cells, therefore, IL-6 is a key cytokine [8], although the pathological roles of Th17 cells in human diseases are still obscure.

IL-6 is responsible for both systemic and local inflammation. Systemically, IL-6 induces inflammatory symptoms such as fever, general fatigue, and anorexia as well as laboratory abnormalities, including increase in acute phase proteins such as C-reactive protein (CRP), serum amyloid A, and fibrinogen, decrease in serum albumin, and hypoferremic anemia through inducing the secretion of hepcidin, an iron regulatory peptide hormone [11]. In the affected joints, IL-6 causes angiogenesis through the induction of vascular endothelial growth factor (VEGF) production [12], which is necessary to oxygenate the hypertrophic synovial tissue in RA [13–15]. VEGF also increases vascular permeability and mediates inflammation. IL-6, in the presence of soluble IL-6 receptor (sIL-6R), induces the differentiation of osteoclast precursor cells to mature osteoclasts, which results in bone absorption and joint destruction of RA [16]. These evidences have encouraged us to develop an IL-6 inhibitor as a therapeutic agent.

Tocilizumab is a humanized antihuman IL-6R monoclonal antibody [17], which specifically blocks IL-6R and developed as a therapeutic agent for IL-6-related diseases. In April 2008, tocilizumab was approved for RA and juvenile idiopathic arthritis (JIA) in Japan, and a license application has been submitted in the USA and Europe.

Blocking IL-6R with tocilizumab

IL-6R complex is composed of 80 kDa IL-6-binding receptor (IL-6R) and 130 kDa non-IL-6-binding but signal-transducing molecule called gp130 [18]. There are two forms of IL-6R, membrane IL-6R and sIL-6R which circulates in blood and synovial fluids. IL-6 can bind both forms of IL-6R, and IL-6/IL-6R complex is made. Then, the complex associates with gp130, which transduces IL-6 signal into the cells. Tocilizumab recognizes both forms of IL-6R and competitively inhibits IL-6 binding to IL-6R [19], and consequently inhibits the biological activity of IL-6.

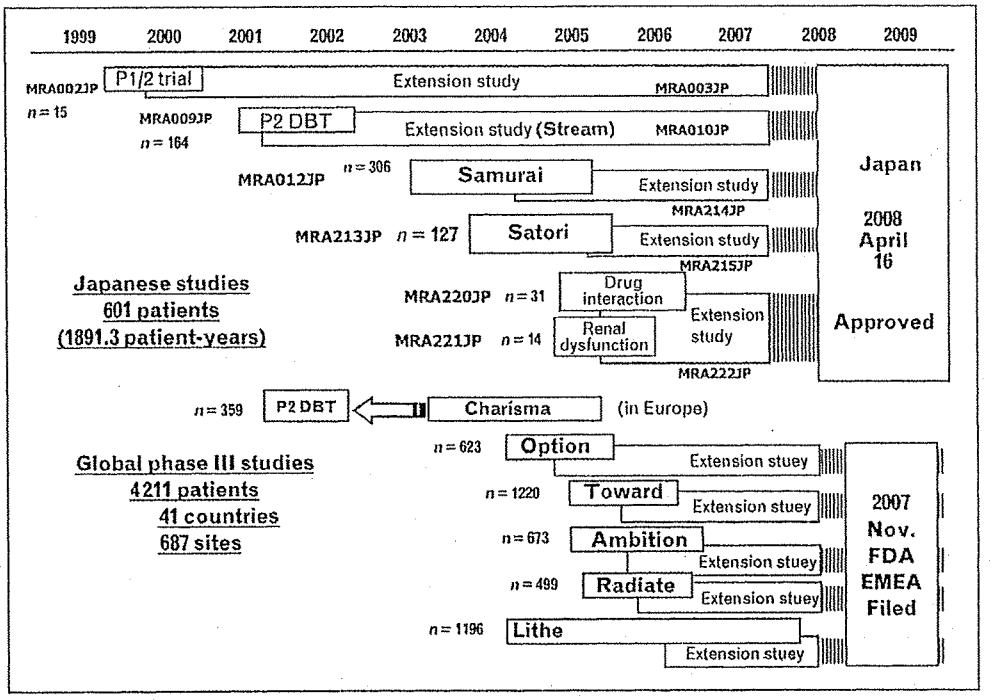
When IL-6R is completely blocked with tocilizumab, an increase in the serum IL-6 levels is observed [20]. This is explained by the inhibition of IL-6R-mediated consumption of IL-6. Although serum IL-6 levels increase by tocilizumab treatment, the activity of IL-6 is completely inhibited as long as IL-6R is blocked by tocilizumab [20]. It is noteworthy that the IL-6 levels increasing after tocilizumab administration correlate well with the disease activities and reflect the amount of endogenously produced IL-6 [20].

Clinical value of tocilizumab

The clinical value of tocilizumab has been evaluated in a series of clinical trials with various study designs all over the world. Overviews of clinical studies are shown in Fig. 1 and Tables 1 and 2. Phase I/II studies started in 1999 both in Japan and the UK. The Japanese phase II study showed that tocilizumab monotherapy (intravenous infusion of tocilizumab 8 mg/kg body weight every 4 weeks) significantly reduced disease activity of RA [21]. Similar efficacy was confirmed in the Chugai Humanized AntiRheumatic Interleukin Six Monoclonal Antibody (CHARISMA) phase II study in the UK [22], in which the safety and efficacy of tocilizumab was evaluated with or without methotrexate (MTX), an anchor drug for RA therapy. Both studies demonstrated that intravenous administration of tocilizumab (8 mg/kg) every 4 weeks is a recommended regimen.

Japanese phase III trials mainly consisted of two studies: Study of Active controlled TOcilizumab monotherapy for Rheumatoid arthritis patients with an Inadequate response to methotrexate (SATORI) [23•] and Study of Active controlled Monotherapy Used for Rheumatoid Arthritis, an IL-6 Inhibitor (SAMURAI) [24••]. The SATORI trial showed that tocilizumab monotherapy was an efficacious treatment for patients with an inadequate response to low-dose MTX (8 mg/week), and switching MTX therapy to tocilizumab monotherapy was well tolerated [23•]. Impairment of physical function was also improved. In addition, it was confirmed that blocking IL-6R by tocilizumab significantly decreased serum VEGF levels in association with improvement in disease activity. The tOcilizumab Pivotal Trial in methotrexate Inadequate respONders (OPTION) study is the first multinational study to assess the therapeutic efficacy of tocilizumab in RA patients with moderate-to-severe disease despite MTX treatment [25••]. Combination therapy of tocilizumab and stable prestudy dose of MTX was efficacious for RA patients with inadequate response to MTX [25••]. In this study, health-related quality of life was assessed using Medical Outcomes Study 36-Item Short-Form General Health Survey and Functional Assessment of Chronic Illness Therapy—Fatigue assessment. Tocilizumab improved health-related quality of life more than placebo. TOcilizumab in combination With traditional DMARD therapy (TOWARD), the second global trial, assessed the combination of tocilizumab with stable prestudy dose of traditional disease-modifying antirheumatic drugs (DMARDs) [26•]. At week 24, response rates of a 20% improvement in RA sign and symptom according to American College of Rheumatology (ACR) criteria (ACR20), ACR50, and ACR70 in the tocilizumab group were superior to those of the DMARD group, respectively. Thus, the addition of tocilizumab to the traditional DMARDs is effective.

Figure 1 Overview of clinical studies of tocilizumab



AMBITION, Actemra versus Methotrexate double Blind Investigate Trial In mONotherapy; CHARISMA, Chugai Humanized AntiRheumatic Interleukin Six Monoclonal Antibody; DBT, double-blind trial; EMEA, European Medicines Agency; FDA, Food and Drug Administration; LITHE, tociLIzumab safety and THE prevention of structural joint damage trial; OPTION, tOcilizumab Pivotal Trial in methotrexate Inadequate respONders; RADIATE, Research on Actemra Determining efficacy after Anti-TNF failurEs; SAMURAI, Study of Active controlled Monotherapy Used for Rheumatoid Arthritis, an IL-6 Inhibitor; SATORI, Study of Active controlled TOcilizumab monotherapy for Rheumatoid arthritis patients with an Inadequate response to methotrexate; STREAM, Long-term Safety and efficacy of Tocilizumab, an antiinterleukin-6 REceptor Monoclonal antibody, in patients with rheumatoid arthritis; TOWARD, TOcilizumab in combination With traditional DMARD therapy.

Table 1 Efficacy of tocilizumab therapy in phase III studies

	ACR20, % (*P*)	ACR50, % (*P*)	ACR70, % (*P*)	DAS28 remission, % (*P*)
SAMURAI trial				
Tocilizumab (8 mg/kg)	78 (<0.001)	64 (<0.001)	44 (<0.001)	59 (<0.001)
DMARDs	34	13	6	3
SATORI trial				
Tocilizumab (8 mg/kg)	80 (<0.001)	49	40	43 (<0.001)
MTX	25	11	6	2
OPTION study				
Tocilizumab (8 mg/kg)	59 (<0.0001)	44 (<0.0001)	22 (<0.0001)	27 (<0.0001)
Tocilizumab (4 mg/kg)	48 (<0.0001)	31 (<0.0001)	12 (<0.0001)	13 (=0.0002)
Control	26	11	2	1
TOWARD study				
Tocilizumab (8 mg/kg)	61 (<0.0001)	38 (<0.0001)	21 (<0.0001)	30 (<0.0001)
Control	25	9	3	3
RADIATE study				
Tocilizumab (8 mg/kg)	50 (<0.001)	29 (<0.001)	12 (0.001)	30 (0.001)
Tocilizumab (4 mg/kg)	30 (<0.001)	17 (<0.001)	5 (0.1)	8 (0.053)
Control	10	4	1	2
AMBITION				
Tocilizumab (8 mg/kg)	70 (<0.0001)	44 (0.0023)	28 (0.0002)	34
MTX	53	34	15	12
LITHE				
Tocilizumab (8 mg/kg)	56 (<0.0001)	36 (<0.0001)	20 (<0.0001)	47 (<0.0001)
Tocilizumab (4 mg/kg)	47	29	16	30
Control	25	10	4	8

P value, each of tocilizumab group versus control group, tocilizumab group versus DMRDs group, tocilizumab group versus MTX group. AMBITION, Actemra versus Methotrexate double Blind Investigate Trial In mONotherapy; DMARDs, disease-modifying antirheumatic drugs; LITHE, tociLIzumab safety and THE prevention of structural joint damage trial; MTX, methotrexate; OPTION, tOcilizumab Pivotal Trial in methotrexate Inadequate respONders; RADIATE, Research on Actemra Determining efficacy after Anti-TNF failurEs; SAMURAI, Study of Active controlled Monotherapy Used for Rheumatoid Arthritis, an IL-6 Inhibitor; SATORI, Study of Active controlled TOcilizumab monotherapy for Rheumatoid arthritis patients with an Inadequate response to methotrexate; TOWARD, TOcilizumab in combination With traditional DMARD therapy.

Table 2 Incidence rates of adverse event and serious adverse event observed in phase III studies

	Adverse event (%)	Serious adverse event (%)
SAMURAI trial		
Tocilizumab (8 mg/kg)	89	18
DMARDs	82	13
SATORI trial		
Tocilizumab (8 mg/kg)	92	7
MTX	72	5
OPTION study		
Tocilizumab (8 mg/kg)	69	6
Tocilizumab (4 mg/kg)	71	6
Control	63	6
TOWARD study		
Tocilizumab (8 mg/kg)	73	7
Control	61	4
RADIATE study		
Tocilizumab (8 mg/kg)	84	6
Tocilizumab (4 mg/kg)	87	7
Control	81	11
AMBITION		
Tocilizumab (8 mg/kg)	80	4
MTX	78	3

AMBITION, Actemra versus Methotrexate double Blind Investigate Trial In mONotherapy; DMARDs, disease-modifying antirheumatic drugs; MTX, methotrexate; OPTION, tOcilizumab Pivotal Trial in methotrexate Inadequate respONders; RADIATE, Research on Actemra Determining efficacy after Anti-TNF failurEs; SAMURAI, Study of Active controlled Monotherapy Used for Rheumatoid Arthritis, an IL-6 Inhibitor; SATORI, Study of Active controlled TOcilizumab monotherapy for Rheumatoid arthritis patients with an Inadequate response to methotrexate; TOWARD, TOcilizumab in combination With traditional DMARD therapy.

TNF inhibitors have been used all over the world for patients with RA refractory to DMARDs, including MTX. Even with TNF inhibitors, however, 60% of patients do not reach a 50% improvement in disease activity [1]. In patients with RA refractory to anti-TNF therapy, three treatment options are currently available: switching between TNF inhibitors [27–29], switching to the abatacept, a costimulation blocker for T cells [30], and rituximab, a B cell-depleting anti-CD20 antibody [31]. The Research on Actemra Determining efficacy after Anti-TNF failurEs (RADIATE) study first examined, in a randomized, double-blind, controlled trial, the efficacy and safety of tocilizumab with MTX in patients with active RA who failed at least one TNF inhibitor [32••]. ACR20 was achieved at 24 weeks by 50.0 and 10.1% of patients in the tocilizumab (8 mg/kg) and control groups, and disease activity score (DAS)28 remission (DAS28 < 2.6) was achieved by 30.1 and 1.6%, respectively. Although the efficacy appeared to be a little lower than that observed in the OPTION study, tocilizumab and MTX showed efficacy comparable to that obtained with abatacept and rituximab treatment in a similar population. Furthermore, patients responded regardless of most recently failed TNF inhibitor or the number of previous failed TNF inhibitors, whereas switching between TNF inhibitors usually increases the chance of inadequate response. This implies that targeting IL-6, a molecule different from TNF, is feasible for patients refractory to anti-TNF therapy.

Until now, there has been no biologic agent the efficacy of which in monotherapy is superior to that of MTX in patients who have not previously experienced MTX. The Actemra versus Methotrexate double Blind Investigate Trial In mONotherapy (AMBITION) study showed that tocilizumab monotherapy was superior to MTX monotherapy for alleviating symptoms in patients with active RA who have not failed prior MTX or biologic therapy [33•]. The result of AMBITION confirmed the efficacy of tocilizumab in monotherapy, which has been shown in Japanese studies [21,23•,24••].

In evaluating radiographic outcomes, two studies have been conducted until now, SAMURAI trial and tociLIzumab safety and THE prevention of structural joint damage trial (LITHE) study [34•]. The SAMURAI trial, a Japanese phase III study, evaluated, in a blinded manner, radiological progression on hands and feet of patients with active RA of less than 5-year disease duration using van der Heijde's modified Sharpe score [24•]. In the control DMARD group, dose, type, combinations, and changing of DMARDs could be varied at the discretion of the prescribing physician except for TNF inhibitors or leflunomide. At week 52, significantly more individuals in the tocilizumab monotherapy group had no radiographic progression compared with the DMARD group (56 versus 39%, $P < 0.01$) and improved total Sharpe score. Increase in erosion score and joint space narrowing were also suppressed. The result indicates that tocilizumab, even in monotherapy, retards structural joint damage. The LITHE study is a fifth global study, including 15 countries and enrolling 1196 moderate-to-severe RA patients refractory to MTX treatment and assessing the ability of tocilizumab to inhibit structural joint damage using Genant-modified Sharpe scores during 12 months of therapy. The result of the LITHE study showed that tocilizumab and MTX significantly inhibited the progression of structural joint damage compared with placebo and MTX. Significantly more individuals in the tocilizumab (8 mg/kg) and MTX group showed no progression in total Genant-modified Sharpe scores compared with that of the tocilizumab (4 mg/kg) and MTX group or the placebo and MTX group (85, 81, and 67%, respectively). Both studies demonstrated the efficacy of tocilizumab for preventing structural joint damage in RA.

Long-term Safety and efficacy of Tocilizumab, an antiinterleukin-6 REceptor Monoclonal antibody, in patients with rheumatoid arthritis (STREAM) study is an open-label extension study of tocilizumab enrolling 143 patients who were treated in the Japanese phase II trial [35••]. A total of 94 patients (66%) continually received tocilizumab for more than 5 years. DAS28 remission was achieved in 52 out of 94 patients (55.3%), 78 (88.6%) were able to decrease their corticosteroid dose, and 28 (31.8%) discontinued corticosteroids. Thirty-two

patients (22%) withdrew from the study because of adverse events and one patient (0.7%) because of unsatisfactory response. The serious adverse event (SAE) rate was 27.5 events per 100 patient-years, with 5.7 serious infections per 100 patient-years, based on a total tocilizumab exposure of 612 patient-years. The high rate of continuing tocilizumab therapy implies that tocilizumab therapy not only sustains the efficacy but also has a good safety profile, including less withdrawal due to adverse events.

Safety of tocilizumab

Incidence rates of adverse events observed in phase III studies are summarized in Table 2. Most of the adverse events observed in those studies were mild or moderate [23•,24••,25••,26•,32••,33•,34•]. Regarding the SAE, infections were most frequently observed in every study, and most of the patients recovered by appropriate treatment. There was no infection specific to tocilizumab treatment. Among the SAE reported in the STREAM study, a Japanese long-term study, pneumonia (1.5 events per 100 patients-years), herpes zoster (1.1 events per 100 patients-years), and bronchitis (0.8 events per 100 patients-years) were frequently observed [35••], although the incidence rate of serious infections was comparable to that of TNF inhibitors. As tocilizumab ameliorates inflammatory manifestations and the acute-phase reaction, it also suppresses the symptom and increase in CRP levels caused by pneumonia, which may result in the delay of diagnosis [36]. Therefore, it is necessary to carefully rule out possible infection even though the symptoms are mild. Increase in the incidence of tuberculosis (TB) is often the problem during anti-TNF treatment. In all Japanese clinical studies, two TB patients were reported among 1981 patient-years, and the incidence was not significantly increased in comparison with Japanese RA patients without biologics and the Japanese population [37].

Regarding the incidence of malignancies in patients receiving tocilizumab, only Japanese data have been available until now. The incidence in all Japanese clinical studies was almost equivalent to that in an observational cohort of RA patients or Japanese population data [38].

Drug-related infusion reactions were observed in 0.8–11% of the patients in the phase III clinical studies [23•,24••,25••,26•,32••,33•,34•]. Most of the infusion reactions were mild to moderate.

About the laboratories, increases in alanine aminotransferase or aspartate aminotransferase or both were often reported in the tocilizumab treatment group in every clinical trial; most of the increases were less than three-fold of the upper normal limit, and the values normalized spontaneously or after the cessation of tocilizumab therapy [23•,24••,25••,26•,32••,33•,34•]. There had been no fulminant hepatitis.

Increases in serum cholesterol levels were also frequently reported in every clinical trial. Total cholesterol (TC) levels did not continue to increase and became stable around the upper normal limit in most of the patients in the Japanese studies [23•,24••,35••]. However, incidences of hyperlipidemia were higher in the global studies [25••,26•,32••,33•,34•]. Although cardiovascular complications associated with increases in cholesterol levels have not been reported, excessive levels of TC need to be controlled by lipid-lowering agents.

Transient neutropenia was often reported in every clinical trial, and neutrophil counts were spontaneously recovered [23•,24••,25••,26•,32••,33•,34•,35••]. This may be because of shifting the distribution of neutrophils [39]. Moreover, there was no apparent association between neutropenia and infection. This safety profile of tocilizumab is acceptable compared with the provided benefit.

Tocilizumab therapy for other inflammatory diseases

Systemic-onset JIA (soJIA) is one of common rheumatic diseases in childhood and is characterized by arthritis, spiking fever, pericarditis, skin rash, splenomegaly, and hepatomegaly [40]. Children with soJIA often show growth retardation and developmental abnormality. In addition to these abnormalities, patients with soJIA are often associated with macrophage activation syndrome, which is sometimes fatal. As IL-6 plays a central role in the pathogenesis of this disease, therapeutic values of tocilizumab have been assessed. In a phase III trial for soJIA in Japan, 56 children (aged 2–19 years) with active soJIA refractory to conventional treatments were enrolled [41]. The patients received tocilizumab 8 mg/kg body weight every 2 weeks for 6 weeks in an open-label lead-in phase, and patients who achieved ACR Pediatric 30 response (ACR Pedi 30) and CRP of less than 5 mg/l were randomly assigned to receive placebo or continue tocilizumab treatment for 12 weeks or until withdrawal for rescue medication in a double-blind phase. The primary endpoint of the double-blind phase was an ACR Pedi 30 response and CRP concentration of less than 15 mg/l. At the end of the open-label lead-in phase, ACR Pedi 30, 50, and 70 responses were achieved by 91, 86, and 68% of patients, respectively, and 43 patients continued to the double-blind phase. In the double-blind phase, only 17% of the placebo group maintained an ACR Pedi 30 and CRP less than 15 mg/l compared with 80% in the tocilizumab group ($P < 0.0001$). Furthermore, at week 48 of the open-label extension study following the double-blind phase, ACR Pedi 30, 50, and 70 responses were achieved by 98, 94, and 90% of the patients,

respectively. Most of the adverse events were mild or moderate. In the open-label extension phase of the study, 13 serious adverse events were observed, including bronchitis, gastroenteritis, and an anaphylactoid reaction, all of which improved by adequate treatment. On the basis of the results of clinical trials, tocilizumab was approved for patients with soJIA in April 2008.

Adult-onset Still's disease is an adult disease showing pathological features similar to soJIA in childhood. There are two reports that tocilizumab therapy improved disease activity of patients with adult-onset Still's disease refractory to conventional treatments, including corticosteroids and immunosuppressants [42,43]. As the cytokine profile of adult-onset Still's disease looks similar to that of soJIA, the blockade of IL-6 will be beneficial.

Tocilizumab therapy may also provide a clinical benefit for vasculitis syndromes. A patient with Takayasu arteritis refractory to a conventional immunosuppressive therapy could be successfully treated with tocilizumab [44]. For these diseases, future clinical studies will be required to establish safety and efficacy.

Conclusion

In this article, clinical value of blocking IL-6 receptor with tocilizumab was reviewed. Although a series of clinical studies have demonstrated excellent safety and efficacy of tocilizumab therapy for RA and soJIA, the number of patients in those clinical trials is still limited. Thus, a large registry data will warrant the safety profile of tocilizumab. Nevertheless, tocilizumab will add a promising therapeutic option for patients with RA and other inflammatory diseases.

In future studies, we need to establish a method of how to choose the right drug for the right patient by predicting the efficacy. If such a prediction becomes possible, it will lighten the treatment cost. Moreover, patients will achieve a clinical remission more realistically.

Acknowledgements

Toru Mima, as a medical advisor, received the consultant fee from Chugai Pharmaceutical Co., Ltd. Norihiro Nishimoto, as a medical advisor, received the consultant fee from Chugai Pharmaceutical Co., Ltd. and, as a member of scientific advisory board, received the lecture fee from F. Hoffmann-La Roche Ltd. Norihiro Nishimoto received research grants from Chugai Pharmaceutical Co., Ltd. and Wyeth K.K.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 299).

1 Smolen JS, Aletaha D, Koeller M, *et al.* New therapies for treatment of rheumatoid arthritis. Lancet 2007; 370:1861–1874.

2 Nishimoto N, Kishimoto T. Interleukin-6: from bench to bedside. Nat Clin Pract Rheumatol 2006; 2:619–626.

3 Harrington LE, Hatton RD, Mangan PR, *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005; 6:1123–1132.

4 Langrish CL, Chen Y, Blumenschein WM, *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 2005; 201:233–240.

5 Park H, Li Z, Yang XO, *et al.* A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005; 6:1133–1141.

6 Iwakura Y, Ishigame H. The IL-23/IL-17 axis in inflammation. J Clin Invest 2006; 116:1218–1222.

7 Mangan PR, Harrington LE, O'Quinn DB, *et al.* Transforming growth factor-β induces development of the T_H17 lineage. Nature 2006; 441:231–234.

8 Bettelli E, Carrier Y, Gao W, *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006; 441:235–238.

9 Sakaguchi S. Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004; 22:531–562.

10 Chen W, Jin W, Hardegen N, *et al.* Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-β induction of transcription factor Foxp3. J Exp Med 2003; 198:1875–1886.

11 Nemeth E, Rivera S, Gabayan V, *et al.* IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 2004; 113:1271–1276.

12 Nakahara H, Song J, Sugimoto M, *et al.* Antiinterleukin-6 receptor antibody therapy reduced vascular endothelial growth factor production in rheumatoid arthritis. Arthritis Rheum 2003; 48:1521–1529.

13 Koch AE, Harlow LA, Haines GK, *et al.* Vascular endothelial growth factor A cytokine modulating endothelial function in rheumatoid arthritis. J Immunol 1994; 152:4149–4156.

14 Folkman J. Angiogenesis in cancer, vascular, rheumatoid, and other disease. Nat Med 1995; 1:27–31.

15 Paleolog EM. Angiogenesis in rheumatoid arthritis. Arthritis Res 2002; 4 (Suppl 3):S81–S90.

16 Tamura T, Udagawa N, Takahashi N, *et al.* Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. Proc Natl Acad Sci U S A 1993; 90:11924–11928.

17 Sato K, Tsuchiya M, Saldanha J, *et al.* Reshaping a human antibody to inhibit the interleukin 6-dependent tumor cell growth. Cancer Res 1993; 53:851–856.

18 Taga T, Kishimoto T. gp130 and the interleukin-6 family of cytokines. Annu Rev Immunol 1997; 15:797–819.

19 Paul-Pletzer K. Tocilizumab: blockade of interleukin-6 signaling pathway as a therapeutic strategy for inflammatory disorders. Drugs Today (Barc) 2006; 42:559–576.

20 Nishimoto N, Terao K, Mima T, *et al.* Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease. Blood 2008; 112:3959–3964.

21 Nishimoto N, Yoshizaki K, Miyasaka N, *et al.* Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody. A multicenter, double-blind, placebo-controlled trial. Arthritis Rheum 2004; 50:1761–1769.

22 Maini RN, Taylor PC, Szechinski J, *et al.* Double-blind randomized controlled clinical trial of the interleukin-6 receptor antagonist, tocilizumab, in European patients with rheumatoid arthritis who had an incomplete response to methotrexate. Arthritis Rheum 2006; 54:2817–2829.

23 • Nishimoto N, Miyasaka N, Yamamoto K, *et al.* Study of active controlled tocilizumab monotherapy for rheumatoid arthritis patients with an inadequate response to methotrexate (SATORI): significant reduction in disease activity and serum vascular endothelial growth factor by IL-6 receptor inhibition therapy. Mod Rheumatol 2008 [Epub ahead of print].

This study demonstrated the safety and efficacy of tocilizumab in RA patients with an inadequate response to MTX.

24 •• Nishimoto N, Hashimoto J, Miyasaka N, *et al.* Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor (SAMURAI): evidence of clinical and radiographic benefit from an x-ray reader-blinded randomized controlled trial of tocilizumab. Ann Rheum Dis 2007; 66:1162–1167.

This was the first study to demonstrate the efficacy of tocilizumab for preventing structural joint damage in patients with RA.

25 Smolen JS, Beaulieu A, Rubbert-Roth A, *et al.* Effect of interleukin-6 receptor •• inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. Lancet 2008; 371:987–997.
This was the first global study to demonstrate the additive effect of tocilizumab on MTX.

26 Genovese MC, McKay JD, Nasonov EL, *et al.* Interleukin-6 receptor inhibition • with tocilizumab reduces disease activity in rheumatoid arthritis with inadequate response to disease-modifying antirheumatic drugs: the tocilizumab in combination with traditional disease-modifying antirheumatic drug therapy study. Arthritis Rheum 2008; 58:2968–2980.
This was the global study to demonstrate the additive effect of tocilizumab on the traditional DMARDs.

27 Hyrich KL, Lunt M, Watson KD, *et al.* Outcomes after switching from one antitumor necrosis factor alpha agent to a second antitumor necrosis factor alpha agent in patients with rheumatoid arthritis: results from a large UK national cohort study. Arthritis Rheum 2007; 56:13–20.

28 van Vollenhoven R, Harju A, Brannemark S, Klareskog L. Treatment with infliximab (Remicade) when etanercept (Enbrel) has failed or vice versa: data from the STURE registry showing that switching tumour necrosis factor alpha blockers can make sense. Ann Rheum Dis 2003; 62:1195–1198.

29 Gomez-Reino JJ, Carmona L. Switching TNF antagonists in patients with chronic arthritis: an observational study of 488 patients over a four-year period. Arthritis Res Ther 2006; 8:R29.

30 Genovese MC, Becker JC, Schiff M, *et al.* Abatacept for rheumatoid arthritis refractory to tumor necrosis factor alpha inhibition. N Engl J Med 2005; 353:1114–1123.

31 Cohen SB, Emery P, Greedwald MW, *et al.* Rituximab for rheumatoid arthritis refractory to antitumor necrosis factor therapy: results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks. Arthritis Rheum 2006; 54:2793–2806.

32 Emery P, Keystone E, Tony HP, *et al.* IL-6 receptor inhibition with tocilizumab •• improves treatment outcomes in patients with rheumatoid arthritis refractory to antitumour necrosis factor biologicals: results from a 24-week multicentre randomised placebo-controlled trial. Ann Rheum Dis 2008; 67:1516–1523.
This was the first study to demonstrate the safety and efficacy of combination therapy of tocilizumab and MTX in RA patients with inadequate response to TNF inhibitors.

33 Jones G, Kowenstein JRGuM, Calvo A, *et al.* Tocilizumab monotherapy is • superior to methotrexate monotherapy in reducing disease activity in patients with rheumatoid arthritis: the AMBITON study [abstract]. Ann Rheum Dis 2008; 67 (Suppl):89.
This was the first study to demonstrate the efficacy of tocilizumab monotherapy in patients with RA who have not failed prior MTX therapy.

34 Kremer JM, Fleischmann RM, Halland A, *et al.* Tocilizumab inhibits structural • joint damage in rheumatoid arthritis patients with an inadequate response to methotrexate [abstract]. Arthritis Rheum 2008; 58 (Suppl):L59.
This was the global study to demonstrate the additive effect of tocilizumab on MTX for preventing structural joint damage in patients with RA.

35 Nishimoto N, Miyasaka N, Yamamoto K, *et al.* Long-term safety and efficacy of •• tocilizumab, an antiinterleukin-6 receptor monoclonal antibody, in monotherapy, in patients with rheumatoid arthritis (the STREAM study): evidence of safety and efficacy in a 5-year extension study. Ann Rheum Dis 2008. [Epub ahead of print]
This study demonstrated the long-term safety and efficacy of tocilizumab in patients with RA.

36 Fujiwara H, Nishimoto N, Hamano Y, *et al.* Masked early symptoms of pneumonia in patients with rheumatoid arthritis during tocilizumab treatment: a report of two cases. Mod Rheumatol 2008. [Epub ahead of print]

37 Nishimoto N, Miyasaka N, Yamanaka K, *et al.* Safety profile of tocilizumab in Japanese patients with rheumatoid arthritis: incidences of infections in Japanese long-term clinical studies. Ann Rheum Dis 2008; 67 (Suppl II): 335; abstract.

38 Yamanaka H, Nishimoto N, Inoue E, *et al.* Incidence of malignancies in Japanese rheumatoid arthritis patients treated with tocilizumab in comparison to those in an observational cohort of Japanese patients and a Japanese population database. Ann Rheum Dis 2007; 66 (Suppl 2):122; abstract.

39 Suwa T, Hogg JC, English D, Van Eeden SF. Interleukin-6 induces demargination of intravascular neutrophils and shortens their transit in marrow. Am J Physiol Heart Circ Physiol 2000; 279:H2954–H2960.

40 Yokota S. Interleukin 6 as a therapeutic target in systemic-onset juvenile idiopathic arthritis. Curr Opin Rheumatol 2003; 15:581–586.

41 Yokota S, Imagawa T, Mori M, *et al.* Efficacy and safety of tocilizumab in patients with systemic-onset juvenile idiopathic arthritis: a randomised, double-blind, placebo-controlled, withdrawal phase III trial. Lancet 2008; 371:998–1006.

42 Iwamoto M, Nara H, Hirata D, *et al.* Humanized monoclonal antiinterleukin-6 receptor antibody for treatment of intractable adult-onset Still's disease. Arthritis Rheum 2002; 46:3388–3389.

43 Nakahara H, Mima T, Yoshio-Hoshino N, *et al.* A case report of a patient with refractory adult-onset Still's disease who was successfully treated with tocilizumab over 6 years. Mod Rheumatol 2008. [Epub ahead of print]

44 Nishimoto N, Nakahara H, Yoshio-Hoshino N, Mima T. Successful treatment of a patient with Takayasu arteritis using a humanized antiinterleukin-6 receptor antibody. Arthritis Rheum 2008; 58:1197–1200.

Mod Rheumatol (2009) 19:273–282
DOI 10.1007/s10165-009-0170-4

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

Received: 26 December 2008 / Accepted: 10 March 2009 / Published online: 19 May 2009

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

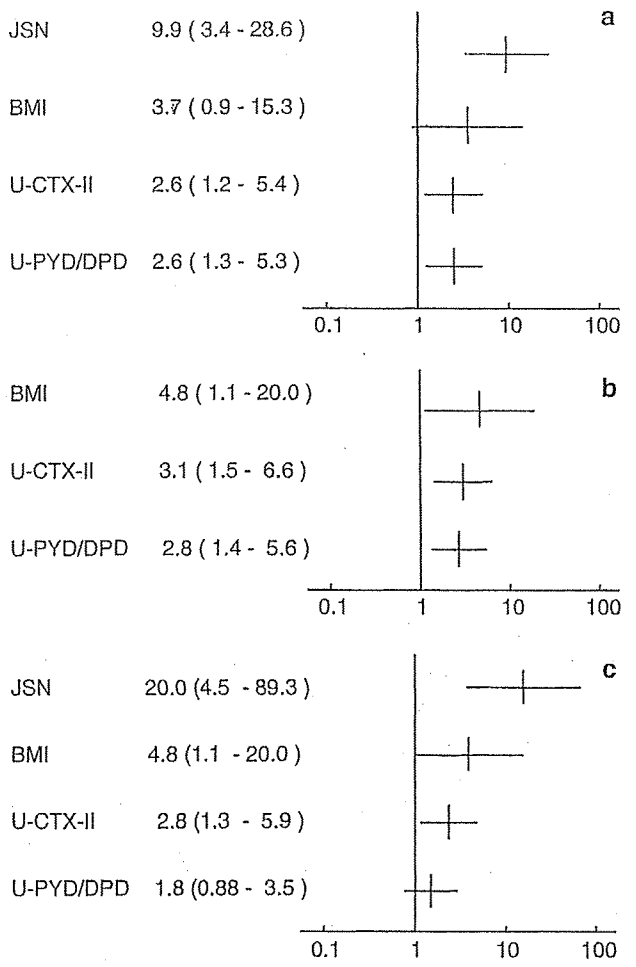


Fig. 1 Odds ratio (95% confidence interval) of radiological progression associated with high baseline joint space narrowing (*JSN*), high urinary C-terminal telopeptide of type II collagen (*U-CTXII*), high urinary total pyridinoline/total deoxypyridinoline (*U-PYD/DPD*), or low body mass index (*BMI*). 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 (ng/mmol/creatinine)	<500	53	327.2 ± 104.6
	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.

Acknowledgments The authors wish to thank Takahiro Kakehi, B.Sc., Tamiko Kuraishi, B.Sc., Yukiyasu Mariko, M.Sc. and Yuichi Kawata, B.Sc., for their valuable assistance with the design and analysis of the study and preparation of this manuscript. This work was supported by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.

References

1. Kuper HH, van Leeuwen MA, van Riel PL, Prevoo ML, Houtman PM, Lolkema WF, et al. Radiographic damage in large joints in early rheumatoid arthritis: relationship with radiographic damage in hands and feet, disease activity, and physical disability. Br J Rheumatol. 1997;36:855–60.
2. Corbett M, Dalton S, Young A, Silman A, Shipley M. Factors predicting death, survival and functional outcome in a prospective study of early rheumatoid disease over fifteen years. Br J Rheumatol. 1993;32:717–23.
3. Drossaers-Bakker KW, de Buck M, van Zeben D, Zwinderman AH, Breedveld FC, Hazes JM. Long-term course and outcome of functional capacity in rheumatoid arthritis: the effect of disease activity and radiologic damage over time. Arthritis Rheum. 1999;42:1854–60.
4. Welsing PM, van Gestel AM, Swinkels HL, Kiemeney LA, van Riel PL. The relationship between disease activity, joint destruction, and functional capacity over the course of rheumatoid arthritis. Arthritis Rheum. 2001;44:2009–17.
5. Plant MJ, Jones PW, Saklatvala J, Ollier WE, Dawes PT. Patterns of radiological progression in early rheumatoid arthritis: results of an 8 year prospective study. J Rheumatol. 1998;25:417–26.
6. Fex E, Jonsson K, Johnson U, Eberhardt K. Development of radiographic damage during the first 5–6 yr of rheumatoid arthritis. A prospective follow-up study of a Swedish cohort. Br J Rheumatol. 1996;35:1106–15.
7. Fuchs HA, Kaye JJ, Callahan LF, Nance EP, Pincus T. Evidence of significant radiographic damage in rheumatoid arthritis within the first 2 years of disease. J Rheumatol. 1989;16:585–91.
8. van der Heijde DM. Joint erosions and patients with early rheumatoid arthritis. Br J Rheumatol. 1995;34:74–8.
9. Scott DL, Grindulis KA, Struthers GR, Coulton BL, Popert AJ, Bacon PA. Progression of radiological changes in rheumatoid arthritis. Ann Rheum Dis. 1984;43:8–17.
10. Gay S, Gay RE, Koopman WJ. Molecular and cellular mechanisms of joint destruction in rheumatoid arthritis: two cellular mechanisms explain joint destruction? Ann Rheum Dis. 1993;52:S39–47.
11. Ochi T, Iwase R, Yonemasu K, Matsukawa M, Yoneda M, Yukioka M, et al. Natural course of joint destruction and fluctuation of serum C1q levels in patients with rheumatoid arthritis. Arthritis Rheum. 1988;31:37–43.
12. Hulsmans HM, Jacobs JW, van der Heijde DM, van Albada-Kuipers GA, Schenk Y, Bijlsma JW. The course of radiologic damage during the first six years of rheumatoid arthritis. Arthritis Rheum. 2000;43:1927–40.
13. van der Heijde DM, van Riel PL, van Leeuwen MA, van 't Hof MA, van Rijswijk MH, van de Putte LB. Prognostic factors for radiographic damage and physical disability in early rheumatoid arthritis. A prospective follow-up study of 147 patients. Br J Rheumatol. 1992;31:519–25.
14. van Zeben D, Hazes JM, Zwinderman AH, Vandenbroucke JP, Breedveld FC. Factors predicting outcome of rheumatoid arthritis: results of a followup study. J Rheumatol. 1993;20:1288–96.
15. Mansson B, Carey D, Alini M, Ionescu M, Rosenberg LC, Poole AR, et al. Cartilage and bone metabolism in rheumatoid arthritis. Differences between rapid and slow progression of disease identified by serum markers of cartilage metabolism. J Clin Invest. 1995;95:1071–7.
16. van der Heide A, Remme CA, Hofman DM, Jacobs JW, Bijlsma JW. Prediction of progression of radiologic damage in newly diagnosed rheumatoid arthritis. Arthritis Rheum. 1995;38:1466–74.
17. Jansen LM, van der Horst-Bruinsma IE, van Schaardenburg D, Bezemer PD, Dijkmans BA. Predictors of radiographic joint damage in patients with early rheumatoid arthritis. Ann Rheum Dis. 2001;60:924–7.
18. Kaltenhauser S, Wagner U, Schuster E, Wassmuth R, Arnold S, Seidel W, et al. Immunogenetic markers and seropositivity predict radiological progression in early rheumatoid arthritis independent of disease activity. J Rheumatol. 2001;28:735–44.
19. Garnero P, Gineyts E, Christgau S, Finck B, Delmas PD. Association of baseline levels of urinary glucosyl-galactosyl-pyridinoline and type II collagen C-telopeptide with progression of joint destruction in patients with early rheumatoid arthritis. Arthritis Rheum. 2002;46:21–30.
20. Boers M, Kostense PJ, Verhoeven AC, van der Linden S. Inflammation and damage in an individual joint predict further damage in that joint in patients with early rheumatoid arthritis. Arthritis Rheum. 2001;44:2242–6.
21. Vittecoq O, Pouplin S, Krzanowska K, Jouen-Beades F, Menard JF, Gayet A, et al. Rheumatoid factor is the strongest predictor of radiological progression of rheumatoid arthritis in a three-year prospective study in community-recruited patients. Rheumatology (Oxford). 2003;42:939–46.
22. Green MJ, Gough AK, Devlin J, Smith J, Astin P, Taylor D, et al. Serum MMP-3 and MMP-1 and progression of joint damage in early rheumatoid arthritis. Rheumatology (Oxford). 2003;42:83–8.
23. Kaufmann J, Kielstein V, Kilian S, Stein G, Hein G. Relation between body mass index and radiological progression in patients with rheumatoid arthritis. J Rheumatol. 2003;30:2350–5.
24. Verstappen SM, Poole AR, Ionescu M, King LE, Abrahamowicz M, Hofman DM, et al. Radiographic joint damage in rheumatoid arthritis is associated with differences in cartilage turnover and

can be predicted by serum biomarkers: an evaluation from 1 to 4 years after diagnosis. Arthritis Res Ther. 2006;8:R31.
25. Meyer O, Nicaise-Roland P, Santos MD, Labarre C, Dougados M, Goupille P, et al. Serial determination of cyclic citrullinated peptide autoantibodies predicted five-year radiological outcomes in a prospective cohort of patients with early rheumatoid arthritis. Arthritis Res Ther. 2006;8:R40.
26. Berglin E, Johansson T, Sundin U, Jidell E, Wadell G, Hallmans G, et al. Radiological outcome in rheumatoid arthritis is predicted by presence of antibodies against cyclic citrullinated peptide before and at disease onset, and by IgA-RF at disease onset. Ann Rheum Dis. 2006;65:453–8.
27. Charni N, Juillet F, Garnero P. Urinary type II collagen helical peptide (HELIX-II) as a new biochemical marker of cartilage degradation in patients with osteoarthritis and rheumatoid arthritis. Arthritis Rheum. 2005;52:1081–90.
28. Garnero P, Jouvenne P, Buchs N, Delmas PD, Miossec P. Uncoupling of bone metabolism in rheumatoid arthritis patients with or without joint destruction: assessment with serum type I collagen breakdown products. Bone. 1999;24:381–5.
29. Young-Min S, Cawston T, Marshall N, Coady D, Christgau S, Saxne T, et al. Biomarkers predict radiographic progression in early rheumatoid arthritis and perform well compared with traditional markers. Arthritis Rheum. 2007;56:3236–47.
30. van der Helm-van Mil AH, van der Kooij SM, Allaart CF, Toes RE, Huizinga TW. A high body mass index is protective on the amount of joint destruction in small joints in early rheumatoid arthritis. Ann Rheum Dis. 2008;67:769–74.
31. Westhoff G, Rau R, Zink A. Radiographic joint damage in early rheumatoid arthritis is highly dependent on body mass index. Arthritis Rheum. 2007;56:3575–82.
32. Brown DA, Moore J, Johnen H, Smeets TJ, Bauskin AR, Kuffner T, et al. Serum macrophage inhibitory cytokine 1 in rheumatoid arthritis: a potential marker of erosive joint destruction. Arthritis Rheum. 2007;56:753–64.
33. Otero M, Lago R, Gomez R, Lago F, Dieguez C, Gomez-Reino JJ, et al. Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and visfatin in patients with rheumatoid arthritis. Ann Rheum Dis. 2006;65:1198–201.
34. Nishimoto N, Hashimoto J, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, et al. Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor (SAMURAI): evidence of clinical and radiographic benefit from an x ray reader-blinded randomised controlled trial of tocilizumab. Ann Rheum Dis. 2007;66:1162–7.
35. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31:315–24.
36. van der Heijde D. How to read radiographs according to the Sharp/van der Heijde method. J Rheumatol. 2000;27:261–3.
37. van der Heijde D, Simon L, Smolen J, Strand V, Sharp J, Boers M, et al. How to report radiographic data in randomized clinical trials in rheumatoid arthritis: guidelines from a roundtable discussion. Arthritis Rheum. 2002;47:215–8.
38. Pincus T, Summey JA, Soraci SA Jr, Wallston KA, Hummon NP. Assessment of patient satisfaction in activities of daily living using a modified Stanford Health Assessment Questionnaire. Arthritis Rheum. 1983;26:1346–53.
39. Yamanaka H, Matsuda Y, Tanaka M, Sendo W, Nakajima H, Taniguchi A, et al. Serum matrix metalloproteinase 3 as a predictor of the degree of joint destruction during the six months after measurement, in patients with early rheumatoid arthritis. Arthritis Rheum. 2000;43:852–8.
40. Posthumus MD, Limburg PC, Westra J, van Leeuwen MA, van Rijswijk MH. Serum matrix metalloproteinase 3 in early rheumatoid arthritis is correlated with disease activity and radiological progression. J Rheumatol. 2000;27:2761–8.
41. Cunnane G, Fitzgerald O, Beeton C, Cawston TE, Bresnihan B. Early joint erosions and serum levels of matrix metalloproteinase 1, matrix metalloproteinase 3, and tissue inhibitor of metalloproteinases 1 in rheumatoid arthritis. Arthritis Rheum. 2001;44:2263–74.
42. Posthumus MD, Limburg PC, Westra J, van Leeuwen MA, van Rijswijk MH. Serum matrix metalloproteinase 3 levels during treatment with sulfasalazine or combination of methotrexate and sulfasalazine in patients with early rheumatoid arthritis. J Rheumatol. 2002;29:883–9.
43. Roux-Lombard P, Eberhardt K, Saxne T, Dayer JM, Wollheim FA. Cytokines, metalloproteinases, their inhibitors and cartilage oligomeric matrix protein: relationship to radiological progression and inflammation in early rheumatoid arthritis. A prospective 5-year study. Rheumatology (Oxford). 2001;40:544–51.
44. Posthumus MD, Limburg PC, Westra J, van Leeuwen MA, van Rijswijk MH. Serum matrix metalloproteinase 3 levels in comparison to C-reactive protein in periods with and without progression of radiological damage in patients with early rheumatoid arthritis. Clin Exp Rheumatol. 2003;21:465–72.
45. Garnero P, Landewe R, Boers M, Verhoeven A, Van Der Linden S, Christgau S, et al. Association of baseline levels of markers of bone and cartilage degradation with long-term progression of joint damage in patients with early rheumatoid arthritis: the COBRA study. Arthritis Rheum. 2002;46:2847–56.
46. Muller A, Jakob K, Hein GE. Evaluation of free and peptide bound collagen crosslink excretion in different skeletal diseases. Ann Rheum Dis. 2003;62:65–7.
47. Astbury C, Bird HA, McLaren AM, Robins SP. Urinary excretion of pyridinium crosslinks of collagen correlated with joint damage in arthritis. Br J Rheumatol. 1994;33:11–5.
48. Black D, Marabani M, Sturrock RD, Robins SP. Urinary excretion of the hydroxypyridinium cross links of collagen in patients with rheumatoid arthritis. Ann Rheum Dis. 1989;48:641–4.
49. Seibel MJ, Duncan A, Robins SP. Urinary hydroxy-pyridinium crosslinks provide indices of cartilage and bone involvement in arthritic diseases. J Rheumatol. 1989;16:964–70.
50. Kaufmann J, Mueller A, Voigt A, Carl HD, Gursche A, Zacher J, et al. Hydroxypyridinium collagen crosslinks in serum, urine, synovial fluid and synovial tissue in patients with rheumatoid arthritis compared with osteoarthritis. Rheumatology (Oxford). 2003;42:314–20.
51. Takahashi M, Kushida K, Hoshino H, Suzuki M, Sano M, Miyamoto S, et al. Concentrations of pyridinoline and deoxypyridinoline in joint tissues from patients with osteoarthritis or rheumatoid arthritis. Ann Rheum Dis. 1996;55:324–7.
52. Eyre DR, Koob TJ, Van Ness KP. Quantitation of hydroxypyridinium crosslinks in collagen by high-performance liquid chromatography. Anal Biochem. 1984;137:380–8.
53. Saito M, Fujii K, Soshi S, Tanaka T. Reductions in degree of mineralization and enzymatic collagen cross-links and increases in glycation-induced pentosidine in the femoral neck cortex in cases of femoral neck fracture. Osteoporos Int. 2006;17:986–95.
54. Bailey AJ, Wotton SF, Sims TJ, Thompson PW. Post-translational modifications in the collagen of human osteoporotic femoral head. Biochem Biophys Res Commun. 1992;185:801–5.
55. Helliwell M, Coombes EJ, Moody BJ, Batstone GF, Robertson JC. Nutritional status in patients with rheumatoid arthritis. Ann Rheum Dis. 1984;43:386–90.
56. Roubenoff R, Roubenoff RA, Ward LM, Holland SM, Hellmann DB. Rheumatoid cachexia: depletion of lean body mass in rheumatoid arthritis. Possible association with tumor necrosis factor. J Rheumatol. 1992;19:1505–10.

57. Munro R, Capell H. Prevalence of low body mass in rheumatoid arthritis: association with the acute phase response. Ann Rheum Dis. 1997;56:326–9.
58. Morgan SL, Anderson AM, Hood SM, Matthews PA, Lee JY, Alarcon GS. Nutrient intake patterns, body mass index, and vitamin levels in patients with rheumatoid arthritis. Arthritis Care Res. 1997;10:9–17.
59. Ebina K, Fukuhara A, Ando W, Hirao M, Koga T, Oshima K, et al. Serum adiponectin concentrations correlate with severity of rheumatoid arthritis evaluated by extent of joint destruction. Clin Rheumatol. 2009;28:445–51.
60. Lindqvist E, Eberhardt K, Bendtzen K, Heinegard D, Saxne T. Prognostic laboratory markers of joint damage in rheumatoid arthritis. Ann Rheum Dis. 2005;64:196–201.
61. Geusens PP, Landewé RB, Garnero P, Chen D, Dunstan CR, Lems WF, et al. The ratio of circulating osteoprotegerin to RANKL in early rheumatoid arthritis predicts later joint destruction. Arthritis Rheum. 2006;54:1772–7.

ARTHRITIS & RHEUMATISM
Vol. 60, No. 12, December 2009, pp 3591–3601
DOI 10.1002/art.25020

Specifically Modified Osteopontin in Rheumatoid Arthritis Fibroblast-like Synoviocytes Supports Interaction With B Cells and Enhances Production of Interleukin-6

Yasuhiro Take,[1] Ken Nakata,[1] Jun Hashimoto,[1] Hideki Tsuboi,[2] Norihiro Nishimoto,[3] Takahiro Ochi,[4] and Hideki Yoshikawa[1]

Objective. **Osteopontin (OPN) is expressed by fibroblast-like synoviocytes (FLS) in rheumatoid arthritis (RA), but its pathologic role is still obscure. The present study was undertaken to analyze the role of OPN in RA by focusing on its effects on cell–cell interactions between FLS and B lymphocytes.**

Methods. **FLS obtained from 10 patients with RA and 10 non-RA subjects and a B lymphocyte cell line were studied. The characteristics of OPN expression by FLS were analyzed by Western blotting, immunoprecipitation, and immunofluorescence studies. In cocultures of FLS and B lymphocytes, the effects of OPN on adhesion of B lymphocytes to FLS and the consequent production of interleukin-6 (IL-6) were analyzed in experiments involving overexpression and knockdown of OPN and inhibitory studies with an OPN-blocking antibody. In vivo, the expression of OPN in RA synovium was examined by immunohistochemistry.**

Results. **A specifically modified 75-kd form of OPN was predominantly expressed in RA FLS, and this was associated with expression of >200-kd thrombin-cleaved OPN that was crosslinked with fibronectin and localized on the surface of the FLS. In FLS–B lymphocyte cocultures, 75-kd OPN–positive FLS produced a significantly higher amount of IL-6 than did 75-kd OPN–negative FLS. When the FLS were separated from B lymphocytes or cultured alone, the production of IL-6 was low and was not significantly different between these 2 culture conditions. Moreover, OPN overexpression enhanced production of IL-6 in 75-kd OPN–positive FLS–B lymphocyte cocultures. Addition of the OPN-blocking antibody inhibited the adhesion of B lymphocytes to FLS. Immunohistochemical analyses revealed that localization of IL-6–positive cells coincided with the sites at which OPN and B lymphocytes were colocalized.**

Conclusion. **Specifically modified 75-kd OPN was expressed by RA FLS. This form of OPN affected FLS–B lymphocyte interactions by supporting the adhesion of B lymphocytes to FLS and enhancing the production of IL-6.**

In patients with rheumatoid arthritis (RA), the synovium of the inflamed joints is the site of a chronic inflammatory reaction, in which leukocytes and macrophages infiltrate, synoviocytes proliferate, and several proinflammatory cytokines (especially, interleukin-6 [IL-6] and tumor necrosis factor α), autoantibodies, and immune complexes are produced (1). In this situation, infiltrating leukocytes and proliferating synoviocytes are thought to interact with each other. Accordingly, several in vitro studies have focused on the interaction between fibroblast-like synoviocytes (FLS) and leukocytes by studying the findings in cocultures of these cells (2–7). Coculture of FLS obtained from RA synovium with B lymphocytes causes adhesion of B lymphocytes to FLS, and consequently this interaction supports the survival of B lymphocytes and also enhances the production of cytokines and immunoglobulin (8–12). The former mechanism is dependent on vascular cell adhesion mol-

Supported by Health and Labour Sciences of Japan research grant 200500732A.

[1]Yasuhiro Take, MD, Ken Nakata, MD, PhD, Jun Hashimoto, MD, PhD, Hideki Yoshikawa, MD, PhD: Osaka University, Suita-city, Osaka, Japan; [2]Hideki Tsuboi, MD, PhD: Osaka Rosai Hospital, Sakai-city, Osaka, Japan; [3]Norihiro Nishimoto, MD, PhD: Wakayama Medical University, Wakayama-city, Wakayama, Japan; [4]Takahiro Ochi, MD, PhD: Osaka Police Hospital, Osaka-city, Osaka, Japan.

Address correspondence and reprint requests to Ken Nakata, MD, PhD, Department of Orthopaedics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita-city, Osaka 565-0871, Japan. E-mail: ken-nakata@umin.ac.jp.

Submitted for publication February 22, 2009; accepted in revised form August 31, 2009.

ecule 1 (VCAM-1) and very late activation antigen 4 (VLA-4) (10), whereas the latter is yet to be determined.

Osteopontin (OPN) is abundant in the bone matrix, where it acts as a bridge between hydroxyapatite and osteoclasts to support bone resorption (13–15). It is also secreted by T lymphocytes and assists in the maturation of B lymphocytes and the migration of macrophages (16–18). These diverse functions are partly explained by the several functional domains of OPN, including integrin-binding, calcium-binding, and heparin-binding sites. Several studies have revealed that the function of OPN depends on posttranslational modifications (19–24). These modifications, which vary between different OPN-expressing cells, consist of phosphorylation at dozens of serine and threonine residues, along with glycosylation (25), sialylation (21), transglutamination (26), and cleavage (27,28). However, the association between the details of posttranslational modification and the function of OPN is still poorly understood.

As for the relationship between OPN and arthritis, OPN-null mice are protected against inflammatory joint destruction in collagen-induced arthritis (29). A blocking antibody directed against the thrombin-cleaved neoepitope of OPN, which cooperates with several integrin receptors as a ligand (30,31), also shows a curative effect on induced arthritis in mice and monkeys (32,33). Indeed, in patients with RA, OPN, especially in the thrombin-cleaved form, is strongly detected in the synovium and synovial fluid of inflamed joints (34–36). In vitro studies on the function of OPN in arthritis have revealed that OPN stimulates the production of several proinflammatory cytokines by mononuclear cells in patients with RA (37), and also that monocytes obtained from mice with induced arthritis show increased migration toward thrombin-cleaved OPN (32). However, these studies were performed using recombinant OPN, which lacks various posttranslational modifications, and thus the posttranslational modification–dependent functions were not taken into account.

Considering these results and the fact that OPN is also strongly expressed in FLS from the RA synovium (35), we hypothesized that RA FLS express a unique form of OPN that acts to stimulate production of cytokines by creating a bridge between FLS and B lymphocytes in cocultures of these cells. The purpose of this study was to analyze the role of OPN in the development of RA, by focusing on the interaction between FLS and B lymphocytes.

PATIENTS AND METHODS

Patients and cell culture. After obtaining the patients' informed consent and securing Institutional Review Board approval, synovial tissue samples were collected from 11 patients with RA, 8 patients with osteoarthritis of the knee, and 2 patients with medial meniscus degeneration who underwent meniscectomy and synovectomy. All of the patients with RA fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) revised criteria for RA (38). FLS were isolated by enzymatic digestion of the synovial tissue from 10 patients with RA and 10 non-RA subjects, as described previously (9). The FLS were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Gibco BRL). Cells from passages 4–10 were used for these experiments. A human B cell line, MC/car, was purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium (Gibco BRL) containing 10% FBS and 1% penicillin/streptomycin.

For coculture experiments, 4×10^4 FLS were plated in 12-well plates and, on the following day, 3.3×10^5 B lymphocytes were added to each well. To prevent cell–cell contact between FLS and B lymphocytes, a Millicell culture insert with 0.4-μm pores (Millipore, Billerica, MA) was used. After incubation for 48 hours, enzyme immunoassay (EIA) was performed to measure the concentrations of IL-6 and OPN in the culture supernatant using a homogeneous time-resolved fluorescence human IL-6 assay kit (CIS Bio International, Saclay, France) and a human OPN assay kit (IBL Japan, Gunma, Japan).

Protein collection, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting. Total cell lysates of FLS were collected by placing the cells in lysis buffer (10× Cell Lysis Buffer: New England Biolabs, Beverly, MA), using 4×10^4 FLS plated in 12-well plates and grown in normal growth medium; some of the FLS had undergone transfection with small interfering RNA (siRNA) or lentiviral infection. We did not add any other reagent to the lysis buffer, which consisted of Tris HCl buffer with detergent, protease inhibitor (leupeptin), and phosphatase inhibitors (sodium pyrophosphate, β-glycerophosphate, and sodium orthovanadate). After 1 freeze–thaw cycle, samples were centrifuged and the supernatant was collected. An aliquot was obtained for protein quantification with the bicinchoninic acid assay, and the remaining supernatant was boiled in SDS sample buffer. As a control, recombinant human OPN was used.

Surface protein was collected from 1.2×10^6 FLS using a Cell Surface Protein isolation kit (Pierce, Rockford, IL). Equal amounts of each sample were separated by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane (Hybond-P; Amersham, Piscataway, NJ), and blocked with 5% bovine serum albumin (BSA)/Tris buffered saline containing 0.1% Tween 20 (TBST). The membrane was then incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-human OPN antibody (O-17; IBL Japan) or mouse anti-OPN N-Half antibody (34E3; IBL Japan) (each at 2 μg/ml), which detects the thrombin-cleaved neoepitope YGLR, mouse anti–β-actin antibody (AC-15; Sigma, St. Louis,

MO) at 1:10,000, or rabbit antifibronectin antibody (H-300; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200. Specificity of the 34E3 antibody was confirmed by Western blotting of thrombin-treated recombinant OPN. After reaction with horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG and enhanced chemiluminescence Western blotting detection reagents (all from Amersham), images of the membrane were captured using a FAS-1000 Lumino Imaging Analyzer (Toyobo, Osaka, Japan).

Immunoprecipitation analysis. Total cell lysates were prepared for immunoprecipitation using RA FLS from a single patient (RA sample 4 in Figure 1A). When the cells had reached 90% confluence, they were collected in a 10-cm dish, and after centrifugation, the resulting supernatant was precleared with ImmunoPure Immobilized Protein A Plus (Pierce), divided into 3 centrifuge tubes, and incubated overnight at 4°C with protein A–Sepharose bound with 10 μg of the anti-OPN antibody (O-17), the antifibronectin antibody, or normal rabbit IgG (Santa Cruz Biotechnology) as a negative control. On the following day, the samples were washed and eluted with SDS sample buffer, and then analyzed by Western blotting.

Immunofluorescence analysis. FLS cultured in Chamber Slides (Nunc, Rochester, NY) were fixed in 4% paraformaldehyde, permeabilized with TBST, blocked with 5% BSA/TBST, and incubated overnight at 4°C with the anti-OPN antibody (O-17) or normal rabbit IgG (Santa Cruz Biotechnology) (each at 2 μg/ml). After the samples were reacted with Alexa Fluor 488–conjugated anti-rabbit IgG at 1:500 (Molecular Probes, Eugene, OR), the fluorescence intensity of the samples was examined under an epifluorescence microscope (Nikon Eclipse 90i; Nikon, Tokyo, Japan).

Transfection with siRNA. Commercially available, predesigned double-stranded RNA (nontargeting siRNA [siCONTROL Pool #1] and siRNA for human secreted phosphoprotein 1 [OPN] and IL-6 [siGENOME SMARTpool siRNA]; Dharmacon, Lafayette, CO) were used for the RNA interference experiments. One microliter of X-TremeGENE reagent (Roche Diagnostic, Penzberg, Germany) and 20 pmoles of the siRNA from either pool were diluted in serum-free medium, and the mixture was then incubated for 15 minutes and added to 4×10^4 FLS in a 12-well plate. Two days after transfection, messenger RNA (mRNA) was collected and reverse-transcribed to first-strand complementary DNA (cDNA) using a high-capacity cDNA reverse transcription kit. Real-time reverse transcription–polymerase chain reaction (PCR) was then performed to evaluate the OPN-knockdown efficacy, using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). Three days after transfection, the cocultures were started.

B lymphocyte adhesion assay. Adhesion of B lymphocytes to FLS was assessed as described previously (11), with some modifications. Briefly, 6.6×10^3 FLS were plated into a 96-well plate. After 3 days, the cells were incubated at 37°C for 2 hours with a polyclonal OPN-neutralizing antibody or normal goat IgG (both from R&D Systems, Minneapolis, MN) at 10 μg/ml. Subsequently, 3.3×10^5 B lymphocytes were added and incubated at 37°C for another 2 hours. B lymphocytes that did not adhere firmly to the FLS were removed by vigorous washing. Three separate fields were viewed under an inverted microscope at a magnification of 100× per well to count the adherent B lymphocytes, with results expressed quantitatively as the mean number of adherent B lymphocytes.

Transglutaminase inhibition. FLS were plated at 4×10^4 cells in 12-well plates, followed by incubation with cystamine sulfate (500 μ*M*) for 2 days to inhibit transglutaminase activity. Subsequently, total cell lysates were collected for further analysis.

Plasmid construction and lentiviral infection. The full-length OPN-coding sequence was amplified by PCR from a human synovial cell cDNA library using the following primers: 5′-CCCTCGAGATGAGAATTGCAGTGATTTGC-3′ (NM_001040058, nt166–186) and 5′-CGGGATCCTTAATTGACCTCAGAAGATGCAC-3′ (NM_001040058, nt1088–1110). The PCR product was digested with *Xho* I and *Bam* HI, and then purified and ligated to pcDNA3.1 (Invitrogen, Carlsbad, CA) for sequencing. The vector was cut with *Pme* I and the OPN insert was obtained by gel extraction/purification, which was then ligated to the lentiviral expression vector pWPI. Lentivirus infection was performed on 4×10^4 FLS in a 12-well plate, as described previously (39,40). Three days after infection, coculture was started for measurement of IL-6 production.

Immunohistochemistry. Specimens of synovial tissue obtained from a patient with RA were fixed in 10% formaldehyde dehydrated, and embedded in paraffin. Sections of the tissue were cut (4 μm thick) on a microtome, and then were analyzed by immunohistochemistry, as described previously (41), using the following primary antibodies: rabbit anti-OPN antibody (O-17) or normal rabbit IgG at 2 μg/ml, mouse anti-CD79α antibody (JCB117; Nichirei, Tokyo, Japan) at 1:50, normal mouse IgG1 (R&D Systems), and goat anti-human IL-6 antibody or normal goat IgG at 2.5 μg/ml.

Statistical analysis. Results are expressed as the mean ± SD of at least 3 independent experiments, if not specified. For statistical comparison, 2-way factorial analysis of variance (ANOVA) (sometimes followed by a Bonferroni post hoc test) was performed using SPSS software (version 16.0; SPSS, Chicago, IL). *P* values less than 0.05 were considered significant.

RESULTS

Detection of specifically modified 75-kd OPN in all 10 RA FLS and 3 non-RA FLS, in conjunction with significantly higher IL-6 production in FLS–B lymphocyte cocultures. Western blotting of the total cell lysates from FLS, using the antibody for the N-terminus of OPN (O-17), showed several bands at 75 kd and also around 54 kd (Figure 1A), which is a well-known characteristic of OPN (42). Double bands around 54 kd, each at an equal density, were detected in every FLS sample. Recombinant human OPN also migrated at around 54 kd. However, 75-kd OPN was predominantly detected in all 10 RA FLS samples (RA samples 1–10 in Figure 1A)

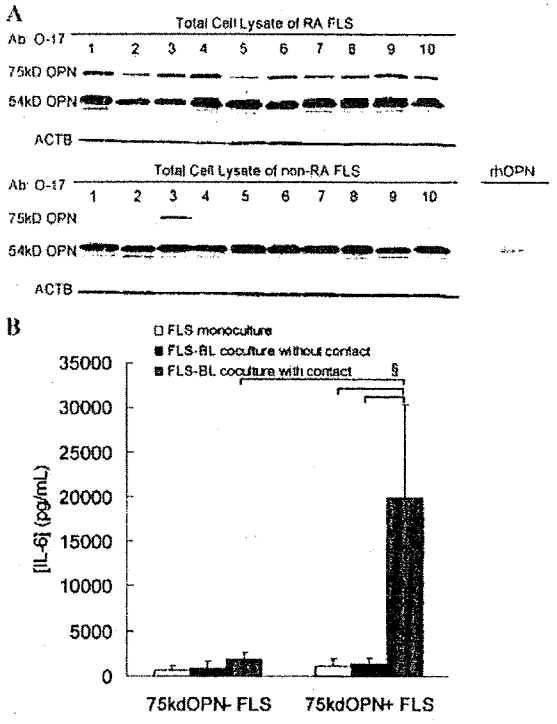


Figure 1. Detection of osteopontin (OPN) and production of interleukin-6 (IL-6) in fibroblast-like synoviocytes (FLS). A, Total cell lysates of rheumatoid arthritis (RA) FLS and non-RA FLS (n = 10 samples each) were assessed using Western blotting with an N-terminus antibody (Ab) against OPN (O-17), in comparison with recombinant human OPN (rhOPN); β-actin (ACTB) served as standard. B, The concentration of interleukin-6 (IL-6) in the culture supernatants of FLS monocultures, FLS–B lymphocyte (BL) cocultures without contact (separated by a Millicell culture insert), and FLS–B lymphocyte cocultures with contact was evaluated by enzyme immunoassay separately in 75-kd OPN–positive FLS (n = 13) and 75-kd OPN–negative FLS (n = 7). Bars show the mean and SD. § = $P < 0.001$ by Bonferroni *t*-test.

and in only 3 non-RA FLS samples (non-RA samples 3, 8, and 9). Thrombin-cleaved OPN was not detected around the reported molecular weight of 30 kd (43).

EIA analysis of the full-length OPN secreted in the culture supernatant showed that the concentration of OPN was 9–18 ng/ml, and this did not differ between RA and non-RA FLS (results not shown). Since the expression of 75-kd OPN was distinct, FLS were grouped into 75-kd OPN–positive and 75-kd OPN–negative FLS.

Production of IL-6 was evaluated by EIA for determination of the IL-6 concentration in the culture supernatants obtained in several culture conditions with FLS and B lymphocytes. In FLS monoculture (i.e., FLS cultured alone), the mean ± SD IL-6 production by the 75-kd OPN–positive FLS (n = 13) and the 75-kd OPN–negative FLS (n = 7) was 1,113.3 ± 818.9 pg/ml and 643.1 ± 576.5 pg/ml, respectively. In FLS–B lymphocyte cocultures without contact (i.e., FLS cocultured with B lymphocytes, but separated by the Millicell culture insert), the IL-6 levels were 1,363.3 ± 714.6 pg/ml in 75-kd OPN–positive FLS and 902.6 ± 772.5 pg/ml in 75-kd OPN–negative FLS. There were no significant differences in IL-6 production among these 4 groups.

However, in cocultures with contact (i.e., FLS and B lymphocytes cultured in contact with each other), IL-6 production was significantly elevated in 75-kd OPN–positive FLS, and showed a tendency to be elevated, although not to a significant extent, in 75-kd OPN–negative FLS. The IL-6 production by the 13 FLS positive for 75-kd OPN cocultured in contact with B lymphocytes was elevated to 19,928.5 ± 10,351.8 pg/ml, which was significantly higher than that in the other 2 culture conditions ($P < 0.001$, by Bonferroni *t*-test) and significantly higher than that in the 7 FLS negative for 75-kd OPN that were in contact coculture with B lymphocytes (910.9 ± 744.1 pg/ml; $P < 0.001$, by Bonferroni *t*-test) (Figure 1B).

Transfection of the FLS with IL-6 siRNA significantly reduced the levels of IL-6 in the 75-kd OPN–positive FLS that were in contact coculture with B lymphocytes. In contrast, when B lymphocytes were cultured alone, IL-6 was not detected in the culture supernatants (results not shown).

Production of >200-kd thrombin-cleaved OPN in the cell surface protein fraction of 75-kd OPN–positive FLS, and cell membrane distribution of OPN. Analysis of cell surface proteins from FLS, using Western blotting with an antibody against the thrombin-cleaved neoepitope of OPN (34E3), detected the presence of >200-kd OPN in the surface fraction of 75-kd OPN–positive FLS. This band was poorly detected in 75-kd OPN–negative FLS or in the nonsurface fraction of FLS (Figure 2A). A similar result was obtained with the O-17 antibody, but the signals were much weaker (results not shown). As a loading control, Western blotting with the antifibronectin antibody probe was performed. When the anti-OPN antibody O-17 was used to analyze the 75-kd OPN–positive FLS by immunofluorescence assay, the distribution of OPN followed the cell outline, regardless of permeabilization (Figure 2B).

Detection of >200-kd OPN in the immunoprecipitant by antifibronectin antibody, and reduction by transglutaminase inhibitor treatment. According to a previous study, OPN appears at >200 kd when it is