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ARTICLE

C-kit positive cellular network in normal human bowel and in motility disorders

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ABSTRACT C-kit positive interstitial cells of Cajal (ICC) appear to have a key role in the normal motility function and development of intestine. They are pacemaker cells, which facilitate active propagation of electrical events and neurotransmission in the bowel wall. The cellular network of ICC is connected by gap junctions to each other and to the smooth muscle cells. Nitrergic innervation is considered to be the most important part of nonadrenergic, noncholinergic innervation in the enteric nervous system. Gut innervation has a complex three-dimensional system that is difficult to appreciate in thin sections. Whole-mount preparation produces a three-dimensional picture to better demonstrate the structure of neuronal networks and their relationship of branching and interconnecting nerve fibres to each other and to the neighbouring tissues. Histochemical and immunohistochemical staining methods combined with whole-mount preparation technique provides a new aspect for studying bowel innervation and distribution of the crucial intracellular molecular architecture. The characteristic profiles of c-kit positive cellular network and nitrergic innervation and their relationship with the smooth muscle fibres in normal gut and in motility disorders provide a morphological basis for investigating intestinal motility disorders. **Acta Biol Szeged 45(1-4):57-64 (2001)**

KEY WORDS

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motility disorders

Although it is now more than one hundred years since Cajal described small fusiform cells with prominent nuclei as forming network in the gastrointestinal tract, many questions about these cells remain unanswered (Rumessen 1993). In the human bowel the interstitial cells of Cajal (ICC) are localized at the level of myenteric plexus between the longitudinal and circular muscle layer, in the deep muscular plexus in the innermost part of the circular muscle layer, and within the circular muscle layer itself (Faussone-Pellegrini 1990; Wester 1999b). Morphological studies have been suggested three major functions for ICCs: (1) as pacemaker cells in the muscles of the gastrointestinal tract, (2) they facilitate active propagation of electrical events, and (3) they mediate neurotransmission (Sanders 1996). Recent reports indicate that transmembrane tyrosine-kinase receptor c-kit is essential for the development and function of the ICCs (Maeda 1992; Rumessen 1996). The immunoreactivity of c-kit is present in various cell types, but in the gut, c-kit is expressed only in ICCs and mast cells (Sanders 1996). ICCs appear to have a key role in the normal function and development of intestine (Hagger 1998; Toma 1999).

The normal motility of the gastrointestinal tract depends on the enteric nervous system (ENS), the muscle layers and the ICCs. It is currently accepted that the cellular network of ICCs is connected by gap junctions to each other and to the smooth muscle cells (Thuneberg 1989; Sanders 1996). Gap junctions are transmembrane channels that allow the exchange of ions, metabolites, and other small molecules (less than 1000 D) including second messengers such as cAMP, inositol triphosphate and Ca^{2+}, between the cytoplasms of adjacent cells (Garfield 1995; Simon 1999). Gap junctional intercellular communication is considered to play a crucial role in the maintenance of homeostasis and morphogenesis, cell differentiation, and growth control in multicellular organisms (Lau 1996; Morley 1997). The basic unit conforming the gap junction channels is a protein called connexin. Fourteen different homologous connexin proteins have been identified and characterized in rodents and eight in humans (Li 1993; Nakamura 1998; Nagaoka 1999). Connexin43 is regarded as the major gap junction protein in mammalians (Nakamura 1998; Mambetisaeva 1999).

The pathophysiology of Hirschsprung's disease (HD) is not fully understood. The most important histological finding in aganglionic colon is the absence of ganglion cells, which normally co-ordinate muscular activity by balancing the motor effects of the preganglionic cholinergic fibres and the inhibitory influence of the postganglionic adrenergic fibres

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(Puri 1993, 1997, 1998). Several authors have examined distribution of c-kit positive cells in the normal and HD bowel and reported marked reduction in c-kit immunopositive cells in the aganglionic bowel (Vanderwinden 1996a; Yamataka 1997; Horisawa 1998).

The whole-mount preparation technique produces a three-dimensional picture to better demonstrate the structure of neuronal networks and their relationship of branching and interconnecting nerve fibres to each other and to the neighboring tissues (Wester 1999). This method is extremely useful for morphological analysis of nerve distribution in luminal organs such as the gastrointestinal tract in both normal condition and in motility disorders (Nemeth 2000b). The optical limitation of conventional light microscopy in the whole-mount preparation technique is that the image viewed is the sum of a sharp-in-focus region and structures outside the focal plane. In confocal laser scanning microscopy, only in-focus light is imaged through a pinhole, whereas out-of-focus light is rejected by the edge of the pinhole. Confocal microscopy thus provides non-invasive optical serial sections through thick biological samples with preserved three-dimensional structure (Delorme 1998).

The aim of this study was to examine the distribution of ICCs and their relationship to the enteric nervous system in normal human gut and in motility disorders.

Materials and Methods

Bowel specimens

Full thickness small and large bowel specimens were obtained at autopsy from 18 children who died of non-gastrointestinal diseases (age: 3 months-12 years). Bowel specimens were opened along the antimesenteric border and were rinsed in PBS and cut into 1x1-cm pieces containing all layers. Subsequently, they were fixed in diluted Zamboni solution (2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer; pH7.3, 900 mOsM), stored overnight at 4ºC, rinsed in phosphate buffer saline for 8 hours at 4ºC and stored at -70ºC in small plastic tubes until use.

Whole-mount preparation

Whole-mount preparation was made in each specimen using fine-pointed forceps, microsurgical scissors and dissection microscope. Initially, the mucosa-submucosa was removed in one layer followed by separation of muscular layers from the submucosal layer. Subsequently, the circular muscle layer was peeled off meticulously fibre by fibre from the longitudinal muscle layer to which the myenteric plexus is adherent. (Fig. 1). The separated layers were fixed without stretching with fine pointed pins on Sylgard silicone elastomer tray (Dow Corning Europe, La Hulpe, Belgium) with the myenteric plexus on the surface of the longitudinal muscle layer. Similar fixation was made on the separated submucosa layer.

Double staining with NADPH-diaphorase histochemistry and c-kit immunohistochemistry

For histochemical staining with NADPH diaphorase, the tissue specimens were incubated in 1 mg/mL β-NADPH (Sigma, Dorset, UK), 0.25 mg/mL nitroblue tetrazolium (Sigma), and 0.5 % Triton-X in 0.05 mol/L Tris-HCl buffer (pH 7.6) at 37ºC until having a robust staining in the nitrergic neurons and nerves. The specimens were then rinsed 3 x 10 min in PBS solution. Thereafter the whole tissue specimens were incubated in mouse c-kit antibody (Novo Castra) in dilution rate 1:50 and 10% normal rabbit serum (DAKO) overnight at 4ºC. After being rinsed in PBS twice the tissue samples were incubated with a biotinylated secondary rabbit anti mouse antibody (DAKO) in dilution 1:200 for 2h. The specimens were rinsed again and incubated in ABComplex/HRP (DAKO) for 60 min and developed in 3.3'-diaminobenzidine tetrahydrochloride (Sigma, London, UK) for about 6 min. Finally the specimens were rinsed and embedded in Glycergel mounting medium (DAKO), covered by glass and investigated with traditional light microscope.

Double immunofluorohistochemistry with nNOS and c-kit

After finishing the whole-mount preparation the specimens were rinsed in PBS solution. Before the standard steps of the immunofluorescein staining procedure the specimens were incubated in 0.5% Triton-X in 0.05 mol/L Tris-HCl buffer (pH 7.6) solution at 37ºC for 4 h. Rinsing twice in PBS the tissues were incubated in nNOS (NOS1) antibody in dilution 1:50 (Santa Cruz, CA, USA) with 10% normal goat serum to prevent the aspecific linking overnight in 4ºC. After being rinsed in PBS twice the tissue samples were incubated with Texas Red labeled goat anti rabbit antibody (Molecular

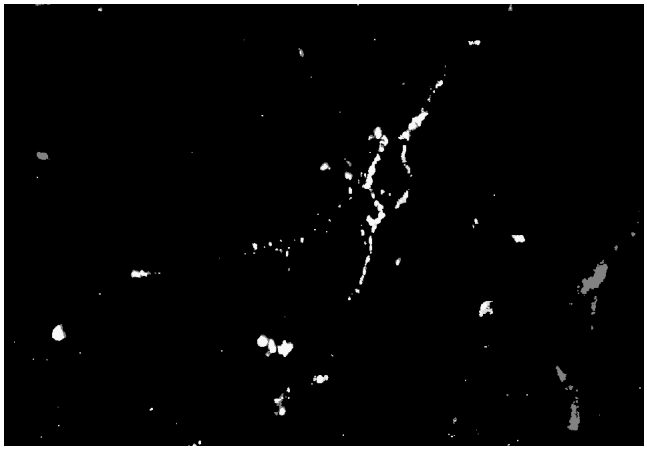

Figure 1. Typical appearance of a c-kit positive Interstital ICCs in the normal human bowel wall using confocal laser scanning microscopy. Original magnification: x 1000.

Probes, Leiden, The Netherlands) in dilution rate 1:50 as a secondary reagent for 2 h at room temperature (RT). Thereafter the whole tissue specimens were rinsed twice and incubated in mouse c-kit antibody (Novo Castra) in dilution 1:50 and 10% normal rabbit serum (DAKO) overnight at 4ºC. As secondary reagent in this step FITC conjugated rabbit anti mouse immunoglobulin (DAKO) was used in dilution 1:20 for 2h at RT. The tissue specimens were embedded into fluorescence mounting medium (DAKO) and investigated with confocal laser scanning microscopy.

Confocal laser scanning microscopy

Specimens were observed using an upright laser scanning confocal microscope (BIO-RAD 2000, Hamil Hamsted, UK) with immersion objectives (x40 NA 0.45, NPL Fluotar or x63 NA 0.75). Tissue specimens were excited using Krypton/Argon laser with excitation and barrier filters set for individual fluorophores according to their specific excitation-emission spectra. (λ= 568 nm, 488 nm, 647 nm). The emitted light was detected by a photomultiplier tube and converted, via an analogue-to-digital converter (BIO-RAD), into a digital pixelated image (512 x 512 picture elements). The detection pinhole was set for use different objectives accordingly. Offset and gain settings were determined at the start of each experiment and kept constant throughout, with laser powered recorded each time.

Three-dimensional pictures were created by overlapping from 12 to 16 images obtained from a single optical section thus obtaining a reconstruction of the sample through its thickness. Serial optical sections were collected at 0.72 μm intervals from the affected area forming a 3-D XYZ image.

Results

Double staining with NADPH-diaphorase histochemistry and c-kit immunohistochemistry

The whole-mount preparation demonstrated the three-dimensional cellular network of c-kit positive cells in the muscle coat of the human bowel. Typical appearance of c-kit positive ICCs was a stellate-like cell with 3-4 long and several short processes protruding in every direction (Fig. 1). The three-dimensional network formed by the c-kit positive cells was mainly located between the longitudinal and circular muscle layers of the bowel wall and at the innermost part of the circular muscle layer (Fig. 2A). This network was very dense between the circular and longitudinal muscle layers. There was also a dense network of the c-kit positive cells at the innermost part of the circular muscle layer. The c-kit positive cells were connected to each other mainly by their long processes. The c-kit positive cells located between the circular muscle fibres had only two long processes running always parallel with the muscle fibres and from the long processes several short processes ran into the muscle fibres making close connection with them. There were no morphological differences observed in the c-kit positive cellular network between the different levels of gastrointestinal tract and in different age groups.

The whole-mount preparation facilitated three-dimensional visualization of the meshlike network of NADPH-diaphorase positive nerve fibres in the myenteric plexus. There was a mesh of nerve bundles with ganglia, containing clusters of ganglion cells between the two muscle layers of the bowel wall. Morphology of the NADPH-diaphorase

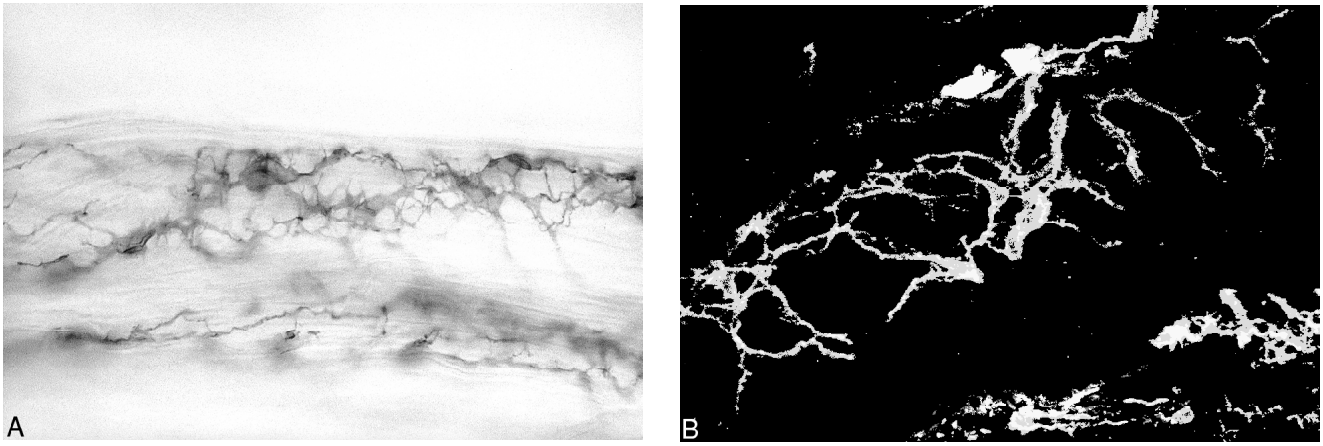

Figure 2. C-kit positive ICCs in the normal human bowel wall as forming a regular cellular network between the smooth muscle fibres **A.** Traditional ABC c-kit immunohistochemistry using light microscopy. Original magnification: x 430. **B.** c-kit immunofluorohistochemistry using confocal laser microscopy. Original magnification: x 430.

positive neuronal network varied between different parts of human gastrointestinal tract. The density of the typical architecture of the meshlike neuronal network increased in distal direction from the duodenum to the large bowel. The dense c-kit positive cellular network, located between longitudinal and circular muscle layer and at the innermost part of circular muscle layer intermingled with the myenteric plexus. Between the circular muscle fibres there was abundance of fine NADPH-diaphorase positive nerve fibres and c-kit positive cellular network running parallel with the muscle. The NADPH-diaphorase positive myenteric plexus was surrounded by a reticular network of c-kit positive ICCs (Fig. 3A).

Double immunofluorohistochemistry with nNOS and c-kit using confocal laser scanning microscopy

In whole-mount preparations immunopositivity to anti nNOS protein antibody was observed between the circular and longitudinal muscle layers as a three-dimensional network of neurons and nerve fibres. The expression of NADPH and the nNOS in enteric neurons and nerves were found in the same location in the entire human gastrointestinal tract. Immunoreactivity to anti c-kit protein antibody was observed in the space between the circular and longitudinal muscle layers surrounding the myenteric plexus (Fig. 3B). The c-kit positive cells formed a dense, three-dimensional network, connected to each other by the long processes (Fig. 2B). C-kit immunopositive cells were also observed between the smooth muscle fibres of the circular muscle layer. The long processes of c-kit positive ICCs ran parallel with the muscle fibres and from the long processes short fine processes made connections with the muscle fibres and c-kit positive cells.

Connexin43

Immunoreactivity to anti-connexin43 antibody was densely and homogeneously distributed as immunopositivity within the whole circular muscle layer. Strong connexin43 immunoreactivity was also seen at the innermost layer of the circular muscle and between the two muscle layers. On double staining the connexin43 expression was present in the cell body and processes of the c-kit positive ICCs (Fig. 4).

Aganglionic segment and transitional zone of Hirschsprung's disease bowel

c-kit

In the aganglionic segment of HD bowel, the c-kit positive ICC were sparse and localised mainly around the nerve trunks between the circular and longitudinal muscle layers (Fig. 5). In the transitional zone c-kit positive ICCs were higher in number than in aganglionic bowel but less than in normal bowel.

Connexin43

There was no expression of anti-Connnexin43 in the circular muscle layer and between the two muscle layers of the aganglionic bowel. In the transitional zone of the HD bowel specimens the immunolocalisation of connexin43 protein was in the processes of the c-kit positive ICCs but not in the cell bodies (Fig. 6).

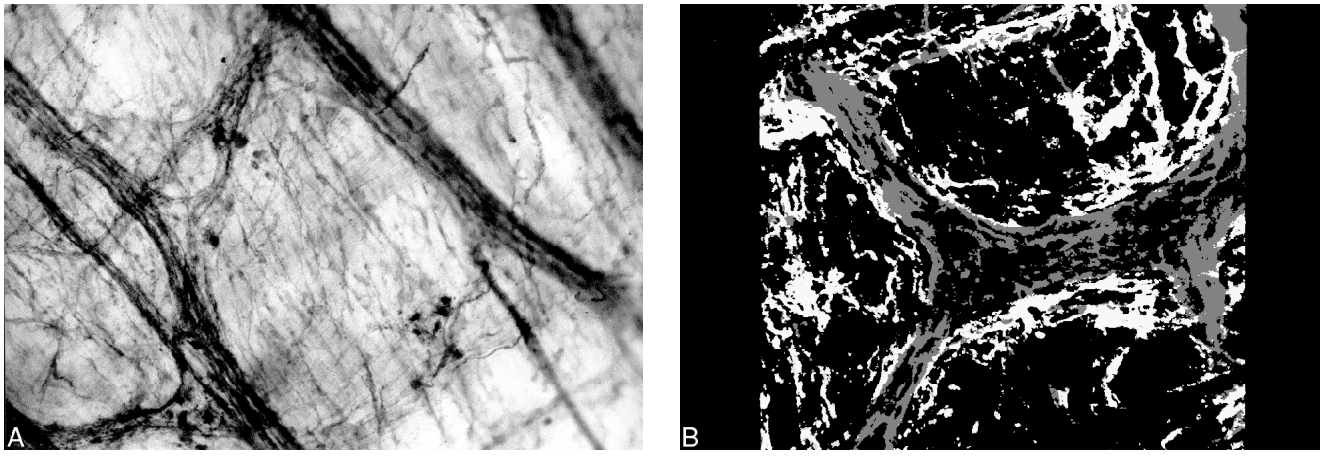

Figure 3. A. Whole-mount preparation of a normal human bowel wall using NADPH-diaphorase histochemistry and c-kit immunohistochemistry and conventional light microscopy. C-kit positive ICCs are forming a dense network surrounding the NADPH diaphorase positive myenteric plexus. Original magnification: x 200. **B.** Whole-mount preparation of a normal human bowel wall using double immunohistochemistry with nNOS and c-kit and confocal laser scanning microscopy. C-kit positive ICCs are forming a regular network around the nNOS positive nerve fibres of the myenteric plexus. Original magnification: x 200.

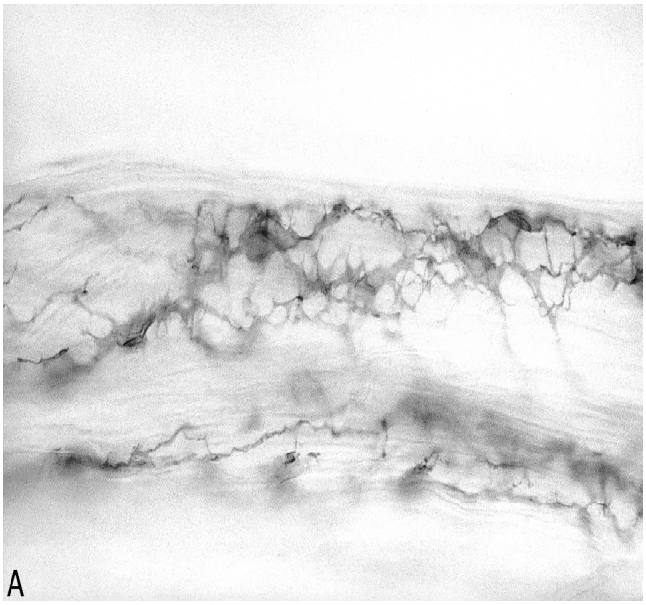

Figure 4. Expression of connexin43 in the ganglionic (normal) part of patient with Hirschsprung's disease. The immunolocalisation of connexin43 is present in the ICCs. Confocal laser scanning microscopy. Original magnification: x 300.

Discussion

Gut innervation has a complex three-dimensional structure that is difficult to appreciate in thin sections, which show only a part of the plexuses, neurons and glial cells. Whole-mount preparation is an elegant technique for the visualization of the myenteric plexus. It provides a method for the study of three-dimensional morphology of the meshwork of nerve fibres and neurons in detail (Fekete 1995; Wester 1999; Nemeth 2000b). Several investigators have used this technique in specimens from the human gastrointestinal tract with various staining methods ranging from silver impregnation to enzyme histochemistry and immunocytochemistry (Ferri 1982; Mebis 1990; Wedel 1998). The optical limitation of conventional light microscopy in the whole-mount preparation technique is that the image viewed is the sum of a sharp-in-focus region and structures outside the focal plane. In confocal laser scanning microscopy, only in-focus light is imaged through a pinhole, whereas out-of-focus light is rejected by the edge of the pinhole. Confocal microscopy thus provides non-invasive optical serial sections through thick biological samples with preserved three-dimensional structure (Delorme 1998).

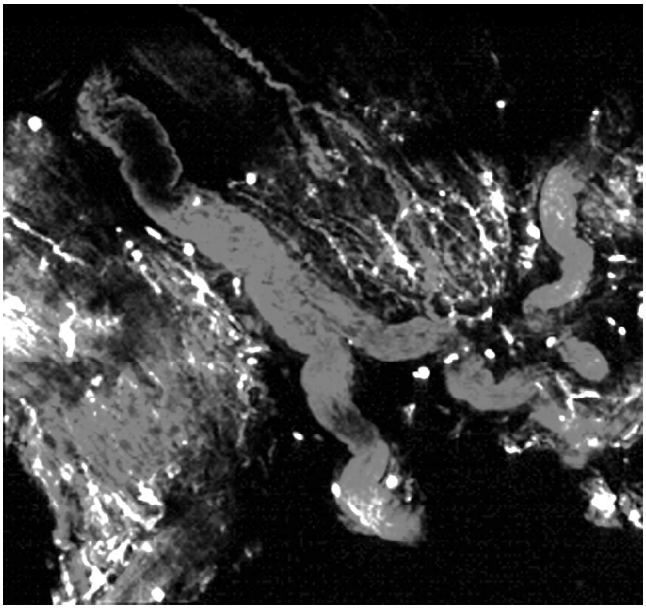

Figure 5. Whole-mount preparation of aganglionic large bowel segment from a patient having Hirschsprung's disease using double immunohistochemistry with nNOS and c-kit. ICCs are present in reduced number around the hypertrophied nerve trunks replacing the normal myenteric plexus Confocal laser microscopy. Original magnification: x 200.

Figure 6. Expression of connexin43 in the transitional zone of large bowel segment from a patient with Hirschsprung's disease. The immunolocalisation of connexin43 is present only in the long processes of ICCs. Confocal laser scanning microscopy. Original magnification: x 430.

Nitric oxide, whose formation is catalyzed by NOS from L-arginine, has been recognized as an inhibitory neurotransmitter to mediate smooth muscle relaxation in the mammalian gastrointestinal tract (Desai 1991). In 1990, Bult et al. first provided evidence that NO is released on stimulation of enteric nonadrenergic noncholinergic (NANC) nerves. Furthermore, the effects of exogenously administered NO or its precursors (L-arginine or nitrosocystein) mimic both the electrophysiological and mechanical effects of NANC nerve stimulation in the intestines of different animal species. In addition, inhibition of NO synthesis by L-arginine analogues or inactivation of NO by oxyhemoglobin have been demonstrated to block NANC nerve-induced relaxation in numerous parts of gastrointestinal system.

The morphology and distribution of ICCs have previously been difficult to study, as standard staining procedures for conventional light microscopy do not disclose this cell type. The c-kit proto-oncogene encodes a transmembrane protein tyrosine kinase receptor and the antibody raised against c-kit protein opened a new chapter in the investigation of c-kit positive cells (Maeda 1992; Sanders 1996). It is well recognised that ICCs are required for the generation of the smooth muscle electrical slow wave (Torihashi 1995). The electrical slow wave determines smooth muscle contractile activity. In the absence of an electrical slow wave, contractile activity is decreased and irregular, resulting in decreased intestinal transit. ICCs are intercalated between nerve terminals and smooth muscle cells, providing a means of transducing signals from neurotransmitters and mediating neurotransmission (Xue 1994). Recently, it has been suggested that ICC may produce NO and amplify inhibitory neurotransmission. Absence or reduction in number of ICCs has been implicated in several disorders of human gastrointestinal motility, including hypertrophic pyloric stenosis, HD, intestinal pseudoobstruction and slow transit constipation (Vanderwinden 1996b; Ekblad 1998; Horisawa 1998; Yamataka 1998; He 2000).

In the present study we combined histochemistry and immunohistochemistry to examine anatomical relationship between nitrergic neuronal network and c-kit positive cellular network in the normal human bowel using whole-mount preparation technique. This technique clearly demonstrated three-dimensional morphology of c-kit positive cellular networks and nitrergic nerves in detail. The distribution of c-kit positive cellular network and nitrergic neuronal network in the muscle layer of bowel was consistent with the previously reported findings (Faussone-Pellegrini 1990; Rumessen 1993; Sanders 1996; Yamataka 1996; Yamataka 1998). The three dimensional network formed by c-kit positive cells was located between the circular and longitudinal muscle layers of the bowel wall, at the innermost part of the circular muscle layer and within the circular muscle layer. The whole-mount preparation elegantly demonstrated nitrergic neuronal network in the myenteric plexus as mesh of nerve fibers with ganglia, containing cluster of ganglion cells between the two layers of the bowel wall.

The association between the c-kit positive cellular network and nitrergic neuronal network was clearly demonstrated by double-labelling histochemistry and immunohistochemistry and by double immunofluorohistochemistry using confocal laser scanning microscopy. Close association was observed between the c-kit positive cellular network and nitrergic neuronal network. The myenteric plexus was surrounded by a dense reticular network of c-kit positive cells. Although the c-kit positive cellular network was closely apposed to and intermingled freely with the myenteric plexus, no evidence indicating direct contact between the two networks could be found either by confocal laser scanning microscopy or by double staining with NADPH-diaphorase histochemistry and c-kit immunohistochemistry. The relationship between nerves and ICC remains unclear. In S_l/S_{ld} mice that do not produce membrane bound stem cell factor, the ligand for kit, small intestinal ICC at the level of the myenteric plexus are absent, suggesting that the stem cell factor is necessary for their development. The major source of stem cell factor is enteric neurons. This would suggest that primary deficit in enteric neurons secreting stem cell factor results in loss or a decreased in the number of ICCs. However, in contrast W/W^1 mutant mice that lack ICCs in the small intestine and have absent small intestinal electrical slow waves and abnormal uncoordinated motility have normal myenteric plexus, suggesting that ICC are not required for the development or maintenance of myenteric plexus (Malys 1996).

The development of double NADPH-diaphorase histochemistry and c-kit immunohistochemistry staining technique in whole mount preparation provides an easy and useful method for investigating c-kit positive cellular network and nitrergic neuronal network in the human bowel wall. In the present study this technique demonstrated close association between these two networks. Since the dense reticular network of ICCs intimately surrounds the nitrergic neuronal plexus and have abundant gap junctions connected with smooth muscle cells and nerve fibres, it is very likely that they play a role as mediator in neurotransmission, possibly the non-adrenergic, non-cholinergic inhibition. There are a number of intestinal motility disorders in which defects in innervation and loss or structural alteration of ICC have been reported, e.g. hypertrophic pyloric stenosis, HD, intestinal pseudo-obstruction and slow-transit constipation. The characteristic profiles of c-kit positive cellular network and nitrergic neuronal network observed in this study provide a morphological basis for investigating intestinal motility disorders.

It is currently accepted that the cellular network of ICCs is connected by gap junctions (Thuneberg 1989; Sanders

1996; Nemeth 2000a). Although the crucial role of gap junctions in the intercellular communication in the gut musculature has been shown by the diffusion experiment using neurobiotin (Farraway 1995), there have been no studies that have revealed the distribution of gap junction protein in the muscular coat in the human colon and also in the ICCs. The contraction relaxation sequence of the smooth muscle cells is a direct consequence of the cyclic depolarisation and repolarisation of membranes of the muscle cells. Gap junctions are sites of propagation or conduction of action potentials between cells of almost all tissues (Garfield 1995; Morley 1997). Gap junctions are intercellular channels that link cells to their neighbours and allow current carrying inorganic ions and small molecules to pass between cells, thereby facilitating electrical and metabolic coupling. The pores of the gap junction channels, which connect the interiors of two cells, are composed of connexins. Connexin43 is reported to be the most important gap junction protein in humans (Mikkelsen 1993; Lau 1996; Nagaoka 1999). By using a type-specific antibody against connexin43, we have demonstrated a unique localisation of gap junction protein connexin43. Double immunostaining revealed that connexin43 expression was present in the cell body and processes of the c-kit positive ICCs. The expression of connexin43 protein is abundant in the ICCs in normal bowel. In the innermost layer of the circular muscle, and between the two muscle layers where the ICCs form a three-dimensional network, strong connexin43 expression was present in the cytoplasm of ICCs and between the ICCs and the smooth muscle fibres. Confocal laser scanning microscopy clearly demonstrated that the gap junction protein connexin43 was present in the circular muscle fibres probably where the ICCs make contact with the muscle fibres.

Some studies have suggested that gap junction channel expression or regulation is altered in various disease states (Garfield 1995; Lau 1996; Mambetisawa 1999; Nemeth 2000a). Changes in gap junction channel composition, or on the levels of connexin expression, have been proposed as determinants for the ability of action potentials to propagate across the neighbouring cells. Immunohistochemical studies have shown that the expression of gap junction channel proteins decreases in the areas affected by myocardial infarction (Lau 1996). Other investigations have shown that chronic exposure of cardiac cells to cAMP can change the level of expression of individual connexins (Morley 1997).

Gap junctions play important roles as sites for the coordination of action of substances, which either stimulate or inhibit the smooth muscle cells to contract. The propagation of electrical signals is key to control of contractility. Lack of conducted action potentials and inactivity of the smooth muscle cells are promoted by decreases in pacemaker activity, excitability of muscle cells and cell-to-cell coupling. Any mechanism that suppresses the generation of action potentials, closes gap junctions, or decreases their numbers inhibits the active events and thereby affects contractility. The lack of expression of connexin43 in the circular muscle layer and between the circular and longitudinal muscle layers in the aganglionic bowel and decreased connexin43 expression in the transitional zone suggest that the intercellular signalling is impaired in Hirschsprung's disease. Lack of gap junctions in HD may prevent exchange of hormones, neurotransmitters and other antagonists between ICCs and smooth muscle cells and thereby level of ability of action potentials to propagate, causing motility dysfunction. Cellular networks of pacemaker activity in intestinal motility are still matter of debate. The hypothesis that gap junctions represent an additional pathway for homotypic and heterotypic cell-cell interactions in the gastrointestinal tract is truly exciting. More connexins such as Cx26, Cx32 and Cx45 are surely involved in the nervous system of bowel under physiological and pathological conditions. Future studies on gap junctional intercellular communication in the bowel wall may provide important clues for the understanding of regulatory mechanism of intestinal motility. These studies range the human motility disorders, genetically induced mouse aganglionosis and the changes of the gap junctional proteins in chemically induced segmental aganglionosis. Specific antibodies and molecular probes for distinct connexins are now available, such that they can be identified by immunocytochemistry, immunoblot and molecular biology strategies. However, functional analysis of gap junction mediated cell-cell communication requires further methodoligical approaches that comprise metabolic cooperation, injection of low-molecular weight dyes and standard electrophysiological techniques.

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