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CXCL14-Like Immunoreactivity Exists in Somatostatin-Containing Cells of Mouse Pancreas

Hirohumi Suzuki[1] and Toshiharu Yamamoto[2]

[1]Department of Biology, Fukuoka University of Education, Akamabunkyo-machi 1–1, Munakata, Fukuoka 811–4192, Japan and [2]Brain Functions and Neuroscience Unit, Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Inaoka-cho 82, Yokosuka, Kanagawa 238–8580, Japan

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Immunohistochemical techniques were employed to investigate the distribution of the chemokine CXCL14, in the mouse pancreas. CXCL14-immunoreactive cells were detected in the peripheral region of the pancreatic islets and were immunoreactive for somatostatin, but not for glucagon, insulin, and pancreatic polypeptide. Immunoelectron microscopy indicated that the CXCL14-like peptide and somatostatin co-existed in the secretory granules. CXCL14, secreted from somatostatin-containing cells, may modulate insulin secretion in a paracrine fashion, and play a novel role in glucose homeostasis in addition to its well-known chemotactic activities.

Key words: CXCL14, BRAK, somatostatin, pancreas, mouse

I. Introduction

Chemokines fundamentally have chemotactic activity mainly for leukocytes and lymphocytes [5]. Among chemokines, CXC chemokines are a major subfamily characterized by two cysteines located at the N-terminal portion where a single amino acid residue is inserted between these cysteines. In mammal, 16 CXC chemokines, namely, CXCL1-CXCL16, have been identified, and these CXC chemokoines are further subdivided into two subfamilies by the presence (ELR^+) or absence (ELR^-) of "Glu-Leu-Arg" residues at the N-terminals [5]. CXCL14, originally termed BRAK, is an ELR^- CXC chemokine and first isolated from human breast and kidney cells [7]. The full-length cDNA of CXCL14 encodes 111 amino acid residues with a putative signal peptide of 34 amino acid residues [1]. In human, CXCL14 mRNA was detected in various tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas [4, 7, 16]. Kurth *et al.* [9] revealed that CXCL14 is a highly selective chemoattractant for human monocytes and that the mobilization responses to CXCL14 are strongly enhanced after treatment of monocytes with prostaglandin E2 or forskolin. Macrophages, one of immune-related cells derived from monocytes, were frequently found with CXCL14-producing fibroblasts, suggesting that CXCL14 is involved in the transformation of tissue macrophages by recruiting extravasated precursors to fibroblasts [9]. In the mammalian adipose tissues, the number of macrophages increases in obesity [10, 22]. A high fat diet (HFD)-fed *CXCL14*-deficient mice was impaired macrophage mobilization in the white adipose tissue (WAT) and improved insulin responsiveness [13]. Macrophages in WAT play an active role in morbid obesity and macrophage-related inflammation activities may contribute to the pathogenesis of insulin resistance induced by obesity [23]. In addition to these chemotactic activities for immune-related cells, the localization of CXCL14 peptide in neurons and endocrine cells [17, 24] implies other physiological roles. In fact, Nara *et al.* [13] reported that serum insulin concentrations of HFD-fed *CXCL14*-deficient mice were significantly lower than those of HFD-fed control mice. Body weight of *CXCL14*-deficient mice was also significantly lower, caused by decreased food intake, compared to control mice [19]. These lines suggest the association of CXCL14 with appetite control.

Molecular biological technique revealed that CXCL14

Correspondence to: Hirohumi Suzuki, Ph.D., Department of Biology, Fukuoka University of Education, Akamabunkyo-machi 1–1, Munakata, Fukuoka 811–4192, Japan. E-mail: spiderhs@fukuoka-edu.ac.jp

is widely conserved in vertebrates (human, cow, pig, rat, mouse, chicken, *Xenopus*, carp, zebrafish) [5, 14]. Although immunohistochemical technique is effective to investigate the distribution of CXCL14 in various tissues of many species, it has been performed rarely to date. In the rat hypothalamus, extensive distribution of CXCL14-immunoreactive neuronal somata and fibers has been described [24]. In the axolotl pituitary, CXCL14-like substance exists in somatotroph and may participate in functions of growth hormone [17]. In this study, we examined CXCL14-like immunoreactivity in the pancreatic islets of mice and we firstly demonstrated that CXCL14-immunoreactivity existed in the cells corresponded to somatostatin-, which is well known to prohibit the secretion of insulin and glucagon, containing cells in the pancreatic islets.

II. Materials and Methods

Animals and fixation

Male albino (BALB/cCrSlc; n=7) and black (C57BL/6NCrSlc; n=7) mice were deeply anesthetized by Halothane Fluothane (Takeda Pharmaceutical Co. Ltd., Osaka, Japan). They were then perfused with 0.85% NaCl, and subsequently, with 4% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer (PB, pH 6.9). The pancreas were rapidly dissected out and fixed for one or two days at 4°C in the same fixative. After fixation, the samples were stored in PB. All procedures were carried out under the approval of the Ethical Committee of Kanagawa Dental University, employing guidelines established by the committee.

Immunohistochemistry

The samples were immersed in 20% sucrose, and cut with a cryostat (HM525; MICROM, Walldorf, Germany) into 6 μm-thick sections that were thaw-mounted on gelatin-coated glass slides.

Immunostaining was performed according to the methods described previously [18]. Briefly, the sections were washed overnight in 0.1 M PB (pH 7.4) containing 0.9% saline (PBS), and incubated with rabbit anti-human CXCL14 antibody (No. 500-P237; PeproTech Inc., Rocky Hill, N.J., USA) diluted to 0.5 μg/ml in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (PBS-BSAT), for 24 hr at 4°C. This antibody was purified by affinity chromatography employing an immobilized human CXCL14 matrix. After washing in PBS, the sections were then incubated with biotinylated goat anti-rabbit IgG (BA-1000; Vector Laboratories, Burlingame, Calif., USA) diluted 1:100 in PBS-BSAT, for 1 hr at room temperature. The sections were then washed again in PBS and incubated with avidin-biotin-horseradish peroxidase complex (ABC; Vector Laboratories) diluted 1:200 in PBS-BSAT, for 30 min at room temperature. After a final wash in PBS, the sections were reacted with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer solution (pH 7.4). Thereafter, sections were counterstained with thionin and coverslipped using Malinol (Muto Pure Chemicals, Tokyo, Japan). Controls for anti-CXCL14 antibody were prepared by omitting the antibody in the first incubation or by using antibody pre-absorbed with recombinant human CXCL14 (5 μg/ml, No. 300-50; PeproTech Inc.). As another control, the antibody was also pre-absorbed with synthetic somatostatin (50 μg/ml, 4023-v; Peptide Institute, Osaka, Japan), because preliminary experiments indicated that CXCL14-immunoreactive cells corresponded to somatostatin-containing cells.

Identification of immunoreactive cell types

To determine the types of CXCL14-immunoreactive cells, double immunofluorescence staining was performed using goat anti-porcine glucagon serum (4660-0610; AbD Serotec, Oxford, UK), guinea pig anti-human insulin serum (Y370; Yanaihara Institute, Fujinomiya, Japan), rat anti-somatostatin serum (NP-105 SSTrat; Protos Biotech Corporation, New York, N.Y., USA), and goat anti-pancreatic polypeptide antibody (SAB2500747; Sigma-Aldrich, Saint Louis, Mo., USA). Immunoreactivities were visualized by fluorescein-conjugated donkey anti-rabbit IgG (AP182F; Millipore, Billerica, Mass., USA) for CXCL14, rhodamine-conjugated donkey anti-goat IgG (AP180R; Millipore) for glucagon and pancreatic polypeptide, Alexa Fluor 555-conjugated goat anti-guinea pig IgG (A21435; Invitrogen Corp., Carlsbad, Calif., USA) for insulin, and rhodamine-conjugated donkey anti-rat IgG (AP189R; Millipore) for somatostatin. To clarify the cross-reaction of anti-somatostatin serum with CXCL14, some sections were processed using the anti-somatostatin serum pre-absorbed with the recombinant human CXCL14 (5 μg/ml; PeproTech Inc.) and immunoreactivity was visualized with DAB.

Immunoelectron microscopy

For the post-embedding method, pancreas dissected out from four similarly perfused mice (two albino and two black) were used. Small blocks of the fixed pancreas were embedded in LR White (London Resin, Berkshire, UK), and ultrathin sections were mounted on nickel grids. For double labeling, sections were incubated with anti-CXCL14 antibody diluted with 0.05 M Tris-HCl buffer (pH 7.2) containing 0.9% saline (TBS), for 2 days at 4°C after blocking non-specific protein binding with normal goat serum (1:10) for 30 min at room temperature. After washing with TBS containing 0.2% BSA (TBS-BSA), the sections were incubated with 5 nm colloidal gold-labeled goat anti-rabbit IgG (EM.GAR 5; British BioCell International, Cardiff, UK) diluted in 0.02 M TBS (pH 8.2) containing 1% BSA, for 1 hr at room temperature. After washing with TBS-BSA, the sections were subsequently incubated with rat anti-somatostatin serum and 15 nm colloidal gold-labeled goat anti-rat IgG (EM.GAT 15; British BioCell

International). After counterstaining with lead citrate for 5 min at room temperature, the sections were examined with an electron microscope (JEM 1220; JEOL, Tokyo, Japan).

III. Results

CXCL14-immunoreactive cells were detected in the peripheral region of the pancreatic islets (Fig. 1A). The majority of the cells were ovoid or ellipsoid in shape with a long axis diameter of 8–20 μm, whereas some immunoreactive cells were dendritic in shape. There were no differences between albino and black mice in view of the CXCL14-immunoreactive cell profiles and distribution patterns. Pre-absorption of the anti-CXCL14 antibody with recombinant CXCL14 abolished these staining profiles in the cells (Fig. 1B). However, pre-absorption of the antibody with synthetic somatostatin had no effect on the staining profiles (Fig. 2A and B). Immunofluorescent microscopy revealed that almost all CXCL14-immunoreactive cells were immuno-positive for somatostatin (Fig. 3A–C); however, a few somatostatin-immunoreactive cells were immuno-negative for CXCL14 (Fig. 3A–C). Pre-absorption of the anti-somatostatin serum with recombinant human CXCL14 had no effect on the somatostatin-immunoreactive staining profiles (data not shown). All CXCL14-immunoreactive cells were immuno-negative for glucagon (Fig. 3D–F), insulin (Fig. 3G–I), and pancreatic polypeptide (Fig. 3J–L). Immunoelectron microscopy revealed co-localization of the 5 nm colloidal gold particles, indicating CXCL14-like immunoreactivity, with the 15 nm colloidal gold particles, indicating somatostatin, on the secretory granules in the somatostatin-containing cells (Fig. 4).

IV. Discussion

In the present study, we have described for the first time the presence of CXCL14-immunoreactive cells, and the co-localization of a CXCL14-like substance with somatostatin, in mouse pancreatic islets, by using a reliable antibody. Heterologous pre-absorption tests have indicated the absence of cross-reaction not only between anti-CXCL14 antibody and somatostatin, but also between anti-somatostatin serum and the CXCL14 peptide. Disappearance of CXCL14-immunoreactive staining profiles by pre-absorption of the anti-CXCL14 antibody with recombinant CXCXL14 indicates that the antibody recognizes mouse CXCL14. The co-localization of CXCL14-like substance and somatostatin immunoreactivity in the same secretory granules suggests that these peptides are secreted together by the somatostatin-containing cells. However, a few somatostatin-immunoreactive cells were immuno-negative for CXCL14 by fluorescent microscopy, suggesting the possibility that some secretory granules contain only somatostatin even within double-labeled cells. Hitherto, chemokines including CXCL14 are mainly known to function as chemoattractants; however, the

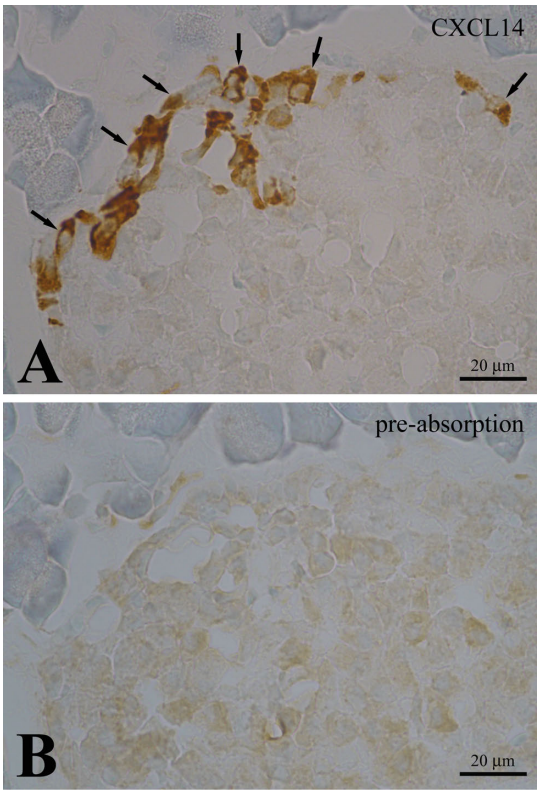

Fig. 1. Serial sections of a mouse pancreatic islet showing CXCL14-like immunoreactivity (**A**), and the effects of pre-absorption with recombinant human CXCL14 (**B**). Arrows in (**A**) indicate CXCL14-immunoreactive cells. Bars=20 μm.

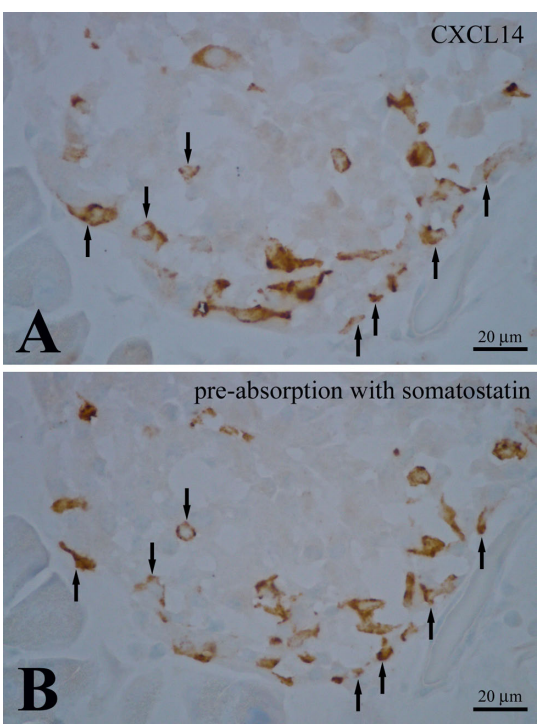

Fig. 2. Serial sections of a mouse pancreatic islet showing CXCL14-like immunoreactivity (**A**), and the effects of pre-absorption with somatostatin (**B**). Arrows indicate identical cells. Bars=20 μm.

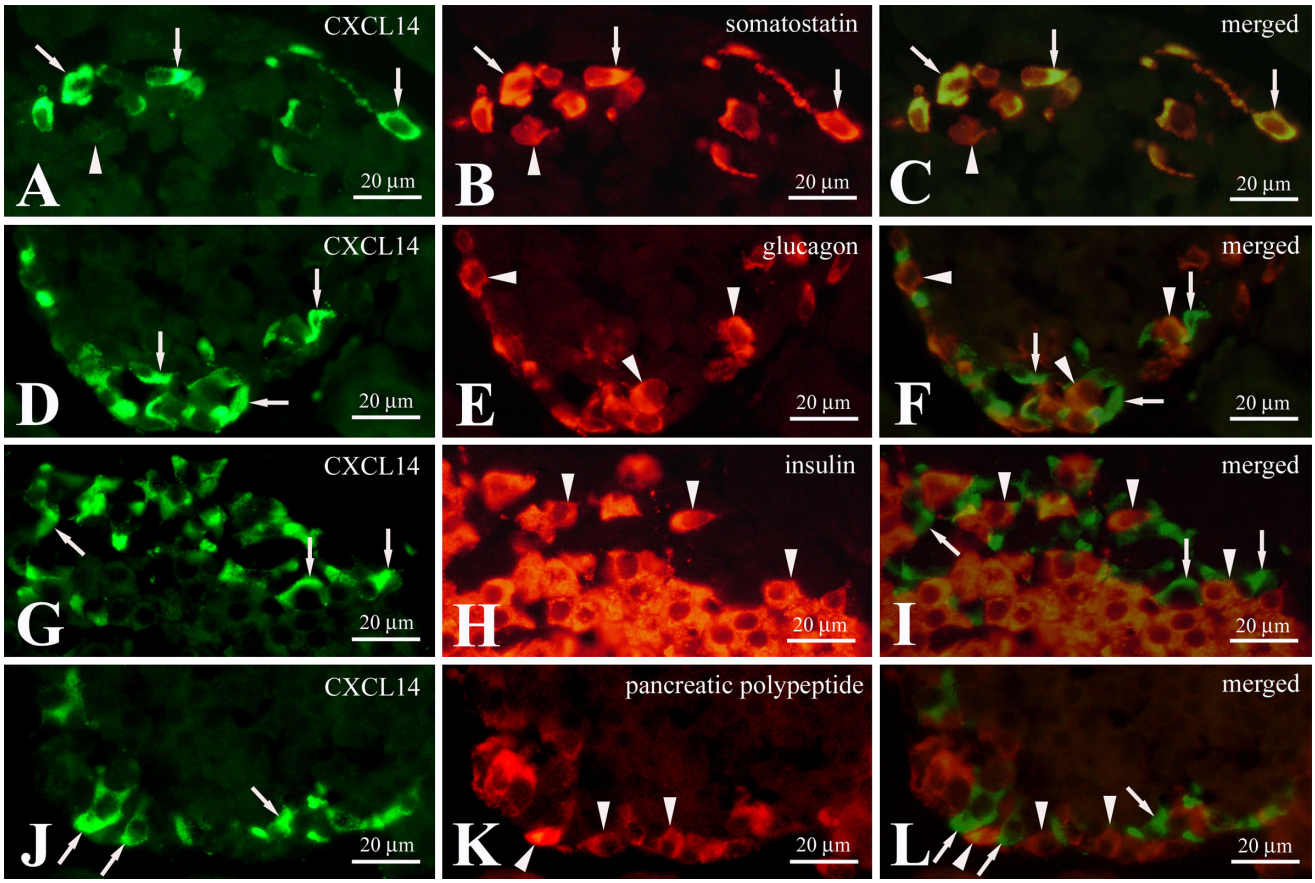

Fig. 3. Immunofluorescent micrographs showing immunoreactivities for CXCL14 (**A, D, G**, and **J**), somatostatin (**B**), glucagon (**E**), insulin (**H**), and pancreatic polypeptide (**K**) in the pancreatic cells. Four sets (**A** and **B**, **D** and **E**, **G** and **H**, **J** and **K**) of identical sections were used. Panel (**C**), (**F**), (**I**), and (**L**) are merged photographs of (**A** and **B**), (**D** and **E**), (**G** and **H**), and (**J** and **K**), respectively. Arrows in (**A–C**) indicate CXCL14-immunoreactive cells that are also immuno-positive for somatostatin. An arrowhead in (**A–C**) indicates a somatostatin-immunoreactive cell that is immuno-negative for CXCL14. Arrows in (**D, F, G, I, J**, and **L**) indicate cells that are immunoreactive for CXCL14 but immuno-negative for glucagon, insulin, and pancreatic polypeptide. Arrowheads in (**E, F, H, I, K**, and **L**) indicate cells that are immunoreactive for major islet hormons except for somatostatin, but immuno-negative for CXCL14. Bars=20 µm.

presence of chemokines in endocrine cells implies other functions associated with the endocrine system. In fact, CXCL12, another ELR$^-$ chemokine similar to CXCL14, stimulates the release of growth hormones from normal as well as cancerous pituitary cells in rats [3, 11]. Growth hormone is a counter-regulatory hormone that antagonizes the hepatic and peripheral effects of insulin on glucose metabolism [12]. Interestingly, we have previously demonstrated the presence of CXCL14 in growth hormone-producing cells in the urodele pituitary [17]; however, it is noteworthy that the animal was not a mammal.

Serum insulin concentrations of HFD-fed *CXCL14*-deficient mice were significantly lower than those of the HFD-fed control mice with and without intraperitoneal glucose injections [13]. CXCL14 co-exisiting with somatostatin might inhibit somatostatin secretion by an autocrine fashion, or intracellularly. Lack of CXCL14 might result in over-secretion of somatostatin, which inhibits insulin secretion as a paracrine agent [2], causing lower levels of plasma insulin. In addition to these intra-insular effects of CXCL14, extra-insular effects were also suggested. HFD-fed *CXCL14*-deficient mice exhibited stronger response to insulin in lowering blood glucose levels than HFD-fed control mice [13]. Intraperitoneal glucose tolerance tests indicated that *CXCL14*-deficient mice were glucose-intolerant under HFD feeding condition [13]. Administration of anti-CXCL14 monoclonal antibody into HFD-induced obese mice increased glucose-intolerance during intraperitoneal glucose tolerance tests [20], similar to *CXCL14*-deficiency [13]. However, monoclonal antibody administration increased insulin resistance when evaluated by intraperitoneal insulin tolerance tests [20]. Although chronic deficiency and acute paucity of CXCL14 may differently affect the insulin resistance, both conditions caused glucose-intolerance suggesting that CXCL14 contribute the persistence of glucose-tolerance by unknown mechanisms. On the other hand, pharmacological experiments conducted in isolated mouse pancreatic islets revealed that ghrelin, secreted by glucagon-containing cells [6, 8], modulated insulin secretion [15]. Exogenous ghrelin suppresses

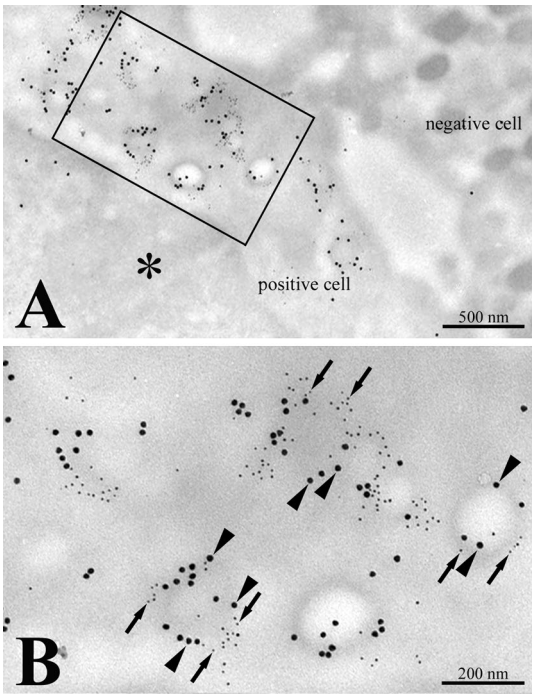

Fig. 4. Low (**A**) and high (**B**) magnification electron micrographs showing CXCL14-like (5 nm colloidal gold particles, arrows in **B**) and somatostatin (15 nm colloidal gold particles, arrowheads in **B**) immunoreactivities in an ultrathin section from the mouse pancreas. * indicates the nucleus of a "positive cell". The boxed area in (**A**) is shown at a higher magnification in (**B**). Bar in (**A**)=500 nm. Bar in (**B**)=200 nm.

glucose-stimulated insulin secretion and worsens intravenous glucose tolerance in healthy humans [21]. CXCL14 secreted from somatostatin-containing cells may participate in the modulation of insulin secretion together with somatostatin and ghrelin, and play an essential role in glucose homeostasis.

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VI. References

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