

## IDENTIFICATION OF THE LOCATION OF THE COLONIC PACEMAKERS: A HISTOLOGIC STUDY

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### 1. ABSTRACT

A recent electrophysiologic study has suggested the existence of 4 pacemakers in the colon which generate the electric waves that are responsible for the colonic motor activity. These pacemakers are located at the cecal pole, the cecocolonic junction, the mid third of the transverse colon and at the colosigmoid junction. We investigated the hypothesis that these pacemaker sites contain ICCs in great numbers and that the electric waves generated from these sites are transmitted by a chain of ICCs to the remaining colon. Specimens were taken from the 4 electrophysiologically defined pacemaker sites and the remaining colon of 24 cadavers. They were subjected to c-kit immunohistochemistry tests. Controls for antisera specificity consisted of tissue incubation with normal rabbit serum substituted for the primary antiserum. The morphometric study was performed by submitting the immunohistochemically-stained sections to image analysis in order to determine the area percent of immuno-reactivity in comparison to the total area of fields examined in the sections. Data obtained from the image analyzer were statistically analyzed. Immunohistochemical and morphometric studies have shown that the 4 electrophysiologically defined pacemaker sites contained a significantly higher proportion of ICCs than the remaining colon, the ICCs at these sites being multipolar and forming a network surrounded by a fibrous tissue wall. In the remaining colon, the ICCs were bipolar and arranged in a linear fashion. The study revealed the existence of a network of ICCs at the 4 pacemaker sites forming the 'pacemaker nodes' (PMNs). In the rest of the colon, a chain of ICCs extended in the colonic wall in a linear fashion forming the 'pacemaker

bundles' (PMBs). We postulate that the colonic electric waves start at the PMNs and spread in the colon along the PMBs.

### 2. INTRODUCTION

The interstitial cells of Cajal (ICCs) are specialized cells existing in the gut. They are located in the myenteric plexus, between the circular and longitudinal muscle layers of the gut and in the innermost part of, and within, the circular muscle layer (1-3). They are considered to be generators of spontaneous pacemaker activity in the smooth muscle layers of the gut (4-8) and may also be involved in neurotransmission (4). ICCs form groups of 2-3 cells each which are connected with analogous groups of 2-3 smooth muscle cells along the ramification of nerve fibers (9). They run singly or in rows inside the muscle sheaths. The ICCs are connected with each other and with the smooth muscle cells by their overlapping contiguous plasma membranes or by elastic bridges (9). Close contact with the nerve endings exists only inside the muscle sheaths. It has been postulated that among all the non-neuronal and non-muscular cell types only the ICCs have cell-to-cell links with smooth muscle cells, nerve cells and nerve endings, supporting the suggested role of ICCs in the pacemaker activity (9, 10). Loss of ICCs is associated with abnormal electric activity in the gut including loss of electric slow waves (11-15).

Recent electrophysiologic studies (16, 17) had suggested the presence of at least 4 pacemakers in the colon that generate the electric waves responsible for the colonic motor activity. It was presumed that these pacemakers were

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located at the cecal pole, the cecocolonic junction, the mid third of the transverse colon and the colosigmoid junction (16, 17). A demonstrable change of the electric wave pattern from one part of the gut to the other occurred at the above mentioned locations. It was postulated that these areas are the potential sites of the colonic pacemakers which discharge electric waves to the related colonic segments (16, 17).

In view of the above mentioned electrophysiologic studies, we hypothesized that the suggested sites of pacemakers contain maximal accumulation of ICCs, and that the generated electric waves are transmitted by the ICCs which are scattered along the gut. This hypothesis was investigated in the current study.

### 3. MATERIAL AND METHODS

#### 3.1. Material

The study comprised 24 cadaveric specimens (14 men, 10 women, mean age  $41.6 \pm 10.4$  SD years, range 27–52). The cadavers had normal gastrointestinal tract. Specimens were taken from the colon from the previously electrophysiologically defined sites of pacemakers and from other parts of the colon. Each specimen was  $1 \times 1$  cm. Two specimens were taken from the lower pole of the cecum (C) and another 2 similar specimens from the distal part of the C, with 2–3 cm apart. Two specimens were taken from the cecocolonic junction and 3 from the ascending colon (AC) with 3–4 cm apart and one from the right transverse (TC). From the mid  $\frac{1}{2}$  of the TC, 3 specimens, 3 cm apart, were taken and another 3 specimens were taken from the left TC and descending colon (DC) with 3 cm apart. Two specimens were taken from the colosigmoid junction and 3 from the sigmoid colon with 2–3 cm apart. After fixation in 10% formalin, the specimens were dehydrated with alcohol, cleared in xylene and then embedded in paraffin wax.

#### 3.2. Methods

Longitudinal sections of  $5 \mu\text{m}$  thickness were cut and put on slides with positive (Poly-lysine) charge. They were stained with H & E, Masson's trichrome and immunohistological staining using c-kit antibody. The sections were then examined by light microscopy. The cells positive for c-kit showed brown deposits and blue nuclei. The c-kit was tested for the positive and negative tissue control.

For immunohistochemical processing, tissue sections were rehydrated in KPBS at room temperature for 20 min., blocked with 10% normal goat serum for 20 min., and incubated overnight at  $4^\circ\text{C}$  with the primary antiserum, a rabbit polyclonal IgG antibody to the human c-kit protein (Oncogene Research Products, Cambridge, MA), diluted 1:100 in KPBS, 0.05% goat serum and 0.1% Triton X-100. The next day, slides were rinsed with KPBS three times (each 10 min) and then incubated with a Cy3-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) at room temperature for 2 hours at a dilution of 1:800 in KPBS, 0.05% goat serum, and 0.1% Triton X-100. Because mast cells also contain the c-kit receptor and thus stain positive with c-kit

antibodies, dual staining with fluorescein-avidin DCS (Vector Laboratories, Burlingame, CA) diluted 1:200 for 2 hours was used to specifically identify mast cell staining. Slides were again washed three times with KPBS and overslipped. They were then imaged using an Olympus Fluoview 500 scanning confocal microscope.

Controls for the specificity of the antisera consisted of incubation of the tissue with normal rabbit serum substituted for the primary antiserum.

#### 3.3. Morphometric Study

Dense brown deposits of a fixed degree and strong immunoreactivity were submitted for image analysis using the Leica imaging system (Leica Qwin, Cambridge, UK). The image analyser was first calibrated to convert the measurement unit produced by the image analyzer program (pixels) into actual micrometer units.

Ten fields from each colonic section were selected and the area as well as the area percent of the dense brown deposits were measured by the image analyzer in relation to a standard measuring frame which was  $4704 \mu\text{m}^2$  using magnification X 400.

$$\text{Area percent} = \frac{\text{area.of.dense.brown.deposit}}{\text{measuringframe.area}} \times 100$$

Three main layers were measured of each colonic segment: myenteric plexus, inner circular and outer longitudinal muscle layers. The area percent obtained from the image analyzer was subjected to statistical analysis.

#### 3.4. Statistical analysis

The results were analyzed statistically using the arithmetic mean (X), standard deviation (SD) and analysis of variance (ANOVA) according to Mould (18). Results were considered significant when probability (P) was  $<0.05$ .

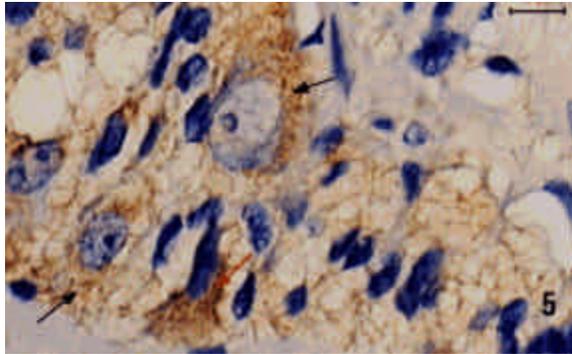
## 4. RESULTS And DISCUSSION

Immunohistochemical studies have shown 2 types of c-kit positive cells in the colon: mast cells and ICCs. The mast cells appeared as rounded cells with rounded nuclei; the cell wall showed no branches. They occurred in all layers of the colon. In contrast, the ICCs had a fusiform shape with dendritic processes and large nuclei (figure 1); they occurred only in the circular and longitudinal muscle layers.

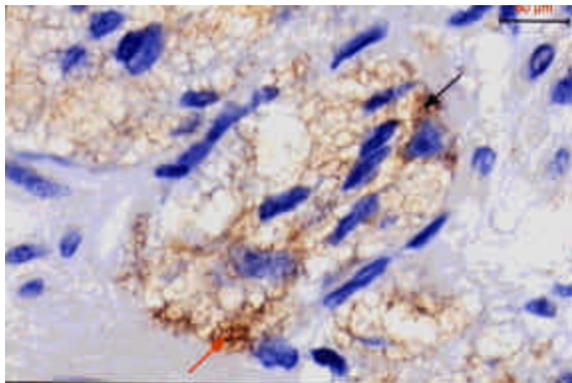
#### 4.1. The ICCs at the electrophysiologic pacemaker sites

Sections from the 4 potential pacemaker sites: cecal pole, cecocolonic junction, mid third of transverse colon and colosigmoid junction, were examined. The ICCs were identified in the intermuscular layer between the circular and longitudinal muscle layers. They formed a network in the intermuscular layer (figure 2). Each ICC had multiple cytoplasmic processes which anastomosed with more than one adjacent cell. The group of cells forming the network was surrounded by a wall of fibrous tissue (figure 3). Amassed ICCs were detected at the aforementioned potential pacemaker sites. We failed to find a connection between these masses of ICCs and the enteric nerve plexus (ENP).

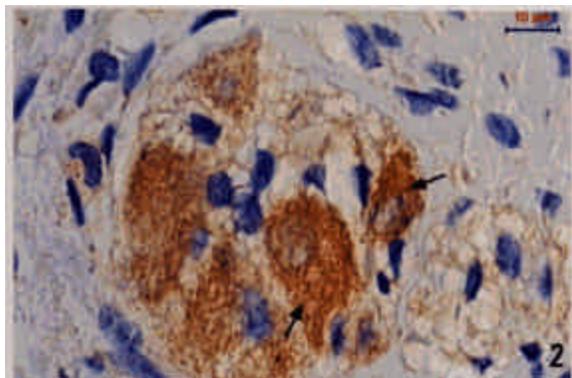
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**Figure 1.** Photomicrograph of a section of the colonic wall showing c-kit-positive ICCs (arrows) with elongated body carrying dendritic processes (c-kit immunostain counterstained with hematoxylin X 400).



**Figure 2.** Photomicrograph of a section of the colonic wall showing c-kit-positive ICCs (arrows) arranged in masses (c-kit immunostain counterstained with hematoxylin X 400).



**Figure 3.** Photomicrograph of a section of the colonic wall showing a mass of c-kit-positive ICCs forming a network. The ICCs (arrows) have multiple cytoplasmic processes which anastomose with the adjacent cells (c-kit immunostain counterstained with hematoxylin X 400).

The ICCs in the cellular network had multipolar dendritic processes, while the cells in the remaining colonic wall were mostly bipolar. In the cellular network one cell was connected to multiple cells by its multiple processes. This differs from the bipolar ICCs where the individual cell was connected to the adjacent cell in a linear fashion by the

polar dendritic processes (figure 4). The linear arrangement of the bipolar cells extended from the cellular network to the next network through the muscle layer in the gut wall (figure 5). Some of the ICCs, during their passage in the colonic musculature, were incorporated in the ENP.

### 4.2. Results of a quantitative morphometric study

Statistical analysis of the data obtained from the image analyzer of the mean area per cent of immunoreactivity for c-kit in the colon is shown in (figure 6). The cecal pole ( $40.6 \pm 7.2$ ) exhibited a significantly higher mean area per cent than the remaining cecum ( $8.4 \pm 1.1$ ,  $p < 0.001$ , figure 6). Similarly in the cecocolonic junction ( $46.2 \pm 8.6$ ), the mean area per cent showed significantly higher readings than the rest of the AC ( $10.2 \pm 1.6$ ) and the right third of the TC ( $9.4 \pm 1.4$ ,  $p < 0.001$ ). Likewise, the mean area per cent of immunoreactivity for c-kit in the mid TC ( $36.7 \pm 6.8$ ) recorded significantly higher values than the rest of the TC ( $8.3 \pm 1.3$ ) and the DC ( $9.4 \pm 1.6$ ) ( $p < 0.001$ , figure 6); in the colosigmoid junction ( $39.8 \pm 6.6$ ) also it was significantly higher than in the sigmoid colon ( $10.3 \pm 1.74$ ,  $p < 0.001$ , figure 6).

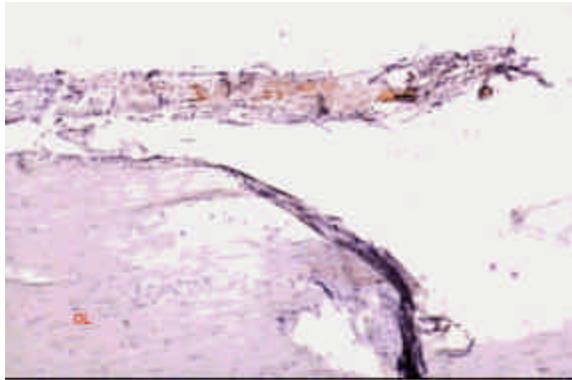
The current study could shed some light on the morphological and morphometric structure of the ICCs at the potential sites of the colonic pacemakers as identified by the electrophysiologic studies (16, 17). The ICCs could not be demonstrated by the routine H & E and Masson's trichrome stains. It was however possible with these stains to demonstrate the histologic structure of the colon as well as the distribution of the enteric nerve plexuses in the colon. Staining with C-kit antibody could detect the ICCs. This method could identify two different c-kit positive cells: mast cells and ICC. The mast cells could be differentiated from the ICCs by their round shape without dendritic processes.

### 4.3. Colonic pacemaker nodes and bundles: a novel concept of colonic motor transmission

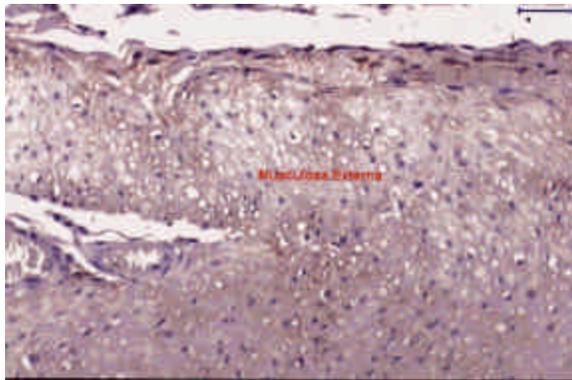
We observed a difference in density and distribution of the ICCs in the colon. The cells accumulated as rounded or oval masses in certain areas in the colon but were arranged in a linear fashion in the remaining colon. We found the ICCs amassed at the previously electrophysiologically determined pacemaker sites which comprise the cecal pole, the cecocolonic junction, the mid transverse colon and colosigmoid junction. The density of the ICCs in these areas was significantly greater than in the rest of the colon. This was evidenced microscopically and confirmed morphometrically by the image analyzer. We give the name "pacemaker nodes" (PMNs) for the masses of ICCs occurring at the above mentioned sites. These PMNs had a spherical or oval shape and contained ICCs of a special type and arrangement. Most of the cells in these nodes were multipolar with multiple processes connecting them with each other in a network arrangement. Each mass was surrounded by a capsule of fibrous tissue.

From these nodes arose a chain of the bipolar ICC type which extended in the colonic wall in a linear, bundle-like arrangement with the cells banded together at their poles. The cell density was significantly less than in the nodes, as could be demonstrated microscopically and

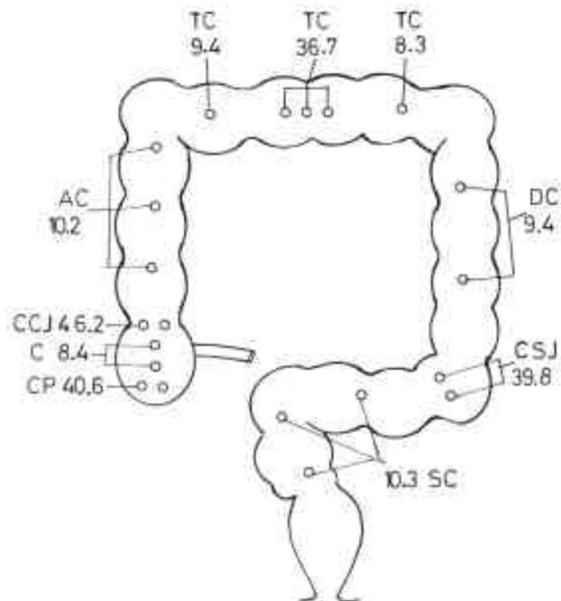
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**Figure 4.** Photomicrograph of a section of the colonic wall showing c-kit-positive ICCs arranged in a linear fashion. Each cell is attached to the adjacent cell by the polar dendritic processes (c-kit immunostain counterstained with hematoxylin X 100).



**Figure 5.** Photomicrograph of a section of the colonic wall showing c-kit-positive ICCs extending along the muscle layer to reach the cellular network (c-kit immunostain counterstained with hematoxylin X 40)



**Figure 6.** Regional distribution (area %) of ICCs in the colon.

by the morphometric assay. These 'pacemaker bundles' (PMBs) extended in the colon from one PMN to the other. The PMNs were located in all the studied specimens in the intermuscular plane, i.e. between the circular and longitudinal muscle layers of the colon, while the PMBs extended in the longitudinal muscle layer. We could not define a relation between the PMNs and the myenteric nerve plexuses. The latter were located in the intermuscular or submucosal layer, while the PMNs were located intermuscularly.

### 4.4. Mode of action of the pacemaker nodes and bundles

Previous studies (4-8, 11-17) have shown that the ICCs evoke electric activity which initiates the colonic motility. We do not know whether these cells initiate the electric waves autonomously or receive stimulation from the enteric nerve plexus (ENP). While the PMNs exhibited no connection with the ENP, the cells of the PMBs were occasionally related to the plexus along their course in the longitudinal muscle layer. We have however demonstrated in preceding studies (19, 20) that paralyzes of the colonic extrinsic innervation or the ENP did not abolish the electric waves but effected a change in the wave parameters and rhythmicity. This would suggest that the electric activity is initiated by the ICCs, but probably under the control of the extrinsic and intrinsic innervation. Other investigators (15, 21) reported that block or removal of ICCs from the gut wall abolished the electric waves of the gut.

### 4.5. Mechanism of colonic giant migrating (mass) contractions: a novel concept

We postulate that the electric waves originate from the PMNs and travel along the colonic wall through the PMBs. Thus, the cecal node effects wave transmission to the cecocolonic junction, while the PMN in the cecocolonic junction starts another wave of electric activity that is carried by the PMB to the PMN of the TC. The latter node would initiate the electric activity that is transmitted by the PMB along the left third of the TC and DC down to the colosigmoid PMN. Subsequently, the colosigmoid PMN starts another wave of electric activity which spreads into the sigmoid colon. These waves of electric activity are responsible for the colonic motility. Thus, the segmental pattern of the colonic contractile activity, which occurs in succession, seems to have its explanation in the pacemaker location within the colon. It further appears to elucidate the mechanism of mass contraction of the colon. In all probability the segmental colonic mass contraction depends on the presence of a pacemaker pertinent to the contractile segment. According to investigators (22, 23), a wave of colonic mass contraction starts in the cecum and involves the right half of the AC to the mid of the TC. The subsequent wave of mass contraction involves the left half of the TC and the DC. The sigmoid colon contracts independently. These physiologic colonic segmental 'en masse' contractions appear to be evoked by the 4 colonic pacemakers located at the above mentioned sites.

In conclusion, the current study could demonstrate immunohistochemically and morphometrically that the ICCs amassed in 'pacemaker nodes' (PMNs) at the electrophysiologically determined pacemaker sites. The

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PMNs contained multipolar ICCs connected with each other in a network-like arrangement. From these PMNs emerged a chain of bipolar ICCs that extended linearly in the colonic wall forming the PMBs. We postulate that the electric waves originate from the PMNs and spread along the colon via the PMBs. This histomorphologic and morphometric study seems to elucidate the mechanism of colonic mass contraction.

### 5. ACKNOWLEDGMENT

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**Key Words:** Electric waves, Interstitial cells of Cajal, C-Kit, Image analyzer, Pacemaker node and bundle

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