

**Mechanisms of diabetes mellitus associated depletion of
interstitial cells of Cajal in the murine stomach**

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Introduction

Interstitial cells of Cajal (ICC) provide the pacemaker activity typical of phasic gastrointestinal muscles of the stomach, small bowel, and colon. Many studies have shown that isolated smooth muscle cells lack spontaneous electrical activity, although ICC display pacemaker activity. The role of ICC in pacemaker activity of gastrointestinal tissue and their role in the mediation of neuromuscular neurotransmission were discovered after several groups recognized that all types of ICC express the gene product of the proto-oncogene *c-kit* that encodes a receptor tyrosine kinase, Kit which is located in the *white spotting (W)* locus. After these early immunohistochemical studies investigations on mutant animals (e.g. *W/W^v* mice) and a variety of other models in which ICC populations are depleted also have proven the fact that ICC are critical players of the gastrointestinal physiology.

In most cases the pathomechanism of ICC loss in different diseases is not known although it would be important to clarify the different steps to prevent or reverse ICC loss. One of the most frequent chronic disease with severe gastrointestinal complications is diabetes mellitus. Although generally considered as a manifestation of irreversible autonomic or enteric neuropathy, diabetic gastropathy is likely to be of multifactorial origin. In spontaneously diabetic NOD/LtJ mice, gastroparesis is associated with depletions of networks of interstitial cells of Cajal (ICC) in the distal stomach. ICC depletion and gastroparesis in NOD/LtJ mice occurs within 1.5 and 3 months after the onset of diabetes and dissecting the relative significance of hyperglycemia and impaired insulin signaling in chronic *in vivo* studies has been notoriously difficult. The first aim of the study was to develop an organotypic culture model that permits the independent control of insulin, glucose, and other growth factor levels over several months and to investigate the mechanism of diabetes-associated ICC depletion in the murine stomach. By using this technique the central role of the lack of insulin/IGF-I has been proven in diabetes associated ICC loss. In the second part of the study by using different approaches (including organotypic cultures) we aimed to explain the mechanisms in detail underline the loss of ICC in this chronic disease.

Materials and methods

Preparation of organotypic cultures

The intact corpus and antrum *tunica muscularis* tissues of juvenile BALB/c mice were pinned onto the surface of 35-mm culture dishes coated with Sylgard 184 and incubated at 37°C in normoglycemic (5.55 mmol/l) M199 medium containing 2% antibiotic-antimycotic and 2 mmol/l L-glutamine. Other supplements (used alone or in combination; see Results) included D-glucose (final concentration: 33.3 mmol/l or 55.5 mmol/l), bovine insulin (5 µg/ml), fetal bovine serum (5%), or murine IGF-I (100 ng/ml). All treatments were started within 48 h after establishing the cultures and maintained throughout the entire experiment. Levels of the supplements were kept constant by changing the culture media every 48 h.

Immunohistochemistry

Cultured and freshly dissected tissues were acetone fixed and immunostained. The following primary antibodies were used: rat IgG2b monoclonal anti- c-Kit clone ACK2 (5 µg/mL), rabbit polyclonal anti-insulin receptor α , anti-IGF-I receptor α , anti-SCF, goat polyclonal anti-insulin receptor β (2 µg/mL), and rabbit polyclonal anti-PGP 9.5 (1:200). Secondary antibodies were conjugated with either Alexa Fluor 488, 594, or Texas red (10 µg/ml). Specificity was verified by omitting the primary or secondary antibodies and by preabsorption with the appropriate blocking peptide. Live immunolabeling was performed by incubating gastric and jejunal *tunica muscularis* tissues with antibodies against SCF (2 µg/mL) and neural cell adhesion molecule (CD56; clone: H28.123; 2 µg/mL) at 4°C for 3 hours. Secondary antibodies were applied after fixation with 4% paraformaldehyde-saline. Confocal images of the whole-mounts were taken with Zeiss LSM 510 Meta Confocal image system. ICC network densities were analyzed quantitatively in superimposed two-dimensional projections of optical sections representing the entire thickness of the whole-mounts.

Purification of ICC by fluorescence-activated cell sorting (FACS)

ICC and macrophages in the intact gastric corpus + antrum tunica muscularis were labeled with Alexa Fluor 488- ACK2 and monoclonal phycoerythrin-cyanine 5 (PC5)-anti- F4/80, respectively. After the tissues were dispersed into single-cell suspensions, macrophages and dendritic cells were labeled with superparamagnetic monoclonal antibodies against CD11b and CD11c, respectively, and the labeling of macrophages was reinforced with a monoclonal PC5-anti-CD11b antibody. Mast cells and other leukocytes were labeled with monoclonal PC5-anti-CD45 antibodies. Macrophages and dendritic cells were then depleted by immunomagnetic selection. ICC were sorted on either a Beckman Coulter EPICS Elite or a Becton Dickinson FACSVantage instrument. A small aliquot of the 20,000 –100,000 ICC harvested in each experiment was reanalyzed by flow cytometry (FCM) on a Beckman Coulter XL/MCL for purity. Approximately 50,000 cells removed before the immunomagnetic depletion were used as unsorted control.

Qualitative and quantitative PCR

Total RNA was isolated using the Trizol reagent than was reverse transcribed with SuperScript II RNase H⁻ Reverse Transcriptase. The cDNA reverse transcription product was amplified with specific primers by PCR. Real-time quantitative PCR was performed by using SYBR Green chemistry on a GeneAmp 5700 sequence detector. Unknown quantities relative to the standard curve for the housekeeping gene β -actin were calculated to obtain transcriptional quantification of gene products. The following PCR primers were used to detect cell type-specific mRNA species: *c-kit* (Y00864, ICC), CD68 (NM_009853; macrosialin, a pan-macrophage marker also expressed by some myeloid-derived dendritic cells); mast cell tryptase (MCT; M57626; mast cells); smooth muscle myosin heavy chain (MyHC; NM_013607; smooth muscle cells); protein gene product 9.5 (PGP 9.5, AF172334, a pan-neuronal marker;); and prolyl-4-hydroxylase (BC018411; fibroblasts). CD34 (NM_133654; fibroblasts, endothelial cells), insulin receptor (Insr; NM_010568), IGF-1-receptor (Igf1r; AF056187), stem cell factor (SCF, NM_013598). When only the presence or absence of certain markers was the question qualitative PCR were used. In this method the amplified products (10 μ l) were separated by electrophoresis on a 2% agarose/1x TAE (Tris, acetic acid, EDTA) gel, and the DNA bands were visualized by ethidium bromide staining.

2.4, *Analysis of gene expression by hybridization*

Affymetrix Mouse Genome 430 2.0 microarrays, which cover the mouse transcriptome (over 39,000 transcripts) on a single array, were used for expression profiling of diabetic and nondiabetic gastric muscles. Total RNA was isolated and purified using Trizol and the RNeasy Mini Kit, respectively. RNA was quantified by spectrophotometry and its quality tested by formaldehyde gel electrophoresis. One-cycle complementary RNA synthesis and hybridization were performed by the Nevada Genomics Center following the manufacturer's protocols. Chips were scanned using an Affymetrix GeneChip 3000 System. Data were analyzed using the Affymetrix GeneChip Operating Software and verified by quantitative RT-PCR.

Electrophysiological recording

Electrical slow-wave activity in cultured and freshly dissected corpus + antrum tissues obtained from 14-day-old mice was analyzed by intracellular recording. Transmembrane potential of circular muscle cells impaled with KCl-filled glass microelectrodes was recorded at $37.5 \pm 0.5^\circ\text{C}$.

Results and discussion

Culturing juvenile (D9-18) gastric *tunica muscularis* tissue for 68-72 days in normoglycemic media resulted a significant decrease in ICC density. Surprisingly, instead of triggering further depletion of ICC loss hyperglycemia partially, but significantly prevented further loss of both ICC classes. By adding insulin or IGF-I to the system, the ICC loss could be completely prevented. Results were obtained by immunohistochemistry techniques were reinforced by the help of quantitative PCR. In cultures where intact ICC network (ie. insulin and IGF-I supplemented media) could be detected we were able to prove the presence of their activity (ie. slow waves) by using electrophysiological recordings. However, under hyperglycaemic conditions (where only partial rescue happened) no functional activity was measured.

Since the lack of insulin and IGF-I is the main cause of ICC loss in diabetic gastroparesis we examined whether ICC is a direct target of these growth factors or their effect can

only be explained by the help of stem cell factor (SCF), the only known growth factor responsible for the development and maintenance of ICC network. For this purpose in the first step we examined the gene expression pattern of FACS purified ICC populations with qualitative PCR. Our results clearly shown that ICC do not have insulin and IGF-I receptor so they are not direct targets of insulin and IGF-I.

The next step was to examine the possible relation of SCF to the lack of insulin and IGF-I. By using immunohistochemistry techniques we mapped the localization of insulin and IGF-I receptors on gastric *tunica muscularis* tissue. We found that both smooth muscle cells (both in circular and longitudinal layers) and the myenteric plexus (including ganglion cells, nerve trunks and intramuscular nerve fibers) possess these receptors (at least insulin receptor α and β and IGF-I receptor α). Similarly to insulin and IGF-I receptors, uniform immunoreactivity for SCF was detected throughout both smooth-muscle layers. However, consistent with earlier reports, only the perikarya of a subset of myenteric neurons contained SCF protein.

The lack of insulin and IGF-I receptors in ICC and their presence in cell types that also express SCF further reinforced our suspicion that the effects of these cytokines on ICC could be mediated by SCF. Therefore, we next investigated whether ICC loss in the stomach of diabetic NOD mice is accompanied by a decline in SCF expression. By using organotypic gastric *tunica muscularis* cultures we found a paralell decline in *c-kit*, myosin haevy chain, and both type of stem cell factor (soluble and membrane bound) gene expression. PGP 9.5 expression was completely lost independently of the treatment. We also approached the problem in *in vivo* experimnets where NOD diabetic mice were used. First, we verified - by immunohistochemical methods - that ICC were clearly reduced in the gastric corpus and antrum of the diabetic animals. Than by using Affymetrix Mouse Gene Chip and RNA hybridization we proved that the decrease in *c-kit* expression was accompanied by a profound reduction in both form of SCF mRNA and similarly to this reduction the myosin haevy chain expresion was also decreased. In contrast, the level of PGP 9.5 (Uchl1) expression does not decrease in gastric tunica muscularis of NOD diabetic animals. By the help of quantitative PCR we also found that the decrease in *c-kit* expression is followed by a similar decrease in SCF (soluble and membrane bound) expression.

Since the changes in the smooth muscle always mirrored the changes in ICC and *Kitl* expression but neuronspecific gene expression remained normal in long-term diabetic NOD mice with depleted ICC and stayed near undetectable levels in the insulin- and IGF-

I- treated organotypic cultures despite the rescue of the ICC network, it clearly indicates that smooth-muscle cells and myenteric neurons, the two main sources of SCF in the gut differ in their relationship to ICC. Therefore, we investigated whether intracellular localization of SCF in myenteric neurons could account for the dramatic dissociation between the fates of neurons and ICC. Live immunolabeling of tunica muscularis tissues from BALB/c and nondiabetic NOD mice only stained the membranes of circular and longitudinal smooth-muscle cells, and no surface labeling of myenteric ganglion cells was detectable. Thus, SCF produced by myenteric neurons may not be available to ICC.

In the last experiment we examined whether immunoneutralization of SCF (the natural ligand for c-Kit) could accelerate the demise of gastric ICC networks in organotypic cultures. Inclusion of polyclonal anti-SCF antibodies (2 µg/mL) in the culture media caused a profound depletion of both main ICC classes and a significant reduction of *c-kit* expression, indicating that loss of SCF *per se* can lead to loss of ICC.

In summary, in this thesis has it been proven that:

- 1, diabetes mellitus associated disruption of interstitial cells of Cajal in the gastric tunica muscularis happens due to the lack of insulin (and/or IGF-I) effect and not because of hyperglycaemia.
- 2, although insulin and/or IGF-I are required for the long-term maintenance of ICC morphology and slow waves, ICC are not direct targets of these hormones.
- 3, absolute or relative insulin or IGF-I insufficiency lead to ICC depletion by reducing SCF expression in smooth muscle cells but not the enteric nervous system.
- 4, insulin and IGF-I signaling are the common denominator of diabetes-associated myopathy and ICC loss.