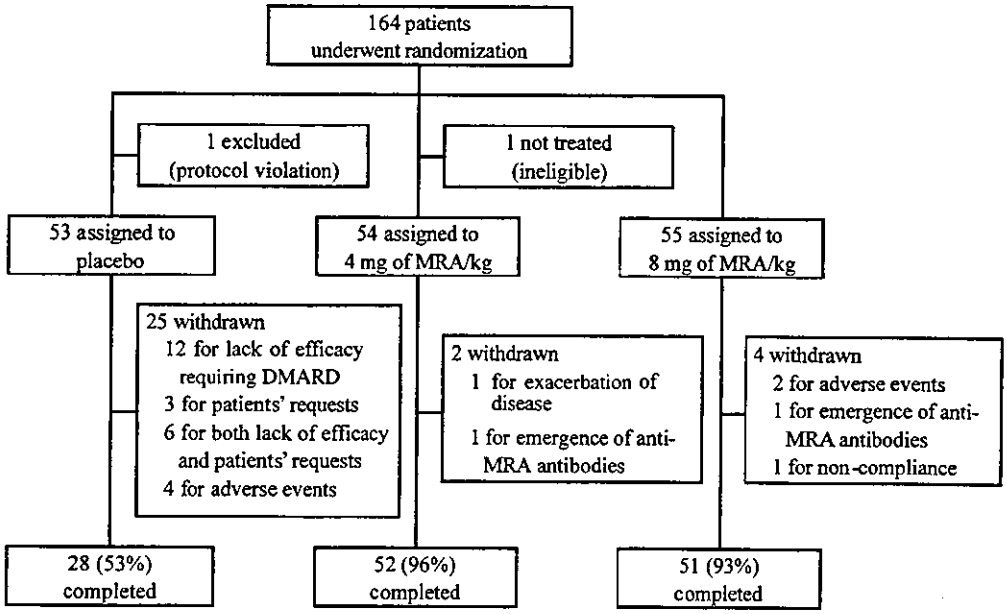

Figure 1. Randomization, reasons for withdrawal, and numbers of patients who completed the trial. MRA = humanized anti–interleukin-6 receptor antibody; DMARD = disease-modifying antirheumatic drug.

variables within the ACR core set (21). Statistical analyses were performed with SAS version 8.2 TS2M0 (SAS Institute, Cary, NC). The incidences of improvements were analyzed by use of the chi-square test. The differences among groups of DAS28 scores and the individual variables of the ACR core set were analyzed by use of Student's *t*-test.

We determined that a sample size of 39 patients per group was needed in order to obtain 80% power to detect a

Table 1. Characteristics of patients at study entry*

	Treatment group		
	Placebo (n = 53)	4 mg/kg MRA (n = 54)	8 mg/kg MRA (n = 55)
Age, median (range) years	53.0 (31–73)	53.5 (21–74)	56.0 (25–74)
No. of men/no. of women	14/39	14/40	9/46
Functional class†			
I	3	3	3
II	35	36	35
III	15	15	17
IV	0	0	0
RA stage†			
I	0	3	0
II	13	14	12
III	21	20	24
IV	19	17	19
Duration of disease, median (range) years	8.4 (0.7–52.7)	7.3 (0.6–35.8)	8.3 (1.3–45.7)
No. of failed DMARDs, median (range)	5 (1–10)	4 (2–8)	5 (1–11)
Tender joint count, mean ± SD	18.2 ± 8.4	19.1 ± 9.0	17.8 ± 9.8
Swollen joint count, mean ± SD	14.1 ± 6.1	16.0 ± 9.1	13.6 ± 6.9
ESR, mean ± SD mm/hour	68.7 ± 31.2	71.2 ± 29.1	67.4 ± 30.9
CRP level, mean ± SD mg/dl	5.5 ± 4.2	4.7 ± 2.9	4.5 ± 3.3

* MRA = humanized anti–interleukin-6 receptor antibody; RA = rheumatoid arthritis; DMARDs = disease-modifying antirheumatic drugs; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein.
† RA functional status determined by American College of Rheumatology criteria. RA stage determined by Steinbrocker criteria.

statistically significant ($P < 0.05$) difference in incidences between the placebo group and the 8-mg group by use of the two-sided chi-square test, where response rates in the population were assumed to be 20% and 50% in the placebo and 8-mg groups, respectively. We decided to recruit 45 patients per group to allow for anticipated withdrawals.

ANAs, anti-DNA antibodies, serum MRA levels, and anti-MRA antibodies. ANAs were measured indirectly by fluorescent antibody with HEp-2 cell substrate; a titer of ≥1:40 was considered positive. Anti-DNA antibodies were measured by ^{125}I radiobinding assay; a value of ≥6.0 IU/ml was considered positive.

Serum MRA levels were assessed by enzyme-linked immunosorbent assay (ELISA). Briefly, 100 μl of recombinant human sIL-6R (1 μg/ml) was added to the wells of an immunoplate precoated with MT18 and incubated at room temperature for 2 hours. After washing, bound MRA was measured using alkaline phosphatase (AP)–conjugated goat anti-human IgG. The calorimetric reaction was measured with a microplate reader.

Serum anti-MRA antibodies neutralizing MRA activities were measured by ELISA. Briefly, serum was added to wells coated with 100 μl of Fab fragment of MRA (0.2 μg/ml) and incubated for 2 hours. After washing, biotin-conjugated Fab fragment of MRA was added and developed with AP conjugated to streptavidin. IgE-type anti-MRA antibodies were also measured by ELISA. In this case, whole MRA was used because an antigen coated each cup, and enzyme-linked anti-IgE antibodies were used as second antibodies.

RESULTS

Characteristics of the patients. One hundred sixty-four patients were enrolled in the study (Figure 1). After enrollment, 1 patient was found to be ineligible (due to exacerbation of renal disease) for entry into the study and was withdrawn before administration of drug or placebo. Another patient was judged to be ineligible because of intravenous infusion of prednisolone during the washout period. A total of 162 patients (37 men and 125 women) were included in the full analysis set. The baseline demographic and clinical data are summarized in Table 1.

The groups were well matched according to their pretreatment characteristics or baseline levels of disease activity. The median age of the groups was 54 years, and the median duration of disease was 7.6 years. A median of 4–5 DMARDs had previously been tried unsuccessfully in the patient population. Furthermore, all patients had active disease in terms of high counts of swollen and tender joints and increased ESRs and CRP levels. Therefore, the population of included patients probably had relatively severe disease. Ninety-five percent of the patients completed MRA treatment (96% in the 4-mg group and 93% in the 8-mg group) compared with 53%

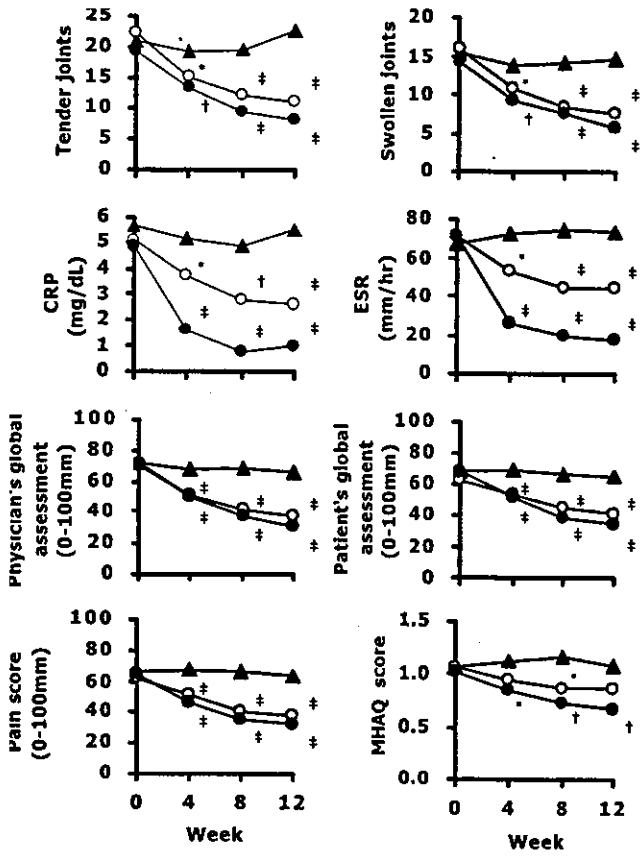

Figure 2. Rheumatoid arthritis disease activity assessments. Solid triangles indicate placebo-treated group. Open circles indicate group treated with 4 mg/kg humanized anti–interleukin-6 receptor antibody (MRA). Solid circles indicate 8 mg/kg MRA–treated group. Values are the mean for each group at each time point. * = $P < 0.05$; † = $P < 0.01$; ‡ = $P < 0.001$ versus placebo, by Student's *t*-test. CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; M-HAQ = modified Health Assessment Questionnaire.

of the patients receiving placebo. Among the 109 patients receiving MRA, 6 withdrew (2 for emergence of anti-MRA antibodies, 2 for adverse events, 1 for exacerbation of disease, and 1 for noncompliance because of changing an NSAID). Among the 53 patients receiving placebo, 25 withdrew (12 for lack of efficacy that required introduction of a DMARD at the discretion of the treating physician, 3 at the patients' requests, 6 for both lack of efficacy and patients' requests, and 4 for adverse events).

Efficacy. MRA treatment significantly improved all measures of disease activity in the ACR core set, and a dose-response relationship was observed between the 4-mg and 8-mg groups (Figure 2). The efficacy was apparent at week 4 and became most pronounced at the

Table 2. Percentage of responders according to the American College of Rheumatology (ACR) improvement criteria and the Disease Activity Score in 28 joints (DAS28)*

	Treatment group			*P*		
Response criteria	Placebo (n = 53)	4 mg/kg MRA (n = 54)	8 mg/kg MRA (n = 55)	Placebo vs. 8 mg/kg MRA	Placebo vs. 4 mg/kg MRA	4 mg/kg MRA vs. 8 mg/kg MRA
ACR improvement†						
ACR20	11.3	57.4	78.2	<0.001	<0.001	0.020
ACR50	1.9	25.9	40.0	<0.001	<0.001	0.118
ACR70	0.0	20.4	16.4	0.002	0.001	0.589
DAS28‡						
Good	0.0	5.6	18.2	0.001	0.085	0.042
Good or moderate	18.9	72.2	90.9	<0.001	<0.001	0.012

* Except where indicated otherwise, values are the percentage of patients achieving a given response. MRA = humanized anti–interleukin-6 receptor antibody; ACR20 = 20% improvement in disease activity according to ACR criteria.
† See ref. 19.
‡ See refs. 20 and 22.

end of treatment (week 12). In particular, complete normalization of the CRP level was observed in 76% and 26% of the patients in the 8-mg and 4-mg groups, respectively, while the CRP level was normalized in only 1.9% of patients in the placebo group. Seventy-eight percent of the 8-mg group achieved at least an ACR20 response compared with 57% of the 4-mg group ($P = 0.02$) and 11% of the placebo group ($P < 0.001$) (Table 2). There were also significantly more ACR50 and ACR70 responses in the 8-mg group than in the placebo group ($P < 0.001$ and $P = 0.002$, respectively). The efficacy was also confirmed by the percentages of patients in the DAS28 categories (20); the incidence of "good or moderate" (22) was 91% in the 8-mg group compared with 72% in the 4-mg group ($P = 0.012$) and 19% in the placebo group ($P < 0.001$) (Table 2).

In addition to the ACR core set of disease activity measures, considerable improvement occurred in platelet counts and in levels of hemoglobin, fibrinogen, serum amyloid A, and albumin (Table 3). Interestingly, fibrinogen levels and platelet counts remained in the low-to-normal range. Rheumatoid factors were positive in 102 of 109 patients (94%) in the MRA groups at baseline. Their titers decreased significantly in the 8-mg group (Table 3), and 3 patients became negative for rheumatoid factors at week 12. However, there was no statistically significant correlation between the decrease in rheumatoid factor titer and ACR response rate in this 3-month study. MRA treatment also significantly increased serum levels of the bone formation markers osteocalcin and PICP and simultaneously decreased levels of the bone absorption markers urinary pyridinoline and deoxypyridinoline.

Safety. Treatment tolerance of MRA was good. The incidences of adverse events were 56%, 59%, and 51% of the patients in the placebo, 4-mg, and 8-mg groups, respectively, and were not dose dependent. Most of the adverse events were mild and acceptable relative to the benefit provided. Table 4 shows the adverse events appearing in >3% of patients in this study. These adverse events did not require cessation of MRA treatment. Upper respiratory infection (common cold) was the most common adverse event overall, but the incidences were similar among the groups. Skin eruptions were reported in 5 patients in the MRA groups. Skin eruptions in 2 of these patients were classified as unrelated to MRA. The other 3 patients had mild and transient redness or papule of the skin. All 5 patients continued MRA treatment, and no exacerbation was observed. Infusion reactions, defined as any adverse experience occurring during or after the infusion on the treatment day, were found in 15%, 13%, and 16% of the patients in the placebo, 4-mg, and 8-mg groups, respectively. Most of them were mild and transient. Sleepiness (2.8%), mild headaches (2.8%), and increases in blood pressure (2.8%) were observed. Three patients had transient increases in blood pressure after infusion (138/75 mm Hg before, 165/80 mm Hg after; 134/82 mm Hg before, 140/90 mm Hg after; 150/88 mm Hg before, 192/104 mm Hg after), and 2 of these patients had had hypertension before entry into the study.

Five serious adverse events were reported in this study: 3 in the MRA group (2.8%) and 2 in the placebo group (3.8%). One patient died of reactivation of chronic active Epstein-Barr virus (EBV) infection and consequent hemophagocytosis syndrome 61 days after receiving a single 8-mg/kg dose of MRA. She showed fluctuating liver function and CRP levels that were inversely correlated with white blood cell counts, and she had increased EBV DNA in plasma before enrollment.

Table 3. Laboratory parameters*

	Treatment group		
	Placebo (n = 53)	4 mg/kg MRA (n = 54)	8 mg/kg MRA (n = 55)
Hemoglobin, gm/dl			
Baseline	11.3 ± 1.5	11.3 ± 1.6	11.3 ± 1.1
Week 12	11.2 ± 1.5	12.2 ± 1.5†	12.8 ± 1.3†
Platelets, $\times 10^4/\mu l$			
Baseline	36.6 ± 13.0	36.4 ± 8.7	32.6 ± 9.2
Week 12	38.5 ± 11.9	29.6 ± 8.8†	21.8 ± 6.5†
Fibrinogen, mg/dl			
Baseline	470.2 ± 118.1	480.0 ± 110.0	463.8 ± 102.1
Week 12	487.9 ± 130.1	396.2 ± 130.3†	231.6 ± 103.3†
Serum amyloid A, mg/ml			
Baseline	383.3 ± 343.0	404.9 ± 355.6	364.8 ± 288.6
Week 12	391.8 ± 375.2	202.0 ± 267.9†	75.0 ± 259.9†
Albumin, gm/dl			
Baseline	3.6 ± 0.4	3.6 ± 0.4	3.5 ± 0.4
Week 12	3.7 ± 0.4†	4.0 ± 0.4†	4.2 ± 0.4†
Rheumatoid factors, IU/ml			
Baseline	337.6 ± 364.8	297.9 ± 377.7	345.5 ± 436.2
Week 12	348.2 ± 402.6	261.2 ± 415.5	235.6 ± 365.6†
Osteocalcin, ng/ml			
Baseline	5.8 ± 2.8	5.7 ± 2.8	5.1 ± 2.2
Week 12	6.2 ± 3.1	6.6 ± 3.1†	6.5 ± 2.7†
PICP, ng/ml			
Baseline	135.4 ± 65.7	127.0 ± 63.5	117.2 ± 47.7
Week 12	144.3 ± 77.8	146.4 ± 70.6†	166.4 ± 78.7†
Urinary pyridinoline, μmoles/mole creatinine			
Baseline	60.3 ± 32.6	58.8 ± 32.7	58.5 ± 29.8
Week 12	62.6 ± 30.9	49.0 ± 29.5†	47.7 ± 24.2†
Deoxypyridinoline, μmoles/mole creatinine			
Baseline	7.8 ± 3.4	7.6 ± 3.5	7.4 ± 3.1
Week 12	7.6 ± 3.8	6.6 ± 3.2†	6.9 ± 3.1†

* Values are the mean ± SD. MRA = humanized anti–interleukin-6 receptor antibody; PICP = C-terminal type I procollagen propeptide.
† $P < 0.05$ versus baseline of each assessment, by paired t-test.

Two weeks after injection of the study drug, her liver function became worse in association with the EBV DNA increase in the plasma, followed by the hemophagocytosis syndrome. Another patient in the 8-mg group was hospitalized because of allergic pneumonitis after completion of the 3 doses of MRA. One patient in the 4-mg group was hospitalized because of infection secondary to a grade 2–3 burn on the leg, but she continued MRA treatment. Both patients were cured by medication. In the placebo group, a subarachnoid hemorrhage and a fracture of the neck of the femur were reported as serious adverse events.

Table 4. Adverse events observed in at least 3% of patients*

	Treatment group		
	Placebo (n = 53)	4 mg/kg MRA (n = 54)	8 mg/kg MRA (n = 55)
Common cold	7 (13.0)	9 (16.7)	5 (9.1)
Headache	1 (1.9)	2 (3.7)	3 (5.5)
Pruritus	3 (5.6)	3 (5.6)	2 (3.6)
Skin eruption	1 (1.9)	2 (3.7)	3 (5.5)
Stomatitis	2 (3.7)	3 (5.6)	4 (7.3)
Fever	1 (1.9)	3 (5.6)	3 (5.5)

* Values are the number (%) of patients. MRA = humanized anti–interleukin-6 receptor antibody.

For the laboratory profiles, abnormalities were observed in 41%, 57%, and 76% of patients in the placebo, 4-mg, and 8-mg groups, respectively. Lipid metabolism–related reactions such as increases in total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were common in the MRA groups (Figure 3). A blood cholesterol increase was observed in 48 of 109 patients (44.0%) in the MRA groups. Total cholesterol levels did not continue increasing, but became stable at a certain level in the extension study (data not shown). No cardiovascular complications were observed in association with the increase in total cholesterol. Liver function disorders were observed in 14 of

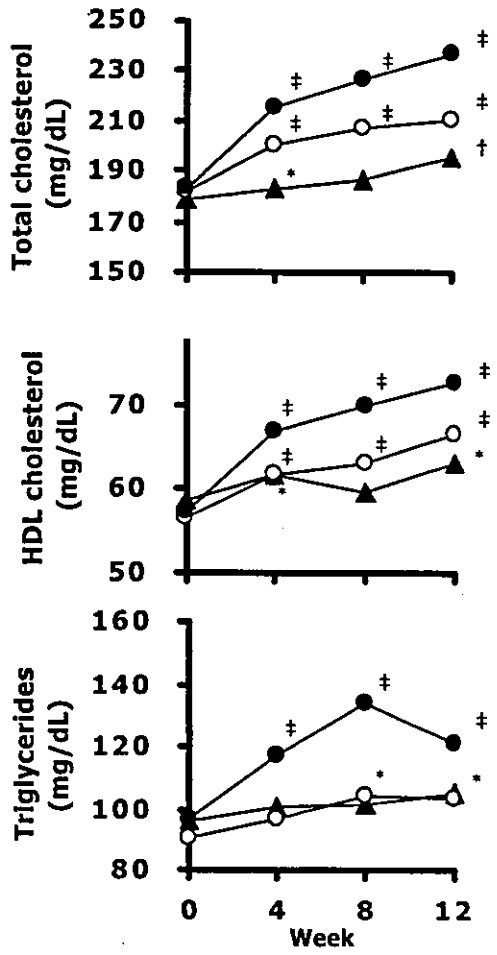

Figure 3. Changes from baseline in serum levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides in patients with rheumatoid arthritis. Solid triangles indicate placebo-treated group. Open circles indicate group treated with 4 mg/kg humanized anti–interleukin-6 receptor antibody (MRA). Solid circles indicate 8 mg/kg MRA–treated group. Each parameter was compared with its baseline value. Values are the mean for each group at each time point. * = $P < 0.05$; † = $P < 0.01$; ‡ = $P < 0.001$ versus baseline, by paired *t*-test.

109 patients (12.8%) in the MRA groups. An increase to grade 2 in alanine aminotransferase was observed in 2 patients, and the others had grade 1 according to the World Health Organization (WHO) guideline. An increase to grade 1 in aspartate aminotransferase was observed in 8 patients. These increases were transient and normalized with repeated administration of MRA. Decreases in white blood cell counts were observed in 17 of 109 patients (15.6%) (to grade 3 in 1 patient, to grade 2 in 5 patients, and to grade 1 in the other patients according to the WHO guideline). The decreases in white blood cell counts recovered without any treatment in all patients, mostly within 2 weeks. Only 1 patient stopped taking the study drug. There was no serious infection associated with transient neutropenia.

ANAs were positive in 69 of 109 patients (63.3%) at baseline. Eight patients became negative for ANAs at week 12 (according to the LOCF method), while 6 patients became positive. Anti-DNA antibodies were positive in 7 of 109 patients (6.4%) at baseline. Five patients became negative for anti-DNA antibodies at week 12 (according to the LOCF method), and only 1 patient became positive. Anti-MRA antibodies were detected in 2 patients who received MRA (1.8%), and although these patients were asymptomatic, they were withdrawn according to the study protocol.

DISCUSSION

This randomized, double-blind, placebo-controlled trial provided evidence for a rapid reduction in disease activity in response to MRA in patients with active RA. The efficacy was dose related, and 8 mg/kg of MRA provided marked clinical benefit. The success in the treatment of RA with MRA confirmed that IL-6 plays important pathologic roles in RA.

Recently, biologic agents targeting tumor necrosis factor (TNF) have been used successfully to treat RA (23–26). However, ~30% of patients failed to respond to the TNF inhibitors. Further study will be required to establish whether MRA is effective for those patients.

MRA showed benefit in some safety profiles. MRA did not induce anti-DNA antibodies, while anti-TNF therapy induced anti–double-stranded DNA antibodies in 16% of patients (23). The benefit of a humanized antibody was also demonstrated in the repetitive treatment, because anti-MRA antibodies were detected in <2% of MRA-treated patients without requiring the use of immunosuppressive agents such as methotrexate. Although serious infections were rare in repetitive treatment with MRA, 1 patient died of reactivation of chronic active EBV infection and consequent hemophagocytosis syndrome. After that event, we screened all patients who were receiving MRA in this and other studies (>200 patients) for plasma EBV DNA, and the patient who died was the only one with detectable EBV DNA in the plasma (data not shown).

We have detected EBV DNA in whole blood (including white blood cells) in some other patients; however, those patients have not experienced any severe adverse events during MRA treatment for more than 1 year in the extension study. Furthermore, blood EBV

DNA became undetectable during MRA treatment in some patients. Although we do not currently know the exact mechanism for the reactivation of chronic active EBV infection, we can exclude such a patient if we carefully examine eligibility, especially regarding existing infection. A long-term safety study is also required, since some serious infections associated with TNF inhibitors were reported after they became commercially available (27,28).

MRA markedly improved inflammation markers, such as CRP levels and ESRs, in a manner similar to that of TNF and IL-1 inhibitors (23,29,30). Specifically, MRA completely normalized CRP levels in 76% of patients in the 8-mg group. These results indicate that IL-6 is a major cytokine responsible for acute-phase protein production in RA. IL-6 was also proven to be a potent regulator of lipid metabolism in vivo. Dose-dependent increases in total cholesterol, HDL cholesterol, and triglycerides were observed and were above the normal range in some patients. This finding is concordant with previous reports that administration of recombinant IL-6 decreased serum cholesterol in cancer patients (31,32) and that IL-6–deficient mice showed an increase in triglycerides and very-low-density lipoprotein in the blood in association with suppressed energy expenditure and increased food intake, with no increase in HDL cholesterol (33). However, MRA treatment also increased HDL cholesterol. The precise mechanisms of this phenomenon are uncertain, but total cholesterol levels did not continue increasing, and no cardiovascular complications were observed. Therefore, the findings do not preclude further use of MRA for RA. Since RA patients reportedly have an increased risk of developing cardiovascular complications (34), we are examining the safety issue of cardiovascular complications after long-term treatment in the extension study.

A mild and transient decrease in white blood cell counts was sometimes observed in the MRA groups. A similar phenomenon was reported in clinical studies with other biologic agents such as anti-CD20 antibody (35) and anti-TNFα antibody (36). Therefore, the phenomenon is not specific to MRA treatment. Most of the liver function disorders observed in the MRA groups were also mild and transient. However, we should be careful when we use MRA in combination with methotrexate.

This clinical trial indicates an acceptable safety profile relative to the clinical benefit. Further studies are therefore required to determine the long-term safety and efficacy of MRA as well as the feasibility of preventing joint damage in RA patients.

ACKNOWLEDGMENTS

The authors wish to thank Takahiro Kakehi, BSc, Toru Suwabe, MSc, and Paul Langman, PhD, for their valuable assistance with the design and analysis of the study and preparation of the manuscript. The members of this study group were as follows: Takao Koike, MD (Hokkaido University, Hokkaido, Japan); Takeshi Sasaki, MD (Tohoku University, Miyagi, Japan); Yuichi Takahashi, MD (Tohoku Employees' Pension Welfare Hospital, Miyagi, Japan); Shu-ichi Ikeda, MD (Shinshu University, Nagano, Japan); Takayuki Sumida, MD (University of Tsukuba, Ibaraki, Japan); Junichi Masuyama, MD (Jichi Medical School, Tochigi, Japan); Michito Hirakata, MD (Keio University, Tokyo, Japan); Hiroshi Hashimoto, MD (Juntendo University, Tokyo, Japan); Naoyuki Kamatani, MD (Tokyo Women's Medical University, Tokyo, Japan); Shunsei Hirohata, MD (Teikyo University, Tokyo, Japan); Shigemasa Sawada, MD (Nerima Hikarigaoka Nihon University Hospital, Tokyo, Japan); Hirobumi Kondo, MD (Kitasato University, Kanagawa, Japan); Shoichi Ozaki, MD (St. Marianna University, Kanagawa, Japan); Eiji Sugiyama, MD (Toyama Medical and Pharmaceutical University, Toyama, Japan); Tsuneyo Mimori, MD (Kyoto University, Kyoto, Japan); Taro Kuritani, MD (NTT West Osaka Hospital, Osaka, Japan); Masaki Suemura, MD (Nissay Hospital, Osaka, Japan); Nobuaki Nakano, MD (Kyowakai Hospital, Osaka, Japan); Teruaki Hamano, MD (Hyogo College of Medicine, Hyogo, Japan); Yasuhiko Yoshinaga, MD (National Sanatorium Minami-Okayama Hospital, Okayama, Japan); Seizo Yamana, MD (Higashi-Hiroshima Memorial Hospital, Hiroshima, Japan); Kiyoshi Takasugi, MD (Dohgo Spa Hospital, Ehime, Japan); Kazuyoshi Saito, MD (University of Occupational and Environmental Health, Fukuoka, Japan); Katsumi Eguchi, MD (Nagasaki University, Nagasaki, Japan); Takemasa Matsuda, MD (Kagoshima Red Cross Hospital, Kagoshima, Japan).

REFERENCES

1. Harris ED Jr. Rheumatoid arthritis: pathophysiology and implications for therapy. N Engl J Med 1990;322:1277–89.
2. Nishimoto N, Kishimoto T, Yoshizaki K. Anti-interleukin 6 receptor antibody treatment in rheumatic disease. Ann Rheum Dis 2000;59:121–7.
3. Feldmann M, Brennan FM, Foxwell BM, Maini RN. The role of TNFα and IL-1 in rheumatoid arthritis. Curr Dir Autoimmun 2001;3:188–99.
4. Guerne PA, Zuraw BL, Vaughan JH, Carson DA, Lotz M. Synovium as a source of interleukin 6 in vitro: contribution to local and systemic manifestations of arthritis. J Clin Invest 1989;83:585–92.
5. Hirano T, Matsuda T, Turner M, Miyasaka N, Buchan G, Tang B, et al. Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. Eur J Immunol 1988;18:1797–801.
6. Houssiau FA, Devogelaer JP, van Damme J, de Deuxchaisnes CN, van Snick J. Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides. Arthritis Rheum 1988;31:784–8.
7. Dasgupta B, Corkill M, Kirkham B, Gibson T, Panayi G. Serial estimation of interleukin 6 as a measure of systemic disease in rheumatoid arthritis. J Rheumatol 1992;19:22–5.
8. Madhok R, Crilly A, Watson J, Capell HA. Serum interleukin 6

levels in rheumatoid arthritis: correlations with clinical and laboratory indices of disease activity. Ann Rheum Dis 1993;52:232–4.
9. Sack U, Kinne RW, Marx T, Heppt P, Bender S, Emmrich F. Interleukin-6 in synovial fluid is closely associated with chronic synovitis in rheumatoid arthritis. Rheumatol Int 1993;13:45–51.
10. Uson J, Balsa A, Pascual-Salcedo D, Cabezas JA, Gonzalez-Tarrio JM, Martin-Mola E, et al. Soluble interleukin 6 (IL-6) receptor and IL-6 levels in serum and synovial fluid of patients with different arthropathies. J Rheumatol 1997;24:2069–75.
11. Kotake S, Sato K, Kim KJ, Takahashi N, Udagawa N, Nakamura I, et al. Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. J Bone Miner Res 1996;11: 88–95.
12. Wendling D, Racadot E, Wijdenes J. Treatment of severe rheumatoid arthritis by anti-interleukin 6 monoclonal antibody. J Rheumatol 1993;20:259–62.
13. Sato K, Tsuchiya M, Saldanha J, Koishihara Y, Ohsugi Y, Kishimoto T, et al. Reshaping a human antibody to inhibit the interleukin 6-dependent tumor cell growth. Cancer Res 1993;53: 851–6.
14. Choy EH, Isenberg DA, Garrood T, Farrow S, Ioannou Y, Bird H, et al. Therapeutic benefit of blocking interleukin-6 activity with an anti–interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Arthritis Rheum 2002;46:3143–50.
15. Nishimoto N, Maeda K, Kuritani T, Deguchi H, Sato B, Imai N, et al. Toxicity, pharmacokinetics, and dose finding study of repetitive treatment with the humanized anti-interleukin 6 receptor antibody, MRA, in rheumatoid arthritis. Phase I/II clinical study. J Rheumatol 2003;30:1426–35.
16. 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.
17. Hochberg MC, Chang RW, Dwosh I, Lindsey S, Pincus T, Wolfe F. The American College of Rheumatology 1991 revised criteria for the classification of global functional status in rheumatoid arthritis. Arthritis Rheum 1992;35:498–502.
18. 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.
19. Felson DT, Anderson JJ, Boers M, Bombardier C, Furst D, Goldsmith C, et al. American College of Rheumatology preliminary definition of improvement in rheumatoid arthritis. Arthritis Rheum 1995;38:727–35.
20. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.
21. Felson DT, Anderson JJ, Boers M, Bombardier C, Chernoff M, Fried B, et al. The American College of Rheumatology preliminary core set of disease activity measures for rheumatoid arthritis clinical trials. Arthritis Rheum 1993;36:729–40.
22. Van Gestel AM, Prevoo ML, van 't Hof MA, van Rijswijk MH, van de Putte LB, van Riel PL. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis: comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism criteria. Arthritis Rheum 1996;39:34–40.
23. Maini R, St Clair EW, Breedveld F, Furst D, Kalden J, Weisman M, et al. Infliximab (chimeric anti-tumour necrosis factor α monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. Lancet 1999;354:1932–9.
24. Moreland LW, Schiff MH, Baumgartner SW, Tindall EA, Fleischmann RM, Bulpitt KJ, et al. Etanercept therapy in rheumatoid arthritis. Ann Intern Med 1999;130:478–86.
25. Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, et al. Infliximab and methotrexate in the treatment of rheumatoid arthritis. N Engl J Med 2000;343:1594–602.
26. Bathon JM, Martin RW, Fleischmann RM, Tesser JR, Schiff MH, Keystone EC, et al. A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis. N Engl J Med 2000; 343:1586–93.
27. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor α-neutralizing agent. N Engl J Med 2001;345:1098–104.
28. Lee JH, Slifman NR, Gershon SK, Edwards ET, Schwieterman WD, Siegel JN, et al. Life-threatening histoplasmosis complicating immunotherapy with tumor necrosis factor α antagonists infliximab and etanercept. Arthritis Rheum 2002;46:2565–70.
29. Charles P, Elliott MJ, Davis D, Potter A, Kalden JR, Antoni C, et al. Regulation of cytokines, cytokine inhibitors, and acute-phase proteins following anti-TNF-α therapy in rheumatoid arthritis. J Immunol 1999;163:1521–8.
30. Bresnihan B, Alvaro-Gracia JM, Cobby M, Doherty M, Domljan Z, Emery P, et al. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. Arthritis Rheum 1998;41:2196–204.
31. Weber J, Yang JC, Topalian SL, Parkinson DR, Schwartzentruber DS, Ettinghausen SE, et al. Phase I trial of subcutaneous interleukin-6 in patients with advanced malignancies. J Clin Oncol 1993;11:499–506.
32. Van Gameren MM, Willemse PH, Mulder NH, Limburg PC, Groen HJ, Vellenga E, et al. Effects of recombinant human interleukin-6 in cancer patients: a phase I-II study. Blood 1994;84: 1434–41.
33. Wallenius V, Wallenius K, Ahren B, Rudling M, Carlsten H, Dickson SL, et al. Interleukin-6-deficient mice develop mature-onset obesity. Nat Med 2002;8:75–9.
34. Del Rincon I, Williams K, Stern MP, Freeman GL, Escalante A. High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. Arthritis Rheum 2001;44:2737–45.
35. Tobinai K, Kobayashi Y, Narabayashi M, Ogura M, Kagami Y, Morishima Y, et al, the IDEC-C2B8 Study Group. Feasibility and pharmacokinetic study of a chimeric anti-CD20 monoclonal antibody (IDEC-C2B8, rituximab) in relapsed B-cell lymphoma. Ann Oncol 1998;9:527–34.
36. Paleolog EM, Hunt M, Elliott MJ, Feldmann M, Maini RN, Woody JN. Deactivation of vascular endothelium by monoclonal anti–tumor necrosis factor α antibody in rheumatoid arthritis. Arthritis Rheum 1996;39:1082–91.

J Am Soc Nephrol 13: 2488–2496, 2002

Human Platelets Stimulate Mesangial Cells to Produce Monocyte Chemoattractant Protein-1 via the CD40/CD40 Ligand Pathway and May Amplify Glomerular Injury

TAKAHARU TANAKA,* TAKASHI KUROIWA,* HIDEKAZU IKEUCHI,* FUMIE OTA,* YORIAKI KANEKO,* KAZUE UEKI,* YOSHITO TSUKADA,* IAIN B. MCINNES,† DIMITRIOS T. BOUMPAS,‡ and YOSHIHISA NOJIMA*

**Third Department of Internal Medicine, Gunma University School of Medicine, Maebashi, Gunma, Japan; †Centre for Rheumatic Diseases, Glasgow Royal Infirmary, University of Glasgow, Glasgow, Scotland; ‡Divisions of Internal Medicine, Rheumatology, Clinical Immunology, and Allergy, University of Crete Medical School, Heraklion, Greece.*

Abstract. Platelets are thought to play an important role in the initiation and the progression of a variety of glomerulonephritides. This study examined whether platelets induce production of monocyte chemoattractant protein-1 (MCP-1), a chemokine involved in leukocyte recruitment and glomerular injury, by cultured human mesangial cells (MC). To this end, platelets isolated from normal human donors were cocultured with MC at various ratios. MCP-1 synthesis was evaluated by quantitative real-time PCR and enzyme-linked immunosorbent assay. Platelets at 1:100 ratio (MC to platelets) induced an approximately 20-fold increase in mesangial MCP-1 mRNA and protein expression through an obligatory cell-to-cell contact–dependent mechanism. Importantly, blockade of the CD40/CD40 ligand (CD40L) pathway with neutralizing antibodies decreased MCP-1 production by approximately 60%. It was confirmed that CD40 was functionally expressed on MC. Gel-shift assays and inhibitors of phosphorylation were used to demonstrate that activation of p38 mitogen-activated protein kinase, protein tyrosine kinases, and nuclear factor–κB activation were essential for MCP-1 production. These data indicate that platelet/MC contact stimulates the production of MCP-1 and may contribute to glomerular inflammatory responses by recruiting leukocytes from the peripheral blood.

Platelets are thought to play an important role in the initiation and the progression of glomerular injury in a variety of glomerulonephritides (GN) (1). Platelets are among the first cells to infiltrate the glomeruli, as shown in a variety of animal models, including Habu snake venom–induced proliferative GN (2), experimental diabetic nephropathy (3), or immune complex nephritis (4,5). Improvement of renal function and reduced glomerular cell proliferation was observed in immune complex nephritis (4) and a model of diabetic nephropathy (3) after treatment with anti-platelet antibodies. Depletion of platelets decreased the release of inflammatory mediators in acute nephrotoxic serum nephritis in rats (6). In humans, circumstantial data, such as decreased platelet survival and increased renal platelet sequestration, suggest a role of platelets in the pathogenesis of diffuse proliferative lupus GN (7). *In vitro* and *in vivo* studies have shown that growth factors found in abundance in platelets, such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), induce mesangial proliferation and matrix accumulation respectively that are observed in the course of glomerular injury (8). Antiplatelet agents improve long-term renal function in membranoproliferative GN (9).

In addition to soluble factors, platelets may also mediate glomerular injury by direct cell contact with glomerular mesangial cells (MC). Structural characteristics in glomeruli, such as defects of glomerular basement membrane between MC and blood lumen and porous glomerular endothelial cells, render direct contact between platelets and MC possible. In addition, injured glomerular endothelial cells in GN may further facilitate direct contact between platelets and MC. Coculture experiments have shown that contact with platelets induces chemokine production by vascular endothelial cells or myeloid leukocytes (10–12). Similar interactions between platelets and MC could therefore feasibly promote glomerular inflammation.

A recent study (13) has identified that platelets express CD40 ligand (CD40L) on their surface. CD40L, a transmembrane protein structurally related to tumor necrosis factor–α (TNF-α), was originally identified on activated T cells. Interaction of CD40L on T cells with CD40 on B cells is of paramount importance for the development and function of the humoral immune system (14). CD40 is also found on monocytes, macrophages, and endothelial cells, suggesting that

Received December 6, 2001. Accepted June 14, 2002.
Correspondence to Dr. Takashi Kuroiwa, Third Department of Internal Medicine, Gunma University School of Medicine, 3-39-15 Showa, Maebashi, Gunma 371-8500, Japan. Phone: 81-27-220-8166; Fax: 81-27-220-8173; E-mail: tkuroiwa@med.gunma-u.ac.jp
1046-6673/1310-2488
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000029588.07166.20

CD40L has a broader immunologic function *in vivo* (15). Upregulation of CD40 on MC has been observed in renal biopsies of patients with inflammatory glomerulonephritides such as lupus nephritis and IgA nephropathy (16). CD40/CD40L interactions between infiltrating mononuclear cells and resident renal cells are thought to play an important role in the pathogenesis in immune-mediated glomerulonephritis (17). Interestingly, CD40L-dependent platelet/endothelial cell interactions may induce the latter to secrete chemokines and express adhesion molecules (13). Similar platelet/MC interactions have not previously been explored.

Monocyte chemoattractant protein-1 (MCP-1) plays a crucial role in the pathogenesis of immune-mediated glomerulonephritides. Cultured renal parenchymal cells, including MC and renal tubular epithelial cells, produce MCP-1 in response to proinflammatory cytokines (18–20). Inhibition of MCP-1 by neutralizing antibodies attenuates macrophage influx in various experimental nephritides, decreases histologic glomerular damage, and reduces proteinuria (21–23). MCP-1–deficient lupus-prone mice demonstrate improved survival and a dramatic reduction in macrophage recruitment, renal pathology, and proteinuria. Notably renal Ig/C3 deposits are not diminished in MCP-1–deficient lupus-prone mice, suggesting an uncoupling of the inflammatory responses from deposition and activation of immune reactants (24).

We used coculture experiments and inhibitors of phosphorylation in this study to address whether platelets induce MCP-1 production by cultured MC and to identify the mechanisms involved in this process. We now report that platelets induce MCP-1 production in part through the CD40/CD40L pathway and identify key molecules involved in intracellular signaling pathways in MC.

Materials and Methods

Antibodies and Cytokines

The following Abs, recombinant cytokines, and proteins were used in this experiment: mouse monoclonal anti-human CD154 (CD40L) (Ancell, Baypor, MN); mouse monoclonal anti-human CD62P (P-selectin) (R&D systems, Minneapolis, MN); mouse monoclonal anti-β1-integrin (clone M13, kindly provided by Dr. Kenneth Yamada, NIDR, NIH, Bethesda, MD); mouse monoclonal anti-human CD40 (Biosource, Camarillo, CA); biotin-conjugated goat anti-mouse immunoglobulins; R-PE-conjugated goat anti-mouse immunoglobulins (Dako, Carpinteria, CA); recombinant human TNF-α (rTNF-α); rIFN-γ (both from R&D systems); and trimeric human CD40L/leucine-zipper fusion protein (rCD40L) (a gift from Immunex, Seattle, WA).

Preparation of Platelets

Platelets were carefully isolated as described elsewhere with slight modification (25). In brief, platelet-rich plasma (PRP) was isolated from freshly drawn citrated whole blood after centrifugation (200 × *g* for 20 min). PRP was recentrifuged (500 × *g* for 20 min) in the presence of acid-citrate-dextrose (ACD; 15% vol/vol). The supernatant was discarded, and Tris-EDTA saline (10 mM Tris, 1 mM EDTA, and 150 mM NaCl) was used to resuspend the platelet pellet. Platelet suspension was centrifuged (500 × *g* for 20 min), the supernatant was discarded, and the platelet pellet was resuspended in phosphate-buffered saline (PBS). For the experiment that used the pre-stimulated platelets (Figure 1B and 3), platelets were activated with 0.2 U/ml of human thrombin (Sigma, St. Louis, MO) or 1 μM of ADP (Sigma) for 10 min, washed twice with PBS, and then resuspended in PBS. In other experiments (Figure 3B), platelets were fixed for 10 min in 1% paraformaldehyde (Sigma) in PBS at 4°C with gentle agitation; this preserves membrane integrity but prevents secretory activity. To test the isolation-induced activation of platelets, P-selectin expression was examined by FACS analysis and found to be less than 5% (data not shown). In contrast, after the stimulation with thrombin or ADP, more than 90% of cells were positive for P-selectin. Contamination of leukocytes was assessed microscopically and found to be negligible.

Human Mesangial Cells (MC)

Human MC were established and characterized as reported previously (26). Cells were cultured in Waymouth medium (Life Technologies, Grand Island, NY) supplemented with 17% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomy-

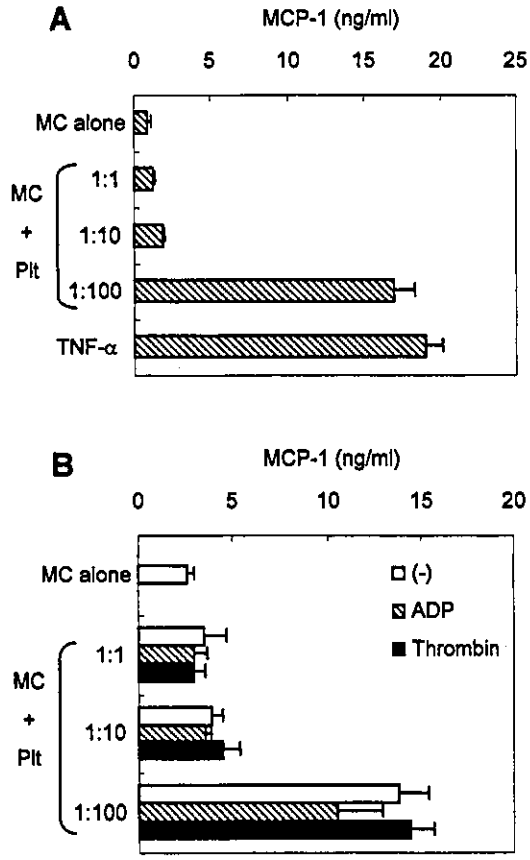

Figure 1. (A) Platelets induce monocyte chemoattractant protein-1 (MCP-1) upregulation by mesangial cells (MC) at the ratio of 1:100. (B) Prestimulation of platelets with either thrombin or ADP is not required. In panel A, platelets were isolated from normal human donors and cocultured with MC at various ratios for 24 h. Alternatively, MC were stimulated with 10 ng/ml of tumor necrosis factor–α (TNF-α) for 24 h (A). In panel B, platelets were pre-stimulated with 0.2 U/ml of thrombin or 1 μM ADP, washed, and then cocultured with MC. MCP-1 production in supernatants was determined by enzyme-linked immunosorbent assay (ELISA). Data are means ± SE from three separate experiments.

cin, 2 mM L-glutamine, 2 mM sodium pyruvate, 1% (vol/vol) nonessential amino acids, and 26 μg/ml of bovine insulin (all Life Technologies). Three independent cell lines were employed during passages 5 through 12.

Platelet/MC Coculture

Platelets were prepared to 1, 10, or 100 × 10^5 /ml in Waymouth medium containing 1% FBS. MC were harvested at 80% confluence and cultured in 24-well plates at 6 × 10^4 cells/0.6 ml per well in 1% FBS/Waymouth medium in duplicate for 16 h. Subsequently prepared 0.6 ml of platelets was added to 6 × 10^4 of MC, resulting in 1:1, 1:10, or 1:100 ratio of coculture (MC:platelets). In the experiments examining the effect of cell contact, identical parallel cultures were established in which platelets were separated from MC by a 0.4-μm pore size semipermeable membrane (Biocoat, Becton Dickinson Labware, Bedford, MA) while sharing the same medium. In the experiments, which examined the effect of rTNF-α, rIFN-γ, and rCD40L, MC were thawed in a 24-well plate at the same concentration with coculture experiment for 16 h for the adherence. Then, MC were further incubated at final volume of 1.2 ml/well in the presence or absence of various cytokines for 24 h. After 24-h incubation at 37°C, MCP-1 synthesis in supernatants and MC CD40 expression were determined by enzyme-linked immunosorbent assay (ELISA, R&D systems) and FACS analysis, respectively.

Inhibition of Chemokine Synthesis by Antibodies or Chemical Inhibitors

In the blocking experiment with neutralizing Abs, coculture of MC and unstimulated platelets was carried out at the ratio of 1:100 (MC:platelets) in the presence or absence of 10 μg/ml Abs against P-selectin, β1-integrin, CD40L, or IgG_1 of irrelevant specificity (Sigma). All antibodies were added to MC simultaneously with platelets. In the experiments that used pyrolidine derivative of dithiocarbamate (PDTC, Sigma), SB203580, PD98059 (both from Calbiochem-Novabiochem, La Jolla, CA), and genistein (Sigma), MC were preincubated with those inhibitors for 2 h, and then washed four times with medium to remove added inhibitors completely. Coculture of MC and unstimulated platelets were subsequently performed at 1:100 ratio for 24 h. MCP-1 synthesis was determined by ELISA in the supernatants after 24 h.

Quantitative Real-Time PCR

MCP-1 mRNA expression in coculture was determined by real-time PCR, following the manufacture's protocol. Briefly, total RNA was isolated from coculture of MC and unstimulated platelets (1:100 ratio) in 6-well plates after a variety time of incubation using RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was synthesized from 2 μg of total RNA by Superscript preamplification system for first strand cDNA system (Life Technologies), using oligo-dT primers. Subsequently, real-time PCR was performed in the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Systems, Foster City, CA) using the SYBR Green I PCR kit (Perkin Elmer Applied Systems). Each reaction contained 25 μl of the 2× SYBR green Master Mix, 300 nM primers (MCP-1, forward: 5'-GAT CTC AGT GCA GAG GCT CG-3', reverse: 5'-TGC TTG TCC AGG TGG TCC AT-3'; β-actin, forward: 5'-GAA CTT TGG GGG ATG CTC GC-3', reverse: 5'-CGG GAA ATC GTG CGT GAC AT-3') (27), 5 μl of a 1:10 dilution of the cDNA prepared above, and water to 50 μl. The reactions were incubated at 94°C for 10 min to activate the Amplitaq Gold polymerase (Perkin Elmer Applied Systems) followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C. The ABI Prism 7700 Sequence Detection System software determined relative mRNA expression of MCP-1 and β-actin in each samples, based on the standard curve described below. MCP-1 mRNA expression in each sample were finally determined after correction with β-actin expression. Standard curve was generated as follows (28). In brief, cDNA was prepared from MC stimulated with 100 ng/ml of LPS (Sigma) for 8 h. Using this cDNA as template, PCR products for MCP-1 and β-actin were prepared with same primers. Each reaction contained 5 μl of the 10× PCR buffer; 500 nM forward and reverse primers; 0.5 μl of *Taq* Gold polymerase; 1 μl of the cDNA; and water to 50 μl. The reactions were incubated at 94°C for 10 min to activate the Amplitaq Gold polymerase followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s 72°C. After confirming specific single band on the agarose gels, serial dilutions (tenfold) of these PCR products (10^{-5}~10^{-9}) were prepared and then amplified simultaneously with samples from coculture using SYBR green. A standard curve was determined by the ABI Prism 7700 Sequence Detection System software. The expression of MCP-1 and β-actin in each sample was quantitated in separate wells with the respective primers. No PCR products were detected in the real-time PCR procedure without reverse transcription, indicating that the contamination of genomic DNA was negligible. Gels of the PCR products after quantification of MCP-1 or β-actin by real-time PCR showed a single band (152 and 711 bp, respectively) with the expected size (data not shown).

Flow Cytometry

CD40 expression on MC was determined by FACS analysis. Briefly, MC were harvested with trypsin/EDTA and stained for CD40 expression by incubating the cells on ice with anti-human CD40 antibody or an isotype-matched control, followed by R-PE–conjugated goat anti-mouse immunoglobulins. CD40 expression was analyzed using CELLQUEST (Becton Dickinson).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were isolated by the methods described previously with slight modification (29). Briefly, cocultures of MC and unstimulated platelets were performed at the ratio of 1:100 in 75-cm^2 flask for 1 h. Alternatively, MC were incubated for 1 h with 10 ng/ml of TNF-α or 100 ng/ml of LPS (Sigma) instead of platelets. After the treatment, cells were washed with ice-cold PBS, harvested by scraping, then spun-down by centrifugation. Pellets were resuspended in 1 ml of hypotonic buffer (10 mM Hepes, pH 7.9, containing 10 mM NaCl, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). After 15-min incubation on ice, 60 μl of 10% NP-40 was added. Cells were then vortexed vigorously for 15 s and then centrifuged for 1 min at 12,000 rpm at 4°C. After removing supernatants, the pellets were resuspended in 500 μl of extraction buffer (20 mM Hepes, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). After incubation for 15 min on ice, samples were vortexed vigorously for 15 s and then centrifuged at 12,000 rpm for 10 min. The supernatants containing nuclear protein were used for EMSA after determining protein concentration with the Brad-ford method (BioRad Protein Assay kit; BioRad Laboratories, Hercules, CA). EMSA of nuclear factor-κB (NF-κB) was carried out by use of Gel Shift Assay Systems (Promega, Madison, WI), following manufacture's protocol. Briefly, double-stranded oligonucleotide containing the NF-κB binding element (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was end-labeled using [γ−32P]ATP (NEN Life Science Products, Boston, MA) and T4 polynucleotide kinase and then purified through G-25 spin columns (BioRad). Nuclear extracts (2 μg of protein) were incubated with radiolabeled probes for 20 min

at room temperature in 10 μl of binding buffer (10 mM Tris-HCL, pH 7.5, containing 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 M DTT, 0.05 mg/ml poly (dI-dC)(dI-dC), 4% glycerol). Specific controls included unlabeled NF-κB or Sp-1 (5'-ATT CGA TCG GGG CGG GGC GAG C-3') consensus oligonucleotides at 100-fold excess. The samples were separated on a nondenaturing 4% polyacrylamide gel in Tris-Borate buffer, dried, and then analyzed by autoradiography.

Statistical Analysis

All experiments were repeated at least three times. Results are presented as the mean ± SEM from three separate experiments where indicated. Statistical significance, where indicated, was determined by *t* test. A value of $P < 0.05$ was considered to represent a statistically significant difference between two groups.

Results

Platelets Induce Upregulation of MCP-1 Production by MC

We first examined whether platelets induce MCP-1 production by MC. Platelets purified from normal human subjects were cocultured for 24 h with MC at various ratios. Platelets, at 1:100 ratio (MC:platelets), enhanced MCP-1 production by 18-fold above the basal level released by MC alone (Figure 1A). This was comparable to levels induced by recombinant TNF-α. We did not observe further increases in MCP-1 production by higher ratios up to 1:500 (data not shown).

We next examined the effect of prestimulation of platelets with ADP or thrombin. Platelets were isolated and stimulated with thrombin or ADP for 10 min. Activation of platelets was verified by the induction of P-selectin expression (data not shown). After washing, platelets were cocultured with MC at various ratios as before. Pretreatment of platelets with thrombin or ADP did not further enhance the expression of MCP-1 (Figure 1B), indicating that specific prior activation of platelets was not required. Stimulated platelets alone did not synthesize MCP-1 (data not shown).

We next determined whether the upregulation of MCP-1 production occurred at the transcriptional level. Platelets (without prior stimulation) were cocultured with MC at the ratio of 1:100. At various time points, cells were harvested and mRNA was extracted and quantified by real-time PCR. Unstimulated MC expressed low levels of basal MCP-1 mRNA. Platelets enhanced MCP-1 mRNA expression 22-fold at 6 h, which was sustained up to 12 h (Figure 2). Taken together, these data demonstrate that platelets induce MCP-1 upregulation by MC at both protein and mRNA levels. The magnitude of this response could not be further enhanced by specific prior *in vitro* platelet stimulation. This may have reflected activation of critical pathways during platelet isolation. This could have varied between donors, we therefore elected to stimulate platelets (judged by P-selectin expression) in subsequent mechanistic experiments to ensure maximal stimulation had been achieved in each experiment.

Direct Cell-to-Cell Contact between Platelets and MC Is Essential for MCP-1 Production

Stimulation of MC by platelets could be mediated either by direct cell-to-cell contact or through soluble factors. We therefore determined whether platelets could induce MC MCP-1 release when separated by a semipermeable membrane that prevents direct cell-to-cell contact. Platelets were incubated with thrombin for 10 min and then cocultured with MC for 24 h at a 1:100 ratio in contact with or separated from MC. As shown in Figure 3A, separation of platelets from MC abrogated MCP-1 upregulation by MC, suggesting a role for cell-surface molecules. ADP-stimulated platelets also failed to induce MCP-1 when separated from MC (data not shown). These data implicate direct cell-to-cell contact in platelet-mediated MCP-1 upregulation in MC.

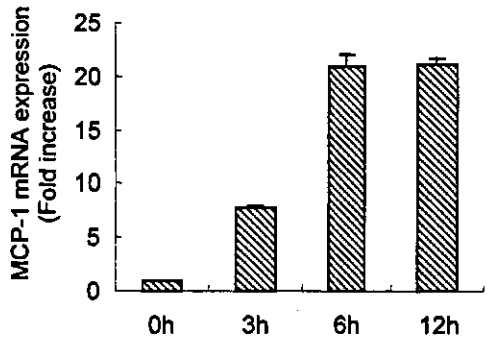

Figure 2. Platelets induce upregulation of MCP-1 mRNA by MC. MC were cocultured with platelets at the ratio of 1:100 for various times, and then MCP-1 and β-actin mRNA expressions were analyzed by real-time PCR. Fold increase in MCP-1 expression at each time point, relative to 0 h, is shown after correction with β-actin expression. Data are means ± SE from three separate experiments.

To address this further, platelets activated with thrombin were fixed with PFA to preserve membrane integrity but prevent secretion of soluble factors. Fixed, activated platelets were cocultured with MC at the ratio of 1:100 for 24 h. Figure 3B demonstrates that fixed activated platelets did not induce MCP-1 upregulation. Fixed activated platelets failed to secrete MCP-1 production, even at the ratio of 1:500 (data not shown). This suggests that cell-surface molecules alone are not sufficient and that soluble factors from activated platelets are also required for MCP-1 synthesis by MC.

CD40/CD40L Pathway is Involved in Platelet-Mediated MCP-1 Production by MC

We next sought to identify molecules involved in platelet/MC interactions. P-selectin plays a crucial role in leukocyte or endothelial cell activation by platelet binding (11,12), and β1-integrins are implicated in platelet interactions with extracellular matrix (30). Activated platelets also express surface CD40L through which platelets can induce proinflammatory responses by endothelial cells (13). We therefore examined the effect of neutralizing P-selectin, β1-integrins, and CD40L in coculture experiments. Platelets were cocultured with MC at the ratio of 1:100 in the presence or absence of antibodies for 24 h. Inhibition of CD40/CD40L reduced MCP-1 induction by approximately 60%, whereas blocking of P-selectin or β1-integrins did not significantly affect the results (Figure 4). Although immunohistochemical analysis has shown that CD40

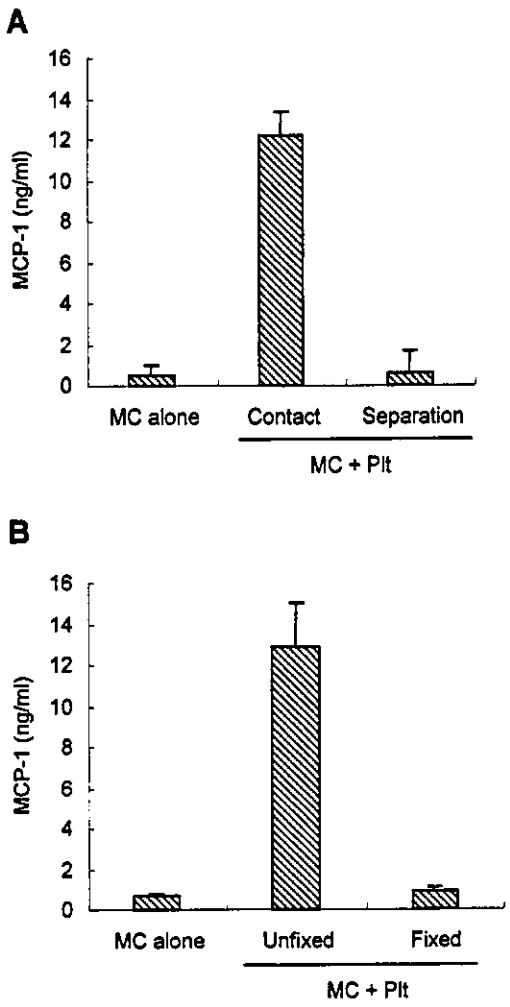

Figure 3. (A) Direct cell-to-cell contact between platelets and MC is essential for MCP-1 upregulation. (B) Fixed activated platelets do not upregulate MCP-1 production by MC. In panel A, MC were cocultured in contact with or separated by a porous membrane insert from pre-stimulated platelets at 1:100 ratio for 24 h. In panel B, platelets were stimulated with thrombin and fixed with 1% PFA to preserve membrane integrity but prevent secretion of soluble factors. MC were subsequently cocultured with fixed or unfixed activated platelets at 1:100 ratio for 24 h. MCP-1 production in supernatants was determined by ELISA. Data are means ± SE from three separate experiments.

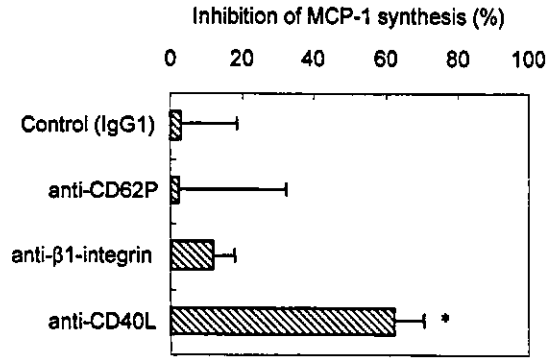

Figure 4. Inhibition of MCP-1 synthesis in MC/platelet coculture by neutralizing Abs. MC were cocultured with platelets at 1:100 ratio for 24 h in the presence or absence of neutralizing Abs for 24 h. Percent inhibition was calculated as follows: $100 - 100 \times$ (MCP-1 synthesis with neutralizing Ab/MCP-1 synthesis without neutralizing Ab). Data are means ± SE from three separate experiments. * Satistically significant difference as compared with control ($P < 0.01$).

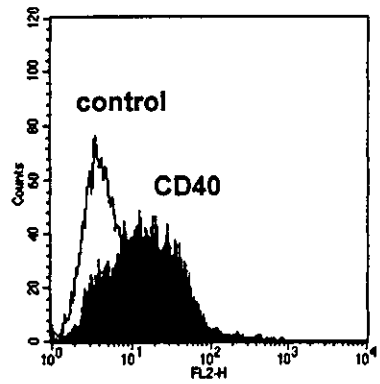

Figure 5. MC constitutively express CD40. MC were stained with anti-human CD40 antibody or an isotype-matched control, followed by R-PE-conjugated goat anti-mouse immunoglobulins. CD40 expression was determined by FACS analysis. Representative data of three distinct experiments are shown. The left-hand histogram represents the staining with control Abs.

is expressed on MC in renal biopsy from patients with a variety of glomerulonephritides (16), its expression on cultured MC has not been identified. Therefore we examined CD40 expression on MC by FACS analysis, and we found that MC constitutively expressed CD40 (Figure 5). In addition, inflammatory cytokines, such as TNF-α and interferon-γ (IFN-γ), or coculturing with platelets at the ratio of 1:100 increased CD40 expression (Table 1). To further examine the role of CD40 for MC activation, we stimulated MC with recombinant CD40L (rCD40L). MC were stimulated with 3 μg/ml of rCD40L for 24 h in the presence or absence of 500 U/ml of IFN-γ, which is known to function synergistically with CD40L in the monocyte/macrophage activation (15). In preliminary studies, the optimal concentrations of rCD40L and IFN-γ were determined. Although rCD40L alone did not upregulate MCP-1, rCD40L in combination with IFN-γ enhanced MCP-1 synthesis by twofold above basal levels in response to IFN-γ alone (Figure 6). These data suggest that CD40 is expressed on MC and is involved in their activation by platelets to release MCP-1.

Platelets Induce Mesangial MCP-1 Upregulation through NF-κB Activation

MCP-1 expression is regulated both in a stimulus-specific and a tissue-specific manner (31). NF-κB plays an essential role for MCP-1 upregulation (32). We performed gel-shift assays to examine the molecular mechanisms by which platelets activate MC. Platelets significantly enhanced NF-κB activation above basal levels detected in resting MC (Figure 7A). Binding was inhibited by adding cold probe against NF-κB,

Table 1. Effect of cytokines or coculture with platelets on MC CD40 expression[a]

Stimuli	CD40 (MFI)
Medium	21.0
rTNF-α (10 ng/ml)	51.1
rIFN-γ (500 U/ml)	43.3
Platelets (MC:platelets = 1:100)	119.7

[a] Shown is the MFI of CD40 expression on mesangial cells (MC) after culturing for 24 h in the presence or absence of rTNF-α, rIFN-γ, or a 100 excess of platelets. Similar results were obtained from three different experiments.

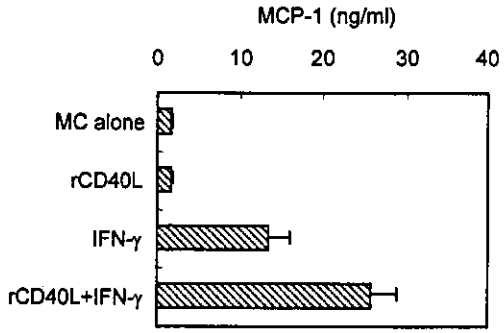

Figure 6. rCD40L enhances MCP-1 production by MC in the presence of interferon-γ (IFN-γ). MC were cultured to adherence at 6×10^4 cells/0.6 ml per well in 24-well plates in duplicate for 16 h. Cells were subsequently stimulated with rCD40L (3 μg/ml), IFN-γ (500 U/ml), or both. After 24 h, MCP-1 synthesis was determined by ELISA. Data are mean ± SE from three separate experiments.

but not against SP-1, indicating specificity. To further investigate the involvement of NF-κB activation, we used PDTC, a specific inhibitor of NF-κB. PDTC reduced MCP-1 upregulation in a dose-dependent manner (Figure 7B). Collectively these data demonstrate that platelet contact induce NF-κB activation in MC, which in turn is essential for MCP-1 synthesis.

Platelets Induce MCP-1 Production through p38 MAPK and PTK

Previous studies have shown that p38 MAPK activation is required in cytokine-induced MCP-1 expression (33,34). Similarly TNF-α or interleukin-1 (IL-1) induces MCP-1 production through protein tyrosine kinases (PTK), but not protein kinase C (PKC) or cAMP-protein kinase A (PKA) (35,36). To examine whether platelets induce MCP-1 synthesis by MC through activation of p38 MAPK and/or PTK, we used SB203580 or genistein, which are inhibitors of p38MAPK or PTK, respectively. As a control we used PD98059, an inhibitor of ERK1/2. MC were preincubated with these inhibitors for 2 h and then washed extensively with medium. In preliminary studies, the concentrations of inhibitors to achieve the maximal effects were established. Treatment with those inhibitors did not alter the basal expression of MCP-1 by MC. Platelets were

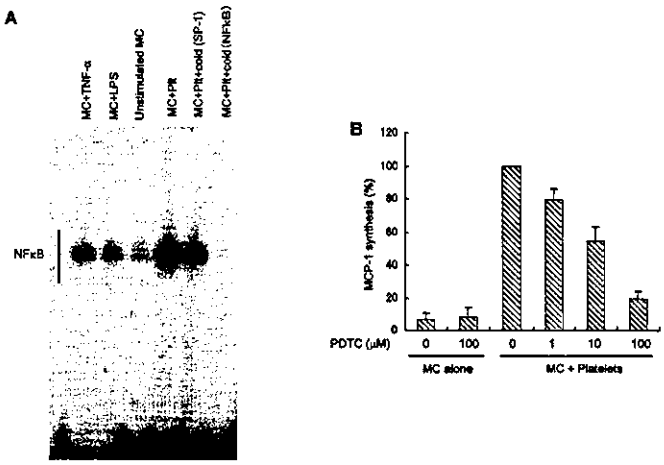

Figure 7. (A) Platelet contact induces nuclear factor–κB (NF-κB) activation by MC. (B) PDTC, the specific inhibitor of NF-κB, reduced MCP-1 production in coculture in a dose-dependent manner. In panel A) MC were cocultured with platelets at 1:100 ratio for 1 h. Alternatively, MC were incubated in the presence or absence of LPS (100 ng/ml) or TNF-α (10 ng/ml) for 1 h. Nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) to examine NF-κB activation. Specificity was checked with an unlabeled probe (cold) against NF-κB or Sp-1. In panel B, MC pretreated with various concentrations of PDTC were cocultured with platelets at the ratio of 1:100 for 24 h. MCP-1 production was determined by ELISA. MCP-1 production in coculture without PDTC is shown as 100%. Data are means ± SE from three separate experiments.

added to inhibitor-treated MC at a final ratio of 1:100. SB203580 and genistein reduced MCP-1 production by 40 and 70%, respectively (Figure 8), whereas PD98059 had no effect. These data suggest that platelets induce MCP-1 synthesis by MC through activation of p38 MAPK and PTK.

Discussion

MCP-1 is known to play a predominant role in monocyte/macrophage recruitment into glomeruli in a variety of glomerulonephritides. Macrophages infiltrating glomeruli in turn stimulate resident glomerular cells to induce (*1*) mesangial proliferation and matrix expansion through macrophage-derived growth factors, such as PDGF and TGF-β, and (2) upregulation of adhesion molecule and chemokine secretion through inflammatory cytokines, such as TNF-α and IL-1β, to facilitate further infiltration of leukocytes. Herein, we report that platelets induce MCP-1 production by MC in part through interactions involving CD40/CD40L and that activation of NF-κB, p38 MAPK, and PTK are involved in this process. Whereas an 100-fold excess of platelets is required to induce this phenomenon, these findings demonstrate a novel mechanism that could be important *in vivo*. Under pathologic conditions whereby the composition of the extracellular matrix may be altered and inflammatory molecules and other cells, such as macrophages, T cells, or blood-derived polymorphonuclear cells, are present, a smaller number of platelets might be enough to stimulate MC. Our findings indicate that platelets are not only regulators of intraglomerular coagulation but also

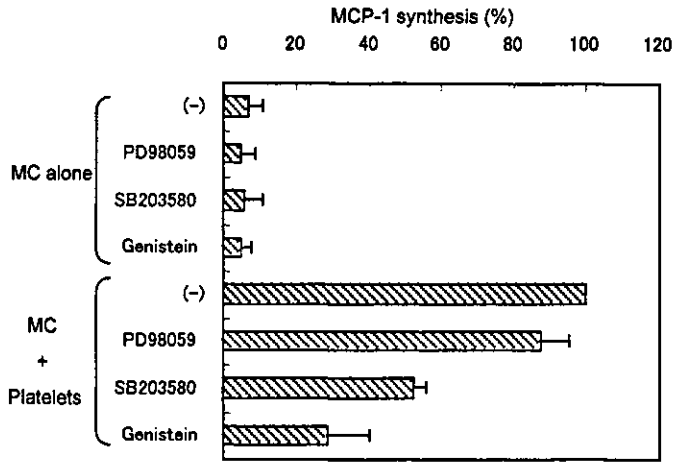

Figure 8. p38 MAPK and protein tyrosine kinases (PTK) are involved in mesangial MCP-1 upregulation induced by platelet binding. MC were pretreated with 50 μg/ml PD98059, SB203580, or genistein for 1 h. In preliminary studies, the concentrations of inhibitors to achieve the maximal effects were determined. Medium was changed four times to remove added inhibitors completely. Subsequently, coculture of MC and platelets were performed at the ratio of 1:100. ELISA determined MCP-1 synthesis after 24 h. MCP-1 production in coculture without inhibitors is shown as 100%. Data are means ± SE from three separate experiments.

key modulators of glomerular inflammatory responses. Platelet-mediated MCP-1 production by MC may be an important mechanism whereby platelets contribute to the amplification and progression of glomerular injury.

Previous studies exploring the pathogenesis of vascular inflammation and atherosclerosis have documented activation of vascular endothelial cells or myeloid leukocytes by platelet binding (10–12). In these studies, contact with activated platelets was shown to induce MCP-1 production by endothelial cells or leukocytes. Coculture of platelets and MC resulted in upregulation of osteopontin, cyclooxygenase 2 (COX2), and MCP-1 mRNA expression by MC (37). In the present study, we have considerably extended such observations and have defined potential pathways whereby such effects are mediated. We show that MC express CD40 *in vitro* and that such expression is functionally involved in cell-to-cell interactions. Critically however, we demonstrate that both cell-to-cell contact and soluble factors were implicated. Thus, pre-stimulated platelets did not induce MCP-1 by MC when separated from MC, indicating that direct contact between platelets and MC is essential. The most plausible explanation for this is that signals mediated by cell contact are indispensable for MCP-1 production. The effects of direct cell-to-cell contact may be bidirectional, resulting in stimulation of platelets to release soluble mediators or upregulate adhesion molecule expression (such as CD40L and P-selectin) and thus further amplify the platelet-induced stimulation of MC. Commensurate with this, rCD40L in combination with IFN-γ enhanced MCP-1 production by MC, whereas rCD40L alone was ineffective (Figure 6). These data indicate that functional CD40 is expressed on cultured MC and that signals mediated by CD40 enhance MCP-1 production.

As platelet expression of CD40L increases upon activation of platelets with ADP or thrombin, we expected, but did not find, higher levels of MC MCP-1 production induced by activated platelets. This may have reflected isolation effects that varied from donor to donor. In addition, MC produce extracellular matrix (including collagen, an activator of platelets) in their surrounding microenvironment, and this by itself may be enough to induce maximum CD40L expression on platelets. Alternatively, in the presence of other stimuli, baseline levels of CD40L similar to those found on unstimulated platelets may be sufficient for optimal activation of MC.

Several studies have shown that CD40/CD40L interaction plays important roles in the pathogenesis of immune-mediated glomerulonephritides. In animal models of lupus nephritis, anti-CD40L antibodies ameliorate nephritis even when administered after disease onset (38). Our data suggest that the protective effect of anti-CD40L in this disease may partly reflect inhibition of MCP-1 production after platelet/MC interactions. CD40L-positive leukocytes are rarely observed in glomeruli as compared with the interstitium in proliferative lupus nephritis (16), further strengthening the idea that platelet-bound CD40L might be the predominant ligand source for CD40 on MC. A functional soluble form of CD40L has recently been reported (39,40), suggesting that platelets may stimulate MC through either soluble or cell-surface CD40L. In contrast to data obtained in other cell types (11,12), blockade of P-selectin on platelets did not decrease MCP-1 production in coculture. Although we used doses of the anti–P-selectin mAb that can be expected to almost completely block surface-expressed P-selectin, mesangial production of MCP-1 was not significantly decreased. Considering the redundancy between P-selectin and other similar adhesion molecules, these data do not exclude a modest contribution by P-selectin.

MCP-1 expression is regulated in both a stimulus-specific and a tissue-specific manner (31). Although several lines of evidence suggest that NF-κB activation is essential, recent studies have shown that MCP-1 induction by PDGF-BB is dependent on proximal Sp-1 but not on NF-κB activation (32). We have demonstrated that platelet binding induces high levels of NF-κB activation that in turn is required for MCP-1 production. Platelets were surprisingly more potent in NF-κB activation than TNF-α or LPS. This also might have resulted from strong and rather unique effects of the direct cell-to-cell contact, together with soluble inflammatory mediators. Accordingly in our previous study, which examined the interaction between monocytes and MC, both cell-to-cell contact and soluble factors were required for the maximum activation of the latter (18). Other studies have shown that platelet contact induces chemokine or adhesion molecule expression by endothelial cells or leukocytes through NF-κB activation (10,12). NF-κB plays a critical role in inflammatory response through induction of a variety of proinflammatory genes. Thus platelet binding to MC may lead to the amplification of inflammatory responses through the expression not only of MCP-1 but also of other inflammatory molecule expression dependent upon

NF-κB. Ligation of CD40 by CD40L activates NF-κB through TNF receptor–associated factors (TRAF) (41). It is thus possible that CD40L upregulates MCP-1 through NF-kB activation; alternatively, activated platelets contain IL-1–like activity which is also a strong inducer of NF-κB activation (42).

Previous studies have demonstrated that TNF-α induces MCP-1 production by HUVEC through p38 MAPK, but not through other MAPK, such as Jun N-kinase (JNK) or ERK1/2 (33). We have identified that p38 MAPK is involved in platelet-mediated MCP-1 production by MC as treatment of MC with a specific inhibitor of p38 MAPK decreased MCP-1 by 50% (Figure 7). Although it is still unclear how activation of p38 MAPK leads to induction of MCP-1 expression, a recent study suggested that p38 MAPK is involved in the activation of NF-κB in the cytoplasm as well as in modulating its transactivating potential in the nucleus (43). The finding that an inhibitor of PTK reduced MCP-1 production by 70% demonstrates that PTK also play a role in platelet-mediated MCP-1 induction by MC. Ligation of CD40 is reported to activate p38 MAPK and PTK, such as lyn, in other types of cells (44,45). Therefore, we have also examined if rCD40L alone can directly induce their activation by Western blot using specific antibodies against phospho-p38 MAP kinase (Cell Signaling Technology, Beverly, MA) or phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY). rCD40L alone did not induce any detectable upregulation of p-p38 MAPK and phosphotyrosine (data not shown). This does not completely exclude the possibility that CD40L activates at low levels p38 MAPK and PTK, considering the sensitivity of Western blotting and the absence of co-stimulation from activated platelets.

In summary, we have shown that platelet contact induces mesangial expression of MCP-1, in part through the activation of CD40/CD40L, NF-κB, p38 MAPK, and PTK. As MCP-1 plays a key role in the infiltration of macrophages into inflamed glomeruli and glomerular injury, we suggest that platelet/MC adhesion may represent an important mechanism for the amplification and the perpetuation of glomerular inflammatory responses in a variety of glomerulonephritides. Inhibition of the interaction between platelets and MC could be of potential therapeutic benefit.

Acknowledgment

This work was supported in part by a grant from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

References

1. Johnson RJ: Platelets in inflammatory glomerular injury. *Semin Nephrol* 11: 276, 1991
2. Barnes JL, Hastings RR, De-La-Garza MA: Sequential expression of cellular fibronectin by platelets, macrophages, and mesangial cells in proliferative glomerulonephritis. *Am J Pathol* 145: 585, 1994
3. Young BA, Johnson RJ, Alpers CE, Eng E, Gordon K, Floege J, Couser WG, Seidel K: Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* 47: 935, 1995
4. Johnson RJ, Garcia RL, Pritzl P, Alpers CE: Platelets mediate glomerular cell proliferation in immune complex nephritis induced by anti-mesangial cell antibodies in the rat. *Am J Pathol* 136: 369, 1990
5. Johnson RJ, Pritzl RI, Ida H, Alpers CE: Platelet-complement interactions in mesangial proliferative nephritis in the rat. *Am J Pathol* 138: 313, 1991
6. Wu X, Pippin J, Lefkowith JB: Platelets and neutrophils are critical to the enhanced glomerular arachidonate metabolism in acute nephrotoxic nephritis in rats. *J Clin Invest* 91: 766, 1993
7. Barnes JL, Venkatachalam MA: The role of platelets and polycationic mediators in glomerular vascular injury. *Semin Nephrol* 5: 57, 1985
8. Couser WG: Pathogenesis of glomerular damage in glomerulonephritis. *Nephrol Dial Transplant* 13[Suppl 1]: 10, 1998
9. Donadio JV Jr, Anderson CF, Mitchell JC III, Holley KE, Ilstrup DM, Fuster V, Chesebro JH: Membranoproliferative glomerulonephritis. A prospective clinical trial of platelet-inhibitor therapy. *N Engl J Med* 310: 1421, 1984
10. Gawaz M, Neumann F, Dickfeld T, Koch W, Laugwitz K, Adelsberger H, Langenbrink K, Page S, Neumeier D, Schömig A, Brand K: Activateed platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. *Circulation* 98: 1164, 1998
11. Weyrich AS, Elstad MR, McEver RP, McIntyre TM, Moore KL, Morrissey JH, Prescott SM, Zimmerman GA: Activated platelets signal chemokine synthesis by human monocytes. *J Clin Invest* 97: 1525, 1996
12. Neumann F, Marx N, Gawaz M, Brand K, Ott I, Rokitta C, Sticherling C, Meinl C, May A, Schömig A: Induction of cytokine expression in leukocytes by binding of thrombin-stimulated platelets. *Circulation* 95: 2387, 1997
13. Henn V, Slupsky JR, Gräfe M, Anagnostopoulos I, Förster R, Müller-Berghaus G, Kroczek RA: CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391: 591, 1998
14. Kroczek RA, Graf D, Brugnoni D, Giliani S, Korthuer U, Ugazio A, Senger G, Mages HW, Villa A, Notarangelo LD: Defective expression of CD40 ligand on T cells causes "X-linked immunodeficiency with hyper-IgM (HIGM1)." *Immunol Rev* 138: 39, 1994
15. Stout RD, Suttles J: The many roles of CD40 in cell-mediated inflammatory responses. *Imunol Today* 17: 487, 1996
16. Yellin MJ, D'Agati V, Parkinson G, Han AS, Szema A, Baum D, Estes D, Szabolcs M, Chess L: Immunohistologic analysis of renal CD40 and CD40L expression in lupus nephritis and other glomerulonephritides. *Arthritis Rheum* 40: 124, 1997
17. Kuroiwa T, Schlimgen R, Illei GG, McInnes IB, Boumpas DR: Distinct T cell/renal tubular epithelial cell interactions define differential chemokine production: Implications for tubulointerstitial injury in chronic glomerulonephritides. *J Immunol* 164: 3323, 2000
18. Kuroiwa T, Lee EG, Danning CL, Illei GG, McInnes IB, Boumpas DR: CD40 ligand-activated human monocytes amplify glomerular inflammatory responses through soluble and cell-to-cell contact-dependent mechanisms. *J Immunol* 163: 2168, 1999
19. Rovin BH, Yoshimura T, Tan LC: Cytokine induced production of monocyte chemoattractant protein-1 by cultured human mesangial cells. *J Immunol* 148: 2148, 1992
20. Prodjosudjadi W, Gerritsma JS, Klar-Mohamad N, Gerritsen AF, Bruijn JA, Daha MR, van Es LA: Production and cytokine-mediated regulation of monocyte chemoattractant protein-1 by human proximal tubular epithelial cells. *Kidney Int* 48: 1477, 1995

21. Wada T, Yokoyama H, Furuichi K, Kobayashi K, Harada K, Naruto M, Su SB, Akiyama M, Mukaida N, Matsushima K: Intervention of crescentic glomerulonephritis by antibodies to monocyte chemotactic and activating factor (MCAF/MCP-1). *FASEB J* 10: 1418, 1996
22. Lloyd CM, Minto AW, Dorf ME, Proudfoot A, Wells TNC, Salant DJ, Gutierrez-Ramos J: RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. *J Exp Med* 185: 1371, 1997
23. Wenzel U, Schneider A, Valente AJ, Abboud HE, Thaiss F, Helmchen UM, Stahl RAK: Monocyte chemoattractant protein-1 mediates monocyte/macrophage influx in anti-thymocyte antibody-induced glomerulonephritis. *Kidney Int* 51: 770, 1997
24. Tesch GH, Maifert S, Schwarting A, Rollins BJ, Kelley VR: Monocyte chemoattractant protein 1-dependent leukocytic infiltrates are responsible for autoimmune disease in MRL-Fas^{lpr} mice. *J Exp Med* 190: 1813, 1999
25. Miller K, Dresner IG, Michael AF: Localization of platelet antigens in human kidney disease. *Kidney Int* 18: 472, 1980
26. Silver BJ, Jaffer FE, Abboud HE: Platelet-derived growth factor synthesis in mesangial cells: Induction by multiple peptide mitogen. *Proc Natl Acad Sci USA* 86: 1056, 1989
27. Dumoulin FL, Nischalke HD, Leifeld L, Bussche A, Rockstroh JK, Sauerbruch T, Spengler U: Semi-quantification of human C-C chemokine mRNAs with reserve transcription/real-time PCR using multi-specific standards. *J Immunol Method* 241: 109, 2000
28. Nagasawa Y, Takenaka M, Matsuoka Y, Imai E, Hori M: Quantitation of mRNA expression in glomeruli using laser-manipulated microdissection and laser pressure catapulting. *Kidney Int* 57: 717, 2000
29. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T:The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell* 78: 773, 1994
30. Watson SP: Collagen receptor signaling in platelets and megakaryocytes. *Thromb Haemost* 82: 365, 1999
31. Ueda A, Okuda K, Ohno S, Shirai A, Igarashi T, Matsunaga K, Fukushima J, Kawamoto S, Ishigatsubo Y, Okubo T: NF-κB and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. *J Immunol* 153: 2052, 1994
32. Ping D, Boekhoudt G, Boss JML Trans-Retinoic acid blocks platelet-derived growth factor-BB-induced expression of the murine monocyte chemoattractant-1 gene by blocking the assembly of a promoter proximal Sp1 binding site. *J Bio Chem* 274: 31909, 1999
33. Goebeler M, Kilian K, Gillitzer R, Kunz M, Yoshimura T, Brocker EB, Rapp UR, Ludwig S: The MKK6/p38 stress kinase cascade is critical for tumor necrosis factor-alpha-induced expression of monocyte-chemoattractant protein-1 in endothelial cells. *Blood* 93: 857, 1999
34. Rovin BH, Wilmer WA, Danne M, Dickerson JA, Dixon CL, Lu L: The mitogen-activated protein kinase p38 is necesssary for interleukin 1beta-induced monocyte chemoattractant protein 1 expression by human mesangial cells. *Cytokine* 11: 118, 1999
35. Rovin BH, Tan LC: Role of protein kinase pathways in IL-1-induced chemoattractant expression by human mesangial cells. *Kidney Int* 46: 1059, 1994
36. Pai R, Ha H, Kirschenbaum MA, Kamanna VS: Role of tumor necrosis factor-α on mesangial cell MCP-1 expression and monocyte migration: mechanisms mediated by signal transduction. *J Am Soc Nephrol* 7: 914, 1996
37. Goppelt-Struebe M, Wiedemann T, Heusinger-Ribeiro J, Vucadinovic M, Rehm M, Prols F: Cox-2 and osteopontin in cocultured platelets and mesangial cells: Role of glucocorticoids. *Kidney Int* 57: 2229, 2000
38. Kalled SL, Cutler AH, Datta SK, Thomas DW: Anti-CD40 ligand antibody treatment of SNF1 mice with established nephritis: Preservation of kidney function. *J Immunol* 160: 2158, 1998
39. Graf D, Muller S, Korthauer U, van Kooten C, Weise C, Kroczek RA: A soluble form of TRAP (CD40 ligand) is rapidly released after T cell activation. *Eur J Immunol* 25: 1749, 1995
40. Pietravalle F, Lecoanet-Henchoz S, Blasey H, Aubry J, Elson G, Edgerton MD, Bonnefoy J, Gauchat J: Human native soluble CD40L is a biologically active trimer, processed inside microsomes. *J Biol Chem* 271: 5965, 1996
41. Arch RH, Gedrich RW, Thompson CB: Tumor necrosis factor receptor-associated factors (TRAFs)—A family of adapter proteins that regulates life and death. *Genes Dev* 12: 2821, 1998
42. Hawrylowicz CM, Santoro SA, Platt FM, Unanue ER: Activated platelets express IL-1 activity. *J Immunol* 143: 4015, 1989
43. Schulze-Osthoff K, Ferrari D, Riehemann K, Wesselborg S: Regulation of NF-κB activation by MAP kinase cascades. *Immunobiology* 198: 35, 1997
44. Craxton A, Shu G, Graves JD, Saklatvala J, Krebs EG, Clark EA: p38 MAPK is required for CD40-induced gene expression and proliferation in B lymphocytes. *J Immunol* 161: 3225, 1998
45. Ren CL, Morio T, Fu SM, Geha RS: Signal transduction via CD40 involves activation of *lyn* kinase and phosphatidylinositol-3-kinase, and phosphorylation of phospholipase Cγ2. *J Exp Med* 179: 673, 1994

連載講座

企画：竹内　勤+

T細胞内のシグナル伝達分子機構

第11回

T細胞副刺激分子 Inducible Costimulator とその異常

針谷正祥*

Activation-inducible lymphocyte immuno-mediatory molecule/Inducible Costimulator は，活性化T細胞に発現される新しいCD 28 ファミリー分子であり，T細胞副刺激分子として作用し，T細胞の増殖およびサイトカイン産生を促進する．ICOS は実験的脳脊髄炎，関節炎，気管支喘息などの動物モデルで病態への関与が示されており，免疫応答の時相により抑制的または促進的に作用する．今後，ヒトの免疫性疾患における ICOS の関与が明らかにされ，新規治療標的としての評価が進むことが期待される．

はじめに

特異抗原に対するT細胞の反応性は，抗原提示と同時にT細胞に与えられる副刺激の種類・強さによって大きく影響される．すなわち，T細胞の活性化にはCD 28 ファミリーまたはTNF ファミリーに属する副刺激分子からの second signal が必須と考えられている．本稿では，近年同定された副刺激分子である Inducible Costimulator (ICOS)の機能について主として免疫性疾患モデルにおける解析結果を中心に紹介し，ヒトの免疫性疾患とのかかわりを考えてみたい．ICOS の遺伝子・蛋白構造およびその基本的な機能については，すぐれた総説があるのでそちら[1)~3)]を参照して頂きたい．

〔キーワード〕
Inducible Costimulator
副刺激分子
CD 28 ファミリー
B7 ファミリー
自己免疫疾患

1．ICOS とそのリガンドの発見

Activation-inducible lymphocyte immuno-mediatory molecule/Inducible Costimulator (AILIM/ICOS：ICOS)は，活性化T細胞に発現される新しいCD 28 ファミリー糖蛋白として1999年に同定された．CD 28 と同様に ICOS はT細胞の増殖およびインターロイキン(IL)-4，IL-5，IL-10，IFN-γ，TNF-α，GM-CSF などのサイトカイン産生を促進するが，IL-2 産生誘導能はCD 28 に比較して低い[4)5)]．さらにノックアウトマウスの解析から，ICOS がリンパ組織の胚中心形成・T細胞のクローン増殖・抗体産生・抗体産生のクラススイッチなどにおいて重要なはたらきをしていることが明らかにされた[6)]．ICOS のリガン

*HARIGAI Masayoshi/東京医科歯科大学医学部附属病院臨床試験管理センター/膠原病・リウマチ内科

ドはB7RP-1(B7-H2, GL50, LICOSともよばれている)であり，CD28やCTLA-4のリガンドであるCD80，CD86にはICOSは結合しない．B7RP-1とCD80，CD86のあいだには約20%の相同性を認め，B7RP-1はB細胞および単球に構成的に発現している[7][8]．

2．免疫性疾患モデルにおけるICOSの発現と機能

上述のようなICOSの発現・機能上の特徴から，ICOSが免疫性疾患の病態形成に関与している可能性が考えられた．実際に，免疫性疾患モデル動物を用いたICOSの *in vivo* での機能解析結果が多数報告されている．以下に，脳脊髄炎，関節炎，気管支喘息の各モデル動物を用いた実験結果を紹介し，ICOSと免疫性疾患の関連性を考察する．

1）実験的自己免疫性脳脊髄炎モデル

C57BL/6マウスにMyelin Oligodendrocyte Glycoprotein(MOG)ペプチドを完全Freudアジュバント(Complete Freud's Adjuvant：CFA)とともに免疫すると7～10日後に実験的自己免疫性脳脊髄炎(Experimental Autoimmune Encephalomyelitis：EAE)を発症する．この実験系においては，CD28-B7間の相互作用が必須であることが知られている．一方，129/svマウスに同様な免疫操作をおこなってもEAEは誘導されない．すなわち，129/svマウスはMOG-induced EAEに抵抗性を示す．C57BL/6 x 129 F2マウスでは，両マウスの表現型を反映して，軽度のMOG-induced EAEが誘導され，病理組織学的には脊髄および脳組織に軽度の細胞浸潤が認められる．ICOS遺伝子をノックアウトしたC57BL/6 x 129 F2マウス(ICOS$^{-/-}$C57BL/6 x 129 F2)を作成しMOGペプチドを投与したところ，非常に重篤なEAEが誘導され，半数のマウスが発症後死亡した(図1)．病理組織学的にも広汎な細胞浸潤を伴い，脳組織中により多くのIFN-γ産生CD4陽性細胞が認められた．MOGペプチドで免疫したICOS$^{-/-}$C57BL/6 x 129 F2およびC57BL/6 x 129 F2から得た脾細胞を *in vitro* でMOGペプチドにより再刺激し培養上清中のサイトカインを測定したところ，いずれのマウスでもIL-4の産生は認められず，IL-10とIFN-γは同程度に産生された．しかしIL-13の産生はC57BL/6 x 129 F2のみで認められ，ICOS$^{-/-}$C57BL/6 x 129 F2ではまったく産生されなかった．IL-13はTh2サイトカインとして抗炎症作用を有するとともにLewis ratにおいてEAE発症を抑制することが知られており，IL-13産生低下がICOS$^{-/-}$C57BL/6 x 129 F2におけるMOG-induced EAE増悪の原因の1つであると考えられた．これらの実験結果から，ICOSはMOG-induced EAEの発症を負に制御するはたらきを有することが示された[6]．

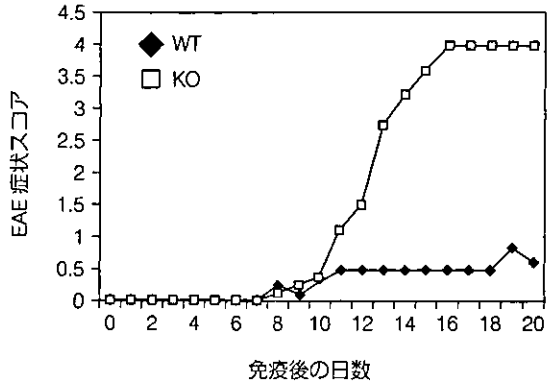

図1．ICOSノックアウトマウスにおけるMOG-induced EAEの増悪(Dong, C. *et al*, 2001[6]より改変引用)
C57BL/6 x 129 F2マウス(WT)では，両マウスの表現型を反映して，軽度のMOG-induced EAEが誘導される．ICOS－/－C57BL/6 x 129 F2(K/O)ではしMOGペプチド投与により非常に重篤なEAEが誘導され，半数のマウスがEAE発症後死亡した．

SJLマウスにプロテオリピド蛋白質(proteolipid protein：PLP)をCFAとともに免疫すると，約10日後からEAEを発症する(PLP-in-

表 1. 抗 ICOS 中和抗体投与による PLP-induced EAE 発症への影響
(Rottman, J. B. *et al*, 2001[9]より改変引用)

抗 ICOS 中和抗体	EAE 発症頻度	EAE の clinical score
投与なし	10/10	3.0+/−0.6
1〜10 日後まで投与	10/10	5.0+/−0.0
9〜20 日後まで投与	5/10	1.0-3.0

duced EAE). 脳組織では, 免疫前には ICOS の発現を認めなかったが, EAE が発症する免疫 10〜20 日後にかけて浸潤 T 細胞における ICOS の持続的な発現亢進を認めた. 一方, B 7 RP-1 は免疫前から軽度発現し, ICOS と同様な継時的変化で発現が亢進した. PLP-induced EAE における ICOS の役割を検討するため, 免疫後 10 日間抗 ICOS 中和抗体を投与した場合と, 免疫後 9〜20 日後まで抗 ICOS 中和抗体を投与した場合について EAE の発症を検討した. 免疫後 10 日間抗 ICOS 中和抗体を投与すると EAE の重症度は悪化し, 病理組織学的にも細胞浸潤が増強することが明らかとなった. 一方, EAE が発症してくる直前の免疫 9〜20 日後に抗 ICOS 中和抗体を投与すると, 発症率は 50%に低下し, 重症度も改善することが示された(表 1)[9].

PLP-induced EAE では Th 1, Th 2 T 細胞のいずれも誘導され, 最終的には Th 1 T 細胞優位となり EAE の発症に関与すると考えられている. 抗 ICOS 中和抗体の投与による PLP-induced EAE の改善または増悪機序と Th 1/Th 2 バランスの関係を検討するため, 免疫 12 日後の脾細胞を分離後 *in vitro* で PLP により再刺激し, 培養上清中の IFN-γ を測定した. 抗 ICOS 抗体非投与群に比較して, 免疫後 10 日間抗 ICOS 中和抗体を投与したマウスの脾細胞の IFN-γ 産生は有意に高値を示し, 免疫 9〜20 日後に抗 ICOS 中和抗体を投与したマウスの脾細胞の IFN-γ 産生は有意に低値を示した. また, PLP に対する T 細胞増殖反応も前者で有意に高く, 後者で有意に低かった. さらに IgG 1 サブクラスの抗 PLP 抗体を測定したところ, 免疫後 10 日間抗 ICOS 中和抗体を投与したマウスでは抗体非投与群に比較して有意に血中抗体価が低下していた. これらの実験結果から, 免疫後 10 日間の antigen priming phase に ICOS -B 7 RP-1 系を阻害すると, PLP に対する免疫応答が Th 1 有意に偏移し PLP-induced EAE を増悪させ, 一方, 免疫 9〜20 日後の efferent phase に ICOS-B 7 RP-1 系を阻害すると PLP に対する T 細胞増殖と IFN-γ 産生が低下し PLP-induced EAE が改善すると考えられる.

ミエリン塩基性蛋白質(Myelin Basic Protein : MBP)特異的 T 細胞レセプター(T Cell Receptor : TCR)トランスジェニック B 10.PL マウスの T 細胞(MBP-TCR トランスジェニック T 細胞)を *in vitro* で MBP にて刺激後, 同系マウスに移入することにより EAE を誘導できる(MBP -induced EAE). MBP-TCR トランスジェニック T 細胞を *in vitro* で MBP により刺激すると, 細胞増殖, IFN-γ・IL-10 産生誘導を認めたが, これらの反応は ICOS-Ig の共存により強く抑制された. また ICOS-Ig 存在下で MBP-TCR トランスジェニック T 細胞を MBP で刺激するとメモリー T 細胞(CD 45 RB^{lo})のアポトーシスが選択的に誘導された. MBP-TCR トランスジェニック T 細胞を ICOS-Ig 存在下で *in vitro* で刺激後に同系マウスに移入すると, MBP-induced EAE の発症がほぼ完全に抑制されたが, その機序としては上記の ICOS-Ig の作用が考えられた. また, MBP-induced EAE の発症後に ICOS-Ig を投与

すると，EAEの臨床スコアが有意に改善した[10]．

これらの3つのEAEモデルから，ICOS-B7 RP-1系はEAE誘導期には発症を抑制する方向に作用し，EAE発症後には病態を増悪させるはたらきを有することが示された．

2）関節炎モデル

コラーゲン誘発関節炎(Collagen-Induced Arthritis：CIA)は，DBA/J1マウスにタイプIIコラーゲンを免疫し，3週間後に再免疫することにより誘導される関節炎モデルである．3週間後の再免疫時をday 0とし，抗B7h/B7RP-1中和抗体(100 μg/マウス)をday -1, 1, 3, 5に腹腔内投与(予防的投与)すると発症時期が有意に遅延し(コントロール抗体4.9+/−1.3日，B7h/B7RP-1中和抗体6.3+/−2.9日)，関節炎スコアは有意に改善した(コントロール抗体11.8+/−3.6日，B7h/B7RP-1中和抗体6.3+/−3.8日)．パンヌス形成・炎症細胞浸潤・軟骨破壊などの病理組織学的所見およびX線上の骨びらんも，B7h/B7RP-1中和抗体投与により有意に改善した．さらに，関節炎発症後にB7h/B7RP-1中和抗体投与を開始したところ(治療的投与，day 5・7・9・11に投与)，予防的投与と同様な関節炎の改善を認めた．滑膜組織において，$CD4^+$細胞および$CD4^-$細胞にICOS発現を認め，B細胞およびマクロファージの一部にB7h/B7RP-1の発現が認められた．CIAの病態形成には炎症性サイトカインが深く関与しているが，B7h/B7RP-1中和抗体予防投与により，CIA罹患関節における腫瘍壊死因子α (Tumor Necrosis Factor α：TNFα)，IL-1β，IL-6の発現がmRNAレベルで有意に抑制された．また，CIAマウスからリンパ節を採取し，リンパ節細胞を *in vitro* でタイプIIコラーゲンにより刺激すると，細胞増殖・IFN-γ産生・IL-10産生が誘導されるが，B7h/B7RP-1中和抗体予防投与後のマウスから得られたリンパ節細胞では，これらの反応が有意に低下していた．

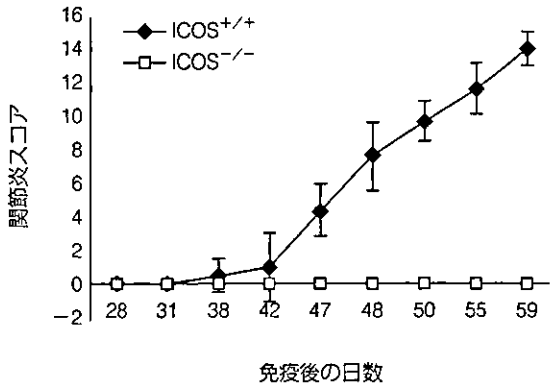

図2. ICOSノックアウトマウスはCIA抵抗性を示す
(Nurieva, R. I. *et al*, 2003[12]より改変引用)
DBA/J1-ICOS−/−F6マウスに，タイプIIコラーゲンをday 0, day 21, day 42に免疫して関節炎を誘導した．DBA/J1-ICOS+/+F6マウスでは，day 31以降関節炎が誘導されたがDBA/J1-ICOS−/−F6マウスでは関節炎は完全に抑制された．

血清中の抗タイプIIコラーゲン抗体価はIgG1・IgG2a・IgG2bサブクラスともにB7h/B7RP-1中和抗体予防投与により有意に低下した．したがって，ICOS-B7RP-1系阻害は，炎症性サイトカイン発現抑制とTh1およびTh2細胞応答抑制の両者により，抗関節炎作用を発揮すると考えられる[11]．

CIAにおけるICOS-B7RP-1系の役割がICOSノックアウト($ICOS^{-/-}$)マウスを用いて調べられている．$ICOS^{-/-}$マウスをDBA/J1マウスに6回backcrossさせ(DBA/J1-$ICOS^{-/-}$F6)，タイプIIコラーゲンをday 0, 21, 42に免疫して関節炎を誘導した．DBA/J1-$ICOS^{+/+}$F6マウスでは，day 31以降関節炎が誘導されたがDBA/J1-$ICOS^{-/-}$F6では関節炎は完全に抑制された(図2)．また病理組織学的な検討でも，DBA/J1-$ICOS^{+/+}$F6で認めらたパンヌス形成・軟骨破壊・骨びらんなどの所見はDBA/J1-$ICOS^{-/-}$F6ではまったく認められなかった．また，IgG2aサブクラスの抗タイプIIコラーゲン抗体はDBA/J1-$ICOS^{-/-}$F6で有意に低下していた．タイプII