Chemerin, a Novel Adipokine That Regulates Adipogenesis and Adipocyte Metabolism

Obesity is an alarming primary health problem and is an independent risk factor for type II diabetes, cardiovascular diseases, and hypertension. Although the pathologic mechanisms linking obesity with these co-morbidities are most likely multifactorial, increasing evidence indicates that altered secretion of adipose-derived signaling molecules (adipokines; e.g. adiponectin, leptin, and tumor necrosis factor α) and local inflammatory responses are contributing factors. Chemerin (RARRES2 or TIG2) is a recently discovered chemoattractant protein that serves as a ligand for the G protein-coupled receptor CMKLR1 (ChemR23 or DEZ) and has a role in adaptive and innate immunity. Here we show an unexpected, high level expression of chemerin and its cognate receptor CMKLR1 in mouse and human adipocytes. Cultured 3T3-L1 adipocytes secrete chemerin protein, which triggers CMKLR1 signaling in adipocytes and other cell types and stimulates chemotaxis of CMKLR1-expressing cells. Adenoviral small hairpin RNA targeted knockdown of chemerin or CMKLR1 expression impairs differentiation of 3T3-L1 cells into adipocytes, reduces the expression of adipocyte genes involved in glucose and lipid homeostasis, and alters metabolic functions in mature adipocytes. We conclude that chemerin is a novel adipose-derived signaling molecule that regulates adipogenesis and adipocyte metabolism.

White adipose tissue, in addition to serving an important metabolic role, is an active endocrine organ that secretes a number of signaling peptides with diverse biological functions (1–3). These signaling molecules, collectively termed adipokines, include the following: cytokines and related proteins (leptin, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and chemokine (c-c motif) ligand 2 (CCL2)); proteins of the fibrinolytic cascade (plasminogen activator inhibitor-1); complement and complement-related proteins (adipsin, acylation-stimulating protein, and adiponectin); vasoactive proteins (renin, angiotensinogen, and angiotensin I and II) and other biologically active peptides such as resistin (4–11). Adipokines have important autocrine/paracrine roles in regulating adipocyte differentiation and metabolism and local inflammatory responses (12–15). Adipokines also have important roles in the regulation of systemic lipid and glucose metabolism through endocrine/systemic actions in the brain, liver, and muscle (6, 16, 17). The secretion and/or serum level of many adipokines is profoundly affected by the degree of adiposity (18–22). This has led to the hypothesis that, in obesity, dysregulation of pro-inflammatory/-diabetic and anti-inflammatory/-diabetic adipokine secretion may serve as a pathogenic link between obesity and type II diabetes and cardiovascular diseases (7, 10, 14, 23). The identification and characterization of novel adipokines will further our understanding of the endocrine function of white adipose tissue, providing novel molecular targets for the development of treatment strategies for obesity and related diseases.

Chemerin (RARRES2 or TIG2) is a recently discovered chemoattractant protein that serves as a ligand for the G protein-coupled receptor CMKLR1 (ChemR23 or DEZ) and has a role in adaptive and innate immunity (24–30). Chemerin is secreted as an 18-kDa inactive pro-protein and undergoes extracellular serine protease cleavage of the C-terminal portion of the protein to generate the 16-kDa active chemerin, which is present in plasma, serum, and hemofiltrate (26–29, 31). The estimated concentration of active chemerin in plasma and serum, respectively, was 3.0 and 4.4 nM in humans and 0.6 and 0.5 nM in mice (28). Consistent with the properties of a proinflammatory adipokine, chemerin expression is profoundly affected by the degree of adiposity (18–22). This has led to the hypothesis that, in obesity, dysregulation of pro-inflammatory/-diabetic and anti-inflammatory/-diabetic adipokine secretion may serve as a pathogenic link between obesity and type II diabetes and cardiovascular diseases (7, 10, 14, 23). The identification and characterization of novel adipokines will further our understanding of the endocrine function of white adipose tissue, providing novel molecular targets for the development of treatment strategies for obesity and related diseases.

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# The abbreviations used are: TNFα, tumor necrosis factor-α; IL, interleukin; shRNA, short hairpin RNA; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; IBMX, isobutylmethylxanthine; RNAi, RNA interference; m.o.i., multiplicity of infection; QPCR, quantitative PCR; ANOVA, analysis of variance; KLH, keyhole limpet hemocyanin; VEH, vehicle; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PPARγ, peroxisome proliferator-activated receptor γ; CE, chemerin; CR, CMKLR1; LZ, LacZ; HSL, hormone-sensitive lipase.

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secreted protein, active chemerin is abundant in ascites fluid from ovarian cancer patients (1.8–7 nM) and synovial fluid from arthritic patients (22 nM) (27). However, the tissues or cells responsible for chemerin secretion into these biological fluids have not been established. Initial screening studies in our laboratory identified high expression of chemerin and CMKLRI in white adipose tissue of mouse. These data suggested that white adipose tissue is a source and target for chemerin signaling. Based on our initial gene expression data, observations that chemerin is a secreted protein (24, 26, 27) and the fact that white adipose tissue is an important endocrine organ (32, 33), we have proposed and tested the hypothesis that chemerin is an adipokine with a regulatory role in adipogenesis or adipocyte function.

EXPERIMENTAL PROCEDURES

Animal Protocol and Housing—The Dalhousie University Committee on Laboratory Animals approved experimental procedures involving mice according to the guidelines of the Canadian Council on Animal Care. C57/BL/6J mice were bred in-house, in the Carleton Campus Animal Care Facility. Lepob/ob mice on a C57/BL/6J background and their littermate controls were obtained from The Jackson Laboratories (Bar Harbor, MR). The mice were kept on a 12-h day-night cycle, were housed in cages lined with pine bedding, and had free access to water and Purina mouse chow.

RNA Isolation and QPCR Analysis—Adult male mice were anesthetized with 50 mg kg⁻¹ of sodium pentobarbital. Tissues were isolated and snap-frozen in liquid nitrogen. For adipose tissue fractionation, freshly isolated epididymal (visceral) fat pads were placed in 5 ml of ice-cold DMEM with 1% BSA and 2 mg/ml of collagenase II and minced with scissors. The tissue was incubated for 15 min at 37 °C with intermittent pipetting, diluted into 10 ml of ice-cold DMEM and filtered through 70-μm nylon mesh, and centrifuged at 2200 rpm for 5 min to separate the adipocyte (buoyant) and stromal vascular fractions (pellet). Total RNA was isolated from each fraction using the RNeasy mini kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer’s instruction. Total RNA from tissues (5 μg) or cells (0.5 or 1.0 μg) was reverse-transcribed using Stratascript™ reverse transcriptase (Stratagene, Cedar Creek, TX), and 1 μl of the cDNA product was amplified by quantitative PCR using 125 nM gene-specific primers (supplemental Table 1) in a total volume of 20 μl with Brilliant SYBR Green QPCR master mix (Stratagene) using a Stratagene MX3000p thermocycler (34). Relative gene expression was normalized to ribosomal polymerase II (rplII) or cyclophilin A (CypA) expression using the ΔΔCt method (35).

Mouse Adipocyte Cell Culture—3T3-L1 preadipocytes were obtained from the American Tissue Culture Collection (Manassas, VA) and grown according to standard protocols (36). Confluent preadipocytes were differentiated in adipocyte media (DMEM, 10% FBS, 850 nM insulin, and 0.1% penicillin/streptomycin) supplemented with 250 nM dexamethasone and 100 μM isobutylmethylxanthine (IBMX) for 3 days. After this time, the cells were maintained in adipocyte media, which was changed every 2 days. All media were phenol red-free. Adipocyte-conditioned media used for Western blots, CMKLRI1 activation, or the chemotaxis assay were obtained by replacing the regular adipocyte media with serum-free adipocyte media for a period of 24 h. For ERK1/ERK2 phosphorylation studies, adipocyte media were replaced with fresh media 4 h prior to the assay. At the time of the assay fresh adipocyte media containing (0.2, 1.0, or 10 nM) chemerin were added to the cells. Between 2 and 30 min thereafter, media were removed, and 100 μl of 1× Laemmli buffer was added to stop the reaction and lyse the adipocytes. Oil Red O staining of adipocytes and quantification of extracted dye were carried out as described previously (36).

Western Blotting—We generated polyclonal rabbit antibodies against a synthetic peptide region of mouse chemerin (supplemental Fig. 1). The immunizing peptide (CLAFQEIGVDRA-EEV) corresponded to amino acids 47–60 of the predicted mouse chemerin protein and was conjugated through a non-native N-terminal cysteine (indicated as boldface C) to keyhole limpet hemocyanin (KLH) (Sigma Genosys). Rabbits were given a primary immunization by subcutaneous injection of 150 μg of the peptide-KLH conjugate in Freund’s complete adjuvant. Subsequent immunizations with the peptide-KLH conjugate in Freund’s incomplete adjuvant were administered 3, 5, and 7 weeks later. 10 days after the final immunization, the rabbits were exsanguinated by cardiac puncture. The specificity of the antisera was confirmed by testing immunoreactivity against recombinant mouse chemerin (R & D Systems, Minneapolis, MN) and COS7 cells that were transiently transfected with a chemerin-pFLAG-CMV-5a construct or the control vector pFLAG-CMV-5a (Stratagene) (supplemental Fig. 1). For Western blots, 100 μl of 24-h conditioned adipocyte media were added to 20 μl of 6× SDS loading buffer. Fifteen μl of the solution was separated on a 12.5% polyacrylamide gel and transferred overnight to a nitrocellulose membrane. Blots were blocked (1 h) in 3% skim milk in pH 7.5 Tris-buffered saline with 0.05% Tween (TBST) and then incubated with protein A-purified rabbit anti-chemerin antisera (1:200) in 3% skim milk/TBST for 2 h at room temperature and then horseradish peroxidase-conjugated mouse anti-rabbit IgG secondary antibody (1:25,000) for 1 h at room temperature in 3% skim milk/TBST. Immunoreactivity was detected by incubation with fluorescent ECL-plus™ reagent (GE Healthcare) and visualized directly with a Storm 840 PhosphorImager (GE Healthcare). A similar protocol using 1:200 dilutions of P-ERK (E-4) and ERK (D-2) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect extracellular-related kinase 2 (ERK2) and phosphorylated ERK1 and ERK2 in whole cell lysates from chemerin-treated adipocytes.

Aequorin Assay—The aequorin assay is a cell-based, bioluminescence reporter gene assay used to detect CMKLRI1 activation (27). CMKLRI1-CHO-K1 cells (Euroscreen, Belgium) express human CMKLRI, Goi₁₆ (G protein), and an intracellular reporter gene (mitochondrial aequorin) that is activated by the Ca²⁺ influx produced when chemerin binds to CMKLRI1 activating Goi₁₆. The control CHO-K1 cells (Euroscreen, Belgium) express the Goi₁₆ protein and mitochondrial aequorin only and do not respond to chemerin. Control CHO-K1 cells were maintained in complete Ham’s F-12 with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml Zeocin. The media supplemented with the antibiotic G418 (400 μg/ml)
were used for maintenance of the CHO-CMKLR1 cells. Cells in mid-log phase, grown in media without antibiotics for 20 h prior to the test, were detached with PBS containing 5 mM EDTA, centrifuged and suspended (5 × 10⁶ cells/ml) in 0.1% BSA media (DMEM/Ham’s F-12 with HEPES, without phenol red) plus 5 µM coelenterazine h (Sigma) and incubated for 4 h at room temperature on an orbital shaker. Cells were then diluted 1 in 10 with 0.1% BSA media and incubated for 1 h. For each measurement 50 µl of cell suspension (25,000 cells) was added into each well of the plate containing 50 µl of diluted conditioned media or recombinant chemerin. The emitted light was recorded for 20 s at 469 nm following the injection of cells. The intensity of emitted light was normalized to the response produced by the Ca²⁺ ionophore digitonin (50 µM).

Cell Migration Assay—Conditioned serum-free media from preadipocytes and adipocytes were tested for the ability to stimulate migration of the murine pre-B lymphoma cell line L1.2 stably transfected with human CMKLR1 (L1.2-CMKLR1) or empty vector (L1.2-pcDNA3). The cells were maintained as described previously (30). All assay incubations were performed under standard conditions (37 °C in 95% air, 5% CO₂). The L1.2 cells were plated at a density of 1 × 10⁵ cells/ml and were treated with 5 mM n-butyric acid 24 h prior to experimentation. Purified mouse chemerin and conditioned (24 h) serum-free media samples from preadipocyte or 13-day mature adipocyte cell cultures was diluted 1:100 into the chemotaxis medium (600 µl final volume) consisting of phenol red-free RPMI 1640 medium and 1% fetal bovine serum and was incubated for 30 min under standard conditions. Transwell inserts (5 µm pore size) containing 250,000 L1.2-CMKLR1 or L1.2-pcDNA cells in 100 µl of chemotaxis media were added to wells containing the media samples and incubated for 3 h. The inserts were removed, and cells that migrated into the lower chamber were labeled for 3 h with calcein-AM. The calcein-AM-labeled cells were diluted 1:10 in PBS and fluorimetrically measured (485 nm excitation and 520 nm emission) using a Cary spectrofluorometer. A standard curve was generated from calcein-AM labeling of known quantities of either cell type and was used to quantify the total number of cells (%) input migration) that migrated toward the test samples.

Immunohistochemistry—Preadipocytes were plated on collagen-coated glass coverslips and differentiated according to the standard protocol. On day 8, the cells were rinsed in PBS and fixed in 4% paraformaldehyde. Fixed cells were rinsed in PBS and incubated with 0.1% Triton X-100 (3 min) followed by incubation in standard blocking solution (10% goat serum, 1% bovine serum albumin in phosphate-buffered saline) for 1 h, 1:200 dilution of anti-human CMKLR1 monoclonal antibody (clone 84939, R & D Systems) for 2 h, 1:200 dilution of Alexa Fluor 488 goat anti-mouse IgG3 (Invitrogen) for 1 h, and counterstained with Hoechst 33258 (Sigma) 1 µg/ml in PBS for 5 min. Slides were washed in PBS and mounted with fluorescent mounting medium (DakoCytomation, Carpinteria, CA). Images were captured on a Zeiss Axiosvert 200 inverted microscope equipped with an AxioCam camera system (Zeiss Canada, Toronto, Ontario, Canada).

Adenoviral Small Hairpin Loop RNA Interference (shRNA)—shRNA vectors were constructed using the Block-it™ adeno-viral RNAi expression system (Invitrogen) according to the manufacturer’s instructions. Single-stranded oligonucleotides (supplemental Table 1B) for shRNA were designed using Block-it™ RNAi designer (Invitrogen). Positive adenoviral clones, mchemerin-pAD-shRNA (CE-shRNA), mCMKLR1-pAD-shRNA (CR-shRNA), and LacZ-pAD-shRNA (LZ-shRNA) were amplified in the HEK-293A producer cell line. Viral copy number in crude lysates was determined by quantitative PCR amplification. A poly-L-lysine (M, 30,000–70,000)-assisted procedure was used to transduce confluent preadipocytes or adipocytes (37). Crude adeno viral lysates (m.o.i. 100–1000) were added to poly-L-lysine (0.5 µg/ml) Opti-MEM mix and incubated for 100 min at room temperature. The m.o.i. refers to the ratio of viral copy/cell number. One-day post-confluent preadipocytes were washed once with PBS, and 500 µl of the transduction mixture was added to each well (12-well plate) and incubated under standard conditions for 2 h followed by addition of 1 ml of DMEM with 0.2% BSA and incubated overnight. The next day media were replaced with normal preadipocyte media for 6 h. At this time the normal adipocyte differentiation and maintenance protocol was followed. Using the same protocol, adipocytes at day 4 post-differentiation were transduced with the crude adeno viral lysates. For the lipolysis assays, day 7 adipocytes were switched to DMEM + 0.1% BSA with or without 0.2 or 1.0 nM chemerin. 24 h later, media were replaced with DMEM + 0.1% BSA with or without 2 µM isoproterenol or 100 µM IBMX. Glyceral released into the media over a period of 2 or 4 h was measured using a lipolysis assay kit (ZenBio) according to the product instructions.

Adiponectin Measurements—An enzyme-linked immunosorbent assay (adiponectin, Quantikine kit, R & D Systems) was used to measure adiponectin levels in 24-h conditioned serum-free media from adipocytes 5 or 8 days post-differentiation.

Human Cells and RNA—Human preadipocytes and adipose tissue RNA were commercially available (ZenBio, Chapel Hill, NC). Human liver and placenta RNA were purchased from Stratagene. Superlots of cryopreserved human subcutaneous preadipocytes (ZenBio) contained preadipocytes pooled from six female donors aged 35–45 with an average body mass index of 29. The preadipocytes were seeded on 12-well plates according to the manufacturer’s instructions. To induce differentiation, the preadipocytes were incubated with 1 ml of adipocyte differentiation media (ZenBio, Research Triangle Park, NC) for 7 days. Thereafter, adipocytes were maintained in DMEM/F-12 with 10% FBS, 850 nM insulin. Oil Red O staining and preparation of cells for RNA extraction were performed as described for the 3T3-L1 cells.

Statistical Analysis—All data are expressed as mean ± S.E. of 3–4 separate measurements unless otherwise stated in the figure legends. A one- or two-way analysis of variance (ANOVA) was used for multiple comparison procedures. A Tukey’s test was used for post hoc analysis of the significant ANOVA. A difference in mean values between groups was considered to be significant when p ≤ 0.05.

RESULTS

Using quantitative real time PCR analysis, we determined that murine chemerin mRNA was most highly expressed in
white adipose tissue, liver, and placenta with intermediate expression in the ovary (Fig. 1A). chemerin mRNA levels in other tissues were less than 5% of that in liver. Expression of CMKLR1 mRNA was highest in white adipose tissue, followed by intermediate levels in lung, heart and placenta (Fig. 1B). By comparison, CMKLR1 expression in other tissues was very low. The adipose organ consists of white and brown adipose, which is contained within various visceral and subcutaneous depots. The white adipose represents the vast majority of adipose tissue in the body and stores energy as triglyceride (38). Conversely, the brown adipose is specialized for energy dissipation through nonshivering thermogenesis (39). We found that chemerin and CMKLR1 were expressed to a similar degree in epididymal, perirenal and mesenteric (visceral), and inguinal (subcutaneous) white adipose tissue depots (Fig. 1C). In comparison, brown adipose (intrascapular depot) only expressed low levels of chemerin and CMKLR1 and suggests a primary function for chemerin and CMKLR1 in energy-storing white adipose tissue opposed to thermogenic brown adipose tissue. Within epididymal white adipose tissue, chemerin and CMKLR1 expression was enriched 2-fold in adipocytes compared with the stromal vascular fraction (Fig. 1D). Differential expression of the adipocyte markers leptin and adiponectin and stromal vascular expressed genes tnf-α and mac-1 confirmed effective separation of adipocytes from stromal vascular cells. Based on these initial gene expression data and previous observations that chemerin is a secreted protein (24, 26, 27), we proposed the hypothesis that white adipocytes are a source and target for chemerin signaling.

We tested this hypothesis using the well established 3T3-L1 adipocyte cell culture model (40) (supplemental Fig. 2). chemerin expression was lowest in undifferentiated cells but increased dramatically with adipocyte differentiation, and by day 13 was 60-fold higher as compared with undifferentiated cells (Fig. 2A). Similarly, CMKLR1 expression was lowest in undifferentiated 3T3-L1 cells but increased progressively to levels 300-fold higher in 13-day differentiated cells versus undifferentiated cells (Fig. 2B). Overall, chemerin and CMKLR1 exhibited a similar temporal pattern of expression to that of the established adipocyte differentiation markers PPAR and leptin (supplemental Fig. 2, D and E) (27, 29, 31). A protein corresponding to the active 16-kDa form of chemerin was detected by Western blotting of conditioned 3T3-L1 adipocyte media as early as day 5 after differentiation. Consistent with the mRNA levels, chemerin secretion increased with adipogenesis (Fig. 2, the reference tissue (expression = 1.0) to which all other tissues were compared. C, comparison of chemerin and CMKLR1 expression in visceral (epididymal, perirenal, and mesenteric) white adipose, subcutaneous (inguinal) white adipose, and brown adipose tissue depots. The epididymal adipose served as the reference (expression = 1.0) to which all other adipose depots were compared. *, p = 0.05 compared with chemerin or CMKLR1 expression in all white adipose tissue depots, ANOVA followed by Tukey’s HSD test. D, expression of chemerin and CMKLR1 mRNA, adipocyte marker genes (leptin and adiponectin) and stromal vascular marker genes (tnf-α and mac-1) were measured in adipocyte and stromal vascular fractions (SVF) of white adipose tissue (WAT). White adipose tissue served as the reference fraction (expression = 1.0) to which the adipocyte and stromal vascular fractions were compared. *, p = 0.05 compared with stromal vascular fractions, ANOVA followed Tukey’s HSD test. Each bar represents the mean ± S.E. of 3–4 samples (A, B, and D) or 8–9 samples pooled from two experiments (C).
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C and D). Histological analysis of mature adipocytes (day 8) with an anti-CMKLR1 antibody demonstrated intense immunoreactivity (Fig. 2E) localized to the cell periphery (white arrowheads). Counterstaining of cell nuclei with Hoechst 33258 confirmed that anti-CMKLR1 immunoreactivity was localized to the non-nuclear regions of these cells. Immunofluorescence was virtually undetectable in cells incubated with the IgG3 control antibody (Fig. 2F).

Although the differentiation-dependent expression and secretion of proteolytically processed chemerin strongly supported an adipokine-like function for this protein, it remained unresolved whether adipocyte-derived chemerin was biologically active. To address this question, we used an aequorin cell-based, reporter gene assay to measure activation of CMKLR1 (27). Both human (κm = 48 ± 12 pm) and mouse (κm = 119 ± 29 pm) chemerin were potent activators of the CMKLR1-aequorin reporter assay (supplemental Fig. 2F). Using this assay, activation of CMKLR1 by 3T3-L1 adipocyte-conditioned media was detected as early as 3 days after inducing differentiation and increased further with adipocyte maturation (Fig. 2G and supplemental Fig. 2G). This result was consistent with the differentiation-dependent expression of chemerin mRNA and secreted protein.

Chemerin stimulates chemotaxis of dendritic cells and macrophages that express CMKLR1 and may be responsible for recruitment of these cells to sites of inflammation (27, 30, 41). Using a chemotaxis chamber assay, we determined that media (1:100 dilution) from 13-day adipocytes produce a 4-fold higher migration of CMKLR1-expressing L1.2 cells as compared with pcDNA-transfected L1.2 cells (Fig. 2H). Migration of CMKLR1-expressing L1.2 cells toward adipocyte media were 3-fold higher than the basal migration toward preadipocyte media. Consistent with these data, recombinant mouse chemerin (0.1 to 1 nM) stimulated migration of CMKLR1-expressing L1.2 cells in a dose-dependent fashion but had no effect on empty vector pcDNA-transfected L1.2 cells (supplemental Fig. 2H). These observations confirmed the presence of functionally active chemerin in adipocyte cell culture media.

A number of adipokines act in a local autocrine/paracrine fashion to regulate adipogenesis and adipocyte metabolism (15, 42, 43). Although chemerin signaling pathways are not well established, CMKLR1 activation is reported to increase intracellular Ca2+ concentrations and phosphorylation of p42 (ERK2) and p44 (ERK1) MAPKs (27). This latter effect may be relevant to adipocyte function as ERK1/2 signaling is involved in adipogenesis and lipolysis pathways (44, 45). Thus, we used ERK1/2 phosphorylation to determine whether adipocytes were responsive to chemerin. Mouse chemerin (0.2 nM) applied to adipocytes transiently and reversibly stimulated (4–5-fold) ERK1/2 phosphorylation (Fig. 2F). At higher (1 or 10 nM) concentrations, chemerin produced a lower response and suggests desensitization and/or inhibition of signaling at higher concentrations. Nonphosphorylated ERK2 served as a loading control, and its expression was similar in all groups. These findings strongly support a potential local/autocrine signaling effect of adipocyte-derived chemerin.

Given the profound increase of chemerin secretion and CMKLR1 expression early in adipocyte differentiation, we
FIGURE 3. Knockdown of chemerin and CMKLR1 impairs differentiation of 3T3-L1 preadipocytes into adipocytes. Confluent preadipocytes were transduced with crude viral lysates that contained 100–1000 m.o.i. of mchemerin-shRNA (CE 100–CE 1000), mCMKLR1-shRNA (CR 100–CR 1000), or control LacZ-shRNA (LZ 100–LZ 1000) followed by the standard adipocyte differentiation protocol. UD and VEH represent undifferentiated preadipocytes and nontransduced preadipocytes, respectively. chemerin (A), CMKLR1 (B), and PPARγ (C) mRNAs were measured by QPCR on day 5 after inducing differentiation and expressed relative to VEH control. The relative mRNA expression is shown as the mean ± S.E. of 4 or 5 samples pooled from two separate experiments (A and C) or 7 samples pooled from three separate experiments (B). Representative Western blot analysis of chemerin protein (D) and determination of chemerin activity (E) by the aequorin bioassay in 24-h conditioned adipocyte media on day 5 post-differentiation. Representative Oil red O staining of neutral lipid (F) and phase contrast images (200) (G) were measured on day 8 after differentiation. Fibroblast cell, white arrowhead; lipid droplets, black arrow. Adiponectin levels in 24-h conditioned serum-free media from adipocyte 5-day post-differentiation (H). *, p < 0.05 compared with VEH or respective LacZ control; †, p < 0.05 compared with VEH, ANOVA followed by Tukey’s HSD test.
hypothesized that an autocrine/local function of this signaling pathway is to regulate adipocyte differentiation. To address this, confluent preadipocytes were transduced with adenoviral vectors expressing shRNA for chemerin (CE), CMKLR1 (CR), or LacZ (LZ; to control for nonspecific effects of viral transduction and shRNA expression) for a period of 24 h. After this time, differentiation media were added to the cells, and differentiation was allowed to proceed as normal. Consistent with our earlier experiments, chemerin and CMKLR1 expression were about 15-fold higher in the day 5 nontransduced vehicle (VEH) control cells compared with the undifferentiated cells. CE- or CR-shRNA transduction of preadipocytes produced a dose-dependent reduction of the mRNA level of the respective target genes (Fig. 3, A and B) as well as secretion of bioactive chemerin into media (Fig. 3, C and D) compared with VEH-treated or LZ-transduced cells. Expression of PPARγ (Fig. 3C) as well as Oil Red O staining of neutral lipid (Fig. 3F) measured 8 days after inducing differentiation was also markedly reduced by 1000 m.o.i. CE- and CR-shRNA treatments. Phase contrast images of live unstained cells taken at day 3–5 and 8 after inducing differentiation demonstrate the overall time course of cellular changes during the differentiation period (supplemental Fig. 3). Morphological changes produced by CMKLR1 and chemerin shRNA treatment were obvious by day 4 after inducing differentiation (supplemental Fig. 3). By day 8, it was readily apparent that cells treated with CE-shRNA remained primarily fibroblast-like (Fig. 3G, white arrow), whereas cells treated with CR-shRNA displayed a mixture of abnormally large cells with perinuclear lipid accumulation (black arrow) and fibroblast-like cells (white arrowhead). Adiponectin secretion into adipocyte media was also reduced by CE- and CR-shRNA treatment (Fig. 3H). By comparison, LZ-shRNA treatment did not affect any of these parameters, indicating that the adenoviral transduction alone did not affect the adipocyte differentiation program. Consistent with the abrogation of adipocyte differentiation, the expression of a number of genes was reduced by these treatments (Fig. 4). Chemerin and CMKLR1 knockdown decreased perilipin (60%), glucose transporter-4, (GLUT4; 80%), adiponectin (50–75%) and hormone-sensitive lipase (HSL; 40–60%) expression compared with vehicle and/or LZ control. Glycerol phosphate acetyltransferase, diacylglycerol 3-phosphate acetyltransferase-2 (DGAT2), and...
TNFα expression were unaffected by CMKLR1 or chemerin knockdown, although TNFα displayed a trend toward increased levels in the CR-shRNA treated cells. Fatty-acid synthase was significantly reduced by CE-shRNA treatment compared with the LZ-shRNA control. The finding that CE-shRNA treatment partially decreased CMKLR1 expression and

**Figure 5. Post-differentiation knockdown of chemerin does not alter adipocyte morphology.** Four days after initiating differentiation of preadipocytes, the cells were transduced with crude viral lysates that contained 300–3000 m.o.i. of CE-shRNA (CE 300–CE 3000), CR-shRNA (CR 300–CR 3000), or control LZ-shRNA (LZ 300–LZ 3000). UD and VEH represent undifferentiated preadipocytes and nontransduced preadipocytes, respectively. Analyses were performed on day 8 post-differentiation. chemerin (A), CMKLR1 (B), and PPARγ (C) mRNA were measured by QPCR and expressed relative to VEH control. The relative mRNA expression is shown as the mean ± S.E. of 6–7 replicates pooled from three experiments. Representative Western blot detection of chemerin protein (D) and chemerin activity (E) determination in 24-h conditioned adipocyte media. Representative Oil red O staining of neutral lipid (F) and phase contrast images of adipocytes (200) (G). The effect of CE- and CR-shRNA (1000 m.o.i.) treatment on adiponectin secretion into adipocyte media over a period of 24 h (H). *, p < 0.05 compared with VEH and respective LZ-shRNA controls, one-way ANOVA followed by Tukey’s HSD test.

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that CR-shRNA treatment produced a partial loss of chemerin expression and activity is also consistent with the impairment of adipocyte differentiation caused by these treatments. Overall, these findings indicated that chemerin/CMKLR1 signaling is critical very early in the adipocyte differentiation process.

To further investigate this early requirement for chemerin/CMKLR1 signaling, preadipocytes were incubated for 3 days in differentiation media followed by transduction with the adenoviral shRNA on day 4. When this post-differentiation protocol was followed, CE-shRNA or CR-shRNA reduced chemerin (≈97%) and CMKLR1 (≈85%) mRNA levels, respectively, compared with the nontransduced VEH or LZ-shRNA control (Fig. 5, A and B). Furthermore, chemerin knockdown at this stage did not alter CMKLR1 expression, and CMKLR1 knockdown did not alter chemerin expression. Western blot (Fig. 5D) and aequorin assay (Fig. 5E) for bioactive chemerin in adipocyte media on day 8 post-differentiation confirmed a complete loss of both chemerin protein and activity with CE-shRNA but not with CR-shRNA treatment. In contrast to pre-differentiation knockdown, post-differentiation reduction of chemerin or CMKLR1 expression had no overt effect on adipocyte differentiation or phenotype as indicated by PPARγ expression (Fig. 5C), neutral lipid accumulation (Fig. 5F), cell morphology (black arrows; Fig. 5G), or adiponectin secretion (Fig. 5H). Thus, for normal adipogenesis, there is an essential requirement for chemerin and CMKLR1 within but not after the first 3 days of the adipocyte differentiation process.

Although post-differentiation knockdown of chemerin and CMKLR1 had no overt effect on adipocyte phenotype, a number of adipocyte-expressed genes were differentially affected by these treatments (Fig. 6). Chemerin knockdown reduced perilipin, GLUT4, adiponectin, and leptin expression compared with VEH, LZ-shRNA, and CR-shRNA treated cells. GLUT4 and DGAT2 expression were higher with CMKLR1 knockdown as compared with the LZ controls. In comparison, HSL, GPAT, IL6, and TNFα were not affected by chemerin or CMKLR1 knockdown. Given the effects on a number of key adipocyte genes, we hypothesized that the chemerin-CMKLR1 pathway modulates the metabolic function of mature adipocytes. Post-differentiation knockdown of chemerin, but not CMKLR1, reduced basal lipolysis by 50–55% as measured by glycerol release into the adipocyte cell media (Fig. 7, A and B). Pretreatment with 0.2 or 1 nM chemerin for 24 h did not restore basal lipolysis in the chemerin knockdown cells nor did it alter basal lipolysis in the control, LZ-shRNA, and CR-shRNA treated cells (Fig. 7, A and B). Treatment with 2 μM isoproterenol stim-
The expression or secretion of a number of adipokines is dysregulated in obesity (18–22). Thus, we have examined if the expression of chemerin and CMKLR1 is altered in adipose tissue obtained from leptin-deficient (Lep<sup>ob/ob</sup>) obese mice as compared with lean wild-type mice. Chemerin expression in visceral and subcutaneous white adipose tissue was similar in 11-week-old lean and obese mice (Fig. 8A). In contrast, chemerin expression was up-regulated (5-fold) in the brown adipose tissue of the ob/ob mice. Furthermore, in ob/ob mice, the expression of chemerin in brown adipose tissue was now comparable with the level of that gene in visceral and subcutaneous white adipose tissue. In ob/ob compared with wild-type mice, CMKLR1 expression was similar in visceral and subcutaneous white adipose depots and was 3-fold higher in brown adipose tissue (Fig. 8B). In contrast to chemerin and CMKLR1, the expression of the brown adipose marker gene, uncoupling protein-1 (UCP-1) was reduced by 70% in the brown adipose of the ob/ob mice (Fig. 8C).

To determine whether white adipose expression and function of chemerin and CMKLR1 are conserved and relevant to humans, we performed gene expression profiling in human adipose tissues, preadipocytes, and adipocytes. Similar to our findings in mouse, chemerin and CMKLR1 were highly expressed in subcutaneous adipose tissue from two human donors (Fig. 9, A and B). Comparatively lower expression of chemerin was detected in human liver, ovarian carcinoma cells, hepatic carcinoma cells, and placenta but was not detectable in dendritic cells. Subcutaneous white adipose tissue, liver, and placenta had similar expression of CMKLR1 mRNA and were higher than expression in dendritic cells. In primary human adipocytes chemerin and CMKLR1 expression was increased 3- and 15-fold, respectively, as compared with preadipocytes (Fig. 9, C and D). Expression of the adipogenesis marker PPARγ was markedly increased (30-fold) in differentiated cells compared with preadipocytes (Fig. 9E). The differentiation-dependent increase of chemerin and CMKLR1 expression was qualitatively similar to that seen for mouse adipocytes supporting a conservation of function for mice and humans. Similar to our experiments in mouse adipocytes, we used ERK1/2 phosphorylation as a marker of CMKLR1 activation in human adipocytes. Treatment of human adipocytes with recombinant human chemerin increased (5-fold) phosphorylation of ERK1 and ERK2 MAPKs (Fig. 9F). The stimulatory effect was maximal with 1 nM chemerin, and nonphosphorylated ERK2 was similar in all treatment groups. Overall, these data support that expression of chemerin and CMKLR1 is conserved and relevant in human adipose tissue.

**DISCUSSION**

Here we provide the first report that white adipose tissue expresses high levels of chemerin and its cognate receptor CMKLR1 in the mouse. Consistent with these in vivo data, we report the novel finding that as 3T3-L1 cells mature into adipocytes, the cells express increasing amounts of chemerin and CMKLR1 mRNA and secrete greater amounts of bioactive chemerin. Taken together, these findings strongly suggest that white adipocytes serve as both a primary source of chemerin secretion as well as a target for autocrine/paracrine chemerin signaling. The data derived from our loss of function experiments confirm this and provide compelling evidence that a crit-
/events of wild-type mice served as the reference tissue (expression formed on log10 transformed sample data. The log10 transformation produced similar variances in each sample were compared. *, wild-type (epididymal) and subcutaneous (inguinal) white adipose tissue, and brown adipose tissue of 11-week-old

FIGURE 9. Human chemerin and CMKLR1 are highly expressed in human subcutaneous adipose tissue and primary adipocytes. The relative expressions of human chemerin (A) and CMKLR1 (B) mRNA were determined in liver, subcutaneous white adipose tissue (SC WAT) from two human donors, ovarian carcinoma cells (OVA), hepatoma (HepG2) cells, immature dendritic cells (DC), and placenta by real time quantitative PCR. The liver served as the reference tissue (expression = 1.0) to which all other tissues or cells were compared. ND, not detected. Expression of chemerin (C), CMKLR1 (D), and PPARγ (E) genes in 15-day differentiated human subcutaneous adipocytes relative to confluent preadipocytes expressed as mean ± S.E. of three samples. *, p < 0.05 compared with preadipocytes. The effect of recombinant human chemerin (hchem) on the phosphorylation of ERK1 and ERK2 MAPKs in primary human adipocytes was determined by Western analysis (F).

FIGURE 8. Modulation of white and brown adipose tissue expression of chemerin and CMKLR1 mRNA in the ob/ob mouse model of obesity. Relative expression of chemerin (A), CMKLR1 (B), and UCP-1 (C) in visceral (epididymal) and subcutaneous (inguinal) white adipose tissue, and brown adipose tissue of 11-week-old wild-type (WT) and obese (Lepob/ob) mice was determined by real time quantitative PCR. Visceral white adipose tissue of wild-type mice served as the reference tissue (expression = 1.0) to which all other adipose depots were compared. *, p ≤ 0.05 compared with the visceral and subcutaneous white adipose tissue; †, p < 0.05 compared with the brown adipose tissue of wild-type mice, ANOVA, followed Tukey’s HSD test. Because of the unequal variances in the mean CMKLR1 expression in brown versus white adipose tissue, ANOVA was performed on log10 transformed sample data. The log10 transformation produced similar variances in each sample group, which is a requirement for the ANOVA.

Previous studies identified the highest levels of chemerin and CMKLR1 expression in the liver and dendritic cells, respectively (27). As the study by Wittamer et al. (27) did not report adipose tissue expression of these genes, a complete comparative analysis with our data is not possible. However, a comparison of our data with published data for relative gene expression levels of identical tissues in mouse and human provides largely consistent results. For example, similar to our findings, previous studies identified liver among the tissues with highest and lowest expression of chemerin and CMKLR1, respectively (27).

Our finding of much higher expression of chemerin and CMKLR1 in white versus brown adipose tissue of lean mice is consistent with a role for chemerin and CMKLR1 in the development and/or function of the white adipocyte. This idea is further exemplified by our studies in ob/ob mice. For instance, brown adipocytes from leptin (ob/ob)- or leptin receptor (db/ db)-deficient mice develop structural (large, unilocular cells with diffuse mitochondrial organization) and molecular (increased leptin and reduced UCP-1 expression) features consistent with that of white adipocytes (46–49). As a result of these changes, ob/ob and db/db obese mice have a reduced capacity for thermogenesis (50, 51). In agreement with this switch in brown adipocyte phenotypes, we show a substantial reduction in UCP-1 expression levels in brown adipose tissue of ob/ob mice. The elevated expression of chemerin and CMKLR1 in brown adipose of ob/ob mice compared with lean controls is a compelling observation and may reflect or contribute to the conversion of brown adipocyte to white adipose phenotypes in the obese mice.

The conclusion that white adipocytes are a source of active chemerin is directly supported by our observations in 3T3-L1 mouse adipocytes. Importantly, the mature 16-kDa protein, but not the precursor 18-kDa protein, was detected in the media of adipocyte-conditioned media. This implies that adipocytes
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FIGURE 10. The role of chemerin and CMKLR1 in adipose tissue biology. Chemerin and the cognate receptor CMKLR1 are highly expressed in adipocytes (step 1). Chemerin is secreted either in the active form or rapidly activated by extracellular proteolytic processing (step 2). Our findings demonstrate that chemerin and CMKLR1 are required for optimal differentiation (step 3) and that both genes have modulatory effects on the expression of adipocyte genes involved in lipid and glucose metabolism (step 4). Furthermore, secreted chemerin may have a role in mediating recruitment (step 5) of CMKLR1-expressing cells (e.g. macrophages) to adipose tissue. The activation of intracellular ERK1/2 signaling (step 6) upon treatment of adipocytes with chemerin provides evidence for autocrine/paracrine action and is consistent with activation of CMKLR1. However, the presence of additional receptors or additional endogenous ligands for CMKLR1 cannot be ruled out at present. Given our findings in adipocytes, altering chemerin and CMKLR1 may have consequences for alterations in systemic metabolism and lipid homeostasis (step 7).

Many adipokines act in a local autocrine/paracrine fashion to regulate adipocyte differentiation and metabolism (15, 42, 43, 52, 53). The estimated media concentration (390 pM) of chemerin by day 3 post-differentiation was well above the $K_{m}$ value (114 pM) for CMKLR1 activation and was similar to the range of chemerin concentrations previously detected in plasma, serum, and human inflammatory fluids (27, 28). This indicates that secretion of physiologically relevant amounts of active chemerin occurs early in adipocyte differentiation. Our finding that CMKLR1 is highly expressed in mouse adipose tissue and exhibits differentiation-dependent expression in murine and human cultured adipocytes suggests that chemerin may also have autocrine/local actions on adipocytes. Given this temporal pattern of CMKLR1 expression and chemerin secretion, a likely autocrine/local function of this pathway is the regulation of signaling pathways involved in adipogenesis. Consistent with this hypothesis, knockdown of chemerin or CMKLR1 expression in preadipocytes severely impaired subsequent differentiation of those cells into adipocytes and reduced the expression of genes involved in glucose and lipid metabolism. A number of critical events occur within the first 72 h of adipocyte differentiation. Twenty four hours after inducing differentiation of 3T3-L1 cells with IBMX, dexamethasone, and insulin, cells undergo a clonal expansion consisting of 1–2 rounds of cell division prior to subsequent growth arrest and commitment to the adipocyte lineage (54, 55). Reinforcing cascades of early transcriptional regulators, including CEBPα, CEBPβ, CEBPδ, and PPARγ, are also required for adipocyte differentiation during this early critical phase. The finding that chemerin and CMKLR1 knockdown largely abrogates adipocyte differentiation when initiated prior to but not after (at day 4) the onset of differentiation indicates that chemerin/CMKLR1 signaling is essential early in the differentiation process and may contribute to or regulate critical early events in adipogenesis. As an increase in adipocyte cell number is an important process for increasing adipose tissue mass (56–58), our results indicate that chemerin and CMKLR1 could have an important biological role in the formation of white adipose tissue during normal development or in pathological states such as obesity.

Although knockdown of chemerin and CMKLR1 expression markedly reduces adipocyte differentiation, the highest expression of chemerin and CMKLR1 occurs in mature adipocytes. We have also observed that chemerin knockdown in the adipocyte maturation period resulted in lower expression of perilipin, GLUT4, adiponectin, and leptin expression in mature adipocytes. Thus, in addition to fulfilling a vital role in adipocyte differentiation, we hypothesized that this novel adipokine will modulate metabolic pathways in mature adipocytes. In line with this, the absence of chemerin expression resulted in a reduced basal lipolysis and IBMX-stimulated lipolysis rate. Interestingly, if adipocytes were exposed to elevated chemerin levels (1 nM), this could blunt the lipolytic response produced by the $\beta$-adrenergic agonist isoproterenol. Catecholamine stimulation of lipolysis involves signaling through a $\beta$-adrenergic receptor, $G_{s}$ protein, and adenylyl cyclase cascade (59). This results in increased intracellular cAMP and activation of cAMP-dependent protein kinase, which in turn phosphorylates and activates HSL, a key enzyme controlling the mobilization of fatty acids from triglycerides (59). Previous reports indicate that nanomolar concentrations of chemerin decrease intracellular cAMP (27). Thus chemerin could oppose the lipolytic action of catecholamines through the reduction of intracellular cAMP levels. IBMX, a PDE3 inhibitor, induces lipolysis by blocking the degradation of cAMP. The inability of chemerin to inhibit IBMX-stimulated lipolysis also suggests the antilipolytic mechanism lies at a point upstream of cAMP production.

Although both chemerin and CMKLR1 shRNA treatment of preadipocytes impaired subsequent adipocyte differentiation, these treatments also produced differences in cell morphology. Furthermore, post-differentiation knockdown of chemerin and CMKRL1 produced differential effects on gene expression and
lipolysis. A possible explanation for this discrepancy is that CMKLR1 has nonspecific effects on other surface receptors that affect adipocyte differentiation, gene expression, and metabolism. However, based on our findings we cannot rule out the possibility of additional adipocyte-derived ligands for CMKLR1 that have a functional impact in the absence of chemerin or the possibility of chemerin activity against related G protein–coupled receptors (60–62). The latter explanation is supported by the observation that chemerin inhibition of isoproterenol-stimulated lipolysis was not prevented by knockdown of CMKLR1 expression.

Several studies have indicated that the development of insulin resistance and type II diabetes in obesity begins with local adipokine responses (10, 14, 21, 23, 63). In this model, increased release of adipokines (e.g. leptin, TNFα, and CCL2) as well as free fatty acids from triglyceride-overloaded adipocytes stimulate macrophage infiltration and activation of a local inflammatory response. In a feed-forward system, activated macrophages release additional pro-inflammatory molecules that perpetuate the inflammatory response and impair adipocyte sensitivity to insulin (14, 63). We have demonstrated that CMKLR1 is highly expressed in the stromal vascular compartment of white adipose tissue and that adipocyte-cell culture media activated human CMKLR1 and stimulated migration of CMKLR1-expressing L1.2 cells. These observations, together with the finding that mouse macrophages express CMKLR1, bind chemerin, and migrate to the active ligand (41) suggest that adipocyte-derived chemerin could act as a paracrine regulator of recruitment of CMKLR1-expressing of immune cells to white adipose tissue as part of the local inflammatory response that coincides with the development of obesity.

Several studies support the idea that the endocrine/systemic actions of adipokines contribute to obesity-related diseases. For example, adipokines such as resistin, TNFα, and IL6 that promote insulin resistance are elevated in obese humans or in rodent models of obesity (7, 21, 64). Other adipokines such as adiponectin have anti-diabetic and anti-inflammatory actions to decrease muscle and liver triglyceride accumulation and increase insulin sensitivity in muscle (4, 5, 65–67). At variance with other adipokines, adiponectin is decreased in obese humans and rodents, and the administration or overexpression of this protein reverses insulin resistance in adiponectin knock-out mice (65, 66, 68). Considering the high level of expression of chemerin in adipocytes and the increased secretion of chemerin with adipocyte maturation, the adipose depot may represent a modifiable source of chemerin secretion that changes with adipose tissue mass. It will be of great interest to delineate the systemic actions of chemerin on inflammation and metabolic function in other tissues.

In summary, we have provided compelling evidence in support of the identification of chemerin as a novel adipokine. In particular, our finding that chemerin has a regulatory role in adipogenesis and adipocyte metabolism identifies the potential importance of this pathway in adipose tissue biology (Fig. 10). Thus, further characterization of the function of chemerin and CMKLR1 signaling in adipocytes has the potential to lead to novel therapeutic approaches for the treatment of obesity, type 2 diabetes, and cardiovascular disease.

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