SYMPOSIUM REPORT

Interstitial cells of Cajal at the clinical and scientific interface

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Considerable work over the past two decades has determined that interstitial cells of Cajal (ICC) serve as pacemaker cells, conduits for active transmission of electrical slow waves, sites of innervation by peripheral motor neurons, and mechanotransducers. While most of the physiology of ICC has been learned from studies of the cells within the gastrointestinal tract, ICC are found in a variety of smooth muscle tissues and may have analogous or novel physiological functions in those organs. Clinical investigations of muscles from patients with a variety of gastrointestinal motility disorders have raised the exciting possibility that loss of ICC may be responsible for the development of motor dysfunction. This review discusses the development of ICC, the kinds of human disorders in which ICC loss may be important, what factors regulate the ICC phenotype, and what therapeutic approaches might be utilized to restore or regenerate ICC. This field is primed for translational discoveries. ICC are responsible for critical physiological functions in smooth muscle tissues, they are lost in pathophysiological conditions, and it will be important now to decipher the conditions that are responsible for ICC loss and develop new therapies to relieve patients of this problem. Success in this endeavour might improve the quality of life for millions of patients.

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During the past two decades much has been learned about a group of cells, referred to as interstitial cells of Cajal (ICC), that populate smooth muscle organs and have important physiological functions. For example, ICC are pacemaker cells in some organs, generating spontaneous electrical depolarizations that organize contractile activity into patterns of phasic contractions. ICC also provide a pathway for active transmission of electrical activity in smooth muscles, much like the network of Purkinje fibres in the heart. ICC form synaptic contacts with peripheral nerves, and have been shown to mediate, at a minimum, cholinergic and nitrergic neural inputs to smooth muscle cells. Finally, ICC express mechano-sensitive channels and mechanisms that can transduce length changes to mediate responses to stretch. This symposium (entitled Involvement of interstitial cells of Cajal in the control of smooth muscle excitability) covered many of these topics and shed light on the distribution and specific mechanisms of ICC populations in visceral smooth muscle tissues.

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Interest in ICC extends beyond basic biomedical research, however, as contractile disorders of the gastrointestinal tract (and elsewhere) have increasingly been associated with loss of ICC. These cells have important functions in regulation of mechanical activity, so loss of ICC could certainly result in motor dysfunction. However, at present there is an incomplete understanding of the cause-and-effect relationship between loss of ICC and the development of motor symptoms in humans. The next exciting phase of ICC research will include studies to: (i) determine the basis for loss of ICC in pathophysiological conditions; (ii) determine the fate of ICC; (iii) determine the factors that control the ICC phenotype; and (iv) develop methods to stimulate regrowth or repopulation of ICC in muscles depleted of these cells. This brief review discusses the state-of-the-art of research on these topics and outlines directions for future research.

Human motility disorders associated with loss of ICC

The etiologies of many human gastrointestinal motility disorders have not been elucidated. Several disorders, such as gastroparesis, chronic idiopathic intestinal

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pseudoobstruction, achalasia, and chronic constipation have been associated with loss of ICC in dysfunctional regions of the gastrointestinal tract. There is not space here to review these studies, but a more complete description and references to primary publications can be found in our recent review (Sanders *et al.* 2006). The possibility that loss of ICC may underlie several motility disorders has greatly increased interest in this field.

Although little is known about the role of ICC in the bladder, there have also been reports of reduced ICC-like cells in the bladder under pathological circumstances. For example, patients with megacystis-microcolon intestinal hypoperistalsis syndrome were found to have reduced Kit-positive cells, and the authors discussed how absence of ICC might contribute to the voiding dysfunction of this disorder (Piaseczna Piotrowska *et al.* 2004). These authors also found reduced ICC in the ureteropelvic junction (UPJ) in cases of UPJ obstruction and suggested that the lack of these cells might block transmission of peristaltic waves across the UPJ (Solari *et al.* 2003). A recent review gives a description of the ICC in the urogenital tract and the likely consequences of losing these cells (Brading & McCloskey, 2005).

ICC development (how does the ICC phenotype develop?)

Understanding the development of ICC may help unravel the mystery of why these cells are lost in some patients, because some of the same signalling pathways required for development are also necessary for long-term maintenance of the ICC phenotype. Contrary to the original ideas of Cajal, ICC are not derived from the neural crest (Young *et al.* 1996; Lecoin *et al.* 1996; Torihashi *et al.* 1997). Developmental studies have demonstrated that ICC and smooth muscle cells come from common mesenchymal precursors (Torihashi *et al.* 1997; Kluppel *et al.* 1998). The development of ICC appears to depend upon expression of c-*kit*, a protooncogene that encodes the receptor tyrosine kinase, Kit. ICC precursors begin to express Kit at about embryonic day 11 (E11), and signalling via Kit becomes critical for the development of ICC as early as E15 in the mouse (Torihashi *et al.* 1997).

There has been controversy in the literature about the role of Kit in embryonic development of ICC. Electrical rhythmicity develops by E18 in mice, suggesting that functional ICC develop before birth (Torihashi *et al.* 1997). In mice with *lacZ* knocked-in to the Kit locus, *lacZ* was expressed in ICC-like cells at birth, but these mice lacked functional Kit (Bernex *et al.* 1996). LacZ-positive cells were observed in the gastrointestinal tracts of W^{lacZ}/W^{lacZ} mice in places normally inhabited by ICC. Another group studied W^{banded} (W^{bd}) mutant mice (again lacking Kit), and also came to the conclusion that ICC can develop before birth in the absence of functional Kit protein (Kluppel *et al.* 1998). Neither study incorporated assays of ICC function, so it is possible that precursor cells, that normally express Kit, might develop in the absence of Kit signalling, but do these cells actually develop into ICC? We have re-examined this question recently using organotypic cultures and various means of blocking Kit during pre- and postnatal development of ICC (Beckett *et al.* 2006). Small intestinal muscles taken at E17 developed normal pacemaker ICC and electrical rhythmicity within 1–2 days in culture, or before the time of birth. This did not occur in muscles of W/W^{V} mice or in muscles in which Kit was blocked by a neutralizing antibody. These data directly demonstrate that Kit signalling is necessary for development of ICC networks and the onset of electrical rhythmicity.

Stability of the functional ICC phenotype

Kit signalling is also necessary for the maintenance of the ICC phenotype. At birth fully functional pacemaker ICC are present in the proximal small intestine, but blocking Kit signalling with neutralizing antibodies or by imatinib mesylate, a receptor tyrosine kinase inhibitor, causes loss of the ICC phenotype and electrical rhythmicity in murine muscles within a few days (Torihashi *et al.* 1995; Beckett *et al.* 2006). As above, blocking Kit signalling at birth leads to rapid loss of ICC networks and electrical rhythmicity, but as animals mature, longer periods of Kit blockade are required to cause loss of Kit-positive cells and electrical rhythmicity (S. M. Ward and K. M. Sanders; unpublished observations). Kit signalling occurs via binding of Kit to its natural ligand, stem cell factor, which is expressed by smooth muscle cells. A recent study has suggested that reduced stem cell factor expression in diabetes may be responsible ultimately for the loss of ICC in diabetic gastroparesis (Ördög *et al.* 2000; Horvath *et al.* 2006).

Inflammatory factors may also influence the ICC phenotype. Surgical manipulation or resection and inflammation of the gastrointestinal tract caused by endotoxins (e.g. lipopolysaccharide) causes a disruption in motility and up-regulation of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase 2 (COX-2) in resident macrophages at the level of the myenteric plexus in gastrointestinal muscles (Eskandari *et al.* 1997). Resident macrophages lie in close proximity to ICC (ICC-MY; Mikkelsen, 1995). Thus, it is possible that the inflammatory response initiated by bowel resection or infections might have deleterious effects on ICC and contribute to the inhibition of motility in response to inflammation.

Post-operative ileus is a significant complication of gastrointestinal surgery. Thus, we developed a murine model of small bowel resection and found that within several hours of surgery ICC populations were greatly diminished on both sides of a resection site (Yanagida *et al.*

2004). This was accompanied by reduced or absent slow wave activity and reduced responses to transmural nerve stimulation (Yanagida *et al.* 2004). Slow wave amplitude was partially restored by inhibitors of iNOS or COX-2 given *in vitro*, suggesting that NO and eicosanoids may directly reduce the excitability of ICC and smooth muscle cells following resection. Recent studies have shown that loss of ICC and electrical rhythmicity is greatly reduced in small intestinal muscles of iNOS knock-out mice or in animals treated before surgery with iNOS inhibitor drugs (H. Yanagida, S. M. Ward and K. M. Sanders, unpublished observations). NO, especially at the high concentrations produced by iNOS, appears to have deleterious effects on ICC. These data suggest that inflammatory responses may have generally negative effects on ICC populations in smooth muscle tissues.

Animal models of ICC loss

It is impossible to closely monitor progressive loss of ICC in human visceral smooth muscle organs during the development of motor symptoms. Thus, it is difficult to relate the loss of ICC to the development of motility disorders. Animal models in which ICC fail to develop or are lost after maturity may provide a means to investigate why ICC are lost in a variety of circumstances. Animals with genetic mutations, surgical treatments, infections, and treated with chemicals have been shown to display lesions in ICC populations. Loss of ICC correlates with loss of pacemaker activity, propagation defects, reduced neurotransmission, and loss of responses to stretch. Motility disorders also accompany defects in ICC populations, so these animals may serve as excellent models for human motor disorders.

The first animal models studied as models of ICC loss had defective Kit signalling. This was accomplished either by blocking Kit with neutralizing antibodies or by using animals with loss-of-function mutations in c-*kit* or the ligand for Kit, stem cell factor (*steel*). For the latter studies, compound heterozygotes were used (e.g. W/W^V and Sl/Sl^d mutants) because homozygotes with total loss-of-function alleles usually die *in utero* from anaemia. Kit or stem cell factor are functional to some extent in W/W^V and Sl/Sl^d mutants, and these animals display only partial loss of ICC. Intramusular ICC are lost in the stomach, lower oesophageal sphincter and pylorus, but myenteric ICC (ICC-MY) survive in the stomachs of W/W^V mice (although they are reduced in number and functional defects have been reported; Ördög *et al.* 2002). ICC-MY are lost in the small bowel, but the equivalent of ICC-IM in the small bowel, ICC of the deep muscular plexus (ICC-DMP), are essentially normal in appearance (Ward *et al.* 1994). ICC are present in the colons and internal anal sphincters of W/W^V mice, although these cells appear to be reduced in number (S. M. Ward and K. M. Sanders, unpublished observations). W/W^V and Sl/Sl^d animals have a variety of interesting motility disorders and may serve as a general model of ICC-dependent gastroparesis and delayed intestinal transit (e.g. radiograms demonstrate slow and poorly coordinated motility in the small bowel; Der-Silaphet *et al.* 1998). No-one has reported whether W/W^V or Sl/Sl^d mutants display symptoms of constipation or incontinence, and although reduced, the populations of ICC in the colon and internal anal sphincter may be sufficient to accomplish normal colonic motor functions.

Non-obese diabetic (NOD) mice (model of Type I diabetes) have patchy loss of ICC in the gastric antrum and microscopic lesions in the connectivity between enteric nerve terminals and ICC-IM (Ördög *et al.* 2000). These animals have many of the symptoms of diabetic gastroparesis (e.g. delayed gastric emptying, antral hypomotility, and reduced neural regulation of the stomach which could yield poor gastric accommodation reflexes). Studies of NOD mice have provided an important new hypothesis regarding the etiology of diabetic gastroparesis, and this hypothesis has growing support from human studies.

An important area of research will be to determine the short- and long-term consequences of intestinal infections on ICC. Rats infected with *Nippostrongylus brasiliensis* have effects on ICC populations (Faussone-Pellegrini *et al.* 2002), and many other models of infectious disease could be developed to study the link between bowel infections, ICC loss and the development of long-term motility dysfunction.

We have developed a model of partial bowel obstruction that leads to hypertrophy of the smooth muscle layers above the obstruction and progressive loss of ICC with time and proximity to the obstruction (Chang *et al.* 2001). This is an exciting model to follow because controlled loss of ICC can be produced in these animals, and the severity of the lesion has spatial and temporal characteristics. Thus, it may be possible to obtain very precise information about the factors within the microenvironment that are required for maintenance of the ICC phenotype, and how obstruction or hypertrophy alter these factors in a manner that results in loss of ICC. Finally, our model of ICC loss after bowel surgery, as described above, may be an excellent model for effects of inflammation on ICC (Yanagida *et al.* 2004).

What happens to ICC loss during pathological processes?

ICC are lost in a seemingly unrelated group of motility disorders, thus a variety of factors may converge on the mechanisms maintaining the ICC phenotype. The fact that ICC loss is not limited to the gastrointestinal tract may suggest that conditions affecting ICC in the gut may

occur in other visceral organs. It is interesting to note that some gastrointestinal motor disorders have correlates in other visceral smooth muscles (e.g. in diabetes), and one might speculate that systemic regulation of ICC (such as by circulating growth factors or systemic inflammatory mediators) might be a factor in these circumstances.

Our understanding of the maintenance of the ICC phenotype is rudimentary at the present time. As discussed above, high concentrations of NO, as might be produced by iNOS, may have deleterious effects on ICC, but the fate of ICC exposed to high concentrations of NO (i.e. death or redifferentiation) is not known. Loss of ICC when Kit signalling was blocked was studied using mice exposed to neutralizing antibodies. During the period when ICC were lost, morphological and immunohistochemical studies showed that ICC do not die when Kit signalling is blocked, because there was no ultrastructural evidence of cell necrosis or apoptosis (Torihashi *et al.* 1999). After Kit blockade, ultrastructural features developed in remnant Kit-positive cells that are similar to smooth muscle cells, including prominent filament bundles and expression of the muscle-specific intermediate filament protein, desmin, and smooth muscle myosin. These observations led to the proposal that ICC redifferentiate toward a smooth muscle-like phenotype when Kit signalling is blocked. The fate of redifferentiated ICC caused by pathological conditions is difficult, at present, to determine. We cannot identify, with certainty, the cells that were once ICC past the point where Kit expression is lost. Thus, the search is on for an immunological marker protein that is expressed specifically in ICC in normal tissues and retained during redifferentiation (or as Kit expression is lost). Finding such a protein will make identification, isolation and functional studies of altered ICC possible, and help determine whether these cells can, under the proper circumstance, redevelop into functional ICC.

What therapies might be applied to alleviate problems due to loss of ICC?

Actually, ICC display a rather robust degree of plasticity or ability to regenerate. These cells can be lost rather quickly under some circumstance, as described above, but there is hope for regeneration of functional ICC networks if the stimuli causing loss can be understood and reversed. For example, in the model of partial small bowel obstruction described above, removal of the mechanical obstruction caused gradual restoration of functional pacemaker ICC and ICC-dependent neural responses (Chang *et al.* 2001). Restoration of ICC was associated with recovery from the smooth muscle hypertrophy above the obstruction. Thus, it is unclear whether ICC loss is caused by smooth muscle hypertrophy or by the inflammatory response that appears to develop in response to obstruction (Won *et al.* 2006).

In diabetes loss of ICC appears to be due mainly to reduced insulin and IGF-1 that eventually leads to reduced stem cell factor expression by smooth muscle cells (Horvath *et al.* 2006). Thus, Type I diabetes in NOD mice appears to create a situation in which Kit signalling is blocked in adult animals. Reduced sensitivity to insulin or IGF-1 may have similar consequences as reduced secretion, and therefore, Type II diabetes also appears to lead to reduced ICC (see Yamamoto *et al.* 2006). In the case of Type I diabetes, restoration of insulin or IGF-1 greatly muted the loss of ICC (Horvath *et al.* 2006). As we learn more about the factors responsible for initiating ICC loss, it may be possible to reverse or counteract these negative influences, making the microenvironment more compatible with the ICC phenotype.

Techniques for isolating, purifying and culturing ICC (e.g. Koh *et al.* 1998; Ördög *et al.* 2004) may make it possible to isolate cells from healthy regions of smooth muscle tissues, expand the number of cells in culture, and transplant functional ICC back into areas with depleted ICC populations. Here again the necessity for Kit signalling to maintain functional ICC must be addressed, because soluble stem cell factor does not appear to be an adequate stimulus of Kit in ICC (see discussion in Ward *et al.* 1995). Thus, currently, the phenotype of ICC in cell culture is evanescent, even when soluble stem cell factor is added to media (e.g. functional cells and Kit expression decrease within a week in culture and smooth muscle markers increase in ICC during this period; Epperson *et al.* 2000). One report describes growing ICC on a 'feeder layer' of cells that were engineered to express the membrane-bound isoform of stem cell factor (Rich *et al.* 2003). This approach appears to enhance retention of the ICC phenotype during cell expansion. Even if sufficient numbers of ICC can be grown and transplanted, it may be necessary to normalize the adverse factors that originally led to loss of ICC before transplanted ICC can graft and re-establish function in tissues. A recent report describes a population of CD34-positive cells that have a low level of Kit expression in the tunica muscularis of the gut (Redelman *et al.* 2006). These cells may be 'ICC stem cells' that, when properly stimulated, may be capable of regenerating ICC. This is an exciting new direction of investigation that should be pursued actively.

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