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ORIGINAL ARTICLE

A combination of biochemical markers of cartilage and bone turnover, radiographic damage and body mass index to predict the progression of joint destruction in patients with rheumatoid arthritis treated with disease-modifying anti-rheumatic drugs

Jun Hashimoto · Patrick Garnero · Désirée van der Heijde · Nobuyuki Miyasaka · Kazuhiko Yamamoto · Shinichi Kawai · Tsutomu Takeuchi · Hideki Yoshikawa · Norihiro Nishimoto

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Abstract The aim of this study was to evaluate the predictive value of biological, radiological and clinical parameters for the progression of radiographic joint damage in rheumatoid arthritis (RA) patients treated with conventional disease-modifying anti-rheumatic drugs (DMARDs). We analyzed the 145 patients with active RA for less than 5 years who were participating in the prospective 1-year randomized controlled trial of tocilizumab (SAMURAI trial) as a control arm treated with conventional DMARDs. Progression of joint damage was assessed by sequential radiographs read by two independent blinded X-ray readers and scored for bone erosion and joint space narrowing (JSN) using the van der Heijde-modified Sharp method. Multivariate analysis revealed that increased urinary levels of C-terminal crosslinked telopeptide of type II collagen (U-CTX-II), an increased urinary total pyridinoline/total deoxypyridinoline (U-PYD/DPD) ratio and low body mass index (BMI) at baseline were independently associated with a higher risk for progression of bone erosion. In addition to these three variables, the JSN score at baseline was also significantly associated with an increased risk of progression of the JSN score and total Sharp score. High baseline U-CTX-II levels, U-PYD/DPD ratio and JSN score and a low BMI are independent predictive markers for the radiographically evident joint damage in patients with RA treated with conventional DMARDs.

Keywords BMI · CTX-II · Joint destruction · PYD/DPD ratio · Rheumatoid arthritis

J. Hashimoto · H. Yoshikawa
Osaka University Graduate School of Medicine, Osaka, Japan

P. Garnero
Inserm Research Unit 664 and Synarc, Lyon, France

D. van der Heijde
Leiden University Medical Center, Leiden, The Netherlands

N. Miyasaka
Tokyo Medical and Dental University, Tokyo, Japan

K. Yamamoto
The University of Tokyo, Tokyo, Japan

S. Kawai
Toho University Omori Medical Center, Tokyo, Japan

T. Takeuchi
Saitama Medical Center/School, Saitama, Japan

N. Nishimoto (✉)
Laboratory of Immune Regulation, Wakayama Medical University, 105 Saito Bio Innovation Center, 7-7-20 Saito-Asagi, Ibaraki, Osaka 565-0085, Japan
e-mail: norichan@wakayama-med.ac.jp

Introduction

Although rheumatoid arthritis (RA) has features of a systemic disease and capable of exhibiting a variety of extra-articular manifestations, it is predominantly characterized by structural destruction of the joints, leading to functional disability [1–4]. Joint destruction often progresses early in the disease process [5–8], but the process is highly variable from patient to patient [9–12]. The identification of patients with rapid joint destruction very early in the disease process is of critical importance to clinicians wanting to optimize treatment strategies. Indeed, although new biological therapies are highly effective in preserving joint structure, they are expensive and may have side effects.

Thus, targeting these treatments to RA patients manifesting rapid progression of the disease may be beneficial.

Several prospective studies have been performed to identify predictive factors indicative of a worse radiological progression of RA [13–31]. The earlier investigations revealed the importance of the rheumatoid factor (RF), inflammation markers or radiographic damage at baseline [13, 14, 16–18, 20, 21], while more recent ones have identified biochemical markers of bone, cartilage and synovial tissue metabolism and catabolic enzymes as being associated with progression in RA [15, 19, 22, 24, 27–29]. Alternatively, RA is also associated with accelerated atherosclerosis and increased cardiovascular mortality and, recently, it has been shown that macrophage inhibitory cytokine 1 (MIC-1), which is linked to clinical events in atherosclerosis, may be involved in the pathological process of erosive joint destruction [32]. The body mass index (BMI) has also been reported to be associated with the radiographic progression of RA, independent of inflammation markers [23, 30, 31], and recent new information suggests the potential involvement of adipokines as regulators of inflammation in RA [33]. These new findings have lead to the recognition of RA as a disease involving a variety of pathological conditions related with joint destruction and made clinicians aware of the fact that RA is a systemic disease in terms of the pathology of the bone and destruction of cartilage. However, to date, there has been no study that has analyzed concomitantly in the same population the independent contribution of these various anthropometric, clinical, laboratory and radiological features to the prediction of disease progression in RA.

The aims of the study reported here were to determine which combination of a few risk factors identified among a panel of clinical, biological and radiological parameters would be powerful in predicting the radiological progression of bone erosion and joint space narrowing (JSN) in RA patients treated with conventional disease-modifying antirheumatic drugs (DMARDs).

Methods

Patients and protocol

The patient cohort consists of 148 patients with RA receiving conventional DMARDs who participated in the control arm of the SAMURAI trial described in a recent publication [34]. The aim of the SAMURAI, which was a 52-week-long multi-center clinical trial, was to evaluate the effect of tocilizumab on radiological joint damage. Three hundred and six patients with RA diagnosed according to the American College of Rheumatology criteria [35] were randomly assigned to tocilizumab monotherapy (8 mg/kg intravenously every 4 weeks) or conventional DMARDs. For the DMARDs group, the dose, type and combination of DMARDs and/or immunosuppressants could vary according to disease activity at the discretion of the treating physician. The study protocol was approved by the Ministry of Health, Labor and Welfare of Japan, and by the ethical committee at each participating site, and patients gave their written informed consent.

Radiographic assessment

Posteroanterior radiographs of hands and anteroposterior radiographs of feet were performed at baseline and at weeks 28 and 52 or at the last visit for patients who withdrew from the study prior to week 52. Radiographs were scored using the van der Heijde-modified Sharp method [36, 37] for bone erosion, joint space narrowing (JSN) and total sharp score (TSS) independently by two readers who were well trained and competent to score radiographs in accordance with the method. The readers were blinded to the treatment group and chronological order of the films.

Clinical assessment

The Disease Activity Score on 28 joints (DAS28), clinical improvement in signs and symptoms of RA, tender joint count, swollen joint count, and modified health assessment questionnaire (MHAQ) [38] were assessed at baseline.

Laboratory examinations

Fasting blood samples and the second morning urine samples were obtained from all subjects at clinical visits. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were measured in the local clinical test laboratory of each investigation site.

To assess bone formation, we measured serum intact-osteocalcin (OC) using a two-site immunoradiometric assay (Mitsubishi Kagaku Iatron, Japan) and serum bone alkaline phosphatase (bone ALP) by an enzyme-linked immunosorbent analysis (ELISA; Quidel, San Diego, CA). Markers of bone resorption included urinary N-terminal crosslinked telopeptide of type I collagen (U-NTX-I), which was measured by an ELISA (Ostex Int, Seattle, WA), and urinary total deoxypyridinoline (U-DPD) and total pyridinoline (U-PYD), measured by a high-performance liquid chromatography (HPLC) assay. Markers of cartilage synthesis included the N-terminal propeptide of type IIA collagen (PIIANP; Linco, St. Louis, MO) and the C-terminal propeptide of type II collagen (PIICP; IBEX Diagnostics, Montreal, Canada). Cartilage degradation was assessed by the urinary excretion of the C-terminal

crosslinked telopeptide of type II collagen (CTX-II CartiLaps ELISA; NORDIC Biosciences, Herlev, Denmark). Synovial tissue metabolism was assessed by measuring the urinary excretion of glucosyl–galactosyl–pyridinoline (Glc–Gal–PYD) by HPLC, serum matrix metalloproteinase-3 (MMP-3) by ELISA (Daiichi Pure Chemical, Japan) and serum amyloid protein A (SAA) by a latex immunoassay (LIA; Eiken Chemical, Japan). Other measures included serum interleukin-6 (IL-6) using a chemiluminescent enzyme immunoassay (CLEIA) (Fujirebio Japan), RF by LIA (Mitsubishi Kagaku Iatron, Japan), and immunoglobulin G (IgG) by LIA (Eiken Chemical, Japan).

Statistical analysis

For analyzing the correlation between markers at baseline and at the 52-week radiological progression of joint damage, we normalized the markers by logarithmic transformation when needed. First, the markers were selected by Pearson correlation coefficient with TSS, erosion score, and JSN score ($|r| > 0.15$). Then, the predictive factors were selected based on the multivariate regression analysis using the backward elimination method, the forward selection method, and the best-subset selection procedure using Mallows' Cp- adjusted R^2.

The odds ratio of progression in TSS, bone erosion and JSN score according to the levels of these baseline factors were estimated by logistic regression analysis with a 95% confidence interval (95% CI). The progression of joint damage was defined as an increase of TSS of 0.5 or more at 52 weeks.

All statistical analyses were two-sided, and p values <0.05 were considered to be significant. All statistical analyses were carried out using SAS ver. 8.2, TS2MO (SAS Institute, Cary, NC).

Results

One hundred and forty-five patients were included in the intent to treatment (ITT) analyses. Demographics and baseline disease characteristics are shown in Tables 1 and 3. At baseline, the mean age and the disease duration were 53.1 and 2.4 years, respectively. Patients had very active disease, as indicated by a DAS28 score of 6.4 and CRP of 4.9 mg/dl at baseline. The kinds of DMARDs and immunosuppressants used for RA treatment during the study and the number of patients are shown in Table 2.

Bivariate linear correlation analyses showed that baseline values of U-PYD, the ratio U-PYD/DPD, U-CTX-II, U–Glc–Gal–PYD, TSS, erosion score, JSN score, age and BMI were associated significantly with the 1-year increase in all three radiological indices of joint damage, i.e. bone erosion score, JSN score and TSS (Table 3). The baseline levels of U-DPD, S-PIIANP, triglyceride, ferritin also had a significant association with one or two variables among these three radiographic progression parameters (Table 3). None of the clinical indices of disease activity nor the biological parameters of inflammation were associated significantly with radiological progression. In the

Table 1 Baseline demographics, clinical and laboratory characteristics of the patient cohort

Baseline demographics, clinical and laboratory characteristics	Values
Number of patients	145
Age, years (mean)	53.1 ± 12.5
Female, *n* (%)	119 (82.1)
BMI (kg/m^2)	21.8 ± 3.0
RA duration (years)	2.4 ± 1.3
Number of previous DMARDs	2.8
Tender joint count	14.4 ± 7.5
Swollen joint count	11.8 ± 5.8
CRP (mg/dl)	4.9 ± 2.9
DAS28	6.4 ± 0.9
Radiological total Sharp score	30.6 ± 42.0
Radiological bone erosion score	13.9 ± 21.7
Radiological joint space narrowing (JSN) score	16.7 ± 21.8

Values are given as the mean ± standard deviation, unless otherwise indicated

RA Rheumatoid arthritis, *DAS28* Disease Activity Score based on 28 joint counts, *CRP* C-reactive protein, *BMI* body mass index, *DMARDs* disease-modifying anti-rheumatic drugs

Table 2 Number of patients using concomitant drugs related to rheumatoid arthritis during the study

Variables	Number of patients[a]
Corticosteroids	145 (100%)
Methotrexate	123 (84.8%)
Mizoribine	11 (7.6%)
Azathioprine	7 (4.8%)
Ciclosporin	5 (3.4%)
Tacrolimus hydrate	3 (2.1%)
Sulfasalazine	60 (41.4%)
Bucillamine	33 (22.8%)
Sodium aurothiomalate	4 (2.8%)
D-Penicillamine	11 (7.6%)
Actarit	6 (4.1%)
Lobenzarit disodium	2 (1.4%)
Cyclophosphamide	2 (1.4%)
Minocycline hydrochloride	2 (1.4%)

[a] Values are given as the number of patients taking a drug; patients can take more than one drug

Table 3 Baseline patient measurements and Pearson correlation coefficient between the levels of candidate factors at baseline and the changes in radiographic score at week 52

Variables	Levels at baseline (mean ± SD)	*r* value between baseline levels and radiological progression at week 52		
		Total sharp score	Bone erosion score	Joint space narrowing (JSN) score
Bone markers				
Intact-osteocalcin (ng/ml)	5.1 ± 2.1	NS	NS	NS
Bone alkaline phosphatase (U/l)	21.5 ± 6.5	NS	NS	NS
S-NTX-I (nmol BCE/l)	15.8 ± 4.8	NS	NS	NS
U-NTX-I (nmol BCE/mmol creatinine)	62.6 ± 31.9	NS	NS	NS
U-DPD (μmol/mol creatinine)	8 ± 4	0.185*	NS	0.187*
Bone or cartilage markers				
U-PYD (μmol/mol creatinine)	55 ± 37	0.278**	0.253**	0.274**
U-PYD/DPD	7.2 ± 1.8	0.190*	0.180*	0.178*
Cartilage markers				
S-PIIANP (ng/ml)	459.8 ± 210.9	NS	−0.188*	NS
S-PIICP (ng/ml)	819.1 ± 311.6	NS	NS	NS
U-CTX-II (ng/mmol creatinine)	902.5 ± 919.2	0.356***	0.321***	0.356***
Radiographic scores				
Total Sharp score	16.7 ± 21.8	0.323***	0.303***	0.307***
Erosion score	30.6 ± 42.0	0.313***	0.308***	0.282**
Joint space narrowing score	13.9 ± 21.7	0.323***	0.291***	0.322***
Symptoms or functions				
DAS28	6.4 ± 0.9	NS	NS	NS
Objective signs				
Tender joint count	14.4 ± 7.5	NS	NS	NS
Swollen joint count	11.8 ± 5.8	NS	NS	NS
Patients reported functional assessment				
MHAQ	0.90 ± 0.58	NS	NS	NS
Inflammation markers				
CRP (mg/dl)	4.9 ± 2.9	NS	NS	NS
ESR (mm/h)	71 ± 25	NS	NS	NS
MMP-3 (ng/ml)	456.5 ± 347.5	NS	NS	NS
SAA (μg/ml)	347 ± 307	NS	NS	NS
Fibrinogen (mg/dl)	490 ± 96	NS	NS	NS
Interleukin-6 (pg/ml)	60.2 ± 64.9	NS	NS	NS
Synovium degradation marker				
U-Glc–Gal–PYD (nmol/mmol creatine)	11.6 ± 9.3	0.255**	0.238**	0.245**
Hematological parameters				
WBC (/μl)	8,923 ± 2,430	NS	NS	NS
RBC (10^4/μl)	397 ± 38	NS	NS	NS
Hemoglobin (g/dl)	11.3 ± 1.4	NS	NS	NS
Platelet (10^4/μl)	37.2 ± 10.1	NS	NS	NS
Lipid parameters				
Total cholesterol (mg/dl)	182 ± 33	NS	NS	NS
HDL cholesterol (mg/dl)	56 ± 14	NS	NS	NS
LDL cholesterol (mg/dl)	108 ± 27	NS	NS	NS
Triglyceride (mg/dl)	90 ± 35	−0.187*	−0.193*	NS
Other biomarkers				
RF (IU/ml)	247 ± 452	NS	NS	NS

Table 3 continued

Variables	Levels at baseline (mean ± SD)	*r* value between baseline levels and radiological progression at week 52		
		Total sharp score	Bone erosion score	Joint space narrowing (JSN) score
IgG (mg/dl)	1,697 ± 492	NS	NS	NS
Albumin (g/dl)	3.7 ± 0.3	NS	NS	NS
Ferritin (ng/ml)	105 ± 116	NS	−0.182*	NS
Age	53.1 ± 12.5	−0.259**	−0.278**	−0.205*
Gender (M:F)	26:119	NS	NS	NS
Duration of disease	2.4 ± 1.3	NS	NS	NS
Anthropometric factor				
BMI (kg/m^2)	21.8 ± 3.0	−0.298***	−0.257**	−0.311***

NS not significant, *S-NTX* Serum type I collagen cross-linked N-telopeptides, *U-NTX* urinary type I collagen cross-linked N-telopeptides, *U-DPD* urinary deoxypyridinoline, *U-PYD* urinary pyridinoline, *S-PIIANP* serum N-terminal propeptide of type IIA collagen, *S-PIICP* serum C-terminal propeptide of type II collagen, *U-CTX-II* urinary C-terminal telopeptide of type II collagen, *MHAQ* modified health assessment questionnaire, *ESR* erythrocyte sedimentation rate, *MMP-3* matrix metalloproteinase-3, *SAA* serum amyloid protein A, *U-Glc–Gal–PYD* urinary glucosyl–galactosyl–pyridinoline, *IgG* immunoglobulin G, *WBC* white blood cell, *RBC* red blood cell, *HDL cholesterol* high-density lipoprotein cholesterol, *LDL cholesterol* low-density lipoprotein cholesterol

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 4 Multivariate regression analysis relating JSN U-CTX-II, U-PYD/DPD, or BMI to changes in the radiographic scores at 52 weeks

Baseline predictor	Parameter estimate		*p* value
Total Sharp score progression			
JSN	4.88		0.04
PYD/DPD	20.81		0.02
CTX-II	9.41		<0.01
BMI	−0.92		<0.01
R^2		0.24	<0.001
Bone erosion progression			
PYD/DPD	11.20		0.04
CTX-II	5.58		<0.01
BMI	−0.48		0.02
R^2		0.17	<0.001
Joint space narrowing progression			
JSN	2.37		0.04
PYD/DPD	9.62		0.02
CTX-II	4.56		<0.01
BMI	−0.46		<0.01
R^2		0.25	<0.001

JSN Joint space narrowing, *PYD/DPD* logarithmic transformed urinary pyridinoline/deoxypyridinoline ratio, *CTX-II* logarithmic transformed urinary C-terminal telopeptide of type II collagen

multivariate analyses, increased levels of U-CTX-II, an increased U-PYD/DPD ratio and decreased BMI were the only independent predictors of the progression of bone erosion (Table 4). Together, these three variables explained 17% of the interindividual variance in the progression of bone erosion. For the progression of JSN and TTS, baseline JSN was also an independent predictor in addition to U-CTX-II, the U-PYD/DPD ratio and BMI (Table 4).

Logistic regression analysis after the categorization of the four predictive variables with the cut-off value of 500 ng/mmol/creatinine in U-CTX-II, median level for the U-PYD/DPD ratio, two cut-off values of 18.5 and 25 kg/m^2, respectively, in BMI and a 0 or >0 score in JSN score at baseline showed that the odds ratio for a yearly increase of TSS >0.5 was 2.6- to 9.9-fold higher risk in the high-risk group than in patients with low risk levels (Fig. 1a); the respective figures for progression in erosion score and for progression in JSN were 2.8–4.8 and 1.8–20.0, respectively (Fig. 1b, c). Baseline levels in the categorized groups are shown in Table 5.

Discussion

Based on our analysis of a panel of several demographical, clinical and laboratory parameters of disease activity, we found that increased urinary CTX-II, a high PYD/DPD ratio and low BMI were independent predictors of radiological progression in bone erosion and TTS in patients with RA receiving conventional DMARDs and that baseline JSN was also an independent predictor of radiological progression in JSN and TTS. These results suggest that these factors should be useful in identifying patients at high risk.

The bivariate analyses revealed that the baseline levels of U-PYD, the U-PYD/DPD ratio, U-CTX-II, TSS, erosion score, JSN score, U-Glc–Gal–PYD, age and BMI were

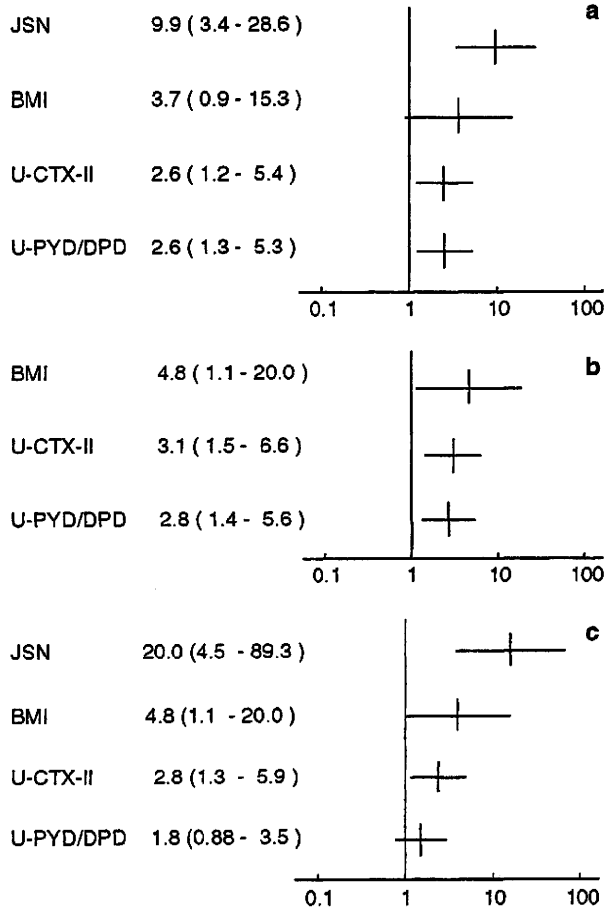

Fig. 1 Odds ratio (95% confidence interval) of radiological progression associated with high baseline joint space narrowing (*JSN*), high urinary C-terminal telopeptide of type II collagen (*U-CTXII*), high urinary total pyridinoline/total deoxypyridinoline (*U-PYD/DPD*), or low body mass index (*BMI*). Progression of joint damage over 1 year was defined as an increase >0.5 U of the total Sharp score (**a**), bone erosion (**b**) or JSN (**c**)

Table 5 Baseline levels in the categorized groups

Variables	Cut-off value	*n*	Mean of baseline value ± SD
JSN	0	30	0
	0<	115	21.1 ± 22.5
U-CTX-II	<500	53	327.2 ± 104.6
(ng/mmol/creatinine)	500≤	88	1,249.0 ± 1,014.9
U-PYD/DPD	<median (6.8)	72	5.8 ± 0.7
	Median (6.8)≤	73	8.6 ± 1.4
BMI (kg/m^2)	<18.5	20	17.5 ± 1.2
	18.5≤, <25	102	21.5 ± 1.6
	25≤	21	27.1 ± 1.7

significantly associated with the 1-year increase in all three indices of TSS, erosion score and JSN score and that the baseline levels of U-DPD, S-PIIANP, triglycerides and ferritin were significantly associated with one or two variables among these three radiographic progression parameters. However, there was no significant association with radiographic progression in the baseline levels of inflammation markers, MMP-3, hematological parameters, patients-reported functional assessments, such as MHAQ, and objective symptomatic scores. Although several previous studies showed that MMP-3 was predictive of radiological progression [22, 29, 39, 40] in RA, our data and those of Cunnane et al. [41] failed to reveal a significant association. Circulating MMP-3 levels have been reported to be significantly decreased after treatment with methotrexate or sulfasalazine or both together [29, 41–44]. These findings suggest that levels of MMP-3 are dependent on the type, duration and intensity of the pharmacotherapy. It is thus possible that differences in the therapeutic regimen between studies may explain some of the inconsistencies in the relation of MMP-3 to progression. Additional factors may include differences in disease duration and activity and variation in assay characteristics, which are not standardized between studies. Consistent with the results of a recent study [29], we confirmed that patient-reported functional assessments and clinical symptomatic indices were not useful in predicting radiological progression.

Inflammation markers, such as CRP and ESR, have been regarded as useful predictors of joint damage in RA. However, our study confirmed the recent findings of Young-Min [29], showing that when novel and more specific markers of joint tissue metabolism were included in the model, these unspecific laboratory tests were no longer predictive. Among these novel tissue turnover markers, the strongest and most consistent association with progression was observed for urinary CTX-II, a biochemical marker of cartilage degradation, a finding consistent with several previous studies involving patients with early RA receiving MTX or etanercept [19], very early RA receiving the COBRA combination therapy or sulfasalazine alone [45] or late RA treated with conventional DMARDs [29]. Taken together, the results from these previous studies and the current one suggest that urinary CTX-II is predictive of radiological progression across patient populations and independent of the type of therapy. We also found that urinary-Glc–Gal–PYD, a specific biochemical marker of synovial tissue metabolism, was associated significantly with radiographical progression in bivariate analysis. This result was consistent with that of a previous study [19] of early RA patients receiving methotrexate or etanercept. However, urinary-Glc–Gal–PYD did not remain in the final panel of predictors after multivariate analysis, confirming

the recent study of Young-Min [29] who showed that Glc–Gal–PYD was predictive in bivariate, but not in multivariate analyses when CTX-II was included in the model. This lack of independent predictive value is likely to be due to the high correlation of Glc–Gal–PYD with CTX-II ($r = 0.61, p < 0.001$) and suggests that in early active RA, degradation of cartilage is closely linked to synovitis. Whether urinary Glc–Gal–PYD could be an independent predictor of progression in late RA or in patients receiving biological therapies remains to be determined.

Previously published cross-sectional studies found an increased urinary PYD/DPD ratio in patients with RA [46–49]. Our study, however, is the first showing that U-PYD/DPD ratio is an independent predictor of radiological progression. Both PYD and DPD are non-reducible cross-links of mature collagen molecules, and they are believed to be important factors for maintaining the structure of the collagen fibril network in the matrix of the various tissues, including bone and cartilage. In healthy tissues, the PYD/DPD ratio is highest in cartilage (ratio: 50), intermediate in synovial tissue and tendons (ratio: 15–16) and lowest in bone (ratio: 3.5) [50–52]. The tissue PYD/DPD ratio can be altered in RA tissue, with the latter showing a higher ratio than healthy synovium [23, 51]. In addition, a high tissue PYD/DPD ratio in bone caused by the overhydroxylation of Lys at the helical cross-linking sites in type I collagen has been observed in the hip fracture cases [53] and osteoporosis [54]. Thus, the PYD/DPD ratio may theoretically provide some indication of the type of articular tissue that is predominantly degraded in RA. In our study, this ratio, but not PYD and DPD separately, was associated with radiological progression of bone erosion and JSN independently of CTX-II, which is a specific marker of cartilage degradation and of Glc–Gal–PYD (a specific marker of synovial metabolism), suggesting indeed the added value of this parameter. One possibility is that this ratio partially reflects structural alterations of bone tissue matrix associated with increased bone fragility, as suggested by some ex vivo biochemical studies [53, 54].

We found that high BMI was correlated negatively with the progression of joint erosion and JSN and that patients with lower values (<18.5), defined as underweight, had a 4.8-fold (95% CI 1.1–20) higher risk than the patients with higher BMI (>25) who were defined as overweight. Previously published reports showed a body weight loss due to disease activity [55–58] in RA, although no significant correlation between BMI and inflammation markers was observed at baseline in our study (data not shown). Our results agree with studies published previously by Kaufmann [23], Westhoff [31] and van der Helm-van Mil [30] which showed that high BMI was protective against the radiological progression in early RA. It has been suggested that the relationships between BMI and joint damage are mediated in part by the adipocytokines secreted by fat tissues. Interestingly, we recently reported that increased serum levels of adiponectin—which is negatively associated with BMI—are associated with a greater overall joint destruction in patients with RA [59]. Using a bivariate analysis, we found that triglycerides, but not total cholesterol and its subfractions were negatively correlated with radiological progression. However, in the multiple variable model, triglycerides were not an independent predictors, possibly because of its positive association with BMI ($r = 0.29, p < 0.001$).

Previously published data showed that high initial radiographical damage evaluated with TSS or the Larsen score was associated with subsequent radiological progression [16, 17] and that the initial erosion score in particular has a predicting value for radiological prognosis [14, 18, 23]. These data were analyzed without biochemical markers of joint tissue turnover as the initial factors; however, we found that baseline radiological joint damage of the extent of JSN was strongly and independently predictive of biochemical markers of joint tissue turnover associated with progression.

We believe that the four independent predictors of radiological progression we identified in this study may reflect different and complementary information of the various pathophysiological processes involved in joint destruction. The baseline Sharp score provides an estimation of the amount of joint destruction that has occurred, on average, during 2.3 years of disease duration before the start of the follow-up. Urinary CTX-II is a dynamic indicator of the rate at which cartilage tissue will deteriorate during the course of the disease. The PYD/DPD ratio may be related to increased bone fragility, and the BMI may provide integrated information on contribution of adipose tissue metabolism to maintain joint tissues health. These four independent predictors were statistically selected using those patients with high disease activity who were participating in the control arm of the SAMURAI study and who had >6 tender joints (of 49 evaluated), >6 swollen joints (of 46 evaluated joints), ESR of >30 mm/h and CRP of >2 mg/dl. These predictors may therefore be beneficial for targeting new biological therapies to patients with rapid progression of joint destruction.

Although our study covered one of the largest ranges of predictive variables for the progression of joint damage ever investigated concomitantly in the same population, due to sample volume limitation we could not analyze a number of the biochemical markers that have been reported to be associated with joint damage in RA, including anti-CCP antibody, cartilage oligomeric matrix protein (COMP) [25, 26, 60], osteoprotegerin (OPG) and Receptor Activator of Nuclear Factor-kappa B Ligand (RANKL) [61]. Our

study included patients with RA within 5 years of disease duration, so it remains to be determined whether the same set of predictive factors will also perform similarly in patients with earlier RA. Furthermore, our study could not clarify the prognostic factors in the each type of DMARDs treatment nor whether CTX-II, the PYD/DPD ratio, the JSN score and BMI predict progression independent of the type of DMARDs treatment, since the dose, type and combination of DMARDs and/or immunosuppressants was varied and changed according to disease activity at the discretion of the treating physician in our study. However, our data could provide the prognostic values of CTX-II, PYD/DPD ratio, JSN score and BMI in the actual clinical practice of RA treatment.

In summary, among of a panel of 40 different variables, we identified baseline joint damage, urinary CTX-II, the PYD/DPD ratio and BMI as strong and independent factors of radiological progression in patients with RA receiving conventional DMARDs. If confirmed in other studies, this set of few variables may be useful to identify patients with RA who are at high risk for disease progression.

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Laboratory and febrile features after joint surgery in patients with rheumatoid arthritis treated with tocilizumab

M Hirao, J Hashimoto, H Tsuboi, et al.

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Extended report

Laboratory and febrile features after joint surgery in patients with rheumatoid arthritis treated with tocilizumab

M Hirao,[1] J Hashimoto,[1] H Tsuboi,[1] A Nampei,[1] H Nakahara,[2] N Yoshio,[2] T Mima,[2] H Yoshikawa,[1] N Nishimoto[2]

[1] Department of Orthopaedics, Osaka University Graduate School of Medicine, Osaka, Japan; [2] Laboratory of Immune Regulation, Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan

Correspondence to:
J Hashimoto, Department of Orthopaedics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan;
junha@ort.med.osaka-u.ac.jp

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ABSTRACT

Objectives: To understand the acute phase responses to surgical intervention in patients with rheumatoid arthritis (RA) treated with the anti-interleukin (IL)6 receptor antibody, tocilizumab.

Methods: In a retrospective 1:1 pair-matched case-control study, 22 tocilizumab-treated RA cases and 22 cases treated with conventional disease-modifying anti-rheumatic drugs (DMARDs) and matched for type of surgery, age and sex were evaluated for body temperature every day, and blood C-reactive protein (CRP) levels and white blood cell (WBC), neutrophil and lymphocyte counts on days −1, 1, 3 and weeks 1 and 2 after joint surgery. Safety issues were also monitored.

Results: No complications of infection or delay of wound healing occurred in either patient group. Tocilizumab partially, but significantly, suppressed the increase in body temperature on postoperative days 1 and 2, compared with DMARDs (average (SD) maximum increase in temperature was 0.45 (0.1)°C in the tocilizumab group and 0.78 (0.1)°C in the DMARD group; $p<0.01$). Tocilizumab completely suppressed the increase in CRP after surgery, whereas all cases treated with DMARDs showed a significant increase of CRP at postoperative day 1 (5.5 (0.6) mg/dl; $p<0.001$). WBC, neutrophil and lymphocyte counts showed no remarkable change after surgery, and there was no significant difference in any cell counts between the patient groups.

Conclusions: Within this small number of cases, safe operations on patients were performed during tocilizumab treatment. Tocilizumab suppressed fever and increase of CRP after surgery, whereas there was no influence on the transition in number of leukocytes. This characteristic postoperative response should be considered during tocilizumab treatment.

Postoperative surgical site infections represent a serious functional and psychological disadvantage in the course of treatment against rheumatoid arthritis (RA), although the incidence of these infrequent complications in joint surgery performed in patients with RA is approximately 1.7% to 7.2%.[1–8] As it is necessary to detect postoperative infections as soon as possible, body temperature, level of C-reactive protein (CRP) and white blood cell (WBC) count, as well as local findings, are considered indicators of infection.

Previously published data show that the cytokine interleukin (IL)6 is upregulated by surgical trauma and involved in the febrile response.[2 3] The acute phase response might be diminished by blocking the synthesis of IL6 and has been reduced in genetically-modified animals that do not produce this mediator.[4 5] CRP is primarily produced in the liver in response to IL6, and CRP synthesis is enhanced synergistically by IL1β through induction of nuclear factor (NF)κB, p50 and p65.[6–8] In addition, IL6 positively regulates numbers of leukocytes and neutrophils, and inhibition of the IL6 receptor leads to a decrease in leukocytes.[9 10]

In recent years, use of tocilizumab, a humanised monoclonal antibody against IL6 receptor, has succeeded in achieving more effective suppression of disease activity of RA, compared to conventional disease-modifying antirheumatic drugs (DMARDs).[11 12] Based on the previous data, it is unknown whether tocilizumab also suppresses surgically-related and infection-related acute phase responses, thereby leading to possible difficulty in early diagnosis of postoperative infection due to lack of observed clinical signs and symptoms in patients treated with tocilizumab. Therefore, it is very important to understand the details of transition of data on blood tests and body temperature after joint surgery in patients with RA treated with tocilizumab. In the current study, we examined postoperative changes in body temperature and blood levels of CRP, WBCs, neutrophils and lymphocytes in patients with RA treated with tocilizumab or conventional DMARDs.

PATIENTS AND METHODS

Patients

A total of 22 joint surgeries in our hospital were performed on patients with RA treated with tocilizumab. In all cases, treatment with tocilizumab (8 mg/kg, every 4 weeks) was continued, and surgery was performed between the administrations of tocilizumab. The most recent infusion was performed 16.1 (9.5) days (range 3 to 27 days) (mean (SD)) before operation. There was no case of postponement of infusion. All surgeries were performed under general anaesthesia. Operations included shoulder arthroplasty (n = 1), total elbow arthroplasty (TEA) (n = 1), total hip arthroplasty (THA) (n = 1), total knee arthroplasty (TKA) (n = 9), total ankle arthroplasty (TAA) (n = 3), foot surgery (n = 5) and hand surgery (n = 2).

Retrospectively, we studied these 22 cases (tocilizumab group) and compared them to 22 operations in patients with RA who received non-biological medication (methotrexate: 6 cases; salazosulfapyridine: 10 cases, bucillamine: 3 cases, D-penicillamine: 1 case, prednisolone: 17 cases)

and who underwent joint surgery. For matching of cases, patients were selected with a 1:1 pair-matched procedure, according to age (range ±5 years) and type and site of surgery.

Body temperature

Body temperature was measured at least three times daily (at 9.00 am, 2.00 pm and 7.00 pm) during hospitalisation. All measurements were performed for 5 min at the external ear canal on the same side. For analysis, we used the peak body temperature at 1 day before surgery, during 1 and 2 days after surgery, 1 week after surgery (week 1) and 2 weeks after surgery (week 2). Expected fever spikes after surgery in the tocilizumab and the DMARD groups were evaluated by change in body temperature, as compared before the operation.

Blood samples

Venous blood samples were obtained before surgery, 1 and 3 days after surgery, 1 week after surgery and 2 weeks after surgery. Samples were collected in EDTA tubes and analysed for WBC counts and segmentation of WBCs (neutrophils and lymphocytes). The change in WBCs, neutrophils and lymphocytes after surgery was evaluated by the percentage changes in these parameters, compared with preoperative values. CRP levels were measured in serum. In our hospital, the normal reference value for CRP was <0.2 mg/dl.

Statistical analysis

All data are expressed as mean (standard error of the mean (SEM)). Differences between the groups were assessed by a post hoc test using SPSS statistical analysis software (SPSS V. 15.0J; SPSS, Chicago, Illinois, USA). A p value of <0.05 was considered statistically significant.

RESULTS

Postoperative complications

No complications of superficial infections, deep infections, or delay in wound healing were observed in either group of cases.

Body temperature

As shown in table 1, there was no significant difference in preoperative body temperature between the tocilizumab and the DMARD groups. Both groups showed significant increases in body temperature, as compared with that of the preoperative day (fig 1). The expected increase in body temperature following surgery was significantly suppressed in the tocilizumab group (fig 1). Average maximum increase in body temperature was 0.78 (0.1)°C in the DMARD group and 0.45 (0.1)°C in the tocilizumab group.

CRP

Preoperative CRP levels in the tocilizumab group were negative in all cases except one, who showed 0.3 mg CRP/dl. All cases in the DMARD group showed positive CRP values, and the mean (SD) value was 3.1(0.6) mg/dl (table 1). After surgery, 18 of the 22 tocilizumab-treated cases showed no postoperative increase in CRP, whereas the remaining 4 cases showed an increase ranging from 0.1 to 1.0 mg/dl. In the DMARD group, all cases showed a significant increase (day 1: 5.5 (0.6) mg/dl; 1 week after surgery: 2.9 (0.5) mg/dl; 2 weeks after surgery: 2.2 (0.5) mg/dl).

WBC counts

As shown in table 1, there was no significant difference in preoperative numbers of WBCs, neutrophils, or lymphocytes between the tocilizumab and the DMARD groups. These cell numbers showed no remarkable changes after surgery in either group. Additionally, no significant difference between the groups was observed in these parameters after surgery. Therefore, cell numbers after surgery were not affected by tocilizumab treatment.

DISCUSSION

An important issue in anti-inflammatory cytokine therapy is the possible risk of infection, because a recent study showed that tumour necrosis factor (TNF)α antagonists increase the risk of infection for musculocutaneous lesions.[13] Reports on small studies involving 12 to 16 cases of joint surgery in patients with RA treated with TNFα antagonists, however, demonstrated no increase in the incidence of postoperative infection.[14 15]

Our hospital's experience with 22 cases of joint surgery in patients with RA treated with tocilizumab also showed no postoperative surgical site infections. Although IL6 knock-out (IL6KO) mice display significantly delayed cutaneous wound healing compared to wild type mice,[16 17] we observed no delay in postoperative wound healing in patients with RA treated with tocilizumab. Because the reported incidence of postoperative infection is around 1.7% to 7.2%,[1-3] the lack of statistical power due to the small number of patients in our study made it impossible to reach a conclusive result as to the influence of tocilizumab treatment on the risk of postoperative infection. However, our experience that there were no postoperative infections in either tocilizumab or DMARD groups suggests the safety of joint surgery in patients with RA during tocilizumab treatment.

High-level production of proinflammatory cytokines including IL6 in the inflamed synovium has a pathological role in systemic manifestations of RA, such as fatigue, fever and laboratory changes.[18] After surgical treatment, immune cells such as macrophages and neutrophils or other cells such as fibroblasts and endothelial cells are activated locally at the surgical site by destruction of tissues, resulting in increased local and serum levels of IL6.[2 19] Therefore, it is plausible that inhibition of IL6 suppresses the systemic inflammatory manifestations of RA and surgery. Indeed, the postoperative fever spike was partially, but significantly, suppressed by tocilizumab treatment, as compared with DMARD treatment in the present study.

IL6 stimulates hepatocytes to produce acute phase proteins such as CRP, fibrinogen, α1-anti-trypsin and serum amyloid A, and simultaneously suppresses albumin production. Previous reports have shown that the increase in CRP and serum amyloid A were normalised after tocilizumab treatment and the decrease in albumin was alleviated after tocilizumab treatment.[10-12 20] In the current study, the preoperative CRP values were normal (<0.2) in 21 out of 22 cases in the tocilizumab group. Furthermore, a postoperative increase in CRP was not observed in the tocilizumab group, whereas in the DMARD group the mean CRP level changed from a preoperative level of 3.1 mg/dl to a maximum level of 5.5 mg/dl 1 day after surgery. This finding clearly indicates that tocilizumab suppressed not only an RA-related increase of CRP, but also a surgery-related increase in CRP. Tocilizumab treatment also may suppress infection-related symptoms after surgery. Although no infection was observed in the patients of this study, we have experienced

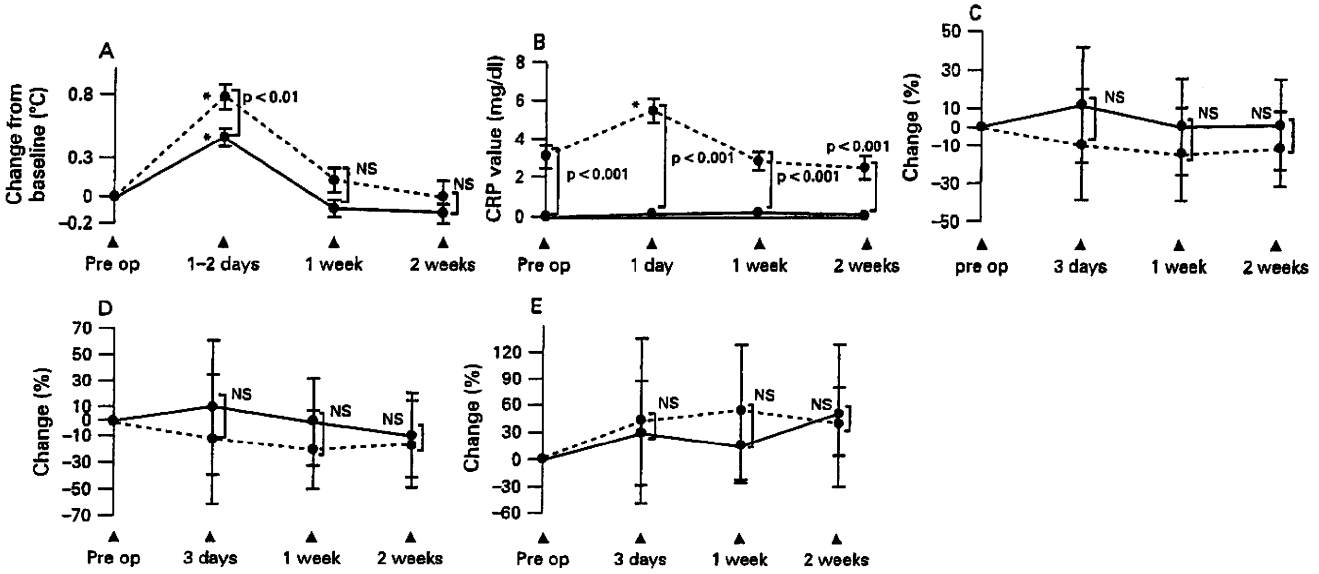

Figure 1 Changes in body temperature, C-reactive protein (CRP) levels and numbers of white blood cells (WBCs; neutrophils and lymphocytes) after surgery. Values are mean (SD). *Significant difference, as compared to values before surgery. NS, no significant difference between the two groups. Dotted lines: disease-modifying antirheumatic drug (DMARD) group. Unbroken lines: tocilizumab group. A. Graph shows the change in body temperature, as compared before surgery. p: p Value for the difference between the DMARD group and the tocilizumab group. B. Graph shows the absolute value of CRP level. p: p Value for the difference between the two groups. C. Graph shows the percentage change in total number of WBCs. D. Graph shows the percentage change in numbers of neutrophils. E. Graph shows the percentage change in numbers of lymphocytes.

some tocilizumab-treated patients with pneumonia without operation. As long as the infection was not severe, serum level of CRP hardly increased while the WBC count did increase. When the patients had severe pneumonia, they showed an increase in CRP level and WBC count (data not shown). This may be explained by the balance between the blood concentration of IL6 and tocilizumab, which competitively binds to IL6 receptor. Severe infection induces a high amount of IL6, which cannot be blocked with usual doses of tocilizumab, resulting in the increase in CRP. Similarly, if the surgical invasion is more severe, CRP would be expected to increase even under tocilizumab treatment. In addition, since the WBC count is less influenced by IL6 than CRP, an increase in WBC count could be a useful sign for possible infection. In the DMARD group, the level of CRP was restored to the basal level within 1 week after surgery, suggesting that surgery-induced inflammation resolves within 1 week in the absence of infection.

A dose-dependent reduction in the neutrophil count following treatment with tocilizumab was reported in patients with RA.[11 12] In the present study, preoperative absolute numbers of WBCs and neutrophils in the tocilizumab group were smaller, but not significantly smaller, than those in the DMARD group. By contrast, preoperative absolute numbers of lymphocytes in the tocilizumab group were larger, but not significantly larger, compared to the DMARD group. No significant postoperative changes in leukocytes, neutrophils, or lymphocytes were observed in either group, and additionally no significant differences between the two groups were seen at any time after surgery. These data suggest that regulation of the increase in leukocytes may depend on not only IL6 but also other cytokines such as granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF).

Table 1 Characteristics of the cases before surgery

Parameter	No. treated with DMARDs	No. treated with tocilizumab	p Value
Body temperature (°C)	36.5 (0.3)	36.6 (0.2)	NS
CRP (mg/dl)	3.1 (0.6)	0.02 (0.02)	<0.001
WBCs/dl	8144 (3229)	7267 (2757)	NS
Neutrophils/ml	6716 (2973)	5424 (2545)	NS
Lymphocytes/ml	978 (413)	1384 (706)	NS
DAS28 (CRP) score	4.4 (0.9)	2.7 (0.7)	<0.001
Prednisolone (mg/day)	7.0 (5.1)	6.5 (5.1)	NS

Values are mean (SD). p Value is for the difference between the DMARD group and the tocilizumab group.
CRP, C-reactive protein; DAS28, 28-joint Disease Activity Score; DMARD, disease-modifying antirheumatic drug; NS, not significant; WBC, white blood cell.

Our data suggest the possibility that tocilizumab might mask the infection-induced increase in CRP and minimise the infection-induced increase in body temperature after orthopaedic surgery in patients with RA. In other words, a small postoperative increase in CRP in patients with RA may be an important sign of the occurrence of non-arthritic types of inflammation, such as postoperative infections. In cases of postoperative increases in CRP in tocilizumab recipients, signs of inflammation in other organs, such as the respiratory system, as well as signs of infection at the surgical site, should be checked. A WBC count may be helpful in knowing whether a concurrent infection is occurring during tocilizumab therapy. Furthermore, serum IL6 should be a good marker for the severity of inflammation because tocilizumab blocks not IL6 itself but the IL6 receptor.[4 5]

In conclusion, although the current study evaluated only 22 cases of orthopaedic surgery in patients with RA treated with tocilizumab and 22 cases of orthopaedic surgery in patients with RA treated with DMARDs, no complications of superficial or deep infection or delay in wound healing after orthopaedic surgery were observed. Additionally, we demonstrated that the increase in CRP was completely suppressed and the rise in body

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temperature was partially suppressed after joint surgery in tocilizumab-treated patients with RA, whereas tocilizumab had no significant influence on the number of leukocytes. Considering these characteristic postoperative responses in patients with RA treated with tocilizumab, we should carefully perform orthopaedic surgery on the patients.

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Differential influences of bucillamine and methotrexate on the generation of fibroblast-like cells from bone marrow CD34+ cells of rheumatoid arthritis patients

Shunsei Hirohata [a,*], Tamiko Yanagida [b], Tetsuya Tomita [c], Hideki Yoshikawa [c]

[a] Kitasato University School of Medicine, Kanagawa 228-8555, Japan
[b] Teikyo University School of Medicine, Tokyo 173-8605, Japan
[c] Osaka University Medical School, Osaka 565-0871, Japan

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ABSTRACT

We have recently demonstrated that bone marrow CD34+ cells from rheumatoid arthritis (RA) patients displayed abnormal capacities to respond to TNF-α and to differentiate into fibroblast-like cells producing MMP-1 (type B synoviocyte -like cells). The current study examined the effects of representative potent disease-modifying antirheumatic drugs, including bucillamine (BUC) and methotrexate (MTX) on the *in vitro* generation of fibroblast-like cells from RA bone marrow CD34+ cells. CD34+ cells purified from bone marrow specimens of 8 patients with active RA were cultured in the presence or absence of pharmacologically attainable concentrations of intramolecular disulfide form of bucillamine (BUC-ID, 3 μM), a major metabolite of BUC or MTX (20 nM). After incubation for 28 days, the generation of fibroblast-like cells was assessed under phase-contrast light microscopy and the concentrations of MMP-1 and VEGF in the culture supernatants were measured by ELISA. BUC-ID, but not MTX, significantly suppressed the generation of fibroblast-like cells from RA bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α ($p=0.024$ as determined by Wilcoxon signed rank test). Accordingly, BUC-ID, but not MTX, significantly suppressed the production of MMP-1 ($p=0.017$) and VEGF ($p=0.017$) by RA bone marrow CD34+ cells, without inhibition of β2-microglobulin production. These results demonstrate that BUC-ID, but not MTX, is a potent inhibitor of differentiation of fibroblast-like cells from RA bone marrow CD34+ cells. Since MTX, but not BUC, has been previously shown to influence on type A synoviocytes, the data provide rationale of combination of BUC and MTX in the treatment of RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of synovial lining cells [1]. Although the dysregulated proliferation of synoviocytes has been suggested to play a pivotal role in synovial hyperplasia [2], it was found that rheumatoid synovium rarely showed evidence of mitosis and that only 4% of rheumatoid synovial cells showed uptake of thymidine [3]. Previous studies have suggested that abnormal myelopoiesis in the bone marrow might play an important role in the pathogenesis of RA [4]. Thus, it has been demonstrated that the generation of CD14 (+) monocyte lineage cells (type A synoviocyte-like cells) from the bone marrow is accelerated in RA patients [5]. In addition, recent studies have disclosed that bone marrow CD34+ cells have enhanced capacity to differentiate into cells with characteristic features of fibroblast-like synoviocytes (type B synoviocytes) in RA [6]. These results raise the possibility that the synovial hyperplasia in RA might be a result of continuous recruitment of bone marrow-derived cells into the synovium [7].

Disease-modifying antirheumatic drugs (DMARDs) have been the mainstay of treatment of RA in recent years. Methotrexate (MTX) clearly has reproducible biological effects on disease activity of RA [8–10] which are sustained over prolonged intervals [11,12]. Although many efforts have been made to explore the mechanisms of action of DMARDs, predominantly on immunocompetent cells, endothelial cells, and synovial cells [13,14], the basis for the efficacy of DMARDs has not been fully elucidated. Recently it has been proposed that DMARDs may work by inhibiting myelopoiesis by supplying fewer inflammatory cells in the inflamed joints [4]. In fact, we have shown that gold sodium thiomalate (GST) as well as MTX inhibits the generation of CD14 (+) cells (type A synoviocyte-like cells) from the bone marrow progenitor cells of RA patients confirming that bone marrow progenitor cells are one of the targets of DMARDs [15,16]. However, the influence of DMARDs, including MTX, on the generation of type B synoviocyte-like cells has not been delineated. The current study therefore examined the effect of MTX and bucillamine (BUC), another potent DMARD, on the *in vitro* generation of type B

* Corresponding author. Department of Rheumatology and Infections Diseases, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan. Tel.: +81 42 778 8111; fax: +81 42 778 9291.
E-mail address: shunsei_tenpoint@yahoo.co.jp (S. Hirohata).

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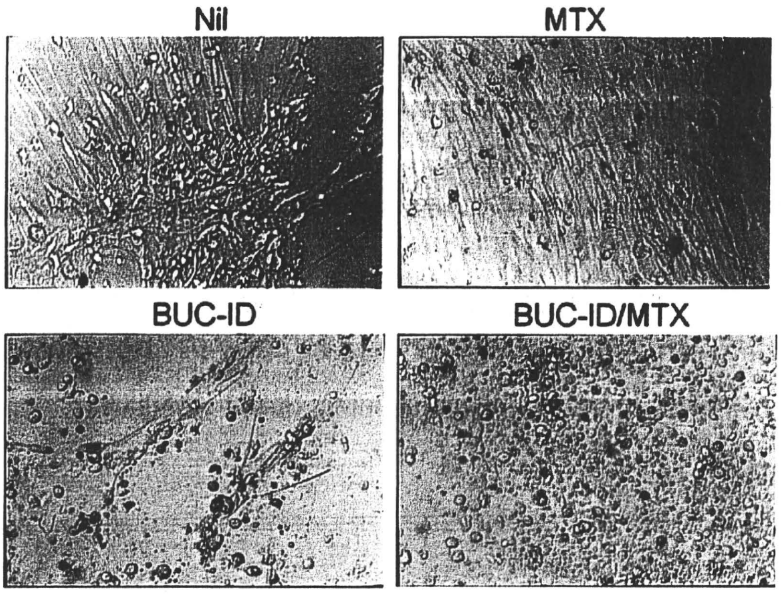

Fig. 1. Morphological changes of CD34+ cells cultured in the presence of SCF, GM-CSF and TNF-α. CD34+ cells from bone marrow of RA patients (1×10^5/well) were cultured in the presence of SCF (10 ng/ml), GM-CSF (1 ng/ml) and TNF-α (10 ng/ml). MTX (methotrexate) (20 nM) or BUC-ID (intramolecular disulfide form of bucillamine) (3 μM) was added where indicated (nil: no addition). After 4 weeks, the morphological changes were determined by phase-contrast microscopy. Original magnification ×50. The data of a representative experiment are shown.

synoviocyte-like cells from bone marrow CD34+ progenitor cells of RA patients.

2. Patients and methods

2.1. Patients

Iliac bone marrow samples were obtained during joint operations from 8 female patients with active RA (mean age 56.6 years, range 43 to 72 years), who gave informed consent. All 8 RA patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for the disease [17]. None of the 8 patients were receiving BUC, whereas 5 of the 8 patients were receiving MTX at the study.

2.2. Reagents

MTX was purchased from Sigma Chemical Co. (St. Louis, MO), and intramolecular disulfide form of BUC (BUC-ID) was a gift of Santen Pharmaceutical Co. (Osaka, Japan). Recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF), stem cells factor (SCF), and tumor necrosis factor-α (TNF-α) were purchased from Pepro Tech EC (London, United Kingdom).

2.3. Culture medium

All cultures were carried out in medium RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with penicillin G (100 U/ml), streptomycin (10 μg/ml), L-glutamine (0.3 mg/ml), and 10% fetal bovine serum (Life Technologies).

2.4. Preparation and culture of bone marrow cells

Heparinized bone marrow aspirates were obtained from the posterior iliac bone. Mononuclear cells were isolated by centrifugation of heparinized bone marrow aspirates over sodium diatrizoate-Ficoll gradients (Histopaque; Sigma Chemical Co., St Louis, MO). CD34+ cells were purified from the bone marrow mononuclear cells by positive selection with magnetic beads (CD34 progenitor cells selection system; Dynal, Oslo, Norway). The cells thus prepared were >95% CD34+ cells and <0.5% CD19+ B cells, as previously described [6]. CD34+ cells were incubated in a 24-well microtiter plate with flat-bottomed wells (No. 3524; Costar, Cambridge, MA) (1×10^5/well) with SCF (10 ng/mL), GM-CSF (1 ng/mL) and TNF-α (10 ng/mL) in the presence or absence of pharmacologically relevant concentrations of BUC-ID (3 μM) [18] and MTX (20 nM) [19]. Preliminary experiments disclosed that MTX as well as BUC-ID in the culture medium was stable after 28 days of incubation, with residual concentrations of approximately 90%. After incubation for 28 days, the cells were observed under phase-contrast microscopy and the culture supernatants were assayed

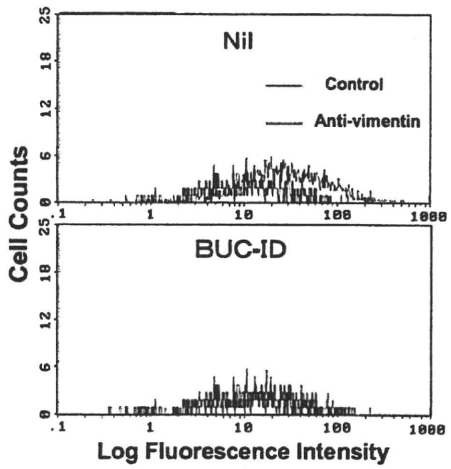

Fig. 2. Expression of vimentin in CD34+ cells cultured in the presence of SCF, GM-CSF and TNF-α. CD34+ cells from bone marrow of RA patients (1×10^5/well) were cultured in the presence of SCF (10 ng/ml), GM-CSF (1 ng/ml) and TNF-α (10 ng/ml). BUC-ID (intramolecular disulfide form of bucillamine) (3 μM) was added where indicated (nil: no addition). After 4 weeks, the cells were harvested and were examined by flow cytometry for the expression of vimentin as described in Materials and Methods.

for matrix metalloproteinase-1 (MMP-1) with the Biotrak human MMP-1 enzyme-linked immunosorbent assay system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and for vascular endothelial growth factor (VEGF) with the human VEGF immunoassay kit (Bio source International, Camarillo, CA). The concentrations of β_2-microglobulin (β_2MG) were determined by a sandwich ELISA as previously described [20].

2.5. Immunofluorescence staining and analysis

Cultured CD34$^+$ cells were fixed with 1% paraformaldehyde in PBS for 5 min at room temperature and were permeabilized in PBS (pH 7.2) containing 2% normal human AB serum, 0.1% sodium azide, and 0.1% saponin (Sigma), followed by staining with fluorescein isothiocyanate (FITC)-conjugated anti-human vimentin mAb (mouse IgG2a; Progen Biotechnik GMBH, Heidelberg, Germany), or FITC-conjugated isotype-matched control mAb (Dako, Glostrup, Denmark). The cells were then analyzed using an EPICS XL flow cytometer (Coulter, Hialeah, FL), as previously described [6].

3. Results

Fig. 1 depicts the representative patterns of the generation of fibroblast-like cells from RA bone marrow CD34+ cells upon stimulation with SCF, GM-CSF and TNF-α for 28 days in the presence or absence of pharmacologically attainable concentrations of MTX and BUC-ID. It is clear that the generation of fibroblast-like cells was suppressed by BUC-ID, but not by MTX. As can be seen in Fig. 2, the expression of vimentin in bone marrow CD34+ cells was markedly suppressed by BUC-ID, confirming that BUC-ID inhibits the generation of fibroblast-like cells. Table 1 compares the effects of MTX and BUC-ID on the generation of fibroblast-like cells from bone marrow CD34+ cells in 8 RA patients. The degree of the generation of fibroblast-like cells was scored as 1 (trace, 0–5%), 2 (mild, 5–30%), 3 (moderate, 30–50%), 4 (strong, >50%), 5 (very strong, with the formation of a cluster or a pile), depending on the observation from two view fields at ×50 magnification. The generation of fibroblast-like cells from RA bone marrow CD34+ cells was significantly suppressed by BUC-ID, but not by MTX. Although 5 of the 8 RA patients were receiving MTX at the study, the in vitro effects of MTX or BUC-ID on the generation of fibroblast like cells were not significantly different irrespective of the administration of MTX.

Table 1
Effects of methotrexate and bucillamine on the generation of fibroblast-like cells from CD34+ cells stimulated with SCF, GM-CSF, and TNF-α[1]

Patients		Generation of fibroblast-like cells[1]			
Age/ gender	DMARDs#	Nil	BUC-ID	MTX	BUC-ID/MTX
60/F	MTX	2	1	1	1
43/F	MTX	5	1	1	1
51/F	MTX	5	2	2	2
72/F	SASP+DPC	4	1	3	1
51/F	MTX	5	5	5	5
46/F	MTX+SASP	4	2	5	1
64/F	None	2	1	4	3
66/F	None	4	4	5	5
	(mean±SD)	(3.875±1.166)	(2.125±1.452)*	(3.25±1.639)	(2.375±1.653)*

[1]CD34+ cells from bone marrow of 8 RA patients (1×10^5/well) were cultured in the presence of SCF (10 ng/ml), GM-CSF (1 ng/ml) and TNF-α (10 ng/ml). MTX (methotrexate) (20 nM) or BUC-ID (intramolecular disulfide form of bucillamine) (3 μM) was added where indicated (nil: no addition). After 4 weeks, the generation of fibroblast-like cells was evaluated and scored as 1 (trace), 2 (mild), 3 (moderate), 4 (strong), or 5 (very strong). Statistical significance was determined with Wilcoxon signed rank test.
#At the study, 6 of the 8 RA patients were receiving DMARDs, including MTX, sulfasalazine (SASP), d-penicillamine (DPC).
*Significant at $p<0.05$.

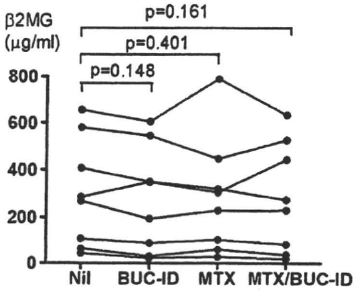

Fig. 3. Concentration of β_2MG in the culture supernatants of CD34+ cells stimulated with SCF, GM-CSF, and TNF-α. CD34+ cells from bone marrow of 8 patients (1×10^5/well) were cultured in the presence of SCF (10 ng/ml), GM-CSF (1 ng/ml) and TNF-α (10 ng/ml). MTX (methotrexate) (20 nM) or BUC-ID (intramolecular disulfide form of bucillamine) (3 μM) was added where indicated (nil: no addition). After 4 weeks, the culture supernatants were harvested and analyzed for β_2MG content by enzyme linked immunosorbent assay. Statistical significance was determined with Wilcoxon signed rank test.

Previous studies disclosed that β_2MG is produced by a number of cell types, including lymphocytes, myeloid cells, and tumor cells [21–23]. The production of β_2MG generally correlates with cell proliferation [21–23]. In fact, the levels of β_2MG in the culture supernatants paralleled the viable cell counts of bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α [24]. To explore whether MTX and BUC-ID might influence the cell out-growth, the effects of these drugs on the production of β_2MG were examined. As shown in Fig. 3, either MTX or BUC-ID did not significantly influence the production of β_2MG by bone marrow CD34+ cells.

Previous studies suggested that the fibroblast-like cells have capacities to produce MMP-1 and VEGF, a feature that is unique to type B synoviocytes [25]. It has been well known that MMP-1 plays an important role in the destruction of cartilage and bone in RA [6]. In addition, VEGF is a key cytokine for angiogenesis, which plays a pivotal role in synovial hyperplasia in RA [26]. We next examined the effects of MTX and BUC-ID on the production of MMP-1 in cultures of RA bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α for 28 days. As can be seen in Figs. 4 and 5, the production of MMP-1 as well as that of VEGF was significantly suppressed by BUD-ID, but not

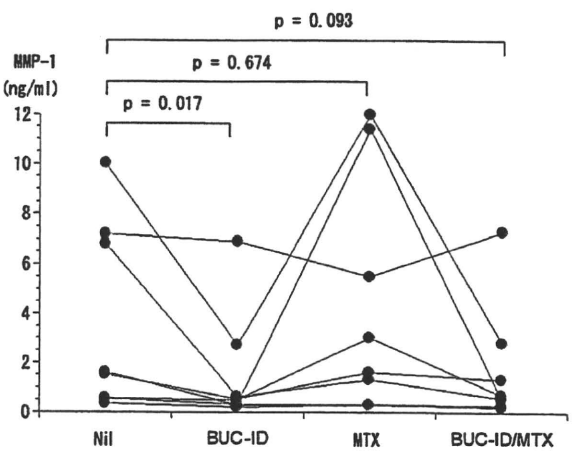

Fig. 4. Concentration of MMP-1 in the culture supernatants of CD34+ cells stimulated with SCF, GM-CSF, and TNF-α. CD34+ cells from bone marrow of 8 patients (1×10^5/well) were cultured in the presence of SCF (10 ng/ml), GM-CSF (1 ng/ml) and TNF-α (10 ng/ml). MTX (methotrexate) (20 nM) or BUC-ID (intramolecular disulfide form of bucillamine) (3 μM) was added where indicated (nil: no addition). After 4 weeks, the culture supernatants were harvested and analyzed for MMP-1 content by enzyme linked immunosorbent assay. Statistical significance was determined with Wilcoxon signed rank test.

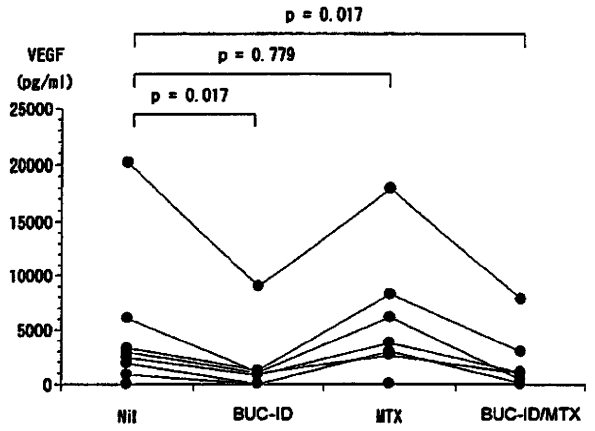

Fig. 5. Concentrations of VEGF in the culture supernatants of CD34+ cells stimulated with SCF, GM-CSF, and TNF-α. CD34+ cells from bone marrow of 8 RA patients (1×10^5/well) were cultured in the presence of SCF (10 ng/ml), GM-CSF (1 ng/ml) and TNF-α (10 ng/ml). MTX (methotrexate) (20 nM) or BUC-ID (intramolecular disulfide form of bucillamine) (3 μM) was added where indicated (nil: no addition). After 4 weeks, the culture supernatants were harvested and analyzed for VEGF content by enzyme-linked immunosorbent assay. Statistical significance was determined with Wilcoxon signed rank test.

by MTX. These results might support the conclusion that BUC-ID, but not MTX, suppress the generation of type B synoviocyte-like cells from bone marrow CD34+ cells in RA.

4. Discussion

Type B synoviocytes, which are also called fibroblast-like synoviocytes, have the morphological appearance of fibroblasts as well as the capacity to produce a variety of factors, including cytokines and matrix metalloproteinases (MMPs), that lead to the destruction of joints [25]. We have recently demonstrated that bone marrow CD34+ cells from RA patients have abnormal capacities to respond to TNF-α and to differentiate into fibroblast-like cells producing MMP-1 [6]. The results in the current studies have disclosed that BUC-ID, but not MTX, significantly suppressed the generation of fibroblast-like cells from RA bone marrow CD34+ cells at their pharmacologically relevant concentrations. Of note, since mesenchymal stem cells have been recently implicated in the generation of the fibroblast-like cells in RA [27], the use of CD34+/CD45− cells would help delineate which bone marrow population is responsible for the production of the fibroblast-like cells. Further studies are required to delineate this point.

MTX has now become internationally the first choice among DMARDs for active RA [8,9]. Previous studies showed that MTX at as little as 5 nM suppressed the growth of differentiated monocytic myeloid cell line THP-1 [28]. In addition, it was found that MTX at 20 nM suppressed the proliferation of human monocytic cells line U937 cells as well as the production of IL-1β by RA bone marrow mononuclear cells [29]. Taken together, these data suggest that MTX might inhibit the generation of type A synoviocytes and their function [16]. On the other hand, BUC-ID at 3μM did not suppress the generation of CD14+ cells from RA bone marrow CD14− cells, suggesting that BUC-ID might not affect the generation of type A synoviocytes and their function in RA [15].

Of note, in the present study, BUC-ID at 3 μM significantly suppressed the generation of fibroblast-like cells irrespective of the presence of MTX in cultures of RA bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α. In addition, BUC-ID inhibited the expression of vimentin, a fibroblastic-specific marker, in cultured bone marrow CD34+ cells. Finally, BUC-ID also suppressed the production of VEGF and MMP-1 in these cultures. These results suggest that BUC-ID might have suppressive influences on the generation of type B synoviocytes and their function in RA. On the other hand, MTX at 20 nM did not suppress either the generation of fibroblast-like cells from RA bone marrow CD34+ cells or the production of MMP-1 and VEGF in these cultures, suggesting that MTX might not affect the generation of type B synoviocytes and their function in RA.

Recent double-blinded, randomized controlled studies have provided evidence that the combination therapy with MTX and BUC resulted in significantly higher clinical efficacy than either monotherapy [30]. Thus, the patients treated with the combination of MTX and BUC showed significantly higher ACR 20 response rate (79.2%) than those treated with MTX alone (43.5%), or with BUC alone (45.8%) in a 96-week trial [30]. As mentioned above, the data in previous and current studies suggest that the targets of MTX and BUC might be different. Thus, MTX appears to suppress the generation and the function of type A synoviocytes, whereas BUC-ID seems to inhibit the generation and the function of type B synoviocytes. Since both type A synoviocytes and type B synoviocytes play an important role in the pathogenesis of RA, these results might account for the clinical efficacy of the combination of MTX and BUC.

It has been shown that MTX effects cellular metabolism at several different steps by inhibition of dihydrofolate reductase, thymidylate synthase, and aminoimidazole carboxamide ribonucleotide transformylase [31]. By contrast, the mechanisms of action of BUD-ID still remain unclear. On the other hand, we have recently revealed that the activation of NFkB1 plays a pivotal role in the abnormal capacity of RA bone marrow CD34+ cells to differentiate into fibroblast-like cells and to produce MMP-1 and VEGF [24]. In this regard, previous studies disclosed that high concentrations of BUC (1 mM) directly inhibited the activation of NFkB in murine system [32]. Since conversion between BUC and BUC-ID might take place in vitro as well as in vitro, it is possible that BUC-ID might also be involved in the inhibition of the activation of NFkB. It is therefore likely that the inhibitory influences of BUC-ID on the generation of fibroblast-like cells from RA bone marrow CD34+ cells might be a result of inhibition of the activation of NFkB. Further studies would be required to explore whether BUC-ID might inhibit the activation of NFkB at pharmacologically relevant concentrations.

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