Original Article

Expression and Function of Chemerin in Synovial Tissues of Patients with Rheumatoid Arthritis

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ABSTRACT

Background: Chemerin is an adipokine that stimulates chemotaxis of cells involved in the innate immune system. We evaluated the role of chemerin in inflammatory arthritides such as rheumatoid arthritis (RA) and osteoarthritis (OA).

Methods: Reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR were used to detect messenger ribonucleic acid (mRNA) for chemerin and chemerin receptor23 (ChemR23) in 20 patients with RA and 10 patients with OA. In addition, the effect of chemerin on matrix metalloproteinase (MMP) production by cultured synovial cells was assessed.

Results: Chemerin mRNA expression was higher in RA synovium than in OA synovium (3.01 ± 3.84 vs 1.00 ± 1.84, $p = 0.024$). ChemR23 mRNA expression was also higher in RA synovium than in OA synovium (2.43 ± 2.95 vs 1.00 ± 1.02, $p = 0.035$). Chemerin induced expression of MMP-1, MMP-3, and MMP-13 mRNA in RA synovium via ChemR23, but did not induce expression of MMP-2, tissue-inhibitor of metalloproteinases (TIMP)-1, or TIMP-2. Chemerin stimulated the extracellular signal-regulated kinase 1/2 (ERK 1/2) pathway via ChemR23. Chemerin-induced MMP-1/3 and MMP-13 expression was suppressed by an ERK1/2 inhibitor and an Akt inhibitor.

Conclusions: Chemerin is produced locally in patients with inflammatory arthritides such as RA, and ChemR23 is an important target of chemerin in synovial tissues of patients with RA. Activated chemerin has a proinflammatory effect in inflamed joints. These findings could lead to development of new therapeutic approaches for inflammatory arthritis.

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KEYWORDS: rheumatoid arthritis, chemerin, ChemR23, matrix metalloproteinase

Chemerin is also known as tazarotene-induced gene 2 (TIG2) and retinoic acid receptor responder 2 (RARRES2) and is a novel chemoattractant that is secreted as an 18-kDa inactive pro-protein. The predicted 371-amino-acid protein has 7 hydrophobic domains.[1)] Prochemerin undergoes extracellular cleavage of its C-terminal portion by serine proteases, yielding 16-kDa active chemerin, which has several isoforms.[1, 2)] Chemerin has been detected at high levels in psoriatic skin and in ascitic fluid from patients with ovarian cancer and liver cancer.[3, 4)] Under nor-

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mal physiologic conditions, chemerin circulates in its inactive form (prochemerin), which is activated through removal of amino acids at the C-terminus by proteases of the coagulation/fibrinolysis system or those derived from neutrophils.[5] An active chemerin isoform of 143 amino acids (chemerin 21-157) has been identified in hemofiltrate and ascites.[4]

Signaling by chemerin is mediated via a 7-transmembrane-spanning G protein-coupled receptor, which is known as chemokine-like receptor-1 (CMKLR1, ChemR23) or chemerinR. Expressions of chemerin and chemerinR messenger ribonucleic acid (mRNA) markedly increase during differentiation of preadipocytes into adipocytes.[6] The ChemR23 receptor is also expressed by professional antigen-presenting cells such as dendritic cells, natural killer cells, and macrophages.[7] Neutrophils are capable of promoting maturation of prochemerin to chemerin, which suggests that the chemerin/chemR23 signaling system is a bridge between innate and adaptive immunity.[8] Because ChemR23 is expressed by both myeloid dendritic cells and plasmacytoid dendritic cells, it may promote adaptive immunity after innate immunity.[9]

Chondrocytes from patients with severe osteoarthritis (OA) were recently shown to express ChemR23 and prochemerin transcripts. Chemerin binds to ChemR23 in chondrocytes, leading to increased phosphorylation of p44/42 mitogen-activated protein kinases (MAPKs) and Akt. Chemerin was also found to regulate production of proinflammatory cytokines and matrix metalloproteases (MMPs).[10] However, the role of chemerin derived from synovial cells has not been clearly evaluated in patients with rheumatoid arthritis (RA).

Chemerin seems to be predominantly produced by adipocytes, and the serum level of chemerin is higher in obese individuals.[11, 12] Chemerin synthesis is up-regulated by proinflammatory cytokines.[13] Chemerin may be produced by adipocytes, as well as by chondrocytes, synovial fibroblasts (SF), and macrophages in RA synovial joints. Chemerin may also be locally up-regulated in obesity and OA, but chemerin production is likely to be more abundant in synovial tissues of patients with RA. High chemerin levels in inflamed synovial joints (such as those of RA patients) may be related to inflammatory changes and cartilage degeneration. That is, chemerin/ChemR23 may regulate chemotaxis and cartilage degradation in synovial joints of patients with RA, in addition to contributing to inflammatory synovitis and joint destruction. However, the expression and role of chemerin and its receptor ChemR23 have not been carefully evaluated in RA patients.

We examined the distribution of chemerin/ChemR23 in synovial tissues and the level of chemerin in the synovial fluid of patients with RA and OA. In addition, the effect of chemerin/ChemR23 on cultured synovial cells was investigated.

Methods

1. Reagents

Interleukin (IL)-1β and tumor necrosis factor (TNF) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Anti-CD3 antibody, anti-CD3, anti-CD8 antibody, anti-CD68 antibody, and anti-von Willebrand factor (vWF) antibody were purchased from Dako Japan Inc. (Tokyo, Japan). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich Japan K. K. (Tokyo, Japan). A chemerin ELISA Kit was purchased from BioVendor GmbH (Heidelberg, Germany). Chemerin was obtained from R&D Systems Inc. A human pro-MMP-1 ELISA kit, total MMP-3 ELISA kit, and human pro-MMP-13 ELISA kit were obtained from R&D Systems Inc. The SensoLyte® 520 MMP Assay Kit was obtained from AnaSpec, Inc. (Fremont, CA, USA). Finally, U0126 [1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadine], was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA), and (−)-deguelin was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

2. Human materials

Patients and clinical samples

Synovial specimens were obtained from 20 patients with RA and 10 patients with OA who were treated at Toho University Omori Medical Center. All RA patients fulfilled the diagnostic criteria of the American College of Rheumatology.[14] OA was diagnosed according to clinical and radiologic criteria.[15] The female/male ratio was 16/4 for RA patients and 8/2 for OA patients. Mean age (±SD) was 57.2±13.3 years for the RA patients and 66.0±12.0 years for the OA patients. Samples of synovial membrane were obtained during surgery for treatment of joint disease, and all patients gave their informed consent for use of their samples in research. In both disease groups, the clinical characteristics of the patients were consistent with their diagnosis. The study protocol was approved by the ethics committee of Toho University (approval number: 19021), and all patients gave written consent for the use of their

tissues in this research.

3. Assessment of chemerin and ChemR23 mRNA expression in synovial tissues by reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR

Total RNA was isolated from synovial tissues by the guanidinium thiocyanate/phenol/chloroform method (Isogen Reagent Kit; Nippon Gene Co., Ltd., Tokyo, Japan). Then cDNA was synthesized from 1 μg of total RNA, using RAV2 reverse transcriptase and oligo (dT) primers (Takara Bio Inc., Otsu, Japan). Reverse transcription-polymerase chain reaction (RT-PCR) was performed to compare chemerin expression levels in cultured synovial cells. The primers for human chemerin (GenBank accession number: BC00069) were 5'-GAAGAAACCCGAG TGCAAAG-3' (sense: 294-313 bp) and 5'-CTTGGAGAAGG CGAACTGTC-3' (antisense: 522-503 bp), and the size of the PCR product was 228 bp. The primers for ChemR23 (GenBank accession number: Y14838) were 5'-ATGGAGGATG AAGATTACAACACT-3' (sense: 175-198 bp) and 5'-CAC AGAGATGCAGCGGTCAGAGC-3' (antisense: 591-569 bp), and the size of the PCR product was 416 bp. Human β-actin (GenBank accession number: M10277) was used as the internal control, with 2 primers (5-CCTCGCCTTTGCC GATCC-3' and 5'-GGATCTTCATGAGGTAGTCAGTC-3') yielding a PCR product of 457 bp. PCR was performed in a reaction mixture containing 25 μM of each primer, 2.5 mM of each dNTP, and 2.5 U of TaqDNA polymerase (Takara Bio Inc.). An automated DNA cycler (Takara Bio Inc.) was used for 40 cycles of denaturation at 95℃ for 30 s, annealing at 56℃ for 30 s, and extension at 72℃ for 45 s. The resulting PCR products were separated by electrophoresis on 2% (w/v) agarose gel and examined under ultraviolet light (using an LAS-3000; Fujifilm Corp., Tokyo, Japan) after staining of gels with ethidium bromide.

Real-time quantitative PCR was performed to compare chemerin and ChemR23 mRNA expression levels in synovial tissues of 20 patients with RA and 10 patients with OA. The primers for chemerin were 5'-TGAGGAGC ACCAGGAGAC-3' and 5'-TTGGAGAAGGCGAACTGTC-3', and the size of the product was 92 bp. The primers for ChemR23 (GenBank accession number: Y14838) were 5'-A AATATCCTGCTTCAACAACTTCA-3' (725-748 bp) and 5'-TGCCGGCTATACCCCACAG-3' (818-800 bp), and the size of the product was 95 bp.

Real-time PCR was done with a real-time PCR kit (qPCR Mastermix for SYBR® Green I; Eurogentec, SA., Liège, Belgium). Amplification was performed, and fluorescence of SYBR® Green dye was identified according to the standard protocol recommended by the manufacturer (2 min at 50℃, 10 min at 95℃, 40 cycles of 95℃ for 15 s each, and annealing for 1 min at 56.9℃ for chemerin and ChemR23). Samples were processed with an ABI Prism® 7700 Sequence Detection System (Applied Biosystems Japan Ltd., Tokyo, Japan), and the calculated cycle threshold (Ct) values were exported to Microsoft Excel. For comparisons between groups, relative mRNA levels were subsequently normalized relative to those found in the patients with OA, which were defined as reference levels (value = 1).

4. Immunohistochemistry for chemerin and surface markers

Synovial tissues were fixed with freshly prepared 4% paraformaldehyde in phosphate buffered saline (PBS). Sections (3 μm) were cut from the tissue blocks and stained with hematoxylin and eosin or examined by immunohistochemistry. Immunoperoxidase staining was done using a VECTASTATIN® Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). Sections were immersed in methanol containing 3% (v/v) H_2O_2 for 20 min to block endogenous peroxidase activity. Then the sections were preincubated with 0.3% (v/v) bovine serum albumin (Sigma-Aldrich Corp.) in PBS for 20 min, followed by incubation with diluted goat serum (1 : 100) for 20 min, after which sections were incubated for 1 h in a humid chamber with a polyclonal rabbit anti-human chemerin antibody (1 : 50 dilution; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). The sections were then washed twice with PBS and incubated for 30 min with biotinylated goat anti-rabbit IgG (1 : 2,000), after which they were again washed twice with PBS. Color was developed with 3,3'-diaminobenzidine (Dojindo Laboratories, Mashikimachi, Kumamoto, Japan).

5. Production of chemerin induced by IL-1β and effect of active chemerin on production of MMP-1, MMP-3, and MMP-13 in cultured synovial cells

RA and OA tissue specimens were obtained from patients who fulfilled the American College of Rheumatology criteria and were undergoing total knee replacement.[13, 14] Synovial tissues were washed and minced in PBS and digested with collagenase (Immuno-Biological Laboratories Co. Ltd., Fujioka, Japan). The cells were pelleted, resuspended in RPMI 1640 medium containing 5% fetal bovine serum (FBS), cultured in a 5% CO_2 incubator at 37℃, and treated with recombinant chemerin or IL-1β at several concentrations. Culture supernatants were harvested after treatment.

Human chemerin was measured in supernatants by using the ELISA kit (Bio Vendor GmbH). Human pro-MMP-1, total MMP-3, and pro-MMP-13 were measured in supernatants with ELISA kits (R&D Systems Inc.) according to the manufacturer's instructions. Total RNA was also isolated from cultured synovial cells using the guanidinium thiocyanate/phenol/chloroform method (Isogen Reagent Kit: Nippon Gene Co., Ltd.), and cDNA was synthesized from 2 μg of total RNA with RAV2 transcriptase and oligo (dT) primers (Takara Bio Inc.). The RT-PCR and real-time PCR primers were 5'-GAGCAAACACATCTGAGGTACAGG A-3' (sense 370-394 bp) and 5'-TTGTCCCGATGATCTCCC CT-3' (antisense 554-535 bp) for human MMP-1 (GenBank accession number: BC013875), 5'-AGATCTTCTTCTTCA AGGACC-3' (sense 1717-1733 bp) and 5'-GGCTGGTCAGT GGCTTGGGGTA-3' (antisense 1941-1920 bp) for human MMP-2 (GenBank accession number: BC002576), 5'-GGTG AGGACACCAGCATGA-3' (sense 76-94 bp) and 5'-TCCCT GGAAAGTCTTCAGC-3' (antisense 1298-1279 bp) for human MMP-3 (GenBank accession number: J03209), and 5'-G CTTAGAGGTGACTGGCAA-3' (sense 246-262 bp) and 5'-CCGGTGTAGGTGTAGATAGGA-3' (antisense 762-742 bp) for human MMP-13 (accession number: BC074808). The RT-PCR primers were 5'-ATCCTGTTGTTGCTGTGGCT GATAG-3 (sense 93-117 bp) and 5'-GGTGGTAACTCTTT ATTTCATGCTG-3' (antisense 782-759 bp) for TIMP-1 (GenBank accession number: X03124), and 5'-AAACGACA TTTATGGCAACCCTATC-3 (sense 444-468 bp) and 5'-A CAGGAGCCGTCACTTCTCTTGATG-5' (antisense 873-849 bp) for TIMP-2 (GenBank accession number: J05593). The amplified cDNA fragments were resolved by electrophoresis on 2% agarose gel and detected under ultraviolet light using an LAS-3000 after staining of the gel with ethidium bromide.

To evaluate expression of mRNAs for MMP-1, MMP-3, and MMP-13, real-time PCR TaqMan® technology was used with a Sequence Detection System (model 7000), according to the manufacturer's recommendations (Applied Biosystems Japan Ltd.). Cells were cultured under various conditions in medium containing 1% (v/v) FBS, and total RNA was extracted with an RNeasy Mini kit (Qiagen K.K., Tokyo, Japan). Synthesis of cDNA was then performed with a SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Corp., Carlsbad, CA, USA). Specific probes for MMP-1, MMP-3, and MMP-13 were obtained from the TaqMan® Gene Expression Assay (Applied Biosystems Japan Ltd.); the ID numbers were Hs00899658_m1 for MMP-1, Hs00968305_m1 for MMP-3, and Hs00233992_m1 for MMP-13. The threshold number of cycles was calculated from the standard curve, and expression of target mRNA was normalized for the expression of β-actin mRNA.

6. Detection of MMP-3 activity in supernatants of cultured synovial cells

We measured total MMP-3 activity in cultured supernatants using a fluorescence peptide (5-FAM/QXL™520 fluorescence resonance energy transfer peptide, SensoLyte® 520 MMP Assay Kit, AnaSpec, Inc.).[15, 16] Supernatants of cell culture media were collected and centrifuged for 10 min at 1000 g, and supernatants were stored at −70℃ until examination. Active MMP-3-containing medium was mixed with MMP-3 fluorescence resonance energy transfer (FRET) substrate and incubated for 1 h, as recommended by the manufacturer. Endpoint fluorescence signals were recorded at extension 490 nm/emission 520 nm by Multireader scan (Dainippon Sumitomo Pharma Co., LTD., Osaka, Japan). Fluorescence readings are expressed in relative fluorescence units (RFU), as recommended by the manufacturer.

7. Investigation of chemerin/ChemR23 signal transduction in synovial cells

Cells were plated in 24-well plates (1×10^5/well) and cultured for 18 h under various conditions in RPMI 1640 medium with 1% (v/v) FBS in a 5% CO_2 incubator. Experiments were performed using triplicate samples from each of 3 patients. Cells were treated with recombinant chemerin (300 ng/ml) and U0126 (a MAPK kinase inhibitor, 0.01 μM) or (−)-deguelin (an Akt inhibitor, 0.1 μM) in RPMI 1640 containing 10% FBS for 18 h. The signal inhibition substrate concentration was selected according to previous reports.[18, 19] After incubation, total RNA was harvested from the cells, and synthesis of cDNA was done as described above. Then the amplified cDNA fragments were resolved by electrophoresis on 2% agarose gel and detected under ultraviolet light after staining of the gel with ethidium bromide.

8. Statistical analysis

Results are expressed as means ± SD. Mean values were compared by the Mann-Whitney test and the Kruskal-Wallis test with post-hoc test. A p value less than 0.05 was considered to indicate statistical significance.

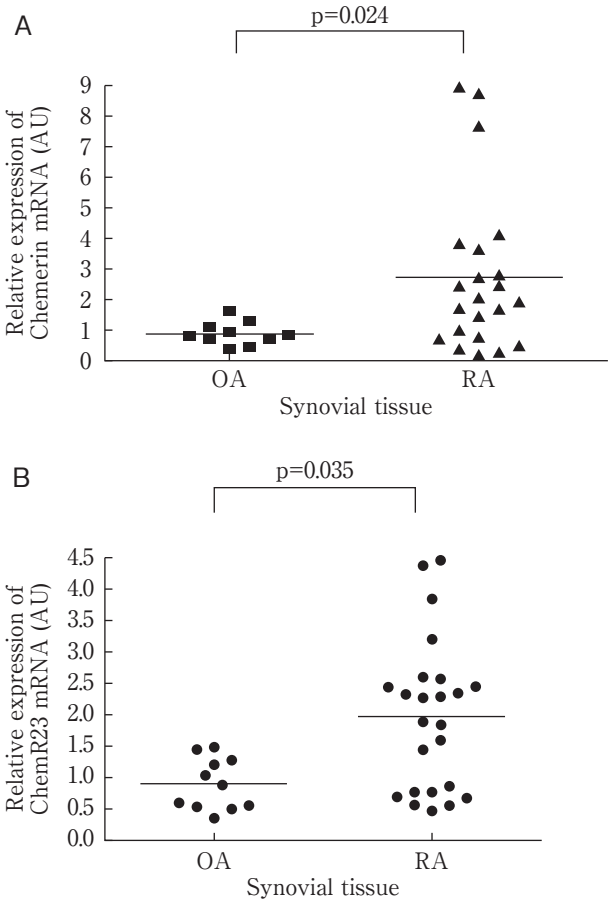

Fig. 1

A. Comparison of chemerin mRNA expression in synovial tissues from patients with RA and OA. Chemerin mRNA expression was significantly higher in RA synovium (3.17 ± 0.684) than in OA synovium (1.01 ± 0.26). Results are means ± standard deviation (SD) (n = 20 for RA, n = 10 for OA). mRNA levels are shown relative to the mean value for OA, which was defined as 1 (mean OA = 1).

B. ChemR23 mRNA expression was significantly higher in RA synovium than in OA synovium. Results are means ± SD (n = 20 for RA, n = 10 for OA). mRNA levels are shown relative to the mean value for OA, which was defined as 1.

mRNA: messenger ribonucleic acid, RA: rheumatoid arthritis, OA: osteoarthritis, ChemR23: chemerin receptor23

Results

1. Chemerin and ChemR23 mRNA expression in synovial tissue

We used real-time quantitative PCR to examine expression of chemerin and ChemR23 mRNA in synovial tissue from 20 patients with RA and 10 patients with OA. Fig. 1A shows higher chemerin mRNA expression in synovial tissues from RA patients than in those from OA patients.

Real-time quantitative PCR revealed 2.5-fold higher expression of ChemR23 mRNA in RA synovium (2.43 ± 2.95) than in OA synovium (1.01 ± 1.0, p = 0.035; Fig. 1B). Chemerin mRNA levels were also higher in RA synovium (3.01 ± 3.84) than in OA synovium (1.02 ± 1.84, p = 0.024; Fig. 1B).

2. Chemerin expression in synovial tissue of patients with RA and OA

We examined expression of chemerin protein in synovial tissue from patients with RA and OA. Chemerin was strongly expressed in the synovial lining (Fig. 2A), where endothelial cells, macrophages, and spindle-shaped fibroblasts all expressed chemerin (Fig. 2B). Macrophages were identified by staining with anti-CD68 antibody, and fibroblast-like cells were identified as spindle-shaped cells that showed negative staining for anti-CD68 and anti-CD3. Vascular endothelial cells were identified using anti-vWF antibody. Chemerin was only weakly expressed in OA tissues (Fig. 2C).

3. Chemerin expression in cultured synovial cells

Chemerin mRNA was detected in cultured synovial cells of patients with RA and OA and was expressed in unstimulated cultured synovial cells of RA and OA patients. IL-1, TNF, and LPS induced chemerin mRNA in synovial cells of patients with RA and OA (Fig. 3A). RT-PCR showed that chemerin mRNA expression was similar in cultured synovial cells from patients with RA and OA.

4. Effect of IL-1β on chemerin expression by cultured RA synovial cells

IL-1β dose-dependently induced chemerin and chemerin mRNA expression by synovial cells (Fig. 3B, C). Chemerin was also detected in cultured supernatants from TNF-stimulated synovial cells. In contrast, ChemR23 expression was not induced by IL-1β stimulation, and ChemR23 was constitutively expressed by cultured synovial cells (data not shown).

5. Effect of recombinant chemerin on MMP-1, MMP-3, and MMP-13 expression

We used RT-PCR to analyze the effect of recombinant chemerin on expression of MMP-1, MMP-2, and MMP-3 mRNA in cultured SF. Physiologic concentrations of chemerin (160 ng/ml and 320 ng/ml) induced MMP-1, MMP-3, and MMP-13 mRNA expression but not MMP-2, TIMP-1, or TIMP-2 expression (Fig. 4A, B). MMP-3 was only detected in culture supernatants of RA synovial cells stimulated by chemerin (Fig. 4C). MMP-1 and MMP-13 were not detected in culture supernatants. MMP-3 enzyme activity was also detected in culture supernatants

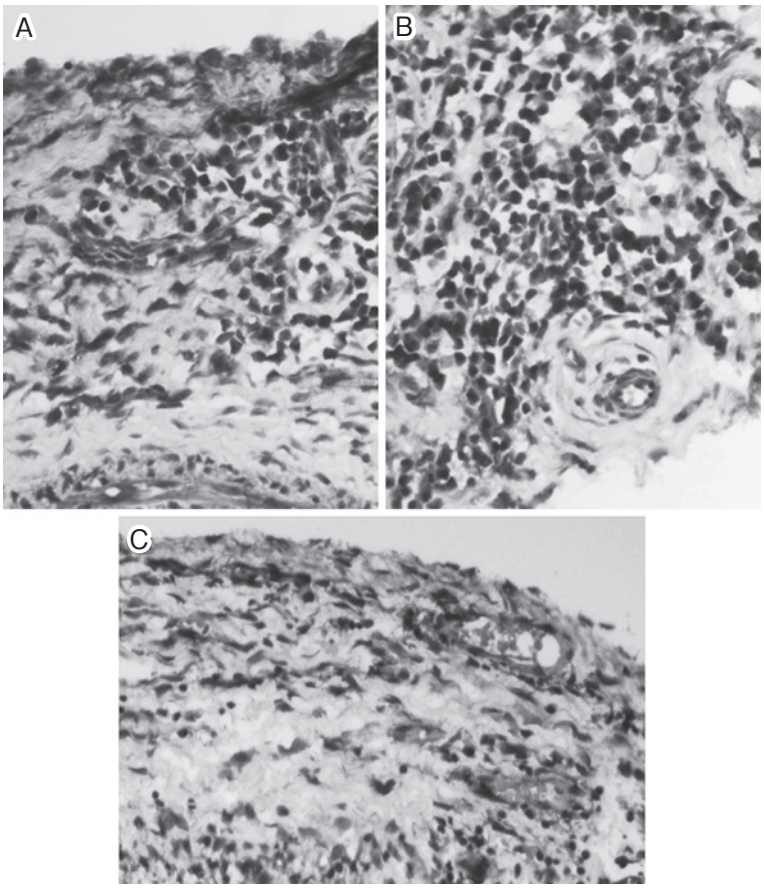

Fig. 2 Immunohistochemistry for chemerin protein in patients with RA and OA.
A. Chemerin is expressed in macrophages and fibroblast-like cells from the synovial lining layer of an RA patient. Hematoxylin and eosin (HE) stain ×200.
B. Chemerin is also expressed by synovial macrophages and fibroblast-like cells, and weakly expressed by vascular endothelial cells. HE stain ×200.
C. Expression of chemerin is weak in cells in the synovial lining of an OA patient. HE stain ×200.
RA: rheumatoid arthritis, OA: osteoarthritis

from RA synovial cells stimulated by IL-1β, and the chemerin MMP-3 activity of IL-1β-stimulated synovial cells (253 ± 43.5 RFU) and chemerin-stimulated synovial cells (168 ± 46.3 RFU) was significantly higher than that of control (138 ± 26.2 RFU) ($p = 0.018$ and $p = 0.049$, respectively; Fig. 4D). U-0126 (10 nM) and (−)-deguelin (100 nM) suppressed up-regulation of MMP-1, MMP-3, and MMP-13 mRNA by chemerin and IL-1β in synovial cells (Fig. 3E).

Discussion

In this study, we found that expression of chemerin and chemerin receptor (ChemR23) mRNA was higher in synovial tissues from RA patients than in those from OA patients. This finding confirms the results of 2 recently published reports,[20, 21] which found that the chemerin receptor, ChemR23, was expressed on synovial cells of patients with RA and that chemerin influenced production of MMPs. In addition, they used immunohistochemistry to show that chemerin was expressed in the synovial lining and in perivascular infiltrate.[20, 21] We used RT-PCR to quantitatively measure chemerin mRNA expression in synovial tissues of patients with RA and OA and detected chemerin in RA synovial tissues. The synovial fluid of patients with RA also contained chemerin.[4–13, 20–22] In addition, we detected a higher chemerin level in synovial fluids from RA patients than in those from OA patients (data not shown). Wittamer et al.[4] reported increased chemerin activity in synovial fluid from RA patients and no activity in synovial fluid from OA patients.

Chemerin is activated by several proteases. Protease ac-

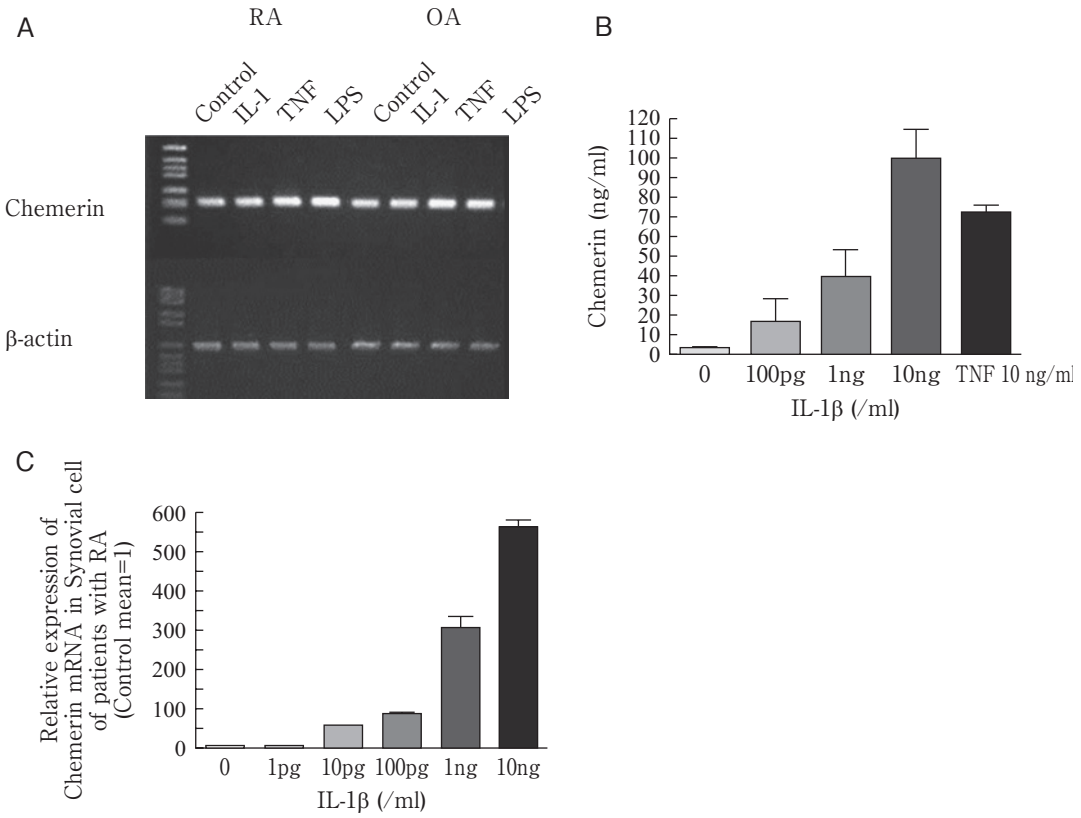

Fig. 3 IL-1β-induced synthesis of chemerin by SF from an RA patient

A. Chemerin mRNA was induced by IL-1β, TNF, and LPS in cultured synovial cells from RA and OA patients. Synovial cells from RA and OA patients were incubated for 18 h with vehicle, IL-1β 10 ng/ml, TNFα 10 ng/ml, or LPS 10 μg/ml. After incubation, total RNA were recovered from several synovial cells, and RT-PCR was performed to determine chemerin mRNA expression. The PCR product of human chemerin had a size of 228 bp. β-actin was used as the internal control and yielded the expected PCR product of 457 bp.

B. IL-1β-induced chemerin synthesis by RA SF.

C. IL-1β-induced chemerin mRNA expression in RA SF. IL-1β induced chemerin production in cultured RA SF. Synovial cells were incubated with vehicle or several concentrations of IL-1β for 18 h. After incubation, culture supernatants were recovered from synovial cells, and chemerin concentrations in the culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA). Complementary deoxyribonucleic acid (cDNA) was synthesized from total RNA separated from SF. Expression of chemerin mRNA was measured by real time PCR, as described in the Methods. Values are means ± standard deviation (SD) (n = 3 for 3 different patients).

SF: synovial fibroblasts, IL: interleukin, RA: rheumatoid arthritis, RNA: ribonucleic acid, mRNA: messenger RNA, TNF: tissue necrosis factor, LPS: lipopoly saccharide,OA: osteoarthritis, PCR: polymerase chain reaction, RT-PCR: reverse transcription-PCR

tivity is increased in highly inflammatory conditions, which suggests that chemerin activity is likely to be higher in RA than in OA.[20] We found that chemerin expression was higher in active inflammatory synovial tissues and in localized macrophages and fibroblastic cells in the synovial lining (Fig. 2A, B). In addition, vascular cells express chemerin.[20,21] Chemerin mRNA expression is increased in patients with inflammatory arthritis such as RA. IL-1 induced chemerin in cultured SF in the present study. TNF and interferon (INF) also induce chemerin protein,[20] and LPS induces chemerin expression in synovial cells. We found that induced levels of chemerin mRNA were similar between cultured synovial cells from patients with RA and OA, which suggests that chemerin level depends on the level of proinflammatory cytokines in RA synovial joints. High levels of several cytokines were observed in the joints of patients with inflammatory conditions such as RA.[22] Inflammatory stimuli may up-regulate

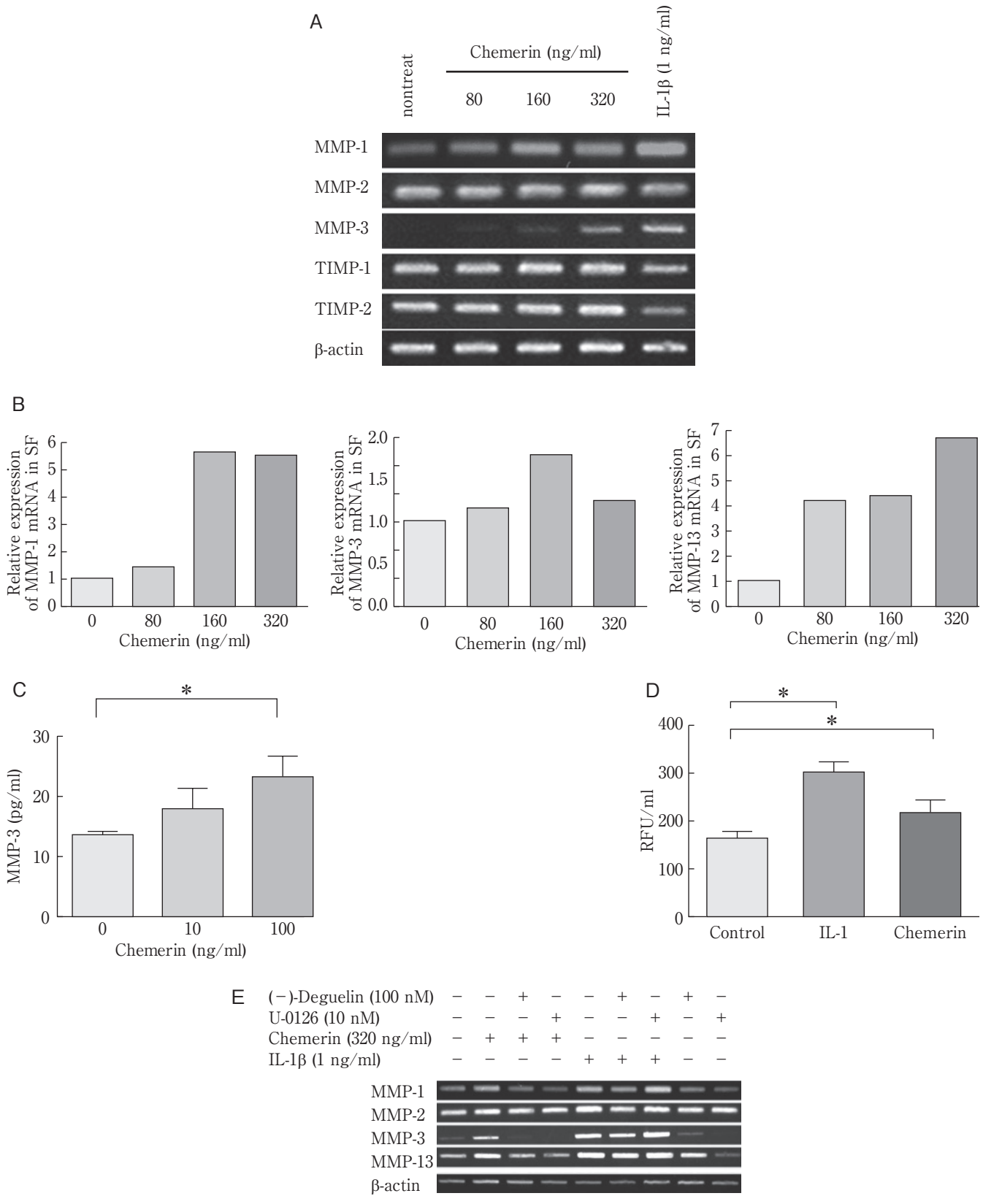
A
nontreat
Chemerin (ng/ml)
80
160
320
IL-1β (1 ng/ml)
MMP-1
MMP-2
MMP-3
TIMP-1
TIMP-2
β-actin
B
Relative expression of MMP-1 mRNA in SF
Chemerin (ng/ml)
Relative expression of MMP-3 mRNA in SF
Relative expression of MMP-13 mRNA in SF
C
MMP-3 (pg/ml)
D
RFU/ml
Control
IL-1
Chemerin
E
(−)-Deguelin (100 nM)
U-0126 (10 nM)
Chemerin (320 ng/ml)
IL-1β (1 ng/ml)
MMP-1
MMP-2
MMP-3
MMP-13
β-actin

Fig. 4

chemerin production in an inflammatory environment such as synovitis in RA.

Pro-inflammatory cytokines such as IL-1β, TNF, and INFγ induce chemerin expression in synovial cells of individuals with inflammatory arthritis such as RA,[20, 21] which suggests that chemerin is an inflammatory factor in synovial cells and contributes to persistent chronic inflammatory synovitis and joint destruction. On the other hand, the chemerin receptor, ChemR23, was constitutively expressed in synovial cells but was not induced by inflammatory cytokines such as TNFα.[20] The effects of chemerin may be regulated by induction of total chemerin synthesis and activation by several proteinases but not by receptor chemR23 expression. Chemerin is an adipokine produced by adipocytes[11] and is elevated in obesity and diabetes mellitus.[23] Chemerin synthesis is up-regulated in adipocytes by proinflammatory cytokines.[13–24] Systemic chemerin is higher in people who are obese and in those who have type 2 diabetes, and it may increase insulin resistance by contributing to insulin signaling abnormalities in adipocytes.[23, 24] Serum chemerin levels in obese individuals were correlated with levels of proinflammatory cytokines.[25, 26] In synovial tissues of RA patients, the principal origin of chemerin may be synovial macrophages and fibroblast-like cells rather than adipocytes, which secrete other adipokines in synovial fluid.[27] Moreover, because serum chemerin concentration did not differ between RA and OA patients and was not correlated with body mass index (unpublished observation), chemerin may be produced locally in inflammatory arthritis such as RA.

Recently, ChemR23 and chemerin transcripts were detected by immunohistochemistry in cultured chondrocytes, and MMP-1 and MMP-3 levels were significantly increased in supernatants of chondrocytes stimulated with recombinant chemerin.[10] MMPs have a key role in remodeling cartilage, and elevated MMP levels are closely related to cartilage damage in both RA and OA.[10] In our study, chemerin (derived from synovial tissues) induced MMP-1, MMP-3, and MMP-13 production by cells cultured from RA synovial tissues. Chemerin expression was higher in RA synovial tissue than in OA synovial tissue, and chemerin concentrations in synovial fluid were also higher in RA. Thus, chemerin derived from synovial tissues may contribute to synovitis and cartilage degradation in RA.

Chemerin induced several MMPs in synovial tissues.

Fig. 4 MMP and TIMP mRNA expression induced by recombinant human chemerin in synovial cells from RA patients.
A. MMP-1, MMP-2, MMP-3, MMP-13, TIMP-1, and TIMP-2 mRNA were detected by RT-PCR. Recombinant human chemerin induced MMP-1, MMP-3, and MMP-13 mRNA after 18 h of incubation.
B. MMP-1, MMP-3, and MMP-13 mRNA induction by human recombinant chemerin in synovial cells from RA patients. SF were incubated with varying doses of recombinant human chemerin (80, 180, or 360 ng/ml) for 18 h in RPMI 1640 medium with 10% FBS. After total RNA was extracted from the cells, complementaly deoxyribonucleic acid (cDNA) was synthesized, and reverse transcriptase-polymerase chain reaction (RT-PCR) for MMP-1, MMP-2, MMP-3, TIMP-1, and TIMP-2 was done as described in the Methods. Expression of target mRNAs was normalized for the expression of β-actin mRNA. All values are the means of duplicate results.
C. Induction of MMP-3 by recombinant human chemerin. SF from RA patients were incubated with chemerin (100 ng/ml) for 18 h. After incubation, culture supernatants were recovered and total MMP-3 concentrations were measured by specific enzyme-linked immunosorbent assay (ELISA). All values are means (n＝3 for 3 different patients). * $p<0.05$, stimulated vs unstimulated.
D. Induction of MMP-3 activity by recombinant human chemerin. SF from RA patients were incubated with rIL-1β (10 ng/ml) or chemerin (100 ng/ml) for 18 h. After incubation, culture supernatants were recovered, and total MMP-3 activity was measured using an fluorescence resonance energy transfer peptide assay. Values are means (n＝3 for 3 different patients). MMP-3 activity was expressed in relative fluorescence units (RFU), according to previous reported methods. * $p<0.05$, stimulated vs unstimulated.
E. Induction of MMP-1, MMP-3, and MMP-13 mRNA by chemerin was suppressed by (−)-deguelin (an Akt inhibitor) and by U0126 (an MAPK kinase inhibitor).
SF from RA patients were incubated with chemerin (320 ng/ml) or IL-1β mixed with an inhibitory substrate, namely, (−)-deguelin (100 nM) or U0126 (10 nM), for 18 h. MMP-1, MMP-2, MMP-3 and MMP-13 mRNA were then detected by RT-PCR as described in the Methods.
MMP: matrix metalloproteinase, TIMP: tissue-inhibitor of metalloproteinase, mRNA: messenger ribonucleic acid, RA: rheumatoid arthritis, RT-PCR: reverse transcription-polymerase chain reaction, SF: synovial fibroblasts, FBS: fetal bovine serum, IL: interleukin, rIL: recombinant IL, MAPK: mitogen-actibated protein kinase

MMPs may also contribute to damaging articular bone and cartilage in rheumatoid arthritis. Chemerin induced MMP-1, MMP-3, and MMP-13 mRNA in cultured synovial cells, and MMP-3 was only detected in culture supernatant induced by chemerin. IL-1β induces MMP-1 and MMP-13 in RA synovial cells.[28] The signal for chemerin-induced production of MMP-3 may be stronger than those for MMP-1 and MMP-13 in RA synovial cells.

Binding of chemerin to ChemR23 increases phosphorylation of p44/42 MAPKs and Akt, while the blocking of MEK-1/2 signaling prevents phosphorylation of p44/42 in chondrocytes.[10] In the present study, MMP-1 and MMP-3 production was blocked by an extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor and an Akt inhibitor of chemerin-induced production of MMPs. These signal transduction pathways are common to chondrocytes and synovial cells, but adipocyte signal transduction systems are different. Chemerin was found to increase basal phosphorylation of Akt in chondrocytes.[10] In addition, chemerin stimulated synovial cells but did not induce Akt phosphorylation.[20] IL-1β induced MMP-3 synthesis via modulation of the nuclear factor kappa-B (NF-κB) and c-Jun N-terminal kinase (JNK) pathways.[29] However, chemerin enhances activation of ERK1/2 and Akt, but not JNK1/2 and NF-κB, in the synthesis of MMP-3 in SF. Chemerin/chemR23 signaling is altered in inflammatory states and in people with metabolic abnormalities.

Chemerin has both inflammatory and anti-inflammatory functions. ChemR23 was reported to influence a receptor for the anti-inflammatory lipid mediator resolvin E_4.[30] In ChemR23 knockout mice, neutrophil infiltration was increased and acute lung injury was detected.[31] The present study found that ChemR23 acts as a receptor for several anti-inflammatory factors. However, it is unclear whether chemerin had anti-inflammatory effects in RA synovitis. More study of the anti-inflammatory effects of chemerin/ChemR23 in RA is warranted.

In conclusion, the present study demonstrated that chemerin was synthesized in synovial tissues of RA patients via the ERK1/2 pathway and bound to its specific receptor, ChemR23. Chemerin/ChemR23 then induced MMP production in synovial tissues of RA patients. These findings could lead to development of new therapies for patients with inflammatory arthritis.

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References

1) Meder W, Wendland M, Busmann A, et al.: Characterization of human circulating TIG2 as a ligand for the orphan receptor ChemR23. *FEBS Lett* **555**: 495–499, 2003
2) Yoshimura T, Oppenheim JJ: Chemerin reveals its chimeric nature. *J Exp Med* **205**: 2187–2190, 2008
3) Albanesi C, Scarponi C, Pallotta S, et al.: Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J Exp Med* **206**: 249–258, 2009
4) Wittamer V, Franssen JD, Vulcano M, et al.: Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. *J Exp Med* **198**: 977–985, 2003
5) Zabel BA, Allen SJ, Kulig P, et al.: Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. *J Biol Chem* **280**: 34661–34666, 2005
6) Yoshimura T, Oppenheim JJ: Chemokine-like receptor 1 (CMKLR1) and chemokine (C-C motif) receptor-like 2 (CCRL2); Two multifunctional receptors with unusual properties. *Exp Cell Res* **317**: 674–684, 2011
7) Parolini S, Santoro A, Marcenaro E, et al.: The role of chemerin in the colocalization of NK and dendritic cell subsets into inflamed tissues. *Blood* **109**: 3625–3632, 2007
8) Zabel BA, Silverio AM, Butcher EC: Chemokine-like receptor 1 expression and chemerin-directed chemotaxis distinguish plasmacytoid from myeloid dendritic cells in human blood. *J Immunol* **174**: 244–251, 2005
9) Wittamer V, Bondue B, Guillabert A, et al.: Neutrophil-mediated maturation of chemerin: A link between innate and adaptive immunity. *J Immunol* **175**: 487–493, 2005
10) Berg V, Sveinbjörnsson B, Bendiksen S, et al.: Human articular chondrocytes express ChemR23 and chemerin; ChemR23 promotes inflammatory signalling upon binding the ligand chemerin$^{21\text{-}157}$. *Arthritis Res Ther* **12**: R228, 2010
11) Goralski KB, McCarthy TC, Hanniman EA, et al.: Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. *J Biol Chem* **282**: 28175–28188, 2007
12) Lehrke M, Becker A, Greif M, et al.: Chemerin is associated with markers of inflammation and components of the metabolic syndrome but does not predict coronary atherosclerosis. *Eur J Endocrinol* **161**: 339–344, 2009
13) Kralisch S, Weise S, Sommer G, et al.: Interleukin-1β induces the novel adipokine chemerin in adipocytes *in vitro*. *Regul Pept* **154**: 102–106, 2009
14) Arnett FC, Edworthy SM, Bloch DA, et al.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* **31**: 315–324, 1988
15) Altman R, Asch E, Bloch D, et al.: Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. *Arthritis*

Rheum **29**: 1039–1049, 1986
16) Stryer L: Fluorescence energy transfer as a spectroscopic ruler. *Annu Rev Biochem* **47**: 819–846, 1978
17) Nagase H, Fields CG, Fields GB: Design and characterization of a fluorogenic substrate selectively hydrolyzed by stromelysin 1 (matrix metalloproteinase-3). *J Biol Chem* **269**: 20952–20957, 1994
18) Dell'Eva R, Ambrosini C, Minghelli S, et al.: The Akt inhibitor deguelin, is an angiopreventive agent also acting on the NF-κB pathway. *Carcinogenesis* **28**: 404–413, 2007
19) Choi YH, Gu YM, Oh JW, et al.: Osterix is regulated by Erk1/2 during osteoblast differentiation. *Biochem Biophys Res Commun* **415**: 472–478, 2011
20) Kaneko K, Miyabe Y, Takayasu A, et al.: Chemerin activates fibroblast-like synoviocytes in patients with rheumatoid arthritis. *Arthritis Res Ther* **13**: R158, 2011
21) Eisinger K, Bauer S, Schäffler A, et al.: Chemerin induces CCL2 and TLR4 in synovial fibroblasts of patients with rheumatoid arthritis and osteoarthritis. *Exp Mol Pathol* **92**: 90–96, 2012
22) Westacott CI, Whicher JT, Barnes IC, et al.: Synovial fluid concentration of five different cytokines in rheumatic diseases. *Ann Rheum Dis* **49**: 676–681, 1990
23) Hu W, Feng P: Elevated serum chemerin concentrations are associated with renal dysfunction in type 2 diabetic patients. *Diabetes Res Clin Pract* **91**: 159–163, 2011
24) Ernst MC, Sinal CJ: Chemerin: At the crossroads of inflammation and obesity. *Trends Endocrinol Metab* **21**: 660–667, 2010
25) Sell H, Divoux A, Poitou C, et al.: Chemerin correlates with markers for fatty liver in morbidly obese patients and strongly decreases after weight loss induced by bariatric surgery. *J Clin Endocrinol Metab* **95**: 2892–2896, 2010
26) Parlee SD, Ernst MC, Muruganandan S, et al.: Serum chemerin levels vary with time of day and are modified by obesity and tumor necrosis factor-α. *Endocrinology* **151**: 2590–2602, 2010
27) Schäffler A, Ehling A, Neumann E, et al.: Adipocytokines in synovial fluid. *JAMA* **290**: 1709–1710, 2003
28) Lee YA, Choi HM, Lee SH, et al.: Hypoxia differentially affects IL-1β-stimulated MMP-1 and MMP-13 expression of fibroblast-like synoviocytes in an HIF-1α-dependent manner. *Rheumatology (Oxford)* **51**: 443–450, 2012
29) Turner-Brannen E, Choi KY, Lippert DN, et al.: Modulation of interleukin-1β-induced inflammatory responses by a synthetic cationic innate defence regulator peptide, IDR-1002, in synovial fibroblasts. *Arthritis Res Ther* **13**: R129, 2011
30) Arita M, Ohira T, Sun YP, et al.: Resolvin E1 selectively interacts with leukotriene B_4 receptor BLT1 and ChemR23 to regulate inflammation. *J Immunol* **178**: 3912–3917, 2007
31) Luangsay S, Wittamer V, Bondue B, et al.: Mouse ChemR23 is expressed in dendritic cell subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. *J Immunol* **183**: 6489–6499, 2009

関節リウマチにおける Chemerin の発現と作用

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要約

目的：関節リウマチ（rheumatoid　arthritis：RA）滑膜病変におけるアディポカイン chemerin の発現と作用について検討した．

方法：RA および変形性関節症（osteoarthritis：OA）滑膜病変における chemerin と chemerin 受容体 chemerin receptor 23（ChemR23）の発現を real-timePCR および免疫組織化学にて測定した．また RA 培養滑膜細胞に interleukin-1（IL-1）を添加し chemerin 産生誘導と chemerin による滑膜細胞からの matrix metalloproteinase（MMP）の誘導を測定した．

結果：RA 滑膜組織は OA 滑膜組織と比較し chemerin，ChemR23 を強く発現していた．IL-1 は滑膜細胞から chemerin を濃度依存的に誘導した．Chemerin は滑膜細胞からの MMP-1,3,13 messenger ribonucleic acid（mRNA）を誘導し，この効果は extracellular signal-regulated kinase 1/2（ERK-1/2），Akt 阻害にて抑制された．

結論：Chemerin は RA 滑膜より産生誘導され，MMP 産生を介して RA 関節炎の病態形成に関与していると考えられた．

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