Roles of stem cell factor on the depletion of interstitial cells of Cajal in the colon of diabetic mice

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Lin L, Xu L-M, Zhang W, Ge Y-B, Tang Y-R, Zhang H-J, Li X-L, Chen JDZ. Roles of stem cell factor on the depletion of interstitial cells of Cajal in the colon of diabetic mice. Am J Physiol Gastrointest Liver Physiol 298: G241–G247, 2010. First published October 29, 2009; doi:10.1152/ajpgi.90706.2008.—The aim of this study was to investigate the effects of stem cell factor (SCF) on interstitial cell of Cajal (ICC) depletion in the colon of diabetic mice. Male C57BL6 mice were treated by a single intraperitoneally injected dose of streptozotocin, and those displaying sustained high blood glucose were selected as diabetes mellitus models. Six groups of mice were used: three groups of normal nondiabetic mice (untreated and treated with IgG or SCF antibody), and three groups of diabetic mice (untreated and treated with vehicle or SCF). Changes of the ICC quantities were analyzed by immunohistochemistry. ICC morphologies were observed with transmission electron microscopy. The SCF levels in sera and colon tissues were detected by ELISA and Western blot, respectively. The nondiabetic mice treated with SCF antibody and the untreated diabetic mice showed decreased SCF levels in the sera and colonic tissues, reduced numbers of ICC, and pathological changes of the ICC ultrastructures, whereas the nondiabetic mice treated with mouse IgG showed no significant changes compared with the nondiabetic mice. The diabetic mice treated with exogenous SCF showed restored SCF levels in both sera and colon tissues and improvement in the numbers of ICC and the damages of ICC ultrastructures, whereas the vehicle control of diabetic mice showed no significant changes compared with the diabetic mice. The blood glucose remained high and unchanged with the treatment of SCF or vehicle in the diabetic mice. These results indicate that diabetic mice show a decline in the number of ICC and impairment in the ultrastructures of ICC, and these abnormalities are attributed to a deficiency in the endogenous SCF but are not related to hyperglycemia. Exogenous SCF partially reverses the pathological changes of ICC in diabetic mice.

diabetes mellitus; colon motility; ultrastructures; gastrointestinal motility

DISORDER OF GASTROINTESTINAL (GI) motility is one of the major complications in diabetes mellitus (DM) with a prevalence of 25–76% (7, 17). GI dysmotility includes gastroparesis, constipation, and diarrhea and affects the quality of life and blood glucose control in patients with DM. The mechanism of dysmotility has been linked to pathology of interstitial cells of Cajal (ICC) in the GI tract. In the patients with type 1 or type 2 DM, a decrease in ICC number and cellular degradation of ICC were reported to be widely spread and observed in the stomach, jejunum, and colon (1, 14, 25).

Recently, ICC have been reported to be the pacemaking cells and to mediate neural transmission (34, 37). A number of studies have shown that abnormalities in ICC, particularly the depletion in the number of ICC, are correlated with various GI diseases such as diabetic dysmotility, chronic intestinal pseudoobstruction, Hirschsprung’s disease, chronic constipation, and achalasia (8, 12, 14, 33, 41). However, the pathological and physiological mechanisms of diabetes-associated loss of ICC are not fully understood.

It is known that ICC survival and functions depend on the activation of c-Kit, a receptor tyrosine kinase integral to ICC (23), by stem cell factor (SCF, Kit ligand). SCF-Kit signaling has been shown to be important for the maintenance of ICC phenotypes, proliferation, and differentiation (35, 42). Reduced SCF was reported in the stomach and colon in the diabetic mice (15, 43). However, the role of SCF in the diabetes-associated loss of ICC is unknown. Therefore the aim of this study was to investigate the role of SCF in the loss of ICC in diabetic mice.

MATERIALS AND METHODS

Animals. Male C57BL mice 4–6 wk of age, weighing 20 ± 2 g, were housed in a specific pathogen-free environment. A mouse model of DM was established by a single-dose intraperitoneal injection of streptozotocin (STZ) 150 mg/kg (4, 21) in the normal mouse. Seventy-two hours later, blood samples were taken daily from the tail vein for the measurement of the fasting blood glucose concentration after 6–8 h of fasting. Once the blood glucose concentration was elevated to and sustained above 12 mmol/l for 1 wk or more, the mouse was considered to be diabetic. The mice in the control group were treated by a single intraperitoneal injection of equal volume of citric acid buffer solution.

Experimental protocol. The mice were divided into six groups of 10 mice each: nondiabetic mice [untreated (NDM), treated with SCF antibody (Antibody-NDM), treated with mouse IgG (IgG-NDM)] and diabetic mice [untreated (DM), treated with SCF (SCF-DM), treated with vehicle (Vehicle-DM)]. The mice in group Antibody-NDM were treated with intraperitoneal injections of 1 mg/kg anti-SCF antibody every other day for a period of 6 wk (5). The mice in group IgG-NDM received a similar regimen of mouse IgG as controls. The mice in group SCF-DM were treated with intraperitoneal injections of exogenous recombinant SCF protein at a dose of 0.2 μg/kg daily for 6 wk (29), whereas the mice in group Vehicle-DM received an identical volume of vehicle (sterile PBS). Nothing was given to the mice groups NDM and DM during the 6-wk treatment period. The blood glucose levels and body weights of all experimental mice were measured weekly. Three days following the completion of the 6-wk treatment, all mice were euthanized for sample collections.

Reagents. STZ was purchased from Sigma (St. Louis, MO). The recombinant mouse SCF, anti-mouse SCF-neutralizing antibody, and SCF ELISA kit were bought from R&D Systems (Abingdon, Oxford-
shire, UK). Antibody against c-Kit and FITC-labeled rat anti-c-Kit monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). An Olympus BX51 microscope (Olympus Optical, Tokyo, Japan) was used to observe the result of immunohistochemistry. A Philips EM-400 transmission electron microscope (Philips, Eindhoven, The Netherlands) was used to observe the ultrastructure of ICC.

Expressions of SCF and c-Kit in colon tissues by Western blot analysis. Tissue samples of about 0.5 g were excised from near proximal colon, cut into pieces of about 0.25 cm², and then grinded into cell suspension with a blade. Crude total proteins were extracted, and protein concentrations were measured by the bicinchoninic acid method. Fifty micrograms of protein were separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a PVDF membrane. After being incubated for 1.5 h in a buffer with bovine serum albumin, the blot was incubated with a SCF antibody (1:500) at 37°C for 1 h and then at 4°C overnight, followed with incubation of a horseradish peroxidase-conjugated secondary antibody at a ratio of 1:2,000 for 2 h. After enhanced chemiluminescence, the blot was subject to autoradiography.

The expression of c-Kit proteins from the same samples were performed similarly with the antibody against c-Kit that was used at a 1:200 dilution (32).

Detection of SCF in the mouse serum by ELISA. To study whether the changes of the SCF level in the colon were parallel to those in serum, 1–2-ml blood samples were taken from the mice before they were euthanized. The serum concentration of SCF was measured by ELISA using a commercial kit according to the manufacturer’s instructions. The standard curve of SCF was determined by the SCF with known concentrations, and the serum concentration of each sample was calculated on the basis of the standard curve.

Expressions of c-Kit in colon tissues by immunohistochemistry. To identify ICC, immunofluorescent staining for c-Kit was used (13). Tissue samples of full thickness of the proximal colon were fixed by 4% paraformaldehyde and then embedded into paraffin. Sections were cut at 4 μm from paraffin-embedded tissue blocks and mounted on gelatin-coated slides. To unmask antigenic sites, sections were boiled in a microwave for 10 min in citrate buffer (0.01 M, pH 6.0) and then allowed to cool for 20 min. To prevent nonspecific absorption of IgG, we incubated the sections in 1% BSA in PBS for 1 h. Sections were allowed to cool for 20 min. To prevent nonspecific absorption of IgG, we incubated the sections in 1% BSA in PBS for 1 h. Sections were then incubated with FITC-labeled rat anti-c-Kit monoclonal antibody overnight at 4°C. Sections were placed in gel aqueous mounting medium with a cover glass and were examined under an Olympus BX51 microscope. Controls were treated by omitting the primary or secondary antibody. No staining was observed under the negative control conditions. Images were taken at a magnification of ×400. In our preliminary experiment, the ICCs were identified as the c-Kit-positive but mast cell tryptase (a marker of mast cell)-negative staining cells from the muscle layer. Accordingly, the c-Kit-positive cells in the muscle layer of the full-thickness colon were counted as ICC. Semi-quantitative analyses were performed by an experienced observer with no knowledge of the study groups in nine nonconsecutive colon sections divided in three slides per animal per group under high-powered microscopic fields (×400).

Table 1. Relative SCF and c-Kit expression levels in colon tissues assessed by the Western blot (SCF/α-tubulin, c-Kit/α-tubulin), SCF in mouse sera by ELISA, and c-Kit-positive cells measured by immunohistochemistry

<table>
<thead>
<tr>
<th></th>
<th>NDM</th>
<th>DM</th>
<th>Antibody-NDM</th>
<th>SCF-DM</th>
<th>IgG-NDM</th>
<th>Vehicle-DM</th>
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<tr>
<td>SCF/α-tubulin</td>
<td>0.921 ± 0.072</td>
<td>0.82 ± 0.11*</td>
<td>0.858 ± 0.056*</td>
<td>1.001 ± 0.016†</td>
<td>0.910 ± 0.077†</td>
<td>0.813 ± 0.082*</td>
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<tr>
<td>SCF in sera, pg/ml</td>
<td>153.1 ± 19.7</td>
<td>120.6 ± 14.8*</td>
<td>89.5 ± 15.5*</td>
<td>189.6 ± 49.9†</td>
<td>149.7 ± 13.3†</td>
<td>117.6 ± 10.1*</td>
</tr>
<tr>
<td>c-Kit-positive cells</td>
<td>82 ± 7</td>
<td>60 ± 5*</td>
<td>62 ± 3*</td>
<td>71 ± 6†</td>
<td>80 ± 5†</td>
<td>58 ± 7*</td>
</tr>
<tr>
<td>c-Kit/α-tubulin</td>
<td>1.013 ± 0.029</td>
<td>0.796 ± 0.072*</td>
<td>0.91 ± 0.376†</td>
<td>0.889 ± 0.042†</td>
<td>1.010 ± 0.035†</td>
<td>0.789 ± 0.081*</td>
</tr>
</tbody>
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Values are means ± SD. *P < 0.05 vs. group NDM; †P < 0.05 vs. group DM. SCF, stem cell factor; NDM, nondiabetic mice; DM, diabetic mice; Antibody-NDM, nondiabetic mice treated with SCF antibody; SCF-DM, diabetic mice treated with SCF; IgG-NDM, nondiabetic mice treated with mouse IgG; Vehicle-DM, vehicle control of diabetic mice.

RESULTS

Body weight and blood glucose. All mice survived and went through the entire protocol. No differences were noted in the baseline body weight before the treatment among the six groups. At the end of the 6 wk, the mean body weights in groups NDM, DM, Antibody-NDM, SCF-DM, IgG-NDM, and Vehicle-DM were 26.6 ± 1.7 g, 19.1 ± 1.4 g, 26.0 ± 1.9 g, 21.8 ± 4.0 g, 27.3 ± 2.1 g, and 18.8 ± 1.6 g, respectively. No difference in the body weight was noted among the three groups of nondiabetic mice or among the three groups of diabetic mice. However, the body weight in the three diabetic groups was significantly lower than that in the three nondiabetic groups (P < 0.05). Meanwhile, the concentrations of fasting blood glucose in nondiabetic mice before and after treatments were 9.1 ± 0.7 mmol/l and 8.4 ± 0.4 mmol/l, respectively in group NDM, 9.9 ± 1.4 mmol/l and 8.6 ± 0.6 mmol/l, respectively in group Antibody-NDM, and 8.8 ± 1.3 mmol/l and 8.6 ± 1.7 mmol/l, respectively in group IgG-NDM. All these values were within the normal range. The fasting blood glucose levels in diabetic mice before and after the 6-wk treatment were 30.2 ± 3.3 mmol/l and 33.3 ± 0 mmol/l, respectively in group DM, 30.1 ± 2.9 mmol/l and 32.7 ± 1.6 mmol/l, respectively in group SCF-DM, and 31.5 ± 2.4 mmol/l and 33.2 ± 1.8 mmol/l, respectively in group Vehicle-DM. All
these values were above the minimal level of DM mice (≥12 mmol/l). The 6-wk treatment did not alter the fasting blood glucose level in any of the six groups of mice, suggesting that SCF played no roles in the regulation of blood glucose.

**SCF protein of colon tissues by Western blot analysis.** As shown in Table 1, the protein expression of SCF was reduced in the untreated diabetic mice, the vehicle control of diabetic mice, and the nondiabetic mice treated with SCF antibody. The treatment of mouse IgG in the nondiabetic mice showed no effects on SCF; the SCF level in group IgG-NDM was similar to that in group NDM. The treatment with SCF increased the SCF level in the diabetic mice compared with the untreated or vehicle-treated diabetic mice (Fig. 1).

**SCF in mouse sera by ELISA.** As shown in Table 1, in parallel to the SCF protein expressions in colon tissues, the SCF concentration in sera was also decreased in the untreated diabetic mice, the vehicle control of diabetic mice, and the nondiabetic mice treated with SCF antibody; no differences were noted among these three groups. The SCF was restored nearly to normal in the SCF-DM mice compared with the NDM mice (P > 0.05). The SCF concentration in group IgG-NDM was not attenuated and comparable to the group NDM, indicating that mouse IgG had no effects on SCF.

**c-Kit levels in colon tissues by immunohistochemistry.** As shown in Table 1 and Fig. 2, the untreated diabetic mice and the nondiabetic mice treated with SCF antibody showed a significant decrease in c-Kit immunoreactivity, compared with the normal nondiabetic mice (P < 0.05). However, no differ-
ence was observed between the normal nondiabetic mice and the diabetic mice treated with SCF ($P > 0.05$) and between the untreated diabetic mice and the nondiabetic mice treated with SCF antibody ($P > 0.05$). Treatment of IgG in the normal mice and the vehicle treatment in the diabetic mice did not alter the number of ICC. These findings indicated that diabetes led to a decrease in the number of c-Kit-positive cells, and the decrease was attributed to a loss in SCF. Exogenous SCF increased the level of reduced c-Kit-positive cells in the diabetic mice.

$c$-Kit levels in colon tissues by the Western blot analysis. As shown in Table 1 and Fig. 3, although the c-Kit protein in the

![Correlation between c-Kit and SCF](image)

**Fig. 4.** Correlations between c-Kit expressions and SCF expression assessed by the Western blot analysis. There was a significant and high positive correlation between the c-Kit level and the SCF level in every group. Each point represents the value from each individual mouse.
nondiabetic mice treated with SCF antibody was decreased compared with the nondiabetic mice \((P < 0.05)\), it was higher than that in the DM mice \((P < 0.05)\) (see Table 1). The SCF treatment in the diabetic mice increased the c-Kit protein \((P < 0.05, \text{SCF-DM vs. DM})\); however, the c-Kit protein was still lower than that in the nondiabetic mice \((P < 0.05)\), suggesting a partial normalization of the c-Kit protein with SCF in the diabetic mice. A significant correlation was noted between the c-Kit protein expression and the SCF level in every group of the mice as shown in Fig. 4; that is, the higher the SCF level was, the more c-Kit was expressed.

**ICC ultrastructures by the transmission electron microscopy.** In the nondiabetic mice, ICC displayed normal morphology, shown by the intact nuclear membrane structures, abundant cytoplasmic contents, and organelles including rough endoplasmic reticulum and mitochondria. They were also shown to be closely connected to neighboring nerve fibers and smooth muscle cells (Fig. 5A). After induction of diabetes or SCF antibody treatment, some ICC with damaged features were identified in the mouse colon with cytoplasmic alterations. The cytoplasm was either completely empty or contained few swollen mitochondria, cisternae of the endoplasmic reticulum, and lysosomes. However, no apoptotic ICC were identified according to universally recognized criteria in regards to the nuclear alterations (Fig. 5, B and C) (20). The treatment of IgG in the nondiabetic mice and the treatment of vehicle in the diabetic mice did not lead to any changes in the morphological features compared with the corresponding untreated groups. However, the treatment with SCF in the diabetic mice obviously reverted the injuries in the colon of diabetic mice (Fig. 5D).

**DISCUSSION**

The blood glucose in the all three groups of diabetic mice was significantly higher than that in the three nondiabetic groups. The diabetic mice showed a reduced body weight compared with the nondiabetic mice. Compared with the NDM mice, the diabetic mice showed a reduced expression of SCF in both the serum and the colon, a reduced number of ICC, damages to the ICC ultrastructures, and reduced c-Kit protein. Interestingly, similar reductions in the expression of SCF and in the number of ICC were noted in the nondiabetic mice treated with SCF antibody. Treatment with exogenous SCF improved the diabetes-induced abnormalities in ICC in the diabetic mice. The treatment with SCF or SCF antibody had no effects on blood glucose.

Reduction in the number of ICC has been reported in patients with diabetes (19, 27) as well as in animal models of diabetes (9, 15, 43). The impairment in ICC was reported to be correlated with dysmotility of the gut in diabetic animals (9, 43). In a few recent studies, a loss of SCF was also observed in diabetic animals; one study reported a significant and substantial loss of SCF in the stomach of nonobese diabetic mice (15), and another study reported a reduction of SCF in db/db mice with a mild loss in the stomach but a more significant loss in the distal small intestine and the colon (43). It was speculated that the loss of SCF might be responsible for the loss of ICC. However, no solid evidence was provided because a causative effect of SCF on the loss of ICC could not be established by merely determining the loss of SCF and loss of ICC in diabetic animals. In this study, in addition to assessing the losses of SCF and ICC in diabetic animals, two more groups of animals were added, namely a group of nondiabetic mice treated with SCF antibody and a group of diabetic mice treated with SCF. The causative effect of SCF on the loss of ICC could be established if the normal mice treated with SCF antibody showed impairment in ICC, whereas the diabetic mice treated with SCF showed improvement in ICC. To the best of our knowledge, this was the first study to systematically investigate the role of SCF in the loss of ICC in diabetes.

Loss of ICC was reported in nonobese diabetic mice, db/db mice, and STZ-induced diabetic rats. In this study, we used STZ-induced diabetic mice. The model of diabetes was established successfully. To provide comprehensive assessment of

![Ultrastructures of ICC.](http://ajpgi.physiology.org/)
the ICC in the colon, three different methods were used including Western blot, immunohistochemistry, and morphological examination. The Western blot analysis revealed a 21.5% loss of ICC, and the immunohistochemistry showed a 24.5% loss of ICC in the diabetic mice, compared with the control mice. The morphological examination showed damages in the ultrastructures of the ICC. These findings were in agreement with previous findings in STZ-induced diabetic rats (9). In the previous study, ICC subtypes were examined in the colon of the STZ-induced diabetic rats; both intramuscular ICC and submuscular plexus ICC were scarce, and the area occupied by intramuscular ICC and submuscular plexus ICC was significantly lower compared with the control rats.

Accompanying the loss of ICC, the SCF was also reduced in the STZ-induced diabetic mice in this study. The reduction in SCF was 11% on the basis of the Western blot analysis and 21% according to the ELISA method. These findings were consistent with the previously reported loss of SCF in nonobese diabetic mice and db/db mice (15, 43). To investigate the role of the SCF on the loss of ICC, we treated the diabetic mice with exogenous SCF for 6 wk and the nondiabetic mice with SCF antibody for the same period. Interestingly, in the diabetic mice treated with SCF, the SCF levels were brought to normal (similar to the control mice) and impairment of ICC was significantly improved. However, the improvement was about 50% according to the Western blot analysis and the immunohistochemistry. More interestingly, the nondiabetic mice treated with SCF antibody showed an effective depletion of SCF that was not less than that in the diabetic mice. Similar impairment in ICC was observed in these SCF antibody-treated mice. The decrease in ICC was the same as that in the diabetic mice on the basis of the immunohistochemistry but was less than that in the diabetic mice according to the Western blot analysis. These findings demonstrated that 1) the reduction of ICC in the diabetic mice was attributed to the loss of SCF and 2) the SCF played a partial role in the maintenance of ICC because the same degree of SCF loss in the control mice resulted in a partial loss of ICC compared with the diabetic mice and because the restoration of SCF in the diabetic mice with the treatment of SCF only partially improved the ICC damages in the diabetic mice. It is known that SCF is required for long-term ICC survival and function (2, 10, 16, 18, 28, 36, 39, 40, 42). ICC express c-Kit (CD117), a receptor of SCF. The binding of SCF to c-Kit may activate tyrosine kinase, and a series of downstream signaling events ultimately affect c-Kit-positive cell fates such as proliferation and differentiation. SCF, as a ligand of c-Kit receptor, is a multifunctional growth factor controlling cell proliferation, differentiation, and migration (11).

There are controversial reports regarding the role of SCF-Kit system on the development, survival, and maintenance of the ICC. One study showed that the inhibition of phosphatidylinositol 3-kinase downstream signaling element linking Kit receptors caused a decrease of ICC in the jejunal and antral of BALB/c mice from birth to 30 days after birth. It was concluded that SCF-Kit signaling was important for development and maintenance of ICC phenotypes and functions in both prenatal and postnatal mice (38). Another in vitro study measured ICC in the gastric corpus and antrum of BALB/c mice by Kit immunohistochemistry and confocal microscopy and reported no changes in the structure and distribution of gastric ICC at the myenteric plexus during postnatal development (days 0–50) (28). It was therefore speculated that SCF-Kit signaling seemed necessary for ICC prenatal development but not for ICC proliferation and phenotype maintenance after birth. On the contrary, another study suggested that SCF-Kit signaling was not essential for prenatal development, proliferation, and survival because the WlacZ mutation mice with the insertion of a lacZ into the first exon of c-Kit gene did not show any difference in ICC between the c-Kit-null and normal prenatal mice (3). However, another study indicated that SCF-Kit signaling might be required for maintaining phenotypes of ICC only after birth but not during embryonic development (22). In this present study, SCF was found to play a crucial role in the survival and maintenance of ICC in the colon in adult mice.

In conclusion, diabetic mice show a decline in the number of ICC and impairment in the ultrastructures of ICC, and these abnormalities are partially attributed to a deficiency in the endogenous SCF but not to hyperglycemia. Exogenous SCF partially reverses the pathological changes of ICC in diabetic mice.


