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Mod Rheumatol (2009) 19:329–333
DOI 10.1007/s10165-009-0158-0

CASE REPORT

Pharmacokinetic study and Fcγ receptor gene analysis in two patients with rheumatoid arthritis controlled by low-dose infliximab

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Received: 10 November 2008 / Accepted: 30 January 2009 / Published online: 3 March 2009

Abstract The main aim of this study is to investigate the pharmacokinetics of infliximab and Fcγ receptor (FcγR) polymorphism in two patients with rheumatoid arthritis (RA) who were well controlled by low-dose infliximab. A 57-year-old woman (Patient 1) and a 67-year-old woman (Patient 2) had active RA despite methotrexate and prednisolone treatments. They improved after the addition of infliximab (3 mg/kg), but developed pneumonia and sepsis, respectively. Although the infliximab doses were reduced to 1.5 mg/kg and 1 mg/kg, respectively, clinical improvements were maintained. Blood samples were obtained at 1 h after infliximab administration and at eight weeks (just before the next dose). The elimination half-life was determined by the serum concentration of infliximab. We also analyzed the polymorphisms of FcγRIIA, FcγRIIIA, and FcγRIIIB for the genomic DNA samples from the two patients and three controls. Amplification of the FcγR-genomic regions in allotype-specific polymerase chain reactions was used to distinguish the genotypes. Decresed clearance of infliximab was proven by a pharmacokinetic study of these patients under low-dose infliximab therapy. 131H/H (FcγRIIA) and 176F/F (FcγRIIIA) were detected in both patients. NA1/NA2 and NA2/NA2 (FcγRIIIB) were detected in Patients 1 and 2, respectively. These patients were well controlled over the long term by low-dose infliximab. The mechanism of the reduced clearance of infliximab might possibly be explained in part by the FcγR polymorphisms.

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Keywords Infliximab · Pharmacokinetics · Fcγ receptor · Polymorphism · Rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that eventually causes the destruction of cartilage and/or bone [1]. The pathogenesis of RA is considered to be related to several proinflammatory cytokines, including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) [2]. TNF-α inhibitors have become available in recent years and are reported to prevent or alleviate inflammation and joint destruction in RA patients [3–5]. The TNF-α inhibitor infliximab is a chimeric anti-human TNF-α monoclonal antibody composed of the variable region of a mouse anti-human TNF-α monoclonal antibody and the constant regions of human IgG1, including the Fc region [6]. It is reported to be effective for RA, and a dose of 3 mg/kg or more is usually administered every four or eight weeks [3].

When human IgG binds to a target antigen and forms an immune complex, it is degraded through Fcγ receptor (FcγR)-mediated phagocytosis [7]. The FcγR is categorized into subclasses I, II, and III, and then these subclasses are further categorized into a number of subtypes. Polymorphisms of various FcγR subtypes have already been reported [8]. The affinity of FcγR binding to the Fc region of IgG differs between these polymorphisms, and differences in the binding of IgG to the Fc region may affect the clearance of immune complexes. This suggests that the clearance of antibody preparations (including infliximab) from the blood will vary between FcγR polymorphisms.

Here we report on two patients with RA who were well controlled for a long period by low doses of infliximab.

Accordingly, we investigated the pharmacokinetics of infliximab and the FcγR polymorphisms in these two patients.

Patients and methods

Subjects

We investigated two RA patients who were well controlled by low-dose infliximab therapy. Both patients fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA [9]. In order to assess FcγR polymorphisms, three healthy volunteers were also investigated. This study was approved by the Ethics Committee of Toho University School of Medicine (No. 17043). We explained the objectives of the study to the patients and controls, and obtained their written informed consent to participation.

Patient 1

The patient was a 57-year-old woman with RA for nine years. Despite treatment with methotrexate (6 mg/week) and prednisolone (5 mg/day), disease activity persisted. An increased dose of methorexate could not be administered due to the elevation of serum hepatic enzyme, despite the coadministration of folic acid (5 mg/week). The disease activity score 28 (DAS28) [10] was 5.42 before starting treatment with infliximab. Serum laboratory data in Patient 1 showed normal hepatic and renal functions. With the addition of infliximab at 3 mg/kg, her condition showed improvement. However, the patient suffered from pneumonia at one week after the second dose of infliximab. Her pneumonia responded to antimicrobial therapy and the patient wished to resume treatment with infliximab. It was restarted at 48 days after the second dose, with 1.5 mg/kg being administered every two months. Symptoms of RA were controlled well (DAS28 was 2.73) at six months after the resumption of infliximab therapy.

Patient 2

The patient was a 67-year-old woman with RA for 14 years. She was taking methotrexate (13 mg/week), folic acid (5 mg/week) and prednisolone (7 mg/day), but her disease remained active. When the dose of methotrexate was increased, serum hepatic enzymes became elevated. The DAS28 was 5.00 before she started treatment with infliximab. Serum laboratory data in Patient 2 showed normal hepatic and renal functions. With the addition of infliximab (3 mg/kg), her symptoms began to improve. The patient suffered from *Escherichia coli* sepsis at two weeks after the second dose of infliximab, which resolved after antimicrobial therapy. Because she wished to resume treatment with infliximab, it was restarted at 63 days after the second dose. With the administration of 1 mg/kg every two months, her symptoms improved. The dose of prednisolone was reduced from 7 to 2 mg/day while her symptoms remained under control (DAS28 was 3.94) at six months after resumption of infliximab.

Serum infliximab concentration profile

In Patient 1, blood samples were taken before and 1 h after (C1h) the fifth dose of infliximab, and also just before the sixth dose (Ctrough). In Patient 2, blood samples were taken before and 1 h (C1h), three weeks, and seven weeks after the fifth dose, as well as just before the sixth dose (Ctrough). Sera separated from the blood samples were sent to the laboratory of Tanabe R&D Service Co., Ltd. Serum infliximab concentration was measured in this laboratory by an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody specific for the TNF-binding site of infliximab [11]. This ELISA system had also been used to measure the serum infliximab concentrations of the patients involved in a clinical trial in Japan [12]. The detection limit of the assay was 0.1 μg/ml.

Analysis of Fcγ receptor polymorphism

DNA was extracted from whole-blood samples of the two patients and three controls using an extraction kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Genotyping of FcγRIIA 131H/R polymorphism was performed as reported by others [13]. Briefly, DNA fragments were amplified by the polymerase chain reaction (PCR) using the following primers: 5′-CTG AGA CTG AAA ACC CTT GGA ATC-3′ (sense) and 5′-GCT TGT GGG ATG GAG AAG GTG GGA TCC ATA-3′ (antisense). The amplification procedure consisted of initial denaturation for 5 min at 95°C, followed by 33 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 40 s. Then final extension was done at 72°C for 5 min, yielding a PCR product of 231 bp. This fragment was digested by NdeI to a 200-bp fragment if 131H was present, and was not digested if 131R was present. Digested fragments were subjected to electrophoresis on 3% agarose gel and stained with ethidium bromide, after which the bands were visualized under ultraviolet (UV) light.

Genotyping of FcγRIIIA 176 V/F polymorphism was performed according to the method reported by others [14]. Briefly, DNA fragments were amplified by the first PCR using the following primers: 5′-ATA TTT ACA GAA TGG CAC AGG-3′ (sense) and 5′-GAC TTG GTA CCC AGG TTG AA-3′ (antisense). Amplification involved initial denaturation for 10 min at 95°C, followed by 35 cycles of

denaturation at 95°C for 1 min, annealing at 56°C for 1.5 min, and extension at 72°C for 1.5 min, with final extension at 72°C for 8 min. The product was a 1.2-kb fragment containing the polymorphic site of FcγRIIIA. The primers for the second PCR were 5′-ATC AGA TTC GAT CCT ACT TCT GCA GGG GGC AT-3′ (sense) and 5′-A CG TGC TGA GCT TGA GTG ATG GTG ATG TTC A C-3′ (antisense). The second PCR involved initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 64°C for 1 min, and extension at 72°C for 1 min. Then final extension was done at 72°C for 9.5 min, yielding a 94-bp fragment. This was digested with NlaIII to a 72-bp fragment if 176 V was present, and was not digested if 176F was present. Digested fragments were subjected to electrophoresis on 3% agarose gel, stained with ethidium bromide, and visualized under UV light.

Genotyping of FcγRIIIB-NA1/NA2 was performed according to the method reported by others [15]. DNA fragments were amplified by PCR using an NA1-specific sense primer (5′-CAG TGG TTT CAC AAT GTG AA-3′) or an NA2-specific sense primer (5′-CAA TGG TAC AGC GTG CTT-3′) and an antisense primer (5′-ATG GAC TTC TAG CTG CAC-3′). PCR involved initial denaturation for 3 min at 94°C, 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 2 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR product was a 141-bp fragment in the presence of NA1 or a 219-bp fragment in the presence of NA2. Products were subjected to electrophoresis on 3% agarose gel, stained with ethidium bromide, and visualized under UV light.

Results

Pharmacokinetics

The pharmacokinetic profiles of infliximab in Patients 1 and 2 are shown in Table 1. Concentration versus time curves for the serum infliximab levels in both patients are displayed in Fig. 1. The C1h values of infliximab in the two patients receiving 1.5 mg/kg or 1 mg/kg of infliximab were lower than the median C1h reported for RA patients receiving 3 mg/kg of infliximab in a clinical trial performed in Japan [12]. In contrast, the Ctrough values of infliximab measured in these patients were equivalent to or higher than the median value determined in the clinical trial. The elimination half-life ($t_{1/2}$) of infliximab for these two patients was longer than the median $t_{1/2}$ value observed in the clinical trial.

Table 1 Pharmacokinetic data for the present two patients and a Japanese clinical trial

	C1h (μg/ml)	Ctrough (μg/ml)	$t_{1/2}$ (h)
Patient 1 (1.5 mg/kg)	35.6	1.2	274.8
Patient 2 (1 mg/kg)	24.3	0.5	245.3
Trial median[a]	53.3	0.5	159.5
Interquartile range	(46.7–60.5)	(<0.1–1.0)	(45.6–232.8)

C1h, infliximab concentration at 1 h after injection; Ctrough, infliximab concentration at 8 weeks after injection

[a] Data were obtained during a clinical trial of Japanese RA patients receiving 3 mg/kg of infliximab [12]

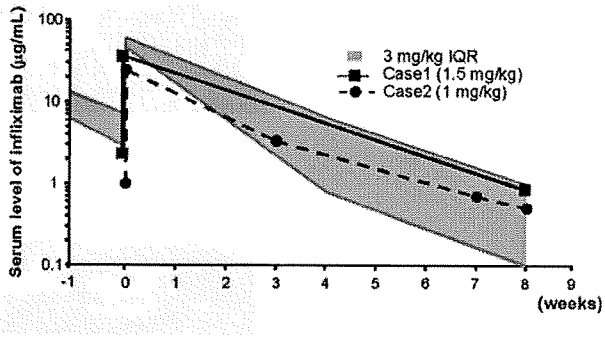

Fig. 1 Concentration versus time curves for infliximab in our two patients. Patient 1: *closed squares and an unbroken line*. Patient 2: *closed circles and a dashed line*. The interquartile range (IQR) for the Japanese clinical trial lies *between the gray lines*

Fcγ receptor polymorphism

The results obtained by the analysis of FcγR polymorphisms in the two patients and three controls are shown in Fig. 2. The FcγR polymorphism profiles of the two patients are summarized in Table 2. The polymorphisms of FcγRIIA and FcγRIIIA were identical in these two patients, being 131H/H and 176F/F, respectively. In contrast, the polymorphism of FcγRIIIB differed between the two patients.

Discussion

We experienced two RA patients who were well controlled over a long period by low-dose infliximab therapy. In these patients, symptoms were relieved by administration of infliximab at 1.5 or 1 mg/kg, which was one-half or less of the usual infliximab dose approved in Japan (3 mg/kg). To determine the reason for the efficacy of low-dose infliximab therapy in these two patients, we first investigated the pharmacokinetics of infliximab.

We found that the serum infliximab concentrations of both patients were similar to the median serum infliximab level in RA patients who were administered 3 mg/kg of infliximab in a Japanese clinical trial [12]. St. Clair et al. [16] measured serum infliximab concentrations in RA

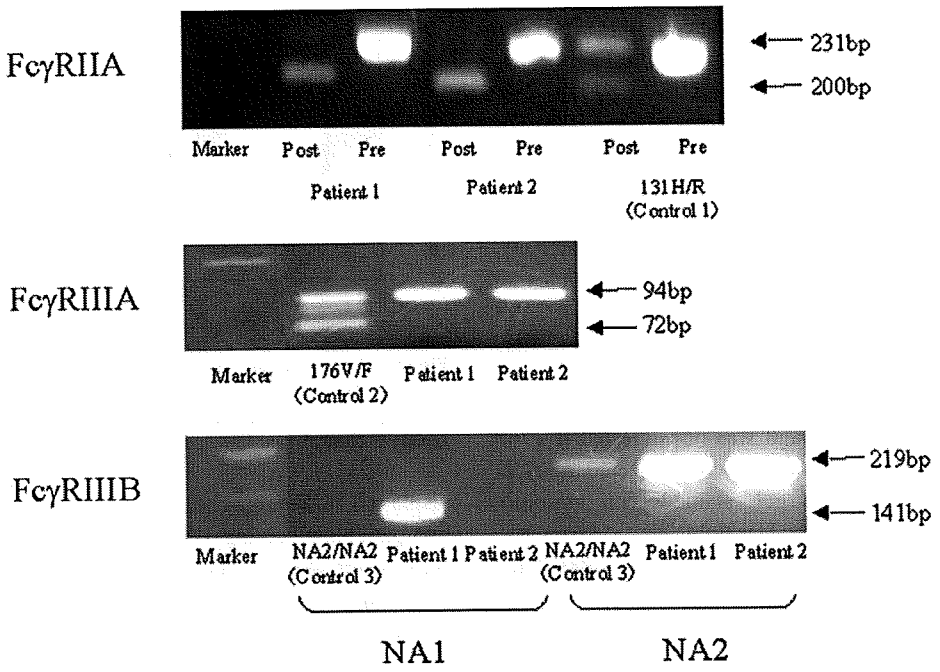

Fig. 2 Allotyping of the Fcγ receptors (FcγRIIA, FcγRIIIA, and FcγRIIIB) in the two patients and three controls. For FcγRIIA, 231-bp fragments are detected in two patients and Control 1 before the digestion of NdeI (Pre). After the digestion of NdeI (Post), a 200-bp fragment is detected in the two patients (131H/H), and both 200-bp and 231-bp fragments are detected in Control 1 (131H/R). For FcγRIIIA, a 94-bp fragment is detected after the digestion of NlaIII in the two patients (176F/F). Both 72-bp and 94-bp fragments are detected after the digestion of NlaIII in Control 2 (176 V/F). For FcγRIIIB, 141-bp and 219-bp fragments are detected after PCR using the NA1- and NA2-specific primers in Patient 1 (NA1/NA2). Only a 219-bp fragment is detected after PCR using the NA2-specific primer in Patient 2 and Control 3 (NA2/NA2)

Table 2 Fcγ receptor polymorphisms in the present two patients

	Patient 1	Patient 2
FcγRIIA	131H/H	131H/H
FcγRIIIA	176F/F	176F/F
FcγRIIIB	NA1/NA2	NA2/NA2

patients receiving infliximab in the ATTRACT study. When they separated the patients into three groups with infliximab Ctrough values of <0.1, 0.1–10, and >10 μg/ml, the number of patients who achieved an ACR20 response at 54 weeks after the start of treatment was positively correlated with the Ctrough value. Wolbink et al. [17] also reported that the mean Ctrough of infliximab in good/moderate responders (3.6 μg/ml) was significantly higher than that of nonresponders (0.5 μg/ml) when they evaluated patients by the European League Against Rheumatism response criteria with DAS28. These two studies demonstrated a close relationship between the serum infliximab concentration and improvement in RA symptoms.

In our two patients, the $t_{1/2}$ of infliximab was longer than the median $t_{1/2}$ determined in a Japanese clinical trial (Table 1) [12]. Also, their relatively high Ctrough levels despite low-dose therapy might be explained by a decrease in infliximab clearance. This antibody contains the Fc region of human IgG [6]. When human IgG binds to an antigen and forms an immune complex, it is degraded through FcγR-mediated phagocytosis [18]. In addition, it was also reported that there were no correlations between serum trough concentrations of infliximab and renal and hepatic functions [12]. Therefore, it is assumed that infliximab binds to TNF-α, forms an immune complex, and is degraded by the same mechanism.

The affinity of FcγR for IgG is influenced by the FcγR genotype. The genotype of FcγRIIIA in both of our patients was 176F/F. Tutuncu et al. [19] investigated FcγRIIIA polymorphism in 35 RA patients and found that 11 patients with the 176F/F genotype were all responders. Since the affinity of FcγRIIIA-176F for IgG is lower than that of FcγRIIIA—176V, the clearance of infliximab may be decreased. According to Kyougoku et al. [20], 53.7% of Japanese RA patients have the FcγRIIIA-176F/F genotype. Although the existence of FcγRIIIA-176F/F may possibly be important for explaining our results in two patients, further investigations of extended numbers of patients will be needed.

The 131H genotype of FcγRIIA increases the binding affinity for IgG compared with the 131R genotype [21]. In

the case of FcγRIIIB, affinity for IgG is increased by NA1 compared with NA2 [21]. Our two patients had a high affinity (131H/H) of FcγRIIA for IgG and different polymorphisms (NA1/NA2 and NA2/NA2) of FcγRIIIB. These results also suggest that these polymorphisms of FcγRIIA and FcγRIIIB did not have much influence on infliximab clearance. However, haplotypes formed by different genotypes of FcγRs, such as FcγRIIA-131H and FcγRIIIA-176F, may possibly suggest a disturbed clearance of infliximab.

The decrease in infliximab clearance may have been caused by a reduced production of TNF-α, the target antigen of this antibody. We did not measure TNF-α levels in our two patients. Petrovic-Rackov et al. [22] reported that there was a significant positive correlation between DAS28 and the TNF-α levels in blood and synovial fluid. The DAS28 scores of both patients were high before the administration of infliximab, so there is little possibility that they had low serum levels of TNF-α.

Acknowledgments The present study was supported in part by a grant from Nakatomi Welfare and Science to SK and by a Project Research Grant No. 19-23 from Toho University School of Medicine to SN. SK received a reseach grant from Mitsubishi Tanabe Pharma Corporation (Tokyo, Japan), the manufacturer of infliximab. No conflict of interest has been declared by the other authors. We wish to thank Ms. Sonoko Sakurai for secretarial assistance.

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Prostaglandins & other Lipid Mediators 89 (2009) 26–33

Contents lists available at ScienceDirect

Prostaglandins and Other Lipid Mediators

Prostaglandin E_2 activates Rap1 via EP2/EP4 receptors and cAMP-signaling in rheumatoid synovial fibroblasts: Involvement of Epac1 and PKA

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ARTICLE INFO

Article history:
Received 12 February 2009
Received in revised form 2 March 2009
Accepted 14 March 2009
Available online 25 March 2009

Keywords:
Rheumatoid arthritis
Inflammation
Prostaglandin E_2
Rap1
Exchange protein directly activated by cAMP
Protein kinase A

ABSTRACT

The small GTPase Rap1 is implicated in a variety of cellar functions. In this study, we investigated the effect of prostaglandin E_2 (PGE_2) on Rap1 activation in rheumatoid synovial fibroblasts (RSF). Rap1 was expressed in RSF, and GTP-bound active Rap1 (GTP-Rap1) was rapidly increased by PGE_2. The effect of PGE_2 was mimicked by an EP2 receptor agonist, an EP4 agonist and a cAMP-elevating agent forskolin with association to the increase of cAMP, but not by an EP1 or an EP3 agonist. RSF expressed the downstream signaling partners of cAMP, exchange protein directly activated by cAMP (Epac1) and protein kinase A (PKA). Both 8-pCPT-2-O-Me-cAMP (an Epac-specific cAMP analog) and 6-Bnz-cAMP (a PKA-specific cAMP analog) activated Rap1 in RSF. Activation of Rap1 by PGE_2 via cAMP-signaling may play an important role in the articular pathology of rheumatoid arthritis (RA).

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of synovial tissues associated with loss of cartilage, erosion of juxtaarticular bone and joint destruction. In RA, the synovial membrane is transformed to a hypertrophic inflammatory tissue with changes of phenotype of resident synovial fibroblasts and endothelial cells in addition of infiltration of macrophages, neutrophils, T- and B-cells. Rheumatoid synovial tissue characteristically overgrows and destroys articular cartilage and erodes juxtaarticular bone [1]. High concentrations of PGE_2 have been detected in the synovial fluid of patients with RA, and cytokine-activated synovial cells are a primary source of PGE_2 in affected joints [2]. Production of PGE_2 is preferentially increased in RA and other inflammatory conditions as a result of the sequential activation of two cytokine-inducible enzymes, cyclooxygenase-2 (COX-2; also known as PGH synthase-2) and microsomal prostaglandin E synthase-1 (mPGES-1) [3–8]. COX-2 and mPGES-1 are highly expressed in inflamed synovial tissues in RA [5,9,10]. Recent studies using mPGES-1 null mice showed a significant reduction of collagen-induced arthritis [11,12] and collagen antibody induced arthritis [13]. Furthermore, PGE_2 is specifically implicated in the symptoms of arthritis because neutralizing antibody against PGE_2 is able to inhibit acute and chronic inflammation in the rat adjuvant arthritis model [14]. Taken together, these previous findings suggest that PGE_2 is a critically important pro-inflammatory mediator in arthritis [5,15–18].

PGE_2 exerts its effects through a family of four different G protein-coupled receptors, EP1–4 [19]. Among these, the EP2 and EP4 receptors increase cAMP via activation of adenylate cyclase [20]. To date, most cAMP-mediated effects of PGE_2 via EP2/4 have been explained by the classic downstream target, protein kinase A (PKA), which phosphorylates downstream targets, such as the cAMP response element binding protein (CREB), in a variety of mammalian cells. However, PKA-independent actions of cAMP have been recognized in various experimental systems [21,22].

Recently, exchange protein directly activated by cAMP (Epac, also designated cAMP–GEFs) has been identified as novel targets for cAMP [23–25]. At least two forms of Epac, Epac1 and Epac2, have been identified and characterized. The Epac proteins contain a guanine nucleotide-exchange factor (GEF) domain which activates the small GTPase Rap1 (a member of the small GTPase Ras family) by directly converting GDP-bound inactive form to GTP-bound active form in response to increases of intracellar cAMP. Several important biological effects on various cellar functions including adhesion, phagocytosis, secretion, proliferation, differentiation, morphogenesis, migration and spreading in mammalian cells have been attributed to Rap1 [26–30]. Rap1 has been reported

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doi:10.1016/j.prostaglandins.2009.03.001

to be activated by Epac in a cAMP-dependent but PKA-independent manner [31]. However, another recent study demonstrated cAMP-dependent Rap1 activation by PKA signaling via C3G (Crk SH3 domain GEF) [32,33], the first identified Rap1-specific GEF [34].

In the present study, we demonstrate for the first time that Rap1 is functionally expressed in RSF and it is activated by PGE_2 via EP2 and EP4 receptors in a cAMP-dependent manner. We also demonstrate that Rap1 is activated via both Epac1- and PKA-mediated signaling in RSF. Our findings provide further insight into the role of PGE_2 in RA.

2. Materials and methods

2.1. Materials

PGE_2 and an enzyme-linked immunosorbent assay (ELISA) kit for cAMP were purchased from Cayman Chemical Co. (Ann Arbor, MI). Rabbit anti-human EP2 and EP4 receptor polyclonal antibodies were kindly gifted from Cayman Chemical Co. EZ-Detect™ Rap1 Activation Kit for Rap1 pull-down assay and bicinchoninic acid (BCA) protein assay reagent were purchased from Pierce Biotechnology Inc. (Rockford, IL). Rabbit anti-human Epac1 polyclonal antibody and normal mouse IgG were purchased from Upstate (Chicago, IL). Goat anti-human Epac2 polyclonal antibody, rabbit anti-human MAP1A polyclonal antibody, rabbit anti-human PKAα cat polyclonal antibody, rabbit anti-human C3G polyclonal antibody, rat cerebellum extract and lysates of human renal adenocarcinoma Caki-1 cells and neuroglioma H4 cells were purchased from Santa Cruz (Santa Cruz, CA). Rabbit anti-human phospho-CREB (Ser133) polyclonal antibody and rabbit anti-human CREB polyclonal antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG and HRP-conjugated rabbit anti-goat IgG were purchased from Jackson ImmunoResearch (West Grove, PA). The PKA-specific cAMP analog, 6-Bnz-cAMP (N^6-benzoyladenosine-3′,5′-cyclic monophosphate), and Epac-specific cAMP analog, 8-pCPT-2-O-Me-cAMP (8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate), were purchased from Biolog Life Science Institute (Hayward CA). Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Calbiochem (San Diego, CA). A selective EP1 receptor agonist (ONO-DI-004, (17S)-2,5-ethano-6-oxo-17,20-dimethylPGE_1), an EP2 receptor agonist (ONO-AE1-259, (16)-9-deoxy-9β-chloro-15-deoxy-16-hydroxy-17,17-trimethylene-19,20-didehydro PGE_1), an EP3 receptor agonist (ONO-AE-248, 11,15-O-dimethyl PGE_2), and an EP4 receptor agonist (ONO-AE1-329, 16-(3-methoxymethyl)phenyl-ω-tetranor-3,7-dithia PGE_1) were provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). The polyvinylidene difluoride (PVDF) membrane and enhanced chemiluminescence (ECL) reagent were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). SuperScript® First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR), Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). TRIpure reagent (Roche Applied Science, Indianapolis, IN) and HotStarTaq polymerase (Qiagen, Valencia, CA) were purchased from the indicated companies.

2.2. Preparation of RSF from patients with RA

Synovial tissues were obtained at the time of joint replacement surgery from patients with RA who fulfilled the revised American Rheumatism Association criteria for this disease [35]. Experiments were carried out according to a protocol that was approved by the institutional review board of the University of Kentucky. RSF were prepared by a previously described method [3]. Briefly, minced synovial tissues were digested overnight with 1 mg/ml collagenase (Type I, Sigma, St. Louis, MO) in DMEM in a humidified 5% CO_2 incubator at 37 °C and the isolated cells were cultured in 175 cm^2 culture flasks in DMEM supplemented with 20% FBS, L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml). At greater than 95% confluency, the adherent RSF were passaged by digestion with 0.05% trypsin/EDTA and used between passages 4 and 6 for all experiments. Cells were plated into 6-well plates (3×10^5 cells/well) or 75 cm^2 culture flasks (2×10^6 cells/flask) in DMEM containing 20% FBS. Cells were starved for 72 h in serum-free DMEM and then incubated with or without various stimuli.

2.3. Detection of GTP-Rap1 by Rap1 pull-down assay

Rap1 pull-down assay was performed by using EZ-Detect™ Rap1 Activation Kit according to the manufacturer's recommendation. Briefly, after RSF were made quiescent by culturing in serum-free DMEM for 72 h, cells were incubated with various stimuli. After washing with ice-cold PBS, cells were lysed in the provided lysis/wash/buffer with protease cocktail inhibitor and then lysates were centrifuged at 16,000 × g for 15 min at 4 °C. The protein content of the supernatant was determined using the BCA protein assay reagent with bovine serum albumin as standard. Equal amounts of protein (750 μg protein) were subjected to affinity precipitation of GTP-Rap1 using the EZ-Detect™ Rap1 Activation Kit, following by Western blotting with provided antibody (1:1000 dilution). To assess the levels of total Rap1, cell lysates adjusted for protein concentration were directly applied to Western blotting without pull-down assay.

2.4. Measurement of intracellular cAMP concentration

Intracellular cAMP concentration was measured with an ELISA kit according to the manufacturer's recommendation by a previously described method [36]. Briefly, after starved in serum-free DMEM for 72 h, cells were stimulated under various conditions. Next, cells were lysed in 0.1N HCl by incubation for 20 min at room temperature. After centrifugation of the lysate at 1000 × g for 10 min at 4 °C, cAMP concentration in supernatants was measured with an ELISA. The protein amount was determined using the BCA protein assay reagent.

2.5. RT-PCR

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 from the cells 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 oligonucleotide primers for EP receptor subtype [37,38], Epac [39], and MAP1A (light chain 2, LC2) and cycle number as follows: human EP1 receptor primers (34 cycles, product size 519 bp), sense 5′-CTC GCC GCG CTG GTG TGC AAC ACG C-3′ and antisense 5′-GGC CTC CCA GGC GCT CGG TGT TAG G-3′; human EP2 primers (34 cycles, product size 510 bp), sense 5′-TTC ATC CGG CAC GGG CGG ACC GC-3′ and antisense 5′-GTC AGC CTG TTT ACT GGC ATC TG-3′; human EP3 primers (34 cycles, product size 398 bp), sense 5′-TGT GTC GCG CAG CTA CCG GCG-3′ and antisense 5′-CGG GCC ACT GGA CGG TGT ACT-3′; human EP4 receptor primers (34 cycles, product size 366 bp), sense 5′-CCT CCT GAG AAA GAC AGT GCT-3′ and antisense 5′-AAG ACA CTC TCT GAG TCC T-3′; human Epac1

primers (40 cycles, product size 304 bp), sense 5′-GCT TCC TCC ACA AAC TCT CA-3′ and antisense 5′-AAC GCT GCC ATC ACC TCT CT-3′; human Epac2 primers (30 cycles, product size 333 bp), sense 5′-AGC CTT ATC CCA TCT TTC TA-3′ and antisense 5′-CTG ACT GTA TTC GCC TCC AC-3′; human MAP1A (LC2) primers (30 cycles, product size 392 bp), sense 5′-AAG GTT CAG GGG CGA GTA G-3′ and antisense 5′-CCA TTG GCA GGG TCA TTC C-3′. After initial denaturation at 95 °C for 15 min, PCR involved amplification cycles of 30 s at 95 °C, 30 s at 56 °C (for EP1–4, Epac2 and MAP1A) or 52 °C (for Epac1), and 45 s at 72 °C, followed by elongation for 5 min at 72 °C. The amplified cDNA fragments were resolved by electrophoresis on 2% (w/v) agarose gel and were visualized under UV light using a BioRad Chemidoc Apparatus (Hercules, CA) after staining of the gel with ethidium bromide.

2.6. Western blot analysis

Cells were lysed in Tris-buffered saline (TBS) containing 0.1% sodium dodecyl sulfate (SDS). After determining the protein content by BCA protein assay reagent with bovine serum albumin as standard, cell lysates adjusted to equal equivalents of protein were applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for electrophoresis and then the proteins were electroblotted onto PVDF membrane. After blocking the membranes in 10 mM TBS containing 0.1% Tween-20 (TBS-T) containing 5% skim milk, the membranes were probed with the respective antibodies (1:200 for Epac1; 1:500 for EP2, EP4, Epac2, MAP1A, PKAα cat and C3G) in TBS-T. After washing the membranes with TBS-T, the membranes were incubated with HRP-conjugated secondary antibody (1:10,000 in TBS-T containing 5% skim milk) for overnight at 4 °C. After further washing with TBS-T, protein bands were visualized with an ECL Western blot analysis system using a BioRad Chemidoc Apparatus (BioRad, Hercules, CA). For detection of phospho- and total CREB, cells were lysed in buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EGTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 1% Triton X-100, 10% glycerol, 0.5% deoxycholic acid, 0.1% SDS, 50 mM glycerophosphate, 3 mM Na_3VO_4 and 10 μg/ml aprotinin). Cell lysates adjusted to equal equivalents of protein were applied to SDS-PAGE and followed by Western blotting with anti-phospho-CREB antibody (1:500) for phosphorylated CREB or anti-CREB antibody (1:500) for total CREB.

2.7. Statistical analysis

All data are expressed as mean ± S.E.M. Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of Rap1 and its activation by PGE_2 in RSF

To elucidate whether Rap1 exists and can be activated in RSF, we first examined the activation of Rap1 in the presence of GTPγS (a substrate for GTP) or GDP in vitro with lysates isolated from RSF. As shown in Fig. 1A, the levels of GTP-Rap1 (GTP-bound active form of Rap1) were increased by incubation with GTPγS and decreased in the presence of GDP, indicating that Rap1 is functionally expressed in RSF. We next examined the effect of PGE_2 on the activation of Rap1 in cultured RSF. As shown in Fig. 1B, the levels of GTP-Rap1 were rapidly increased from 30 to 120 min after PGE_2 stimulation and were decreased at 240 min, without changes in the levels of total Rap1. GTP-Rap1 was increased by PGE_2 in a concentration dependent manner (Fig. 1C).

3.2. Contribution of PGE_2 receptor subtypes in Rap1 activation in RSF

Since PGE_2 stimulated GTP-Rap1 in RSF, we next determined the PGE_2 receptor subtypes, EP1–4, in activation of Rap1. As shown

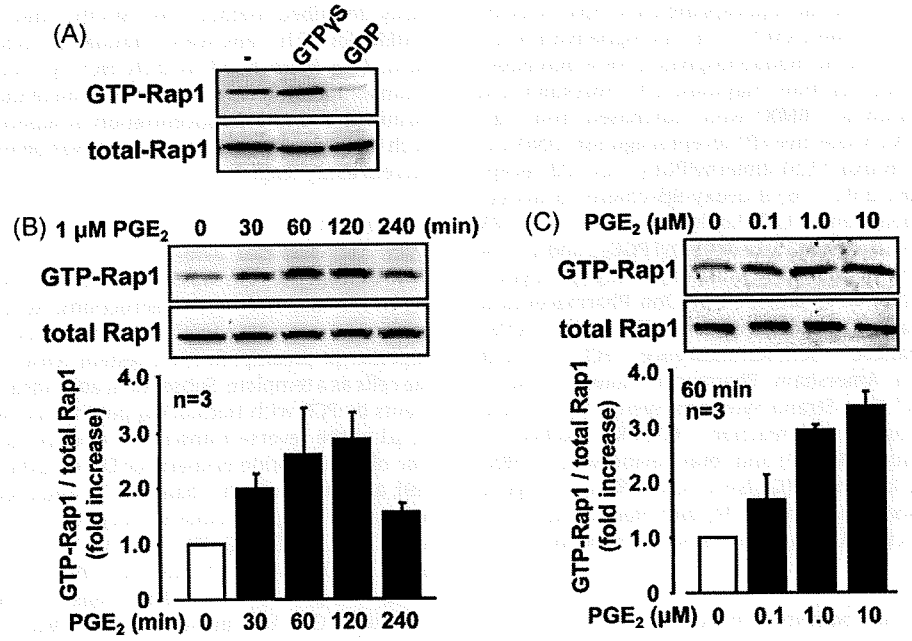

Fig. 1. Expression of Rap1 and its activation by PGE_2 in RSF. (A) Cell lysates from RSF were treated in vitro with GTPγS (10 μM) or GDP (100 μM) for 30 min at 30 °C, according to the EZ-Detect™ Rap1 Activation Kit manufacturer's protocol. GTP-Rap1 was detected by Rap1 pull-down assay followed by Western blotting as described in Section 2. Total Rap1 levels are also shown. (B and C) Serum-starved RSF were stimulated with or without PGE_2 (0.1–10 μM) for the time period as indicated and cell lysates were subjected to Rap1 pull-down assay. Rap1 activation indicates the ratio of GTP-Rap1 against total Rap1 and a value of "1" was assigned to the value of 0 min (B) or value of non-stimulation (C). Blots are representative data from the indicated number of patients with RA in each panel.

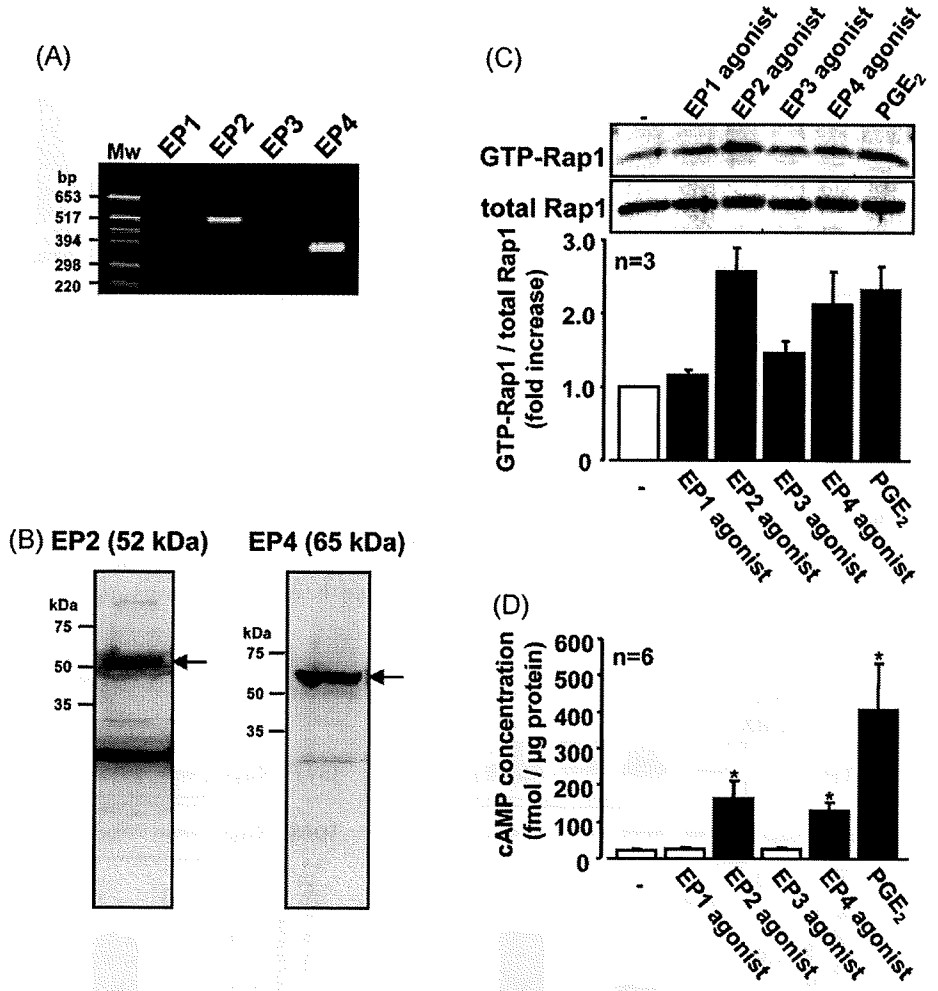

Fig. 2. Contribution of PGE_2 receptor subtypes in Rap1 activation in RSF. (A) mRNA expression of EP1, EP2, EP3 and EP4 receptors in RSF were evaluated by RT-PCR. Only EP2 and EP4 mRNA was detected. Results are representative examples from three patients with RA. (B) Protein expression of EP2 and EP4 receptors in RSF was analyzed by Western blotting. Blots are representative data from three patients with RA. (C) Serum-starved RSF were incubated for 60 min with or without PGE_2 (1 μM) or EP receptor agonists (10 μM). Cell lysates were subjected to Rap1 pull-down assay. Blots are representative data and values are expressed as means ± standard errors from the number of patients with RA. (D) RSF were incubated for 30 min with or without PGE_2 (1 μM) or EP receptor agonists (EP1–4, 10 μM). Levels of cAMP were measured by ELISA. Values are expressed as means ± standard errors from the number of patients with RA. A significant difference from the value of non-stimulation determined by Student's *t*-test is indicated with a single asterisk ($P < 0.05$).

in Fig. 2A, mRNA for the EP2 and EP4 receptors was co-expressed in RSF, whereas mRNA for the EP1 and EP3 receptors was not detected. While, EP1 and EP3 mRNA was detectable in some lines of cultured chondrocytes from patients with osteoarthritis (data not shown). In addition, we detected immunoreactive bands of the expected size for EP2 (52 kDa) and EP4 (65 kDa) in RSF by Western blotting (Fig. 2B). We next determined the effect of specific EP receptor agonists on Rap1 activation. The specificity of each EP receptor agonist used in this study was confirmed by Suzawa et al. using a binding assay for the respective receptor subtypes expressed in CHO cells [40]. As shown in Fig. 2C, an EP2 receptor agonist (ONO-AE1-259) and an EP4 receptor agonist (ONO-AE1-329), as well as PGE_2, up-regulated the levels of GTP-Rap1 in RSF. However, an EP1 receptor agonist (ONO-DI-004) and an EP3 receptor agonist (ONO-AE-248) did not. Increased levels of cAMP were also observed under the stimulation by EP2 agonist and EP4 agonist but not by EP1 agonist and EP3 agonist (Fig. 2D). These data suggested that PGE_2 activates Rap1 via the EP2 and EP4 receptor subtypes associated with an increase in cAMP.

3.3. *Effect of cAMP-elevating agents on Rap1 activation in RSF*

PGE_2 caused a rapid increase in the levels of intracellar cAMP with maximal levels at 15 min after PGE_2 stimulation (Fig. 3A), and we observed a concentration-dependent effect of PGE_2 on the increase in the elevation of cAMP in RSF (Fig. 3B). The PGE_2-mediated change in cAMP mirrored the time and concentration change to Rap1 following treatment with PGE_2 as shown in Fig. 1. To further confirm the importance of cAMP in mediating the effects of PGE_2 on activation of Rap1, we examined the effect of IBMX (a phosphodiesterase inhibitor) on PGE_2-induced Rap1 activation. As shown in Fig. 4A, increased levels of GTP-Rap1 by PGE_2 were enhanced in the presence of IBMX, which was correlated with further increased levels of cAMP (Fig. 4B). To determine whether other cAMP-elevating agent induced the same biological effects as PGE_2 on Rap1 activation, we examined the effect of forskolin (a direct activator of adenylate cyclase). Forskolin also increased GTP-Rap1 concomitant with increasing cAMP levels (Fig. 4C and D). These data indicate that cAMP is involved in the PGE_2-induced Rap1 as a second messenger in RSF.

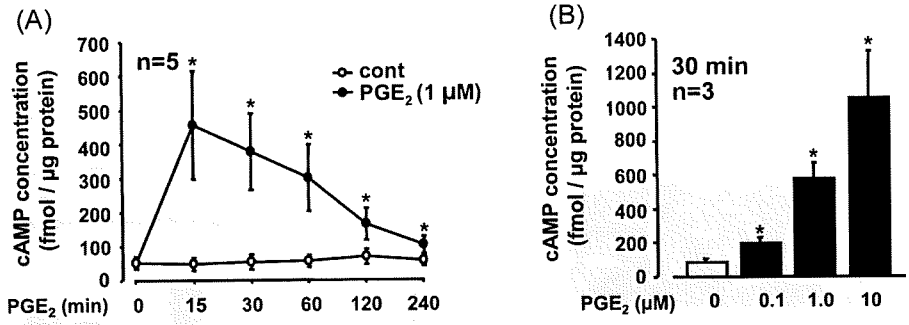

Fig. 3. Increase of cAMP by PGE_2 in RSF. Serum-starved RSF were stimulated with or without PGE_2 (0.1–10 μM) for the time period as indicated. Levels of cAMP were measured by ELISA. A significant difference from the value of 0 min (A) or non-stimulation (B) determined by Student's *t*-test is indicated with a single asterisk ($P < 0.05$). Values are expressed as means ± standard errors from the number of patients with RA indicated in each panel.

3.4. *Expression profile of cAMP-dependent Rap1 signaling molecules in RSF*

We next investigated further the downstream targets of cAMP-signaling responsible for the activation of Rap1 in PGE_2-stimulated RSF. As shown in Fig. 5A and B, Epac1 mRNA and protein were expressed in RSF, while Epac2 expression was absent. MAP1A, an Epac1-associated microtubular protein, was also detected (Fig. 5A and B). Interestingly, Epac1 is represented by double bands at both the protein and mRNA levels. Kooistra et al. previously reported the double bands of Epac1 protein in human umbilical vein endothelial cells (HUVEC) and Ovcar3 cells (ovarian carcinoma cell line) [41]. They also reported that both bands were abolished by Epac1 siRNA, suggesting the existence of two variants of Epac1. We also detected

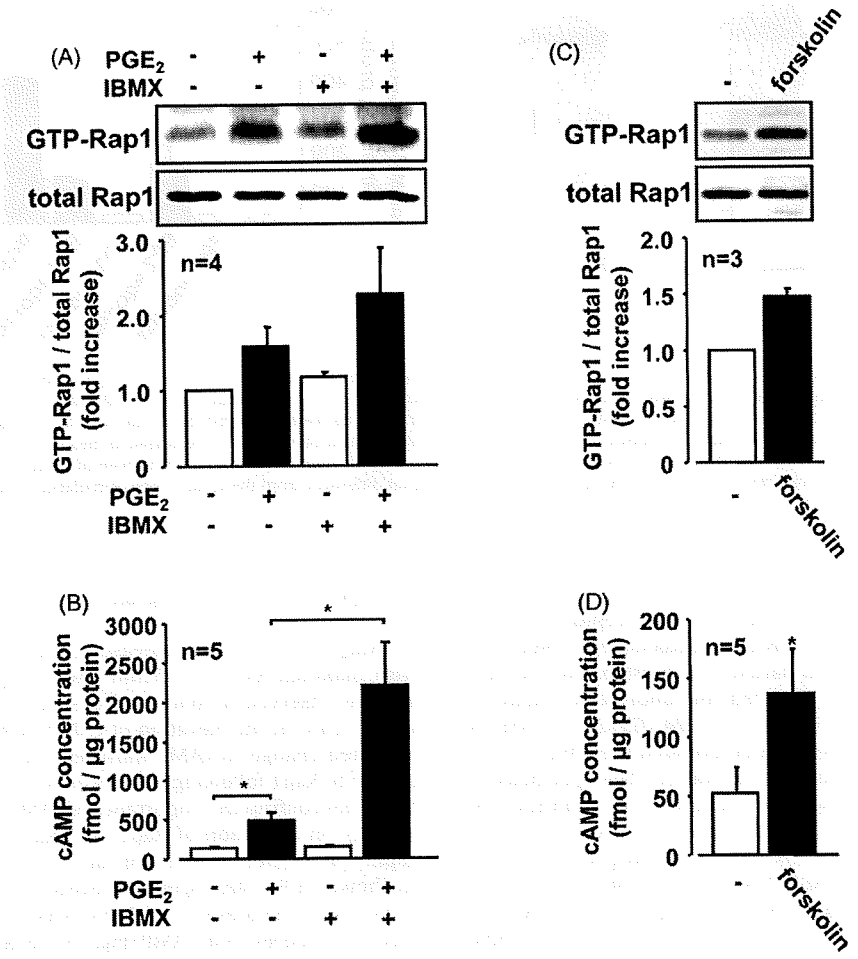

Fig. 4. Effect of cAMP-elevating agents on Rap1 activation in RSF. (A) Serum-starved RSF were stimulated with or without PGE_2 (1 μM) for 60 min in the presence or absence of IBMX (100 μM). Lysates from the cells were subjected to Rap1 pull-down assay. (B) cAMP levels in cell lysates from RSF stimulated with or without PGE_2 (1 μM) for 30 min in the presence or absence of IBMX (100 μM) were analyzed by ELISA. (C) Rap1 activation was analyzed by detecting GTP-Rap1 with lysates from RSF stimulated with forskolin (10 μM) for 60 min. (D) cAMP levels in cell lysates from RSF stimulated with or without forskolin (10 μM) for 30 min were analyzed by ELISA. Blots are representative data and values are expressed as means ± standard errors from the number of patients with RA indicated in each panel. A significant difference between two groups (B and D) determined by Student's *t*-test is indicated with a single asterisk ($P < 0.05$).

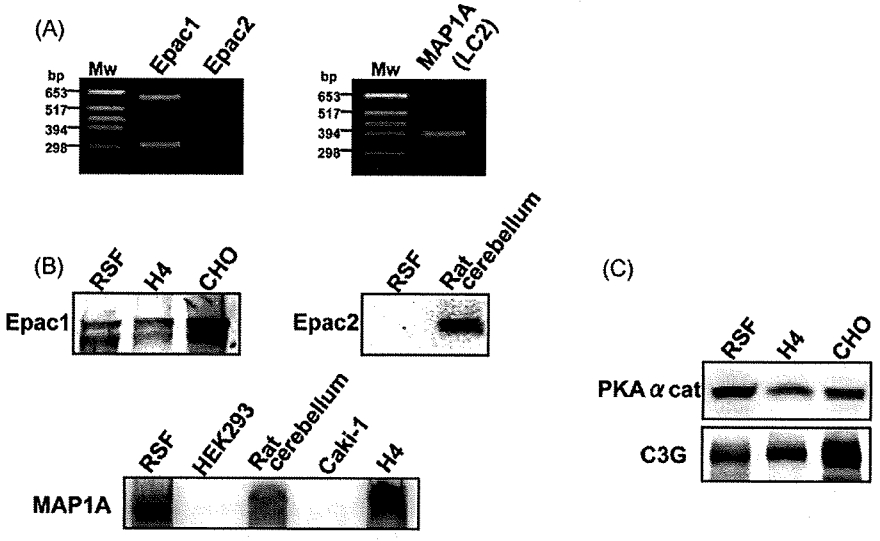

Fig. 5. Expression profile of cAMP-dependent Rap1 signaling molecules in RSF. (A) mRNA expressions of Epac1, Epac2 and MAP1A in RSF were detected by RT-PCR. (B) Protein expression of Epac1, Epac2, and MAP1A were determined by Western blotting. (C) Protein expression of PKAα cat and C3G was determined by Western blotting. Results are representative examples from two to four patients with RA. H4 cells, CHO cells, rat cerebellum extract were used as positive controls. HEK293 cells and Caki-1 cells were used as negative controls.

protein expression of PKAα cat (a catalytic subunit of PKA) and C3G, also known as a Rap1-specific GEF, in RSF (Fig. 5C).

3.5. *Effect of Epac-specific and PKA-specific cAMP analog on activation of Rap1 and CREB in RSF*

Distinct roles for Epac and PKA in Rap1 signaling have been previously described by using highly specific cAMP analogs, Epac-specific 8-CPT-2Me-cAMP and PKA-specific 6-Bnz-cAMP [42]. Thus, to assess whether Rap1 activation in RSF is linked with Epac- and/or PKA-mediated signaling pathway in RSF, we examined the effect of these analogs on Rap1 activation. Since it is well known that CREB phosphorylation is mediated by PKA signaling but not by Epac signaling in various cell types, we also evaluated analog specificity by examining the effect of these agonists on phosphorylation of CREB. The Epac-specific cAMP analog increased the levels of GTP-Rap1 (Fig. 6A), but had no effect on the phosphorylation of CREB (Fig. 6B). On the other hand, the PKA-specific cAMP analog increased both the levels of phospholyrated CREB (Fig. 6B) and GTP-Rap1 (Fig. 6A). These results suggest that cAMP-dependent activation of Rap1 is mediated by Epac signaling, but also by PKA signaling, in RSF.

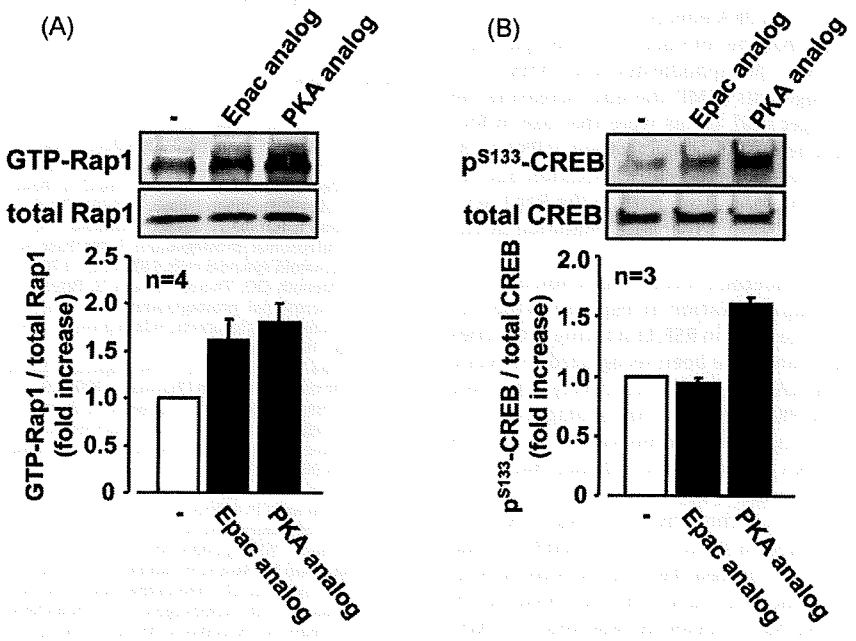

Fig. 6. Effect of Epac-specific and PKA-specific cAMP analog on activation of Rap1 and CREB in RSF. (A) Serum-starved RSF were stimulated with 8-CPT-2-O-Me-cAMP (Epac-specific cAMP analog, 500 μM) and 6-Bnz-cAMP (PKA-specific cAMP analog, 500 μM) for 45 min. Lysates from the cells were subjected to Rap1 pull-down assay. (B) Serum-starved RSF were stimulated with 8-CPT-2-O-Me-cAMP (Epac-specific cAMP analog, 500 μM) and 6-Bnz-cAMP (PKA-specific cAMP analog, 500 μM) for 10 min. Levels of phospho-CREB and total CREB were analyzed by Western blotting. Blots are representative data and values are expressed as means ± standard errors from the indicated number of patients with RA in each panel.

4. Discussion

The novel findings of this study are that Rap1 is functionally expressed in RSF and is activated by PGE_2 due to increased cAMP via the Gαs-coupled EP2 and EP4 receptors. In addition, cAMP-dependent Rap1 activation is mediated by both Epac1 and PKA in RSF.

Rap1 is strongly implicated in the regulation of cytoskeletal structure and cell–cell adhesion in mammalian cells [26,27,43]. In RA, cell–cell adhesion mediated by cadherin-11, a member of the cadherin superfamily, is thought to be essential for synovial tissue organization [44,45] and cadherin-11 plays an important role in the response to experimental arthritis [46]. In addition, cell–cell contact between T-cells and adherent RA synovial cells mediates Rap1 inactivation and regulates production of reactive oxygen species in T lymphocytes after exposure to inflammatory cytokines [47].

We clearly demonstrate a role for cAMP-signaling including its downstream partners, Epac and PKA, in Rap1 activation in RSF. RSF expressed Epac1, but not Epac2, and an Epac-specific cAMP analog led to the activation of Rap1 in RSF. However, we also found that Rap1 was also activated by a PKA-specific cAMP analog. These results demonstrate that cAMP-dependent Rap1 activation is mediated not only by Epac1, but also by PKA in RSF. Most reports have implicated Epac as responsible for cAMP-dependent Rap1 activation in other cell types. However, a recent study also proposed that Rap1 could be activated by PKA signaling via C3G [32,33]. Indeed, we observed C3G protein expression in RSF in the present study. Wang et al. [33] demonstrated the existence of distinct pools of Rap1 that can be selectively activated by either PKA or Epac, which may differentially distinguish downstream effects of Rap1. In fact, their report clearly showed that Epac1 activated a perinuclear pool of Rap1 and this did not result in ERK activation; however, the addition of a membrane-targeting motif to Epac1 resulted in relocation of Epac1 from its normal perinuclear localization to the plasma membrane and revealed an ability to activate ERK in a cAMP/Rap1-dependent manner. Further assessment of the subcellular localization of Epac1 and/or other downstream effectors associated with Epac1/Rap1 in RSF may provide further understanding of independent Epac and PKA effects.

A recent report suggested that the interaction of the Epac agonist, 8-CPT-2-O-Me-cAMP, with phosphodiesterases (PDEs) may inhibit the hydrolysis of endogenous cAMP, thereby increasing the levels of this second messenger [48]. If this were the case in RSF, the expected outcome might be the indirect activation of PKA by 8-CPT-2-O-Me-cAMP. However, we detected Rap1 activation but not PKA activation by 8-CPT-2-O-Me-cAMP, suggesting the Rap1 activation by agonistic effect on Epac rather than PDE inhibition by the agonist in this study.

The present study suggests that the coordinated action of Epac1 and PKA are involved in the Rap1 activation in response to the elevation of cAMP after exposure to PGE_2 in RSF. Enhancing properties of Epac signaling to PKA signaling have been recognized in several cellular events. For example, cAMP analogs selectively activating Epac synergize strongly with PKA-specific cAMP analog to induce neurite outgrowth in rat neuronal pheochromocytoma PC-12 cells [42]. In addition, Epac and PKA cooperatively enhance functional gap junction neoformation in cardiomyocytes [49].

Since cAMP is the key mediator in the activation of Rap1 by PGE_2, we expected the receptors linked to stimulation of cAMP, EP2 and EP4, would be involved. This was indeed the case. Actions of PGE_2 via EP2 and EP4 have been implicated in a number of critical mechanisms critical to arthritis. We have previously reported that PGE_2 positively auto-regulates the expression of mPGES-1 which is a terminal enzyme for PGE_2 biosynthesis, via activation of EP2 and EP4 receptors in RSF under interleukin (IL)-1β-stimulated condition [38]. PGE_2 also regulates the production of cytokine and growth factor such as IL-6, vascular endothelial growth factor, parathyroid hormone-related peptide, and macrophage colony stimulating factor though the activation of EP2 and EP4 receptors in IL-1-stimulated synovial fibroblasts [50,51]. Furthermore, previous *in vivo* studies have been demonstrated that EP2 and/or EP4 null mice are resistant to chronic inflammation of joints in the experimental arthritis models including collagen antibody induced arthritis [52] and collagen-induced arthritis [53].

It is likely that PGE_2 exerts its effects on inflammation and on synovial morphology through activation of Rap1 and perhaps other small GTPases that regulate cytoskeletal organization, migration, and activation status via mitogen activated protein kinases, known to be highly expressed in RA synovial tissue [54]. The PGE_2-induced changes in RSF morphology and cytoskeleton organization could have significant consequences [55,56]. PGE_2 has been previously shown to regulate migration of variety of cells associated with changes of cell morphology and cytoskeleton organization via cAMP-signaling and small GTPases [57–61]. In a separate study, we showed morphologic changes, redistribution of cadherin-11 on the plasma membrane, and dissociation of the actin cytoskeleton after treatment with PGE_2 or a combination of PKA and Epac agonists (data not shown). Since cadherin-11 deficiency is associated with altered response to an inflammatory insult, PGE_2 could mediate some of its pro-inflammatory effects by directly altering the architecture and function of synovial tissue via Rap1. Further understanding of these key pathways may yield new therapeutic targets in patients with RA.

Acknowledgments

We thank Ono Pharmaceutical Co. Ltd. (Osaka Japan) for providing us with PGE_2 receptor subtype agonists (ONO-DI-004, ONO-AE1-259, ONO-AE-248, and ONO-AE1-329), and Cayman Chemical Co. (Ann Arbor, MI) for providing us with rabbit anti-human EP2 and EP4 receptor polyclonal antibodies.

This work was supported by the NIH/NIAMS R01 AR049010, the Arthritis Foundation Biomedical Sciences Grant and Travel Award from the Japanese Society of Clinical Pharmacology and Therapeutics.

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—Regular Articles—

Preliminary Study to Identify the Predictive Factors for the Response to Methotrexate Therapy in Patients with Rheumatoid Arthritis

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(Received January 5, 2009; Accepted April 15, 2009; Published online April 21, 2009)

To identify the major factors predicting the response to Methotrexate (MTX) therapy in rheumatoid arthritis (RA) patients, we evaluated the relationship between the response to MTX and factors such as the concentration of MTX-polyglutamates (MTX-PGs) in erythrocytes (RBCs), genotypes of thymidylate synthase (TYMS) 5′-UTR (2R/3R) and 3′-UTR (−6/+6), 5,10-methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C, and other patient-related factors. Thirty-six Japanese RA patients were enrolled in this cohort study. The concentrations of MTX-PGs in RBCs were measured, and polymorphisms were determined using PCR-RFLP method. As an indicator of the accumulated capacity of MTX-PGs in the RBCs of each patient, the MTX dose/MTX-PGs (AC-MPG, l/week) was calculated. The response to MTX therapy was assessed using the MTX dose for a ≥50% decrease in CRP level (MTX dose for 50% CRP, mg/week), and the relationships between MTX dose for 50%CRP and various other factors were evaluated using multiple linear regression analysis. The MTX dose was 6.9±0.3 mg/week and the MTX-PGs concentration in RBCs was 97.3±8.1 nmol/l (n=36, blood samples=95, mean±S.D.). The range of MTX dose for 50%CRP was 2.0–13.0 mg/week. Most individual AC-MPG levels showed no change during the evaluation period (coefficient of variation=5.9%). Based on the results of multiple linear regression analysis, AC-MPG, TYMS 3′-UTR (−6/+6), and ESR at the start of MTX therapy were associated with the MTX dose for 50%CRP. AC-MPG, TYMS 3′-UTR (−6/+6), and ESR might be the major predictive factors for the response to MTX therapy in Japanese RA patients.

Key words——rheumatoid arthritis; methotrexate; methotrexate-polyglutamates; polymorphism; clinical trial

INTRODUCTION

Methotrexate (MTX) is the most widely used disease-modifying antirheumatic drug (DMARD) in the treatment of rheumatoid arthritis (RA) and is regarded as a key drug in the 2002 update of the RA treatment guidelines from the American College of Rheumatology subcommittee.[1] However, it is recognized that there are large individual differences in the optimal dose of MTX in RA patients.[2] In the USA and many European countries, the recommended general target dose of MTX is 15–20 mg/week, but the individual optimal dose is the range of 5–20 mg/week.[3] In Japan, the approved maximum dose of MTX is 8 mg/week, but in practice 2–20 mg/week of MTX is prescribed based on individual sensitivity to and tolerance of MTX.[4] Differences in the optimal MTX dose mainly reflect individual differences in the response to MTX therapy. The reasons for those individual differences in the response to MTX therapy are thought to be different concentrations of intracellular MTX-polyglutamates (MTX-PGs),[5,6] different enzyme activity at MTX-active sites,[5,7–9] and other patient-related factors[10] such as body weight, renal function, disease severity, *etc.*

It was confirmed that MTX exerts its antirheumatic effects by adding up to six glutamates to MTX (MTX-PG_{1-6}) *via* folypolyglutamyl synthase (FPGS) in target cells (mononuclear cells, lymphocytes, or synovial cells) which are retained intracellularly over a long period.[11–14] The concentration of MTX-PGs in erythrocytes (red blood cells; RBCs) is reported to reflect that in target cells.[6] Therefore, the concentration of MTX-PGs in RBC is used as a substitute for the MTX-PGs concentration in target cells.[5,6] We measured the concentration of MTX-PGs in RBCs and evaluated the relationship between the response to MTX and the concentration of MTX-PGs in

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RBCs.

Two genetic polymorphisms that influence enzyme activity were reported in both the thymidylate synthase (TYMS) and 5,10-methylenetetrahydrofolate reductase (MTHFR) genes at MTX-active sites.[15-18,22] The two or three 28-bp tandem repeats in the TYMS 5′-untranslated region (UTR) act as an enhancer of the TYMS promoter, and increasing the number of repeats leads to stepwise increases in TYMS mRNA expression.[17] The 6-bp deletion/insertion was located in the TYMS 3′-UTR, 447 bp downstream from the stop codon, and the 6-bp deletion expresses less TYMS mRNA than the 6-bp insertion.[18] The MTHFR C677T polymorphism consists of a C>T change resulting in an alanine to valine substitution that renders the enzyme more thermolabile.[15] In the MTHFR A1298C polymorphism, the A>C change causes a glutamine to alanine substitution and leads to reduced enzyme activity.[16] Several studies evaluated the relation between these genetic polymorphisms and MTX therapeutic effects,[5,7-9] but the indicator of the therapeutic effects and the evaluated genotype site differed in every study. Because the mechanisms of action of MTX are complex, the relation between the above mentioned-factors and the response to MTX therapy should be evaluated not individually but comprehensively. Therefore, the purpose of this preliminary study was to identify the major predictive factors of the individual response to MTX therapy in RA patients by evaluating the concentration of MTX-PGs in RBCs, the four polymorphisms of the TYMS and MTHFR genes, and other patient-related factors.

PATIENTS AND METHODS

Study Design This cohort study was conducted from July 2004 to August 2006 at a single investigational site, Kitasato Institute Hospital (Tokyo, Japan).

Participants Patients met the 1987 American College of Rheumatology criteria, and they were receiving MTX or scheduled to start MTX therapy.

The use of low-dose oral corticosteroid (predonisolone, <10 mg/day) and nonsteroidal antiinflammatory drugs (NSAIDs) was allowed, but during the evaluation period the dose was changed as little as possible. For the prevention of adverse effects induced by MTX, all patients were administered folic acid (5 mg/week). This study protocol was approved by the Institutional Review Board of the Kitasato Institute Hospital and written informed consent was given by all patients prior to enrollment.

Patient Background and Predictive Factors from Patient History The duration of RA, previous use of DMARDs, age at which MTX administration was initiated, gender, body weight, laboratory data; serum C-reactive protein (CRP) concentration, erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), mean corpuscular volume (MCV), and serum creatinine clearance (Scr) were obtained from the medical records.

Determination of MTX-PGs Concentration in RBCs The concentration of MTX-PGs in RBCs was evaluated more than twice per patient at approximately 3-month intervals. For the determination of MTX-PGs in RBCs, heparinized peripheral whole blood (5 ml) from RA patients who had been receiving the same dose of MTX for more than 1 month were collected more than 3 days after MTX administration. All collected blood samples were stored immediately at 4°C and RBCs and plasma were separated within 48 h in a 10-min centrifugation step.[19] RBCs and plasma were stored at −80°C until analysis. MTX-PGs concentrations in RBCs were determined using the modified method of Dervieux *et al.*[19] In brief, the MTX-PGs in hemolyzed RBCs were converted to MTX in the presence of plasma γ-glutamyl hydrolase and mercaptoethanol at 37°C. Then MTX was purified in a perchloric acid deproteinization step, followed by solid-phase extraction. The concentration of MTX was measured by using a TDX analyzer (Abbott Japan, Tokyo). The quantification limit and coefficient of variation (CV) of this analytical method are 30 nmol/l and <12.4%, respectively. Using the values of MTX-PGs concentration in RBCs from each patient, the MTX dose/MTX-PGs (AC-MPG, l/week) was calculated as an indicator of the accumulated capacity (*i.e.*, clearance) of MTX-PGs in the RBCs from each patient. The lower AC-MPG means the higher accumulated capacity of MTX-PGs in RBCs (*i.e.*, lower clearance of MTX-PGs in RBCs).

Genotype Determination Peripheral blood samples (5 ml) for genetic analysis were collected in tubes containing EDTA-2Na at the time of the first assessment and stored at −20°C until DNA extraction. DNA was extracted using the agglutination partition method (Sepa Gene, Sanko Junyaku, Ltd, Tokyo, Japan). The MTHFR C677T polymorphism

was detected using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, as reported previously.[15] Individuals with the 677CC genotype presented one fragment (198 bp), those with the 677CT genotype presented two fragments (175 and 198 bp), and those with the 677TT genotype presented one fragment (175 bp) when visualized on 3% agarose gels. The MTHFR A1298C polymorphism was detected using a PCR-RFLP method, as reported previously.[16] Individuals with the 1298AA genotype presented five fragments (56, 31, 30, 28, and 18 bp), those with the 1298AC genotype presented six fragments (81, 56, 31, 30, 28, and 18 bp), and those with the 1298CC genotype presented four fragments (81, 31, 30, and 18 bp) when visualized on 10–25% gradient polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan). The TYMS 5′-UTR polymorphism was detected using a PCR-RFLP method, as reported previously.[20] Individuals with the 2R2R genotype presented one fragment (210 bp), those with the 2R3R genotype presented two fragments (210 and 238 bp), and those with the 3R3R genotype presented one fragment (238 bp) when visualized on 3% agarose gels. The TYMS 3′-UTR polymorphism was also detected using a PCR-RFLP method, as reported previously.[21] Individuals with the +6-bp/+6-bp genotype presented two fragments (88 and 70 bp), those with the +6-bp/−6-bp genotype presented four fragments (152/158, 88, and 70 bp), and those with the −6-bp/−6-bp genotype presented one fragment (152 bp) when visualized on 3% agarose gels.

Statistical Analysis The response to MTX therapy in each RA patient was assessed using the MTX dose resulting in a ≥50% decrease in the serum CRP level (MTX dose for 50%CRP, mg/week). The Spearman's rank correlation coeficient was used to investigate the relationship between AC-MPG and MTX dose for 50%CRP. Relationships between genotypes and MTX dose for 50%CRP were analyzed using analysis of variance (ANOVA) or Student's *t*-test. The genetic differences in enzyme activities in MTX-active sites in each patient were evaluated using a pharmacogenetic index, which was the sum of four homozygous variant genotypes (TYMS 5′-UTR 2R/2R, TYMS 3′-UTR −6/−6, MTHFR 677 T/T, and MTHFR 1298 C/C). The relationship between the pharmacogenetic index and MTX dose for 50%CRP was analyzed using ANOVA.

Multiple linear regression analysis was performed to identify the predictive factors of individual response to MTX therapy. The explanatory variables for the dependent variable (*i.e.*, MTX dose for 50% CRP) were AC-MPG, four polymorphisms (TYMS 5′-UTR 2R/3R, TYMS 3′-UTR −6/+6, MTHFR C677T, and MTHFR A1298C), CRP, RF, MCV, and Scr levels and ESR at the start of MTX therapy, duration of disease, previous use of DMARDs, age at which MTX administration was initiated, gender, and body weight. The method of forward-backward stepwise selection was used for the selection of explanatory variables for multiple linear regression analysis. Multicolinearity was evaluated by Pearson correlation coefficient of a correlation matrix.

All statistical analyses were carried out using Statistical Analysis System softwere (14.0J; SPSS, Chicago, IL). A p-value of less than 0.05 was considered to represent a statistically significant difference.

RESULTS

Thirty-six RA patients (31 women and 5 men) were enrolled from July 2004 to August 2006 at the Kitasato Institute Hospital, Tokyo. Twenty-eight patients who were receiving MTX and 8 patients in whom MTX therapy initiation was deemed necessary were included in this study. All patients received folic acid (5 mg/week) from the initiation of MTX therapy. The number of patients with Steinbrocker stage I to IV were 5, 10, 9, and 12, respectively.

Factors Predicting Response to MTX Treatment Data on factors predicting the response to MTX therapy are summarized in Table 1. The median duration of RA was 4.0 years, and 91.7% of patients had used DMARDs previously. The median age at which MTX therapy was initiated was 61.0 years. The median levels of CRP, RF, MCV, and Scr and median ESR at the start of MTX therapy were 1.9 mg/dl, 78.6 IU/ml, 89.0 fl, and 0.6 mg/dl and 51.5 mm/h, respectively. No patient had impaired renal function.

At the start of MTX therapy, 50% of patients were receiving low-dose oral corticosteroids and other DMARDs, and all patients had received NSAIDs. Because the body weight at the start of MTX therapy could not be obtained from the medical records, it could not be assessed. Adverse effects during the evaluation period were observed in only 1 of the 36 patients.

Table 1. Factors Predicting Response to Methotrexate in RA Patients

n	36
Women (%)	86
CRP (mg/dl)*	1.9 (0.4–12.8)
ESR (mm/h)*	51.5 (13.0–143.0)
RF (IU/ml)*	78.6 (0.0–811.0)
MCV (fl)*	89.0 (75.7–101.1)
Scr (mg/dl)*	0.6 (0.4–1.0)
Previous use of DMARDs (%)	91.7
Duration of disease (yr)	4.0 (0.0–36.0)
Age at MTX initiation (yr)	61.0 (26.0–86.0)

Values are expressed as medians; figures in parentheses are ranges (min-max).
* Level and ESR at the initiation of MTX therapy.

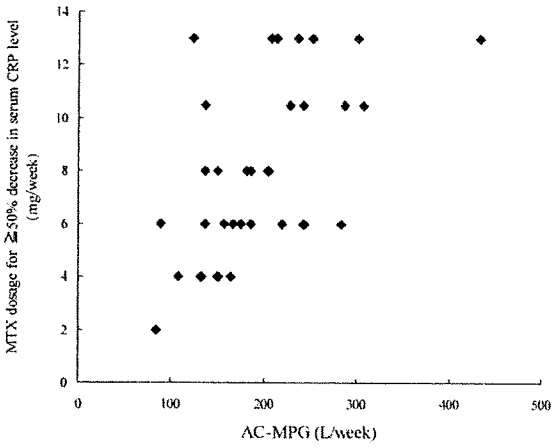

Fig. 1. Linear Regression of AC-MPG and MTX Dose for 50 % Decrease in Serum CRP Level in 36 Japanese RA Patients
AC-MPG=MTX dose/RBC MTX-PGs conc. Ratio. A linear regression of $r=0.59$ and $r^2=0.34$ ($p<0.001$) was obtained.

Relationship between Concentration of MTX-PGs in RBCs and MTX Dose for ≥50% Decrease in Serum CRP Level Ninety-five blood samples (mean 2.6 times/patient) were collected, and the MTX-PGs concentration in RBCs (mean±S.D.) was 97.3±8.1 nmol/l at the 6.9±0.3 mg/week MTX dose (n=36). Mean AC-MPG in the 36 patients ranged from 85.2–432.3 l/week, and there was a difference of about 5.1-fold among them. The mean CV of AC-MPG was 5.9% (n=36), and most individual AC-MPG values did not change during the evaluation period. Therefore, AC-MPG was deemed patient-intrinsic value, and it was used as an explanatory variable of the MTX dose for 50%CRP. The relationships between individual AC-MPG and MTX dose for 50% CRP are shown in Fig. 1. The MTX dose for 50% CRP ranged from 2.0 to 13.0 mg/week, and there were differences of approximately 7-fold among patients. The higher the AC-MPG, the higher the MTX dose for 50%CRP became. The analysis revealed a good correlation between AC-MPG and MTX dose for 50%CRP ($p<0.001$). Therefore, AC-MPG was used as an explanatory variable of the MTX dose for 50%CRP. In all samples, the MTX concentrations in plasma were measured using a TDX analyzer, but all MTX concentrations were below the limit of determination.

Relationships between Genetic Polymorphisms of TYMS or MTHFR and MTX Dose for ≥50% Decrease in Serum CRP Level The four polymorphisms of TYMS and MTHFR were investigated in the 36 RA patients, and the distribution of TYMS 5′-UTR (2R/3R), TYMS 3′-UTR (−6/+6), MTHFR C677T, and MTHFR A1298C polymorphisms are summarized in Table 2. Allelic frequencies (%) were TYMS 5′-UTR 3R(86)>2R(14), TYMS 3′-UTR 6-bp deletion(65)>insertion(35), MTHFR 677C (61)>T(39), and 1298A(76)>C(24), respectively. No patient had TYMS 5′-UTR 2R/2R.

The relationships between the four polymorphisms of TYMS or MTHFR and MTX dose for 50%CRP were evaluated in three groups (homozygous mutant type, heterozygous mutant type, and homozygous wild type), two groups (without/with homozygous wild type) by every polymorphism site, or three groups classified by the pharmacogenetic index. The MTX dose for 50%CRP (mean±S.D.) in patients without the TYMS 3′-UTR +6 allele was 6.5±2.4 mg/week and in those with the TYMS 3′-UTR +6 allele it was 8.8±3.6 mg/week, and the difference was statistically significant (p=0.039). However, no significant difference was found in any other analyses.

Identification of Major Predictive Factors for Response to MTX Therapy in RA Patients To identify the predictive factors of MTX dose for ≥50 %CRP, we performed multivariate regression analysis including AC-MPG, the four polymorphisms of TYMS and MTHFR, and other factors. Each genotype was classified into two groups (with/without homozygous wild type), and the pharmacogenetic index was classified into two groups (0 or 1 and 2). The multivariate regression analysis was performed with 15 explanatory variables. No patient had TYMS 5′-

Table 2. Distribution of TYMS and MTHFR Gene Polymorphisms in RA Patients

	Genotype frequency (%)			Allele frequency (%)	
TYMS 5′-UTR	*2R/2R*	*2R/3R*	*3R/3R*	*2R*	*3R*
	0(0.0)	10(27.8)	26(72.2)	14	86
TYMS 3′-UTR	*+6/+6*	*+6/−6*	*−6/−6*	*+6*	*−6*
	4(11.1)	17(47.2)	15(41.7)	35	65
MTHFR 677	*C/C*	*C/T*	*T/T*	*C*	*T*
	16(44.4)	12(33.3)	8(22.2)	61	39
MTHFR 1298	*A/A*	*A/C*	*C/C*	*A*	*C*
	22(61.1)	11(30.6)	3(8.3)	76	24

Values for genotype frequency are expressed as number of patients (%).

Table 3. Multiple Regression Analysis of MTX Dosage for ≥50% Decrease in Serum CRP Level

	Dependent variable MTX dosage for ≧50% decrease in serum CRP level	
	β	*p*-value
Explanatory variable		
AC-MPG	0.590	<0.001
TYMS3′-UTR without+6-bp allele	−0.268	0.039
ESR*	0.293	0.025
R^2	0.51	

R^2, coefficient of determination. *β*, standard partial regression coefficient.
* ESR at the initiation of MTX therapy.

UTR 2R/2R, and therefore genotypes were divided into 2R/3R and 3R/3R. The results of this analysis are shown in Table 3. In the model, three variables, AC-MPG level, TYMS 3′-UTR (−6/+6), and ESR at the initiation of MTX therapy were major predictive variables of the MTX dose for 50%CRP ($p<0.001$, $p=0.039$, and $p=0.025$, respectively). The AC-MPG level was the most influential explanatory variable for the MTX dose for 50%CRP ($\beta=0.590$). The model-explained variance of the MTX dose for 50%CRP was 51.0%. In this analysis, multicolinearity was recognized between CRP and ESR ($r=0.794$). Therefore, basing on the results of coefficient of determination (R^2), ESR was adopted as the explanatory variable.

DISCUSSION

In this study, we evaluated the relationships between the individual response to MTX and various associated factors in 36 Japanese RA patients, and identified AC-MPG level, TYMS 3′-UTR (−6/+6), and ESR at the initiation of MTX therapy as the major predictive factors for the response to MTX.

The range of MTX dose for 50%CRP which was used as the indicator of the response to MTX therapy[23] was 2.0–13.0 mg/week, with differences of approximately 7-fold among patients. Half of our 36 RA patients were receiving low-dose oral corticosteroids and all were receiving NSAIDs at the start of MTX therapy. However, all continued the same dose of oral corticosteroids and NSAIDs until a ≥50% decrease in the serum CRP level had been achieved, therefore, it was thought that concomitant use of these drugs did not affect in the evaluation of MTX dose for 50%CRP.

The distribution of the allelic frequencies of TYMS 5′-UTR (2R/3R), TYMS 3′-UTR (−6/+6), MTHFR C677T, and MTHFR A1298C in these 36 Japanese RA patients was similar to that in our previous report on 102 healthy Japanese adults.[22] In the evaluation of the relationship between MTX dose for 50%CRP and the genetic polymorphisms of TYMS or MTHFR, there was a significant difference in MTX dose for 50%CRP between patients with and without the TYMS 3′-UTR +6 allele ($p=0.039$). Kumagai *et al.*[8] evaluated the relationship between the response to MTX and TYMS genotype in 105 Japanese RA patients with a history of MTX administration. They reported that a better response to MTX was seen in patients with the TYMS 3′-UTR −6 allele. Our finding supports their result.

In the evaluation of the relationships between the MTX dose for 50%CRP and genetic polymorphisms