal BE (3401 (402) pg/g; p=0.04) (fig 2). In keeping with our previous findings, IL-4 protein expression was increased in the BE samples (mean 1272 (304) pg/mg) compared with the adjacent squamous oesophageal mucosa (660 (59) pg/mg) (p<0.005). However, there was no difference in the levels of IL-4 expression in the distal compared with the proximal BE segment (p>0.05) (data not shown).

Distribution of Barrett’s metaplastic subtypes within BE segment

There was no difference in the proportion of gastric compared with intestinal metaplasia within any part of the BE segment (p=0.95) (fig 3A), independent of the presence of dysplasia (p=0.58). The predominant type of intestinal metaplasia was type II (incomplete small intestinal-type with non-sulphated sialomucins) but in the presence of dysplasia type III metaplasia (features similar to colonic epithelium with sulphated mucins) predominated (p<0.05) (figs 3B, 4). There was no relationship between the subtypes of intestinal metaplasia and the position within the BE segment or segment length (p>0.5) (data not shown).

IL-10 expression

In view of the propensity for increased IL-10 distally where cancers predominate, we compared expression in dysplastic BE specimens, IL-10 expression was confined to the inflammatory cell infiltrate in non-dysplastic BE specimens, IL-10 was strongly expressed by both epithelial and inflammatory cells in the presence of dysplasia (fig 5).
Effect of acid and bile salts on cytokine expression

The effect of acid and bile on IL-1β expression in BE organ cultures was dependent on the pattern of exposure. In BE there was a trend for IL-1β expression to be increased by pulsatile acid and reduced by continuous acid exposure (p=0.1, data not shown). Exposure of the squamous and Barrett’s oesophageal mucosa to bile had a much more pronounced effect but due to interpatient variation this did not reach statistical significance (fig 6). IL-1β expression was increased by exposure of the squamous epithelium to any amount of bile exposure (p=0.07). In contrast, IL-1β expression in BE was only increased by pulsatile exposure (p=0.09). There was no significant effect of acid and bile on IL-4 expression (data not shown).

Cytokine expression in squamocolumnar cell cultures

A simplified in vitro coculture model was used to mimic the squamocolumnar junction. Both IL-8 and IL-1β expression decreased as the individual cell cultures reached confluency (fig 7). In contrast, expression of these cytokines significantly increased as the cocultures reached confluency (p<0.0001 compared with single cultures). Furthermore, IL-1β expression more than doubled in the coculture at 72 hours compared with maximal expression seen in the single cultures at 12 hours (p<0.0001) (fig 7A).

DISCUSSION

Our results demonstrate that there is an inflammatory gradient within BE. Inflammation is maximal at the new squamocolumnar junction with associated oesophagitis and is characterised by increased expression of the proinflammatory cytokines IL-1β and IL-8. The proximal inflammation is not explained by the distribution of metaplastic subtypes.
However, proinflammatory cytokine expression is variably induced by intermittent exposure of the BE to acid and bile, and significantly increased by squamocolumnar interactions. In contrast, the distal BE segment is characterised by a relatively non-inflamed columnar epithelium with associated high levels of IL-10 expression.

The propensity for inflammation to occur proximally is interesting given that exposure to refluxate is maximal at the GOJ and decreases towards the mouth. The degree of inflammation is independent of PPI medication although there is a trend towards reduced proximal inflammation in patients on PPIs. It is possible that bile salts are partly responsible for proximal inflammation and furthermore data suggest that the standard dose of PPI medication may not completely suppress acid reflux in approximately 30% of BE patients.

The inflammatory gradient is also independent of the metaplastic subtypes. Traditionally, BE was thought to contain a gastric fundic-type of epithelium near the GOJ, specialised intestinal epithelium at the squamocolumnar junction, and a cardiac-type mucosa in between. The presence of gastric-type epithelium distally would be expected to be more highly adapted to an acidic environment. However, our results demonstrated that BE is a mosaic of metaplastic subtypes in keeping with evidence that mucosal injury at the gastric cardia is highly localised to the squamocolumnar junction of patients with GORD. Furthermore, in this study the biopsies containing squamous mucosa alone were not particularly inflammatory.

The relative lack of inflammation in the distal portion of BE near to the GOJ where exposure to refluxate is maximal would suggest that BE is an adaptive response to gastro-oesophageal reflux exposure (fig 1). The presence of the potent anti-inflammatory/Th-2 cytokine IL-10 may play a causal role in the prevention of a proinflammatory response distally (fig 2), or alternatively Th-2 expression may be a secondary response by the columnar BE cells to gastro-oesophageal reflux.

Increased expression of IL-10 distally is interesting in view of the association between IL-10 and carcinogenesis. IL-10 may act as an immune escape mechanism for tumour cells by inhibiting MHC class II dependent antigen presentation and Th-1 cytokine production. In this study, patients with high grade dysplasia had increased epithelial and inflammatory cell expression of IL-10 (fig 5). However, the number of patients in this cohort is too small to determine the extent to which these factors may influence the site predilection for carcinogenesis within BE.

In the future, a greater understanding of the site specific role of cytokines in BE and the effect of refluxate on cytokine expression may enable us to develop a more effective therapeutic approach to the prevention of the inflammatory and neoplastic complications of this disease.

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REFERENCES

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