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Apoptotic frequencies are inversely related to levels of cell survival proteins in neoplastic development in patients with Barrett's oesophagus

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Summary. *Purpose:* We aimed here to assess the apoptotic cell frequency in Barrett's oesophagus tissue and the histological series leading to adenocarcinoma. In parallel, we also aimed to quantify the expression of several anti-apoptotic proteins. The purpose was to assess the role of these anti-apoptotic proteins in apoptosis resistance across this well defined neoplastic series. Methods: Apoptotic bodies were manually counted in 74 biopsy tissue sections after robust training (and the use of CD45 and caspase 3 as internal controls). Immunohistochemistry was used to measure the expression of BCL-XL, XIAP and BCL-2 in a subset of the same sections. *Results:* Apoptotic frequency dropped from 0.2% in the Barrett's oesophagus and low grade dysplasia groups to <0.1% in the high grade dysplasia and adenocarcinoma groups. In parallel a marked increase in BCL-XL expression as the tissue progressed towards adenocarcinoma was observed. Moreover a non-significant increase in XIAP expression was detected. BCL-2 expression was low and did not change during the histological advancement. *Conclusions:* Anti-apoptotic protein expression increased across the histological series to cancer in Barrett's oesophagus. This occurred in an inverse manner to the level of apoptotic cells. Apoptotic resistance may drive neoplastic development in the oesophagus and BCL-XL may be prime target for therapy.

Key words: Barrett's oesophagus, apoptosis, BCL-XL, NF-kB

«Le frequenze apoptotiche sono inversamente collegate ai livelli di proteine sopravvissute nella cellula durante lo sviluppo neoplastico in pazienti portatori di esofago di Barrett»

Riassunto. *Scopo:* Si è inteso valutare la frequenza apoptotica delle cellule nel tessuto dell'esofago di Barrett e i quadri istologici che portano all'adenocarcinoma. In parallelo, si è anche cercato di quantificare l'espressione di molteplici proteine anti-apoptotiche, allo scopo di valutare il ruolo di queste proteine nella resistenza apoptotica attraverso la ben definita successione neoplastica. *Metodi:* Dopo aver svolto un tirocinio esperto (e con l'utilizzo di CD45 e caspasi 3 come controllo interno) i corpi apoptotici sono stati manualmente conteggiati in 74 sezioni di tessuto ottenute tramite biopsia. E' stata utilizzata l'immunoistochimica per misurare l'espressione di BCL-XL, XIAP e BCL-2 in un sottoinsieme delle stesse sezioni. *Risultati:* La frequenza apoptotica è diminuita dal valore di 0.2% nell'esofago di Barrett e nei gruppi con displasia a basso grado, fino al valore di 0.1% nella displasia ad alto grado e nei gruppi di adenocarcinomi. In parallelo si è evidenziato un marcato incremento nell'espressione BCL-XL mentre le lesioni tessutali sono progredite verso l'adenocarcinoma. Si è riscontrato inoltre un aumento non significativo nell'e-

spressione XIAP. L'espressione BCL-2 era bassa e non cambiava durante lo sviluppo neoplastico. *Conclusioni:* L'espressione della proteina anti-apoptotica è aumentata in tutti i quadri istologici di cancro nell'esofago di Barrett, mentre si è verificato un andamento inverso a livello delle cellule apoptotiche. La resistenza apoptotica può guidare lo sviluppo neoplastico nell'esofago e il BCL-XL può considerarsi il primo obiettivo della terapia.

Parole chiave: esofago di Barrett, apoptosi, BCL-XL, NF-kB

Introduction

Barrett's oesophagus (BO) is the only known precursor to oesophageal adenocarcinoma (ADC), a type of cancer whose incidence is dramatically increasing in the West (1). BO occurs secondary to chronic reflux disease and is thought to be driven by chronic exposure to stomach acid and duodenal contents (e.g. bile acid).

A key aspect in OA development is the acquisition of a cell survival phenotype. This survival phenotype is a key hallmark of cancer in general (2). Apoptosis is thought to represent a protective mechanism with respect to cancer since damaged or aged cells are eliminated before they are allowed to seed neoplastic development. Apoptosis is an intricately controlled process important in embryonic development (e.g. in origin of fingers in mammals) and in maintaining cellular homeostasis. Two main types of apoptotic pathways are present, those extrinsically controlled (e.g. by immune surveillance) whereby signalling pathways (e.g. involving TNF and Fas-ligand) induce apoptosis in targeted cells. Or secondly, intrinsic pathways, whereby cells take the initiative themselves to commit suicide once internal damage is detected (e.g p53 dependent apoptosis as a result of excess DNA damage). Both extrinsic and intrinsic pathways utilise similar effector proteins (caspases), but they have distinct signalling pathways and specific caspase subtypes. Anti-apoptotic factors exist to counterbalance apoptotic processes to ensure that apoptosis is only invoked when necessary. These anti-apoptotic factors may prevent the intrinsic apoptotic pathways by preventing mitochondrial release of pro-apoptotic cytochrome C (e.g. Bcl-2 and Bcl-xL competing with Bax). However, anti-apoptotic factors may also block caspase proteins, the ultimate effectors of apoptosis (e.g. inhibitors of apoptosis (iap) like Xiap).

In OA development, it has previously been shown that apoptotic cell numbers drop during neoplastic development (3) suggesting that oesophageal tissues acquire anti-apoptotic (cell survival) phenotypes. Consistent with this survival phenotype, it has separately been suggested that several of the anti-apoptotic proteins are over-expressed at various stages in OA development (e.g. Bcl-2 and Bcl-xL) (4-9). Furthermore, there has been a recent suggestion that polymorphisms in apoptotic genes may predispose some reflux patients to ADC formation (10). However, the link between apoptosis and survival protein levels has not been demonstrated in the same patients. Hence, our aim here was to investigate the levels of key survival proteins in parallel to measuring the frequency of apoptotic cells across the series of BO to ADC.

A key pathway leading to cell survival in cancer cells involves the NF-kB transcription factor. NF-kB, once activated, is capable of up-regulating several proteins that block apoptosis. In OA development this activation of NF-kB has been suggested to be critical in terms of blocking apoptosis (11). NF-kB regulated anti-apoptotic proteins include Bcl-xL and Xiap. NF-kB also plays other roles in neoplastic development by promoting cell cycle progression and increasing proliferative signalling. We and other Authors have previously shown that NF-kB activity increases during neoplastic development in BO (12, 13) and it was also elegantly demonstrated that NF-kB activity was a poor prognostic indicator for patients with OA (12). The long standing reflux of gastric juices (gastric acid and bile acid) into the lower third of the oesophagus causes the accumulation of reactive oxygen species (ROS) (13, 14). ROS may well be crucial in the development of Barrett's oesophagus (BO) and ADC in part by activating NF-kB and leading to an anti-apoptotic phenotype being invoked. Hence, it has been

suggested that anti-oxidants may protect against OA development in BO patients, by promoting apoptosis induction, through NF-kB inhibition.

Cancer development in Barrett's oesophagus is a multi step cancer that takes place in four different stages; known as: Barrett's oesophagus (BO), Low grade dysplasia (LGD), High grade dysplasia (HGD) and ultimately Barrett's adenocarcinoma (ADC). However, this classification is histologically based, and used by clinicians to stage a patient's neoplastic development. A major problem in managing Barrett's oesophagus is not knowing which patients will eventually get cancer. Thus, the scientific community is seeking molecular markers that would achieve an early diagnosis for those who are at high cancer risk. The central role that apoptosis plays in cancer development may suggest that apoptosis related proteins could provide such markers.

Materials and Methods

Tissue collection

All biopsies were collected from patients with Barrett's oesophagus or adenocarcinoma, obtained from Abertawe BroMorgannwg University Health Board from the year 2002 to 2008. Ethical approval was obtained from Dyfed Powys local research ethics committee prior to the start of the study.

Haematoxylin and Eosin (H&E) staining

Paraffin sections were de-paraffinised at 37°C and were haematoxylin stained by using xylene and then a descending alcohol series (100, 95, and 70%) then placed in Haematoxylin stain for 3-5 minutes. Sections were placed in running tap water for 5 minutes and then counterstained in eosin for 30-45 seconds. Slides were then allowed to dry and mounted using DPX mounting media and a cover slip. A total of 74 Barrett's patient's tissues with different histologies were used for this study. Apoptotic cells were sought in each of the 74 biopsy sections (sometimes multiple histologies were present in each section, marked by an expert histopathologist). One slide per patient was assessed for apoptotic bodies (n=74). However, in some cases more than one histology was present on each slide and so these different histological features were separately scored. Frequencies of apoptotic cells were calculated by dividing the total number of apoptotic cells by the total number of epithelial cells scored per each grade and multiplied by 100%. Approximately 5,000 to 10,000 epithelial cells were scored per section and only glandular (columnar) epithelial cells were scored. For the adenocarcinomas, selected areas of marked tumour were scored for apoptotic cells.

Immunohistochemistry (IHC)

A total of 55 Barrett's tissues (4 μm thickness) at different histological stages were used for the study. For Bcl-xL staining, 30 sections were used: Barrett's, n=10, HGD, n=10, AD, n=10; for Xiap there were 25 sections were used: BO, n=6, LGD, n=4, HGD, n=6, AD, n=9. Since fewer sections were available for Xiap (due to exhaustion of the samples), LGD sections were also included. For Bcl-xL and Xiap IHC, a Ventana Benchmark XT automated stainer (Illkirch, France) was used. Biotin Blocking Kit was used prior to IHC to avoid any false positive results. The antibodies used were Mouse anti-Xiap (Mouse IgG1, hILP), BD Transduction Laboratories) and anti- Bcl-xL (54H6, Rabbit mAb, Cell Signaling Technology). Control slides from cervical cancer and colon cancer tissues were used as positive controls in these studies. The antibody dilutions used were as follows Xiap 1:250 and Bcl-xL 1:300. All stained slides were counterstained with a light Haematoxylin staining for 60 seconds. Olympus microscopes (CH30 and CH2) were used for all histological assessment and examinations at both Swansea School of Medicine and Singleton hospital department of Pathology. All scoring and assessments were carried out "blind" to avoid any bias on results outcomes. IHC scoring for protein expression was set as follows: [+] for poor expression, [++] for moderate expression and [+++] for strong expression. For Bcl-2 analysis, a separate group of 86 patient samples (18 squamous, 27 BO, 12 LGD, 9 HGD, 20 ADC) were used following very similar protocols to those outlined above. The Bcl-2 antibody

(Bcl-2 oncoprotein, Dako clone 124) was used at a dilution of 1:50. Fishers exact test was applied for all H&E staining and in apoptosis assessment, also Dunnett's t-test (2 sided) was applied on H and E results.

Results

Studies on the apoptotic cell frequency were carried out here on routine paraffin embedded tissue sections (4 μm) which were H&E stained. Clinical samples from routine endoscopic investigations carried out at Abertawe BroMorgannwg University Health Board (ABMUHB) between 2002 and 2006 were used in this study. Endoscopic biopsies obtained from 74 Barrett's oesophagus patients who had not undergone radio/chemotherapy before the endoscopy were identified. The sections identified had different disease stages Barrett's oesophagus (BO, n=19); low grade dysplasia (LGD, n=19); high grade dysplasia (HGD, n=17) and Barrett's adenocarcinoma (ADC, n=19).

CD45 immunostaining was initially used to identify other cells that might interfere with the identification of apoptotic cells (e.g. lymphocytes). This was undertaken to ensure that the scored apoptotic cells were not artefacts (see Figure 1 panel A and B). Caspase 3 staining was also used to confirm the correct identification of apoptotic cells (Figure 1, panel C and D). The H&E stained sections of the 74 patients were then investigated using light microscopy for the presence of apoptotic cells (Figure 1, panel E and F). Two scorers assessed the sections and only cells identified as apoptotic cells by both observers were included in the study. The exact histology of the samples was correlated with the apoptotic frequency after scoring the tissue sections was complete (e.g. the scorers were semi-blinded to the histology prior to apoptosis analysis).

Final results of apoptotic frequencies (%) for each histological grade were calculated as a mean of the apoptotic frequencies found at histology relative to non-apoptotic cells. The apoptotic frequencies at different histological grades were compared against each other. Results illustrated in Figure 2, show a close range of frequencies of apoptotic cells in the first two stages BO and LGD (0.21% and 0.20%) while a significant decrease appears in the other two grades HGD and ADC (0.071% and 0.065%). Therefore, the results from early histological grades (BO & LGD) were pooled together as were the latter grades (HGD & ADC) and similarly treated. *P-values* obtained by 2-tail Fisher exact test comparing apoptotic frequencies in the early histologies (BO & LGD) versus the late histologies (HGD & ADC) were performed. This showed a p-value < 0.0003. Statistical significance was also seen between BO and ADC and LGD and ADC (p-value < 0.005 and < 0.02 respectively) when using Dunnetts t-test. Therefore, the apoptotic frequency appears to decrease across the histological stages in the progression to adenocarcinoma.

Immunohistochemistry (IHC) for Bcl-xL and Xiap expression

Bcl-xL and Xiap protein expression was identified in a subset of patients used for the above histological assessment of apoptosis. The IHC was carried out in histological samples of 30 patients in the case of Bcl-xL (BO=10, HGD=10 and ADC=10, Table 1, Figure 3), but in only 25 histological samples for Xiap due to the exhaustion of certain patient biopsy samples (BO=6, LGD=4, HGD=6 and ADC=9) (Table 2). IHC staining was manually evaluated with light microscopy. The intensity of Bcl-xL immunostaining increased with advancement of the histology (Figure 3, Table 1). The intensity of Bcl-xL staining steadily increased from BO to HGD and ADC tissues. Significantly more highly stained sections (intensity = 3+) in the ADC samples compared to the BO samples (0/10 v 9/10) when using a 2-tailed Fishers exact test (p=0.011) were used. Bcl-xL protein immunostaining was seen as mainly cytoplasmic staining for oesophageal epithelium but some membrane staining was also noticed. Stromal areas were generally negative for staining.

Xiap showed lower levels of staining overall compared to Bcl-xL, but this could be antibody specific. For Xiap immunostaining, the intensity also increased from BO to dysplasia and ADC tissues

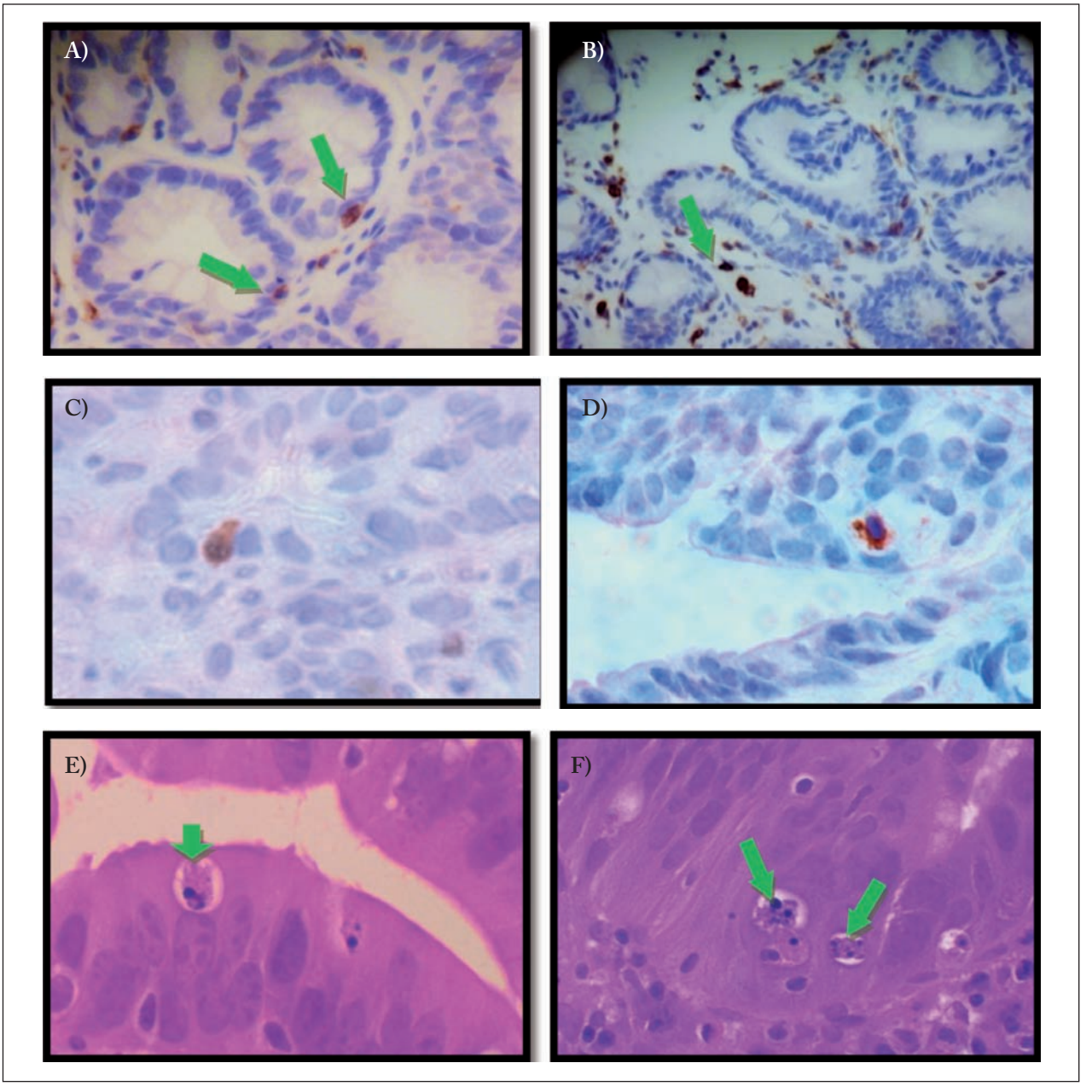

Figure 1. Slides A&B show CD45 immunostaining for lymphocyte identification. Migrating lymphocytes were found in-between epithelial cells and appeared to be overlapping the epithelium. Slides C&D show caspase 3 staining to confirm the identification of apoptotic cells. Slides E&F show H&E stained sections from patient's with Barrett's oesophagus. Green arrows show apoptotic cells/bodies.

(Table 2), but no significant changes in protein expression were observed in the Xiap stained sections, which could be due to lower numbers of samples being analysed. When pooling the numbers of highly stained sections from BO and LGD, a significant difference when compared to the ADC samples (0/10 v 6/9) using the 2-tailed Fishers exact test was observed (p=0.024) Xiap stained both cytoplasm and nucleus and again no obvious stromal staining was noted.

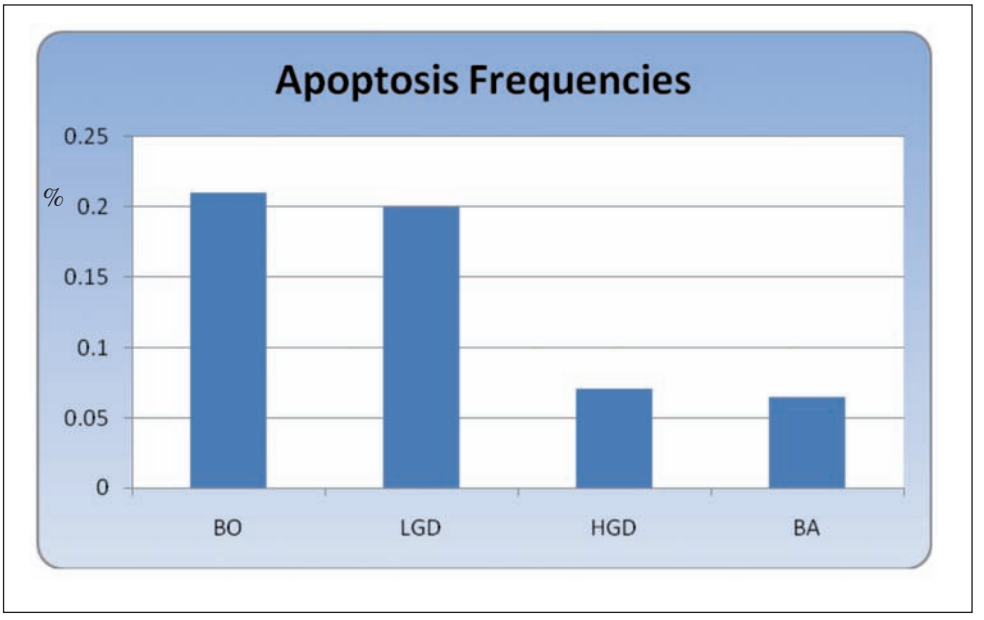

Figure 2. This graph illustrates the frequency of apoptotic cells (y-axis, as a %) scored from 74 different patient's biopsies (BO=19, LGD=19, HGD=17, ADC =19). The frequency of apoptotic cells/bodies seen in this study are higher in BO and LGD histologies, however, this frequency decreased at HGD and ADC stages. Results show similar frequencies of apoptotic cells in BO and LGD (0.21% and 0.20%) while a significant decrease noted in the other two grades; HGD and ADC (0.071% and 0.065%).

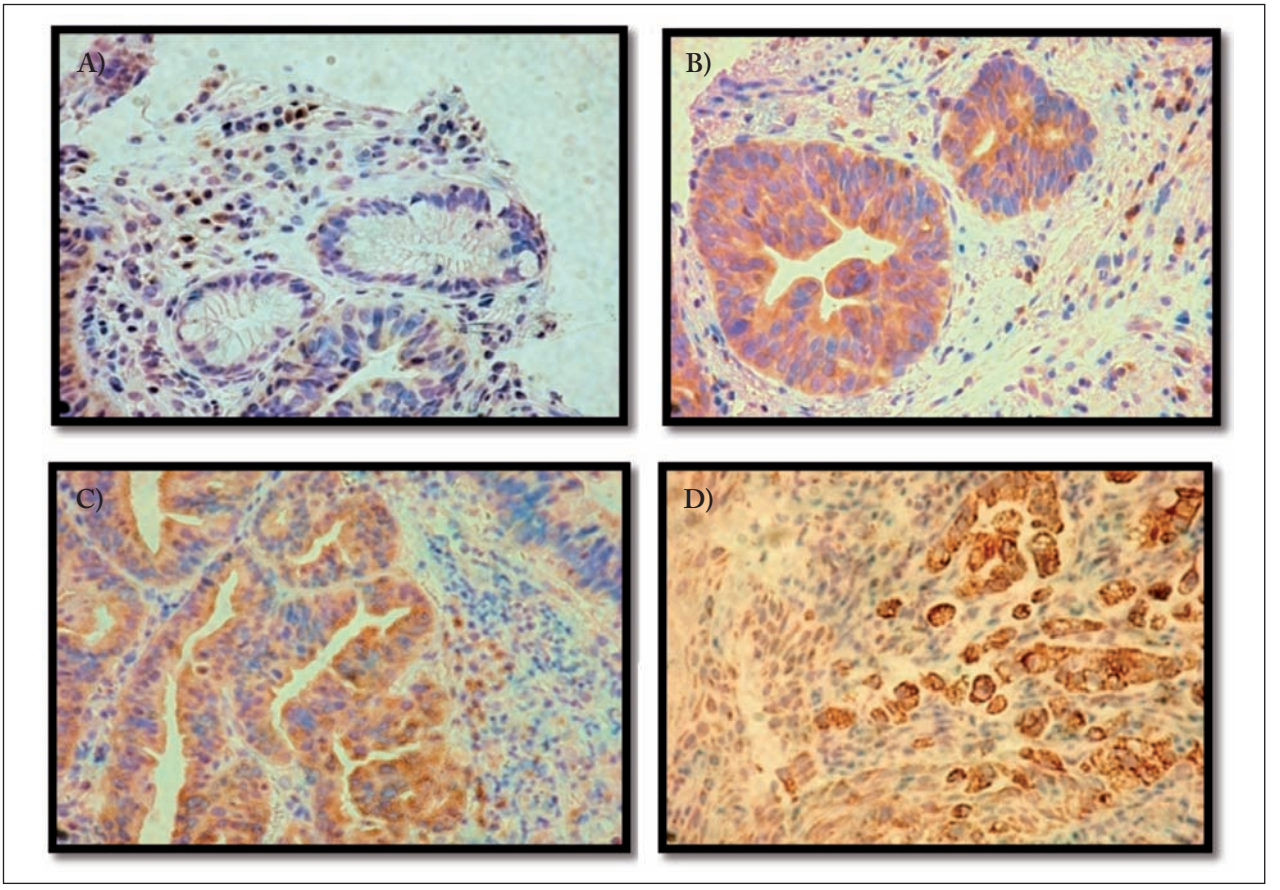

Figure 3. Bcl-xL staining in Barrett's oesophagus shows higher expression at adenocarcinoma stage. Slide A was scored [+], diagnosis was L/HGD on same slide. Slide B was scored [++]; diagnosis was HGD. Slide C was given [++] score, diagnosis was HGD, slide D was scored [+++] diagnosis was ADC.

Table 1. Bcl-xL staining patterns in the histological series leading to adenocarcinoma

Staining Intensity Bcl-xL	Patient's samples		
	BO (n=10)	HGD (n=10)	ADC (n=10)
-/+	5	1	0
++	5	8	1
+++	0	1	9

Table 2. Xiap staining patterns in the histological series leading to adenocarcinoma

Staining Intensity Xiap	Patient's samples			
	BO (n=6)	LGD (n=4)	HGD (n=6)	ADC (n=8)
-/+	3	2	1	0
++	3	2	4	2
+++	0	0	1	6

Real-time PCR analysis of Bcl-xL and Xiap mRNA levels also showed increased expression of both genes in ADC tissue compared to matched squamous tissue, but the 4 -5 fold increases on average were not significant and as numbers of samples were low (3 samples of each for Bcl-xL and 6 of each for Xiap), the data is not shown here.

Discussion

We show here that as Barrett's oesophagus progresses to adenocarcinoma, a significant reduction in the number of apoptotic cells resident in the columnar epithelium is shown. This drop in apoptotic cell number is coupled with increased expression of 2 anti-apoptotic proteins (Bcl-xL and Xiap). It is likely that the 2 observations are linked as they were identified in the same tissues. Hence, up-regulation of anti-apoptotic factors may block apoptosis and this may promote neoplastic development in the oesophagus.

Our data supports previous studies on apoptosis levels in BO and ADC. Katada *et al.* (1997), reported many apoptotic cells present in the luminal surface of inflamed oesophageal tissue, but very rarely seen in BO, dysplastic tissue or cancer tissue (4). A more detailed study by Whittles *et al.* (1999), showed a similar drop in the apoptotic frequency during neoplastic progression in BO (3). Interestingly, their apoptotic frequencies were similar to those reported here. They found average apoptotic frequencies in BO to be 0.46, which fell to 0.29 in dysplastic tissue and ranged from 0.43 in well differentiated ADC to 0.14 in poorly differentiated ADC. Our figures of 0.2% in BO falling to <0.1 in HGD and ADC are not dissimilar. Interestingly, Whittles *et al.*, also showed apoptotic frequencies to vary across the length of the crypt in the columnar tissues with the highest levels (1.99) being found at the luminal surface and the lowest levels (0.026) below the base of the crypt. We did not record the location of the apoptotic cells in relation to the crypt here since most sections were cut obliquely and hence crypt architecture was lost.

We show here that in the same histological sections, as apoptosis levels fall, a rise in the protein expression of the anti-apoptotic proteins Bcl-xL and Xiap is present. This is consistent with these proteins actively blocking apoptotic pathways and resulting in the drop in the number of apoptotic cells. Bcl-xL staining was noted to be stronger than Xiap and the histology-related increase in expression was more noted with the former. However, as with all IHC studies, a clear reliance on the specific antibody used is shown and hence, it is possible that the Bcl-xL staining was stronger due to the antibody being better. Previous studies have suggested that Bcl-xL protein levels increase during carcinogenesis in BO. Iravani *et al.*, (2003) showed that Bcl-xL staining was detectable in 27% of BO, 60% LGD, 71% HGD and 59% of ADC (9). Soslow *et al.* (1999), also showed increasing Bcl-xL expression with histological progression in BO (7). Sarbia *et al.* (1998), further showed that Bcl-xL was expressed in 97% of ADC and the level of expression was inversely proportional to response to chemotherapy (5). In the published literature on Xiap protein expression in BO or ADC very little is published.

Both these anti-apoptotic factors are believed to be controlled by the transcription factor NF-kB. Hence it is interesting to note numerous studies now suggesting that NF-kB levels and activity rising during neoplastic development in BO (12, 13). Hence, it is possible that NF-kB activation (by reflux

components, by oxidative stress and by the inflammatory response in the epithelial cells) leads to up-regulation of these anti-apoptotic factors and is directly responsible for a reduction in the levels of apoptosis during carcinogenesis in BO. This link between NF-kB and cell survival has been shown to be important in the colon and indeed is thought to stem from bile salt induced activation of NF-kB (15). Further studies monitoring NF-kB activity, alongside anti-apoptotic protein levels would help decipher this link.

Another anti-apoptotic protein (Bcl-2) has also been implicated in many cancers (e.g. gastric and colorectal cancer), but the story in BO-ADC is controversial. Whilst some reports suggest a role for Bcl-2 expression in BO, others do not. For example, Katada *et al.* (1997), showed increases in Bcl-2 expression from BO (72% cases positive) to LGD (100% cases positive) to HGD (25% cases positive) and ADC (40% cases positive) (4). Rioux-Leclercq *et al.* (1999), also saw Bcl-2 expression in LGD tissue (84% of cases) but not in HGD or ADC (6). Whilst Van der Woude *et al.* (2002), saw Bcl-2 only in immune cells (8). Goldblum and Rice (1995) and Lauwers *et al.* (1997), showed that Bcl-2 expression was not elevated in BO or ADC tissues (16, 17). Due to this discrepancy we further examined the role of Bcl-2 in BO using IHC in 86 tissue sections across the histological series. This data is shown in Table 3. We saw very low levels of Bcl-2 staining across the board, with few sections showing moderate or strong staining. Furthermore, no increase in Bcl-2 expression in the histological progression from BO to ADC is observed and hence we suggest that Bcl-2 is not driving the anti-apoptotic phenotype in BO.

The strengths of this study are the reasonably high number of samples used for apoptosis identification (n=74) and the co-ordination of the histology with expert histopathologists. The weaknesses of the study include the fact that not all sections used for apoptosis scoring could be studied by IHC for anti-apoptotic protein levels. This was due to exhaustion of some tissues and so could not be avoided.

Table 3. Bcl-2 staining patterns in the histological series leading to adenocarcinoma.

Staining Intensity Bcl-2	Patient's samples				
	Sq (n=18)	BO (n=27)	LGD (n=12)	HGD (n=9)	ADC (n=20)
-/+	18	25	12	8	19
++	0	0	0	0	0
+++	0	2	0	1	1

In conclusion, we show here that levels of apoptotic cells drop during histological progression in Barrett's oesophagus. This is accompanied by increased expression of Bcl-xL and Xiap proteins. Hence, these NF-kB controlled anti-apoptotic factors may be responsible for the death of apoptotic cells in later histologies of this neoplastic series. Our data suggests that Bcl-2 is not involved in blocking apoptosis in BO. In terms of therapeutic developments, clearly targeting the apoptotic machinery would be beneficial to prevent carcinogenesis. It has been noted that even modest reductions in Bcl-xL expression may protect against neoplastic development (18) and hence blocking anti-apoptotic signalling may be a promising avenue for anti-cancer research. With this in mind it is interesting to note that a recent study by ourselves showed that supplementation of Barrett's patients with curcumin led to increased apoptosis levels in the columnar epithelium (19).

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