CCR2 knockout exacerabtes cerulein-induced chronic pancreatitis with hyperglycemia via decreased GLP-1 receptor expression and insulin secretion

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Am J Physiol Gastrointest Liver Physiol 304: G700–G707, 2013. First published February 28, 2013; doi:10.1152/ajpgi.00318.2012.—Glucagon-like peptide-1 (GLP-1) promotes insulin release; however, the relationship between the GLP-1 signal and chronic pancreatitis is not well understood. Here we focus on chemokine (C-C motif) ligand 2 (CCL2) and its receptor (CCR2), which regulates various immune cells, including macrophages, to clarify the mechanism of GLP-1-mediated insulin secretion in chronic pancreatitis in mice. One and multiple series of repetitive cerulein administrations were used to induce acute and chronic cerulein pancreatitis, respectively. Acute cerulein-administered CCR2-knockout (KO) mice showed suppressed infiltration of CD11b+/Gr-1high cells, but not CD11b-/Gr-1low cells, in pancreatic islets compared with their WT counterparts. Furthermore, although serum GLP-1 levels in chronic cerulein-administered WT and CCR2-KO mice were comparably upregulated after cerulein administrations, GLP-1 receptor levels in pancreases of chronic cerulein-administered CCR2-KO mice were comparably upregulated after cerulein administrations. However, chronic cerulein-administered CCR2-KO mice showed significantly increased infiltration of CD11b+/Gr-1high macrophages and pancreatic inflammation and significantly upregulated insulin secretion compared with paired wild-type (WT) mice. Nevertheless, chronic cerulein-administered CCR2-KO mice showed significantly increased infiltration of CD11b+/Gr-1low macrophages and pancreatic inflammation compared with paired WT mice. Therefore, animal models are often used to investigate the relationship between insulin secretion and chronic pancreatitis. Cerulein-induced pancreatitis is a standard model for investigating acute pancreatitis (12) and chronic pancreatitis (24). However, the mechanism of insulin secretion in the chronic model of the disease is unclear.

Infiltration of mononuclear cells is a hallmark of pathogenesis of various immune diseases. Among them, macrophages play a key role in innate immunity for the maintenance of homeostasis, for example inflammation dampening via the production of anti-inflammatory cytokines such as IL-10 and TGF-β, debris scavenging, angiogenesis, and wound healing (9, 11). Chemokine (C-C motif) ligand 2 (CCL2) is a small cytokine of the CC chemokine family, and its receptor (CCR2) plays an important role in the recruitment of monocytes, memory T cells, and peritoneal macrophages in inflammatory tissues (1, 19, 22). Recently we showed that CCL2-deficient mice are resistant to acute cerulein pancreatitis by affecting the migration of pathological TNF-α-producing CD11bhigh/CD11c+/Gr-1low macrophages (16). However, no studies on the role of the CCR2/CCL2 axis and its involvement in insulin secretion in chronic pancreatitis have been reported to date.

Glucagon-like peptide-1 (GLP-1), secreted from intestinal L cells, is associated with increased intracellular cyclic AMP, leading to insulin release from β-cells during hyperglycemia and suppression of inappropriate glucagon secretion from α-cells. Consistent with this mechanism, a long-acting GLP-1 analog, liraglutide, has been developed for the treatment of type 2 diabetes (14). However, the relationship between GLP-1 and diabetes with chronic pancreatitis has not been clarified.

The aims of this study were to clarify the role of the CCR2/CCL2 axis in the pathogenesis of chronic cerulein pancreatitis and to examine the mechanisms underlying insulin secretion and GLP-1 signals in chronic pancreatitis.

MATERIALS AND METHODS

Animals. Breeding pairs of CCR2-deficient mice (knockout) (CCR2-KO) on a C57BL/6J background (Jackson Laboratory, Bar Harbor, ME) were used. The breeding pair was generated as described previously (24). Animals were also provided by Jackson Laboratory. CCR2-KO mice were aged 10–15 weeks old when used for experiments. C57BL/6J WT and CCR2-KO mice were obtained from Jackson Laboratory. For further details, see the online supplement.

IN THE YEAR 2000, more than 171 million people worldwide were reported to have diabetes; it is estimated that this number will almost double by 2030 (23). Diabetes is a serious metabolic disorder that causes hyperglycemia as a result of poor insulin production and insulin resistance. Clinical evidence shows that diabetes occurs in the late phase of chronic pancreatitis. Numerous clinical cases of chronic pancreatitis and insulin therapy have been studied; however, the relationship between chronic pancreatitis and insulin secretion is still unknown.

Therapies for chronic pancreatitis include changing patients’ alcohol habits and prescribing drugs of digestive enzymes and/or analgesic drugs, which are usually effective to control the symptoms. Because chronic pancreatitis is rarely treated with surgery, specimens of pancreatic β-cells from patients with chronic pancreatitis are difficult to obtain; histological investigation of such tissue is consequently challenging. Therefore, animal models are often used to investigate the relationship between insulin secretion and chronic pancreatitis. Cerulein-induced pancreatitis is a standard model for investigating acute pancreatitis (12) and chronic pancreatitis (24). However, the mechanism of insulin secretion in the chronic model of the disease is unclear.

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Harbor, ME) and wild-type (WT) C57BL/6J mice (Charles River Laboratories Japan, Tokyo, Japan) were used. All mice were maintained and bred at the animal facilities at Keio University, Japan, and all experiments were approved by and performed according to the guidelines of the Experimental Animal Committee of Keio University, School of Medicine.

Mouse models of acute and chronic cerulein pancreatitis. Acute cerulein pancreatitis was induced in 8-wk-old male mice with seven hourly intraperitoneal injections of cerulein (American Peptide, Sunnyvale, CA), at a dose of 50 µg/kg. Chronic cerulein pancreatitis was induced by six hourly intraperitoneal injections of cerulein, at a dose of 50 µg/kg, given twice per week for 10 wk (25). Control animals were injected with the same volume of physiological saline. Mice were euthanized with an overdose of pentobarbital. Blood and pancreatic tissues were collected 1 h after the final cerulein injection for acute pancreatitis and at 8:00 in the morning 1 wk after the final cerulein injection for chronic pancreatitis.

Histological analyses, including interstitial space, necrosis, islet cell, and fibrotic tissue using hematoxylin and eosin (HE) stain and Masson-Trichrome stain, were quantified using a LuminaVision analysis system (Mitani, Fukui, Japan) (10, 13).

Blood examination. Concentrations of serum amylase (AMY) and lipase (LIP) were measured by a commercial laboratory (SRL, Tokyo, Japan). Plasma glucose was measured with a OneTouch UltraVue meter (Johnson & Johnson, New Brunswick, NJ), serum insulin with an insulin measuring kit (Morinaga Institute of Biological Science, Kanagawa, Japan), and plasma GLP-1(7–36) and GLP-1(9–36) with an enzyme immunoassay kit (Yanaihara Laboratory, Shizuoka, Japan), according to manufacturers’ instructions.

Immunohistochemistry. Pancreatic tissue fixed with paraffin was deparaffinized in xylene, hydrated with ethanol, and finally rinsed in PBS. Anti-insulin antibody (Dako, Glostrup, Denmark) and GLP-1R antibody (Abcam, Cambridge, UK) were used as primary antibodies and were incubated with a mixture of two fluorescence-conjugated secondary antibodies (FITC-conjugated goat anti-rabbit antibodies for insulin (Abcam) and Alexa-568-conjugated goat anti-rabbit antibody for GLP-1R (Invitrogen, Carlsbad, CA)) in PBS for 30 min at room temperature.

Western blotting. Pancreatic tissues taken from the mice after euthanasia were immediately frozen in liquid nitrogen and stored at −80°C. The tissues were homogenized with RIPA buffer (Thermo Scientific, Rockford, IL) containing protease inhibitors (Thermo Scientific). After being centrifuged for 15 min at 4°C at 14,000 revolution/min, the supernatant was transferred to new tubes, and protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL). Samples were run on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). The membranes were incubated with primary antibodies against GLP-1R, insulin, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Flow cytometry analysis. Fresh pancreatic tissue was minced and digested with 3 mg/ml collagenase A (Roche, Mannheim, Germany) for 15 min at 37°C. The digest was filtered through a 40-µm cell strainer and washed with Hank’s balanced salt solution containing 1.5% FCS, as described previously (16). Viability was ascertained using with 7-AAD and annexin V (BD Pharmingen, Franklin Lakes, NJ) double staining; specimens with ≥80% living cells were selected for examination. Isolated cells were incubated with anti-CD16/CD32 antibody (BD Pharmingen) to prevent nonspecific antibody binding. Surface antigens were stained with CD11b-phycocerythrin-Cy7 and Ly6C-allophycocyanin-Cy7 (Gr-1, clone: RB6–8C5; BD Pharmingen). Flow cytometry was performed on a FACSCanto II/TM (BD
**Biosciences, San Jose, CA**. All data were reanalyzed with FlowJo Version 7.2.5 software (TreeStar, Ashland, OR).

**Glucose tolerance test and insulin sensitivity.** Glucose tolerance test (GTT) was performed to measure changes in blood glucose concentrations after an intravenous injection of 20% glucose at 2 g/kg. Insulin sensitivity was measured by examining the changes in blood glucose levels after an intravenous injection of regular insulin Novolin R (Novo Nordisk Pharma, Bagsvaerd, Denmark), at a dosage of 0.75 U/kg. GTT was performed on WT and CCR2-KO mice with chronic pancreatitis treated with and without the GLP analog, liraglutide (Novo Nordisk Pharma). For 3 days before the GTT, liraglutide was injected intraperitoneally twice each day at dosages of 100 μg/kg body wt on the first day, 200 μg/kg on the second day, and 300 μg/kg on the final day.

**Quantitative RT-PCR analysis with isolated islet cells.** Islet RNA was extracted with Trizol (Invitrogen) from isolated mouse islet cells, as previously described (8) using collagenase-p (Roche, Basel, Switzerland). Reverse transcription and quantitation of mRNA expression were performed as previously described (20) using the SYBR Premix Ex Taq (Perfect Real Time) kit in a Thermal Cycler Dice Real Time system (TaKaRa Biologicals, Ohtsu, Shiga, Japan).

**Statistical analysis.** All data are expressed as means ± SD. The two-tailed nonparametric test (unpaired t-test) was used for statistical analysis. P < 0.05 were considered statistically significant.

**RESULTS**

**CCR2-KO mice were resistant to acute cerulein pancreatitis.** Acute cerulein pancreatitis in WT and CCR2-KO mice was analyzed with HE staining of pancreatic tissues, and serum AMY and LIP levels were measured. We confirmed that both WT and CCR2-KO mice were healthy until they were 40 wk old (data not shown). Saline-administered WT and CCR2-KO mice showed no pancreas abnormalities. In contrast, cerulein-administered WT mice developed acute pancreatitis with edema and infiltration of inflammatory cells into the pancreas; however, the severity in cerulein-administered CCR2-KO mice was markedly reduced (Fig. 1A). Consistently, absolute numbers of inflammatory cells (Fig. 1B), serum AMY (Fig. 1C), serum LIP (Fig. 1D), necrotic cells (Fig. 1E), and interstitial space (Fig. 1F) in cerulein-administered CCR2-KO mice were...
significantly less than in cerulein-administered WT mice, whereas no differences were observed between the two types in the paired saline-administered mice.

CCR2-KO mice were sensitive to chronic cerulein pancreatitis. In contrast to the saline-administered mice, long-term administration of cerulein for the chronic model induced severe edema, infiltration of inflammatory cells, and fibrosis (Fig. 2A). Surprisingly, however, contrary to the results observed in acute cerulein pancreatitis, cerulein-administered CCR2-KO mice developed severe pancreatitis compared with the WT mice (Fig. 2A). The absolute numbers of inflammatory cells (Fig. 2B), the serum AMY (Fig. 2C) and LIP (Fig. 2D), and interstitial space (Fig. 2F) in cerulein-administered CCR2-KO mice were significantly higher than in cerulein-administered WT mice, whereas no differences were observed between the two mouse types in the paired saline-administered mice. Necrotic cells and fibrosis space did not significantly differ between cerulein-administered WT and CCR2-KO mice (Fig. 2E and G). As with the acute model (Fig. 1), the body weight of cerulein-administered WT mice decreased after the first sequential cerulein injection, whereas their cerulein-administered CCR2-KO counterparts maintained their body weight (Fig. 2E). However, 1 wk after the onset of chronic cerulein pancreatitis, the body weight of cerulein-administered WT mice started to increase more rapidly than that of the cerulein-administered CCR2-KO mice and, at 7 wk after the first injection, was significantly higher than that of the cerulein-administered CCR2-KO mice, which had more severe pancreatitis. The body weight of WT mice and CCR2-KO mice that received saline injections for 10 wk did not differ.

CD11b<sup>−/−</sup>/Gr-1<sup>−/−</sup> and CD11b<sup>−/−</sup>/Gr-1<sup>high</sup> macrophages markedly accumulated in CCR2-KO mice receiving long-term cerulein. Phenotypes of inflammatory cells in the pancreas were analyzed with flow cytometry. Consistent with our previous results (16), in the acute cerulein pancreatitis model, the absolute number of CD11b<sup>−/−</sup>/Gr-1<sup>low</sup> cells in the pancreas of cerulein-administered WT mice was significantly higher than in the cerulein-administered CCR2-KO mice (Fig. 3, C and D). The saline-administered WT and CCR2-KO mice showed no significant differences in inflammatory cells (Fig. 3, A and B). In the chronic cerulein pancreatitis model, CD11b<sup>−/−</sup>/Gr-1<sup>low</sup> macrophages disappeared; however, the absolute cell numbers of CD11b<sup>−/−</sup>/Gr-1<sup>−/−</sup> and CD11b<sup>−/−</sup>/Gr-1<sup>high</sup> macrophages in pancreases of cerulein-administered CCR2-KO mice were significantly higher than in cerulein-administered WT mice (Fig. 3, E and F).
In chronic pancreatitis, results were the reverse of those in the acute cerulein pancreatitis model; chronic cerulein-administered CCR2-KO mice showed lower blood glucose than paired WT mice, but the difference between WT and CCR2-KO mice was not significant. The sizes of islets in mice with cerulein-induced chronic pancreatitis were not different between WT and CCR2-KO mice with and without cerulein-induced chronic pancreatitis (Fig. 5B).

GLP-1 receptor expression was downmodulated in CCR2-KO mice receiving long-term cerulein. One mechanism of insulin secretion from pancreatic β-cells involves the GLP-1 signal (5). Therefore, serum GLP-1 and GLP-1 receptor in the pancreas were measured. To exclude differences in food intake among the mice, tissues and blood samples were taken following overnight fasting. GLP-1 levels in chronic cerulein-administered WT and CCR2-KO mice were found to be greater than in the paired saline-administered mice (Fig. 6A). However, there were no differences in GLP-1 levels between WT and CCR2-KO mice, irrespective of saline or cerulein administration (Fig. 6A). In contrast, GLP-1R expression in the pancreas of chronic cerulein-administered WT and CCR2-KO mice was significantly lower than in the paired saline-administered mice, but the decrease was greater in cerulein-administered CCR2-KO mice, resulting in a significant difference between cerulein-administered WT and CCR2-KO mice, whereas the insulin levels were not different between these groups (Fig. 6, B and C). The immunohistochemistry results for insulin and GLP-1R in pancreatic tissues also showed lower GLP-1R expression in CCR2-KO mice, irrespective of saline or cerulein administration (Fig. 6A).

**CCR2-KO mice that received long-term cerulein developed severe hyperglycemia with less insulin production.** Concentrations of blood glucose and serum insulin were measured in mice that had in acute and chronic cerulein pancreatitis and given unrestricted access to food and water. In the acute pancreatitis model, cerulein-administered CCR2-KO mice showed lower blood glucose than did paired WT mice, but the saline-administered WT and CCR2-KO mice did not differ (Fig. 4A). Consistently, serum insulin concentrations in cerulein-administered CCR2-KO mice were significantly higher than in their paired WT counterparts, but there was no difference between the saline-administered groups of mice (Fig. 4B). In the chronic pancreatitis model, results were the reverse of those in the acute pancreatitis model; chronic cerulein-administered CCR2-KO mice showed higher blood glucose (Fig. 4C) and less insulin secretion (Fig. 4D) compared with the chronic cerulein-administered WT mice.

We next conducted GTTs to confirm glucose intolerance in the chronic pancreatitis models. The GTT showed greater glucose levels in the chronic cerulein-administered CCR2-KO mice than in the chronic cerulein-administered WT mice (Fig. 4E), but no differences in GTT between WT and CCR2-KO mice without cerulein pancreatitis were observed. On the other hand, insulin sensitivity in cerulein-administered mice was not different in the two types of mice (Fig. 4F), indicating that poor insulin secretion is the major cause of glucose intolerance in chronic cerulein-administered CCR2-KO mice.

**Islet number and size did not decrease in mice receiving long-term cerulein.** Because CCR2-KO mice had more severe pancreatitis than WT mice, the number and size of islets in chronic cerulein pancreatitis were counted in each mouse. The number of islets from mice with cerulein-induced chronic pancreatitis was greater than that from paired mice without cerulein (Fig. 5A), but the difference between WT and CCR2-KO mice was not significant. The sizes of islets in mice with cerulein-induced chronic pancreatitis were not different between WT and CCR2-KO mice with and without cerulein-induced chronic pancreatitis (Fig. 5B).
Fluorescence-activated cell sorting analysis showed cell numbers significantly higher, in mice with liraglutide treatment (Table 1). Inflammatory cell infiltration of pancreas was higher, but not significantly different, and the severity of pancreatitis was not different, and inflammation by densitometry. Mice were treated with cerulein injections or saline injections for 10 wk. Tissue and blood samples were taken from mice after overnight fasting. Blots were representative of 5 independent experiments. D: Immunohistochemistry for insulin and GLP-1R in chronic cerulein pancreatitis. Shown are micrographs of pancreatic tissues (×400) with insulin antibody with FITC (green), and GLP-1R antibody with Alexa-568 (red). E: quantization of mRNA expression for GLP-1R in isolated islet cells. The amount of mRNA was normalized to 18S mRNA. \( N = 5 \), \( * P < 0.05 \).

**DISCUSSION**

Although chronic pancreatitis is closely related to insulin insufficiency and diabetes mellitus (2), the molecular relationship between insulin secretion and chronic pancreatitis is unclear (17). This study demonstrates that CCR2-KO mice receiving long-term cerulein develop more severe chronic pancreatitis via 1) downmodulation of GLP-1R expression, 2) decrease in insulin release, and 3) hyperglycemia. It also provides the first evidence of a protective role for the CCR2/CCL2 axis against diabetes.

The CCR2/CCL2 axis plays a pivotal role in the pathogenesis of many immune diseases characterized by mononuclear cell infiltration, including chronic pancreatitis (16). First, our present study showed that CCR2-KO mice develop less severe acute cerulein pancreatitis with decreased accumulation of CD11b\(^+\)/Gr-1\(^{low}\) macrophages. This was not surprising, as we previously showed that CCL2-KO mice developed less severe pancreatitis with less infiltration of TNF-\(\alpha\)-expressing CD11b\(^+\)/Gr-1\(^{low}\)/F4/80\(^{high}\) macrophages into the pancreas. Taken together, we conclude that CCR2\(^+\) macrophages are involved in the pathogenesis of pancreas tissue damage, at least in the acute phase of pancreatitis. However, it was very surprising that, compared with WT mice, CCR2-KO mice reproducibly developed more severe chronic cerulein pancreatitis with accumulation of CD11b\(^+\)/Gr-1\(^{low}\) and CD11b\(^+\)/Gr-1\(^{high}\), but not CD11b\(^+\)/Gr-1\(^{low}\), macrophages. Together with the histological findings on the long-term cerulein-administered CCR2-KO mice, these results suggest that CCR2-KO mice develop chronic pancreatitis with increased accumulation of macrophages, which may be one of the mechanisms for the development of chronic pancreatitis.

**Table 1.** **Chronic cerulein pancreatitis and liraglutide treatment**

<table>
<thead>
<tr>
<th></th>
<th>CP</th>
<th>CP + Liraglutide</th>
<th>( P ) Value</th>
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<tbody>
<tr>
<td>AMY, IU/ml</td>
<td>560 ± 73</td>
<td>610 ± 82</td>
<td>N.S.</td>
</tr>
<tr>
<td>LIP, IU/ml</td>
<td>32 ± 5.6</td>
<td>33 ± 7.1</td>
<td>N.S.</td>
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<tr>
<td>Cell Infiltration Cells per 100 Acinar Cells</td>
<td>20 ± 9</td>
<td>30 ± 14</td>
<td>N.S.</td>
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<tr>
<td>Necrotic Cells per 100 Acinar Cells</td>
<td>0.25 ± 0.05</td>
<td>0.26 ± 0.05</td>
<td>N.S.</td>
</tr>
<tr>
<td>Interstitial Space, %</td>
<td>11 ± 4.9</td>
<td>10 ± 4.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fibrosis Space, %</td>
<td>9.9 ± 2.9</td>
<td>9.6 ± 3.4</td>
<td>N.S.</td>
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Values are means ± SD. Wild-type (WT) mice were treated with cerulein injections (CR\(^+\)) for 10 wk, and then treated with the glucagon-like peptide 1 (GLP-1) analog, liraglutide, for 3 days. Levels of serum amylase (AMY) and lipase (LIP) were measured in blood taken from mice on the day after the final liraglutide injection. Total number of inflammatory cells in each high-power field (HPF), total number of necrotic cells per 100 acinar cells, percentage of interstitial space in each HPF, and percentage of fibrosis space in each HPF, were averaged by analyzing each pancreas in 10 fields. \( N = 5 \). CP, cerulein pancreatitis; N.S., not significant.
mice, it seems that CCR2+ cells (yet unidentified) emerge at the late phase of pancreatitis to mediate tissue repair of chronic pancreatitis, but lack of these cells may allow the accumulation of other immune components, including CD11b+/Gr-1− and CD11b+/Gr-1high macrophages. Further studies are warranted to clarify the protective role of CCR2+ monocytes in chronic pancreatitis.

A previous study showed that insulin secretion in rats with chronic pancreatitis was significantly lower than in controls (4). We found a consistent inverse relationship between levels of chronic pancreatic damage and insulin secretion, as insulin secretion was markedly impaired in the chronic cerulein-administered CCR2-KO mice but well-preserved in acute cerulein-administered CCR2-KO mice. This also suggests that a CCR2-dependent tissue repair system arises after acute inflammation to maintain insulin secretion. Presently, the mechanism of decreased insulin secretion in chronic pancreatitis is unclear. We confirmed that plasma GLP-1 levels in chronic pancreatitis increased to similar levels in both WT and CCR2-KO mice, as reported previously (6). However, this is the first report to show a significant decrease in GLP-1R expression in chronic cerulein-administered CCR2-KO mice compared with paired WT mice. Therefore, we conclude that, in chronic cerulein-administered CCR2-KO mice, which lack such a CCR2-dependent tissue repair system at the late phase of inflammation, other mechanisms induce the downmodulation of GLP-1R expression.

Last, the clinical relevance of this study should be debated. In this study, we also showed that treatment with a GLP-1 analog diminished the differences in GTT between chronic cerulein-administered WT and CCR2-KO mice, indicating that GLP-1 treatment is appropriate for patients with diabetes secondary to chronic pancreatitis, who clinically appear to have impaired insulin secretion (21). Moreover, the finding that GLP-1 analog treatment improves GTT in a mouse model of chronic pancreatitis is new. Therefore, we propose that GLP-1 treatment improves GTT in a mouse model of chronic pancreatitis model with diabetes induced by cerulein plus stress in rat. Dig Dis Sci 40: 2356–2363, 1995.


