CXCL5 inhibits excessive oxidative stress by regulating white adipocyte differentiation

Dabin Lee a,b, Kang-Hoon Lee a, Dong Wook Kim a, Sanghyuk Yoon a, Je-Yoel Cho a,b,*

a Department of Biochemistry, BK21 Plus and Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, Seoul, 08826, Republic of Korea
b Comparative Medicine Disease Research Center, Seoul National University, Seoul, 08826, Republic of Korea

ARTICLE INFO

Keywords: Chemokine CXCL5 KO mice Fat cell Adipogenesis Oxidative stress ROS

ABSTRACT

Chemokines have been well-documented as a major factor in immune cell migration and the regulation of immune responses. However, recent studies have reported that chemokines have diverse roles, both in immune cells and other cell types, including adipocytes. This study investigated the molecular functions of C-X-C motif chemokine ligand 5 (CXCL5) in white adipose cells using Cxcl5 knock-out (KO) mice fed a high-fat diet (HFD). The expression of Cxcl5 decreased by 90% during adipocyte differentiation and remained at a low level in mature adipocytes. Moreover, adipogenesis was enhanced when adipocytes were differentiated from the stromal vascular fraction (SFV) of Cxcl5 KO mice. Feeding an HFD increased the generation of reactive oxygen species (ROS) and promoted abnormal adipogenesis in Cxcl5 KO mice. Oxidative stress and insulin resistance occurred in Cxcl5 KO mice due to decreased antioxidant enzymes and failure to remove ROS. These results indicate the principal roles of CXCL5 in adipogenesis and ROS regulation in adipose tissue, further suggesting that CXCL5 is a valuable chemokine for metabolic disease research.

1. Introduction

In modern populations worldwide, the incidence of metabolic diseases is increasing due to irregular lifestyles and unhealthy eating habits [1,2]. In particular, obesity is a primary driver of many metabolic issues, including inflammation, insulin resistance, Type 2 diabetes, high blood pressure, and cardiovascular disease [3,4]. To better understand and overcome obesity, it is important to study adipose tissues and their differentiation mechanisms.

White adipose tissue (WAT) has been recognized primarily as an organ that stores energy in triglycerides and releases it as free fatty acids. However, current knowledge has expanded the functions of WAT from local energy storage to the secretion of several factors that play a role in several pathophysiological processes, including immune response, hypertension, glucose homeostasis, and cancer. WAT is composed of complex cell types, including adipocytes and immune cells [5]. Cytokines and proteins are secreted mainly from the immune cells surrounding adipocytes to regulate immune responses [6]. In addition, some cytokines, known as adipokines, are secreted by adipocytes and regulate metabolic processes such as food intake, energy homeostasis, adipocyte differentiation, insulin sensitivity, and immune responses. Well-known adipokines include leptin, adiponectin, tumor necrosis factor α (TNFα), and interleukin 6 (IL-6), which are involved in fat metabolism and immune response [7–11].

Chemokines and adipocyte differentiation are an area of active research. A chemokine is a small cytokine that stimulates immune cells’ chemical attraction, produced by varied cells in response to infection and other inflammatory stimuli [12]. More than 50 chemokines have been identified in humans and classified into four groups according to the location of preserved cysteine residues (CXCL, CCL, CL, and CX3CL) [12]. In a recent study, CXCL3 and CXCR2 were reported as chemokines and receptors that promote adipocyte differentiation [13,14].

CXCL5 is one of the CXC chemokines secreted by cells when stimulated by inflammatory cytokines such as interleukin 1 (IL-1) and TNFα [15]. CXCL5 stimulates neutrophils and induces an inflammatory response [16]. However, the secretion of CXCL5 in adipocytes and its role is unclear. Here, we generated Cxcl5 knock-out (KO) mice with the CRISPR/Cas9 system and elucidated the role of CXCL5 in epididymal white adipose tissue (eWAT).

* Corresponding author. Department of Biochemistry, College of Veterinary Medicine Seoul National University, Gwanak-ro1, Gwanak-gu, Seoul, Republic of Korea.
E-mail address: jeycho@snu.ac.kr (J.-Y. Cho).

https://doi.org/10.1016/j.redox.2022.102359
Received 18 May 2022; Received in revised form 27 May 2022; Accepted 30 May 2022
Available online 3 June 2022
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2. Material and methods

2.1. Animals

Cxcl5 KO mice were generated by deleting exon 1 in the Cxcl5 gene, as previously described [17]. Mice were maintained under a 12 h light/dark cycle in a controlled-temperature room (22 °C). Five-week-old mice were fed either a 10% normal-fat diet (NFD, control) or a 60% high-fat diet (HFD) (Research diet, USA) for 13 weeks. The Seoul National University Institutional Animal Care and Use Committee (IACUC) approved all animal experiments and protocols (SNU-160825-2-1).

2.2. Adipose Stromal Vascular Fraction (SVF) isolation and adipocyte differentiation

Primary adipocytes were obtained from subcutaneous fat from 4-week-old mice. Subcutaneous fat was minced in collagenase buffer and digested at 37 °C with constant agitation at 160 rpm for 25 min. Digestion was stopped by adding 10 ml serum-containing medium. After filtration through a cell strainer (100 μm), the solution was centrifuged for 5 min at 1,500 rpm, the supernatant was removed, and the pellet was resuspended. Again, cells were filtered through a 40-μm cell strainer and digested at 37 °C for 2 h. The solution was centrifuged for further use. Cells were seeded after centrifugation. Isolated preadipocytes were induced to differentiate using MDI media (10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, 10 μg/ml insulin) and 1 μM rosiglitazone. After 2 days, the medium was replaced with a medium containing 10% FBS with 1 μg/ml insulin and changed with 10% FBS media every 2 days. Differentiation of 3T3-L1 preadipocytes was performed following a previous study [17].

2.3. Oil red O staining and triglyceride assay

Differentiated adipocytes were fixed with 10% formalin in PBS for 1 h at room temperature and washed with 60% isopropanol. Cells were stained with Oil Red O solution for 30 min and washed with water. Stained cells were eluted with 100% isopropanol for 10 min, and the absorbance was checked at 500 nm. According to the manufacturer’s protocol, the triglyceride level was measured using a Triglyceride Quantification Kit (BM-TGR-100, BIOMAX, KR).

2.4. Reactive Oxygen Species (ROS) measurement

ROS production was induced by treatment with 200 or 300 μM of H2O2 for 24 h. The level of intracellular ROS was measured via cell-permeant 2,7′-dichlorofluorescin diacetate (H2DCFDA) staining. Green fluorescence was detected by microscopy (ECHO, US). To increase the accuracy of the data, pictures were taken at random. And all pictures were analyzed for fluorescence intensity using ImageJ software (Fiji ImageJ).

2.5. LC-MS/MS analysis

eWAT was dissected from wild-type (WT), and Cxcl5 KO mice fed an HFD for 13 weeks, and total protein was extracted from the tissues. The proteins were digested with trypsin. Digested peptides were labeled with isobaric tags for relative and absolute quantitation (iTRAQ) (AB Sciex, Framingham, MA) to normalize protein amounts quantitatively. Then, anion exchange-based fractionation was performed to identify more proteins. The fractionation was performed by following the protocol by Matthias et al. [18]. LC-MS/MS analysis was performed with Q Exactive (0726090, Thermo Scientific, US). Using the raw data obtained after LC-MS/MS analysis, proteins were identified with MaxQuant (version 1.5.8.3) and normalized with Scaffold Q+ software (Scaffold 4.7.5; Proteome Software, Portland, OR).

2.6. RNA isolation and quantitative RT-PCR

Total RNA was isolated with TRIzol (15596018, Ambion, US) from cells or tissues, and reverse transcription was performed using Omniscript (205113, QIAGEN, DE) according to the manufacturer’s protocol. SYBR green (S7563, Invitrogen, US) and GO Taq (M8298, Promega, US) were used for quantitative real-time PCR with specific primers (listed in Table S1). The expression of all target genes was normalized with 36b4.

2.7. Western blot and histological analysis

Proteins were extracted using 4% SDS Tris/HCl pH lysis buffer for tissue samples and RIPA buffer for cells (89900, Thermo Scientific, US). Tissue samples were homogenized before lysing at 100 °C, and clear supernatant was obtained by centrifugation for further use. Cells were lysed in RIPA buffer by incubation for 30 min on ice followed by sonication. After that, the lysate was centrifuged for 10 min at 14,000 rpm. Western blot protocol was performed as detailed previously paper [17] to detect the expression of PPARγ (2430, Cell signaling, US), PRDX4 (P3352-25L, BIOMOL, DE), p-AKT (9271S, Cell signaling, US), Total AKT (9272, Cell signaling, US), p-JNK (9255S, Cell signaling, US), Total JNK (9252S, Cell signaling, US), and α-tubulin (LF-MA0117, AB Frontier, KR). Histological analysis was performed following previous studies [17].
2.8. Luciferase assay

HEK293T cells were plated on 24-well plates at a density of 50–60% confluence and transiently transfected with PPARγ, retinoid X receptor α (RXRα), β-galactosidase, and DR-1 DNA plasmid by the lipofectamine 3000 (L3000001, Thermo Scientific, US) according to the manufacturer’s protocol. Forty-four hours after transfection, the cells were treated with rosiglitazone, alone or with recombinant CXCL5, for 24 h. The transcription efficiency of the reporter gene was confirmed through luciferase activity normalized to β-galactosidase activity.

2.9. Intraperitoneal Glucose Tolerance Test (IPGTT)

Mice fasted for 16 h before intraperitoneal (i.p.) injection with 2.0 g/kg glucose. Approximately 20 μl of blood was collected from the mice at 0, 15, 30, 60, 90, and 120 min after glucose injection, and blood glucose was measured using an Accu-check Performa glucometer (Roche).

2.10. Thiobarbituric Acid Reactive Substance (TBARS) assay and cholesterol assay

A TBARS assay was performed using a TBARS Assay Kit (BO-TBR-200, BIOMAX, KR) to measure the level of lipid peroxidation in the adipose tissue of the mice fed an HFD. Both esterified and unesterified cholesterol (free cholesterol) were measured in serum using a Total cholesterol assay kit (BM-CHO-100, BIOMAX, KR). Both analyses were performed according to the manufacturer’s protocols.

3. Results

3.1. Proteomic analysis revealed that the function of Cxcl5 in adipocytes reversely associates with fatty acid metabolic process including oxidation-reduction

We performed a proteomic analysis of epididymal white adipose tissue (eWAT) obtained from WT and Cxcl5 KO mice to explore the roles of the chemokines expressed in adipocytes. The eWAT was isolated from 18-week-old WT and Cxcl5 KO mice, and the tissue was prepared for proteomics analysis (Fig. 1A). A total of 1,765 proteins were identified; differentially expressed proteins (DEPs) are represented by the heatmap and volcano plot in Fig. 1B and C. A total of 136 proteins were significantly different (66 upregulation and 70 downregulation) in Cxcl5 KO compared with WT mice (Tables S2 and 3). The up-and-downregulated DEPs were subjected to gene ontology (GO) and network enrichment analyses separately. The terms enriched in upregulated DEPs suggested that many proteins are functionally associated with actin filament organization, fragmentation, and actin-binding; these functions determine cell shape, nuclear shape, cell spreading, and cell stiffness affect cell differentiation (Fig. 1D and E). Interestingly in the enrichment analysis, serum response factor (SRF) was enriched in the upregulated proteins of Cxcl5 KO, as confirmed in the transcription factor-target interaction database for humans and mice in TRRUST (transcriptional regulatory relationships unraveled by sentence-based text mining) (Fig. 1F). It is well known that SRF induces actin cytoskeleton organization together with myocardin-related transcription factor A (MRTFA), a co-activator of SRF [19] and that it inhibits brown fat or beige fat differentiation [20,21).

The DEPs downregulated in Cxcl5 KO were enriched in lipid metabolism and oxidoreductase (Fig. 1G and H). It is meaningful for the clinical approach because the proteins related to fat metabolism, including cholesterol transport and metabolism, were decreased (Fig. 1H). Proteins downregulated in Cxcl5 KO eWAT were associated with PPARγ and SREBP1 transcription factors in TTRUST database (Fig. 1I). Since PPARγ and SREBP1 are essential transcription factors for the differentiation of white adipocytes, it is postulated that Cxcl5 could influence the differentiation of adipocytes.

3.2. Depletion of Cxcl5 enhances adipogenesis

We thus confirmed the role of Cxcl5 in adipocyte differentiation. Since Kabir et al. previously reported on the expression of chemokines during adipogenesis [9], the changes in Cxcl5 expression were examined during the differentiation of 3T3-L1 cells into adipocytes. Adipocyte maturation was measured by the accumulation of lipid droplets and changes in the expression of marker genes such as Pparγ (pro-liferator-activated receptor-gamma), C/ebp (CCAAT-enhancer-binding protein), Glut4 (glucose transporter type 4) (Fig. 2A and B). During differentiation, Cxcr2 expression dramatically increased beginning on induction day 4, while the expression of its ligands, such as Cxcl1, Cxcl3, and Cxcl5, varied (Fig. 2C). Cxcl2 was not detected during differentiation, and Cxcl3 expression increased, while Cxcl1 and Cxcl5 decreased during adipogenesis. Notably, although the expression of Cxcl5 decreased by more than 90% beginning early in the process (Day 2), the roles of Cxcl5 in the differentiation of adipocytes are still unclear.

We then examined the effect of Cxcl5 depletion on adipocyte differentiation using Cxcl5 KO mice. A stromal vascular fraction (SVF) was isolated from the WAT of both WT and Cxcl5 KO mice to differentiate into adipocytes (Fig. 2D). Interestingly, significant increases were found in the number of mature adipocytes and the deposits of lipid and fat in Cxcl5 KO (Fig. 2E). This observation was also validated by the higher absorbance of the cells stained with Oil Red O (Fig. 2F) and the higher triglyceride concentration in Cxcl5 KO (Fig. 2G). Most of all, significant increases in the expression of adipogenesis marker genes such as Pparγ, Fas (fatty acid synthase), Serp1-c (sterol regulatory element-binding protein 1-c), Acaca (acyl-CoA carboxylase alpha), Nox4 (NADPH Oxidase 4), and Adipoq (adiponectin) in Cxcl5 KO indicated that Cxcl5 deficiency enhances adipogenesis. Except for Nox4, all other markers were significantly up-regulated in Cxcl5 KO mice in the early stage of differentiation SVF (Fig. 2H). It is of note that the marker genes were decreased thereafter, because adipose tissue is mainly composed of mature adipocytes after rapid differentiation. Typically, the difference in gene expression between WT and Cxcl5 KO was largest after six days of differentiation (Fig. 2G). PPARγ protein levels were also significantly up-regulated in adipocyte differentiation of Cxcl5 KO (Fig. 2I). These results strongly suggest that Cxcl5 negatively regulates adipocyte differentiation.

3.3. CXCL5 treatment inhibits adipogenesis

We hence conducted an experiment to introduce recombinant CXCL5 to clarify the relationship between CXCL5 and adipogenesis. 3T3-L1 cells were treated with recombinant CXCL5 during adipocyte differentiation for 6 days. As expected, it was confirmed that abundant CXCL5 inhibited the differentiation of preadipoctyes into adipocytes. The decreases in lipid droplets in cells and the absorbance value for Oil red O staining in the recombinant CXCL5 treated group demonstrated that CXCL5 is negatively associated with adipogenesis (Fig. 2A and B). Also, the triglycerides level was lower in adipocytes treated with recombinant CXCL5 than in control (Fig. 2C). The adipogenesis-associated genes Pparγ, Srebp1-c, and Glut4 were significantly down-regulated by CXCL5 treatment during adipocyte differentiation (Fig. 2D). It was demonstrated that CXCL5 negatively affects adipocyte differentiation, we then tested whether CXCL5 regulates PPARγ, a master regulator in adipocyte differentiation. A luciferase reporter assay of the transcriptional activity
Fig. 1. Adipogenesis was high but the expression of lipid metabolism-related proteins was low in Cxcl5 KO mice. A) Schematic diagram of proteomics analysis using eWAT from WT and Cxcl5 KO mice. B) Heatmap and C) volcano plots of proteins with a more than 1.2-fold increase (orange) or decrease (purple) in Cxcl5 KO mice. GO analysis of proteins (top 5) differentially expressed in the eWAT of Cxcl5 KO mice. GO analysis results of D) upregulated and G) downregulated proteins. The Network between GO terms in DEP with E) upregulated and H) downregulated eWAT in Cxcl5 KO mice. Enrichr analysis results from TRRUST transcription factors 2019 database for transcription factors of F) upregulated and I) downregulated proteins in Cxcl5 KO mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Fig. 2. Cxcl5 mRNA expression is dramatically downregulated during adipogenesis. A) 3T3-L1 adipocytes were differentiated by treatment with MDI (0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/mL insulin) for 2 days. Next, after incubation for the next 2 days in a medium containing insulin, the medium was replaced with Dulbecco’s modified eagles medium (DMEM) medium and exchanged once every two days until differentiation was complete. B) mRNA expression of the adipogenic marker gene. C) Changes in mRNA expression of Cxcr2 and its ligands during the differentiation of adipocytes. D) Schematic diagram of the separation of SVF from adipose tissue. E) Differentiation of SVF isolated from WT and Cxcl5 KO mice into adipocytes was confirmed by Oil red O staining. Scale bar = 200 μm. F) Stained cells were dissolved with isopropanol, and the OD measured at 500 nm. G) Triglyceride concentrations of WT and Cxcl5 KO during adipocyte differentiation. H) Expression of adipogenic gene mRNA in WT and Cxcl5 KO during adipocyte differentiation. After adipocyte induction I) PPARγ protein expression levels in WT and Cxcl5 KO. Statistical analysis was performed using two-tailed unpaired Student’s t-tests. *p < 0.05, **p < 0.01 and ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
of PPARγ was induced via treatment with rosiglitazone, the ligand of PPARγ, alone or with recombinant CXCL5 (Fig. 3E). Luciferase activity was inversely related to the amount of CXCL5 used to treat the cells (Fig. 3F). These results suggest that CXCL5 inhibits the transcriptional activity of PPARγ in a ligand-binding, domain-dependent manner. Our data strongly indicate that CXCL5 negatively regulates the differentiation of adipocytes.

3.4. Adipocytes differentiated from Cxcl5 KO mice have high ROS

Since numbers of GO terms associated with fatty acid or lipid metabolism, including oxidation-reduction, and a fatty acid β-oxidation, were enriched in the down-regulated DEP of Cxcl5 KO (Fig. 1F) and a solid association was reported between excessive fat accumulation and high production of ROS [22], we measured the amount of ROS in both Cxcl5 KO and WT. H2DCFDA staining showed that the basal level of intracellular ROS was higher in Cxcl5 KO than in WT (Fig. 4A). We also measured the transcription levels of antioxidant genes such as the Prdx (peroxiredoxin) gene group, Sod1 (superoxide dismutase), Cat (catalase), and Gpx3 (glutathione peroxidase3). Of note, several antioxidant genes were increased in Cxcl5 KO compared to WT at the beginning of differentiation and dramatically downregulated at the end of differentiation (Fig. S1). Furthermore, in the oxidative stress condition of exposure to H2O2, ROS accumulation increased robustly in Cxcl5 KO cells. H2DCFDA stained the most brightly on the Cxcl5 KO cells upon exposure to H2O2 (Fig. 4A). It was confirmed again in the gene expression levels of antioxidant enzymes. As shown in Fig. S1, most antioxidant enzymes, including Prdx1, decreased at the transcription level in Cxcl5 KO. H2O2 induced significant increases in the expression of some of these enzymes, such as MnSod, in WT but not in Cxcl5 KO (Fig. 4C). Most of the genes, including Prdx2,3,4,5, Sod1, Cat, and Nrf2, were maintained or slightly increased when WT adipocytes were treated with H2O2, but they were still expressed at low levels in Cxcl5 KO adipocytes. Western blots of representative proteins PRDX4 and MnSOD show that the antioxidant enzymes downregulated in Cxcl5 KO are less responsive to H2O2 than in WT adipocytes (Fig. 4B).

Conversely, the generation of ROS and the expression of antioxidant enzymes were confirmed when adipogenesis while treated with CXCL5. As a result, ROS production was decreased when differentiated while treated with CXCL5 (Fig. 4D and E), but there was no difference in the expression of antioxidant enzymes (Fig. 4F). On the other hand, we also tested if reducing ROS during adipogenesis can restore normal adipogenesis in Cxcl5 KO by antioxidant treatment (Fig. S2). Expectedly, antioxidants treatment retarded or blocked adipogenesis in a concentration-dependent manner. Since ROS effects on adipogenesis were too obvious to hypothesize that Cxcl5 directly regulates antioxidant enzyme expression, we thus propose that Cxcl5 depletion promotes the differentiation of adipocytes, resulting in substantial ROS accumulation that induces chronic oxidative stress.
3.5. HFD amplified the systemic oxidative stress caused by impaired antioxidant enzymes in Cxcl5 KO mice

Since our results showed that CXCL5 is strongly associated with a high level of ROS and fat metabolism in adipocyte tissue, we fed a HFD to Cxcl5 KO mice for 13 weeks (Fig. 5A). Based on the previous results, we hypothesized that Cxcl5 KO mice fed with HFD would exhibit the disturbance of ROS scavenging or oxidative-stress-related proteins and thus an increased oxidative stress condition. Fig. 5A illustrates the scheme of the experiment. After eWAT tissue sampling, the proteins were digested by the in-solution digestion method. Digested peptides were labeled with iTRAQ to normalize protein amounts quantitatively. After labeling the fragmented peptides with the iTRAQ isobaric mass tags, the sample was fractionated into six by anion exchange fractionation. After that, mass spectrometry was performed to obtain comparative proteome quantitation. A total of 1,781 proteins was identified in the eWAT (Table S4). To understand the role of CXCL5 in HFD-mediated ROS, we sorted out the proteins that were upregulated in WT HFD but not in the NFD or Cxcl5 KO HFD groups (Fig. 5B). A total of 324 proteins were retrieved and subjected to GO analysis. We found that the protein reduced in Cxcl5 KO was enriched with proteins involved in responses to oxidative stress and cellular redox homeostasis, consisting of antioxidant proteins that scavenge ROS (Fig. 5C and Table 1). The data show that the eWAT of Cxcl5 KO fed HFD poorly expresses the antioxidant proteins and thus has increased ROS with a more severe oxidative stress condition.

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Fig. 5. Increased oxidative stress due to decreased antioxidant proteins in Cxcl5 KO mice. A) Schematic diagram of proteomic analysis of WT and Cxcl5 KO mice fed an NFD or HFD. B) Heatmap with a pattern that increases in WT HFD but decreases in Cxcl5 KO HFD. C) GO analysis of proteins that decreased in Cxcl5 KO HFD eWAT among the list of proteins increased more than 1.2 times in WT HFD eWAT compared to WT eWAT. D) Lipid peroxidation evaluated by the TBARS assay in eWAT of WT or Cxcl5 KO mice fed NFD and HFD. E) Western blot of phosphorylation AKT (Ser473) and phosphorylation JNK in eWAT proteins from WT or Cxcl5 KO mice fed NFD and HFD. F) Western blot of phosphorylation AKT (Ser473) and total AKT in 3T3-L1 cells treated with CXCL5 or insulin or treated together. G) mRNA expression of Il-6, Tnfα, Adipoq, Glut4, and Glut5. The 36b4 gene was used for internal controls for RNA and protein. Statistical analysis was performed using two-tailed unpaired Student’s t-tests. *p < 0.05 and **p < 0.01.
markers such as these data indicate that CXCL5 have a positive effect on ROS and insulin pathways in WT and phosphorylated AKT [23,24], we thus examined these two signaling pathways in WT and Cxcl5 KO mice with different diet conditions. The data clearly showed that phosphorylated AKT Ser473 decreased and phosphorylated JNK increased in the eWAT of Cxcl5 KO, and the difference was more significant in the HFD groups (Fig. 5E). In addition, when Cxcl5 was treated with insulin in 3T3-L1 cells, p-AK Ser473 signaling was further activated than insulin alone (Fig. 5F). Together these data indicate that Cxcl5 has a positive effect on ROS and insulin signaling regulation. Furthermore, the expression of inflammation markers such as Tnfα and Il-6 was higher in the eWAT of Cxcl5 KO, while Adipoq expression was lower (Fig. 5G). These results suggest that the accumulation of ROS in the Cxcl5 KO mice fed an HFD due to their impaired antioxidant enzyme activities results in the increased inflammatory response in the eWAT.

3.6. HFD combined with Cxcl5 deficiency increased insulin resistance and cholesterol in the blood

Since all of the results indicated that Cxcl5 deficiency may cause functional damage in eWAT, we characterized Cxcl5 KO mice regarding metabolic-related phenotypes. Unexpectedly, a significant difference in body weight was observed between the Cxcl5 KO and WT groups fed an HFD, but lean and fat mass/body weight, free body fluid, and amount of eWAT tissue weight/body weight (TW/BW) did not significantly differ (Fig. 6A–C). Also, there was no significant difference in histology between WT and Cxcl5 KO mice fed an NFD. However, distinct differences in histology were observed in the groups fed a HFD; Cxcl5 KO mice fed an HFD exhibited larger adipocytes than WT mice fed a HFD (Fig. 6D). Accurate measurements of the size of the adipocytes confirmed that the adipocytes were significantly larger in Cxcl5 KO mice fed an HFD (Fig. 6E). In particular, the numbers of macrophages that penetrated the adipose tissue and stained crown-like structures were significantly higher (Fig. 6E). We also performed a histological analysis of other adipocyte tissues (inguinal white adipose tissue (iWAT), brown adipose tissue (BAT)), and the liver. As in the eWAT, the adipocytes were enlarged in Cxcl5 KO mice fed an HFD (Figs. S3A and B). Moreover, a fatty liver was found in Cxcl5 KO mice, and it became severe in the Cxcl5 KO group fed an HFD (Fig. S3C). Fat accumulation was confirmed in the liver of Cxcl5 KO mice, and when the expression levels of Irs1,2 and Glut4 in the liver were checked, they were not significantly different from those of the WT HFD group (Fig. S4). The blood glucose level was higher in Cxcl5 KO than in WT in the groups fed an NFD (Fig. 6F), and in the intraperitoneal glucose tolerance tests, blood glucose levels were significantly higher in the Cxcl5 KO mice fed an HFD (Fig. 6G). These results suggest that oxidative stress caused by Cxcl5 deficiency triggers insulin resistance. In addition, consistent with previous findings that ROS induces intracellular lipid accumulation and causes an increase in cholesterol [25], total cholesterol level was significantly higher in Cxcl5 KO than in WT mice when feeding either an NFD or an HFD. Cholesterol presents in the body in two different forms: esterified cholesterol and free cholesterol. However, only free cholesterol is biologically active and has cytotoxicity [26], so the concentration of free cholesterol was measured separately. Notably, the free cholesterol level was significantly higher in Cxcl5 KO mice regardless of diet (Fig. 6H). Altogether, the increase in ROS and decrease in antioxidant proteins caused by Cxcl5 deficiency induces insulin resistance and increases cholesterol in the body, suggesting that fat metabolism and the metabolic system in the body were damaged in Cxcl5 KO mice.

4. Discussion

The adipose tissue in the body is a vital organ that stores extra energy. However, improper enzyme functions in this tissue cause excessive lipid accumulation, resulting in various metabolic diseases. Thus, proper differentiation of adipocytes and accumulation and decomposition of fat help maintain energy homeostasis in the body [27]. Recent studies, including ours, have suggested the involvement of Cxcl5 in adipogenesis and lipid metabolism [17]. Cxcl5 is known as a chemokine secreted in inflammatory diseases and is highly expressed in metabolic diseases. For example, Cxcl5 is highly expressed in obese or diabetic patients and is associated with hypercholesterolemia. It has been reported that the in vivo concentration of Cxcl5 is abnormally higher than physiological concentrations in patients with these diseases [28-30]. Conversely, we have previously reported that Cxcl5 is required for normal lipid metabolism and that UCp1 is less expressed in Cxcl5 KO individuals under cold stress conditions [17]. As such, there is still confusion regarding the role of Cxcl5, but in this study, we describe Cxcl5 as a factor regulating healthy differentiation in adipocytes. Our results seem contradictory to the previous study that Cxcl5 promotes insulin resistance [28], but there are differences between the two studies. First, there is a big difference in the target cell type expressing Cxcl5. The previous study focused on the M1 macrophage, but the current study on adipocytes. Second, experimental conditions are different. This study focused on the adipocyte differentiation condition instead of inflammatory condition that was tested in the previous study. Third, from the adipogenesis point of view, the previous study looked at the adipose tissue, which means that differentiation was already done, and the Cxcl5 level would be low. On the contrary, the current study focused on the role of Cxcl5 in the early stage of adipocyte differentiation using the Cxcl5 KO mice. The cells have low Cxcl5 levels, and adipocyte differentiation was not occurring appropriately.

This study suggests that Cxcl5 is involved in adipocyte differentiation and expansion and consequently regulates antioxidant enzymes. Our data confirmed that pre-adipocytes differentiation into mature adipocytes was increased in Cxcl5 KO mice (Fig. 1). Cxcl5 expression must be downregulated for healthy differentiation of adipocytes. Contrary to the well-known inflammatory response of Cxcl5, during the normal differentiation of adipogenesis, Cxcl5 might not participate in the inflammatory response. The role of Cxcl5 that we have identified is to induce the healthy differentiation of adipocytes. As shown in Fig. 2, the expression of various chemokines and their receptors is regulated during adipocyte differentiation. When adipocyte differentiation occurs, Cxcl1 or Cxcl5 decreases, but the expression of Cxcl3 or Cxcr2 increases. These results are consistent with studies showing that Cxcl3 and Cxcr2 induce adipogenesis [13,14]. Conversely, Cxcl5, which was reduced during adipocyte differentiation, inhibited the differentiation in our data. Accordingly, if we focused on the end stage of adipocyte differentiation or fully differentiated adipocyte, transgenic mice overexpressing Cxcl5 can be a better mouse model than the Cxcl5 KO mice model. However, we think that the Cxcl5 KO mice model is better since we looked at an early stage of adipogenesis. Moreover, we also conducted the

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Table 1

| Official gene symbol | Gene name | Difference |
|----------------------|-----------|------------|
| DLH                  | dihydroprolidase dehydrogenase | 2.502939   |
| PRDX1                | peroxiredoxin 1 | 1.626282   |
| PRDX2                | peroxiredoxin 2 | 1.621552   |
| PRDX3                | peroxiredoxin 3 | 1.780662   |
| PRDX4                | peroxiredoxin 4 | 3.837177   |
| PRDX5                | peroxiredoxin 5 | 2.43681    |
| PRDX6                | peroxiredoxin 6 | 1.647107   |
| P4HB                 | prol 4-hydroxylase, beta polypeptide | 2.236035   |
| PDEA3                | protein disulfindase isomerase 3 | 1.634867   |
| PDEA4                | protein disulfindase isomerase 4 | 1.612836   |
| PDEA6                | protein disulfindase isomerase 6 | 1.541046   |
| TXNDC5               | thioridoxin domain containing 5 | 1.615277   |
| TXNLI                | thioridoxin-like 1 | 2.829663   |
overexpression experiment by treating the recombinant CXCL5 protein, and successfully showed that CXCL5 inhibits adipocyte differentiation. In Cxcl5 KO cells, differentiation markers are expressed very highly compared to WT in the early stage of differentiation, and show a decreasing pattern in the late stage of differentiation (Fig. 2H). At the end of differentiation, a decrease in adiponectin, a hormone that helps metabolism and a differentiation marker, was confirmed. It was not only at the cellular level but also in the Cxcl5 KO eWAT tissue (Fig. 5 G).

Eventually, in Cxcl5 KO, differentiation occurs rapidly, and a lot and adipose tissue is abnormally formed which is considered to eventually show an insulin resistance phenotype. Additionally, we suggested the mechanism of CXCL5 in the regulation of adipogenesis related gene expression via PPARγ. There were two possible ways in which CXCL5 could regulate PPARγ function. We first showed that PPARγ expression was increased in Cxcl5 KO. The other finding was that treatment with CXCL5 inhibits the transcriptional activity of PPARγ via the influence of

Fig. 6. Increased insulin resistance and cholesterol due to fat accumulation during irregular regional differentiation in Cxcl5 KO mice. A) Graphs of body weight gain when fed an NFD or HFD for 16 weeks. B) Body composition of WT and Cxcl5 KO mice fed an NFD or HFD. Representative images of C) weight of eWAT/body weight and D) eWAT H&E staining of WT and Cxcl5 KO mice fed an NFD or HFD. Scale bar = 200 μm. E) The diameter of the lipid droplet in H&E staining of eWAT. F) Non-fasting blood glucose levels in WT and Cxcl5 KO mice fed an NFD or HFD. G) Intraperitoneal glucose tolerance test (IPGTT) results for WT and Cxcl5 KO mice fed an HFD for 16 weeks. (n = 8) IPGTT measurement was performed for up to 120 min by injecting 2 g glucose/kg body weight into the fasting mouse for 16 h. H) Determination of total and free cholesterol in the serum of mice fed an NFD or HFD. Statistical analysis was performed using two-tailed unpaired Student’s t-tests. *p < 0.05 and **p < 0.01.
the ligand-binding domain. Further study of the molecular functions of CXCL5 regarding its influence on PPARγ activity will be guaranteed.

Numerous antioxidants function to remove ROS in the body [31]. Changes in antioxidant enzymes have several implications. When ROS is low, the expression of antioxidant enzymes is low. Conversely, when ROS is too high and intracellular regulation is impossible, the expression of antioxidant enzymes may be low [32]. Impaired adipogenesis in Cxcl5 KO triggers ROS accumulation, leading to oxidative stress in adipocytes (Fig. 4A). As a result, it also fails to regulate the expression of antioxidant enzymes (Fig. 4B, C, Fig. S1). As a feedback action, adipocytes from Cxcl5 KO mice were better differentiated with more ROS accumulation than adipocytes from WT mice (Figs. 2E and 4A). As a consequence of the above functions, Cxcl5 deficiency led to inflammatory reactions and insulin resistance in Cxcl5 KO mice. Although there is no significant difference in the weight or weight of fat, there are many other values and features that should be considered for abnormalities in adipocytes and adipogenesis. We showed that the overall glucose concentration was not properly regulated in the Cxcl5 KO HFD group in IPGTT data and confirmed that the ROS was high and crown-like structures were more visible in the eWAT fed Cxcl5 KO HFD; these were found not only in eWAT but also in BAT and the liver (Fig. S3). It is meaningful because oxidative stress in the liver is a crucial mechanism in developing non-alcoholic fatty liver disease [33]. These characteristics were more severe in the Cxcl5 KO group fed an HFD because Cxcl5 deficiency makes it more challenging to regulate ROS, so it is expected that fatty liver formation is severe in Cxcl5 KO. These results suggest that it is worth studying the relationship between the mechanisms of Cxcl5 and the fatty liver. ROS increases during metabolism even in brown adipose tissue, but in Cxcl5 KO mice, ROS increases further, and the lipid droplets of BAT become larger, as seen in eWAT. ROS is regulated by UCP1, which is highly expressed in BAT [34], and it is expected that ROS might not be properly regulated in Cxcl5 KO due to the decrease of UCP1 [17]. Lastly, free cholesterol and total cholesterol were the highest in Cxcl5 KO HFD.

The current study strongly suggests that ROS accomplished more active adipocyte differentiation in Cxcl5 KO cells. Previous studies have shown that, among many other factors, ROS promotes adipocyte differentiation [22,35]. Since too much ROS accumulation will eventually damage the cells, more studies are required to determine the levels of ROS needed for better differentiation of adipocytes. Altogether, our study identified CXCL5 as a novel adipokine involved in adipocyte differentiation and acts as an important factor in regulating fat metabolism. We observed the difference between WT and whole-body Cxcl5 KO in adipose tissue and performed the present study targeting white adipose tissue. To specify the function of CXCL5 in adipose tissue, we isolated SVF cells from adipose tissue from Cxcl5 KO mice. Therefore, although we generated and used whole-body Cxcl5 KO mice, we demonstrated the function of CXCL5 in a cell unit. As a future study, it is worthwhile to study the specific role of Cxcl5 in metabolic-related tissues through tissue-specific KO mouse studies. This study presents a new perspective on treating obesity and metabolic diseases through the understanding of CXCL5 in adipocyte differentiation.

5. Conclusions

This study demonstrated that CXCL5 has an essential role in adipogenesis in adipose tissue. Furthermore, HFD in Cxcl5 KO mice promoted abnormal adipogenesis and further increased ROS severity. It was explained by showing that the expression of antioxidant enzymes also decreased and failed to remove ROS promptly. Consequently, oxidative stress and insulin resistance have occurred in Cxcl5 KO mice. These results showed an essential role in the adipogenesis and ROS regulation of CXCL5 in adipose tissue, further suggesting that CXCL5 is a valuable chemokine for metabolic disease research.
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