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RESEARCH

Biphenotypic Surface Epithelial Cells in the Gastrointestinal Tube with Mixed Epithelial-Myofibroblastic Differentiation: A Paradigm

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Abstract Epithelial cells and myofibroblasts are well-characterized histomorphological elements of tissues. They are distinguished from one another on the basis of topography and of differences in cytokeratin (CK) and α-smooth muscle actin (SMA) expression. Certain epithelial cells exhibit CK / SMA co-expression. This study aimed to define the immunophenotypical characteristics of these biphenotypic cells with respect to cytodifferentiation (broad spectrum of CKs, SMA), cell-cell interaction (E-cadherin, adenomatous polyposis coli - APC, β-catenin), and cell survival (cyclooxygenase-2 - Cox-2). At the routine gastrointestinal pathology service of the Department of Pathology, University of Szeged, tissue samples were identified from instances of cervical inlet patch (n=5), Barrett's esophagus (n=5), gastritis (n=5), fundic gland polyp (n=2), gastric neoplastic polyp (n=1), inflammatory bowel disease (n=5), and colonic neoplastic polyp (n=3). that contained epithelial cells expressing SMA. These biphenotypic cells were further immunophenotyped. Foregut-derived biphenotypic cells expressed CKs 7 and 20, while hindgut-derived biphenotypic cells expressed only CK 20. Subepithelial myofibroblasts adjacent to biphenotypic epithelium expressed Cox-2, SMA, and β-catenin, as did biphenotypic cells. Myofibroblasts, however, did not express CKs. In neoplastic polyps, APC expression weakened as cytologic atypism increased, while intermingled biphenotypic cells in neoplastic glands overexpressed APC, as did myofibroblasts beneath. CK subspecies expression in biphenotypic cells reflects embryonic development of the gastrointestinal tract. The immunophenotyping analysis addresses bidirectional (via transdifferentiation from epithelia into myofibroblasts or vice versa) formation of biphenotypic cells within damaged epithelium, a phenomenon that must be further analysed.

Keywords Myofibroblast · Actin · Stem cell

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Introduction

Myofibroblasts (MF) are members of unique cell populations found in several places, among them the eye (corneal MF), lung (intersitial cells), kidney (mesangial and interstitial cells), liver (perisinusoidal stellate cells), pancreas (periacinar stellate cells), and urogenital tract (peritubular and stromal cells). In the intestines, MF are mainly located at the periglandular/subepithelial region (periglandular and subepithelial MF) but their derivatives have a role in the pacemaker motility of the gastrointestinal tract as Cajal's intersitial cells [12, 13]. The origin of MF is still unclear, as is whether stem cell, fibroblast, smooth muscle cells, or transiently activated MF may represent stages in pathways for myofibroblastic differentiation [11]. However, renal tubular epithelial and mesothelial transdifferentiation to MF is described [5, 10]. Morphologically, MF are defined in general by SMA and vimentin co-expression. Ultrastructural analysis reveals prominent actin bundles with gap junctions [16].

Generally, MF share certain functions in growth, repair, and fibrogenesis. However, they have many organ-specific functions that confer heterogeneity. In the normal intestine,

periglandular MF (intestinal subepithelial myofibroblasts - ISEMF) drive primitive epithelium into organogenesis and cytodifferentiation. They also may have roles in basal membrane formation, absorption, water and electrolyte transport, mucosal protection, and contraction (by a fine ISEMF network) [15]. Their functions become more important in pathologic circumstances such as injury, healing, inflammation, fibrosis in gastric ulcers and inflammatory bowel disease, and neoplasia, as in polyps and cancers [1, 7, 14].

While interactions between ISEMF and epithelial cells are at the front line of research, cells with mixed epithelial-myofibroblastic differentiation have never been investigated in the context of gastrointestinal histopathology. In material accessioned via our routine gastrointestinal histopathology service (~6,000 biopsy cases / year) we identified SMA-expressing cells in the epithelial lining of the gastrointestinal tract. Their presence raised questions about the connection between ISEMF and epithelial cells in various gastrointestinal disorders. Therefore, the aim of this study was to define the phenotypical characteristics of SMA-expressing epithelial cells using antibodies against a spectrum of cytokeratins (CKs) and, using immunohistochemical analysis, to map cyclooxygenase-2 (Cox-2) expression topographically and to assess E-cadherin / adenomatous polyposis coli (APC) / β-catenin interactions among cells expressing SMA, cells not expressing SMA, and ISEMF.

Materials and Methods

Gastrointestinal biopsy specimens (n=115) consecutively evaluated in the Department of Histopathology of the University of Szeged were routinely processed from formalin into paraffin, sectioned at 4 um, stained with hematoxylin / eosin (HE), Giemsa technique, and periodic acid – Schiff technique / Alcian blue (PASAB), and immunostained for SMA (clone 1A4/asm-1; LabVision, Fremont, CA) contained cells consistent with MF. These specimens included instances of cervical inlet patch (n=5), Barrett's esophagus (n=5), gastritis (n=5), fundic gland polyp (n=2), gastric neoplastic polyp (n=1), inflammatory bowel disease (n=5) and colonic neoplastic polyp (n=3). In cervical inlet patch samples, oxynto-cardiac mucosa was investigated; in Barrett's esophagus samples, mingled oxynto-cardiac mucosa and metaplastic specialized intestinal mucosa was investigated; and in gastritis samples, antral-oxyntic mucosa was investigated. The gastric neoplastic polyps were flat tubular adenomata with high grade dysplasia. The inflammatory bowel disease samples, all colonic, included 3 instances of Crohn's disease and 2 of ulcerative colitis. The colonic neoplastic polyps were tubular (n=1), tubulovillous (n=1) and flat tubular (n=1) adenomata, all with high grade dysplasia. Additional sections, also at 4 um, of these specimens were immunostained using antibodies against CK 5/6, CK7, CK18, CK 20, HBME-1 (antimesothelial monoclonal antibody), Cox-2, E-cadherin, β-catenin, and APC (Table 1).

Antigen retrieval, tissue processing, and visualisation were conducted following manufacturers' instructions. Samples of normal gastric cardia, oxyntic and antral gastric mucosa, and small and large intestinal mucosa served as controls. The control samples were obtained from patients suspected of gastrointestinal disease; two pathologists independently assessed the samples as free from any gastroenterological disease.

Certain regions of paraffin-embedded tissue containing biphenotypic cells were postfixed in 0.5% osmium tetroxide and re-embedded into epoxy resin (Embed 812; Electron Microscopy Sciences, Hatfield, PA). Semithin sections (1 μm) were stained with toluidine blue. After selection of representative regions, thin sections (70 nm) were stained with uranyl acetate (0.25%) and Reynolds' lead citrate (26.6 g lead nitrate, 35.2 g sodium citrate and 160 ml sodium hydroxide – 1 M in 1,000 ml solution). Ultrastructural investigation was carried out using a Philips E010 transmission electron microscope (FEI, Eindhoven, The Netherlands).

Table 1 Primary antibodies employed from Labvision (Fremont, USA) and Dako (Glostrup, Denmark). For visualisation, high-affinity polymer (Envision®; Dako) was used for 30 min. Sections were counterstained with hematoxylin

Primary antibody	Clone	Source	Antigen retrieval	Diltution	Incubation time
CK5/6	D5/16B4	(Labvision)	Citrate	1:40	30′
CK7	OVTL1230	(Dako)	Trypsine	1:300	30′
CK18	DC10	(Labvision)	Citrate	1:200	30′
CK20	Q6	(Labvision)	Citrate	1:100	30′
HBME-1	HBME-1	(Dako)	Citrate	1:50	30′
Cox-2	SP21	(Labvision)	Citrate	1:200	30′
E-cadherin	SPM471	(Labvision)	Citrate	1:100	50′
β-catenin	17C2	(Labvision)	Citrate	1:30	30′
APC	Polyclone	(Labvision)	Citrate	1:100	60′

Results

The results are shown in Table 2 and Fig. 1. In the epithelium of all foregut-derived samples, CKs, Cox-2, β-catenin, and E-cadherin markers were focally positive but lacked SMA expression. This was only found in biphenotypic cells, which consistently and more pronouncely expressed all the other markers as well. Moreover, biphenotypic cells showed lighter cytoplasm at Giemsa staining (Fig. 1b) being distinguishable from other cells even on light microscopy.

Foregut-derived MFs consistently expressed SMA and Cox-2, with focal cytoplasmic APC/β-catenin expression. They did not mark for CKs or E-cadherin.

With mid- and hindgut epithelium, in specimens with changes of inflammatory bowel disease and colorectal adenoma, epithelial cells showed consistent mild positivity of all investigated markers except SMA and CK7. The biphenotypic cells of mid- and hindgut epithelium displayed pronounced positivity for SMA and for all of the other investigated markers, but exhibited no staining for CK7. Mid- and hindgut derived MFs, like their foregut counterparts, showed consistent SMA and Cox-2 expression, and focal cytoplasmic APC/β-catenin expression, without marking for CKs or E-cadherin.

In dysplastic adenomata of fore-, mid- and hindgut, APC expression gradually decreased in the epithelial cells of dysplastic glands as cytologic atypism worsened. This differed from the few intermingled biphenotypic cells in the same glands, which overexpressed APC. CK5/6 and HMBE-1 were consistently absent from all biphenotypic cells. Sections stained with PASAB technique, as well as materials examined ultrastructurally, were focally deficient in basement membrane beneath biphenotypic cells.

Discussion

Origin of Biphenotypic Cells

Studies have demonstrated that pericapillary pericytes and the ISEMF with which they are connected, located at the furthest depths of the foveolae, form not a static but a dynamic network [9, 13]. This network provides a microenvironment ("niche") for primitive epithelial elements, helping them to differentiate and to proliferate. The exact origin of MF is still a matter for debate. Connective tissue fibroblasts can be induced to transdifferentiate into ISEMF by soluble factors such as platelet derived growth factor and transforming growth factor-β. Smooth muscle cells are also suspected to be able to differentiate into ISEMF, although this process is not yet fully defined. Stellate MF, regarded as multipotent cells reacting to various noxious stimuli and to factors like connective tissue growth factor, insulin-like growth factor, and transforming growth factor-β, also can form a MF network [11].

Another little-known point is the connection between stem cells and MF. Whether MF arise from progenitor (stem) cells of the neuroepithelial crest during development [8] is not fully understood, but in mice capillary pericytes may constitute a transitional status between hemopoetic stem cells and MF [4].

A provocative aspect in our study is our demonstration that epithelial cells are endowed with myofibroblastic features. The sources of these biphenotypic cells can only be hypothesised; possibly they arebidirectional (via transdifferentiation from epithelia into MF or vice versa). That basement membrane was not found underlying biphenotypic cells (Fig. 1t) and that MF contain

Table 2 Antigen expression profiles (++ pronounced; + moderate; +/− focal; - negative) in the epithelial elements (E), SMA-expressing biphenotypic cells (BC), and myofibroblasts (MFs) in cervical inlet patch (CIP; *n*=5), Barrett's esophagus (BE; 5), gastritis (G; 5), inflammatory bowel disease (IBD; 5), fundic gland polyp (FGP; 2), and gastric and colorectal adenomatous polyp (GAP and CAP; 2 and 3, respectively)

Case	SMA	CK5/6	CK7	CK18	CK20	HBME-1	Cox-2	E-cadherin	β-catenin	APC
CIP/BE/G/FGP										
E	−	−	+/−	+/−	+/−	−	+	+/−	+/−	+/−
BC	+	−	++	++	++	−	++	++	++	+/−
MF	+	−	−	−	−	−	+	−	+/−	+/−
IBD										
E	−	−	−	+	+	−	+	+	+	+
BC	+	−	−	++	++	−	++	++	++	+
MF	+	−	−	−	−	−	++	−	+/−	+/−
GAP / CAP										
E	−	−	G+/CR−	+	+	−	+	+	+	+
BC	+	−	G+/CR−	++	++	−	++	++	++	++
MF	+	−	−	−	−	−	++	−	+/−	+/−

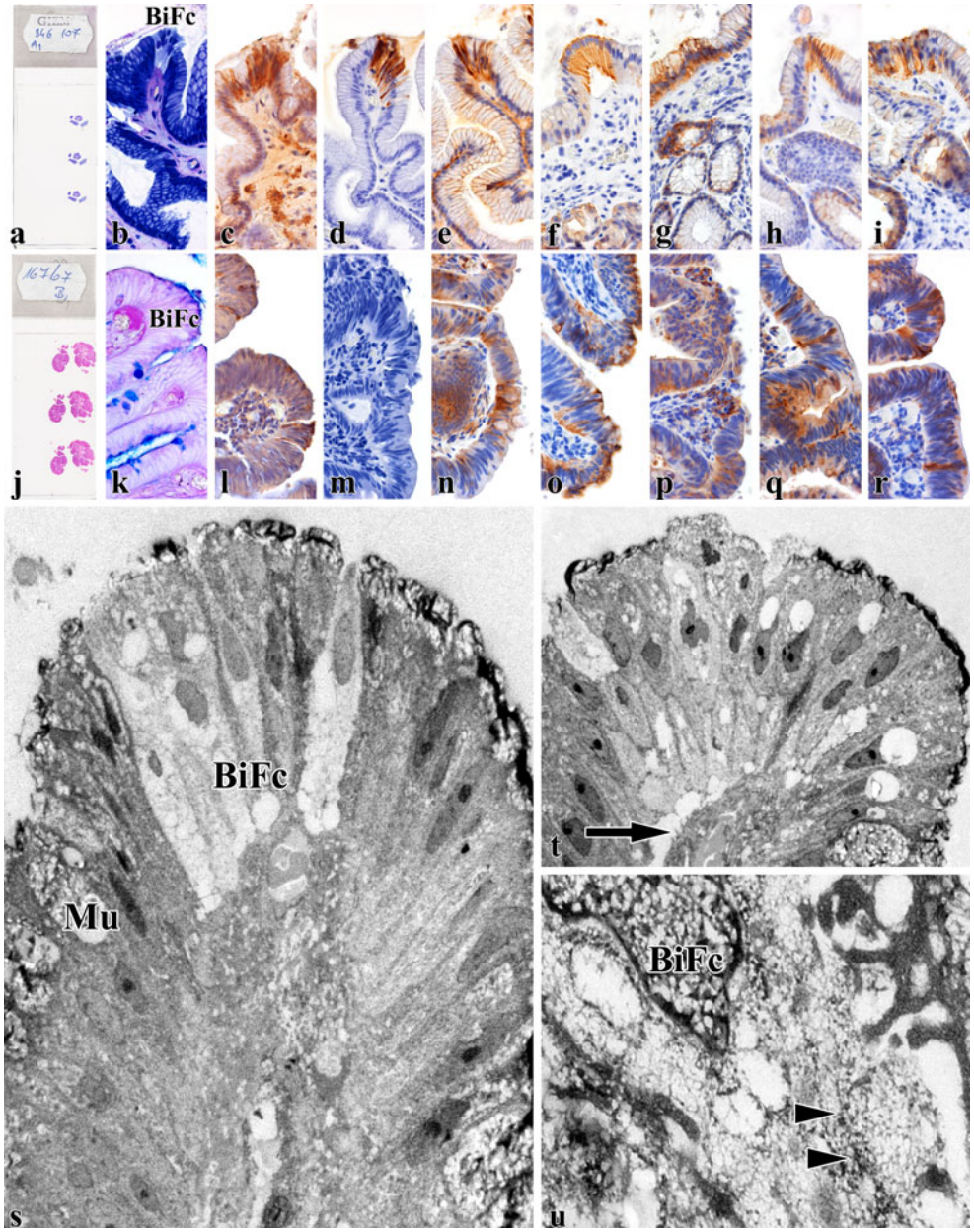

Fig. 1 Two characteristic examples of morphology and immunoexpression profile of biphenotypic cells. 346/07 (**a**), gastric cardiac mucosa, exhibits elongated foveolar cells whose cytoplasm stains less than does that of neighboring cells (**b**). On immunostaining, these cells mark for SMA (**c**) and mark strongly for CK7 (**d**), CK20 (**e**), and CK18 (**f**), as well as for Cox-2 (**g**). The biphenotypic cells also express β-catenin (**h**) and E-cadherin (**i**). In 167/07 (**j**), a colorectal adenomatous polyp, biphenotypic cells are less evident, but the SMA (**k**), CK20 (**m**), CK18 (**n**), Cox-2 (**o**), β-catenin (**p**), and E-cadherin (**q**) expression profile in biphenotypic cells is similar to that of foregut-derived biphenotypic cells, although those in the hindgut lack CK7 expression (**l**). APC expression in intermingled biphenotypic cells within the adenomatous polyp is preserved, in contrast with less pronounced expression of APC in dysplastic epithelial cells (**r**). Ultrastructurally, the cytoplasm of biphenotypic cells (BiFc - s) is more electron-lucent than is that of nearby mucinous cells (Mu - s). The basal membrane is discontinuous, with absence below the BiFc cells (→ **t**). In biphenotypic cells artefactually degenerated actin fibers are identified (▲; **u**), with a prominent intercytoplasmic filament meshwork (top right; u). Scanned slides (1×), a, j; 400×, b (Giemsa) and c-r (avidin-biotin reaction, hematoxylin counterstain); 1,100×, s and t (osmium tetroxide / uranyl acetate / lead citrate); 34,000×, u (as s and t)

lytic (metalloproteinase) enzymes [17] suggest that the close interaction between epithelial and myofibroblastic elements may involve digestion of the interposed basement membrane.

The first hypothetical step may be upward migration from beneath, followed by MF basement membrane digestion. After MF insert themselves among epithelial cells, MF may produce strong intercellular adhesion

molecules (E-cadherin - β-catenin signal) and CKs as part of settling down in epithelium.

This view of stem cell – epithelium metamorphosis is completely different from the view that derives epithelial stem cells from the base of the crypt [2]. In this second (opposite) hypothetical scenario, biphenotypic cells leave the epithelium, lose CK markers during epithelium - MF transdifferentiation, and downmigrate into the lamina propria. The latter sequence is exemplified by renal tubular epithelial or mesothelial transdifferentiation into MF in case of injury [5, 10]. Although these views are hypotheses, MF are known to cluster under injured epithelium close to biphenotypic cells, playing an important role in regeneration [13].

Expression Profile of Biphenotypic Cells

As far as β-catenin and E-cadherin signals are considered, in normal adult epithelial cells β-catenin is connected either to E-cadherin or to APC; otherwise it is degraded. *APC* is a tumor suppressor gene whose protein product, APC, interacts with β-catenin [3]. APC inhibits the intracytoplasmic and intranuclear accumulation of β-catenin, preventing cell cycle promotion. Sporadic gastric and colorectal adenomata as well as adenomas in familiar adenomatous polyposis usually harbor loss of function mutation in *APC*, resulting in the nuclear accumulation of β-catenin. β-catenin, together with E-cadherin, also plays a crucial role in development and cell-cell interaction of tissues in fetal life [6]. That biphenotypic cells showed increased expression of cytoplasmic β-catenin and E-cadherin reflects the importance of tight cell-cell interactions in damaged epithelium. The glandular epithelium of the examined adenomata showed increased cytoplasmic expression of β-catenin with focal intranuclear accumulation of this protein, whilst the biphenotypic cells in the same lesions displayed no intranuclear β-catenin accumulation. Of further interest is that APC expression in intermingled biphenotypic cells of adenomata was preserved, a finding in contrast with decreased expression of APC in dysplastic epithelial cells. This means that biphenotypic cells are resident immigrants rather than clonal members of adenoma epithelial-cell populations. Overexpression of Cox-2 by stromal myofibroblasts in adenomatous polyps and colorectal adenocarcinomas may encourage epithelial resistance to apoptosis [1, 15]. In our study selective overexpression of Cox-2 in both ISEMF and biphenotypic cells suggested increased resistance to apoptotic injury.

Although expression of β-catenin, E-cadherin and Cox-2 in biphenotypic cells was similar, regardless of in which part of the gut they were, with respect to CKs these cells exhibited differences that can perhaps be ascribed to organogenesis. CK7 expression in foregut-derived gastrointestinal tract biphenotypic cells, with lack of CK7 expression in mid- and hindgut-derived gastrointestinal tract biphenotypic cells, indicates that these cells exhibit CK expression patterns expected for these portions of the gut. This organogenic fidelity was also confirmed by CK 20 that showed positivity throughout the gut in non-biphenotypic (normal and neoplastic) cells similarly to biphenotypic cells that had consistent marked expression of CK20 in all parts of the gut, nevertheless, the non-biphenotypic cells had a tendency to increase their CK20 expression toward to hindgut.

Further Significance and Implications of Biphenotypic Cells

In this study, we investigated biphenotypic cells through the lens of classical histomorphology and immunohistochemistry. Our findings and hypotheses thus operate at the level of routine histopathology. To gain further information, using molecular biological methods, on the genesis of these biphenotypic cells will be important. Clonality investigations could clarify connections between ISEMF and biphenotypic cells in the setting of repair as well as in neoplastic conditions, with further therapeutical implications.

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