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Expression of connexins 26, 32 and 43 in the human colon - an immunohistochemical study

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Abstract: Gap junctional intercellular communication (GJIC) is a mechanism for direct cell-to-cell signalling and is mediated by gap junctions (GJs), which consist of proteins called connexins (Cxs). GJIC plays a critical role in tissue development and differentiation and is important in maintenance of tissue homeostasis. The purpose of the study was to evaluate the expression of Cx26, Cx32 and Cx43 in the human colon. Surgical specimens were obtained from patients who underwent surgical resection of colorectal tumours. Tissue samples (50 cases) were collected from normal colon, at the maximum distance from the tumor. Using antibodies for Cx26, Cx32 and Cx43, immunohistochemical detection was made. In epithelial cells, strong Cx26 immunoreactivity was found, whereas Cx32 and Cx43 were sparsely distributed. Strong Cx43 immunostaining in muscularis mucosae was observed. In the circular layer of muscularis externa, expression of Cx43 and Cx26 was seen, but only in the portion closest to the submucosa. No immunoreactivity was found in the longitudinal muscle layer. Small vessels stained positively only for Cx43. Furthermore, there was no difference in staining between samples derived from various sections of the colon. This study showed immunohistochemically for the first time the expression of Cx26 in human colon mucosa.

Key words: Connexins - Colon - Immunohistochemistry

Introduction

Gap junctions are structures localized at the plasma membranes that allow the exchange of ions, nucleotides, metabolites and other small molecules (less than 1 kDa) including second messengers such as cAMP, IP_3 and Ca^{2+} between adjacent cells, thereby facilitating electrical and metabolic coupling [3, 11]. Gap junctions consist of two hemichannels called connexons, and each hemichannel is composed of six transmembrane proteins - connexins (Cxs). Gap junctions may be heterotypic (each connexon composed of different Cx isotypes) or heteromeric (each connexon composed of more than one Cx isotype) [11]. The composition and organisation of gap junction channel subunits can play a critical role in determining the properties of these channels (altered conductance, altered pH sensitivity). The connexins belong to a multigene family of transmembrane proteins and now up to 20 different connexin isoforms have been established in humans [27]. Multiple connexins are often expressed in the same tissue [18] or even in the same cell [2]. Gap junctional intercellular communication (GJIC) plays a critical role in tissue development and differentiation and is important in maintenance of tissue homeostasis.

The digestive tract gap junctions are located between various kinds of cells in each layer of the wall [13]. There is now strong evidence that gap junctions can play a role in controlling tissue development [17] and growth of tissues [24], in conduction of electrical activity between cells (cardiac or smooth muscle cells) [22], in control of intestinal motility [14] and in exchange of biochemical information between adjacent cells through exchange of ions, small molecules and second messengers [3, 11]. On the other hand, only a few studies showed the distribution of connexins in the normal human large bowel [4, 13, 15]. To our knowledge, the present paper is the first description of Cx26 expression in colon mucosa.

Materials and methods

Surgical specimens used in this study were obtained from 50 patients who underwent surgical resection of colorectal cancer. Tissue samples were derived from the colon (ascending, descending, transverse or sigmoid colon), at the maximum distance (usually 10-20 cm) from the tumor. They were collected immediately after tumor removal, fixed in 10% buffered formaldehyde solution, embedded in paraffin

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at 56˚C according to standard procedures and then cut into 5 µm thick sections in direct series. Histopathological examination was performed using standard hematoxylin-eosin staining.

Tissues selected for immunohistochemical studies did not reveal significant pathological changes under microscopic examination. Cx26 was detected with a goat polyclonal antibody sc-7261 (Santa Cruz Biotechnology, USA) at dilution 1:400. This antibody recognises amino acids in the amino terminus of Cx26 and is recommended for detection of human Cx26. Cx32 was detected with a goat polyclonal antibody sc-7258 (Santa Cruz Biotechnology, USA) at dilution 1:200. This antibody recognises amino acids in the carboxyl terminus of human Cx32. Cx43 was detected with a goat polyclonal antibody sc-6560 (Santa Cruz Biotechnology, USA) at dilution 1:300. This antibody recognises epitope mapping at the C-terminus and it is recommended for detection of human Cx43. Immunohistochemical studies, including negative controls with omission of the primary antibodies, were performed as described previously [10]. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 min. After rinsing in phosphate-buffered saline (PBS), the sections were incubated with antibodies at 4˚C overnight using a staining chamber (The Binding Site, UK). Primary antibodies were diluted in PBS with a proper 1.5% normal blocking serum. After rinsing in three changes of PBS, a streptavidin-biotin-peroxidase complex technique was used to reveal antibody-antigen reactions (LSAB+ Kit, Dako, Denmark) according to the protocol provided by the manufacturer. Staining was routinely developed using 3,3'-diaminobenzidine as a chromogen (Dako, Denmark). Expression of Cxs in studied structures of the human colon was classified using a three-point scale: (-) no immunoreactivity; (+/-) weak immunoreactivity observed in a part of studied cases; (+) strong immunoreactivity observed in all studied cases.

Results

Distribution of Cx26

Strong immunoreactivity to Cx26 was observed between epithelial cells in crypts of the majority of studied cases (Figs. 1a,b), in others moderate or weak immunostaining pattern was seen. At the luminal surface of colon mucosa weak immunoreactivity in a part of studied cases was seen. No immunostaining was found in the muscularis mucosae, whereas punctuate immunoreactivity was seen in the circular layer of muscularis externa especially at the inner border of the circular muscle layer near the submucosa (Fig. 1g). Positive reaction for Cx26 was not detected in the longitudinal muscle layer. No apparent immunostaining was found in endothelial or smooth muscle cells of colon vessels. The summary of immunohistochemical staining for the studied connexins is presented in Table 1.

Distribution of Cx32

Between epithelial cells of the crypts and in the superficial epithelium of the colonic mucosa, weak immunoreactivity appeared as dots (Figs. 1c,d). Similar to Cx26, no immunostaining of Cx32 was observed in the muscularis mucosae. In contrast to Cx26, no immunoreactive sites were seen in the muscularis externa of the colon wall. In the longitudinal muscle layer, no positive reaction was revealed for this protein. Furthermore, no positive reaction to Cx32 could be detected in colon vessels.

Table 1. Immunohistochemical staining for connexins 26, 32 and 43 in different compartments of the human colon.

	Cx26	Cx32	Cx43
Epithelium - surface	+/-	+	+/-
Epithelium - crypts	+	+	+
Muscularis mucosae		–	+
Muscularis externa - circular layer	+	–	+
Muscularis externa - longitudinal teniae coli	–	–	–
Arterioles	–	–	+

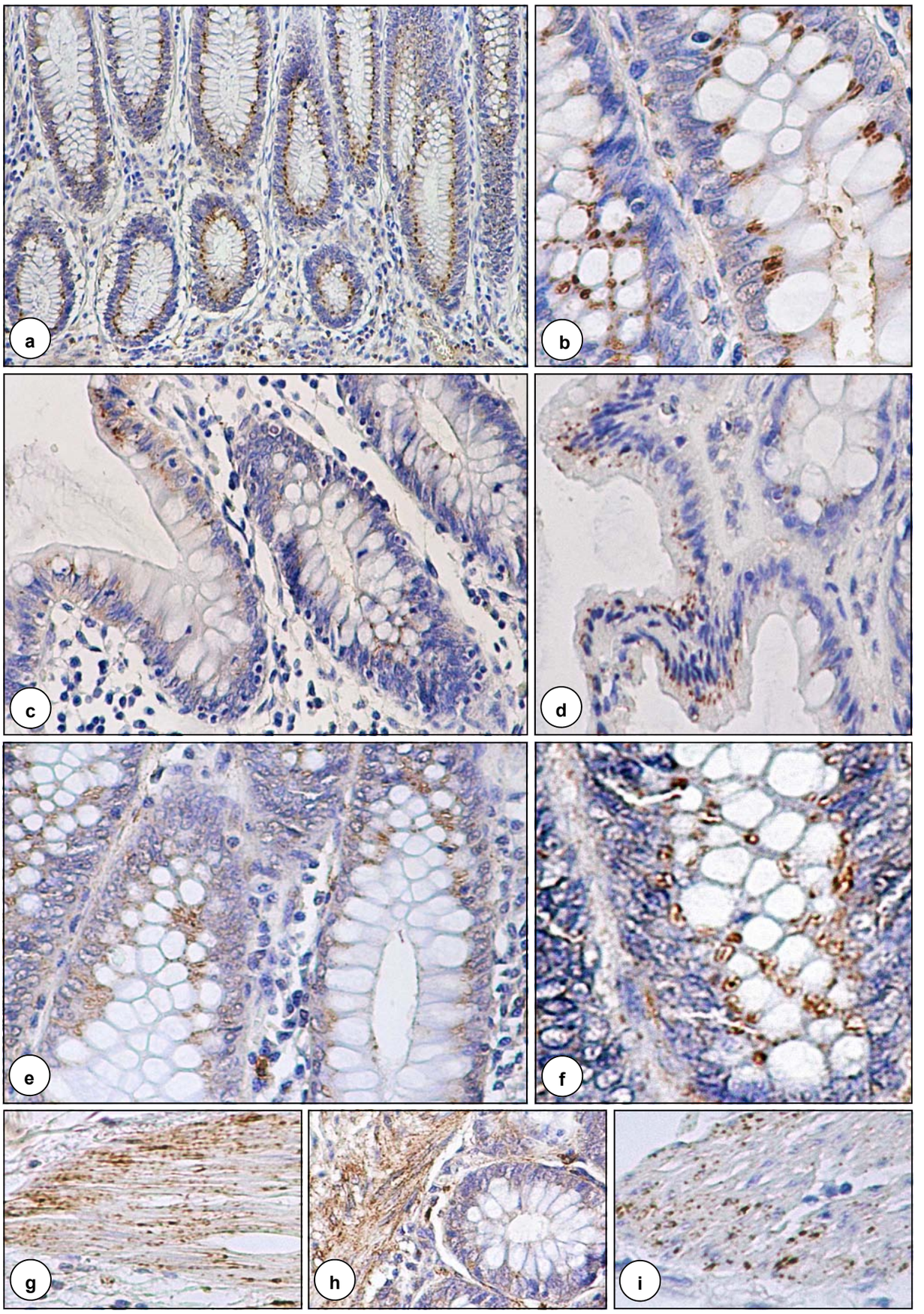
(-) no immunoreactivity; (+/-) weak immunoreactivity observed in a part of studied cases; (+) strong immunoreactivity observed in all studied cases.

Distribution of Cx43

Punctuate staining aggregations were observed between epithelial cells of the crypts (Figs. 1e,f). At the luminal surface of colon mucosa weak immunoreactivity in a part of studied cases was seen. A strong immunoreaction was present in muscularis mucosae (Fig. 1h). In the circular layer of muscularis externa, punctuate staining was observed particularly on the inner border of the circular muscle layer, near the submucosa (Fig.1i), although in some cases a significant level of staining was seen on the outer border of the circular muscle layer. The longitudinal muscle layer did not show apparent immunoreactivity for Cx43. In contrast to Cx26 and Cx32, reactivity with anti-Cx43 antibody was found in the colon arterioles.

We also observed focal cytoplasmic as well as paranuclear immunostaining of all studied connexins in epithelial cells, especially in the superficial epithelium of the colonic mucosa. There was no difference in expression of all studied connexins between samples derived from various sections of the colon. The studied markers were not detected in control samples, where immunostaining was performed with the omission of the primary antibodies.

Figs. 1a-f. Immunoreactivity for Cx26 (**a,b**), Cx32 (**c,d**) and Cx43 (**e,f**) in the mucosa of the normal human large intestine. Immunopositive Cxs are mainly distributed between epithelial cells. In the superficial epithelium, a weak cytoplasmic inmmunostaining for Cx32 is focally present (c). a: × 100, b: × 600, c: × 200, d,e: × 400, f: × 600. **Fig. 1g.** Immunoreactivity for Cx26 on the inner border of the circular muscle layer near the submucosa. × 200. **Figs. 1h,i.** Immunoreactive sites of Cx43 in the muscularis mucosae (h) and on the inner border of the circular muscle layer (i). h: × 200, i: × 400.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Discussion

Using a variety of techniques such as transmission electron microscopy, Lucifer yellow transfer and immunohistochemistry, gap junctions in the digestive tract of mammalian species have been identified. However, these studies were conducted mainly in animal tissues. In the human colon mainly ultrastructural studies of gap junctions distribution were performed [19]. Immunohistochemical studies in the human colon were restricted to Cx43 and Cx32 [4, 13, 15]. The present study demonstrated immunohistochemically for the first time the expression of Cx26 in human colon mucosa.

It has been demonstrated that expression of Cx26 in the epithelium of the mammary gland [23], endometrial epithelium [20], urothelium [6] and in the epithelium of vocal folds [21] is present, but we have shown for the first time the expression of Cx26 in mucosa of the colon. Furthermore, similarly to Dubina *et al*. [4], significant immunoreactivity to Cx32 and Cx43 between epithelial cells was seen. In addition, we also observed strong immunoreactivity to Cx43 in muscularis mucosae. Immunohistochemical analysis revealed weak expression of evaluated Cxs in paranuclear area as well as in the cytoplasm of epithelial cells in a part of studied cases. We suppose that during biosynthesis of Cxs or degradation of connexons, the used antibodies can give positive reaction with some epitopes, which exist in the moment of reaction. Many studies that were performed in various tissues, as well as cell lines, detected connexin polypeptides in the endoplasmic reticulum and the Golgi apparatus, where synthesis of connexins, posttranslational processing and maturation into gap junction channels take place [5]. Cytoplasmic localization of Cxs at the luminal surface of colonic epithelium can result from accumulation of degraded gap junction proteins during replacement of epithelial cells, but this process is still poorly understood and needs further studies.

Since gap junctions are sites of propagation or conduction of action potentials between cells [28], it is possible that they participate in generation of rhythmical peristaltic movements, as a result of contributing to the production of synchronous contraction in the muscle layer. The most common connexin in smooth muscle cells is Cx43. Previous studies have shown the presence of Cx43 between smooth muscle cells of the urinary bladder [16], myometrium [9], vessels [8, 26] and in the muscularis externa of the digestive tract in humans [13, 15] and animals [14]. Parallel to Nemeth *et al*. [15], in our study strong punctate reaction to Cx43 was seen in the circular muscle layer, especially near the submucosal border and in some cases in the outer division of the circular muscle layer. This is in agreement with previous electron-microscopic analysis, which revealed the presence of gap junctions in the circular muscle layer [1]. On the other hand, Mikkelsen *et al*. [13] have demonstrated no immunoreactivity to Cx43 in the entire circular muscle of the human colon, but we suppose that this might be primarily due to the use of more diluted antibodies (1:1000-1:3000) in their studies (compared to our higher concentrations). There was no evidence concerning expression of Cx26 in the muscularis externa of the human large intestine. The present study demonstrates for the first time the presence of Cx26 in the circular layer of muscularis externa, however we suggest that these observations need a better evidence in the future.

It is currently accepted that gap junctional intercellular communication plays a crucial role in the control of vascular integrity and vasomotor responses [7, 25]. In vasculature, three connexins (Cx37, Cx40 and Cx43) are reported to be present [12], but Cx43 seems to be dominant in vascular myocytes [8] and in endothelial cells [29]. Van Kempen *et al*. [25] found distribution of Cx37, Cx40 and Cx43 in aorta and coronary artery of several mammalian species. Furthermore, Wang *et al*. [26] showed the presence of Cx43 transcripts in cultured myocytes and frozen tissues from human vessels. Our findings of Cx43 expression in smooth muscle and endothelial cells of colon arterioles are consistent with these reports.

In conclusion, many physiological roles have been proposed for gap junctions in the digestive tract such as maintenance of tissue homeostasis, regulation of tissue development, electrical and metabolic coupling as well as regulation of cellular growth and differentiation. This study thus shows that all examined connexins exist within colon mucosa, and may participate in all above mentioned physiological processes through creation of an intercellular communication network.

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