of TYMS/MTHFR, the MTX dose for 50%CRP tended to be lower in patients with the homozygous mutant-type TYMS 3′-UTR (−6/+6) or MTHFR A1298C than in patients without. There was a significant difference in the MTX dose for 50%CRP between patients with and without TYMS 3′-UTR (−6/+6) ($p=0.039$), although there was no significant difference in it between patients without and with MTHFR A1298C ($p=0.38$). This result might be explained by the differences in the distribution of TYMS 3′-UTR (−6/+6) and MTHFR A1298C in Japanese. In previous study of ours of healthy Japanese volunteers,[22] the genotype frequency of MTHFR 1298 C/C was 13.7%, whereas that of TYMS 3′-UTR −6/−6 was 35.3%.

As a result of multivariate regression analysis, AC-MPG level, TYMS 3′-UTR (−6/+6), and ESR at the initiation of MTX therapy were major predictive variables for the MTX dose for 50%CRP. The β value of AC-MPG and TYMS 3′-UTR (−6/+6) in univariate regression analysis was 0.591 and −0.346, respectively, and these variables are thought to be major independent predictive factors for the response to MTX therapy. To the best of our knowledge, this is the first report on predicting the response to MTX therapy including factors such as the concentration of MTX-PGs in RBCs, genetic polymorphisms of MTX-active sites, and other patient-related factors. The MTX dose for 50%CRP could not be predicted based on ESR and CRP and RF levels alone, although 51% of individual differences in the MTX dose for 50% CRP could be explained by considering the factors of AC-MPG and the genotype of TYMS 3′-UTR (−6/+6). However, their predictive ability was insufficient for clinical use. Therefore, further assessment of the relationships between the response to MTX therapy and other associated factors including body weight, number of tender and swollen joints, and matrix metalloproteinase-3 level reflecting the degree of joint destruction[24] must be made.

Our study had three limitations. First, there was no patient with the TYMS 5′-UTR 2R/2R genotype, and therefore the relationship between TYMS 5′-UTR 2R/2R and MTX dose for 50%CRP could not be assessed. Second, this study was conducted only among Japanese RA patients. Ethnic differences were reported in the distribution of TYMS 5′-UTR (2R/3R), TYMS 3′-UTR (−6/+6), and MTHFR C677T.[22] Therefore, the major predictive factors may differ by ethnic group. Third, this study was a preliminary trial. Therefore, additional data will be needed to demonstrate conclusive results.

The polymorphisms of dihydrofolate reductase (DHFR)[25] and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC),[5] which are MTX and MTX-PGs-active sites, γ-glutamyl hydrolase,[26] which catalyzes the conversion from MTX-polyglutamates to MTX, and reduced folate carrier (RFC)-1,[27] which is responsible for the intracellular transport of MTX, were not analyzed in this study. It has been reported that these genetic polymorphisms influence enzyme activity, and therefore they are also expected to be associated with individual differences in the response to MTX therapy. Dervieux *et al.* reported that the number of homozygous mutant types of TYMS, RFC-1, and ATIC was related to the effect of MTX in RA patients.[5] Therefore, further investigation of these genetic polymorphisms must be performed in the future.

In conclusion, AC-MPG, TYMS 3′-UTR (−6/+6), and ESR at the initiation of MTX therapy might be the major predictive factors of the response to MTX therapy in Japanese RA patients. Genotyping can be carried out before the start of MTX therapy, and the AC-MPG level can also be estimated using one blood sample 1 month after receiving the same dose of MTX. Therefore, the evaluation of AC-MPG and TYMS 3′-UTR (−6/+6) would be useful for individualized MTX therapy in RA patients.

Acknowledgement This study was supported in part by Grants for Encouragement of Research, Japan Research Foundation for Clinical Pharmacology.

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Prostaglandin E_2 Differentially Modulates Proinflammatory/Prodestructive Effects of TNF-α on Synovial Fibroblasts via Specific E Prostanoid Receptors/cAMP[1]

Elke Kunisch,[2]* Anne Jansen,*† Fumiaki Kojima,† Ivonne Löffler,[3]‡ Mohit Kapoor,† Shinichi Kawai,§ Ignacio Rubio,‡ Leslie J. Crofford,† and Raimund W. Kinne*

The present study investigated the influence of PGE_2, E prostanoid (EP) receptors, and their signaling pathways on matrix metalloproteinase (MMP)-1 and IL-6 expression in synovial fibroblasts (SFs) from rheumatoid arthritis (RA) patients. RASFs expressed all four EP receptors, with selective induction of EP2 by TNF-α. TNF-α time-dependently increased intracellular cAMP/protein kinase A signaling (maximum, 6–12 h) and PGE_2 secretion (maximum, 24 h). PGE_2 and the EP2 agonists butaprost or ONO-AE1-259 ((16)-9-deoxy-9β-chloro-15-deoxy-16-hydroxy-17,17-trimethylene-19,20-didehydro PGE_1), in turn, induced a rapid, time-dependent (maximum, 15–30 min) increase of cAMP. Additionally, cyclooxygenase-2 inhibition by NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide) reduced the TNF-α-induced increase in IL-6 mRNA/protein, which was restored by stimulation with PGE_2 or EP2, EP3, and EP4 agonists. In contrast, TNF-α-induced MMP-1 secretion was not influenced by NS-398 and diminished by PGE_2 via EP2. Finally, 3-isobutyl-1-methylxanthine enhanced the effects of PGE_2 on MMP-1, but not on IL-6 mRNA. In conclusion, PGE_2 differentially affects TNF-α-induced mRNA expression of proinflammatory IL-6 and prodestructive MMP-1 regarding the usage of EP receptors and the dependency on cAMP. Although specific blockade of EP2 receptors is considered a promising therapeutic strategy in RA, opposite regulation of proinflammatory IL-6 and prodestructive MMP-1 by PGE_2 via EP2 may require more complex approaches to successfully inhibit the cyclooxygenase-1/2 cAMP axis. *The Journal of Immunology,* **2009, 183: 1328–1336.**

In rheumatoid arthritis (RA),[4] activated synovial fibroblasts (RASFs) contribute to the inflammatory/destructive potential of the aggressive synovial tissue by producing proinflammatory mediators and matrix-degrading enzymes including matrix metalloproteinases (MMPs) (1, 2, 3, 4, 5, 6). Under the influence of proinflammatory cytokines, for example, TNF-α or IL-1β, RASFs secrete PGE_2 (7, 8, 9). PGE_2 can raise cAMP levels in RASFs (10, 11, 12, 13, 14), and cAMP is involved in the IL-1β-induced expression of several target genes, e.g., IL-6, M-CSF, and vascular endothelial growth factor (11). PGE_2 belongs to the family of prostanoid, autocrine, and paracrine lipid mediators released by cells following mechanical injury or stimulation with cytokines or growth factors. The synthesis of the prostanoid is catalyzed by the cyclooxygenase (COX) pathway (15).

PGE_2 mediates its biological functions via binding to four types of membrane-bound, G protein-coupled receptors termed E prostanoid (EP)1 to EP4 (15, 16). Following ligand binding, the EP receptors activate different signal transduction pathways. EP1 raises intracellular Ca^{2+}, whereas EP3 reduces or increases cAMP by activating inhibitory G (G_i) or stimulatory G (G_s) proteins depending on the particular splice variant expressed by the cell (17). The EP2 and EP4 receptors increase intracellular cAMP by activating adenylate cyclase via G_s proteins. However, differences in the strength of G_s coupling, activation of other signal transduction pathways, agonist-induced desensitization, and agonist-induced internalization result in a differential response of the target cell to a ligand-induced activation of the EP2 or EP4 receptors (18).

Human RASFs have consistent mRNA expression for the PGE_2 receptors EP2 and EP4, while there have been inconsistent reports of EP1 and EP3 mRNA expression (10, 19, 20, 21). Surprisingly, although EP2 and EP4 receptors are regarded as attractive pharmacological targets for RA treatment, the exact role of cAMP or other signals issued by PGE_2-challenged EP receptor subtypes, as well as their influence on the effects stimulation with TNF-α or

*Experimental Rheumatology Unit, Department of Orthopedics, University Hospital Jena, Jena, Germany; †Department of Internal Medicine, Division of Rheumatology, Kentucky Clinic, University of Kentucky, Lexington, KY 40536; ‡Institute of Molecular Cell Biology, Center for Molecular Biomedicine, Friedrich Schiller University Jena, Jena, Germany; and §Department of Internal Medicine, Division of Rheumatology, Toho University School of Medicine, Tokyo, Japan

Received for publication March 11, 2009. Accepted for publication May 8, 2009.

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[1] The study was supported by the German Federal Ministry of Education and Research (BMBF; Grants FKZ 01ZZ9602, 01ZZ0105, and 010405 to R.W.K., Interdisciplinary Center for Clinical Research (IZKF) Jena, including a grant for junior researchers to E.K.; Grants FKZ 0312704B and 0313652B to R.W.K., Jena Center for Bioinformatics; Grant 01GS0413, NGFN-2 to R.W.K.), the German Research Foundation (DFG; Grants KI 439/7-1 and KI 439/6-1 to R.W.K.), National Institutes of Health/National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant 1 R01 AR049010 to L.J.C., and a grant for the advancement of female scientists to Elke Kunisch (LUBOM Thuringia). Anne Jansen was supported by a stipend from the Friends of the Friedrich Schiller University Jena, as well as by travel allowances from Jenapharm and the Boehringer Ingelheim Foundation.

[2] Address correspondence and reprint requests to Dr. Elke Kunisch, Experimental Rheumatology Unit, Department of Orthopedics, University Hospital Jena, Klosterlausnitzer Strasse 81, D-07607 Eisenberg, Germany. E-mail address: elke.kunisch@med.uni-jena.de

[3] Current address: Department of Internal Medicine III, University Hospital Jena, Jena, Germany.

[4] Abbreviations used in this paper: RA, rheumatoid arthritis; RASF, rheumatoid arthritis synovial fibroblast; MMP, matrix metalloproteinase; COX, cyclooxygenase; EP, E prostanoid; NS-398, *N*-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide; IBMX, 3-isobutyl-1-methylxanthine; PKA, protein kinase A.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900801

IL-1β, remains largely unknown. The present study sought to analyze the involvement of PGE_2-dependent cAMP signaling in TNF-α-induced proinflammatory IL-6 and/or prodestructive MMP-1 effector functions of RASFs.

Materials and Methods

Materials

Rabbit anti-human polyclonal Abs against the EP1, EP2, EP3, and EP4 receptors, PGE_2, NS-398 (*N*-(2-Cyclohexyloxy-4-nitrophenyl)-methanesulfonamide), butaprost, sulprostone, and an ELISA kit for cAMP were purchased from Cayman Chemical. A competition assay for the detection of PGE_2 was obtained from Biotrak (Amersham Pharmacia Biotech). Research grade 3-isobutyl-1-methylxanthine (IBMX) was purchased from Calbiochem. Selective EP1 (ONO-DI-004, (17*S*)-2,5-ethano-6-oxo-17, 20-dimethyl PGE_1), EP2 (ONO-AE1-259, (16)-9-deoxy-9β-chloro-15-deoxy-16-hydroxy-17,17-trimethylene-19,20-didehydro PGE_1), EP3 (ONO-AE-248, 11,15-*O*-dimethyl PGE_2), and EP4 (ONO-AE1-329, 16-(3-methoxymethyl)phenyl-ω-tetranor-3,7-dithia PGE_1) receptor agonists were provided by Ono Pharmaceutical. Recombinant human TNF-α was purchased from R&D Systems, DMEM from Invitrogen, FCS from Cambrex Bio Science, TriPure reagent from Roche Applied Science, and HotStarTaq polymerase from Qiagen.

Patients, tissue digestion, and cell culture

Synovial tissue from RA patients was obtained during open joint replacement/arthroscopic synovectomy from the Clinic of Orthopedics, Eisenberg, Germany. All patients fulfilled the respective American Rheumatism Association criteria (22). The study was approved by the Ethics Committee of the University Hospital Jena (Jena, Germany), and patient informed consent was obtained. Immediately after synovectomy, tissue was placed in culture medium at ambient temperature and subjected to digestion within 2 h.

RA synovial samples were digested, subsequently cultured for 7 days, and RASFs were negatively isolated as previously described (23, 24). RASFs were cultured in the virtual absence of contaminating nonadherent cells and macrophages. Third-passage cells were used for all experiments. Stimulation of the cells was performed in DMEM/0.2% lactalbumin hydrolysate. *Mycoplasma* contamination of the cells was excluded by 4′-6-diamidino-2-phenylindole (DAPI) staining.

Cell stimulation

For kinetic analysis of the TNF-α-induced expression of EP receptors, RASFs (2.5×10^5 cells/well of a 12-well plate) were allowed to adhere for 24 h in DMEM/10% FCS at 37°C and 5% CO_2. Thereafter, cells were stimulated with 10 ng/ml TNF-α (R&D Systems). After stimulation for 0, 1, 2, 4, 8, 10, and 24 h, the cells were lysed in buffer for RNA isolation.

For analysis of intracellular cAMP and protein kinase A (PKA) substrate phosphorylation, as well as mRNA and protein expression of proinflammatory/prodestructive IL-6 and MMP-1, RASFs (4×10^5 cells/well of 6-well plates or $1.5–2.0 \times 10^5$ cells/well of a 12-well plate) were allowed to adhere for 24 h in DMEM/10% FCS at 37°C. Thereafter, cells were pretreated with 1 μM NS-398 for 30–45 min followed by treatment with TNF-α (10 ng/ml), PGE_2 (1 μM), the EP2 receptor agonist butaprost or the EP3/EP1 agonist sulprostone (each 1 μM), or selective EP agonists (EP1, EP2, EP3, and EP4; ONO; 10 μM). In selected experiments (see Figs. 2, *A* and *B*, and 4, as well as supplemental Fig. 2),[5] 500 μM IBMX was added to each well 4 h before the end of the experiment to increase the signal for intracellular cAMP production and PKA substrate phosphorylation; in other experiments, the results were compared for cultivation with and without prior coincubation with IBMX (see Figs. 2*E* and 6, 100 μM IBMX; supplemental Fig. 1, *A* and *B*, 500 μM; and supplemental Fig. 1*C*, 100 μM IBMX). Supernatants of the cells were collected for analysis of protein secretion.

Analysis of EP receptor, MMP-1, and IL-6 expression by real-time RT-PCR

Total RNA was isolated from RASFs using a commercially available RNA isolation kit (Macherey & Nagel) and 1 μg was reverse-transcribed using SuperScript II reagents (Invitrogen). EP1, EP2, EP3, EP4, MMP-1, and IL-6 mRNA expression was analyzed by real-time PCR in a RealPlex PCR machine (Eppendorf). PCR reactions were performed in a total volume of 20 μl in 96-well plates containing a reaction mix of HotMaster DNA polymerase (0.05 U; Eppendorf), 10× *Taq* buffer with 15 mM magnesium (Eppendorf), $MgCl_2$ (final concentration, 3.5 mM; Invitrogen), dNTPs (0.4 mM; Roche), BSA (40 ng/ml), SYBR Green (1/1250; SYBR Green I, 10,000 concentrate; Molecular Probes), sense and antisense primers (each 0.3 μM), and cDNA. To normalize the amount of cDNA in each sample, the housekeeping gene aldolase was also amplified. The sequences of the PCR primers used in this study and the real-time PCR conditions are described in Tables I and II. The fluorescence emitted by dsDNA-bound SYBR Green was measured once at the end of each additional heating step and continuously during the melting curve program. The concentration of EP1, EP2, EP3, EP4, MMP-1, and IL-6 mRNA in each sample was calculated by the RealPlex software using an external standard curve. Product specificity of the real-time PCRs was confirmed by 1) melting curve analysis (see Table II), 2) agarose gel electrophoresis, and 3) cycle sequencing of the PCR products.

For conventional RT-PCR of EP receptor expression, PCR reactions were performed in a total volume of 50 μl containing a reaction mix of *Taq*-polymerase (50 mU/μl; Jena Bioscience), 10× PCR buffer, 4% DMSO, dNTP (50 μM), as well as sense and antisense primers (each 0.5 pmol; Jena Bioscience). For EP1 and EP3 PCR, 5% 5× Q-Solution (Qiagen) was added to the mix. The sequences of the PCR primers used in this study and the PCR conditions are stated in Tables I and II. Verification of the PCR products was performed by cycle sequencing.

RT-PCR for MMP-1 and IL-6

For conventional RT-PCR of MMP-1 and IL-6, RNA from the cells was extracted with TriPure reagent according to the manufacturer's instructions. Reverse transcription was performed according to the manufacturer's instructions using a SuperScript preamplification system with 1 μg of total RNA as a template. Subsequent amplifications of the cDNA fragments by PCR with HotStarTaq polymerase were performed using 0.5 μl of the reverse-transcribed mixture as a template with specific primers and PCR conditions as mentioned in Tables I and II. The amplified cDNA fragments were resolved electrophoretically on 2% agarose gels and then visualized under UV illumination using a Bio-Rad ChemiDoc apparatus after staining with ethidium bromide.

Analysis of EP receptor and MMP-1 protein in RASFs by Western blot

For the analysis of EP receptor protein, 35 μg of protein from nonstimulated or TNF-α-stimulated RASFs (30 h) was separated by denaturing SDS-PAGE (12%) and transferred onto blotting membranes (Hybond-C Extra; Amersham Life Sciences). In the case of MMP-1, cell culture supernatant was used. After blocking with 2.5% skim milk in Tris-buffered saline-Tween 20 (10 mM Tris, 150 mM NaCl, 0.1% Tween 20 (pH 7.4)), membranes were probed overnight at 4°C with specific primary Abs against the EP1, EP2, EP3, or EP4 receptors (Cayman Chemical) or against MMP-1 (clone 50647; R&D Systems), washed, and incubated with HRP-conjugated goat anti-rabbit IgG as a secondary Ab. Proteins were visualized by chemiluminescence (Supersignal West chemiluminescent substrate; Pierce). The intensity of each band was quantified using an integration image software (Scion Corporation).

cAMP measurements

Intracellular cAMP was determined in RASFs using either the cAMP enzyme immunoassay kit (Cayman Chemical) or the cAMP [^{3}H] assay system TRK 432 (Amersham Bioscience). Samples were prepared exactly as described by the manufacturer.

Phosphorylation of PKA substrates

Phosphorylation of PKA substrates was determined by Western blot analysis of RASFs with an anti phospho-PKA substrate Ab (clone 100G7; Cell Signaling Technology). The blots were subsequently reprobed with β-actin to ascertain equal protein loading.

IL-6 measurements

Human IL-6 was measured in diluted cell culture supernatants using a quantitative sandwich enzyme immunoassay (OptEIA; BD Biosciences). A wavelength of 450 nm with a wavelength correction at 570 nm was used. Sample concentrations of IL-6 were determined by comparison with a standard curve (range, 2.34–300 pg/ml).

PGE_2 measurements

PGE_2 concentrations in the supernatants of TNF-α-stimulated cells were determined using a competition assay (sensitivity, 40 pg/ml PGE_2, Biotrak;

[5] The online version of this article contains supplemental material.

Table I. *Sequences of PCR primers used in this study*

Gene	Primer: Real-Time PCR	Primer: Conventional PCR	kb
Aldolase			
Sense	5′-tcatcctcttccatgagacactct-3′		313
Anti-sense	5′-attctgctggcagatactggcataa-3′		
GAPDH			
Sense		5′-cca ccc atg gca aat tcc atg gca-3′	606
Anti-sense		5′-tct aga cgg gag gtc agg tcc acc-3′	
Sense		5′-tcagcaatgcctcctgcac-3′	250
Anti-sense		5′-ccagtgagcttcccgttcag-3′	
IL-6			
Sense	5′-atgaactccttctccacaagcg-3′	5′-atgaactccttctccacaagcgc-3′	199/627
Anti-sense	5′-ctcctttctcagggctgag-3′	5′-gaagagccctcaggctggactg-3′	
MMP-1			
Sense	5′-gacctggaggaaatcttgc-3′	5′-aactctggagtaatgtcacac-3′	321/584
Anti-sense	5′-gttagcttactgtcacacgc-3′	5′-attcgtaagcagcttcaagcc-3′	
EP1			
Sense	5′-cttgtcggtatcatggtggtgtc-3′		317
Anti-sense	5′-ggttgtgcttagaagtggctgagg-3′		
EP2			
Sense	5′-ccacctcattctcctggcta-3′		216
Anti-sense	5′-cgacaacagaggactgaacg-3′		
EP3			
Sense	5′-cttcgcataactggggcaac-3′		300
Anti-sense	5′-tctccgtgtgtgtcttgcag-3′		
EP4			
Sense	5′-tggtatgtgggctggctg-3′		429
Anti-sense	5′-gaggacggtggcgagaat-3′		

Amersham Pharmacia Biotech). The OD in each well was determined at 450 nm. The concentration of PGE_2 was determined by comparison with a standard curve (range, 50–6400 pg/ml).

Statistical analysis

The data were expressed as means ± SEM. Significance was tested using the nonparametric Mann-Whitney *U* test. Differences were considered statistically significant for $p \leq 0.05$. Analyses were performed using the SPSS 13.0 program.

Results

Role of PGs in TNF-α-induced IL-6 and MMP-1 mRNA expression and secretion in RASFs

Exposure of RASFs to TNF-α led to a marked induction of IL-6 mRNA expression and secretion (Fig. 1, *A* and *C*). To test the involvement of PGs in this process, a pharmacological approach was used to inhibit COXs. The COX-2-selective inhibitor NS-398 significantly reduced TNF-α-induced IL-6 mRNA expression and protein secretion, pointing to an enhancing role of COX-2-derived PGs in this process. In line with this notion, NS-398-blocked expression and secretion of IL-6 was restored by simultaneous administration of exogenous PGE_2 (Fig. 1, *A* and *C*). Taken together, these data point to a critical role of PGs, possibly PGE_2, as modulators of the proinflammatory actions of TNF-α.

As observed for IL-6, TNF-α also significantly induced the mRNA expression and secretion of MMP-1 in RASFs (Fig. 1, *B* and *D*). However, NS-398 did not significantly reduce, but even numerically enhanced, MMP-1 mRNA expression and secretion. Concordantly, addition of exogenous PGE_2 significantly reduced the NS-398-enhanced mRNA expression and secretion of MMP-1 upon TNF-α stimulation (Fig. 1, *B* and *D*). Therefore, PGE_2 and possibly other PG species appear to have critical and partially opposite effects on proinflammatory and prodestructive signaling by TNF-α in RASFs.

TNF-α activates the cAMP/PKA signaling pathway in RASFs

TNF-α induced a gradual, time-dependent increase in cAMP levels that reached a maximum after 8–10 h of stimulation (Fig. 2*A*). The

Table II. *Real-time and conventional PCR conditions*

Gene	Real-Time PCR: Initial Denaturation (°C; min)	Denaturation (°C; s)	Annealing (°C; s)	Elongation (°C; s)	Melting (°C; s)	Conventional RT-PCR: Initial Denaturation (°C; min)	Denaturation (°C; s)	Annealing (°C; s)	Elongation (°C; s)	Terminal Elongation (°C; min)	No. Cycles
Aldolase	95; 10	95; 20	58; 20	68; 20	82; 8						
GAPDH						94; 3	94; 60	59; 60	72; 60	72; 7	25
						95; 15	95; 30	56; 30	72; 45	72; 5	26
IL-6	95; 15	95; 10	62; 10	68; 20	81; 8	95; 15	95; 30	56; 30	72; 45	72; 5	25
MMP-1	95; 10	95; 15	58; 15	68; 20	81; 8	95; 15	95; 30	56; 30	72; 45	72; 5	26
EP1	95; 10	95; 20	60; 10	68; 20	83; 8	95; 2	95; 45	59; 45	72; 60	72; 5	35
EP2	95; 10	95; 15	60; 15	68; 20	Not performed	95; 2	95; 45	61; 45	72; 60	72; 5	35
EP3	95; 10	95; 20	60; 15	68; 20	83; 8	95; 2	95; 45	59; 45	72; 60	72; 5	40
EP4	95; 15	95; 15	60; 15	68; 20	81; 8	94; 3	95; 60	60; 45	72; 60	72; 7	35

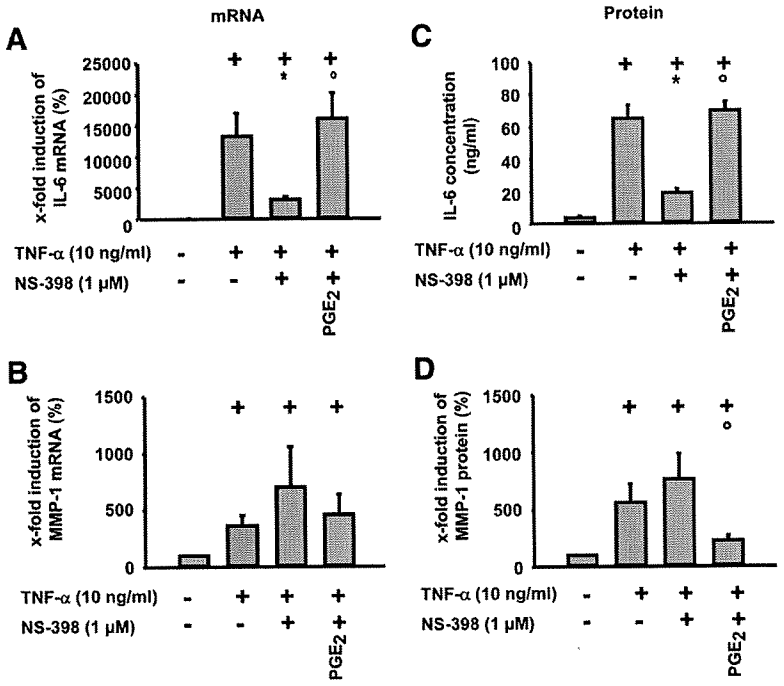

FIGURE 1. Influence of PGE_2 on TNF-α-induced IL-6 and MMP-1 mRNA expression and protein secretion in RASFs. Cells were stimulated with TNF-α (10 ng/ml) in the absence or presence of NS-398 and PGE_2 (1 μM each) for 24 h. IL-6 and MMP-1 mRNA expression was analyzed by real-time PCR (*A* and *B*). IL-6 secretion was analyzed by ELISA (*C*), and MMP-1 secretion was analyzed by Western blot (*D*); means ± SEM for six patients with RA; +, $p \leq 0.05$ Mann-Whitney *U* test vs control; *, $p \leq 0.05$ Mann-Whitney *U* test vs TNF-α; ○, $p \leq 0.05$ Mann-Whitney *U* test vs TNF-α/NS-398.

β-adrenoreceptor agonist isoproterenol, a well-known cAMP-elevating agent, was used as a positive control in this and forthcoming experiments. To confirm with an independent approach that TNF-α addressed the cAMP/PKA signaling cassette, the phosphorylation status of PKA target proteins was assessed using a phosphorylation-specific Ab that selectively detects the minimum RRXS/T consensus target sequence for PKA in its phosphorylated state. The corresponding experiment (Fig. 2*B*) illustrated a time-dependent increase in the phosphorylation of multiple PKA targets, in full agreement with the cAMP measurements, and thus confirming that TNF-α activates the cAMP/PKA pathway.

Since TNF-α induced both cAMP/PKA signaling and PGE_2 release with similar kinetics (Fig. 2*A–C*) and PGE_2 increased cAMP in RASFs (Fig. 2*D*; enhanced by IBMX, Fig. 2*E*), PGs may mediate the activation of the cAMP/PKA pathway by TNF-α.

TNF-α induces the expression of EP2 receptors in RASFs

PCR of total RNA preparations from RASFs was performed and mRNA for all four EP receptors was detected in these cells (Fig. 3, *A* and *B*). Intriguingly, TNF-α induced a time-dependent increase in EP2 mRNA (maximum, 10 h), as assessed by both real-time (Fig. 3*A*) or conventional PCR (Fig. 3*B*). In contrast,

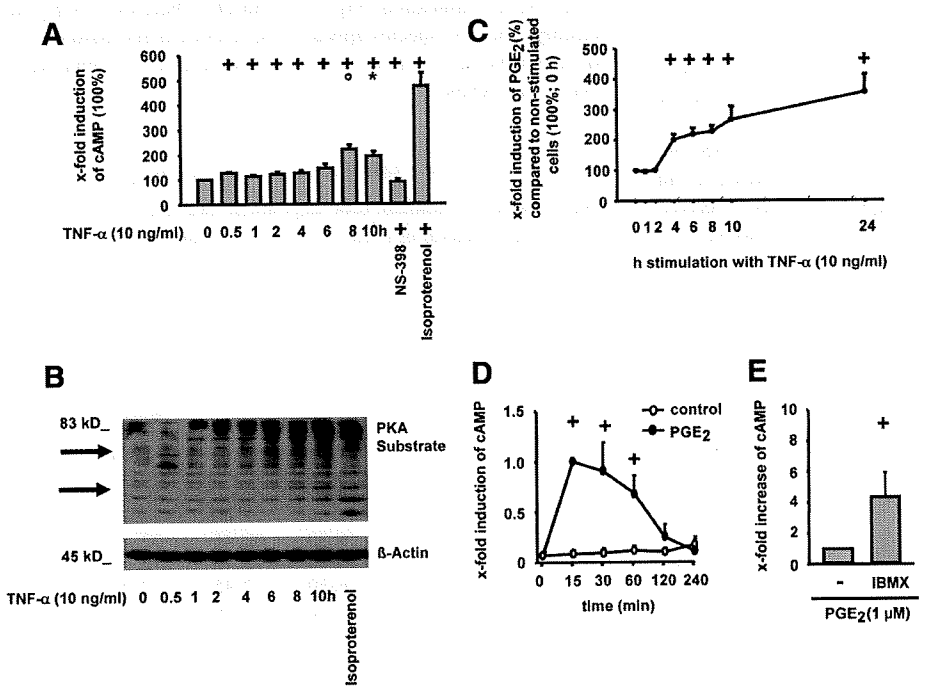

FIGURE 2. Influence of TNF-α on intracellular cAMP levels, PKA substrate phosphorylation, and PGE_2 secretion in RASFs. RASFs were stimulated with TNF-α (10 ng/ml; different time points), TNF-α/NS-398 (1 μM; 10 h), or isoproterenol (100 μM; 10 min) in the presence of IBMX (500 μM; *A* and *B*) or the absence of IBMX (*C*). Alternatively, cells were stimulated with PGE_2 for different time points (*D*) or for 30 min with/without IBMX (100 μM) (*E*). Intracellular cAMP was determined by RIA (*A*) or ELISA (*D*), PKA substrate phosphorylation by Western blot (*B*), and PGE_2 secretion by ELISA (*C*); means ± SEM for three patients with RA; +, $p \leq 0.05$ Mann-Whitney *U* test vs 0 h (*A*, *C*, and *D*) or vs without IBMX (*E*); ○, $p \leq 0.05$ Mann-Whitney *U* test vs ≤4 h (*A*); *, $p \leq 0.05$ Mann-Whitney *U* test vs 6 h (*A*).

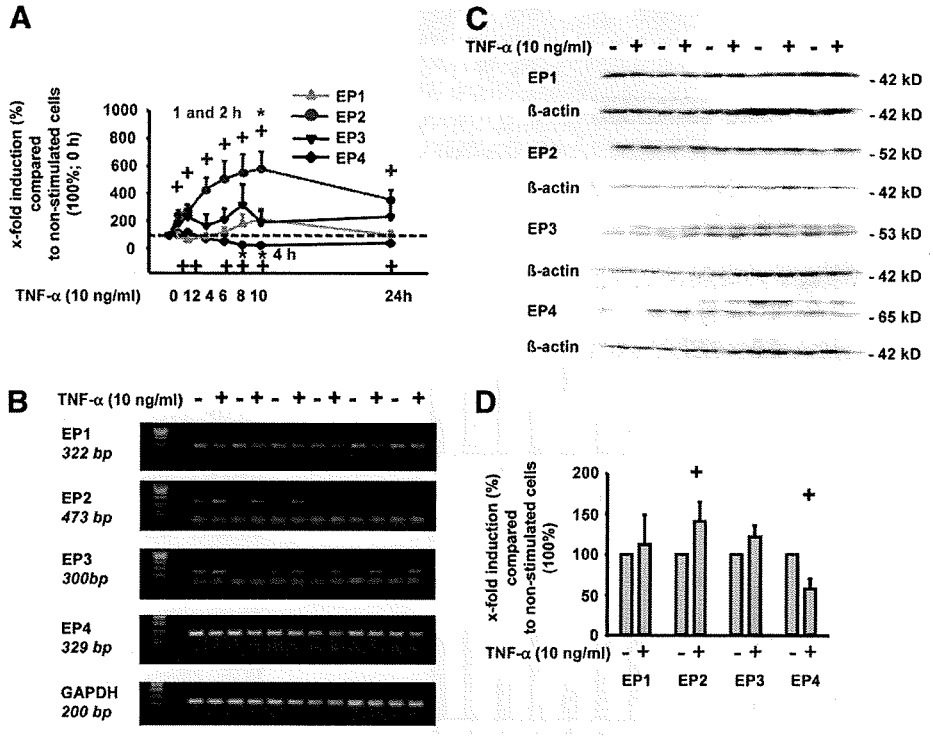

FIGURE 3. Influence of TNF-α on the EP receptor expression in RASFs. RASFs were stimulated with 10 ng/ml TNF-α for different time points (*A*), 8 h (*B*), or 30 h (*C* and *D*). EP receptor expression was analyzed by quantitative real-time RT-PCR (*A*) or conventional RT-PCR (*B*). To analyze the influence of TNF-α on EP receptor protein levels, protein extracts were subjected to Western blot analysis using specific Abs against the EP1, EP2, EP3, and EP4 receptors (*C*; quantification in *D*); means ± SEM for five patients with RA; +, $p \leq 0.05$ Mann-Whitney *U* test vs the 0 h time point (*A*) or vs culture without TNF-α (*D*); *, $p \leq 0.05$ Mann-Whitney *U* test vs indicated time points (*A*).

stimulation with TNF-α reduced EP4 mRNA levels and left EP1 and EP3 mRNA unchanged.

To confirm these data, Western blots of cell lysates were performed. As shown in Fig. 3*C*, RASF extracts contained all four EP receptor proteins (i.e., EP1 (42 kDa), EP2 (52 kDa), EP3 (53 kDa), and EP4 (65 kDa)). In agreement with the PCR data, TNF-α significantly up-regulated EP2 protein expression following stimulation for 30 h (1.4-fold; see quantification in Fig. 3*D*). In contrast, EP4 protein was significantly down-regulated (43% reduction), whereas EP1 or EP3 protein levels were not altered by TNF-α stimulation.

PGE_2 and selective EP receptor agonists modulate cAMP/PKA pathway activation by TNF-α and TNF-α-induced secretion of IL-6 and MMP-1 in RASFs

COX-2 inhibition with NS-398 completely prevented the increase in cAMP levels induced by TNF-α (Fig. 4*A*), further underlining

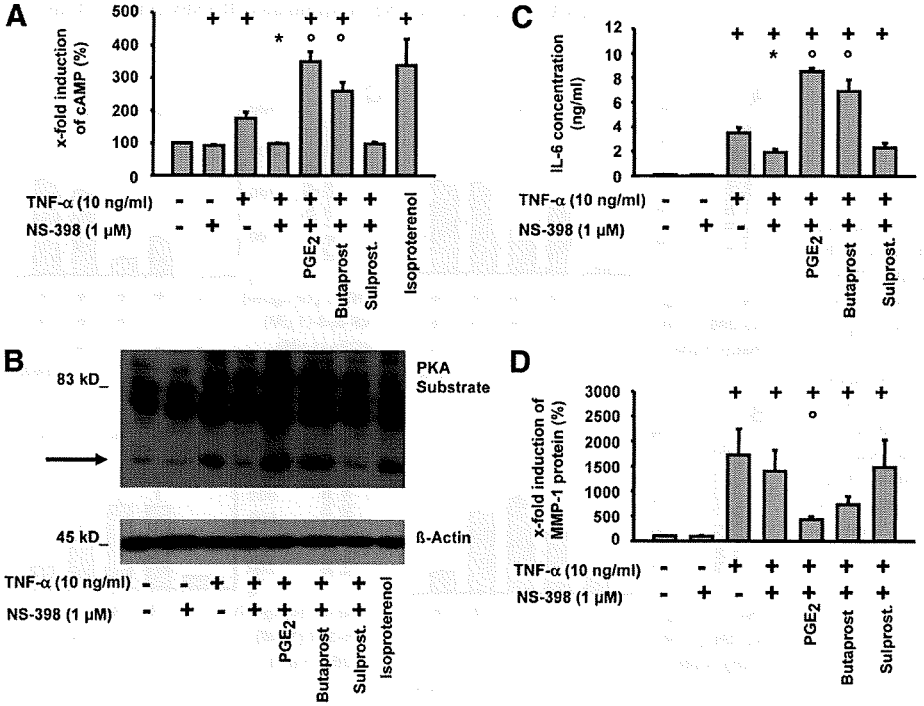

FIGURE 4. Role of EP2 and EP3 receptors in TNF-α-induced cAMP production, PKA substrate phosphorylation, and IL-6 or MMP-1 secretion in RASFs. Cells were stimulated with TNF-α (10 ng/ml) in the absence or presence of NS-398, PGE_2, butaprost, or sulprostone (Sulprost.; 1 μM each) with IBMX for 10 h. Intracellular cAMP was determined by RIA (*A*) and PKA substrate phosphorylation by Western blot (*B*); IL-6 secretion was analyzed by ELISA (*C*) and MMP-1 secretion by Western blot (*D*); means ± SEM for three patients with RA; +, $p \leq 0.05$ Mann-Whitney *U* test vs control; *, $p \leq 0.05$ Mann-Whitney *U* test vs TNF-α; ○, $p \leq 0.05$ Mann-Whitney *U* test vs TNF-α/NS-398.

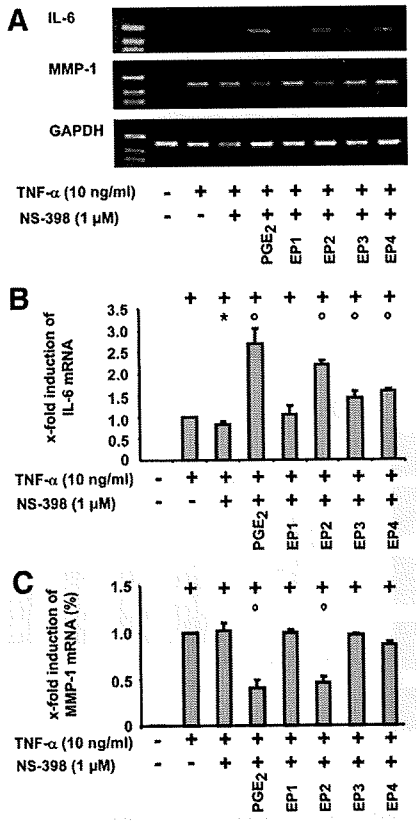

FIGURE 5. Effect of selective EP receptor agonists on the regulation of MMP-1 and IL-6 (RT-PCR). *A*, RASFs were harvested at 24 h after TNF-α (10 ng/ml) stimulation with or without NS-398 (1 μM), PGE_2 (1 μM), and/or selective agonists of the EP receptors 1–4 (10 μM each). mRNA expression of (*B*) IL-6 and (*C*) MMP-1 was detected by conventional RT-PCR. For each experiment, a value of 1 was assigned to the stimulation with TNF-α. Results are expressed as means ± SEM for three patients with RA; +, $p \leq 0.05$ Mann-Whitney *U* test vs control; *, $p \leq 0.05$ Mann-Whitney *U* test vs TNF-α; ○, $p \leq 0.05$ Mann-Whitney *U* test vs TNF-α/NS-398.

that PG release by TNF-α was involved in the up-regulation of cAMP levels (see also Fig. 2*A*). Accordingly, addition of PGE_2 or the EP2 selective agonist butaprost restored the increase in cAMP levels to a level above that in TNF-α/NS-398-treated cells. In contrast, the EP1/3-specific agonist sulprostone did not revert the blockade exerted by NS-398. All cAMP measurement data were also confirmed by phospho-(PKA-substrate) Western blots (Fig. 4*B*). The effects of PGE_2 were dose-dependent, both in the presence and the absence of NS-398 (supplemental Fig. 2).

To test whether the TNF-α/PGE_2/cAMP axis was physiologically relevant in RASFs, the IL-6 and MMP-1 secretion was examined under the same experimental conditions (Fig. 4, *C* and *D*). Except for the induction of IL-6 by TNF-α in the presence of NS-398, IL-6 production showed a pattern identical to that of cAMP levels and PKA activity (Fig. 4, *A–C*). EP3 signaling induced a marginal, nonsignificant raise of the mean IL-6 concentration compared with that of the TNF-α/NS-398 treatment group, however, with a consistent increase in the paired comparison for all three individual patients (supplemental Fig. 3).

In contrast, PGE_2-elicited signaling and, more specifically, EP2-dependent signals, diminished the MMP-1 secretion induced by TNF-α (Fig. 4*D*), demonstrating that PGE_2/EP2 signals have directly opposite effects on IL-6 and MMP-1 release by RASFs (for PGE_2 effects, see also Fig. 1).

Effects of PGE_2 and selective EP receptor agonists on TNF-α-induced expression of mRNAs for IL-6 and MMP-1 in RASFs

In the case of IL-6, the mRNA expression in comparison with TNF-α-stimulated RASFs in the presence of NS-398 was significantly increased by PGE_2 and selective receptor agonists for EP2 (3.2-fold and 2.7-fold, respectively), but also for EP3 and EP4 (1.7-fold and 1.9-fold, respectively; Fig. 5, *A* and *B*).

The above results for MMP-1 were confirmed with selective EP receptor agonists; that is, in the presence of COX-2 inhibitors only PGE_2 and the EP2 receptor agonist significantly suppressed the mRNA expression in TNF-α-stimulated RASFs (Fig. 5, *A* and *C*).

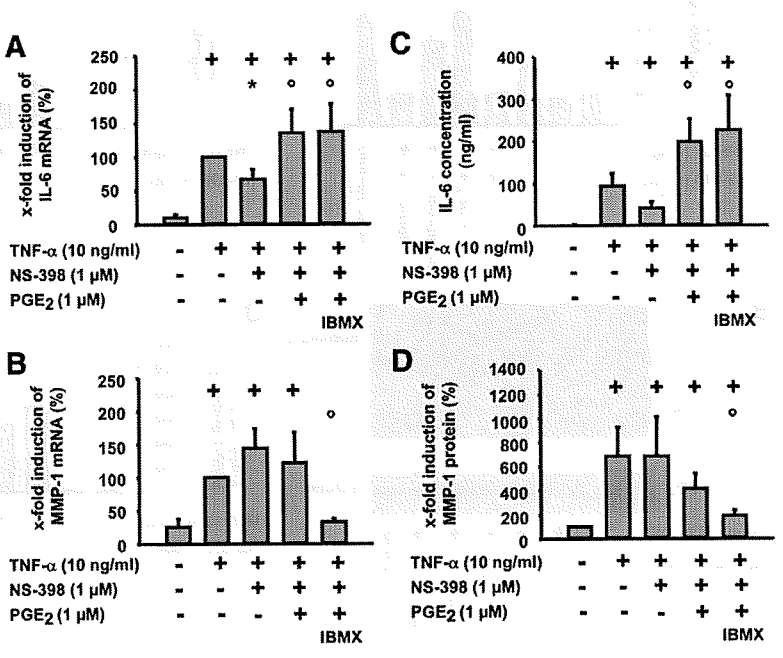

FIGURE 6. Effect of the phosphodiesterase inhibitor IBMX on the regulation of MMP-1 and IL-6 by PGE_2 (RT-PCR). RASFs were harvested at 24 h after TNF-α (10 ng/ml) stimulation with or without NS-398 (1 μM), PGE_2 (1 μM), and/or IBMX (100 μM). mRNA expression of IL-6 and MMP-1 was detected by real-time RT-PCR (*A* and *B*). IL-6 secretion was analyzed by ELISA (*C*), and MMP-1 secretion was analyzed by Western blot (*D*). For each experiment, a value of 100% was assigned to TNF-α. Results are expressed as means ± SEM for 3 patients with RA; +, $p \leq 0.05$ Mann-Whitney *U* test vs control; *, $p \leq 0.05$ Mann-Whitney *U* test vs TNF-α; ○, $p \leq 0.05$ Mann-Whitney *U* test vs TNF-α/NS-398.

Effect of the phosphodiesterase inhibitor IBMX on TNF-α-induced expression of mRNAs for IL-6 and MMP-1 in RASFs

To directly assess the relevance of cAMP for the effects of PGE_2 on the TNF-α-induced gene expression, the degradation of cAMP was inhibited by addition of IBMX. Strikingly, the mRNA expression and secretion of IL-6 remained unaffected (Fig. 6, *A* and *C*), whereas the mRNA expression and secretion of MMP-1 was further suppressed in the presence of IBMX (Fig. 6, *B* and *D*).

Discussion

This study demonstrates for the first time that PGE_2 has opposite effects on MMP-1 and IL-6 synthesis, uses different PGE_2 receptors for these effects, and differentially applies the postreceptor signaling molecule cAMP. Thus, PGE_2 is a differential key mediator of inflammatory/destructive functions in TNF-α-stimulated RASFs and may exhibit both proinflammatory (10, 11, 12, 25) and antidestructive capacities (Ref. 18 and the present study). These bipolar effects of PGE_2 in RASFs may also be the reason for the inefficacy of COX-2 inhibitors to arrest joint destruction and should be considered in future studies focused on the therapeutic inhibition of COX-1/2 in RA.

PGE_2 has opposite effects on the TNF-α-induced protein expression of MMP-1 and IL-6

In RASFs, TNF-α induces the secretion of proinflammatory and prodestructive mediators, for example, IL-6, PGE_2, and MMP-1 (present study and Refs. 9, 26). The stimulatory effect of PGE_2 on the TNF-α-induced IL-6 secretion in RASFs is in agreement with previously published data in IL-1β-stimulated RASFs (11, 14). However, the molecular mechanisms involved in the interplay between PGE_2 and TNF-α for the control of IL-6 secretion in RASFs have so far remained largely undeciphered.

In marked contrast to the effects on IL-6, TNF-α-induced MMP-1 secretion was significantly reduced by PGE_2, a finding also reported in IL-1β-stimulated RASFs (13, 27, 28). This clearly implicates PGE_2 as a negative feedback molecule in the signaling pathway linking TNF-α to MMP-1 production. Whether this involves phosphorylation of p53 or expression of NURR1, or else inhibition of Erk and NF-κB activation by PGE_2, as previously reported in the context of IL-1β/TNF-α signaling, remains to be investigated (13, 29, 31).

Interestingly, in TNF-α-stimulated periodontal ligament fibroblasts PGE_2 down-regulates MMP-13 (but not MMP-1 or MMP-3) (32, 33). This emphasizes the antidestructive properties of PGE_2, but also shows clear differences between fibroblasts of different origin. Contrasting results for IL-6 in periodontal ligament fibroblasts may also indicate cell-specific differences (34).

TNF-α induces an increase of intracellular cAMP and activation of PKA

The present study shows the novel finding that stimulation of RASFs with TNF-α induces an increased production of intracellular cAMP and PKA activity. Similar findings have been previously reported for IL-1β (35), suggesting that the cAMP system represents a critical regulatory pathway in RASFs.

With regard to the increased levels of intracellular cAMP induced by TNF-α, the participation of PGs appears plausible because previous data show an increase of intracellular cAMP in RASFs following PGE_2 stimulation (35, 36). In contrast to the slow effects of TNF-α, the increase of cAMP induced by PGE_2 occurred as early as 15 min after the start of stimulation (see Fig. 2*D*). This difference can be explained by the delayed synthesis of PGE_2 following TNF-α stimulation (Fig. 2*C*) (35). In turn, cAMP may directly contribute to a further increase of PGE_2 synthesis in RASFs (37). Concurrent with the increase of intracellular cAMP, TNF-α induced a phosphorylation of PKA substrates in RASFs in a strictly COX-2-dependent manner (see Fig. 4*B*), showing that the increase in cAMP translated into downstream PKA signaling (supplemental Fig. 4). This establishes PKA as a target of TNF-α (and PGE_2) in RASFs, a notion previously only inferred from the use of pharmacological inhibitors (H89) or activators (Rp-cAMP) (27, 28, 38).

Only the effects of PGE_2 on TNF-α-induced MMP-1 production are cAMP-dependent (but not those on IL-6 production)

Down-regulation of TNF-α-induced MMP-1 expression by PGE_2 was mediated via cAMP. Therefore, the cAMP increase may have partial specificity for the antidestructive properties of PGE_2, because phosphodiesterase IV inhibitors reduce joint damage in arthritis models (39, 40) or RA (41, 42) by further increasing cAMP levels. Also, cAMP-dependent regulation of MMP-1 has been shown after stimulation of RASFs with IL-1β (27), indicating partially common mechanisms for postreceptor signaling of these two pivotal proinflammatory cytokines.

The insensitivity of the augmentation of TNF-α-induced IL-6 expression by PGE_2 to an increase of cAMP suggests a relevance of other pathways. Indeed, cAMP-independent pathways (PI3K/ERK) are involved in the signaling of the EP4 receptor (43). Alternatively, IL-6 expression may only depend on cAMP at very high intracellular concentrations (44).

In contrast to our findings with TNF-α, Inoue et al. have reported that the regulation of IL-1β-induced IL-6 expression involves cAMP-dependent pathways (11) This may indicate specific and differential regulation of IL-6 expression by different proinflammatory cytokines well below the receptor level.

TNF-α differentially regulates the expression of EP receptors

The biological function of PGE_2 is mediated by four membrane-bound receptors (15, 16), all of which are expressed in RASFs (present study and Refs. 10, 11, 12, 45). In agreement with previously published data following IL-1β stimulation, TNF-α up-regulates the expression of the EP2 (and to some degree the EP3) (11, 45). Thus, up-regulation of EP2 and/or EP3 may be a widespread response to proinflammatory signals in RASFs. In contrast to previous reports (11), TNF-α down-regulated EP4. This difference may be explained by the different cytokines used for stimulation or by different culture conditions. The induction of the enzymes involved in the synthesis of PGE_2 (8, 46, 47), in conjunction with the up-regulation of certain EP receptors by proinflammatory cytokines (present study and Refs. 11, 45), suggests that the PGE_2 signaling cascade is tightly controlled by proinflammatory cytokines, not only at the level of PGE_2 synthesis, but also at the level of expression of particular prostanoid receptor subclasses.

Individual PGE_2 receptors differentially modulate the functional effects of TNF-α

Up-regulation of the EP2 receptor by TNF-α points to a prominent role of this receptor for the TNF-α/PGE_2-elicited signal in RASFs. This was confirmed by the PGE_2/TNF-α-induced increase of intracellular cAMP and phosphorylation of PKA substrates via EP2 (butaprost), but not via EP3 (Fig. 4, *A* and *B*), and, to a minor degree, by EP4 (under IBMX; supplemental Fig. 1). The unresponsiveness of intracellular cAMP levels to sulprostone/EP3 stimulation has been reported before for other cell types (48) and likely reflects the fact that EP3 receptors are mostly coupled to G_i proteins. The weaker ability of the EP4 receptor to stimulate cAMP formation compared with EP2 has also been described (45,

50). This may be due to rapid desensitization of the EP4 receptor via internalization (50, 51).

Analysis of the secretion of proinflammatory IL-6 and prodestructive MMP-1 further underlined the dominant role of the EP2 receptor. Although the IL-6 mRNA expression was significantly increased by stimulation of EP2, EP3, and EP4, the magnitude of IL-6 induction via EP2 (>2.5-fold) was larger than via EP3/EP4 (<2.0-fold). This is somewhat in contrast to the results of Inoue et al. (11), who reported that the IL-6 secretion in IL-1α-stimulated RASFs was only induced by agonists for the EP receptors 2 and 4. Explanations include the usage of different proinflammatory cytokines (IL-1β vs TNF-α), different agonist concentrations (20 nM vs 10 μM), and variable EP3 mRNA/protein expression (11). The selective regulation of MMP-1 via the EP2 receptor is a novel observation.

The dominant role of EP2 in the regulation of TNF-α-induced functions of RASFs suggests that the EP2 receptor is a potential therapeutic target in RA. However, the findings presented herein indicate that this point needs to be regarded with caution. In particular, the radically opposite consequences of PGE_2/EP2 signaling on TNF-α-induced IL-6 and MMP-1 secretion suggest that a blockade of EP2 activity, while being beneficial in reducing inflammatory parameters, may on the other hand exacerbate tissue destruction. Also, proinflammatory IL-6 is induced by PGE_2 predominantly via EP2, but to some degree also by EP3 and EP4, making difficult the exclusive targeting of just one EP receptor.

In agreement with the present data, previous reports have also shown an influence of both EP2 and EP4 on the secretion of IL-6 (11). However, the down-regulation of EP4 by TNF-α suggests a minor role of EP4 in RASFs. On the other hand, a possible involvement of the EP4 receptor in the pathogenesis of RA is supported by results in chondrocytes (52) and in animal models (20, 53, 54, 55). Taken together, the present data indicate that the concerted therapeutic manipulation of both the EP2 and EP4 receptors may represent a promising approach for the treatment of RA.

Acknowledgments

Bärbel Ukena, Bianca Lanick (Experimental Rheumatology Unit, University Hospital Jena, Jena, Germany) and Lihua Yang (Kentucky Clinic, University of Kentucky, Lexington, KY) are gratefully acknowledged for technical assistance, and Dr. Ernesta Palombo-Kinne for critical revision of the manuscript. We also thank Ono Pharmaceutical for providing us with PGE_2 receptor subtype agonists (ONO-DI-004, ONO-AE1-259, ONO-AE-248, and ONO-AE1-329).

Disclosures

The authors have no financial conflicts of interest.

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—Notes—

Erythrocyte Methotrexate-polyglutamate Assay Using Fluorescence Polarization Immunoassay Technique: Application to the Monitoring of Patients with Rheumatoid Arthritis

Sachie INOUE,[a] Masayuki HASHIGUCHI,[a] Shinichi KAWAI,[b] and Mayumi MOCHIZUKI*,[a]

[a]*Division for Evaluation and Analysis of Drug Information, Keio University Faculty of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan, and* [b]*Division of Rheumatology, Department of Internal Medicine (Omori), Toho University School of Medicine, 6-11-1 Omori-Nishi, Ota-ku, Tokyo 143-8541, Japan*

(Received January 30, 2009; Accepted April 17, 2009)

Methotrexate (MTX), *i.e.*, MTX-polygluatmate 1 (MTX-PG_1), exerts its antirheumatic effects mainly by ≤ 6 (MTX-PG_{2-7}) *via* folypolyglutamyl synthase in cells. The authors developed a new method using fluorescence polarization immunoassay to determine MTX-PG_{1-7} concentrations in erythrocytes (RBC). MTX-PG_{2-7} in RBC of rheumatoid arthritis (RA) patients receiving MTX was converted to MTX in the presence of plasma γ-glutamyl hydrolase and mercaptoethanol at 37°C. The MTX in RBC was extracted in a perchloric acid deproteinization step then on a solid-phase extraction column. The concentration of MTX was measured by TDX analyzer. The mean MTX recovery rate was 76.1% ($n=8$). The intraday and interday coefficients of variation were $<11.3\%$ ($n=8$) and $<12.4\%$ ($n=3$), respectively, at low and high concentrations (30–300 nmol/l). The calibration curve was linear over the range 30–300 nmol/l. The total concentration of MTX-PGs (mean$\pm$S.D.) in RBC obtained from 95 Japanese RA patient blood samples was 97.3 ± 8.1 nmol/l for the MTX dose of 0.13 ± 0.05 mg/week/kg. This newly developed method for the quantification of MTX-PGs in RBC is sensitive and accurate and can be applied for routine monitoring of MTX therapy in RA patients.

Key words——methotrexate; methotrexate-polyglutamate; rheumatoid arthritis; fluorescence polarization immunoassay

INTRODUCTION

Methotrexate (MTX), *i.e.*, MTX-polyglutamate 1 (MTX-PG_1), is the most widely used disease-modifying antirheumatic drug in the treatment of rheumatoid arthritis (RA).[1,2] However, it is known that there are large interindividual differences in the response to MTX therapy in patients with RA.[3,4] Those differences are mainly thought due to variations in individual pharmacokinetics of MTX.[5] Once MTX is transported into the cell, it progressively adds ≤ 6 MTX polyglutamates (MTX-PG_{2-7}) *via* folypolyglutamyl synthetase (FPGS) and is retained within cells for a long time. On the other hand, MTX-PG_{2-7} is converted back into MTX by γ-glutamyl hydrolase (GGH), and MTX is transported to the extracellular space.[5-8] Therefore intracellular MTX-PG_{1-7} (MTX-PGs) may be the main compounds exerting the antirheumatic effects of MTX; thus monitoring MTX-PGs in erythrocytes (RBC) as a substitute[7,9] for the MTX-PG concentration in target cells such as mononuclear cells, lymphocytes, or synovial cells could be helpful in adjusting the optimal MTX dose in RA patients. Although several methods to determine the concentration of MTX-PGs in RBC[10-12] have been developed, the concentrations of MTX-PGs in RBC are extremely low, and a method to determine MTX-PGs in RBC has not been completely established.

The present report describes a simple and accurate method for determination of MTX-PGs in RBC using the fluorescence polarization immunoassay (FPIA) method and its usefulness in monitoring RA patients receiving MTX.

MATERIALS AND METHODS

Chemicals MTX and lyophilized plasma were purchased from Sigma-Aldrich (Tokyo, Japan). Mercaptoethanol, perchloric acid, potassium hydroxide, dipotassium hydrogenphosphate, potassium dihydrogenphosphate, and methanol were obtained from Wako Pure Chemical Industries (Tokyo, Japan). An Oasis HLB 30 mg/ml solid extraction column was purchased from Waters (Tokyo, Japan).

Drug Solutions MTX was dissolved in 0.1 M potassium hydroxide. After dissolution, stock solu-

*e-mail: mochizuki-my@pha.keio.ac.jp

tions were prepared by diluting to 100 μmol/l in water. Working standard solutions of MTX in water (6, 4, 2, 1, 0.6 μmol/l) were prepared from stock solutions by serial dilution. The 100 μmol/l stock solutions were stable at −80°C for ≥12 months. Drug-free RBC used for validation of the method were obtained from 5 healthy volunteers. Calibrators were prepared by adding known amounts of working standard solutions to 190 μl of blank RBC to obtain the final concentrations of 300, 200, 100, 50, and 30 nmol/l for MTX.

Extraction Procedure Total MTX-PGs in RBC were measured after conversion from MTX-PG_{2-7} to MTX as follows. Four hundred microliters of reconstituted plasma (source of GGH) was added to 200 μl of RBC hemolysates. The RBC were disrupted in a freeze-thaw cycle. After mixing the sample for 30 s, 400 μl of buffer containing 100 mmol/l of potassium phosphate (pH 7.4) and 150 mmol/l of mercaptoethanol was added to the sample. After incubating the sample at 37°C for 12–14 h, MTX-PG_{2-7} was converted to MTX. After incubation and cooling, 100 μl of 70% perchloric acid was added to the mixture. After 10 s of vortex-mixing, the mixture was centrifuged at 10000 rpm for 5 min. Seven hundred fifty microliters of the supernatant solution was injected into the solid-phase extraction sorbent. After washing with 1 ml of 5% methanol and 500 μl of 20% methanol, MTX was eluted with 2 ml of methanol. The eluate was evaporated under nitrogen at 60°C, and the residue was dissolved in 130 μl of drug-free plasma. Next, a 65 μl aliquot was injected into a TDX analyzer (Abbott, Tokyo, Japan).

Patient Samples Ninety-five samples of heparinized peripheral whole blood (5 ml) were obtained from 36 Japanese RA patients comprising 31 women and 5 men whose mean (±S.D.) age and body weight were 58.8±2.5 years and 53.3±1.7 kg, respectively, and who had been receiving the same dose of MTX for>1 month. After a 10-min centrifugation step to separate plasma and RBC, the RBC were washed twice with two volumes of saline. All collected blood samples were stored at 4°C, and RBC and plasma were separated within 48 h.[12] RBC were stored at −8 °C until analysis. This study protocol was approved by the Institutional Review Board of the Kitasato Institute Hospital and written informed consent for participation was given by all patients.

RESULTS

Recovery and Linearity The recovery rate was determined by assaying blank RBC spiked with 5 different known concentrations (30–300 nmol/l) and blank plasma spiked with same amounts of MTX in 8 replicates. The results are shown in Table 1. The recovery rate ranged from 74.6% to 78.1% (76.1±7.5; mean±S.D.; $n=8$). The analysis of calibrators gave a linear curve over the range tested (Fig. 1). The linearity curves were defined by the following equation: $y=0.7658x+2.5629$ ($r^2=>0.999$), where y is the measured concentration of RBC in samples indicated by the TDX analyzer and x is the spiked MTX concentration.

Precision Intraday and interday precision determined by assaying blank RBC spiked with 5 different known concentrations of MTX is shown in Table 2. Intraday precision was assessed by assaying 8 samples at 5 drug concentrations (30, 50, 100, 200, and 300 nmol/l). Interday precision was evaluated by assaying 3 samples at 5 concentrations (30, 50, 100,

Table 1. Methotrexate Recovery Rates

MTX concentration (nmol/l)	Recovery rate (%) (mean±S.D., $n=8$)
30	78.1±8.8
50	77.1±8.6
100	75.0±8.1
200	75.6±6.8
300	74.6±6.2

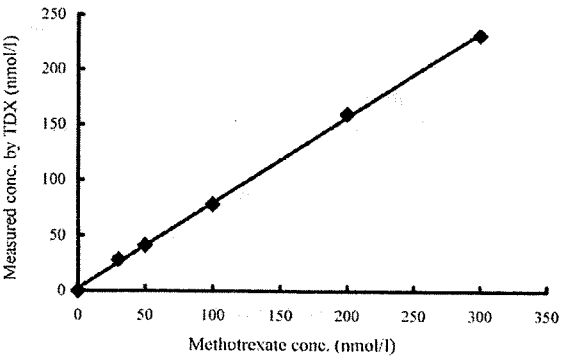

Fig. 1. Linear Regression Curves of the Concentration of Methotrexate Determined by TDX Analyzer and Spiked Methotrexate in the Calibrators

Table 2. Precision of Determination of Methotrexate in Spiked Erythrocytes

Spiked MTX concentration (nmol/l)	Intraday variation (n=8)		Interday variation (n=3)	
	Measured concentration (nmol/l) (mean±S.D.)	CV (%)	Measured concentration (nmol/l) (mean±S.D.)	CV (%)
30	23.44±2.65	11.30	28.33±3.49	12.33
50	38.54±4.31	11.19	40.89±4.95	12.12
100	81.48±8.33	10.23	77.86±6.26	8.04
200	151.25±13.56	8.97	158.49±9.09	5.74
300	223.66±18.50	8.27	230.64±12.36	5.35

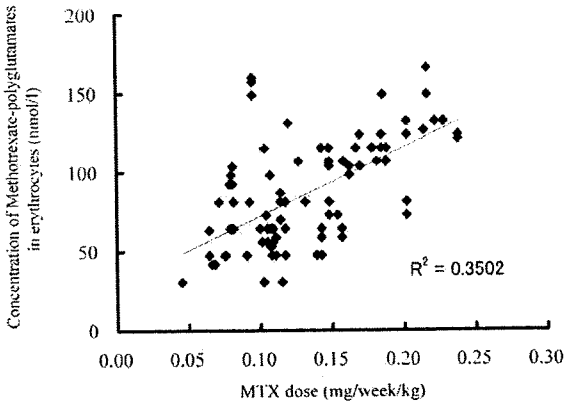

Fig. 2. Methotrexate Polyglutamate Concentration in Erythrocytes *versus* Methotrexate Dose in 95 Blood Samples from Japanese Rheumatoid Arthritis (RA) Patients

200, and 300 nmol/l) on 5 days. Precision was quantitated by calculating intraday and interday coefficients of variation (CVs) according to the formula: [(SD/mean measured concentration)×100]. In the determined range, intraday and interday CVs were < 11.3% and <12.3%, respectively. The limit of quantification for MTX was 30 nmol/l.

Patient Samples The concentrations of MTX formed from MTX-PG_{2-7} by enzymatic conversion could be measured in all 95 blood samples. The mean MTX-PG concentration in RBC was 97.3±8.1 nmol/l for the MTX dose of 0.13±0.05 mg/week/kg (Fig. 2).

DISCUSSION

Although numerous methods have been developed for the determination of plasma MTX concentrations,[13-15] there are few methods for the quantification of MTX-PG concentrations in RBC. Because the RBC matrix is very complex, accurate quantification of MTX-PGs in RBC is difficult. Moreover, in RA patients the MTX dose is markedly lower than that administered to patients with leukemia and other cancers. To overcome these limitations, several investigators attempted to develop methods to determine MTX-PG concentrations in RBC. However, those methods had several disadvantages.[12] The radiochemical ligand-binding assay developed by Kamen *et al.*[10] and Kamen and Winick[16] is time-consuming and requires the use of radioisotopes. An alternative method, the dihydrofolate reductase inhibition enzymatic assay developed by Shroeder and Heinsvig[11] and Imbert *et al.*[17] lacks specificity. The recently developed HPLC-fluorometry approach[12,18] is sensitive and accurate but uses the specific technique of postcolumn photooxidation with ultraviolet irradiation. Therefore we developed an easier-to-use, more accurate method for the quantification of MTX-PGs in RBC. It was confirmed that MTX-PG_{2-7} (the pool of 6 MTX-PGs) was converted completely to MTX in the presence of plasma GGH and mercaptoethanol (the sulfhydryl donor for GGH) with an 8-h incubation step at 37°C in the dark at an RBC concentration of 1000 nmol/l.[19-21] Therefore according to previous reports we judged that MTX-PG_{2-7} in RBC of RA patients receiving MTX was converted completely to MTX under our incubation conditions. In a preliminary study, we examined the experimental conditions for the conversion from MTX-PG_{2-7} to MTX and confirmed that the concentration of MTX reached plateau after incubating RBC samples (n=5/patient) obtained from 3 RA patients receiving MTX at 37°C for 8–14 h in the dark (data not shown). Using a

solid column sorbent, the present method allowed determination of the concentration of MTX-PGs in RBC using the TDX analyzer. It has been confirmed that the cross-reactivity of MTX with a folic acid compound, MTX metabolites, and other chemical analogues was $<1\%$ in the measurement range of the TDX analyzer. Recently, Hayashi *et al.*[24] reported a method for determination of MTX-PGs in RBC using TDX analyzer. This method differs from our developed method in the following respect that they measured all MTX-PG_{2-7} directly without hydrolyzing them to MTX, basing on their observation that anti-MTX monoclonal antibody shows reactivity to MTX-PG_7 as equal to MTX.

Our newly developed method was applied to the quantification of MTX-PGs in RBC of 95 blood samples from 36 Japanese RA patients receiving MTX. The MTX dose ranged from 2.0 to 13.0 mg/week, and the MTX-PGs in all RBC samples could be quantitated.

Our method has certain limitations. This method cannot measure each MTX-PG compound concentration separately. Dervieux *et al.*[22] postulated that longer-chain MTX-PGs (MTX-PG_{4-7}) in RBC might correlate more closely than total MTX-PGs with the response to MTX therapy in RA patients. Another report commented on the relation of the total MTX-PG concentration and therapeutic response to MTX.[23]

In conclusion, we developed an accurate and sensitive method for quantification of MTX-PGs in RBC. This method can be applied to routine monitoring of MTX therapy in RA patients to adjust the individual optimal MTX dose. The TDX analyzer is a commonly used measuring instrument; therefore monitoring using our newly developed method can be adopted to various clinical practices.

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Mod Rheumatol (2009) 19:351–357
DOI 10.1007/s10165-009-0197-6

REVIEW ARTICLE

Japan College of Rheumatology 2009 guidelines for the use of tocilizumab, a humanized anti-interleukin-6 receptor monoclonal antibody, in rheumatoid arthritis

Ryuji Koike · Masayoshi Harigai · Tatsuya Atsumi · Koichi Amano · Shinichi Kawai · Kazuyoshi Saito · Tomoyuki Saito · Masahiro Yamamura · Tsukasa Matsubara · Nobuyuki Miyasaka

Received: 18 March 2009 / Accepted: 8 June 2009 / Published online: 10 July 2009

Abstract The introduction of biological agents targeting tumor necrosis factor-alpha (TNF-α) has brought about a paradigm shift in the treatment of rheumatoid arthritis (RA). Although these anti-TNF agents have excellent efficacy against RA, a substantial number of patients still show inadequate responses. In Western countries, such patients are already being treated with new classes of antirheumatic drugs such as abatacept and rituximab. Tocilizumab (TCZ) is a humanized monoclonal antibody developed in Japan against the human interleukin-6 (IL-6) receptor. TCZ does not only alleviate the signs and symptoms of RA but also seems to prevent progressive bone and joint destruction. However, there is a concern that TCZ might increase the risk of adverse events such as infections since IL-6 plays a pivotal role in the immune system. Calculating the relative risks of specific adverse outcomes with TCZ use remains difficult, due to insufficient patient numbers enrolled in clinical trials to date. This review presents tentative guidelines for the use of TCZ for RA patients prepared by the Japan College of Rheumatology and based on results of clinical trials in Japan and Western countries. The guidelines are intended as a guide for postmarketing surveillance and clinical practice, and will be revised periodically based on the surveillance.

Keywords Rheumatoid arthritis · Tocilizumab · Anti-TNF · IL-6 · Antirheumatics

Introduction

The introduction of biological agents has significantly altered the natural course of rheumatoid arthritis (RA) and

R. Koike (✉) · M. Harigai
Department of Pharmacovigilance, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
e-mail: koike.rheu@tmd.ac.jp

R. Koike
Clinical Research Center, Tokyo Medical and Dental University Hospital, Faculty of Medicine, Tokyo, Japan

R. Koike · M. Harigai · N. Miyasaka
Department of Medicine and Rheumatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

T. Atsumi
Department of Medicine II, Hokkaido University Graduate School of Medicine, Sapporo, Japan

K. Amano
Division of Rheumatology and Clinical Immunology, Saitama Medical Center, Saitama Medical University, Kawagoe, Japan

S. Kawai
Division of Rheumatology, Toho University Omori Medical Center, Tokyo, Japan

K. Saito
The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Japan, Kitakyushu, Japan

T. Saito
Department of Orthopaedic Surgery, Yokohama City University, Graduate School of Medicine, Yokohama, Japan

M. Yamamura
Department of Rheumatology, Aichi Medical University School of Medicine, Aichi, Japan

T. Matsubara
Matsubara Mayflower Hospital, Kato, Japan

associated joint destruction. Anti-tumor necrosis factor-alpha (TNF-α) agents, infliximab and etanercept, show excellent efficacy in Japanese RA patients. However, the use of these anti-TNFs has been linked to potentially serious adverse effects, particularly infections and malignancies. The Japan College of Rheumatology (JCR) therefore released specific guidelines developed specifically for the proper use of these agents [1].

Tocilizumab (TCZ) is a humanized anti-human interleukin-6 receptor (IL-6R) monoclonal antibody developed recently in Japan. TCZ binds to the alpha chain of both membrane-bound and soluble IL-6R, thus blocking IL-6/IL-6R signaling [2, 3]. In the recent Japanese clinical trial of TCZ monotherapy, 80.3% of patients on 8 mg/kg TCZ achieved the American College of Rheumatology improvement criteria 20% (ACR20) response [4]. TCZ does not only suppress the signs and symptoms of RA but also inhibits bone and joint destruction in RA patients [5]. In the European phase III clinical trial, 50.0% of patients on 8 mg/kg TCZ achieved ACR20 at 24 weeks [6]. It is reported that TCZ is effective even in RA patients refractory to anti-TNF biologics [6]. TCZ is also effective against multicentric Castleman disease [7] and juvenile inflammatory arthritis (JIA) [8], and as of January 2009, TCZ has been approved for clinical use against RA as well as these two conditions, but only in Japan.

Since TCZ is a drug with a new mode of action, unexpected adverse effects may be encountered when used in a variety of RA patients in daily practice. Although both efficacy and safety profiles of TCZ for RA have been substantiated in clinical trials, the relatively small patient numbers to date preclude accurate detection and risk assessment of adverse events associated with this drug, particularly rare occurrences. Furthermore, patients enrolled in clinical trials are carefully selected based on both inclusion and exclusion criteria and thus differ from those in daily practice in terms of risk analysis.

The Ministry of Health, Labor, and Welfare (MHLW) commissioned Chugai Pharmaceutical Company to undertake a postmarketing surveillance of the initial 3,000 cases for adverse events. Based on this, and data of clinical trials conducted in Japan and Western countries, the JCR formulated official guidelines for the use of TCZ in Japan [9] (Table 1). The guidelines are equivalent to a "clinical guide to use" and subject to change periodically based on the pharmacovigilance activities in Japan. The JCR also organized a committee for the medical treatment of rheumatic disease. The committee designed a proposal for the initial guidelines on the safe use of TCZ, which were then discussed and approved by the JCR board of directors. The guidelines were subject to the standard journal review process.

Eligibility and inclusion criteria for TCZ

TCZ is recommended for patients who are diagnosed by the American College of Rheumatology 1987 classification criteria for the diagnosis of RA [10], and who show inadequate response despite treatment for at least 3 months with the maximum permissible dose of one of the nonbiologic disease-modifying antirheumatic drugs (DMARDs) [methotrexate (MTX), bucillamine, sulfasalazine, leflunomide, or tacrolimus] rated as "recommendation A level" in the diagnostic manual and evidence-based treatment guidelines [11] developed by the study group of the MHLW, or tacrolimus approved as DMARD only in Japan with some efficacy data [12, 13]. Patients showing inadequate response to the biologic DMARDs approved in Japan (infliximab, etanercept, and adalimumab) are also included. Inadequate response to previous treatment is defined by the presence of at least six tender joints and swollen joints, and either C-reactive protein (CRP) levels of at least 2.0 mg/dl or an erythrocyte sedimentation rate (ESR) of at least 28 mm/h.

To avoid potential opportunistic infections, patients should have a peripheral leukocyte count of 4,000/mm^3 or more, a peripheral lymphocyte count of 1,000/mm^3 or more, and a negative test for blood β-D-glucan. These criteria are similar to those set for the use of anti-TNF agents in RA patients [1] and are based on the important role played by cellular immunity against opportunistic infections caused by *Mycobacterium tuberculosis* or fungi such as *Pneumocystis jiroveci*, and that these infections are likely to occur in patients with low peripheral lymphocyte counts [14]. A test for blood β-D-glucan, a component of fungi, has been included in the diagnosis of fungal infections, especially those with *Pneumocystis jiroveci*.

Exclusion criteria for TCZ

Active infection

TCZ is contraindicated in patients with ongoing infections, as is the case for other biologics. IL-6 is the major inducer of inflammatory responses against infection [15], thus TCZ could mask signs of infection such as fever, general malaise, and elevation of surrogate markers including CRP and ESR, and consequently make early diagnosis of infections more difficult. However, symptoms such as cough, sputum, and dyspnea are not masked with TCZ. Leukocytosis and a left shift in neutrophil count are also not suppressed by TCZ. Furthermore, radiographic analysis can be used to detect early-stage respiratory infections.

As a precaution against tuberculosis, candidate patients for treatment with TCZ must also undergo a thorough review of family and past history of tuberculosis, chest

Table 1 Guidelines for the use of tocilizumab (TCZ) for treatment of rheumatoid arthritis

Eligibility and inclusion criteria

1. Patients fulfilling the American College of Rheumatology 1987 Classification criteria for the diagnosis of rheumatoid arthritis (RA) and showing inadequate response to conventional treatment. Inadequate response of RA to previous treatment is defined as the presence of the following three clinical findings:

   Tender joints ≥6

   Swollen joints ≥6

   ESR ≥28 mm/h or CRP ≥2.0 mg/dl
2. Patients showing inadequate control despite treatment for at least 3 months with standard doses of conventional biological or nonbiological disease-modifying antirheumatic drugs (DMARDs) [e.g., methotrexate (MTX), bucillamine, leflunomide, tacrolimus, sulfasalazine, biological DMARD infliximab, etanercept, and adalimumab]
3. It is recommended that patients should have the following laboratory test results in order to avoid potential opportunistic infections:

   WBC ≥4,000/mm^3

   Peripheral blood lymphocytes ≥1,000/mm^3

   Serum or plasma (1 to >3)-β-D-glucan: negative

Exclusion criteria

1. Active infection

   Bacterial infection

   Mycobacterial infection including latent tuberculosis defined by history, purified protein derivative (PPD) skin test, radiography, or interferon-γ releasing assay

   Chronic active Epstein–Barr virus (CAEBV) infection defined by detection of EBV genome in peripheral blood

   Active hepatitis B virus (HBV) infection
2. History of serious hypersensitivity reaction against TCZ

Dosage of TCZ

Administer 8 mg/kg diluted in 100–250 ml saline by drip infusion every 4 weeks.

When starting infusion, observe the patient carefully. In the absence of abnormal symptoms, drip infusion should be completed in about 1 h.

Cautions

There are several issues related to safety of TCZ. When starting or continuing TCZ therapy, it is necessary to notify and be prepared for the following complications, medical interventions, and/or conditions:

1. *Infection.* Infectious disease, especially respiratory infection, is the most important based on its frequency and severity. When symptoms, such as fever, cough, and dyspnea, occur under TCZ therapy, it is necessary to notify the condition and consult the diagnostic flow chart shown in Fig. 1

   Bacterial pneumonia

   Tuberculosis and nontuberculous mycobacterial infection

   Pneumocystis pneumonia
2. *Bowel perforation and secondary peritonitis.* Several cases with bowel perforation followed by peritonitis have been reported.
3. *Hyperlipidemia.* TCZ therapy is reported to increase serum levels of lipids, cholesterol, and/or triglycerides.
4. *Transient neutropenia.* Neutropenia is reported in patients on TCZ therapy although the majority of cases are transient and often mild in nature.
5. *Use with other DMARDs.* TCZ has been approved for use without concomitant DMARDs in Japan. When considering combining TCZ with other DMARDs, it is necessary to evaluate the indications and to monitor the adverse events with utmost care.
6. *Serious infusion reactions.* Severe infusion reactions may require airway maintenance, oxygen inhalation, subcutaneous epinephrine, and intravenous corticosteroid.
7. *Surgery under TCZ therapy.* Because TCZ might delay wound healing and mask surgical-site infection, surgery should be postponed until clearance of drug from the bloodstream, i.e., for at least 14 days after the last infusion of the drug.
8. *Use during pregnancy and lactation.* Avoid use of TCZ in pregnant and lactating women due to concerns regarding safety for embryos and neonates.
9. *Use in patients with malignancies.* Consider carefully prescription of TCZ for patients with precancerous lesions or recent history of neoplasm because it remains unclear whether TCZ affects carcinogenesis or not.

radiography, and a purified protein derivative (PPD) skin test. Suspected abnormalities on chest radiography should be further investigated by computed tomography of the chest. TCZ is contraindicated in patients with abnormalities on chest radiography such as linear opacities, calcification larger than 5 mm, and pleural thickening suggestive of old tuberculosis, and in individuals with pulmonary or extrapulmonary tuberculosis. However,

treatment with TCZ may be considered together with antituberculosis agents only if the potential benefits outweigh the potential risks. In patients with a strongly positive PPD skin test (presence of induration) or radiographic opacities suggestive of old pulmonary tuberculosis, treatment with isoniazid (0.3 g/day or 5 mg/kg for low-body-weight patients) should be initiated at least 3 weeks prior to administration of TCZ and continued for the subsequent 6–9 months. It is advisable that patients with active nontuberculous mycobacterial infection are excluded from TCZ treatment because it is often difficult to control these infections.

It remains unclear whether blocking the IL-6/IL-6R pathway directly induces the proliferation or activation of herpes group viruses such as Epstein–Barr virus (EBV). However, it would be better for patients with active EBV infection, defined by detection of EBV genes in peripheral blood, to avoid treatment with TCZ. One case died from hemophagocytosis associated with reactivation of EBV following a single infusion of TCZ [16].

It remains controversial whether TCZ also affects viral loads of hepatitis B virus (HBV) and hepatitis C virus (HCV). However, patients with active HBV infection should be excluded because hepatitis B infection is often exacerbated by various immunosuppressants [17].

Past history of serious infusion reaction

Although severe or life-threatening infusion reactions following TCZ administration are rare, a few cases with acute reaction have been reported [4–6, 21]. History of serious infusion reaction against TCZ is a contraindication, in line with the manufacturer's recommendation.

Use of TCZ

The recommended dosage and administration route of TCZ in Japan is 8 mg/kg given once monthly as an intravenous infusion over 1 h. Drug-related infusion reactions were reported in clinical trials, although most were mild [4–6, 21]. Simultaneous administration of MTX is not mandatory, since clinical trials conducted in Japan were performed with monotherapy.

Because TCZ can suppress elevation of CRP and ESR, it is inadequate to evaluate its effectiveness by clinical scores. Rather, it is recommended that any such evaluation should be based on tender joint and swollen joint counts, or on calculated scores that do not rely on CRP or ESR, e.g., the clinical disease activity index (CDAI) [18]. In addition, the efficacy of TCZ should be evaluated after at least 3 months of treatment because stable responses to TCZ were only accomplished after such period in the published clinical trials [4, 5].

Cautions for the safe use of TCZ

Infections

Infections are the most common adverse events during TCZ therapy, although serious outcomes are rare [4–6, 19–22]. In addition, all infections improve by appropriate treatment, and no prolongation of treatment due to infection has been documented. Since pneumonia is a particularly common and serious infection in RA patients [23], the JCR has developed a specific algorithm for the differential diagnosis of pneumonia (Fig. 1).

No specific pathogens were detected in patients with pneumonia or other infections related to TCZ treatment [4–6, 19–22]. No increase in tuberculosis was observed with TCZ treatment [4, 5, 20–22], compared with the anti-TNF agents [24]. However, TCZ therapy may significantly alter the onset or clinical course of pneumonia. There are anecdotal reports that patients presenting with minimal clinical symptoms develop severe pneumonia with shock within 1 day of the start of TCZ therapy [25]. Sufficient evidence indicates that TCZ can suppress the elevation of CRP and fever even in infections. CRP and fever are not suitable markers of early stages of infection. Suppression of inflammatory responses might delay the early diagnosis of infections, especially pneumonia, and both patients and physicians should be aware of this effect during TCZ therapy.

Epidemiological studies have identified the risk factors for infection in RA patients. These include advanced age [23, 26], pulmonary comorbidities [23, 26], corticosteroid use [23, 26], and impaired daily activity [21]. Patients with these risk factors should be considered more susceptible to infections during TCZ therapy, and tapering or ceasing corticosteroid use is strongly recommended. Patients should also be vaccinated prior to or even during TCZ treatment against influenza and pneumococcus if over 65 years old. TCZ does not alter the response to vaccination against influenza or pneumococcus [27, 28].

Bowel perforation

Several cases of bowel perforation followed by peritonitis were reported in the clinical trials of TCZ [29]. Although this adverse event is extremely rare, it is more infrequent with other biologics including anti-TNF agents [30, 31]. Postmarketing surveillance should explore whether this adverse event is specific to TCZ. In this regard, serum IL-6 level was elevated specifically prior to intestinal perforation [32, 33], and it is possible that TCZ could mask the early symptoms of diverticulitis and hamper healing by its inhibitory effect on IL-6/IL-6R signaling.