ORIGINAL ARTICLE

Immunohistochemical study of epithelial-myofibroblast interaction in Barrett metaplasia

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ABSTRACT

Context: Sub-epithelial myofibroblasts are known to influence the biology (proliferation, differentiation and apoptosis) of overlying epithelia. In the intestine, myofibroblasts have been demonstrated to be essential for epithelial differentiation. It is therefore hypothesized that myofibroblasts may also be involved in intestinal metaplasia that is characteristic of Barrett esophagus. **Objective:** This study endeavors to immunohistologically evaluate epithelial-myofibroblast interaction in Barrett's metaplasia. **Materials and Methods:** Nineteen archival esophageal endoscopic biopsies of Barrett's metaplasia were immune-phenotyped for the following epithelial and myofibroblast antigens – cytokeratins (CK) 8, 13, 18, CDX2 (Caudal type homeobox 2), α-smooth muscle actin (SMA). **Results:** α-SMA immunostaining revealed close association between myofibroblasts and metaplastic Barrett's epithelium but not with normal esophageal squamous epithelium. Myofibroblasts were more prominent in dysplastic than in non-dysplastic Barrett metaplasia. CDX2 and CK 8/18, indicators of intestinal differentiation were expressed in Barrett metaplasia but not normal esophageal squamous epithelium, while the reverse was the case for CK 13, which only stained normal esophageal squamous epithelium. **Conclusion:** Although their precise role is yet to be clearly defined, sub-epithelial myofibroblasts are very likely involved in the pathogenesis of Barrett's metaplasia.

KEY WORDS: Barrett's metaplasia, myofibroblasts, α-smooth muscle actin

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INTRODUCTION

Barrett's metaplasia (BM) is an adaptive response of the lower esophagus to gastro-esophageal reflux disease (GORD). Although GORD is quite common especially among Caucasians in the Western World, afflicting up to 44% of the population, only about 10% of them develop Barrett metaplasia.[1,2] Annually, some 0.4-1% of BMs in the Western World become dysplastic and may then progress to adenocarcinoma, herein lies the major significance of BM as esophageal adenocarcinoma is reported to be the fastest rising solid malignancy in the Western world.[3-5]

The association between esophageal disease and gastro-esophageal reflux, was first proposed over a century ago by Tileston,[6] an American surgeon who observed lower esophageal ulcers at post-mortem. However, Norman Barrett, the eminent British thoracic surgeon after whom the disorder is named erroneously thought it was a congenital anomaly (congenital short esophagus) with abnormal extension of the stomach into the mediastinum.[7]

Acid-peptic / bile gastro-duodenal refluxate is noxious to the distal esophageal squamous mucosa and causes chemical injury. Inflammation and healing then follow with proliferating mucosal stem cells - under the influence of chemical mediators -differentiating into gastric/ intestinal type glandular epithelium, which is presumably more resistant to the toxic refluxate.[2,8]

Myofibroblasts are known to be involved in healing, as they are required for granulation tissue formation, synthesis of extracellular matrix, wound contraction and epithelial regeneration.[9] Their role in epithelial regeneration is due to the fact that they elaborate mediators and matrix components that influence the biology (proliferation, differentiation, apoptosis) of the overlying epithelium.[10-14]

As far back as 1997, Duluc *et al.*[15] demonstrated that intestinal myofibroblasts are crucial for the expression of the intestinal differentiation gene *CDX2* (Caudal type homeobox 2). Several other studies have demonstrated expression of this intestinal differentiation gene in Barrett's oesophagus.[16,17] It is therefore hypothesized that myofibroblasts are also involved in the squamous-to-columnar trans-differentiation of Barrett's metaplasia, and possibly the subsequent dysplasia that may ultimately lead to malignancy.

Using appropriate immunophenotypic markers for gastro-intestinal tract (GIT) epithelium (CDX2, cytokeratins [CK] 8, 13, 18 and subepithelial myofibroblasts (Smooth muscle actin -SMA), this study endeavors to immunohistochemically

evaluate the interaction between distal esophageal epithelium and the underlying myofibroblasts in BM.

MATERIALS AND METHODS

This ethically approved study was carried out at the Department of Cancer Studies and Molecular Medicine, University of Leicester, UK. The specimens included 23 endoscopic biopsies from the archives of histopathology department, Leicester Royal Infirmary. Nineteen of the specimens were histologically diagnosed Barrett's metaplasia, while four were normal controls – two esophageal, one gastric cardia and one intestinal.

Four micrometer sections from the archival paraffin embedded blocks were immunophenotyped using the streptavidin biotin technique to determine expression of the following antigens - CDX2, α-SMA, CK8, CK13, and CK18.

The streptavidin reagents and monoclonal antibodies for α-SMA, CK8 and CK18 were products of *Dako AS, Glostrup-Denmark*; while CK13 and CDX2 were from *Biogenex, California-USA.* Streptavidin-Alkaline phosphatase technique was employed for the cytokeratins and α-SMA, whereas streptavidin-horseradish peroxidase was used for CDX2. Antigen retrieval was by microwaving deparafinized rehydrated sections for 10 minutes in 10mM citrate buffer (pH 6).

RESULTS

Of the 19 histologically diagnosed BMs, low grade dysplasia was present in five, and high grade dysplasia / adenocarcinoma in one. The four control intestinal, gastric and esophageal biopsies were histological unremarkable normal specimens. Immunohistochemical expression of the various differentiation antigens by epithelial and subepithelial myofibroblasts cells were as follows [Table 1].

Cytokeratins 8/18

These two cytokeratins which are usually expressed by gastric / intestinal mucosa were expressed in metaplastic Barrett epithelium but not expressed in normal esophageal squamous epithelium [Figure 1a]. Dysplastic BM stained less intensely than non-dysplastic BM.

Cytokeratin 13

This is a marker for esophageal squamous epithelium and was accordingly mainly expressed in the supra-basal squamous cells of normal oesophagus [Figure 1b], but not in BM except in 3 non-dysplastic BM, which exhibited occasional mild staining.

Table 1: Immunophenotype of esophageal biopsies

Immuno-histochemical Antigen	*Normal esophageal squamous epithelium*	*Non-dysplastic BM*	*Dysplastic BM*
CK 8/18	-	++	+
CK13	++	- *	-
SMA**	-	+	++
CDX2	-	+	+

*Mostly negative with occasional mild positive staining, **Staining of myofibroblasts around squamous and Barrett epithelium

α-Smooth muscle actin (SMA)

SMA staining of sub-epithelial myofibroblasts was prominent around metaplastic Barrett's glands [Figure 1c] but not around normal esophageal squamous epithelium [Figure 1d]. α -SMA positive myofibroblasts were even more prominent around high grade dysplastic BM / adenocarcinoma [Figure 1e]. Scant staining around esophageal sub-mucous glands was also present [Figure 1c].

CDX2

This intestinal differentiation transcription factor was expressed in both dysplastic and non-dysplastic BM but not in normal esophageal squamous mucosa [Figure 1f].

DISCUSSION

SMA immunostaining of esophageal biopsies in this study established a close relationship between sub-epithelial myofibroblasts and Barrett metaplastic glands, but not with normal esophageal squamous mucosa. The myofibroblasts were more prominent in dysplastic BM [Figure 1e] than in non-dysplastic BM [Figure 1c].

Metaplastic trans-differentiation from normal esophageal squamous epithelium to intestinal type glands was immunohistologically verified by changes in the expression of epithelial markers - CK 8/18, CDX2 which are normally expressed in the intestine and not in esophageal squamous mucosa, but become manifest in BM. On the other hand, CK13 which is normally expressed in esophageal squamous mucosa was absent or substantially reduced in BM.

Having immunohistologically established an association between sub-epithelial myofibroblasts and Barrett's metaplasia; it becomes pertinent to determine the cause and effect relationship. Are subepithelial myofibroblasts responsible for the squamous-to-glandular trans-differentiation in BM, or are they just part of the stromal reaction to metaplastic Barrett's epithelium following chemical injury?

Published reports on epithelial-myofibroblast interactions in other tissues are available to support either contention; so the truth must lie somewhere in between – a mutually interdependent interaction between myofibroblasts and overlying epithelia.[11,12,18,19]

Myofibroblasts are known to be essential for healing of injured tissues where they elaborate extra-cellular matrix proteins, mediate wound contraction and are required for re-epitheliazation.[9,20] It is this involvement of myofibroblasts in epithelial regeneration during healing, and their well documented influence on gut epithelial biology (differentiation, proliferation,

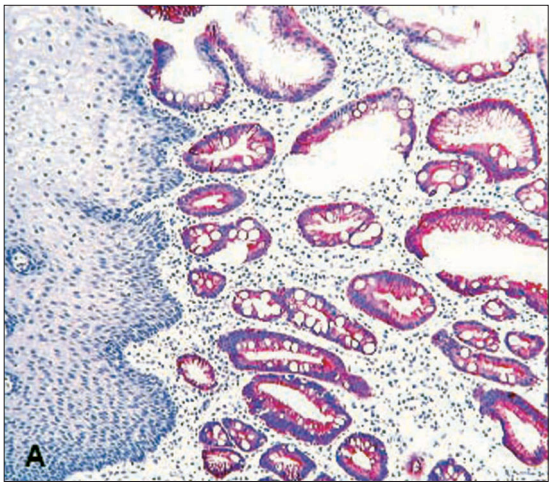

Figure 1a: CK8 (APAAP; x 40) stains BM (right) but not normal squamous epithelium (left).

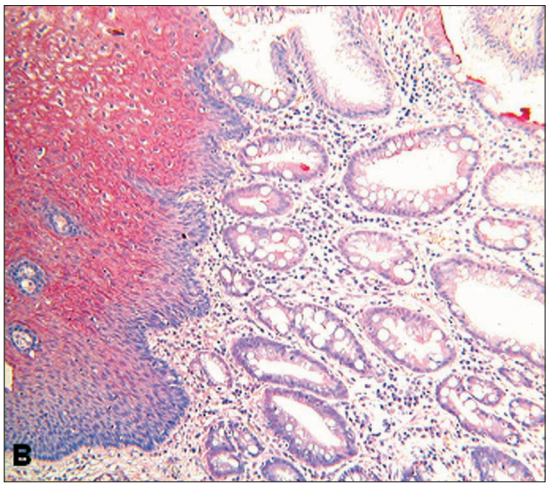

Figure 1b: CK13 (APAAP; x40) stains suprabasal cells in normal esophageal squamous epithelium (right) but not BM (left), except or occasional scanty staining.

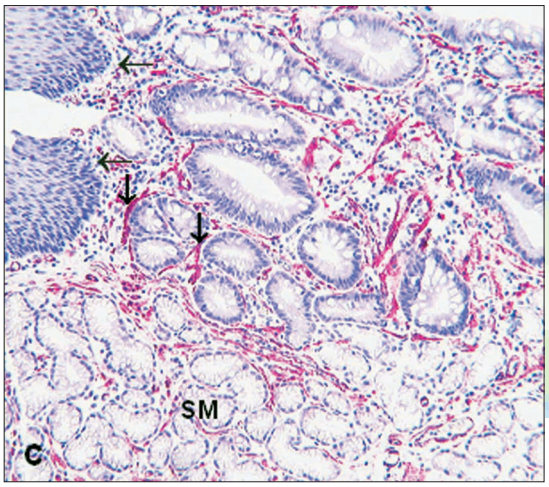

Figure 1c: SMA (APAAP; x25) – myofibroblasts more prominent around BM (down arrows ↓) than around normal squamous mucosa (left arrow ←) and sub mucosal esophageal glands below (SM).

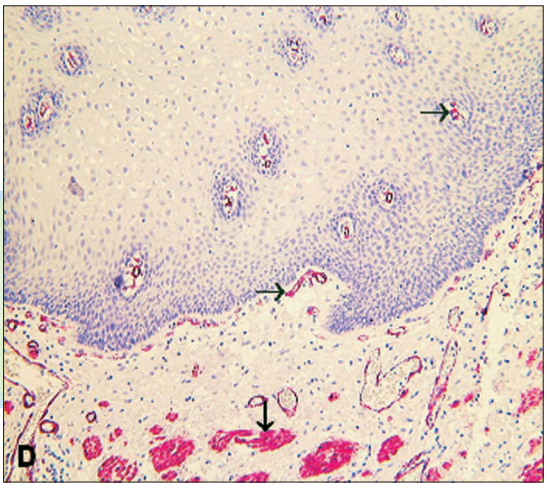

Figure 1d: SMA (APAAP; x25) – Normal squamous mucosa with very virtually no subepithelial myofibroblasts. Part of the muscularis can be seen lower down (down arrow ↓). Vascular smooth muscle is also stained (right arrows →) .

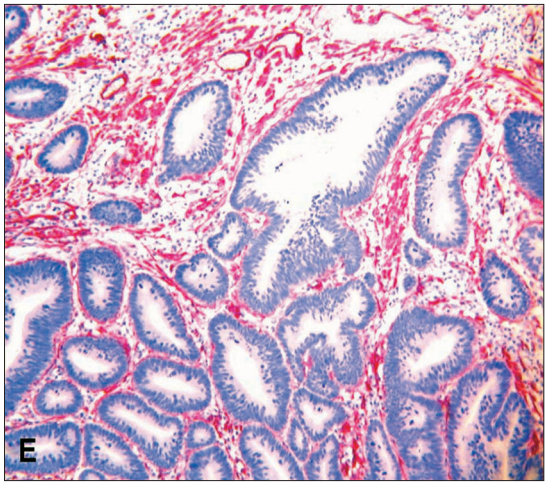

Figure 1e: SMA (APAAP; x40) - very prominent myofibroblasts in high grade dysplasia / adenocarcinoma.

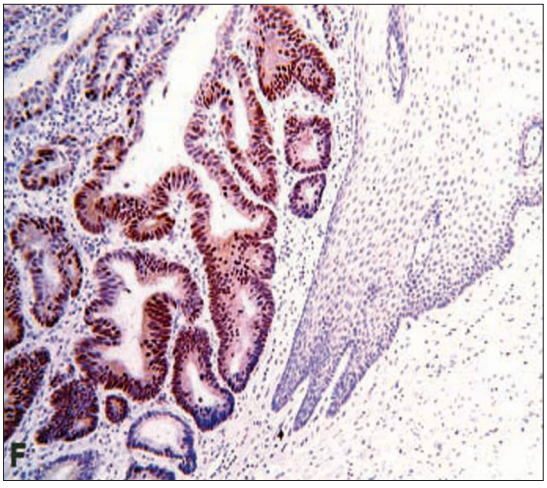

Figure 1f: CDX2 (DAB; x40) stains nuclei of high grade dysplastic BM (left) but not normal squamous epithelium (right)

apoptosis) during embryologic and postnatal life, that has spawned the hypothesis about their postulated involvement in the pathogenesis of BM.[11-15,18,21]

Injured epithelia such as lower esophageal mucosa in GORD, and the accompanying inflammatory leukocytes are known to elaborate chemotactic mediators such as PDGF and TGF-β that recruit fibrocytes and fibroblasts to the mucosal injury and then induce their trans-differentiation to myofibroblasts.[9]

This recruitment of fibrocytes and fibroblasts to injured mucosa, and their subsequent trans-differentiation to myofibroblasts is crucial because sub-epithelial myofibroblasts are not usually associated with normal esophageal squamous epithelium [Figure 1d].

The myofibroblasts together with inflammatory cells in turn elaborate mediators that are essential for epithelial regeneration and differentiation.[9] These mediators include bone morphogenetic factor (BMF-4), epidermal growth factor (EGF), hepatocyte growth factor, keratinocyte growth factor, epimorphin and COX-2 derived prostaglandins (PGE1/PGE2).[13-15,18,22-24] Some of the mediators like epimorphin, PGE1/2 and EGF are known to induce glandular differentiation and/or mucin secretion , which in the oesophagus would result in the intestinal type glandular metaplasia typical of Barrett's esophagus.[18,23,25,26]

The growth/differentiation factors induce enterocyte differentiation by activating the intestinal differentiation gene CDX2 via p38 MAPK signal transduction.[27] CDX2 gene encodes a transcription factor that effects intestinal differentiation by transcribing intestine specific genes such as villin, MUC2, claudin and sucrase-isomaltase amongst others.[28,29,31] In this study and several others, CDX2 has been shown to be expressed in Barrett's metaplasia.[16,17]

Since intestinal myofibroblasts have been demonstrated to be essential for epithelial CDX2 expression, it can be surmised that myofibroblasts in Barrett's esophagus play a similar role in the development of intestinal metaplasia.[15]

In addition to the paracrine effect on epithelial proliferation and differentiation, subepithelial myofibroblasts may exert direct influence on the overlying epithelium through direct inductive contact. Electron microscopy reveals that these myofibroblasts and the overlying gut epithelium both extend cytoplasmic processes through fenestrations in the basal lamina and underlying collagen table thereby establishing direct physical contact.[11,32,33]

This obviously suggests some form of inductive contact although the interacting cell-surface molecules and signaling pathways involved are yet to be defined.

Studies with malignant cells suggest that trans-membrane cell adhesion molecules like cadherins and IgCAMs may be involved in such contact signaling.[34] Mechanisms similar to this malignant contact signaling may be operational in Barrett's, as myofibroblasts in this study were observed to be more prominent in dysplastic (premalignant) BM than in non-dysplastic BM. Thus myofibroblasts may not just be involved in the development of Barrett's esophagus but also in its malignant progression.

In spite of the ample evidence incriminating sub-epithelial myofibroblasts in the pathogenesis of BM, further studies are required to define their precise role as numerous studies suggest other molecular mechanisms may be involved. For instance some studies implicate acid and bile in GORD refluxate as direct upregulators of CDX2 and COX2 genes, which respectively mediate intestinal differentiation and epithelial proliferation in BM.[8,35-37]

Other reports ascribe the development and progression of Barrett metaplasia to mutations and epigenetic alterations in the lower esophageal epithelium.[38-41]

Ascertaining the interaction of these varied pathogenic mechanisms in the development of Barrett metaplasia will provide better understanding of this disease that would hopefully translate into improved therapeutic intervention and management.

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