BARRETT'S OESOPHAGUS

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

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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 culture, 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.[1 2] Complications arising in BE are relatively uncommon but they may result in substantial patient morbidity and mortality.[3–5] 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,[6] and oesophageal adenocarcinomas tend to be located distally.[7 8] 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 twice as common as oesophageal adenocarcinomas (>2 cm from the GOJ).[9 10] It has been suggested that a predilection for cancers to occur in the distal Barrett's segment may explain why the correlation between segment length and carcinoma is not as strong as expected.[11 12]

The aetiopathogenesis of inflammatory and neoplastic complications in BE is poorly understood. One factor may be the degree and pattern of epithelial exposure to specific components of gastroduodenal refluxate.[13–15] The pattern of exposure to refluxate is not uniform along the length of the oesophagus and mucosal exposure time depends on factors such as the volume of the refluxate and the length of transient lower oesophageal sphincter relaxations (TLOSR). Hence it has been demonstrated by dual pH studies that exposure to reflux is less in the proximal compared with the distal oesophagus.[16]

The high repetitive exposure of the distal oesophagus to refluxate may be important with regards to carcinogenesis.[17 18] For example, there is evidence to suggest that intermittent exposure of the BE to acid and bile salts, as occurs during multiple TLOSRs, may cause a hyperproliferative epithelial cell response.[19–21] Although not yet proved, these findings suggest that complete acid (and bile) suppression using high dose proton pump inhibitors (PPIs) may be important to reduce progression to cancer in these patients.[22]

At a cellular level, the specific cytokine milieu may be important in the pathogenesis of complications within BE.[23 24–26] Furthermore, we have recently demonstrated that the cytokine milieu in BE is distinct compared with oesophagitis and uninflamed normal squamous oesophagus.[27 28] In BE the inflammatory profile is characterised by a relative increase in the Th-2 type cytokines interleukin (IL)-4 and IL-10, compared with the proinflammatory nature of oesophagitis. These studies were performed using biopsies from the midpoint of the BE segment. However, given the tendency for strictures to occur proximally and given the heterogeneity of BE on a cellular and molecular level,[29–31] we hypothesise that there may be regional variations in cytokine expression.

Therefore, this study was designed to test the hypothesis that proximal-distal variations in the inflammatory response of BE may be germane to the distribution of inflammatory and malignant complications. Firstly, we have compared the degree of inflammation and the production of cytokines in the proximal compared with the distal segment of BE. Secondly,

Abbreviations: GOJ, gastro-oesophageal junction; GORD, gastro-oesophageal reflux disease; BE, Barrett's oesophageal epithelium; PPI, proton pump inhibitor; TLSOR, transient lower oesophageal sphincter relaxation; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction.

Table 1 Endoscopic degree of inflammation

	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	p Value
No of patients	29	6	7	4	4	
PPI	18	4	3	2	1	0.59
Segment length (cm)	6.6 (0.51)	6.0 (0.63)	6.14 (0.46)	5.5 (1.23)	9.3 (2.18)	0.25

The revised Savary-Miller classification was used to determine the degree of endoscopic inflammation. Neither a proton pump inhibitor (PPI) (χ^2) nor segment length (ANOVA) significantly affected the degree of endoscopic inflammation.

Table 2 High grade dysplasia and cancer

Patient No	Surveillance	Tumour stage	BE length (cm)	Mid tumour (cm from GOJ)
1	Yes	High grade dysplasia	8	4.0—multifocal
2	Yes	T2, N0 adenocarcinoma	6	1.0—distal
3	No	T2, N1 adenocarcinoma	5	0.5—distal
4	No	T1, N0 adenocarcinoma	3	2.5—proximal
5	No	T1, N0 adenocarcinoma	4	0.5—distal
6	No	T2, N0 adenocarcinoma	5	1.0—distal

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.

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.[32 33] Duodenal biopsies served as control intestinal epithelium. Quadrantic biopsies were taken from Barrett's segment starting 2 cm above the GOJ to ensure clearance of any associated hiatus hernia (these biopsies are referred to as "distal"). Further biopsies were then taken every 2 cm up the segment.[34] Biopsies referred to as "proximal" were those taken closest to the new squamocolumnar junction (1–2 cm below the Z line depending on the segment length and the irregularity of the junction). In addition, biopsies were taken from the squamous mucosa 2 cm above the new squamocolumnar junction.

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 (developed for use in gastritis) was employed to formally assess the histopathological degree of inflammation within glandular BE.[35] 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.[36 37]

Immunostains for types of metaplasia

Alcian blue staining was used to determine the proportion of intestinal versus gastric metaplasia (70 slides from 35 patients). The stains outlined below were used in order to determine the predominant subtype of intestinal metaplasia at each level of the BE segment (120 slides from 20 patients with long segments (>5 cm).

(A) Alcian blue pH 2.5/periodic acid-Schiff (AB pH 2.5/PAS).[38]

(B) High iron diamine/alcian blue pH 2.5 (HID/AB pH 2.5).[38 39]

(C) Periodate borohydride-PAS (PB/KOH/PAS) reaction.[40]

The mean proportion of gastric versus intestinal epithelium in the proximal and distal segments was quantified from the number of goblet cells per unit area with the aid of a graticule (at least three low power fields on at least three sections per biopsy). In addition, the predominant intestinal subtype was compared at each level of the BE segment.

Immunohistochemistry

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.[28]

Organ culture and ELISA

Organ culture was performed for 20 consecutive BE patients in an oxygen enriched environment, as described previously.[41] 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.[19 42] Tissues were exposed to acid (0.1 M hydrochloric acid) or to bile acids (sodium glycocholate and taurocholate, glycocholic acid, and deoxycholate (Sigma, St Louis, Missouri, USA) to a total final concentration of 1 mM; pH 7.4), either continuously or as a one hour pulse.[19 21] Cytokine concentrations in the supernatant were measured by ELISA assays for IL-8, IL-10 (R&D antibodies), IL-1β, and IL-4 (R&D, Duo-ELISA kit), according to the manufacturer's instructions. Cytokine concentrations

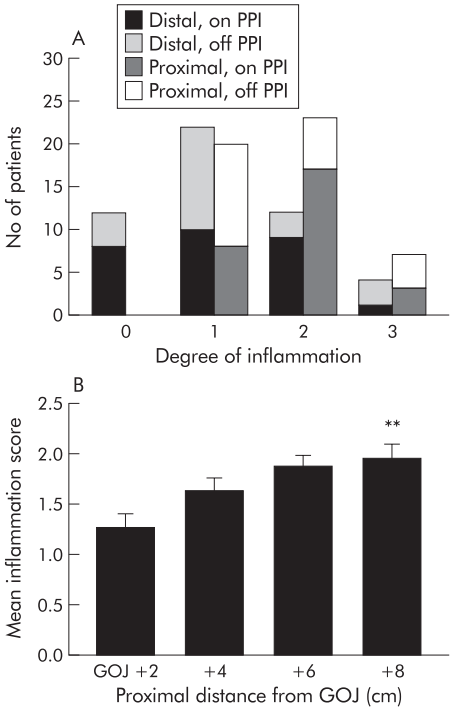

Figure 1 (A) Degree of light microscopic inflammation (graded 0–3 using a visual analogue scale) in the proximal compared with the distal Barrett's oesophageal epithelium (BE) segment (n=50 patients) (p=0.001 proximal compared with distal segment). The bars are subdivided to highlight the number of patients 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.

were expressed as per biopsy weight which has been shown to correlate well with tissue protein content.[43]

Cell culture

TE7 cells[20 44] 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 penicillin, 100 μg/ml streptomycin, and 1 mM glutamine in 25 cm^3 tissue culture dishes. For cocultures, OE-21 and TE7 cells were seeded in equal density onto the culture dish which was verified 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.[27]

Competitive RT-PCR for a panel of cytokines

mRNA expression of IL-1β and IL-8 in cell lines were quantified 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.[34] All reagents for RT-PCR were purchased from Life Technologies (Paisley, UK). The ethidium bromide stained bands were quantified using Kodak Electrophoresis Documentation and Analysis System 120 (EDAS) software (Eastman Kodak Company, Rochester, New York, USA).[45]

Statistical analysis

Data are expressed as mean (SEM). The χ^2 test was used to compare the effect of clinical variables on inflammation grades. The Student's paired *t* test was used to identify differences 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

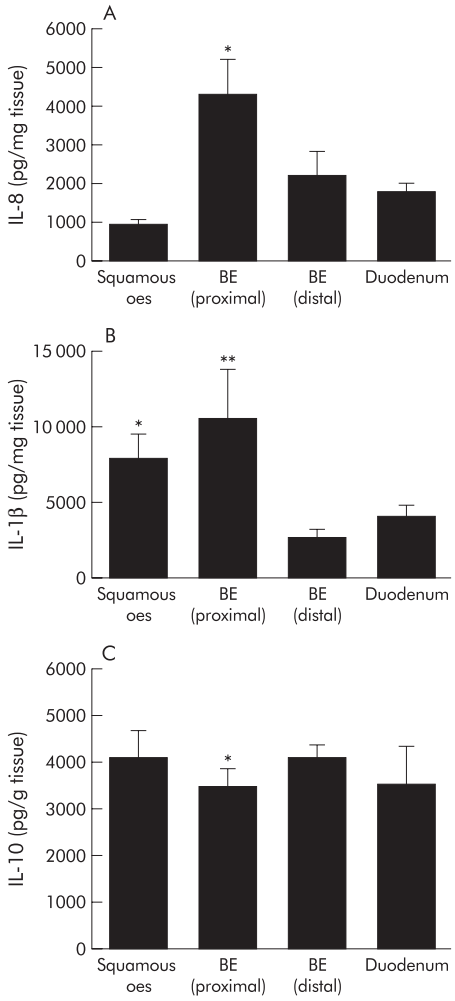

Figure 2 Following organ culture, expression of interleukin (IL)-8 (A), IL-1β (B), and IL-10 (C) were determined by ELISA. Biopsies from squamous oesophagus, proximal Barrett's oesophageal epithelium (BE), distal BE, and duodenum in the same patients are compared (at least 10 patients per cytokine). 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.[46] 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 malignant) 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 suppressant medication ($p>0.5$) (table 1).

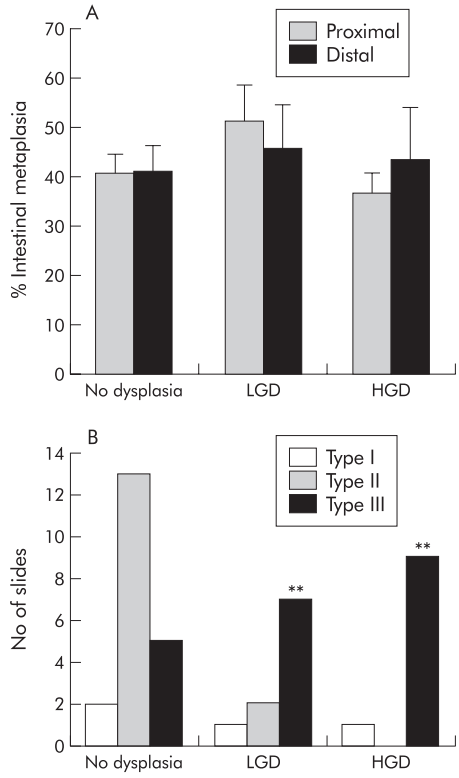

Figure 3 Intestinal metaplastic versus gastric subtypes were determined by mucin staining (A). There was a mosaic of intestinal and gastric subtypes along the length of the Barrett's oesophageal epithelium (BE) segment, independent of the degree of dysplasia: no dysplasia (n=10), low grade dysplasia (LGD) (n=8), and high grade dysplasia (HGD) (n=6) (p>0.5). The predominant intestinal subtype (type I, complete small intestinal-type; type II, incomplete small intestinal-type; type III, colonic-type) is shown for the same patient groups according to the degree of dysplasia (B). **p=0.01 compared with the other metaplastic subtypes in patients with the same degree of dysplasia.

There were 8/50 patients with low grade dysplasia and six patients with high grade dysplasia or adenocarcinoma (table 2). The carcinomas originated in the distal BE segment in 4/6 of these patients (table 2).

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=0.02) segment. Similarly, expression of the pro-inflammatory cytokine IL-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). IL-1β expression was also significantly increased in the inflamed squamous mucosa above the BE segment (7838 (1638) pg/mg) (p=0.01).

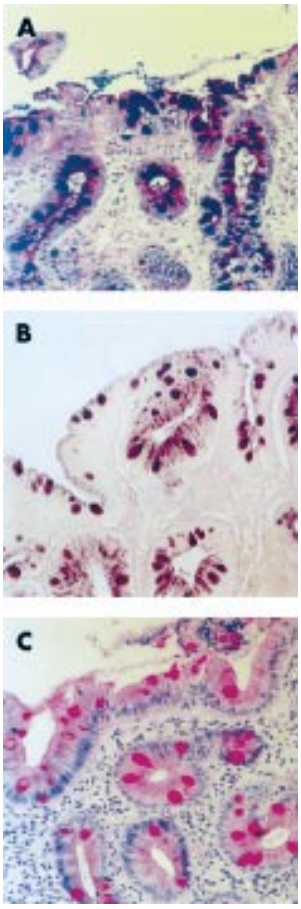

Figure 4 Representative staining pattern for type III intestinal metaplasia. (A) Blue alcian blue/periodic acid-Schiff (AB/PAS) staining for acidic mucins. (B) Brown high iron diamine/AB staining for sulphated mucins. In addition, there are sulphomucin positive non-goblet columnar cells. (C) Red periodate borohydride-PAS staining for O-acetylated sialomucins.

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,[28] 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 compared with non-dysplastic BE specimens. Whereas 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).

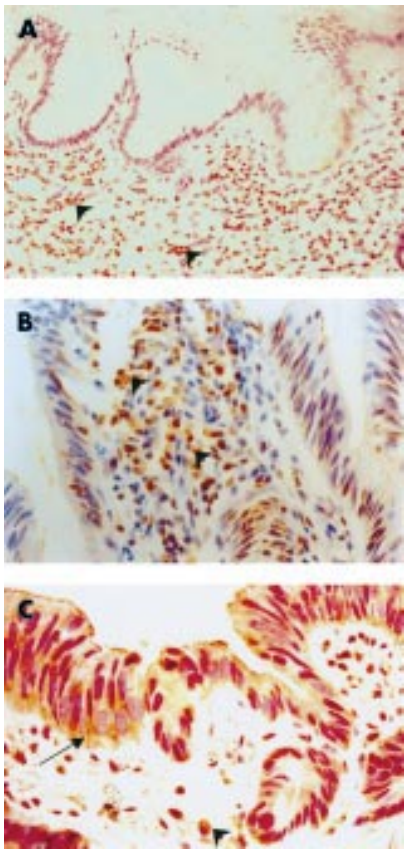

Figure 5 Representative immunohistochemical staining for interleukin 10. (A) Positive inflammatory cells (arrowheads) in the lamina propria of non-dysplastic Barrett's oesophageal epithelium (BE) (magnification ×20). In dysplastic BE (B and C; magnification ×40) there are a large number of positive inflammatory cells in the lamina propria (arrowheads in (B) and (C)), as well as marked patchy cytoplasmic epithelial staining (arrow in (C)).

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).

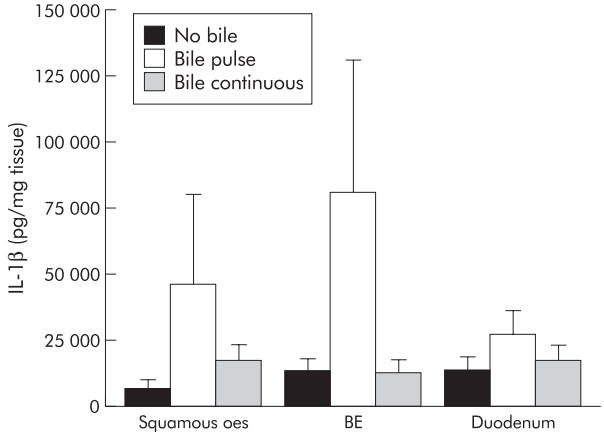

Figure 6 Effect of explant exposure to bile in either a pulsatile or continuous fashion (n=11). Following 24 hours of explant culture, cytokine expression in the supernatant was determined by ELISA. BE, Barrett's oesophageal epithelium; IL-1β, interleukin 1β.

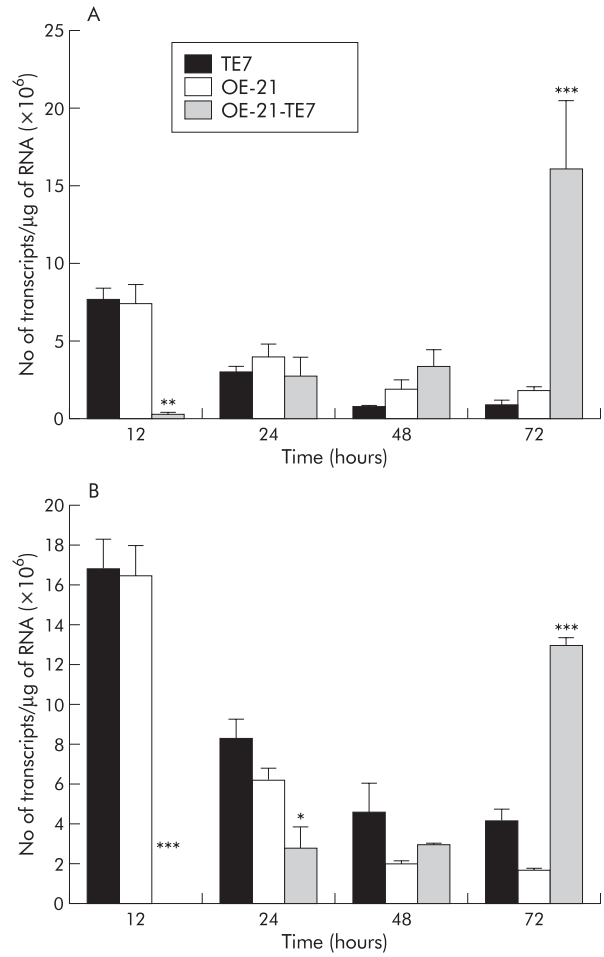

Figure 7 Cytokine mRNA expression was quantified by competitive reverse transcription-polymerase chain reaction for interleukin (IL)-1β (A) and IL-8 (B). Cells were seeded at time=0 and reached confluency by 72 hours. TE7 (Barrett's carcinoma cell line) and OE-21 (squamous carcinoma cell line) were compared when cultured separately and as a coculture. *p=0.017, **p<0.001, and ***p<0.0001 compared with single cultures at the same time point.

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.[16] 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.[48 49]

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.[50] 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 another systematic biopsy study[51] and the known heterogeneity of BE on a cellular and molecular level.[30 52 53]

However, our data suggest that pulsatile exposure of BE and proximal squamous mucosa to bile salts induce the expression of IL-1β. However, this did not reach statistical significance due to the large interpatient variability (fig 6). In addition, the marked effect of the interaction between the squamous and columnar epithelial cell types may be important in the generation of a proinflammatory response at this site (fig 7). The importance of squamocolumnar cell interactions is 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 inflamed.[54]

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.[55–58] 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.[25] 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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