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Apoptotic Cell Death of Human Interstitial Cells of Cajal

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Abstract

Interstitial cells of Cajal (ICC) are specialized mesenchyme-derived cells that regulate contractility and excitability of many smooth muscles with loss of ICC seen in a variety of gut motility disorders. Maintenance of ICC numbers is tightly regulated, with several factors known to regulate proliferation. In contrast, the fate of ICC is not established. The aim of this study was to investigate whether apoptosis plays a role in the regulation of ICC numbers in the normal colon. ICC were identified by immunolabeling for the c-Kit receptor tyrosine kinase and by electron microscopy. Apoptosis was detected in colon tissue by immunolabeling for activated caspase-3, terminal dUTP nucleotide end labeling, and ultrastructural changes in the cells. Apoptotic ICC were identified and counted in double labeled tissue sections. Apoptotic ICC were identified in all layers of the colonic muscle. In the muscularis propria $1.5 \pm 0.2\%$ of ICC were positive for activated caspase-3 and in the circular muscle layer $2.1 \pm 0.9\%$ of ICC were positive for TUNEL. Apoptotic ICC were identified by electron microscopy. Apoptotic cell death is ongoing in ICC. The level of apoptosis in ICC in healthy colon indicates that these cells must be continually regenerated to maintain intact networks.

Keywords

caspace; colon; gastrointestinal motility; electron microscopy; TUNEL

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INTRODUCTION

Interstitial cells of Cajal (ICC) are specialized, mesenchyme-derived cells that form networks of cells in the muscularis propria of the gastrointestinal tract. ICC appear to have at least four separate physiological functions. ICC pace the intestine (1-3), amplify neuronal signals (4,5), act as mechanosensors (6) and set the smooth muscle membrane potential gradient (7). Furthermore, ICC-like cells can be identified outside the gastrointestinal tract. The urinary tract (8), vascular smooth muscle (9) and uterus (10) contain ICC-like cells and, like gut ICC, these cells can also generate pacemaker potentials (8,11).

Given the important role ICC play in the control of motility, it is not surprising that ICC numbers are tightly controlled. Activation of the c-Kit receptor (12), release of neuronal nitric oxide (13), and activation of the 5HT_{2B} receptor (14) increase proliferation of ICC in mice. Conversely, insults to the gastrointestinal tract can decrease ICC numbers. Other animal studies have shown that within weeks of an insult, ICC networks can be depleted and then repopulate over a period of up to two months after the insult is removed (15,16).

Loss of ICC has been associated with several motility disorders including gastroparesis, intestinal pseudo-obstruction and slow transit constipation (17-19). The mechanism for loss of ICC in humans has not been demonstrated and while loss of ICC is well documented in motility disorders and in animal models of disease, it is not clear if there is also ongoing ICC loss and turnover in non-diseased tissue. Apoptosis, a well recognized mechanism of cell death in a wide variety of cell types, such as intestinal mucosa, has not to date been shown to occur in ICC.

The aim of this study was to determine whether loss of ICC occurs through an apoptotic cell death process in non-diseased human intestine. In this study, we demonstrate expression of the apoptotic markers activated caspase-3 and terminal dUTP nucleotide end labeling (TUNEL) as well as morphological changes consistent with apoptosis in the normal human colon. These data provide novel evidence indicating an ongoing turnover of normal ICC networks. Some of this work has been previously reported in abstract form (20,21).

MATERIALS AND METHODS

Patients

Tissues for these studies were obtained from patients at Mayo Clinic under protocols approved by the institutional review board. These tissues had not been previously studied. All enrolled patients had non obstructive colon cancer and underwent a partial colectomy for their disease (Table 1). The patients had no gastrointestinal motility problems prior to surgery and were not diabetic. Tissue samples were collected at least 5 cm away from the edge of the tumor. The colon was reported as visually and pathologically normal at the time of surgery with no evidence for dilatation or ischemia. Tissues were processed for either activated caspase-3, TUNEL or both (Table 1). We also used tissues from 3 additional normal colons to investigate whether apoptotic ICC could be identified by electron microscopy. Two were descending colons from a 45yr old female and a 68yr old male, both with sigmoid colon cancer and the third one was an ascending colon from a 75yr old female with cecal cancer.

Methods

Immunohistochemistry for activated caspase-3 and c-Kit bright field microscopy—These tissues were collected in the operating room and immediately fixed in formalin and embedded in paraffin. Detection of activated caspase-3 was achieved using antibodies (Biocare Medical, Concord CA) to an epitope that is generated when the intact

caspase-3 is activated by cleavage (22). Five or ten μm thick sections from formalin fixed, paraffin embedded blocks were labeled for examination under light microscopy according to a standard procedure. c-Kit was detected using an antibody from MBL (Nagoya, Japan). Positive controls for c-Kit and caspase-3 as well as negative controls (no primary antibody) were included in each run. Activated caspase-3 positive ICC were counted under a bright field upright BX51 WI microscope at 600X magnification using a 60X, 0.90 NA water objective (Olympus, Melville, NY). Positive labeling for activated caspase-3 positive cells was confirmed by examining the cells for fluorescence of Vulcan Fast Red™ (Biocare) emitted at 645 nm when excited at 560 nm. For each section, the area of smooth muscle in the images was measured by digital morphometry with commercial imaging software (Metamorph, Universal Imaging, Downingtown, PA or Analyze™, Mayo Foundation, Rochester, MN). ICC were identified by their shape and c-Kit immunoreactivity. The number of activated caspase-3 positive ICC was counted from every region of the smooth muscle (typically 360 fields per specimen). The slides were examined in random order and activated caspase-3 positive cells counted. Data were then expressed as the number of positively labeled cells per mm^3 smooth muscle. Ten μm sections were prepared from 4 control tissues to identify ICC and count the proportion of ICC that were positive for activated caspase-3.

Fluorescence immunohistochemistry for TUNEL and c-Kit—To minimize the effects of ischemia, tissues for these experiments were collected in the operating room and immediately placed in ice-cold F12 medium, dissected and flash frozen in isopentane on dry ice. Fluorescence immunohistochemistry for c-Kit (using the antibody from MBL) and TUNEL was done on 10-12 μm sections using a modification of the procedure published by the manufacturer of the TUNEL kit (TACS sTdT, Trevigen Inc, Gaithersburg, MD). To counterstain nuclei, sections were mounted in SlowFade™ containing 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI, Molecular Probes, Eugene, OR).

Multiple control experiments were done to exclude non-specific labeling and false positive results. Positive control experiments for TUNEL included an intrinsic positive control in the mucosa or gastrointestinal lymphoid tissue of each specimen where apoptotic cells were always observed. TUNEL was done separately on some specimens to confirm that the amount of TUNEL-positive cells obtained in the single labeled tissues was similar to the amount of TUNEL positive cells in tissues subjected to the longer double label technique. Six to twelve slides with 1-3 non-adjacent sections on each slide were examined from each tissue for evidence of TUNEL labeled ICC using a laser scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany) at 400X and 1000X magnification. Cells were counted under epifluorescence illumination and deemed to be TUNEL positive only if the fluorescein signal colocalized with the DAPI signal in the nucleus of the cells. This was confirmed by collecting confocal images using the optimal pinhole size for the 100 X, 1.4 NA, oil objective at 543 nm (z axis step 0.4 μm). TUNEL positive ICC were identified by their location, characteristic shape and immunoreactivity for c-Kit. The slides were examined in random order.

We counted all TUNEL-positive cells and specifically ICC that were TUNEL positive from all the 450 μm diameter non-overlapping fields in the circular muscle regions of each section. This was achieved by using a computer controlled motorized stage. To allow comparison between tissues and between different labeling methods, data were converted to cells per mm^3 of tissue by correcting for the calibrated area of the visual field for each objective.

The following lasers and emission and excitation filters were used to visualize the labeled structures and collect images: TUNEL positive nuclei were labeled with fluorescein (Ex

488, Em 505-550), c-Kit positive cells were labeled with Cy3 (Ex 543, Em 560-615) and nuclei were labeled with DAPI (Ex 364, Em 385-470). Images were collected in multi-track mode to minimize cross over between channels. The confocal images were imported into Analyze™ and were reconstructed in three dimensions using volume rendering as previously reported (23). Positively labeled structures were mapped as binary objects in each of the images by thresholding each image based on the brightness of the voxels to remove background fluorescence. The object maps were then combined for each of the fluorophores (DAPI, fluorescein and Cy3) to establish the extent of colocalization between the positively labeled structures. To illustrate the colocalization, a two dimensional rendering of the volume was created showing the objects as partially transparent.

Electron Microscopy—Tissues were processed according to a standard procedure. The colon was cut into strips 10 mm long that contained the muscularis propria and a small portion of the submucosa. The strips were fixed for 6 hours in a solution of 2% glutaraldehyde 0.1M in cacodylate buffer, pH 7.4. After four rinses in cacodylate-buffered solution containing 0.22M sucrose, the strips were post-fixed for 1 hour in 1% OsO₄ in 0.1M phosphate buffer. After a rinse for 30 minutes in ddH₂O, the strips were stained *en bloc* in 2% uranyl acetate for 30 minutes at 55°C and rinsed in ddH₂O for 10 minutes. The strips were then dehydrated in graded ethanol and embedded in Spurr using flat moulds in order to obtain full-thickness sections with the circular muscle in cross-section. Semi-thin sections were stained with a solution of toluidine blue in 0.1M borate buffer and then observed under a light microscope. Ultra-thin sections of the selected areas were obtained with the LKB NOVA ultramicrotome using a diamond knife and stained with a saturated solution of uranyl acetate in methanol (50:50) for 12 minutes at 45°C, followed by an aqueous solution of concentrated bismuth subnitrate for 10 minutes at room temperature. These sections were examined under a JEOL 1010 electron microscope and photographed.

Statistical Analysis—We used commercial software (InStat, Graphpad Inc., San Diego, CA) for statistical tests. Data were compared by unpaired, two-tailed t tests and non-parametric Mann-Whitney tests where the data did not follow a normal distribution. Where population data are compared, they are expressed as means with standard errors. A $P < 0.05$ was taken to be statistically significant.

Materials—Unless indicated, reagents were from Sigma-Aldrich (St Louis, MO).

RESULTS

ICC were identified by their location in the muscularis propria and myenteric plexus regions and by the presence of two or more processes projecting from the cell body. Activated caspase-3 immunoreactivity was present in a subset of these ICC (Figure 1a,b). Also, in all sections, the antiserum to activated caspase-3 selectively labeled either epithelial cells in the mucosal crypts or cells in lymphoid tissue in the submucosa as well as other cells in the muscle layers that were not c-Kit positive (Figure 1b). Activated caspase-3 positive ICC in both muscle layers and in the myenteric plexus region were identified by the red reaction product in the cell. This positive immunoreactivity could be confirmed by the fluorescence of the Vulcan Fast Red reaction product (Figure 1a,b). In the same tissue section, positively labeled ICC could be distinguished from caspase negative ICC (Figure 1b). Activated caspase-3 positive ICC had other indications of cellular injury including shorter processes and a condensed cytoplasm when compared to caspase negative ICC. The number of activated caspase-3 positive ICC was 23.1 ± 4.6 cells per mm³ of smooth muscle (mean $\pm$ SEM, n = 7, Figure 1c). The numbers of activated caspase-3 positive ICC varied from tissue to tissue. However, although there was variation in the number of activated caspase-3 positive ICC in tissues from individual patients, no relationship with patient age was seen

(Figure 1c). Thicker sections from 4 patients were examined to estimate the proportion of ICC that were positive for activated caspase-3. In these tissues, 1.5 ± 0.2 % of the ICC were positive for activated caspase-3 (mean $\pm$ SEM, $n=4$ tissues, 2547 ICC from 1002 fields at 600X magnification).

TUNEL was used to confirm that the presence of activated caspase-3 in ICC reflects apoptosis of these cells. Like the activated caspase-3 studies, the presence of TUNEL positive cells in the mucosal crypts and lymphoid tissues of each section was used as a positive control for detection of apoptosis. TUNEL positive ICC were detected in the muscle layers and the myenteric plexus regions of the tissues (Figure 2a-c). These ICC were identified by the colocalization of TUNEL with DAPI positive nuclei in cells that, as is typical for ICC, were c-Kit positive and had two or more processes. Co-localization of TUNEL, DAPI and c-Kit was confirmed by examining each ICC under 1000X optical magnification using confocal microscopy to obtain maximal spatial resolution. Often TUNEL-positive ICC (arrowhead in Figure 2) were closely adjacent to TUNEL-negative ICC (arrows in Figure 2). Therefore, volume rendering and 3D reconstruction was used to confirm this colocalization (Figure 2b,d). In the circular muscle layer, 2.1 ± 0.9 % of ICC were TUNEL-positive (mean $\pm$ SEM, $n=9$ patients, Figure 2e). Similar to the caspase-3 experiments, TUNEL-positive cells that were c-Kit negative were also observed in the tissues. The numbers of these cells were quantified and were five fold higher than the number of TUNEL-positive/c-Kit positive ICC (all TUNEL positive cells: 0.34 ± 0.09 , TUNEL positive ICC: 0.068 ± 0.018 , mean $\pm$ SEM, $n=9$ patients, Figure 2f). The tissues from the two oldest patients contained the highest levels of TUNEL positive ICC (Figure 2e). However, overall, there was no significant relationship between the numbers of TUNEL-positive ICC and the age of the patients. Similarly, there was no relationship between total number of ICC and age (Figure 3, $r^2=0.14$, $P>0.05$).

Using electron microscopy, four populations of ICC, submucosal (ICC-SM), intramuscular (ICC-IM), septal (ICC-SP) and myenteric (ICC-MY) were identified by well established criteria for human colonic ICC (24-26). Some of these ICC showed classical features (Figure 4a) and others also had a hybrid of fibroblast-like features (Figure 4b). Peg-and-socket junctions were common between the ICC and the smooth muscle cells (Figure 4c). At the electron microscopy level, ICC with apoptotic features were identified. These cells were uncommon with most ICC appearing to be normal. The apoptotic ICC identified were mainly located within the circular muscle layer intermingled with the smooth muscle cells. All of them shared the same relationships with SMC (Figures 4d,c). The apoptotic ICC were identified according to universally recognized criteria in regards to the nuclear and cytoplasmic alterations (27). Chromatin was compacted and appeared either filling the entire nucleus that still possessed a nuclear envelope (Figure 3d) or as dark clumps close to remnants of the nuclear envelope (Figure 4e). The cytoplasm was either completely empty or contained few swollen mitochondria, cisternae of the endoplasmic reticulum and lysosomes (Figures 4d,c). As expected from the TUNEL and caspase data, other cells that were not ICC were identified with apoptotic features including smooth muscle cells undergoing apoptosis (Figure 4f).

DISCUSSION

Two findings that shed light on the regulation of ICC numbers were confirmed by two independent histochemical methods and by electron microscopy in this study. , We show that apoptosis occurs as a mechanism for ICC death in the normal human colon and we quantify the number of ICC that are apoptotic in the normal human colon. Taken together, these two findings indicate that human ICC networks are continually turning over in a relatively short time frame.

Data from animal models suggest that a decrease in number of ICC in response to an insult may occur, at least in part, through trans-differentiation, that is the transformation of mature ICC into another non-ICC cell type (28). In human studies, depletion of ICC networks associated with human motility disorders has been shown in many studies (29-31) but no generally applicable mechanism for ICC loss, such as transdifferentiation, has been identified. The current study demonstrates apoptosis as an ongoing process in ICC networks of normal human colon and suggests that this mechanism may also contribute to deplete ICC networks in human motility disorders.

The characteristics of cell death noted in ICC from human colon were similar to the characteristic of apoptosis in other cell types (32). In particular, the injured cells were positive for TUNEL and activated caspase-3 and had evidence of altered processes and shrinkage of the cytoplasm. The presence of apoptotic ICC was confirmed by electron microscopy, which showed nuclear and cytoplasmic alterations in ICC, findings considered specific for apoptosis (27).

The markers chosen for this study are widely accepted as indicators of apoptosis (22,33,34). In order to address potential methodological limitations, we used the complementary approaches of TUNEL, activated caspase-3 immunoreactivity and changes in cell ultrastructure to establish that ICC undergo apoptosis. TUNEL may produce false positive labeling (33) with excessive fixation or prolonged proteinase K treatment (35,36). Therefore, in the TUNEL protocol, we used tissue that was rapidly processed, briefly fixed and not treated with proteinase K. Activated caspase-3 immunoreactivity may also produce falsely positive indications of apoptosis. To ensure that the activated caspase-3 immunoreactivity was not due to inappropriate cross reactivity, we tested antisera from three different commercial sources and used the antibody that gave minimal background staining but clearly recognized apoptotic cells in colonic crypts or gastrointestinal lymphoid tissue. The antibody used has been previously reported to selectively detect apoptotic cells in paraffin embedded human placenta (37) as well as paraffin-embedded tissue from other species (38,39). The detection of ultrastructural changes in ICC morphology that are definitive for apoptosis (27) complete the identification of apoptotic ICC in the normal human colon. This assessment does not preclude the possibility that some caspase or TUNEL-positive cells could still be repaired but taken together with the electron microscopy data, the findings do indicate that apoptosis occurs in ICC

Variability in the levels of apoptotic ICC was observed in the tissues examined and the factors underlying this were not apparent. Age was one factor that we considered since the two oldest patients in our study had by far the highest levels of TUNEL-positive ICC. However, we could not rigorously test for a correlation with age because the age range of the patients from whom most of the specimens were collected was quite narrow (35-55 years). We also detected apoptotic cells that were not immunoreactive for c-Kit in all layers of the muscularis propria at numbers that were approximately 5 times higher than the number of TUNEL-positive ICC.

If the typical time that a dead or dying cell can be detected is known, then the rate of ICC loss can be predicted using a model with two variables: the period of time that the dead or dying ICC expresses markers of cell death; and the proportion of ICC in a population expressing those markers (i.e., TUNEL, caspase-3 and specific structural abnormalities at electron microscopy). The period of time that a dead or dying cell can be detected in gut muscularis propria is not known, but studies in heart and liver indicate that apoptotic cells can be detected for less than 24 hours (40). Taking this conservative value for detection of dead or dying ICC, all ICC will die in less than 6 months when the proportion of cells positive for apoptotic markers is 1%. If 0.1% of ICC are positive for apoptosis at a given

time point, then it will take over 6 years for 90% of ICC to be depleted in the absence of regeneration. Using both activated caspase-3 and TUNEL as markers of apoptosis, approximately 1-2% of ICC are dead or dying in the normal colon. This would deplete ICC networks over a short period of time. As normal appearing ICC networks are well visualized in adults, this suggests that regeneration is required for maintenance of normal ICC networks even in healthy tissue. Recent data from mouse studies support the concept of a tissue resident stem cell that contributes to regeneration of ICC (41) and that regeneration of ICC networks occurs in mouse colon following injury (16). Therefore, similarly to cardiac myocytes (42), apoptosis and cellular regeneration from either progenitors cells and/or proliferation of adult ICC are likely to be ongoing in ICC networks. In this view, a small shift of the balance between death and replenishment will lead to premature loss of ICC contributing to the development of motility disorders. While it is likely that c-Kit positive ICC may proliferate to maintain the ICC networks evidence indicative of proliferation in mature human ICC has not yet been published. Another concept that is raised by apoptotic cell death in ICC is whether apoptosis contributes to modeling of developing ICC networks in response to demand for the functional needs that ICC serve in the gastrointestinal tract. To attain a full picture of the mechanisms underlying turnover of ICC, it is now necessary to assess both rates of cell death and cell replenishment.

In conclusion, this study identified apoptotic ICC in the normal human colon using three separate and well accepted markers of apoptosis. The finding of apoptotic ICC in normal tissue, together with the numbers of apoptotic ICC found, advances the hypothesis that a continual replenishment of ICC is required to maintain normal networks and therefore normal gastrointestinal motility.

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Abbreviation used in this paper

(ICC)	Interstitial cells of Cajal
(SMC)	smooth muscle cell
(NF)	nerve fiber

REFERENCES

1. Thomsen L, Robinson TL, Lee JC, et al. Interstitial cells of Cajal generate a rhythmic pacemaker current. *Nature Med.* 1998; 4:848–51. [PubMed: 9662380]
2. Ward SM, Burns AJ, Torihashi S, Sanders KM. Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine. *J Physiol.* 1994; 480:91–7. [PubMed: 7853230]
3. Huizinga JD, Thuneberg L, Kluppel M, Malysz J, Mikkelsen HB, Bernstein A. W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature.* 1995; 373:347–9. [PubMed: 7530333]
4. Burns AJ, Lomax AE, Torihashi S, Sanders KM, Ward SM. Interstitial cells of Cajal mediate inhibitory neurotransmission in the stomach. *Proc Nat Acad Sci USA.* 1996; 93:12008–13. [PubMed: 8876253]
5. Ward SM, Beckett EA, Wang X, Baker F, Khoyi M, Sanders KM. Interstitial cells of Cajal mediate cholinergic neurotransmission from enteric motor neurons. *J Neuroscience.* 2000; 20:1393–403.

6. Strege PR, Ou Y, Sha L, et al. Sodium current in human intestinal interstitial cells of Cajal. *Am J Physiol.* 2003; 285:G1111–21.
7. Farrugia G, Lei S, Lin X, et al. A major role for carbon monoxide as an endogenous hyperpolarizing factor in the gastrointestinal tract. *Proc Nat Acad Sci USA.* 2003; 100:8567–70. [PubMed: 12832617]
8. Klemm MF, Exintaris B, Lang RJ. Identification of the cells underlying pacemaker activity in the guinea-pig upper urinary tract. *J Physiol.* 1999; 519 Pt 3:867–84. [PubMed: 10457097]
9. Pucovsky V, Moss RF, Bolton TB. Non-contractile cells with thin processes resembling interstitial cells of Cajal found in the wall of guinea-pig mesenteric arteries. *J Physiol.* 2003; 552:119–33. [PubMed: 12897177]
10. Duquette RA, Shmygol A, Vaillant C, et al. Vimentin-positive, c-kit-negative interstitial cells in human and rat uterus: a role in pacemaking? *Biol Reprod.* 2005; 72:276–83. [PubMed: 15385413]
11. Brading AF, McCloskey KD. Mechanisms of Disease: specialized interstitial cells of the urinary tract--an assessment of current knowledge. *Nat Clin Pract Urol.* 2005; 2:546–54. [PubMed: 16474598]
12. Gibbons SJ, Roeder JL, Wouters MM, Farrugia G. Regulation of attachment and proliferation of interstitial cells of Cajal by c-Kit. *Neurogastroenterol Motil.* 2007; 19:23–4.
13. Choi KM, Gibbons SJ, Roeder JL, et al. Regulation of interstitial cells of Cajal in the mouse gastric body by neuronal nitric oxide. *Neurogastroenterol Motil.* 2007; 19:585–95. [PubMed: 17593140]
14. Wouters MM, Gibbons SJ, Roeder JL, et al. Exogenous serotonin regulates proliferation of interstitial cells of Cajal in mouse jejunum through 5-HT2B receptors. *Gastroenterology.* 2007; 133:897–906. [PubMed: 17854596]
15. Chang IY, Glasgow NJ, Takayama I, Horiguchi K, Sanders KM, Ward SM. Loss of interstitial cells of Cajal and development of electrical dysfunction in murine small bowel obstruction. *J Physiol.* 2001; 536:555–68. [PubMed: 11600689]
16. Faussone-Pellegrini MS, Vannucchi MG, Ledder O, Huang TY, Hanani M. Plasticity of interstitial cells of Cajal: a study in the mouse colon. *Cell Tissue Res.* 2006; 325:211–7. [PubMed: 16596393]
17. Forster J, Damjanov I, Lin Z, Sarosiek I, Wetzel P, McCallum RW. Absence of the interstitial cells of Cajal in patients with gastroparesis and correlation with clinical findings. *J Gastrointest Surg.* 2005; 9:102–8. [PubMed: 15623450]
18. Feldstein AE, Miller SM, El-Youssef M, et al. Chronic intestinal pseudoobstruction associated with altered interstitial cells of Cajal networks. *J Pediatr Gastroenterol Nutr.* 2003; 36:492–7. [PubMed: 12658043]
19. He CL, Burgart L, Wang L, et al. Decreased interstitial cell of Cajal volume in patients with slow-transit constipation. *Gastroenterology.* 2000; 118:14–21. [PubMed: 10611149]
20. Gibbons SJ, De Giorgio R, Garrity MM, et al. Increased numbers of activated caspase-3 immunoreactive interstitial cells of Cajal in the colon of patients with slow-transit constipation. *Neurogastroenterol Motil.* 2005; 17:6.
21. Gibbons SJ, Faussone-Pellegrini MS, De Giorgio R, et al. Ultrastructural evidence of apoptosis in interstitial cells of Cajal from the colon of patients with slow transit constipation. *Neurogastroenterol Motil.* 2006; 18:756.
22. Gown AM, Willingham MC. Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. *J Histochem Cytochem.* 2002; 50:449–54. [PubMed: 11897797]
23. Lyford GL, He CL, Soffer E, et al. Pan-colonic decrease in interstitial cells of Cajal in patients with slow transit constipation. *Gut.* 2002; 51:496–501. [PubMed: 12235070]
24. Faussone-Pellegrini MS, Cortesini C, Pantalone D. Neuromuscular structures specific to the submucosal border of the human colonic circular muscle layer. *Can J Physiol Pharmacol.* 1990; 68:1437–46. [PubMed: 2285888]
25. Faussone-Pellegrini MS, Pantalone D, Cortesini C. Smooth muscle cells, interstitial cells of Cajal and myenteric plexus interrelationships in the human colon. *Acta Anat (Basel).* 1990; 139:31–44. [PubMed: 2288187]
26. Faussone-Pellegrini MS, Thuneberg L. Guide to the identification of interstitial cells of Cajal. *Microsc Res Tech.* 1999; 47:248–66. [PubMed: 10602286]

27. Kerr, JFR.; Harmon, BV. Definition and incidence of apoptosis: an historical perspective. In: Tomei, LD.; Cope, FO., editors. *The Molecular Basis of Cell Death*. Cold Spring Harbor Laboratory Press; New York: 1991. p. 5-29.
28. Torihashi S, Nishi K, Tokutomi Y, Nishi T, Ward S, Sanders KM. Blockade of kit signaling induces transdifferentiation of interstitial cells of cajal to a smooth muscle phenotype. *Gastroenterology*. 1999; 117:140–8. [PubMed: 10381920]
29. He CL, Soffer EE, Ferris CD, Walsh RM, Szurszewski JH, Farrugia G. Loss of interstitial cells of cajal and inhibitory innervation in insulin-dependent diabetes. *Gastroenterology*. 2001; 121:427–34. [PubMed: 11487552]
30. Isozaki K, Hirota S, Miyagawa J, Taniguchi M, Shinomura Y, Matsuzawa Y. Deficiency of c-kit+ cells in patients with a myopathic form of chronic idiopathic intestinal pseudo-obstruction. *Am J Gastroenterol*. 1997; 92:332–4. [PubMed: 9040218]
31. Kenny SE, Connell MG, Rintala RJ, Vaillant C, Edgar DH, Lloyd DA. Abnormal colonic interstitial cells of Cajal in children with anorectal malformations. *J Ped Surg*. 1998; 33:130–2.
32. Hengartner MO. The biochemistry of apoptosis. *Nature*. 2000; 407:770–6. [PubMed: 11048727]
33. Negoescu A, Lorimier P, Labat-Moleur F, et al. In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. *J Histochem Cytochem*. 1996; 44:959–68. [PubMed: 8773561]
34. Sanders EJ. Methods for detecting apoptotic cells in tissues. *Histology & Histopathology*. 1997; 12:1169–77. [PubMed: 9302576]
35. Garrity MM, Burgart LJ, Riehle DL, Hill EM, Sebo TJ, Witzig T. Identifying and quantifying apoptosis: navigating technical pitfalls. *Mod Pathol*. 2003; 16:389–94. [PubMed: 12692204]
36. Migheli A, Cavalla P, Marino S, Schiffer D. A study of apoptosis in normal and pathologic nervous tissue after in situ end-labeling of DNA strand breaks. *J Neuropathol Exp Neurol*. 1994; 53:606–16. [PubMed: 7525880]
37. Kenis I, Tartakover-Matalon S, Cherepnin N, et al. Simvastatin has deleterious effects on human first trimester placental explants. *Human Reprod*. 2005; 20:2866–72.
38. Hacker E, Muller HK, Irwin N, et al. Spontaneous and UV radiation-induced multiple metastatic melanomas in Cdk4R24C/R24C/TPras mice. *Cancer Res*. 2006; 66:2946–52. [PubMed: 16540642]
39. Hasina R, Pontier AL, Fekete MJ, et al. NOL7 is a nucleolar candidate tumor suppressor gene in cervical cancer that modulates the angiogenic phenotype. *Oncogene*. 2006; 25:588–98. [PubMed: 16205646]
40. Hayakawa K, Takemura G, Koda M, et al. Sensitivity to apoptosis signal, clearance rate, and ultrastructure of fas ligand-induced apoptosis in in vivo adult cardiac cells. *Circulation*. 2002; 105:3039–45. [PubMed: 12082000]
41. Ordog T, Redelman D, Anderson LR, Lorincz A, Young DL, Horvath VJ. Isolation and characterization of a CD34+ multipotent progenitor cell line from the postnatal murine gastric tunica muscularis. *Gastroenterology*. 2005; 128:A-493.
42. Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. *Circ Res*. 2003; 92:139–50. [PubMed: 12574141]

a)

b)

Fig 1c)

Figure 1. Activated caspase-3 positive interstitial cells of Cajal (ICC) in the human colon
 Representative activated caspase-3 (red staining and red fluorescence indicated by arrowheads) and c-Kit positive ICC (brown staining) are shown from sections of human colon with counterstained nuclei (blue). The left panels in a and b show white light illuminated, bright field images, the middle panels show the fluorescent structures in the same field (Ex 560 nm, Em 645), the right panels are merged images. a) example of an activated caspase-3 positive ICC in circular muscle from a 40 year old male; b) another example of an activated caspase-3 positive ICC in the myenteric plexus region of the colon of a 45 year old female. Arrows in b and c indicate activated caspase-3 positive cells that are not ICC. The asterisks indicate two ICC that are negative for activated caspase-3. Scale bars

= 25 μm . c) Numbers of activated caspase-3 positive ICC expressed as the number of apoptotic ICC per mm^3 of total muscle volume. The values obtained from the individual patient samples are labeled with the ages of the patients. The mean $\pm$ SEM for the 7 patients is shown on the right.

Fig 2e) **Fig 2f)** **Figure 2. TUNEL positive interstitial cells of Cajal (ICC) in the human colon**

Representative images of TUNEL positive and TUNEL negative ICC from sections of human colon. a) A TUNEL-positive ICC (arrowhead) immediately adjacent to a TUNEL-negative ICC in the myenteric plexus region (arrow) and close to other TUNEL-negative ICC (arrow). The top left image in each panel shows Kit immunostaining (red), the top right TUNEL (green), the bottom left DAPI (blue) and the bottom right the merged images. b) 3D volume rendered images of the same image as (a) rotated 90 degrees to show localization of the TUNEL-positive nucleus (green) within the c-Kit immunoreactive ICC (transparent white). c) a TUNEL positive ICC from the circular muscle. The top left image in each panel shows Kit immunostaining (red), the top right TUNEL (green), the bottom left DAPI (blue)

and the bottom right the merged images. d) 3D volume rendered image of the same image as (c) showing localization of the TUNEL image (green) within the c-Kit positive structures (transparent white) shown in two planes 90 degrees rotated from each other. Examples of TUNEL-positive cells are from a 45 year old female. e) Numbers of TUNEL-positive ICC expressed as the % of total number of ICC in the circular muscle layer of each section. On the left is shown the values obtained from individual patient samples labeled with the ages of the patients; on the right is the mean $\pm$ SEM for the 9 patients. f) Numbers of all TUNEL positive cells and TUNEL positive ICC in the circular muscle layer. The numbers of TUNEL-positive cells per field obtained from individual tissue samples are shown labeled with the ages of the patients together with the mean $\pm$ SEM for all the tissues in a separate column.

Figure 3.

Number of circular muscle interstitial cells of Cajal (ICC)/field in the normal human colon plotted against age of the patients. Data are the mean $\pm$ SEM obtained by counting ICC in an average of 132 fields per patient. Each field was 0.95 mm² and 6 to 10 tissue sections were counted for each tissue specimen. The fit to the data did not show a significant change in the number of ICC with age ($r^2 = 0.14$, $P > 0.05$).

Figure 4.

Electron micrographs of interstitial cells of Cajal (ICC) from normal human colon. **a)** Two ICC in the circular muscle layer in contact with smooth muscle cells. These ICC are also close to a bundle of nerve fibers. One of these ICC has the classical features of ICC while the second one (lower ICC) also has several cisternae of rough endoplasmic reticulum. **b)** An ICC also showing fibroblast-like features with an extended rough endoplasmic reticulum and a large Golgi apparatus. This cell is in contact with a smooth muscle cell (*arrow*). **c)** A detail of a peg-and-socket junction (*asterisk*) between an ICC and a smooth muscle cell. **d** and **e**: apoptotic ICC. In **d**, the nucleus of the ICC (*asterisk*) is filled with a condensed chromatin and the cytoplasm is empty. *Arrows* indicate contact areas between the ICC and

neighboring smooth muscle cells. In **e**, an ICC (*asterisks*) whose nucleus shows intensely heterochromatic blocks (*white asterisk*) immersed in a clear matrix. The nuclear envelope is discontinuous and the cytoplasm contains only some lysosomes and few cisternae of the endoplasmic reticulum. The *arrow* indicates a contact area between the ICC and one smooth muscle cell. In **f**, an apoptotic smooth muscle cell. Scale bar: **a**, 1.6 μm ; **b**, 2 μm ; **c**, 0.8 μm ; **d**, 1 μm ; **e**, 1.3 μm ; **f**, 1.3 μm . Interstitial cell of Cajal: ICC, smooth muscle cell, SMC, nerve fiber NF.

Table 1

Details of tissue samples studied for TUNEL and activated caspase expression

Age	Sex	Activ ^d Caspase-3 (bright field)	TUNEL (fluor)	Tissue source
33	M	N	Y	Sigmoid
34	F	Y	N	Sigmoid
35	F	Y	N	Ascending
35	F	Y	N	Sigmoid
40	M	Y	Y	Left colon
40	F	Y	Y	Ascending
42	F	N	Y	Sigmoid
45	F	Y	N	Sigmoid
45	F	Y	Y	Sigmoid
54	M	N	Y	Ascending
56	F	N	Y	Sigmoid
71	M	N	Y	Sigmoid
76	F	N	Y	Sigmoid
		7	9	