adiponectin mRNA were not detectable in human PBMCs.

Real-time PCR was performed to quantitatively evaluate resistin mRNA expression. This showed that stimulation by LPS increased resistin mRNA expression 17-fold ($p < 0.05$) compared with that in unstimulated human PBMCs, and this upregulation of resistin mRNA expression was reversed by dexamethasone in a dose-dependent manner (Fig. 2b).

Furthermore, we examined resistin protein levels in the culture supernatants by ELISA. Resistin protein was detected in the supernatants of unstimulated human PBMCs (0.17 ± 0.01 ng/mL), and its level was increased significantly by LPS stimulation (0.34 ± 0.02 ng/mL, $p < 0.01$). The increase in resistin secretion induced by LPS was inhibited by the addition of dexamethasone to cultures (Fig. 2c).

Discussion

To our knowledge, this is the first study to prospectively investigate the serum levels of 3 adipokines (resistin, leptin, and HMW-adiponectin) before and after glucocorticoid therapy in new patients with systemic autoimmune diseases in the active phase. Serum resistin levels were initially high in these patients and decreased towards normal after glucocorticoid therapy, while serum leptin and HMW-adiponectin levels were initially low and then increased after glucocorticoid therapy. We also found a significant association between the serum levels of resistin and Hs-CRP (an inflammatory marker) in these patients. Furthermore, our in vitro study revealed that dexamethasone inhibited LPS-induced upregulation of resistin mRNA and protein expression by human PBMCs in a concentration-dependent manner.

To evaluate the association of adipokines with inflammation, we performed multivariate analysis, which showed that the serum resistin level was significantly associated with serum Hs-CRP, along with the presence of autoimmune disease and older age. Serum CRP levels in patients with SLE [6] and PM/DM [7] are known to be low regardless of disease activity. In this study, same trends in the serum Hs-CRP levels in these diseases were noted. In addition, serum resistin levels among patients with SLE, vasculitis syndrome, PM/DM, and AOSD were significantly different, and those in patients with SLE and PM/DM tended to be lower than those in patients with vasculitis syndrome and AOSD. These data suggest that an increase in resistin might contribute to inflammation in patients with systemic autoimmune diseases.

Assessment of carotid plaque and IMT by ultrasonography provides a noninvasive and reliable measurement of

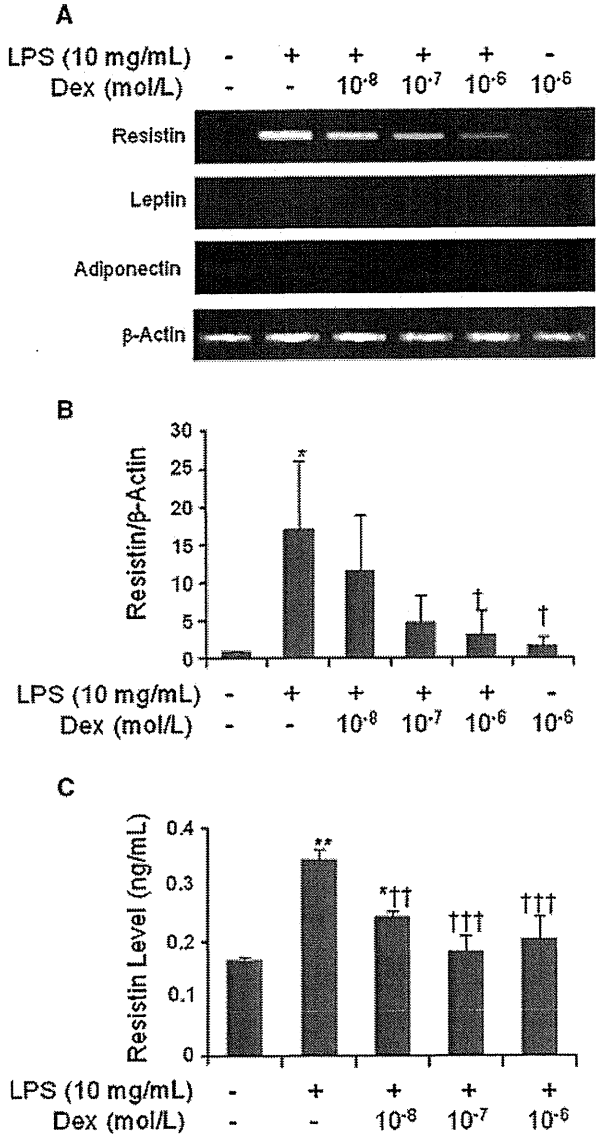

Fig. 2 Effects of lipopolysaccharides (LPS) and dexamethasone (Dex) on adipokine gene expression and secretion in human peripheral blood mononuclear cells (PBMCs). **a** Effects of LPS and Dex on adipokine mRNA gene expression in human PBMCs detected by reverse transcription-polymerase chain reaction (PCR) analysis. **b** Real-time-PCR study of resistin mRNA expression in human PBMCs. LPS-induced upregulation of resistin mRNA expression is inhibited by Dex in PBMCs. Resistin mRNA expression was normalized to that of β-actin mRNA. Fold induction was measured relative to mRNA expression by control PBMCs incubated without LPS or Dex. Data are presented as the mean ± SD ($n = 3$). **c** Resistin levels in culture supernatants of human PBMCs. LPS-induced upregulation of resistin secretion into culture supernatants is inhibited by Dex. Resistin was measured by enzyme-linked immunosorbent assay. Data are presented as the mean ± SD ($n = 3$). Significance was evaluated by ANOVA, followed by Bonferroni's post hoc analysis for pairwise comparison when the main effect of ANOVA was significant. $^{*}p < 0.05$; $^{**}p < 0.001$ compared with unstimulated cells; $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$; $^{\dagger\dagger\dagger}p < 0.001$ compared with LPS-stimulated cells

the systemic burden of atherosclerosis [8]. In our patients (mean age 58.3 years), there was a high prevalence of carotid artery plaque (68.2 %), a finding compatible with previous reports of a similar prevalence of carotid plaque in patients with SLE (mean age 52 years) [9] and patients with giant cell arteritis (mean age 79 years) [10]. The median IMT of our patients was also consistent with that for patients with SLE [9] and patients with giant cell arteritis [10].

Ross [11] suggested that chronic inflammation contributes to accelerated atherogenesis and plays a role in all stages of atherosclerosis. Cho et al. [12] demonstrated that resistin can accelerate the progression of atherosclerotic plaque by aggravating inflammation of the vessel wall through stimulating monocyte infiltration, and by activating of endothelial cells and smooth muscle cells. In the present study, we found a significant association between IMT and age or hypertension, well-known atherosclerotic risk factors, but we could not find any significant relationship between premature atherosclerosis and serum adipokine levels.

Resistin was originally identified as a 12.5 kDa polypeptide expressed and secreted by white adipose tissue [13]. Human adipocytes express resistin at very low levels, if at all, whereas high levels are expressed by PBMCs (especially monocytes and T lymphocytes), macrophages, neutrophils, and bone marrow cells that take part in the inflammatory response [14–17]. We found high serum resistin levels in our patients with active disease, suggesting an association of resistin with the inflammatory processes related to autoimmune diseases. Previous cross-sectional studies suggested that serum resistin levels were not increased in SLE patients compared with those in control subjects [18–20]. In this study, however, the serum resistin level of 18 patients with SLE was significantly higher than that of healthy controls, and the high resistin level of the patients declined after glucocorticoid therapy. We recently reported that serum resistin levels were elevated in patients with Kawasaki disease. They were decreased by the intravenous administration of immune globulin [21]. Therefore, the discrepancy between our findings and previous reports might be due to differences in disease activity or treatment, since most of the subjects enrolled in the previous studies were outpatients who were already on immunosuppressive therapy, including glucocorticoids.

We also investigated the association of resistin with inflammation by performing an in vitro experiment using human PBMCs, since they contain monocytes and T lymphocytes, which are reported to produce resistin [17]. Our study showed that LPS upregulated the expression of resistin mRNA and protein in human PBMCs. Such findings may help to explain hyperresistinemia in our patients with acute inflammation. Induction of resistin by LPS may be partly mediated by the activation of nuclear factor-κB (NF-κB), activator protein 1 (AP-1), or mitogen-activated protein kinase (MAPK) [22, 23]. Although the significance of hyperresistinemia in patients with autoimmune diseases has not yet been clarified, Tarkowski et al. [24] reported that resistin induced the production of proinflammatory cytokines such as IL-6 and IL-8 by interacting with Toll-like receptor 4 (TLR4). Thus, it is possible that resistin plays an important role in inflammation associated with systemic autoimmune diseases.

In the present study, we demonstrated that resistin expression was downregulated by glucocorticoids both in vivo and in vitro. The promoter region of the human resistin gene contains binding sites for proinflammatory transcription factors such as cRel (one of 5 NF-κB subunits) and AP-1, but has no glucocorticoid response elements (GREs) [25]. Accordingly, glucocorticoid therapy might inhibit resistin expression by repressing the activation of these transcription factors through the glucocorticoid receptor [26].

Previous cross-sectional studies of serum leptin levels in patients with SLE have not obtained definitive results. Some studies have shown that leptin levels were higher in patients than in healthy control subjects [18, 20, 27–29], while others have found that leptin levels did not differ between patients and healthy controls [30]. On the other hand, our prospective study demonstrated that serum leptin levels were lower in patients with active autoimmune diseases than in healthy controls, and that the leptin levels of the patients increased after glucocorticoid therapy. Kümpers et al. [31] performed a prospective study of serum leptin levels in patients with antineutrophilic cytoplasmic antibody (ANCA)-associated vasculitis receiving glucocorticoid therapy. They found lower leptin levels in the ANCA-associated vasculitis patients than in healthy controls, and there was an increase in leptin after glucocorticoid therapy, consistent with our results.

In this study, we found low leptin levels in patients with active autoimmune diseases. It has been reported that long-term exposure to proinflammatory cytokines such as IL-1β or TNFα cause suppression of leptin protein and gene expression in adipose tissue [32–34], while acute stimulation by proinflammatory cytokines increases leptin release in vitro [32, 33]. Serum leptin levels were high in some of our patients but low in most of them. Thus, it is possible that these variations in serum leptin levels were caused by different inflammatory states.

Previous studies have found that glucocoricoids increase the serum leptin level in healthy humans [35, 36]. It was also reported that glucocorticoids upregulate leptin expression by cultured human adipose tissue [37]. These results support our finding that serum leptin levels

increased after glucocorticoid therapy in patients with systemic autoimmune diseases. It has been demonstrated that the human *obese* (*ob*) gene, the product of which is leptin, contains GREs in the promoter region [38], suggesting that glucocorticoids may activate *ob* gene transcription by interacting with these GREs.

In this study, the baseline serum HMW-adiponectin level (before glucocorticoid therapy) was lower in our patients with autoimmune diseases than in the healthy control subjects. However, previous cross-sectional studies of the serum total adiponectin level in SLE patients have yielded conflicting results, and some studies have indicated that serum adiponectin levels were higher in SLE patients than in healthy controls [18, 19, 28]. In the present study, the serum HMW-adiponectin level in the 18 patients with SLE was significantly lower than that in the healthy controls. The discrepancy between this finding and previous reports might be explained by differences in disease activity or therapy, as was discussed for resistin. Furthermore, total adiponectin (containing different isoforms) was measured in previous studies [18, 19, 28], whereas we measured the HMW fraction of adiponectin. It is known that HMW-adiponectin is the more active and clinically relevant form of this protein [39, 40]. The serum HMW-adiponectin level in male patients tended to be higher than that in healthy controls. Since multiple regression analysis in this study showed that the serum HMW-adiponectin level was positively associated with age, this trend might be explained by the increased age of the male patients.

Bruun et al. [41] have reported the reciprocal suppression of adiponectin and proinflammatory cytokines (such as TNFα and IL-6) in cultured human adipose tissue, and they also noted the same effect for serum levels in vivo. Therefore, an increase in proinflammatory cytokines might have suppressed adiponectin production in our patients with active autoimmune diseases. Adiponectin may have a protective effect against atherosclerotic vascular changes [42]. Systemic autoimmune diseases are known to be associated with an increase in premature atherosclerosis and with increased mortality due to cardiovascular diseases [8, 43, 44]. Thus, hypoadiponectinemia might be an important cardiovascular risk factor in patients with systemic autoimmune diseases.

In this study, serum HMW-adiponectin levels were found to be increased after glucocorticoid therapy. Both adiponectin and HMW-adiponectin levels have previously been reported to increase with oral glucocorticoid therapy in healthy subjects [45] and patients with type 2 diabetes [46]. Adiponectin and proinflammatory cytokines suppress each other [41], while glucocorticoids inhibit the production of proinflammatory cytokines such as TNFα [47]. Thus, the reduction in proinflammatory cytokine levels resulting from glucocorticoid therapy may have led to an increase in HMW-adiponectin in our patients with systemic autoimmune diseases.

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Conflict of interest None.

References

1. Lago F, Dieguez C, Gómez-Reino J, Gualillo O. Adipokines as emerging mediators of immune response and inflammation. Nat Clin Pract Rheumatol. 2007;3:716–24.
2. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. Nature. 2006;444:847–53.
3. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem. 1972; 18:499–502.
4. Kuzuya T, Nakagawa S, Satoh J, Kanazawa Y, Iwamoto Y, Kobayashi M, et al. Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. Diabetes Res Clin Pract. 2002;55:65–85.
5. Kumeda Y, Inaba M, Goto H, Nagata M, Henmi Y, Furumitsu Y, et al. Increased thickness of the arterial intima-media detected by ultrasonography in patients with rheumatoid arthritis. Arthritis Rheum. 2002;46:1489–97.
6. Bertouch JV, Roberts-Thompson PJ, Feng PH, Bradley J. C-reactive protein and serological indices of disease activity in systemic lupus erythematosus. Ann Rheum Dis. 1983;42:655–8.
7. Gabay C, Gay-Croisier F, Roux-Lombard P, Meyer O, Maineti C, Guerne PA, et al. Elevated serum levels of interleukin-1 receptor antagonist in polymyositis/dermatomyositis. A biologic marker of disease activity with a possible role in the lack of acute-phase protein response. Arthritis Rheum. 1994;37:1744–51.
8. Salmon JE, Roman MJ. Subclinical atherosclerosis in rheumatoid arthritis and systemic lupus erythematosus. Am J Med. 2008;121: S3–8.
9. Svenungsson E, Jensen-Urstad K, Heimbürger M, Silveira A, Hamsten A, de Faire U, et al. Risk factors for cardiovascular disease in systemic lupus erythematosus. Circulation. 2001;104: 1887–93.
10. Gonzalez-Juanatey C, Lopez-Diaz MJ, Martin J, Llorca J, Gonzalez-Gay MA. Atherosclerosis in patients with biopsy-proven giant cell arteritis. Arthritis Rheum. 2007;57:1481–6.
11. Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med. 1999;340:115–26.
12. Cho Y, Lee SE, Lee HC, Hur J, Lee S, Youn SW, et al. Adipokine resistin is a key player to modulate monocytes, endothelial cells, and smooth muscle cells, leading to progression of atherosclerosis in rabbit carotid artery. J Am Coll Cardiol. 2010;57:99–109.
13. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, et al. The hormone resistin links obesity to diabetes. Nature. 2001;409:307–12.
14. Patel L, Buckels AC, Kinghorn IJ, Murdock PR, Holbrook JD, Plumpton C, et al. Resistin is expressed in human macrophages

and directly regulated by PPAR gamma activators. Biochem Biophys Res Commun. 2003;300:472–6.
15. Nagaev I, Smith U. Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. Biochem Biophys Res Commun. 2001;285:561–4.
16. Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV, et al. Resistin/Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. Diabetes. 2001;50:2199–202.
17. Kunnari AM, Savolainen ER, Ukkola OH, Kesäniemi YA, Jokela MA. The expression of human resistin in different leucocyte lineages is modulated by LPS and TNFalpha. Regul Pept. 2009; 157:57–63.
18. Chung CP, Long AG, Solus JF, Rho YH, Oeser A, Raggi P, et al. Adipocytokines in systemic lupus erythematosus: relationship to inflammation, insulin resistance and coronary atherosclerosis. Lupus. 2009;18:799–806.
19. De Sanctis JB, Zabaleta M, Bianco NE, Garmendia JV, Rivas L. Serum adipokine levels in patients with systemic lupus erythematosus. Autoimmunity. 2009;42:272–4.
20. Vadacca M, Margiotta D, Rigon A, Cacciapaglia F, Coppolino G, Amoroso A, et al. Adipokines and systemic lupus erythematosus: relationship with metabolic syndrome and cardiovascular disease risk factors. J Rheumatol. 2009;36:295–7.
21. Kemmotsu Y, Saji T, Kusunoki N, Tanaka N, Nishimura C, Ishiguro A, et al. Serum adipokine profiles in Kawasaki disease. Mod Rheumatol. 2012;22:66–72.
22. Lehrke M, Reilly MP, Millington SC, Iqbal N, Rader DJ, Lazar MA. An inflammatory cascade leading to hyperresistinemia in humans. PLoS Med. 2004;1:e45.
23. Shyu KG, Chua SK, Wang BW, Kuan P. Mechanism of inhibitory effect of atorvastatin on resistin expression induced by tumor necrosis factor-alpha in macrophages. J Biomed Sci. 2009;16:50.
24. Tarkowski A, Bjersing J, Shestakov A, Bokarewa MI. Resistin competes with lipopolysaccharide for binding to Toll-like receptor 4. J Cell Mol Med. 2010;14:1419–31.
25. Singh AK, Battu A, Mohareer K, Hasnain SE, Ehtesham NZ. Transcription of human resistin gene involves an interaction of Sp1 with peroxisome proliferator-activating receptor gamma (PPARgamma). PLoS One. 2010;5:e9912.
26. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids—new mechanisms for old drugs. N Engl J Med. 2005;353: 1711–23.
27. Al M, Ng L, Tyrrell P, Bargman J, Bradley T, Silverman E. Adipokines as novel biomarkers in paediatric systemic lupus erythematosus. Rheumatology (Oxf). 2009;48:497–501.
28. Sada KE, Yamasaki Y, Maruyama M, Sugiyama H, Yamamura M, Maeshima Y, et al. Altered levels of adipocytokines in association with insulin resistance in patients with systemic lupus erythematosus. J Rheumatol. 2006;33:1545–52.
29. Garcia-Gonzalez A, Gonzalez-Lopez L, Valera-Gonzalez IC, Cardona-Muñoz EG, Salazar-Paramo M, González-Ortiz M, et al. Serum leptin levels in women with systemic lupus erythematosus. Rheumatol Int. 2002;22:138–41.
30. Wisłowska M, Rok M, Stepień K, Kuklo-Kowalska A. Serum leptin in systemic lupus erythematosus. Rheumatol Int. 2008;28: 467–73.
31. Kümpers P, Horn R, Brabant G, Woywodt A, Schiffer M, Haller H, et al. Serum leptin and ghrelin correlate with disease activity in ANCA-associated vasculitis. Rheumatology (Oxf). 2008;47: 484–7.
32. Bruun JM, Pedersen SB, Kristensen K, Richelsen B. Effects of pro-inflammatory cytokines and chemokines on leptin production in human adipose tissue in vitro. Mol Cell Endocrinol. 2002; 190(1–2):91–9.
33. Zhang HH, Kumar S, Barnett AH, Eggo MC. Tumour necrosis factor-alpha exerts dual effects on human adipose leptin synthesis and release. Mol Cell Endocrinol. 2000;159:79–88.
34. Yamaguchi M, Murakami T, Tomimatsu T, Nishio Y, Mitsuda N, Kanzaki T, et al. Autocrine inhibition of leptin production by tumor necrosis factor-alpha (TNF-alpha) through TNF-alpha type-I receptor in vitro. Biochem Biophys Res Commun. 1998; 244:30–4.
35. Papaspyrou-Rao S, Schneider SH, Petersen RN, Fried SK. Dexamethasone increases leptin expression in humans in vivo. J Clin Endocrinol Metab. 1997;82:1635–7.
36. Newcomer JW, Selke G, Melson AK, Gross J, Vogler GP, Dagogo-Jack S. Dose-dependent cortisol-induced increases in plasma leptin concentration in healthy humans. Arch Gen Psychiatry. 1998;55:995–1000.
37. Masuzaki H, Ogawa Y, Hosoda K, Miyawaki T, Hanaoka I, Hiraoka J, et al. Glucocorticoid regulation of leptin synthesis and secretion in humans: elevated plasma leptin levels in Cushing's syndrome. J Clin Endocrinol Metab. 1997;82:2542–7.
38. Gong DW, Bi S, Pratley RE, Weintraub BD. Genomic structure and promoter analysis of the human obese gene. J Biol Chem. 1996;271:3971–4.
39. Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. J Clin Invest. 2006;116: 1784–92.
40. Haugen F, Drevon CA. Activation of nuclear factor-kappaB by high molecular weight and globular adiponectin. Endocrinology. 2007;148:5478–86.
41. Bruun JM, Lihn AS, Verdich C, Pedersen SB, Toubro S, Astrup A, et al. Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. Am J Physiol Endocrinol Metab. 2003;285:E527–33.
42. Matsuzawa Y, Funahashi T, Kihara S, Shimomura I. Adiponectin and metabolic syndrome. Arterioscler Thromb Vasc Biol. 2004; 24:29–33.
43. Tervaert JW. Translational mini-review series on immunology of vascular disease: accelerated atherosclerosis in vasculitis. Clin Exp Immunol. 2009;156:377–85.
44. Tyrrell PN, Beyene J, Feldman BM, McCrindle BW, Silverman ED, Bradley TJ. Rheumatic disease and carotid intima-media thickness: a systematic review and meta-analysis. Arterioscler Thromb Vasc Biol. 2010;30:1014–26.
45. Rieth N, Jollin L, Le Panse B, Lecoq AM, Arlettaz A, De Ceaurriz J, et al. Effects of short-term corticoid ingestion on food intake and adipokines in healthy recreationally trained men. Eur J Appl Physiol. 2009;105:309–13.
46. Jang C, Inder WJ, Obeyesekere VR, Alford FP. Adiponectin, skeletal muscle adiponectin receptor expression and insulin resistance following dexamethasone. Clin Endocrinol (Oxf). 2008;69:745–50.
47. Smoak K, Cidlowski JA. Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory ignaling. Mol Cell Biol. 2006;26:9126–35.

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.[6]

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.

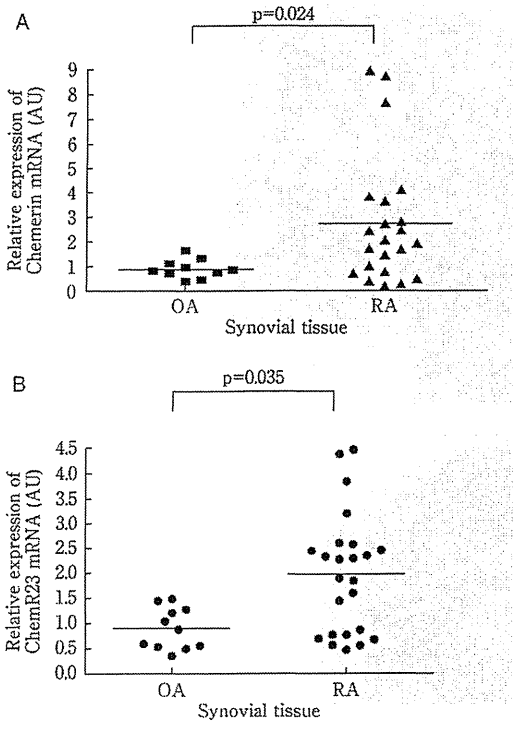

Fig. 1

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

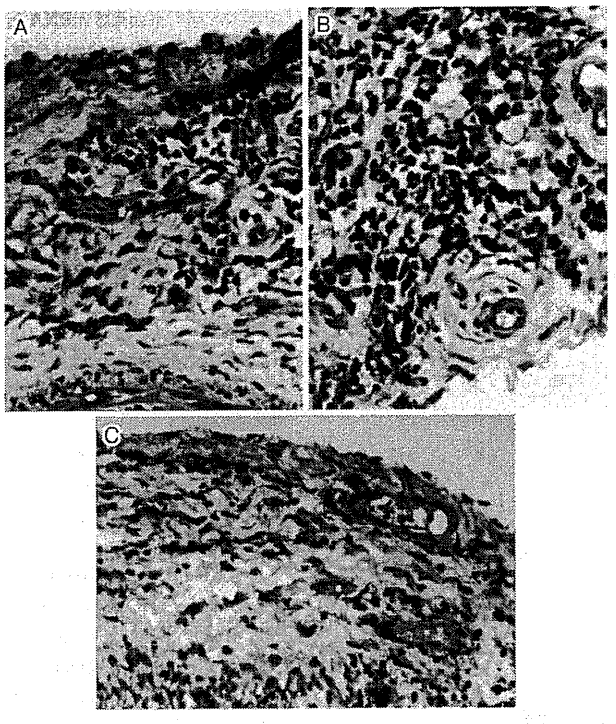

Fig. 2 Immunohistochemistry for chemerin protein in patients with RA and OA.
A. Chemerin is expressed in macrophages and fibroblast-like cells from the synovial lining layer of an RA patient. Hematoxylin and eosin (HE) stain ×200.
B. Chemerin is also expressed by synovial macrophages and fibroblast-like 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.[9] 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-

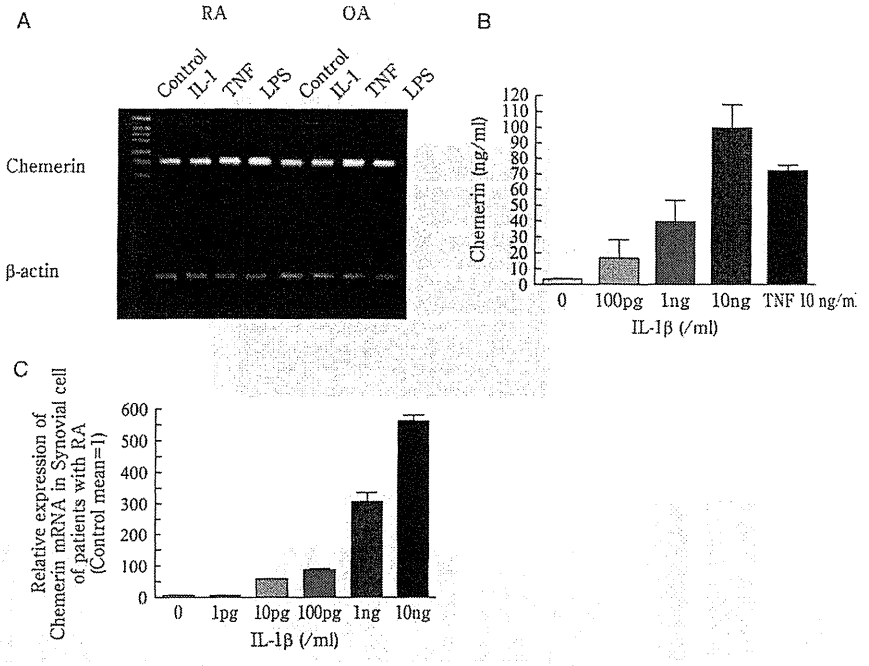

Fig. 3 IL-1β-induced synthesis of chemerin by SF from an RA patient

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

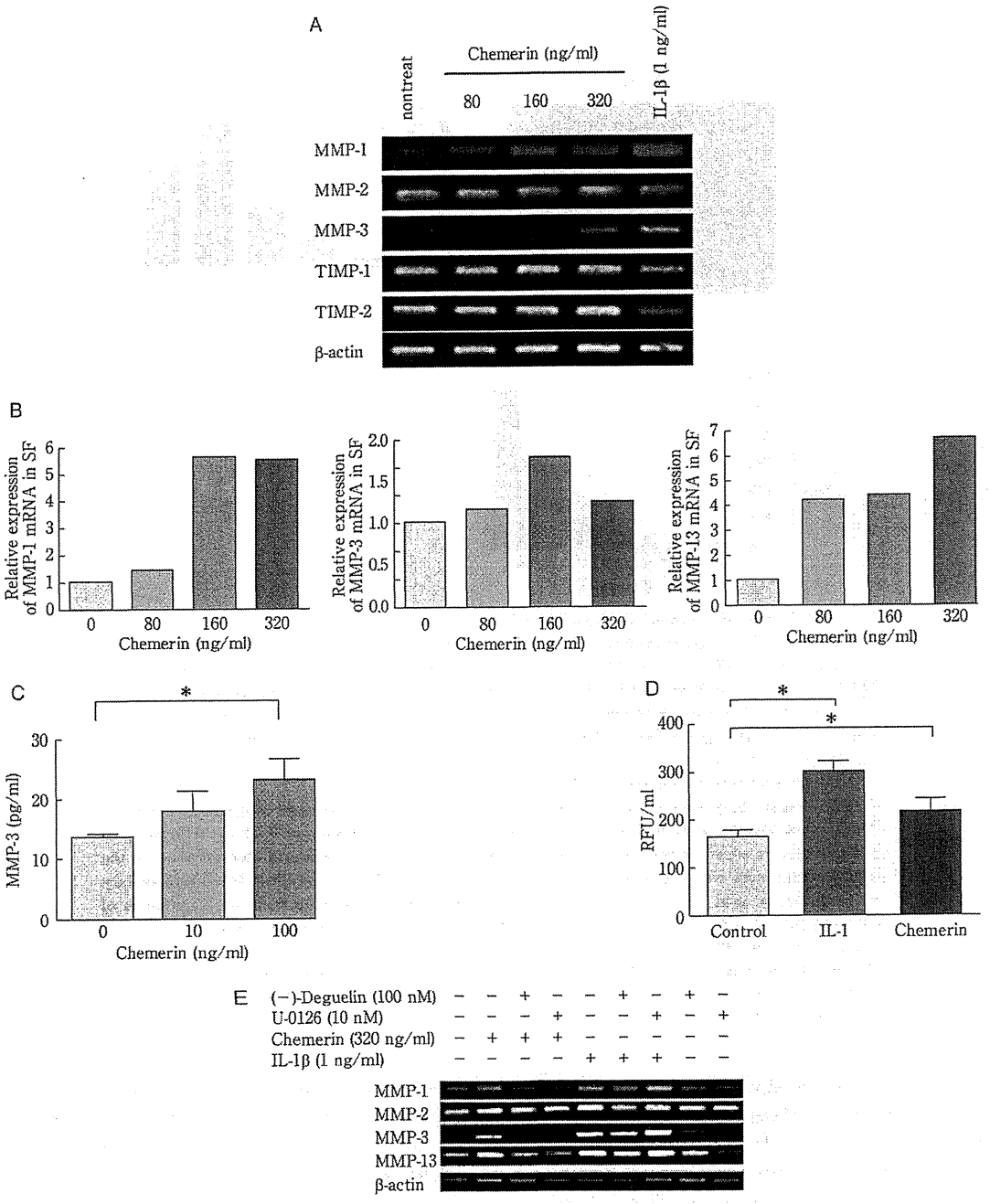
A
nontreat
Chemerin (ng/ml)
80
160
320
IL-1β (1 ng/ml)
MMP-1
MMP-2
MMP-3
TIMP-1
TIMP-2
β-actin
B
Relative expression of MMP-1 mRNA in SF
Relative expression of MMP-3 mRNA in SF
Relative expression of MMP-13 mRNA in SF
0
80
160
320
Chemerin (ng/ml)
C
MMP-3 (pg/ml)
0
10
100
Chemerin (ng/ml)
D
RFU/ml
Control
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.[29] 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_1.[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

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ORIGINAL ARTICLE

Prevention of joint destruction by tacrolimus in patients with early rheumatoid arthritis: a post hoc analysis of a double-blind, randomized, placebo-controlled study

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Abstract
Objectives A multicenter, randomized, double-blind, placebo-controlled study of the oral calcineurin inhibitor tacrolimus was performed in patients with early rheumatoid arthritis who had responded poorly to disease-modifying antirheumatic drugs (DMARDs), and factors related to suppression of joint destruction were investigated.
Methods The change in the total Sharp score (ΔTSS) was assessed by univariate analysis in patients with X-ray films to identify the main determinant of a ΔTSS of <0.5 in week 52. Patients with this factor were then investigated further.
Results Univariate analysis showed that a baseline C-reactive protein (CRP) level of <1.5 mg/dL was the major determinant of ΔTSS <0.5 at week 52 in the tacrolimus group. Detailed analysis of patients with a baseline CRP of <1.5 mg/dL revealed no significant differences in background factors between the two groups. In week 52, ΔTSS was significantly smaller in the tacrolimus group than in the placebo group (2.67 ± 5.40 vs. 8.05 ± 10.32, respectively, $p = 0.017$). Both groups had a similar incidence of adverse reactions.
Conclusions Adding tacrolimus to DMARDs significantly suppressed disease activity and joint destruction in patients with early rheumatoid arthritis, a disease duration ≤3 years, a CRP <1.5 mg/dL, and a poor response to oral DMARDs.

Keywords DMARD · Rheumatoid arthritis · Tacrolimus

Introduction

Tacrolimus is a macrolide antibiotic that was first identified as a metabolic product of the actinomycete *Streptomyces tsukubaensis*. It is a calcineurin inhibitor that shows strong immunosuppressive activity by selectively blocking T-cell activation [1, 2]. Tacrolimus was initially used clinically in Japan in organ transplantation, after which its efficacy for myasthenia gravis, rheumatoid arthritis (RA), lupus nephritis, and ulcerative colitis was also demonstrated.

In Japan, oral tacrolimus was approved for the treatment of RA in April 2005 (it is indicated for patients in whom conventional therapy is inadequate), after its efficacy and safety had been confirmed in clinical studies of RA patients who showed a poor response to disease-modifying antirheumatic drugs (DMARDs) [3, 4]. Recently, tacrolimus has often been used concomitantly with DMARDs, including methotrexate (MTX), and the improvement of symptoms through the use of this concomitant therapy has been reported [5, 6]. However, its effect on joint destruction is yet to be clarified.

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Previously, we performed a double-blind placebo-controlled study [7] to investigate the efficacy and safety of tacrolimus, as well as its capacity to prevent joint destruction, in 123 patients with early RA <3 years in duration, who had a diagnosis of RA based on the 1987 criteria of the American College of Rheumatology (ACR). According to the ACR criteria [9], the ACR20 improvement rate was significantly higher ($p = 0.005$) in the tacrolimus group than the placebo group. Also, according to the European League Against Rheumatism (EULAR) criteria [10, 11], a significantly higher ($p < 0.001$) percentage of the tacrolimus group showed a moderate or good response compared with the placebo group. Furthermore, the percentage of patients with a final disease activity score in 28 joints (DAS28) [12] of <2.6 was significantly higher ($p = 0.005$) in the tacrolimus group than in the placebo group. There was no significant difference between the two groups with regard to the incidence of adverse events or discontinuation due to adverse events.

However, evaluation of joint destruction in week 52 by the modified Sharp method [13] revealed the following results for the tacrolimus group and the placebo group, respectively: the total Sharp score (TSS) was 6.16 ± 10.84 (mean ± SD) versus 7.73 ± 12.23 and the bone erosion score was 2.50 ± 4.56 versus 4.27 ± 7.53 ($p = 0.090$). There were no significant differences in the change in TSS (ΔTSS) between the two groups, but the bone erosion score was lower in the tacrolimus group, suggesting that tacrolimus has the potential to reduce the progression of bone erosion. In the present study, therefore, we performed subanalyses to determine factors related to the prevention of joint destruction by tacrolimus therapy, and we found that tacrolimus suppressed disease activity and joint destruction in patients with early rheumatoid arthritis and lower levels of serum CRP (<1.5 mg/dL).

Materials and methods

Patients and study protocol

The enrollment criteria for this study were as follows: (1) males or females aged 20–65 years with a diagnosis of RA according to the ACR criteria [8]; (2) duration of disease ≥6 to ≤3 years; (3) at least 6 tender joints out of 68 joints surveyed; (4) at least 3 swollen joints out of 66 joints surveyed; (5) a C-reactive protein (CRP) level ≥1.0 mg/dL or erythrocyte sedimentation rate (ESR) ≥30 mm/h; (6) radiographic bone erosion at more than one site in the hands or lower limbs; and (7) current treatment with MTX (6–8 mg/week), salazosulfapyridine (1 g/day), or bucillamine (100–300 mg/day), and a compliance rate of ≥75 % during the minimum administration period (8 weeks before baseline, 8 weeks, or 12 weeks).

The exclusion criteria were (1) previous treatment with tacrolimus; (2) class 4 of Steinbrocker's functional classification; (3) treatment with biological products (infliximab or etanercept) or leflunomide within 12 weeks before the study for suppression of joint destruction; (4) steroid therapy at >7.5 mg/day (as prednisolone equivalent) within 4 weeks before the study, (5) administration of >2 tablets/suppositories of nonsteroidal anti-inflammatory drugs (NSAIDs) daily; and (6) diseases such as renal dysfunction, pancreatitis/impaired glucose tolerance, hyperkalemia, advanced hepatic dysfunction, cardiac disease (ischemic heart disease, arrhythmia requiring treatment, cardiac failure, etc.), severe respiratory disease, severe infection, drug hypersensitivity, or malignancy.

Subjects who fitted the above criteria, and who gave written informed consent, were randomized to a tacrolimus (3 mg/day) or a placebo group. Study drugs were administered once a day after the evening meal for a period of 52 weeks. The dosages of concomitant MTX, salazosulfapyridine, bucillamine, and NSAIDs were not changed, while dose reduction was allowed for steroids, but an increase above the baseline dose was not permitted. Initiation of new antirheumatic drugs or steroids was also not permitted.

At enrollment, in week 28, and in week 52 (or at discontinuation), plain X-ray films of both hands and both lower limbs were taken. Two blinded evaluators employed the modified Sharp method to determine the bone erosion score (ES) and the joint space narrowing (JSN) score from the X-ray films, and the sum of the ES and JSN scores was calculated as the TSS [9, 10]. The change in TSS from baseline (ΔTSS) was used to assess the progression of joint destruction. All participating institutions received the approval of their governing institutional board or equivalent, and the trial was implemented in accordance with the ethical principles of the Declaration of Helsinki and good clinical practice (GCP) guidelines, as well as relevant laws or regulations promulgated by the Institutional Review Boards for clinical trials. This study is registered at ClinicalTrials.gov (NCT00319917).

Statistical analysis

Factors with an influence on the suppression of joint destruction by tacrolimus were extracted by univariate analysis, employing gender (male, female), age (<49, ≥49 years), disease duration (<1.3, ≥1.3 years), stage (stages I/II, stages III/IV), functional class (class 1, classes 2–4), CRP (<1.5, ≥1.5 mg/dL), ESR (<41.5, ≥41.5 mm/h), DAS28-CRP (≤5.1, >5.1), DAS28-ESR (≤5.1, >5.1), TSS (<11.0, ≥11.0), ES (<5.0, ≥5.0), JSN score (<3.5, ≥3.5), yearly progression (<9.2, ≥9.2), rheumatoid factor

(<63.5, ≥63.5 IU/mL), matrix metalloproteinase-3 (MMP-3) (<187.5, ≥187.5 ng/mL), concomitant MTX therapy (yes, no), and the dose of MTX (<8, ≥8 mg/week) at the start of tacrolimus administration. CRP = 1.5, which was the median value of the population, was used in the analysis to keep the number of cases in the two groups uniform. Age, disease duration, CRP, ESR, TSS, ES, JSN, yearly progression, rheumatoid factor, MMP-3, and baseline dose of MTX were analyzed after being dichotomized at the median value (<median, ≥median).

For each factor extracted by univariate analysis, the effect on ΔTSS was compared between the tacrolimus group and the placebo group, and the factors that showed a significant difference between the two groups were selected. Next, the patients in whom a significant difference in these factors was observed were selected and used to perform a comparison between the tacrolimus and placebo groups with respect to each patient background factor, use of DMARDs, dose of DMARDs, changes in the Sharp score in week 52, improvement according to the EULAR criteria, and adverse events.

Changes in Sharp scores were examined by an analysis of variance in relation to the baseline score and the use of MTX as a covariate. The improvement rate according to the EULAR criteria was examined by logistic regression analysis. Background factors and adverse events were compared between the tacrolimus group and the placebo group by Fisher's exact test, the *t* test, or the Wilcoxon rank-sum test. Statistical significance was accepted at $p < 0.05$ (two-sided). Results are reported as the mean ± SD.

Missing radiographic data were compensated for using the linear extrapolation method, while other missing values were compensated for using the last-observation-carried-forward method.

Adverse events were classified by system organ class and preferred terms were taken from the ICH Medical Dictionary for Regulatory Activities (MedDRA Ver.11.1).

Results

Among the 123 randomized patients (61 in the tacrolimus group and 62 in the placebo group) registered in this study, 116 patients (58 in each group) had TSS data (Fig. 1). There were no differences between the background factors of the 116 patients and those of the 123 patients (data not shown). Also, there were no differences in background factors between both groups (tacrolimus and placebo) within this set of 116 patients, and the results were similar to the profile observed for all 123 patients.

Factors that influenced the achievement of ΔTSS <0.5 in week 52 were investigated in the tacrolimus group ($n = 58$) by univariate analysis, and this revealed significant influences of CRP, ESR, and DAS28-CRP (Table 1). When stratified analysis was carried out using these factors, a significant difference in ΔTSS in week 52 between the tacrolimus and placebo groups was (only) observed in the subgroup of patients with a baseline CRP of <1.5 mg/dL.

Among the 116 patients for whom the TSS was calculated, 29 patients from the tacrolimus group and 31 patients

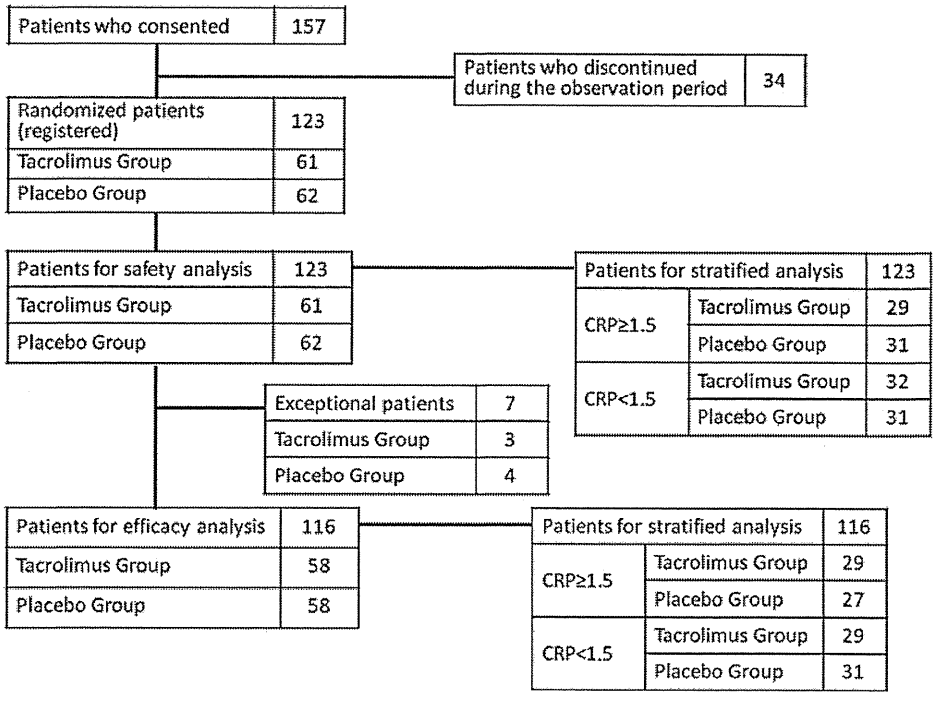

Fig. 1 Patient disposition. One hundred twenty-three RA patients were registered in this study. A total of 123 patients were randomized to either the tacrolimus group (61 patients) or the placebo group (62 patients) for safety analysis. The patients were then stratified according to CRP (<1.5, ≥1.5). Also, a total of 115 patients were randomized to either the tacrolimus group (58 patients) or the placebo group (58 patients) for efficacy analysis. Again, these patients were stratified according to CRP (<1.5, ≥1.5)

Table 1 Univariate analysis of the influence of background factors on the achievement of ΔTSS <0.5 in the tacrolimus group ($n = 58$)

		p value
Gender	Male (0/6) vs. female (14/52)	0.972
Age	<49 (8/29) vs. ≥49 (6/29)	0.541
Disease duration (years)	<1.3 (10/29) vs. ≥1.3 (4/29)	0.073
Stage classification	I, II (10/42) vs. III, IV (4/16)	0.925
Functional classification	1 (3/12) vs. 2–4 (11/46)	0.938
CRP (mg/mL)	<1.5 (11/29) vs. ≥1.5 (3/29)	0.021
ESR (mm/h)	<41.5 (11/29) vs. ≥41.5 (3/29)	0.021
DAS28-CRP	≤5.1 (13/32) vs. >5.1 (1/26)	0.009
DAS28-ESR	≤5.1 (6/15) vs. >5.1 (8/43)	0.103
Total score (modified Sharp method)	<11.0 (8/29) vs. ≥11.0 (6/29)	0.541
Bone erosion score (modified Sharp method)	<5.0 (6/27) vs. ≥5.0 (8/31)	0.751
Joint space narrowing score (modified Sharp method)	<3.5 (9/29) vs. ≥3.5 (5/29)	0.225
Yearly progression	<9.2 (7/29) vs. ≥9.2 (7/29)	1.000
Rheumatoid factor (IU/mL)	<63.5 (8/29) vs. ≥63.5 (6/29)	0.541
MMP-3 (ng/mL)	<187.5 (8/29) vs. ≥187.5 (6/29)	0.541
Concomitant MTX at the start of administration	with (8/39) vs. without (6/19)	0.358
MTX dose at the start of administration (mg/week)	<8 (9/33) vs. ≥8 (5/25)	0.523

CRP C-reactive protein, *ESR* erythrocyte sedimentation rate, *DAS28* Disease Activity Score 28, *MMP-3* matrix metalloproteinase-3, *MTX* methotrexate

from the placebo group had a baseline CRP of <1.5 mg/dL. Among all 123 patients, 32 patients in the tacrolimus group and 31 in the placebo group were subjected to safety analysis. With regard to background factors and concomitant medications, no significant differences were observed between both groups (Table 2).

Analysis of the clinical response of patients with a CRP level of <1.5 mg/dL at baseline was performed. In week 52, the tacrolimus group ($n = 32$) included 18 patients with a good response (56.3 %), 7 patients with a moderate response (21.9 %), and 7 patients with no response (21.9 %). Accordingly, 78.1 % of patients showed a moderate or good response. In the placebo group ($n = 31$), there were 10 patients with a good response (32.3 %), 6 patients with a moderate response (19.4 %), and 15 patients with no response (48.4 %), so 51.6 % of the patients had a moderate response or better. Again, there was a significant difference between the two groups ($p = 0.030$) (Fig. 2).

Evaluation of joint destruction showed the following yearly progression of TSS by week 52 in the tacrolimus group and the placebo group, respectively: ΔTSS was 2.67 ± 5.40 versus 8.05 ± 10.32 ($p = 0.017$), the change in the ES was 1.16 ± 3.10 versus 4.12 ± 6.28 ($p = 0.034$), and the change in the JSN score was 1.52 ± 3.19 versus 3.94 ± 5.14 ($p = 0.050$). ΔTSS and the change in ES were significantly smaller in the tacrolimus group than in the placebo group, and the change in the JSN score was also smaller in the tacrolimus group (Fig. 3a).

When the cumulative probability of ΔTSS up to week 52 was plotted, the cumulative probability of ΔTSS ≤0 was 37.9 % (11/29 patients) in the tacrolimus group and 16.1 % (5/31 patients) in the placebo group (Fig. 3b). This was approximately 1.57 times higher than the cumulative probability of ΔTSS ≤0 of 24.1 % for the tacrolimus group in the overall study (and 1.15 times higher for the placebo group), but this difference between groups was not significant ($p = 0.056$).

Regarding safety, the occurrence of adverse events among patients with a CRP of <1.5 mg/dL was noted in 81.3 % of those from the tacrolimus group (26/32 patients, 102 events) versus 90.3 % of such patients from the placebo group (28/31 patients, 85 events) (Table 3). Assessment according to system organ class showed no significant differences in event incidence between the two groups, and the event incidence revealed by this analysis was similar to that obtained in the overall study. Severe adverse events were observed in one patient from the tacrolimus group (3.1 %) versus seven patients from the placebo group (22.6 %), while discontinuation of administration due to adverse events occurred in two patients (6.3 %) and five patients (16.1 %), respectively.

Discussion

Since joint destruction progresses from the early stage of RA and eventually causes irreversible functional impairment, appropriate diagnosis and early treatment are needed. The 2012 ACR Recommendations [14] state that treatment with DMARDs should be initiated before joint destruction is evident. Moreover, to minimize the progression of joint destruction in patients with a disease duration of six-months or longer, administration of DMARDs alone or concomitantly is recommended, with re-evaluation every

Table 2 Comparison of the patients included in the safety analysis with CRP <1.5 mg/dL

		Tacrolimus group ($n = 32$)	Placebo group ($n = 31$)	p value
Female sex	Patients (%)	28 (96.6)	28 (90.3)	0.613[a]
Age (years)	Mean ± SD	48.6 ± 9.8	51.5 ± 11.6	0.291[b]
Height (cm)	Mean ± SD	156.9 ± 6.1	157.2 ± 8.1	0.866[b]
Weight (kg)	Mean ± SD	52.7 ± 6.0	53.0 ± 10.2	0.881[b]
Disease duration (years)	Mean ± SD	1.5 ± 0.7	1.6 ± 0.7	0.590[b]
Stage classification (stage)				
I (early stage)	Patients (%)	0	1 (3.2)	0.632[c]
II (middle stage)	Patients (%)	21 (72.4)	23 (74.2)	
III (advanced stage)	Patients (%)	8 (27.6)	5 (16.1)	
IV (terminal stage)	Patients (%)	0	2 (6.5)	
Functional classification (class)				
1	Patients (%)	7 (24.1)	7 (22.6)	0.888[c]
2	Patients (%)	22 (75.9)	24 (77.4)	
3	Patients (%)	0	0	
4	Patients (%)	0	0	
Number of painful joints	Mean ± SD	12.1 ± 7.8	11.5 ± 5.2	0.746[b]
Number of swollen joints	Mean ± SD	10.0 ± 5.1	8.5 ± 5.2	0.283[b]
Physical function evaluation by patients	Mean ± SD	0.4 ± 0.4	0.5 ± 0.3	0.214[b]
CRP (mg/dL)	Mean ± SD	0.5 ± 0.4	0.7 ± 0.4	0.257[b]
ESR (mm/h)	Mean ± SD	36.1 ± 18.1	42.4 ± 20.3	0.214[b]
Rheumatoid factor (IU/mL)	Mean ± SD	98.3 ± 126.5	115.9 ± 118.8	0.581[b]
DAS28-CRP				
≤3.2	Patients (%)	2 (6.9)	1 (3.2)	–
>3.2, ≤5.1	Patients (%)	21 (72.4)	25 (80.6)	
>5.1	Patients (%)	6 (20.7)	5 (16.1)	
	Mean ± SD	4.4 ± 0.8	4.3 ± 0.7	0.927[b]
DAS28-ESR				
≤3.2	Patients (%)	0	0	–
>3.2, ≤5.1	Patients (%)	11 (37.9)	11 (35.5)	
>5.1	Patients (%)	18 (62.1)	20 (64.5)	
	Mean ± SD	5.3 ± 0.8	5.2 ± 0.8	0.934[b]
Total score (modified Sharp method)	Mean ± SD (min–max)	15.9 ± 17.0 (2.0–75.0)	16.7 ± 17.1 (0.0–65.5)	0.858[b]
Bone erosion score (modified Sharp method)	Mean ± SD (Min–Max)	9.0 ± 8.3 (2.0–33.0)	7.7 ± 8.2 (0.0–40.5)	0.531[b]
Joint space narrowing score (modified Sharp method)	Mean ± SD (min–max)	6.9 ± 10.6 (0.0–42.0)	9.0 ± 12.1 (0.0–41.0)	0.471[b]
Yearly progression	Mean ± SD (min–max)	10.4 ± 8.7 (1.3–32.1)	10.7 ± 10.9 (0.0–46.0)	0.891[b]
Concomitant agents				
Methotrexate				
Dose (mg/week)	Patients (%)	16 (55.2)	18 (58.1)	1.000[a]
	Mean ± SD	7.0 ± 1.0	7.3 ± 1.0	0.339[b]
Salazosulfapyridine				
Dose (g/day)	Patients (%)	10 (34.5)	6 (19.4)	0.247[a]
	Mean ± SD	1.0 ± 0.0	1.0 ± 0.0	–
Bucillamine				
Dose(mg/day)	Patients (%)	3 (10.3)	7 (22.6)	0.302[a]
	Mean ± SD	166.7 ± 57.7	142.9 ± 53.5	0.545[b]