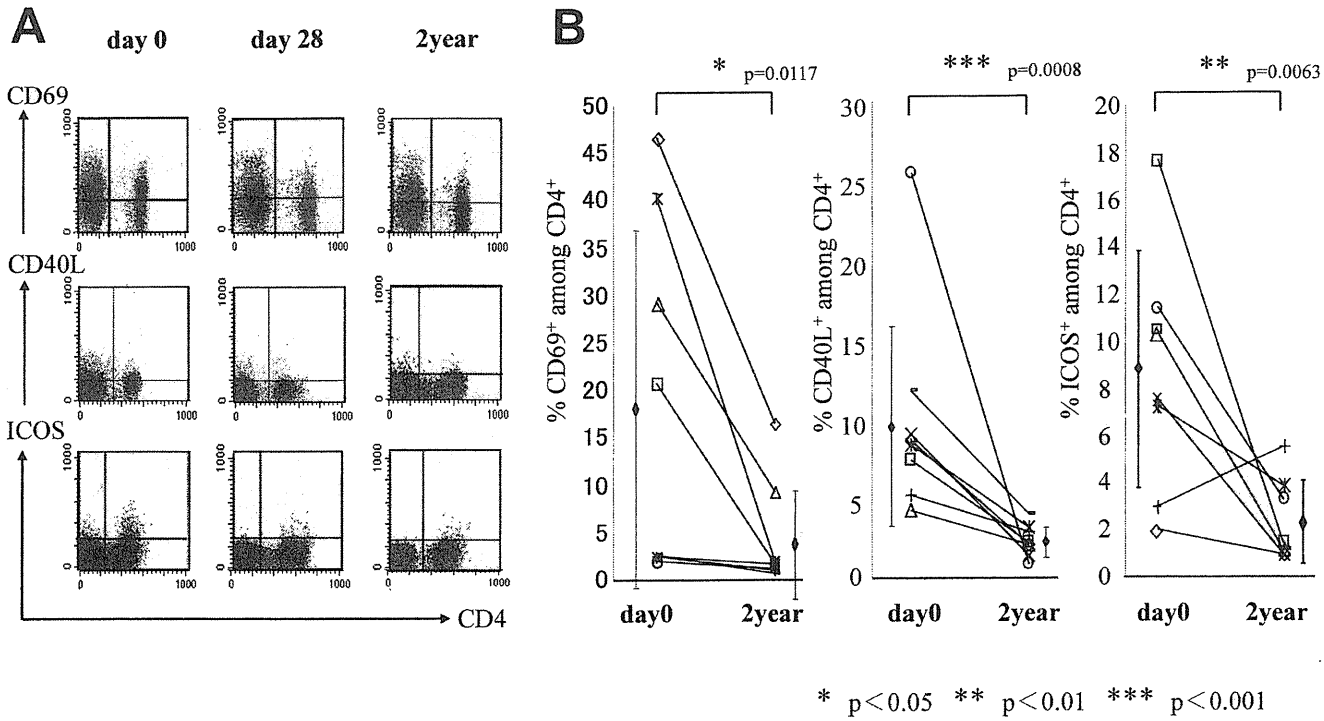

Figure 4. Changes in levels of the T cell activation marker CD69, and of the costimulatory molecules CD40L and inducible costimulator (ICOS) in patients with SLE who have prolonged remission and show major clinical response following rituximab therapy. (A) Cases showing typical courses (x axis, CD4). From top to bottom, changes from baseline to 28 days and 2 years after rituximab treatment in Patient 3 (y axis, CD69), Patient 4 (y axis, CD40L), and Patient 1 (y axis, ICOS). (B) Changes in percentages of CD69-positive cells, CD40L-positive cells, and ICOS-positive cells in the CD4-positive cell population before and 2 years after rituximab treatment in the 8 patients with prolonged remission.

cells, as well as the levels of CD40 and CD80 on the CD19-positive cells, increased, without any significant change in the number of T cells (Figure 5A). This patient was treated again with rituximab. The retreatment resulted in the disappearance again of memory B cells from peripheral blood and a decrease in disease activity. The butterfly rash disappeared, the anti-dsDNA antibody test was negative, and urinary protein excretion and occult blood disappeared.

The patient with T cell-dominant relapse was a 29-year-old woman (Patient 6). Despite intense immunosuppressive therapy, she continued to have central nervous system (CNS) symptoms and a high disease activity level (SLEDAI 9, BILAG 12). The results of tests for anti-dsDNA antibody and anti-Sm antibody were negative. Treatment with rituximab induced remission of SLE along with rapid disappearance of both naive and memory B cells from the peripheral blood. Two years later, however, the disease relapsed and the patient presented with CNS disease manifestations. While no changes in B cells were seen either before or after the relapse, an increase in the population of memory T cells was noted, along with markedly elevated levels of ICOS on CD4-positive cells (Figure 5B). In this patient, disease activity was found to be worse and to involve predominantly T cell abnormalities. Therefore, she was retreated with IVCY, because this drug is considered to be effective against T cells as well. Systemic symptoms, such as fever, malaise, and lymph node swelling, and also the psychiatric symptoms improved with IVCY treatment. In addition, brain perfusion scintigraphy showed an improvement of blood flow, and the level of ICOS on CD4-positive T cells decreased (data not shown).

DISCUSSION

Rituximab has recently been demonstrated to be effective in the treatment of SLE[1,2,3,4,5,6,7,8] and we undertook our study to determine the mechanisms of the longterm remission of SLE induced by rituximab and the relapse after remission. When patients with highly active SLE were treated with rituximab, rapid depletion of CD19+IgD+CD27– naive B cells, CD19+IgD–CD27– memory B cells, and CD19+IgD–CD27+ memory B cells from the peripheral blood was observed, while $CD19^{low}CD27^{high}$ or IgD–CD38+ plasma cells persisted in the blood until Day 28. For patients with clinical remission for about 2 years after rituximab treatment, the plasma cells as well as memory B cells remained depleted or in markedly reduced numbers, although the naive B cells recovered. Analysis of the

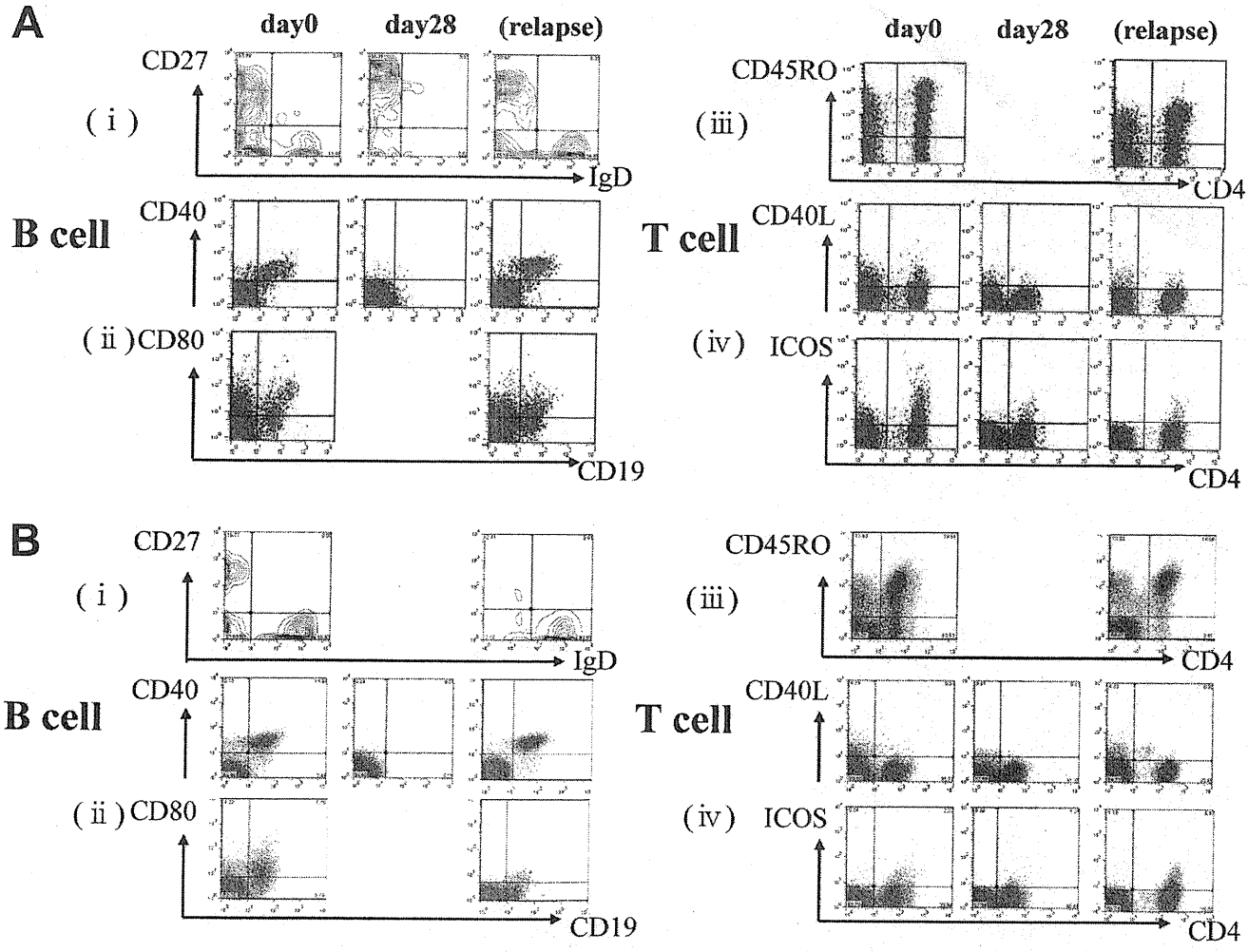

Figure 5. (A) Changes in levels of B cell and T cell surface antigens before and 28 days after treatment, and at the time of SLE relapse, 1.5 years after treatment, in Patient 9, who showed B cell-dominant relapse. (i) Changes in CD19-positive cell subsets (x axis, IgD; y axis, CD27; changes in the numbers of naive B cells, memory B cells, and plasma cells). (ii) Changes in levels of CD40 and CD80, costimulatory molecules expressed on CD19-positive cells. (iii) Changes in the CD4-positive cell subsets (x axis, CD4; y axis, CD45RO; changes in the numbers of naive T cells and memory T cells). (iv) Changes in levels of CD40L and ICOS, costimulatory molecules expressed on CD4-positive cells. (B) Changes in levels of B cell and T cell surface antigens before and 28 days after rituximab treatment, and at the time of relapse, 2 years after treatment, in Patient 6, who showed T cell-dominant relapse. (i) Changes in the CD19-positive cell subsets (x axis, IgD; y axis, CD27; changes in the number of naive B cells, memory B cells, and plasma cells). (ii) Changes in levels of CD40 and CD80. (iii) Changes in the CD4-positive cell subset (x axis, CD4; y axis, CD45RO; changes in numbers of the naive T cells and memory T cells). (iv) Changes in levels of CD40L and ICOS.

changes in the levels of the costimulatory molecules on the B cells revealed that levels of both CD40 and CD80 remained suppressed until 2 years after rituximab therapy.

However, in Patient 9, who showed relapse after prolonged remission, an increase in the percentage of CD19+IgD–CD27– memory B cells and CD19+IgD–CD27+ memory B cells, as well as levels of CD40 and CD80 on these cells, was noted just before the relapse of SLE. Further, in these patients who showed relapse, the anti-dsDNA antibody titers increased, along with development of lupus nephritis as organ involvement, suggesting the correlation between changes in the B cells and the pathophysiology of SLE. Thus, in the patients in whom B cells were successfully depleted by rituximab therapy, the numbers of memory B cells and plasma cells, which express costimulatory molecules, remained suppressed for prolonged periods of time, even though the naive B cells recovered. These findings suggest that reconstitution of the peripheral B cell compartment is crucial for sustaining longterm SLE remission and that recovery of memory B cells expressing costimulatory molecules precedes the SLE relapse.

A significant finding was that rituximab used to produce B cell depletion also affected T cell differentiation and activation. In cases with highly active SLE complicated by

lupus nephritis or CNS disorders, findings suggestive of T cell subset involvement in the pathophysiology of SLE have been reported, such as reduction in the population of naive T cells and an inverse correlation with the antibody-forming potential[23,24,25,26,27]. In patients with sustained SLE remission for 2 years after rituximab treatment, however, there were significant increases in the peripheral blood CD4-positive and CD4+CD45RA+ naive T cells. Further, although no changes were seen in the number of CD4+CD45RO+ memory T cells, the expression of CD45RO decreased ($CD45RO^{bright}$ to $CD45RO^{intermediate}$), suggesting the reduced activation potential of the cells. In fact, reduction or disappearance of the expression of CD69 and the costimulatory molecules CD40L and ICOS was noted. As described, activated B cells in patients with SLE showed enhanced expression of MHC class II antigens and costimulatory molecules and an antigen-presenting potential close to that of dendritic cells, suggesting T cell activation. However, the costimulatory molecule-expressing B cells disappeared, thereby reducing the costimulatory molecule-expressing memory T cells, a change that probably contributes to longterm remission in patients with SLE.

The case of Patient 6 in this study is interesting because the SLE relapse was associated with predominant T cell abnormalities. With regard to the clinical presentation of this patient, there were marked systemic symptoms such as fever (over 38°C), polyarthritis, and lymphadenopathy, along with CNS involvement. However, this patient cannot be viewed as a specific or extraordinary case of SLE. The fact that an increase in the memory T cells and an increase in the levels of ICOS on the CD4-positive cells preceded the relapse of SLE, without any changes in the number of B cells, B cell subsets, or surface antigen expression, indicated that T cell activation was predominantly involved in the SLE relapse.

Many clinical studies revealed that some patients do not benefit at all from peripheral B cell depletion therapy with rituximab[1,2,7,13]. When those findings are considered with our findings, it would appear that the existence of T cell-dependent/B cell-independent abnormalities may be involved in the pathogenesis of SLE. This may reflect the heterogeneity in the pathophysiology of SLE. Indeed, the patient with B cell-dominant relapse in our study responded well to retreatment with rituximab, and a favorable outcome of the patient with T cell-dominant relapse was obtained following treatment with IVCY. Thus, a higher efficacy of B cell-targeted therapy may be obtained in patients with B cell-dominant SLE, while T cell-targeted therapy may be needed for patients with T cell-dominant SLE.

Our findings support the notion that activated T cells, in addition to activated B cells, may be potentially involved in the pathogenesis of SLE, and that interaction between activated B cells and T cells may worsen the pathophysiology of SLE. Depletion of B cells by rituximab may result in the reconstitution of B cells in the peripheral compartment. That could cause inhibition of T cell activation and differentiation mediated by memory B cells, which in turn might lead to longterm remission of SLE.

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REFERENCES

1. Looney RJ, Anolik JH, Campbell D, Felgar RE, Young F, Arend LJ, et al. B cell depletion as a novel treatment for systemic lupus erythematosus: a phase I/II dose-escalation trial of rituximab. Arthritis Rheum 2004;50:2580-9.
2. Leandro MJ, Edwards JC, Cambridge G, Ehrenstein MR, Isenberg DA. An open study of B lymphocyte depletion in systemic lupus erythematosus. Arthritis Rheum 2002;46:2673-7.
3. Rastetter W, Molina A, White CA. Rituximab: expanding role in therapy for lymphomas and autoimmune diseases. Annu Rev Med 2004;55:477-503.
4. Anolik J, Sanz I, Looney RJ. B cell depletion therapy in systemic lupus erythematosus. Curr Rheumatol Rep 2003;5:350-6.
5. Tanaka Y, Yamamoto K, Takeuchi T, Nishimoto N, Miyasaka N, Sumida T, et al. A multicenter phase I/II trial of rituximab for refractory systemic lupus erythematosus. Mod Rheumatol 2007;17:191-7.
6. Tokunaga M, Fujii K, Saito K, Nakayamada S, Tsujimura S, Nawata M, et al. Down-regulation of CD40 and CD80 on B cells in patients with life-threatening systemic lupus erythematosus after successful treatment with rituximab. Rheumatology 2005;44:176-82.
7. Tokunaga M, Saito K, Kawabata D, Imura Y, Fujii T, Nakayamada S, et al. Efficacy of rituximab (anti-CD20) for refractory systemic lupus erythematosus involving the central nervous system. Ann Rheum Dis 2007;66:470-5.
8. Lu TY, Ng KP, Cambridge G, Leandro MJ, Edwards JC, Ehrenstein M, et al. A retrospective seven-year analysis of the use of B cell depletion therapy in systemic lupus erythematosus at University College London Hospital: the first fifty patients. Arthritis Rheum 2009;61:482-7.
9. Anolik JH, Barnard J, Cappione A, Pugh-Bernard AE, Felgar RE, Looney RJ, et al. Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. Arthritis Rheum 2004;50:3580-90.
10. Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, et al. A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus. J Immunol 2007;178:6624-33.
11. Cappione A, Anolik JH, Pugh-Bernard A, Barnard J, Dutcher P, Silverman G, et al. Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus. J Clin Invest 2005;115:3205-16.
12. Jacobi AM, Odendahl M, Reiter K, Bruns A, Burmester GR, Radbruch A, et al. Correlation between circulating CD27 high plasma cells and disease activity in patients with systemic lupus erythematosus. Arthritis Rheum 2003;48:1332-42.
13. Anolik JH, Barnard J, Owen T, Zheng B, Kemshetti S, Looney RJ, et al. Delayed memory B cell recovery in peripheral blood and lymphoid tissue in systemic lupus erythematosus after B cell depletion therapy. Arthritis Rheum 2007;56:3044-56.
14. Desai-Mehta A, Lu L, Ramsey-Goldman R, Datta SK. Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production. J Clin Invest 1996;97:2063-73.
15. Grammar AC, Slota R, Fischer R, Gur H, Girschick H, Yarboro C,

et al. Abnormal germinal center reactions in systemic lupus erythematosus demonstrated by blockade of CD154-CD40 interactions. J Clin Invest 2003;112:1506-20.
16. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, et al. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol 2000;1:475-82.
17. Skok J, Poudrier J, Gray D. Dendritic cell-derived IL-12 promotes B cell induction of Th2 differentiation: a feedback regulation of Th1 development. J Immunol 1999;163:4284-91.
18. Vallerskog T, Gunnarsson I, Widhe M, Risselada A, Klareskog L, van Vollenhoven R, et al. Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE. Clin Immunol 2007;122:62-74.
19. Sfikakis PP, Souliotis VL, Fragiadaki KG, Moutsopoulos HM, Boletis JN, Theofilopoulos AN. Increased expression of the FoxP3 functional marker of regulatory T cells following B cell depletion with rituximab in patients with lupus nephritis. Clin Immunol 2007;123:66-73.
20. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271-7.
21. Hay EM, Bacon PA, Gordon C, Isenberg DA, Maddison P, Snaith ML, et al. The BILAG index: a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus. Q J Med 1993;86:447-58.
22. Bencivelli W, Vitali C, Isenberg DA, Smolen JS, Snaith ML, Sciuto M, et al. Disease activity in systemic lupus erythematosus: report of the Consensus Study Group of the European Workshop for Rheumatology Research. III. Development of a computerised clinical chart and its application to the comparison of different indices of disease activity. The European Consensus Study Group for Disease Activity in SLE. Clin Exp Rheumatol 1992;10:549-54.
23. Morimoto C, Steinberg AD, Letvin NL, Hagan M, Takeuchi T, Daley J, et al. A defect of immunoregulatory T cell subsets in systemic lupus erythematosus patients demonstrated with anti-2H4 antibody. J Clin Invest 1987;79:762-8.
24. Sato K, Miyasaka N, Yamaoka K, Okuda M, Yata J, Nishioka K. Quantitative defect of CD4+2H4+ cells in systemic lupus erythematosus and Sjögren's syndrome. Arthritis Rheum 1987;30:1407-11.
25. Raziuddin S, Nur MA, Alwabel AA. Selective loss of the CD4+ inducers of suppressor T cell subsets (2H4+) in active systemic lupus erythematosus. J Rheumatol 1989;16:1315-9.
26. Tanaka S, Matsuyama T, Steinberg AD, Schlossman SF, Morimoto C. Antilymphocyte antibodies against CD4+2H4+ cell populations in patients with systemic lupus erythematosus. Arthritis Rheum 1989;32:398-405.
27. Mimura T, Fernsten P, Jarjour W, Winfield JB. Autoantibodies specific for different isoforms of CD45 in systemic lupus erythematosus. J Exp Med 1990;172:653-6.

Incidence and Risk Factors for Serious Infection in Patients with Rheumatoid Arthritis Treated with Tumor Necrosis Factor Inhibitors: A Report from the Registry of Japanese Rheumatoid Arthritis Patients for Longterm Safety

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ABSTRACT. ***Objective.*** To compare tumor necrosis factor-α (TNF-α) inhibitors to nonbiological disease-modifying antirheumatic drugs (DMARD) for the risk of serious infection in Japanese patients with rheumatoid arthritis (RA).

Methods. Serious infections occurring within the first year of the observation period were examined using the records for patients recruited to the Registry of Japanese Rheumatoid Arthritis Patients for Longterm Safety (REAL), a hospital-based prospective cohort of patients with RA. The analysis included 1144 patients, 646 of whom were treated with either infliximab or etanercept [exposed group: 592.4 patient-years (PY)]. The remaining 498 patients received nonbiological DMARD with no biologics (unexposed group: 454.7 PY).

Results. In the unexposed group, the incidence rate for all serious adverse events (SAE) was 9.02/100 PY and for serious infections, 2.64/100 PY. In the exposed group, SAE occurred in 16.04/100 PY and serious infections in 6.42/100 PY. The crude incidence rate ratio comparing serious infections in the exposed group with the unexposed group was 2.43 (95% CI 1.27–4.65), a significant increase. A multivariate analysis revealed that the use of TNF inhibitors is a significant independent risk factor for serious infection (relative risk 2.37, 95% CI 1.11–5.05, p = 0.026).

Conclusion. Our study has provided the first epidemiological data on Japanese patients with RA for the safety of TNF inhibitors compared to nonbiological DMARD for up to 1 year of treatment. Anti-TNF therapy was associated with a significantly increased risk for serious infections, compared to treatment with nonbiological DMARD. (First Release April 15 2011; J Rheumatol 2011; 38:1258–64; doi:10.3899/jrheum.101009)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
TUMOR NECROSIS FACTOR-α
DRUG TOXICITY
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The introduction of tumor necrosis factor-α (TNF) inhibitors for treatment of rheumatoid arthritis (RA) is a major therapeutic breakthrough[1]. Because biologics, including TNF inhibitors, have become important and widely used clinical tools for treatment of RA, assessment of their safety is important. There are significant concerns relating to the association between opportunistic infections and TNF inhibitors. One example of this association is the observed reactivation of latent tuberculosis[2]. Serious bacterial, granulomatous, and fungal infections have also been reported to be associated with TNF inhibitor use[3,4].

To develop the safety profiles of biologics, several groups from Europe and the United States have established registries for patients receiving these drugs. Some of these have reported elevated risk for infections in patients with RA treated with biologics, including TNF inhibitors, compared to treatment with nonbiological disease-modifying antirheumatic drugs (DMARD)[5,6,7,8,9,10,11]. To date, there has been no comparable report on the safety of biologics for Asian patients with RA. Because racial and geographic differences occur in morbidities of such infections as *Mycobacterium tuberculosis*, the *Coccidioides* species, and *Pneumocystis jirovecii*, the development of a defined safety profile for treatment with biologics in each geographic area is crucial for clinicians[12,13,14,15].

In Japan, postmarketing surveillance programs of all cases treated with infliximab and etanercept were implemented, revealing several important safety concerns for these TNF inhibitors during the first 6 months of the therapy. These studies identified infection as the most important serious adverse event (SAE) during treatment with the TNF inhibitors[16,17]. These studies, however, had serious deficiencies related to the absence of appropriate comparator groups and the short tracking period. We therefore established the Registry of Japanese Rheumatoid Arthritis Patients for Longterm Safety (REAL) database in 2005 to compare the safety of midterm to longterm treatment with biological DMARD to treatment with nonbiological DMARD.

The primary purpose of our study was to use the REAL database to compare the incidence of serious infections between TNF inhibitor-treated and nonbiological DMARD-treated patients with RA. A second objective was to identify independent risk factors for serious infections in this population.

MATERIALS AND METHODS

Data source. The REAL database is a hospital-based prospective cohort of patients with RA administered by the Department of Pharmacovigilance of the Tokyo Medical and Dental University. The ethics committee of the Tokyo Medical and Dental University Hospital and those of the participating institutions approved our study. Twenty-three institutions participate in REAL, including 15 university hospitals and 8 referring hospitals. Enrollment to the REAL database began in June 2005.

The criteria for admission to the REAL database include those patients (1) meeting the 1987 American College of Rheumatology criteria for RA; (2) ≥ 20 years old and able and willing to provide written informed consent and comply with the requirements of the protocol, or, for those patients < 20 years, having parents or legal guardians willing and able to provide written informed consent and to comply with the requirements of the protocol; and (3) starting treatment with biologics (the exposed group) or nonbiological DMARD (the nonexposed group) at the time of study entry. In addition, patients receiving treatment with nonbiological DMARD at the time of study entry are also enrolled as the nonexposed group. Exclusion criteria include (1) patient participation in a clinical trial for approval of drugs at the time of enrollment or during the followup in the study, and (2) patients withdrawing consent to join the study. We identified all patients who were registered from the participating hospitals of our study to the postmarketing surveillance programs for each biological DMARD that were implemented by the corresponding pharmaceutical companies. Participating physicians at each hospital enrolled all of these patients to the REAL database. In addition, patients who fulfilled the inclusion criteria were consecutively recruited for both groups by participating physicians at each hospital.

Exposed group. Because infliximab was introduced in Japan in 2003, etanercept in 2005, and adalimumab and tocilizumab in 2008, few data for patients receiving adalimumab or tocilizumab were available in the REAL database at the time we conducted our study. We therefore included only

those patients with RA who had started infliximab or etanercept at enrollment in the REAL database. Nonbiological DMARD were used for these patients at the attending physicians' discretion. Six hundred forty-six patients were enrolled in the exposed group. Patients who switched from infliximab to etanercept or etanercept to infliximab were included in the analysis using the combined time of the treatment. For those patients no longer receiving either infliximab or etanercept, only the time of actual use of these TNF inhibitors was analyzed.

Unexposed group. Four hundred ninety-eight patients were enrolled in the unexposed group. At the time of enrollment in our study, 57.6% of the patients in the unexposed group were being treated with methotrexate (MTX), 20.3% with salazosulfapyridine, 18.7% with tacrolimus, and 13.9% with bucillamine. Nonbiological DMARD used in fewer than 10 patients were leflunomide, actarit, gold salt, auranofin, mizoribine, D-penicillamine, and cyclosporine. Sixty-four patients (12.9%) of the unexposed group were given combination therapy with > 1 nonbiological DMARD agent during the observation period. Some patients who were initially enrolled in the unexposed group received biologics when clinically indicated; the time period following this change was excluded from the analysis.

Data collection. Each patient's recorded baseline data included demography, disease activity, comorbidities, treatments, and laboratory data at the start of the observation period. The same followup forms were used for both groups and included queries about RA disease activity, treatments, laboratory data, and occurrence and details of SAE. The followup forms were submitted every 6 months by the participating physicians to the REAL Data Center at the Department of Pharmacovigilance of Tokyo Medical and Dental University. The participating physicians in each hospital confirmed their submitted data to the REAL Data Center. Data were retrieved from the REAL database on November 30, 2008, for our study.

Baseline characteristics of patients. The observation period for 646 patients in the exposed group was 592.4 patient-years (PY). For 498 patients in the unexposed group, the observation period was 454.7 PY. In the exposed group, 300 patients (272.1 PY) received infliximab but not etanercept and 343 patients (320.3 PY) received etanercept but not infliximab. Three patients were switched from infliximab to etanercept during the observation period. The median length of the observation period was 1 year in both groups, and the percentage of patients followed up for a year was 83.1% in the exposed and 82.1% in the unexposed group. Minimal duration of followup was 2 months in the unexposed group and 3 months in the exposed group. The primary reason for not having at least a full year of followup in about 18% of the patients was that they were enrolled in the REAL database for < 1 year before November 30, 2008, when the data were retrieved from the database. Baseline data at the start of the observation period for the patients are shown in Table 1. Compared to the unexposed group, the exposed group was younger ($p < 0.001$), had more severe disease activity ($p < 0.001$), was treated with higher dosages of MTX ($p < 0.001$) and corticosteroids ($p = 0.001$), and had failed a larger number of DMARD ($p < 0.001$). Percentages of the patients on their first DMARD at baseline were 30.1% for the unexposed group and 24.0% for the exposed group ($p < 0.012$). Significantly more patients having comorbidities, including chronic pulmonary diseases ($p = 0.046$) and diabetes ($p = 0.024$), were seen in the exposed group compared to the unexposed group.

Definition of SAE. Our definition of an SAE was based on events described in the report by the International Conference on Harmonization[18]. In addition, bacterial infections that required intravenous administration of antibiotics, as well as opportunistic infections, including tuberculosis, *P. jirovecii* pneumonia (PCP), systemic fungal infection, cytomegalovirus infection, and herpes zoster were also regarded as SAE. The diagnosis of infections was based on a physician's clinical diagnosis, a comprehensive evaluation based on physical findings, laboratory data, and radiological examinations. The detection of infectious pathogens was not mandatory for making a diagnosis of infection. SAE were classified using the System Organ Class (SOC) of the Medical Dictionary for Regulatory Activities (MedDRA; version 11.1).

Table 1. Comparison of patients with rheumatoid arthritis (RA) treated with (exposed) and without (unexposed) the tumor necrosis factor-α (TNF) inhibitors infliximab or etanercept at the start of the observation period. Values are mean ± SD unless otherwise stated.

Characteristics	Exposed Group, n = 646	Unexposed Group, n = 498	p
Age, yrs	58.3 ± 13.2	61.4 ± 12.8	< 0.001
Women, %	82.0	83.3	0.568
Disease duration, yrs	9.5 ± 8.6	9.2 ± 9.2	0.654
Steinbrocker stage (III or IV), %	55.1	43.8	< 0.001
DAS28 (3/CRP)	3.9 ± 1.0, n = 642	2.8 ± 1.0, n = 495	< 0.001
MTX use, %	69.0	60.2	0.002
MTX dose, mg/wk	7.6 ± 2.2	6.4 ± 2.0	< 0.001
MTX dose > 8 mg/wk, %	11.1	5.0	< 0.001
Use of immunosuppressive drugs, except for MTX, %*	3.7	20.5	< 0.001
Corticosteroid use, %**	71.4	62.0	0.001
Prednisolone dose, mg/day	5.7 ± 3.0	4.6 ± 2.1	< 0.001
> 7.5 mg prednisolone/day, %	13.6	3.1	< 0.001
No of failed DMARD	1.6 ± 1.1	1.3 ± 1.1	< 0.001
Chronic pulmonary disease, %***	21.4	16.7	0.046
Diabetes, %	10.7	6.8	0.024

* Including tacrolimus, leflunomide, mizoribine, and cyclosporine. ** Converted to corresponding prednisolone dosage. *** Including interstitial pneumonia, chronic obstructive pulmonary disease, bronchial asthma, prior pulmonary tuberculosis, and bronchiectasis. DAS28: 28-joint count Disease Activity Score; CRP: C-reactive protein; MTX: methotrexate; DMARD: disease-modifying antirheumatic drug. † Number of DMARD that were tried but did not bring about a response.

Statistical analyses. Serious infections observed within the first year of the observation period were analyzed for each patient. The observation period for the present analysis was defined as follows: for patients who initiated treatment with the TNF inhibitors infliximab or etanercept or nonbiological DMARD at the time of study entry, the start of the observation period was the date these agents were first administered; for patients receiving the treatment with nonbiological DMARD at the time of study entry, the start of the observation period was the date of their enrollment in the REAL database. The observations ended 1 year after the start of the observation period, or on the day a patient died or met the exclusion criteria, or for the exposed group, no longer received either infliximab or etanercept, or for the unexposed group, started biologics, whichever came first. Patients were not removed even after the development of SAE as long as they did not meet the above criteria for censoring a patient. Considering the time it takes for pharmacokinetic/pharmacodynamic effects and data to appear from previous studies of at-risk periods[6], we considered any SAE occurring within 90 days after the last administration of infliximab or etanercept that was within the first year of the observation period to be attributable to the effects of the TNF inhibitors. Because the length of the at-risk period (90 days) after the date of discontinuation of treatment is more than 10 times as long as the half-lives of the 2 TNF inhibitors (i.e., 8.1 days for infliximab and 4.8 days for etanercept), we defined the date of drug discontinuation as the date of last administration, instead of the date of the first missed dose, which was the method used by another study[6]. The same number of SAE was found in the exposed group of our study using either definition for the date of drug discontinuation (data not shown). The date of the last administration of infliximab or etanercept was retrieved from medical records and reported by the participating physicians.

The incidence rates (IR) per 100 PY and incidence rate ratios (IRR) with their 95% CI were calculated. For univariate analysis, the chi-squared

test for categorical variables and the Student t-test or Mann-Whitney U tests for continuous variables were used for comparisons among groups. For multivariate analysis, Poisson regression analyses were used to estimate the risk of serious infection with the TNF inhibitors infliximab and etanercept, and to identify any variable having a significant and independent influence on the development of serious infections. Variables that were included in the multivariate analysis were chosen using the results of univariate analysis. The analyses were conducted using SPSS (version 16.0, SPSS Inc., Chicago, IL, USA) and R statistical language software (version 2.8.1, R Foundation for Statistical Computing, Vienna, Austria). All p values were 2-tailed and $p < 0.05$ was considered statistically significant.

RESULTS

Types and incidence rates of SAE. One hundred thirty-six SAE were reported during the observation period, 41 in the unexposed group and 95 in the group exposed to infliximab or etanercept. Based on the SAE categories classified using the SOC, infections and infestations were the most common, followed by injury, poisoning, and procedural complications, in which fractures accounted for 76% (Table 2). In the exposed group, there were 38 serious infections including 25 bacterial, 11 opportunistic (6 cases of herpes zoster, 3 PCP, 1 pulmonary cryptococcosis, and 1 pulmonary nontuberculous mycobacterial infection), and 2 other infections. In the unexposed group, 12 serious infections occurred, including 8 bacterial, 3 opportunistic (1 each PCP, pulmonary tuberculosis, and pulmonary nontuberculous mycobacterial infection), and 1 viral infection. The respiratory system was the most frequent infection site (23 for the exposed group and 9 for the unexposed group), followed by skin and subcutaneous tissue (9 for the exposed and 1 for the unexposed), urinary tract (1 for each group), and bone and joints (1 for each group). The rates of treatment discontinuation after serious infections were 2.19/100 PY in the exposed group and 0.22/100 PY in the unexposed group. The rate ratio comparing the exposed group with the unexposed group was 9.98 (95% CI 1.31–76.29), a significant elevation. On the other hand, the rates of treatment discontinuation after SAE other than serious infections were not statistically different between the 2 groups [1.86/100 PY in the exposed group and 0.66/100 PY in the unexposed group; the rate ratio was 2.81 (95% CI 0.79–10.09)].

Table 2. Categories of serious adverse events (SAE) using the system organ class (SOC).

System Organ Class Allocation	No. SAE in Study Patients: Exposed Group, n = 646	Unexposed Group, n = 498	Total
Cardiac disorders	2	1	3
Endocrine disorders	1	0	1
Eye disorders	1	1	2
Gastrointestinal disorders	6	4	10
General disorders and administration site conditions	2	1	3
Hepatobiliary disorders	4	4	8
Infections and infestations	38	12	50
Injury, poisoning, and procedural complications	12	5	17
Metabolism and nutrition disorders	0	1	1
Musculoskeletal and connective tissue disorders	1	1	2
Neoplasms benign, malignant, and unspecified	4	5	9
Nervous system disorders	1	1	2
Renal and urinary disorders	3	2	5
Reproductive system and breast disorders	1	0	1
Respiratory, thoracic, and mediastinal disorders	14	2	16
Skin and subcutaneous tissue disorders	2	1	3
Vascular disorders	3	0	
Total	95	41	136

In the exposed group, the IR of SAE was 16.04/100 PY and the IR of serious infection was 6.42/100 PY. The crude IRR comparing the exposed group with the unexposed group for SAE was 1.78 (95% CI 1.23–2.57) and for serious infections was 2.43 (95% CI 1.27–4.65); both of these IRR were significantly elevated (Table 3).

Contribution of TNF inhibitors to the development of serious infections. Because the background data of the patients differed considerably between the exposed and unexposed groups (Table 1), we performed univariate analysis to identify candidate risk factors for the development of serious infections (data not shown) and selected age, chronic pulmonary diseases, Steinbrocker stage[19], disease activity, corticosteroid dosage, and MTX dosage as covariates for multivariate analyses. We used the Poisson regression model to evaluate the risk for development of serious infection from the use of TNF inhibitors. The use of TNF inhibitors was found to constitute a significant risk factor for serious infection. The relative risk (RR) was 2.37 (95% CI 1.11–5.05, $p = 0.026$; Table 4).

Among the confounding factors, we found that these factors were independently associated with development of serious infection (Table 4): increasing age (RR 1.82 per 10-year increment; 95% CI 1.32–2.52; $p = 0.00031$), chronic pulmonary diseases (RR 2.61; 95% CI 1.38–4.94; $p = 0.0031$), advanced disease (Steinbrocker stage III or IV; RR 2.07; 95% CI 1.07–3.97; $p = 0.03$), and dosage of MTX > 8 mg/week (RR 2.61; 95% CI 1.40–4.86; $p = 0.0024$). When the dosages of MTX and prednisolone (PSL) were recategorized as MTX use (yes/no), MTX dosage > 6 mg/week (yes/no), PSL use (yes/no), and PSL dosage > 5 mg/day (yes/no), or were used as continuous variables, the analyses gave essentially the same results (data not shown).

Risk factors for infection during treatment with the TNF inhibitors infliximab or etanercept. To identify the risk factors contributing to the development of serious infections during treatment with infliximab or etanercept, we compared the background data of those patients who did or did not develop serious infections, using univariate analyses (Table 5). The patients who developed serious infections

Table 3. Number and incidence of serious adverse events (SAE) in patients with rheumatoid arthritis who were treated with (exposed) and without (unexposed) the tumor necrosis factor-α inhibitors infliximab or etanercept.

Event	Exposed Group, n = 646 592.35 PY	Unexposed Group, n = 498 454.74 PY	Crude IRR (95% CI)
All SAE, no. events	95	41	0
IR (/100 PY)	16.04 (12.81–19.26)	9.02 (6.26–11.78)	1.78 (1.23–2.57)
Serious infection, no. events	38	12	
IR (/100 PY)	6.42 (4.38–8.46)	2.64 (1.15–4.13)	2.43 (1.27–4.65)
Serious respiratory tract infection, no. events	23	9	
IR (/100 PY)	3.88 (2.30–5.47)	1.98 (0.69–3.28)	1.96 (0.91–4.24)

PY: patient-years; IR: incidence rate; IRR: incidence rate ratio.

Table 4. Multivariate analysis of independent risk factors for serious infections in the Registry of Japanese Rheumatoid Arthritis Patients for Longterm Safety (REAL) database. The relative risk (RR) of biologics for development of serious infection for up to 1 year of the observation period was calculated using the Poisson regression model after adjusting for the confounding factors of age, chronic pulmonary disease, Steinbrocker stage, disease activity, corticosteroid dosage, and methotrexate dosage.

	RR (95% CI)	p
TNF inhibitor* (yes)	2.37 (1.11–5.05)	0.026
Age, by decade	1.82 (1.32–2.52)	0.00031
Chronic pulmonary disease (yes)	2.61 (1.38–4.94)	0.0031
Stage III or IV (vs Stage I or II)**	2.07 (1.07–3.97)	0.030
MTX dose > 8.0 mg/wk	2.61 (1.40–4.86)	0.0024
DAS28 (3/CRP)	0.87 (0.66–1.14)	0.31
Prednisolone dose > 7.5 mg/day	1.21 (0.58–2.55)	0.61

* Infliximab or etanercept. ** Steinbrocker classification[19] was used to define RA disease stages. TNF: tumor necrosis factor-α; DAS28: 28-joint count Disease Activity Score; CRP: C-reactive protein; MTX: methotrexate.

Table 5. Comparison of background data for patients with rheumatoid arthritis (RA) who were treated with the tumor necrosis factor inhibitors infliximab or etanercept. Values are mean ± SD, unless otherwise stated.

Factors	Infection, n = 612	Without Infection, n = 34	p
Age, yrs	57.9 ± 13.3	67.1 ± 8.1	< 0.001
Women, %	82.0	82.4	0.961
RA disease duration, yrs	9.3 ± 8.5	13.0 ± 10.2	0.008
Steinbrocker stage (III or IV), %*	53.9	76.4	0.010
DAS28 (3/CRP)	3.9 ± 1.0	3.7 ± 1.2	0.356
MTX dose mg/wk	5.2 ± 3.9	5.6 ± 4.2	0.387
Use of immunosuppressive drugs except for MTX, %**	3.8	2.9	0.636
Corticosteroid use, %	62.0	71.4	0.001
Prednisolone dose, mg/day***	4.0 ± 3.6	4.7 ± 3.4	0.214
Chronic pulmonary disease, %†	20.4	38.2	0.014
Diabetes, %	10.3	17.6	0.143

* Steinbrocker classification[19] was used to define RA disease stages. ** Including tacrolimus, leflunomide, mizoribine, and cyclosporine. *** Converted to corresponding prednisolone dosage. † Including interstitial pneumonia, chronic obstructive pulmonary disease, bronchial asthma, prior pulmonary tuberculosis, and bronchiectasis. DAS28: 28-joint count Disease Activity Score; CRP: C-reactive protein; MTX: methotrexate.

were significantly older ($p < 0.001$) and had longer disease duration ($p = 0.008$) as well as more advanced disease (Steinbrocker stage III or IV; $p = 0.01$). The percentages of patients given corticosteroids and having chronic pulmonary diseases were higher for patients who developed serious infections. The contributions of age, disease duration, corticosteroid use, and chronic pulmonary disease to the development of serious infections in the exposed group were analyzed using the Poisson regression model. This multivariate analysis showed increasing age per 10-year increment (RR 1.97; 95% CI 1.34–2.84) and the use of corticosteroids (RR 2.97; 95% CI 1.04–8.50) to be significantly associated ($p = 0.00058$ and $p = 0.042$, respectively) with the development of serious infection during TNF inhibitor therapy.

DISCUSSION

In our prospective study of a Japanese hospital-based cohort of patients with RA, the multivariate analysis demonstrated that treatment with the biologic TNF inhibitors infliximab or etanercept was associated with an increased risk for serious infections. Increasing age, chronic pulmonary diseases, an advanced disease stage of RA, and dosage of MTX were also identified as independent risk factors for serious infections in this population.

The IR of serious infection in the exposed group (6.4/100 PY; 95% CI 4.4–8.5) is comparable to those reported previously [6.2–6.4/100 PY from a German RA registry and 6.1/100 PY (95% CI 5.7–6.5) from a British RA registry][5,6]. Our data were also consistent with the results of the postmarketing surveillance programs in Japan, which found the IR of serious infection during the first 6 months of anti-TNF therapy was 8.1/100 PY in patients treated with infliximab and 7.7/100 PY for those treated with etanercept[16,17,20]. Schneeweiss, *et al*[8] reported a lower IR for serious infection, 4.8/100 PY (95% CI 3.1–6.6), in patients receiving TNF inhibitors. This difference from our results can probably be

explained by variations in such methodologies as inclusion criteria or definition of infectious events. Schneeweiss, *et al*[8] focused on hospitalizations of elderly patients due to serious bacterial infections while being treated with TNF inhibitors. The IR of SAE and serious infections in the unexposed group of our study were similar to those of other clinical trials conducted in Japan[21,22,23], as well as to those reported from 4 European registries (IR 2.3–3.9/100 PY)[5,6,8,9]. Thus, we postulate that our results did not underestimate the risk of serious infections during treatment with nonbiological DMARD. Examining the infection sites in our study, the respiratory system was the most frequent site for both exposed and unexposed groups, followed by skin and subcutaneous tissue, which is consistent with other epidemiological studies of patients with RA[7,24].

Evaluating patients with RA for predisposing factors for infection prior to initiating TNF inhibitor therapy is important. The independent risk factors identified in our study were in overall agreement with previous reports of predictors of infection among patients with RA[25]. First, the association of corticosteroid use with serious infection, as shown by the multivariate analysis of the exposed group, is consistent with several reports describing corticosteroid use as an important risk factor for infection[8,9]. The relatively low number and rate of serious infections in the unexposed group probably resulted in a lack of enough power to detect the risk from corticosteroid in the analysis of the total population of our study. Second, finding an association between Steinbrocker stage and increased risk for serious infection is also supported by the results of the postmarketing surveillance of infliximab in Japanese patients with RA, which found that Steinbrocker stage III or IV was a predictor for bacterial pneumonia by multiple logistic regression analysis[16]. It has been reported that the Health Assessment Questionnaire (HAQ) score is associated with serious infection in patients with RA[7,11]. Because the HAQ comprises disease activity-related and joint damage-related components[26], it is plausible that joint damage can be a risk factor for serious infection. The results of our study and those of postmarketing surveillance of infliximab in Japan[16] support this concept. Third, we found that MTX dosage was associated with increased risk of serious infection; however, this association disappeared when the unexposed and exposed groups were analyzed separately. According to some reports using cohorts much larger than ours, the immunosuppressive DMARD, such as leflunomide, cyclosporine, and azathioprine, were associated with an increased risk of infection, but MTX was not[8,27]. Others have found the use of MTX to be a risk factor for infection in patients with RA[28]. Further studies are needed to assess any association between MTX dosage and serious infection in a larger number of Japanese patients with RA.

Our study provides the first pharmacoepidemiological evidence of the safety of treatment with the TNF inhibitors infliximab or etanercept in Japanese patients with RA, compared to nonbiological DMARD. In our study cohort, treatment with infliximab or etanercept was associated with increased risk for serious infections when compared to treatment with nonbiological DMARD. The results of our study suggest that careful pharmacovigilance procedures are essential to insure safe use of TNF inhibitors in patients with RA.

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APPENDIX

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REFERENCES

1. Smolen JS, Aletaha D, Koeller M, Weisman MH, Emery P. New therapies for treatment of rheumatoid arthritis. Lancet 2007;370:1861-74.
2. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med 2001;345:1098-104.
3. Wallis RS, Broder M, Wong J, Lee A, Hoq L. Reactivation of latent granulomatous infections by infliximab. Clin Infect Dis 2005;41 Suppl 3:S194-8.
4. Kroesen S, Widmer AF, Tyndall A, Hasler P. Serious bacterial infections in patients with rheumatoid arthritis under anti-TNF-alpha therapy. Rheumatology 2003;42:617-21.
5. Listing J, Strangfeld A, Kary S, Rau R, von Hinueber U, Stoyanova-Scholz M, et al. Infections in patients with rheumatoid arthritis treated with biologic agents. Arthritis Rheum 2005;52:3403-12.
6. Dixon WG, Symmons DP, Lunt M, Watson KD, Hyrich KL, Silman AJ. Serious infection following anti-tumor necrosis factor alpha therapy in patients with rheumatoid arthritis: lessons from interpreting data from observational studies. Arthritis Rheum 2007;56:2896-904.
7. Dixon WG, Watson K, Lunt M, Hyrich KL, Silman AJ, Symmons DP. Rates of serious infection, including site-specific and bacterial intracellular infection, in rheumatoid arthritis patients receiving anti-tumor necrosis factor therapy: results from the British Society for Rheumatology Biologics Register. Arthritis Rheum 2006;54:2368-76.
8. Schneeweiss S, Setoguchi S, Weinblatt ME, Katz JN, Avorn J, Sax PE, et al. Anti-tumor necrosis factor alpha therapy and the risk of serious bacterial infections in elderly patients with rheumatoid arthritis. Arthritis Rheum 2007;56:1754-64.
9. Curtis JR, Patkar N, Xie A, Martin C, Allison JJ, Saag M, et al. Risk of serious bacterial infections among rheumatoid arthritis

patients exposed to tumor necrosis factor alpha antagonists. Arthritis Rheum 2007;56:1125-33.
10. Curtis JR, Xi J, Patkar N, Xie A, Saag KG, Martin C. Drug-specific and time-dependent risks of bacterial infection among patients with rheumatoid arthritis who were exposed to tumor necrosis factor alpha antagonists. Arthritis Rheum 2007;56:4226-7.
11. Askling J, Fored CM, Brandt L, Baecklund E, Bertilsson L, Feltelius N, et al. Time-dependent increase in risk of hospitalisation with infection among Swedish RA patients treated with TNF antagonists. Ann Rheum Dis 2007;66:1339-44.
12. Bergstrom L, Yocum DE, Ampel NM, Villanueva I, Lisse J, Gluck O, et al. Increased risk of coccidioidomycosis in patients treated with tumor necrosis factor alpha antagonists. Arthritis Rheum 2004;50:1959-66.
13. Komano Y, Harigai M, Koike R, Sugiyama H, Ogawa J, Saito K, et al. Pneumocystis jiroveci pneumonia in patients with rheumatoid arthritis treated with infliximab: A retrospective review and case-control study of 21 patients. Arthritis Rheum 2009;61:305-12.
14. Gomez-Reino JJ, Carmona L, Valverde VR, Mola EM, Montero MD. Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk: a multicenter active-surveillance report. Arthritis Rheum 2003;48:2122-7.
15. Harigai M, Koike R, Miyasaka N. Pneumocystis pneumonia associated with infliximab in Japan. N Engl J Med 2007; 357:1874-6.
16. Takeuchi T, Tatsuki Y, Nogami Y, Ishiguro N, Tanaka Y, Yamanaka H, et al. Postmarketing surveillance of the safety profile of infliximab in 5000 Japanese patients with rheumatoid arthritis. Ann Rheum Dis 2008;67:189-94.
17. Koike T, Harigai M, Inokuma S, Inoue K, Ishiguro N, Ryu J, et al. Postmarketing surveillance of the safety and effectiveness of etanercept in Japan. J Rheumatol 2009;36:898-906.
18. U.S. Food and Drug Administration. E2B(R) clinical safety data management: Data elements for transmission of individual case safety reports. [Internet. Accessed Feb 28, 2011.] Available from: http://www.fda.gov/RegulatoryInformation/Guidances/ucm129371.htm
19. Steinbrocker O, Traeger CH, Batterman RC. Therapeutic criteria in rheumatoid arthritis. J Am Med Assoc 1949;140:659-62.
20. Koike T, Harigai M, Inokuma S, Inoue K, Ishiguro N, Ryu J, et al. Safety outcomes from a large Japanese post-marketing surveillance for etanercept [abstract]. Arthritis Rheum 2007;56 Suppl:S182.
21. Kawai S, Hashimoto H, Kondo H, Murayama T, Kiuchi T, Abe T. Comparison of tacrolimus and mizoribine in a randomized, double-blind controlled study in patients with rheumatoid arthritis. J Rheumatol 2006;33:2153-61.
22. Hara M, Abe T, Sugawara S, Mizushima Y, Hoshi K, Irimajiri S, et al. Efficacy and safety of iguratimod compared with placebo and salazosulfapyridine in active rheumatoid arthritis: a controlled, multicenter, double-blind, parallel-group study. Mod Rheumatol 2007;17:1-9.
23. 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.
24. Doran MF, Crowson CS, Pond GR, O'Fallon WM, Gabriel SE. Frequency of infection in patients with rheumatoid arthritis compared with controls: a population-based study. Arthritis Rheum 2002;46:2287-93.
25. Doran MF, Crowson CS, Pond GR, O'Fallon WM, Gabriel SE. Predictors of infection in rheumatoid arthritis. Arthritis Rheum 2002;46:2294-300.
26. Smolen JS, Aletaha D, Grisar JC, Stamm TA, Sharp JT. Estimation of a numerical value for joint damage-related physical disability in rheumatoid arthritis clinical trials. Ann Rheum Dis 2010; 69:1058-64.
27. Wolfe F, Caplan L, Michaud K. Treatment for rheumatoid arthritis and the risk of hospitalization for pneumonia: associations with prednisone, disease-modifying antirheumatic drugs, and anti-tumor necrosis factor therapy. Arthritis Rheum 2006;54:628-34.
28. Greenberg JD, Reed G, Kremer JM, Tindall E, Kavanaugh A, Zheng C, et al. Association of methotrexate and tumour necrosis factor antagonists with risk of infectious outcomes including opportunistic infections in the CORRONA registry. Ann Rheum Dis 2010;69:380-6.

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Activation of the Activin A-ALK-Smad pathway in systemic sclerosis☆

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ABSTRACT

Systemic sclerosis (SSc) is a chronic disease of unknown etiology that is characterized by multiple tissue fibrosis. Transforming Growth Factor-beta (TGF-β) is thought to be the most important mediator that induces fibrosis. However, the molecular mechanisms by which fibrosis is induced have not been fully elucidated. In this study, the role of activin, a member of the TGF-β superfamily, was investigated in the pathogenesis of fibrosis in SSc. Serum activin A levels in patients with SSc were measured by ELISA, and the expression of the activin receptor type IB (ACVRIB/ALK4) and the activity of the signaling pathway via ACVRIB/ALK4 were investigated using western blotting. To evaluate a potential therapeutic strategy for SSc, we also attenuated the ACVRIB/ALK4 pathway using an inhibitor. Serum activin A levels were significantly higher in SSc patients than in normal controls. Activin A and ACVRIB/ALK4 expression were also higher in cultured SSc fibroblasts. Activin A stimulation induced phosphorylation of Smad2/3 and CTGF expression in SSc fibroblasts. Procollagen production and Col1α mRNA also increased upon stimulation by activin A. The basal level of Smad2/3 phosphorylation was higher in cultured SSc fibroblasts than in control cells, and treatment with the ALK4/5 inhibitor SB431542 prevented phosphorylation of Smad2/3 and CTGF expression. Furthermore, production of collagen was also induced by activin A. Activin A-ACVRIB/ALK4-Smad-dependent collagen production was augmented in SSc fibroblasts, suggesting the involvement of this signaling mechanism in SSc. Inhibition of the activin A-ACVRIB/ALK4-Smad pathway would be a new approach for the treatment of SSc.

1. Introduction

Systemic sclerosis (SSc), a systemic disorder characterized by tissue fibrosis of the skin and other organs, is often associated with extensive vascular damage resulting in Raynaud's phenomenon. The molecular mechanisms of fibrosis in SSc have been studied for many years. It is known that increased extracellular matrix (ECM) proteins, particularly type 1 collagen, vascular damage and aberrant immune activation are involved in the pathogenesis of fibrosis. However, the molecular mechanisms responsible have not yet been fully elucidated, although we do know that cytokines and growth factors are critical for the regulation of fibroblast activation. Because of its prominent profibrotic function, the transforming growth factor-beta (TGF-β) signaling pathway has been extensively studied [1–5]. Alterations to the TGF-β signaling pathway, including up-regulation of TGF-β receptor(s), have been reported [1–4] and suggest that the constitutively elevated synthesis of ECM is due to the overactivation of TGF-β signaling. TGF-β binds either to the type III TGF-β receptor (TβRIII), which presents it to the type II receptor (TβRII), or directly to TβRII on the cell membrane. The binding of TGF-β to TβRII then leads to binding of the type I TGF-β receptor (TβRI). This type I receptor is also known as activin receptor-like kinase 5 (ALK5), which is then phosphorylated by the type II receptor. After being activated, the TβRI/ALK5 protein kinase phosphorylates the transcription factors Smad2 or Smad3. Phosphorylated Smad2 or Smad3 bind to Smad4, the common Smad, and the resulting complex moves from the cytoplasm into the nucleus. In the nucleus, the Smad complex interacts in a cell-specific manner with various other transcription factors [6–8] and then trans-activates fibrosis-associated genes such as type I collagen and CTGF [6] [9].

Activin is a TGF-β superfamily member that was originally identified as an inducer of follicle-stimulating hormone (FSH) release from the pituitary. It is also a dimeric protein that consists of two activin β subunits and exists in three distinct forms: activin A (βAβA), activin B (βBβB), and activin AB (βAβB). All of these forms

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initially bind to the type II activin receptor (ACVRII or ACVRIIB) and then recruit the type I receptor (ACVRIB; ALK4). Receptor heterodimerization subsequently results in phosphorylation and activation of Smad2/3, as it does after activation by TGF-β [10]. Activin exerts many functions in cell proliferation, differentiation, apoptosis, metabolism, homeostasis, immune response, wound repair, and endocrine function [10]. With regard to connective tissue function, activin A is strongly expressed in wounded skin and acts in wound repair and skin morphogenesis through stimulation of keratinocytes and stromal cells [11]. Activin also participates in tissue regeneration by accelerating capillary formation and inducing fibroblast growth factor (FGF) expression [12], as demonstrated by the overexpression of activin A in the epidermis of transgenic mice and the resulting improved wound healing and decreased scar formation [11,13]. A series of studies has implicated activin A in the pathogenesis of fibrosis, especially bleomycin-induced pulmonary fibrosis [14–17] and fibrosis of the liver [18,19], pancreas [20], and kidney [21,22]. The mechanism of fibrosis by activin A is speculated to be via CTGF expression [12,23].

There is strong evidence for a genetic role in SSc pathology, but this evidence is still not compelling by traditional genetic principles. cDNA expression microarray analysis revealed that ACVRIB/ALK4 is highly expressed in SSc monozygotic twins [24], which prompted us to investigate the role of activin A in the regulation of SSc fibrosis. We found that activation of the activin A-ACVRIB/ALK4-Smad pathway plays a critical role in fibroblastic change in SSc and that this pathway would be a potential therapeutic target for SSc.

2. Materials and methods

2.1. Study participants

SSc patients were diagnosed using the classification criteria of the American College of Rheumatology and were classified into two subsets—the diffuse cutaneous type and the limited cutaneous type—according to LeRoy's criteria. Patients with overlapping syndromes were excluded from this study. The present study was approved by the ethical committee of our institution (Institute of Rheumatology, Tokyo Women's Medical University), and informed consent was obtained from all patients.

2.2. Cells and cell culture

Skin biopsies from the dorsal forearm were performed as a diagnostic procedure in patients with SSc of the diffuse cutaneous type and from age-and gender-matched healthy volunteers (normal controls) after informed consent. No medical interventions, including administration of corticosteroid or immunosuppressive agents, were performed before skin biopsy. After the skin biopsy specimen had been washed in phosphate-buffered saline (PBS) with penicillin/streptomycin in a Petri dish, the tissues were transferred to a new Petri dish, where minced pieces adhered to the plastic surface. RPMI1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) with penicillin/streptomycin was carefully added into a Petri dish after a 30 min incubation on room air. Minced tissues were cultured at 37 °C with 5% CO_2 for several days. When the cells spread around the minced tissues were of a sufficient quantity, they were detached with 0.05% trypsin and 0.5 mM EDTA and plated in 25 cm^2 culture flasks for proliferation. Cells were passaged at a split ratio of 1–3 after reaching high confluence.

Established fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (Invitrogen). Cells from the third to fifth passages were used in the current study.

2.3. Immunohistochemical staining

Indirect immunoperoxidase staining on formaldehyde-fixed, de-paraffinized tissue sections was performed using the Vectastain Elite kit (Vector, Burlingame, CA) with DAB substrate. Anti-ACVRIB/ALK4 antibody (MAB222, R & D Systems, Minneapolis, MN) was used as the primary antibody at a 1:50 dilution.

For immunocytological staining, cells were fixed with 4% paraformaldehyde and blocked with 5% horse serum. Anti-activin A antibody (AF338, R & D Systems) was used as the primary antibody, which was detected using horseradish peroxidase (HRP)-conjugated anti-rabbit antibody with DAB substrate.

2.4. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNAs were extracted using TRI Reagent (Invitrogen) according to the manufacturer's instructions. For real-time PCR analysis, the RNA was treated with DNase I (Invitrogen), and cDNA was generated using SuperScript III (Invitrogen) with Oligo dT primers. Real-time PCR analysis was carried out on Chromo4 (Bio-Rad, Hercules, CA) using the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) for COL1A and GAPDH.

2.5. Western blotting

Aliquots of 1×10^6 cells were washed with PBS and lysed in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.1% SDS, 0.05% sodium deoxycholate, 5 mM EDTA, and 10 mM Tris–HCl, pH 7.4) containing protease inhibitors. Protein concentration was measured using the DC protein assay (Bio-Rad). After being boiled with SDS sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, and 62.5 mM Tris–HCl, pH 6.8), 30 μg of protein was subjected to SDS-PAGE. To detect ACVRIB/ALK4, cells were directly lysed in SDS sample buffer with ultrasound sonication and then subjected to SDS-PAGE. After transfer to Cellulose Nitrate Membranes (Whatman, GE Healthcare, Pollards Wood, UK), the blots were blocked with 5% skim milk and probed with anti-Smad 2/3 antibody (Cell Signaling, Danvers, MA), anti-phospho-Smad2 (Ser 465/467, Cell Signaling), anti-CTGF (L-20, Santa Cruz, Santa Cruz, CA), anti-ACVRIB/ALK4 (ab78415, Abcam, Cambridge, UK) or anti-β-actin (Sigma–Aldrich, St. Louis, MO) antibodies. Primary antibodies were detected by binding HRP-conjugated anti-rabbit or -mouse second antibody (GE Healthcare) with ECL chemiluminescence (GE Healthcare).

2.6. Measurement of type I procollagen and activin A

Cultured fibroblasts were prepared at a density of 40,000 cells/well in 24-well culture plates with DMEM plus 10% FBS. After 24 h of culture, the medium was removed, and the cells were cultured in serum-free medium (QBSF-5, Sigma–Aldrich). Concentrations of type I procollagen in the fibroblast supernatants were measured using a Procollagen type I C peptide (PIP) EIA kit (TAKARA, Otsu, Japan). The activin A concentration in serum and cultured supernatant was measured using a Quantikine ELISA kit (R & D Systems).

2.7. Reagents

Recombinant human activin A (338-AC) and recombinant human follistatin were purchased from R & D Systems. ALK-4 inhibitor (SB431542) was purchased from TOCRIS Bioscience (Ellisville, MO).

2.8. Statistical analysis

The statistical analysis was carried out using non-parametric *t*-tests. The Wilcoxon test was performed to compare the paired treatments. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. ACVRIB/ALK4 is abundantly expressed in SSc patient fibroblasts

The expression level of ACVRIB/ALK4 was investigated by immunohistochemistry using skin biopsy specimens. Normal control and SSc patient-derived skin specimens both showed positive ACVRIB/ALK4 expression (Fig. 1A), but the amount of expression seen in the SSc-derived skin specimens was relatively higher. To more precisely evaluate the expression of ACVRIB/ALK4, we performed western blotting analysis using cultured fibroblasts established from normal control and sporadic SSc patients. The SSc fibroblasts showed strikingly increased expression of ACVRIB/ALK4 (Fig. 1B), suggesting ACVRIB/ALK4 involvement in SSc pathogenesis.

3.2. Activin a expression is elevated in skin fibroblasts and sera of SSc patients

We also investigated levels of activin A, the ligand for ACVRIB/ALK4, in fibroblasts, cultured serum, and serum from SSc patients. Although activin A was detected in both normal control and SSc-derived cultured fibroblasts (Fig. 2A), activin A secretion into cultured supernatant was much higher in SSc-derived fibroblasts than in control supernatant (Fig. 2B). Additionally, in a clinical setting, serum activin A levels were significantly higher in SSc patients than in normal controls, with diffuse cutaneous SSc having a much higher concentration of activin A serum levels than limited cutaneous SSc (Fig. 2C). These observations suggest that the degree of skin fibrosis may correlate with the level of serum activin.

3.3. The activin A-ACVRIB/ALK4 pathway is activated in SSc fibroblasts

The ACVRIB/ALK4 receptor signals through the Smad pathway, which was investigated using a phospho-specific antibody. Surprisingly, phosphorylation of Smad2/3 was strikingly augmented in SSc fibroblasts without any activin stimulation. The addition of a neutralizing antibody (MAB222) attenuated the phosphorylation of Smad2/3 in normal and SSc fibroblasts, while the addition of the ALK4/5 inhibitor SB431542 completely blocked this process (Fig. 3A). To gain further insight into the role of the activin A-ACVRIB/ALK4 signaling pathway in SSc pathogenesis, normal and SSc-derived fibroblasts were stimulated with activin A. Phosphorylation of Smad2/3 was decreased to undetectable levels after serum starvation with extensive washing with serum-free medium. Subsequent stimulation with activin A-induced phosphorylation of Smad2/3, and the addition of SB431542 blocked phosphorylation of Smad2/3

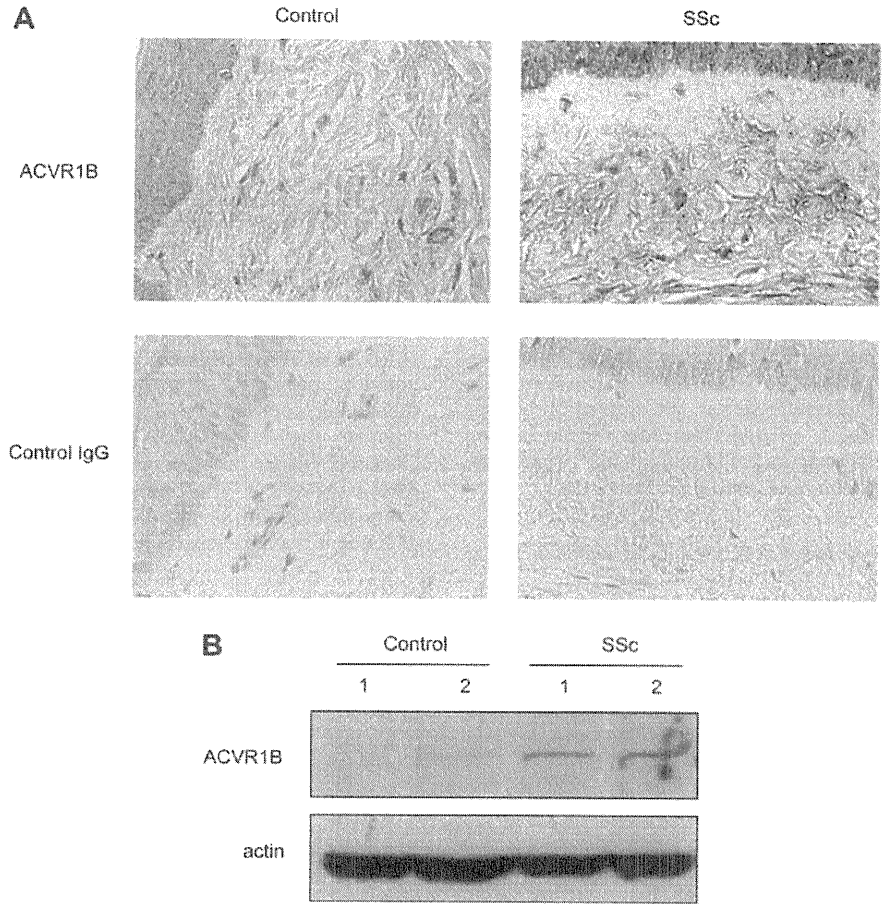


Fig. 1. A, Immunohistochemical analysis of ACVRIB/ALK4 expression in normal and SSc-derived skin specimens. B, Western blot analysis of ACVRIB/ALK4 expression (two normal control-derived fibroblasts and two SSc-derived fibroblasts). Representative data from duplicate experiments are shown.

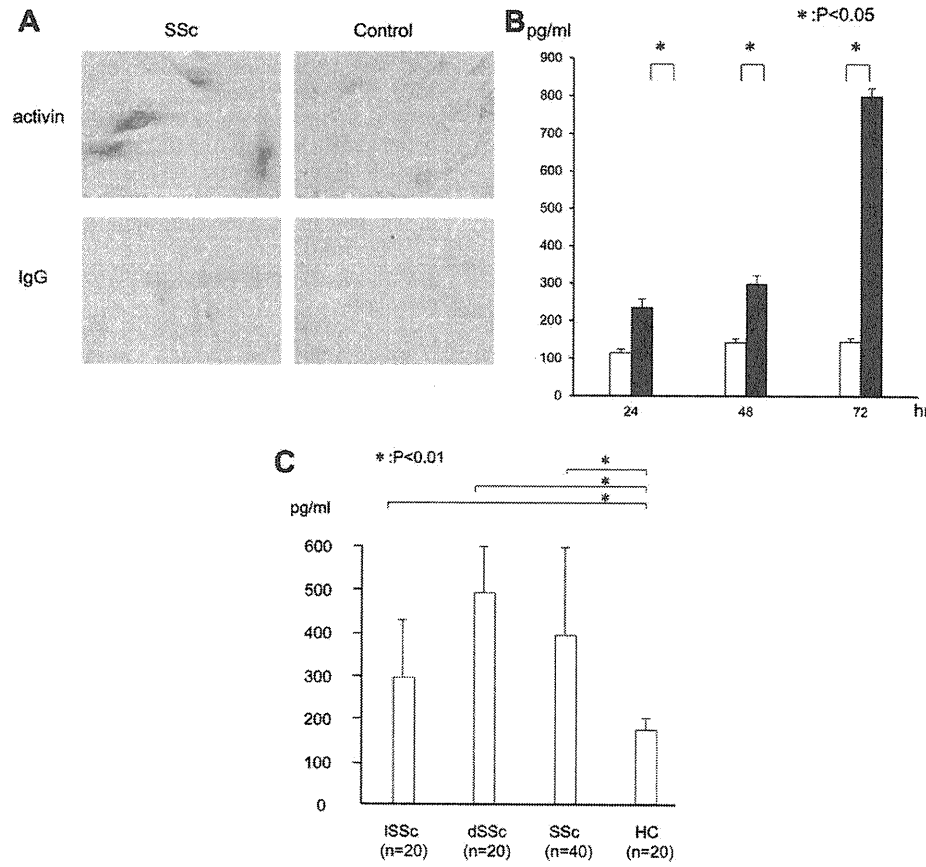


Fig. 2. A, Immunocytological staining of activin A. Normal and SSc-derived fibroblasts were stained with an anti-activin A antibody. Representative data from duplicated experiments are shown. B, The concentration of activin A was measured by an ELISA assay using cultured supernatants at the indicated time point. The open column is cultured supernatant from normal control, and the closed column is cultured supernatant from SSc-derived fibroblasts. C, Serum activin A concentration was measured by an ELISA assay (lSSc, limited cutaneous SSc; dSSc, diffuse cutaneous SSc). Data were obtained from triplicate experiments. The mean values are shown in a bar graph with standard errors.

(Fig. 3B). Interestingly, the phosphorylation of Smad2/3 was augmented in SSc-derived fibroblasts compared to controls. Because CTGF significantly impacts collagen production and is regulated by the Smad2/3 signaling pathway, we measured its expression and found that CTGF expression was also induced by activin A stimulation, again with augmented expression in SSc-derived fibroblasts. These experiments establish an important role for the activin A-ACVRIB/ALK4-Smad pathway in SSc fibroblasts and suggest the autocrine activation of activin A-ACVRIB/ALK4-Smad2/3.

3.4. Effects of activin A on collagen production in normal fibroblasts

Increased CTGF expression following activin A stimulation suggests that the production of procollagen might also be stimulated by activin A. Procollagen production and Col1α mRNA expression were investigated in normal fibroblasts after activin A stimulation, and we found that activin A treatment induced Col1α mRNA expression in a dose- and time-dependent manner and increased the production of procollagen (Fig. 4A and B).

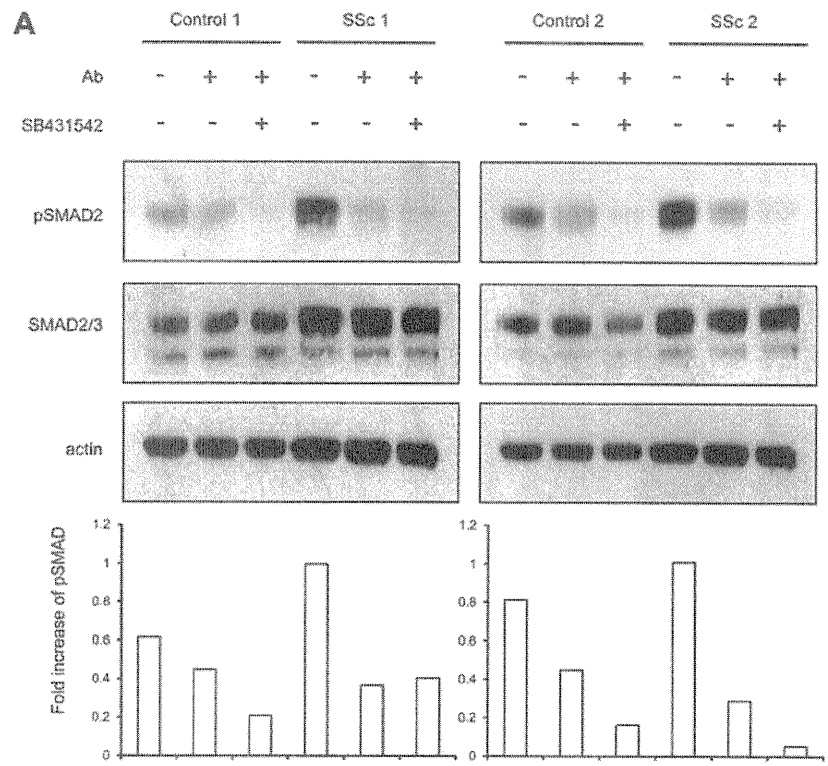
3.5. An ALK inhibitor as a potent therapeutic agent for SSc

Attenuation of Smad pathway activation in SSc fibroblasts, using either an ACVRIB/ALK4 neutralizing antibody (MAB222) or an ALK inhibitor, prompted us to investigate the possibility of a new therapeutic approach for SSc. SB431542 was examined to evaluate whether they are capable of blocking collagen production. SB431542 blocks both the activin A-ACVRIB/ALK4 and the TGF-β-ALK5 pathways. The treatment of fibroblasts with SB431542 attenuated activin A-induced procollagen production in a dose-dependent manner, especially in SSc-derived fibroblasts (Fig. 5).

4. Discussion

The relationship between the TGF-β pathway and fibrosis has been well characterized in patients with SSc [2,3,25–27]. In contrast to our observation of increased expression of activin A-ACVRIB/ALK4 and activation of the downstream Smad pathway in SSc fibroblasts, the production of TGF-β is equivalent between normal

Fig. 3. Ligand-induced activation of the Smad signaling pathway examined by western blot analysis. A, 1×10^6 cells were plated into 6 cm plate the day before experiment. After washing the cell with PBS, cells were serum-starved for 3 h and then treated with PBS or an anti-ACVRIB/ALK4 neutralizing antibody (MAB222) at a concentration of 100 ng/ml, with or without SB431542 (5 μM final) for 1 h. Then, cells were subjected western blotting analysis. B, 1×10^6 cells were plated into 6 cm plate the day before experiment. After washing the cell with PBS, cells were serum-starved and pretreated with DMSO or SB431542 (5 μM final) for 3 h. Cells were then stimulated with PBS or rActivin A at a concentration of 10 ng/ml for 4 h under DMEM with FBS. Then, cells were subjected western blotting analysis. Representative data from triplicate experiments are shown. Phosphrylation of SMAD was quantitated by densitometry (Image J), and values were provided as a bar graph beneath the gel data.


A
Control 1
SSc 1
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Ab
SB431542
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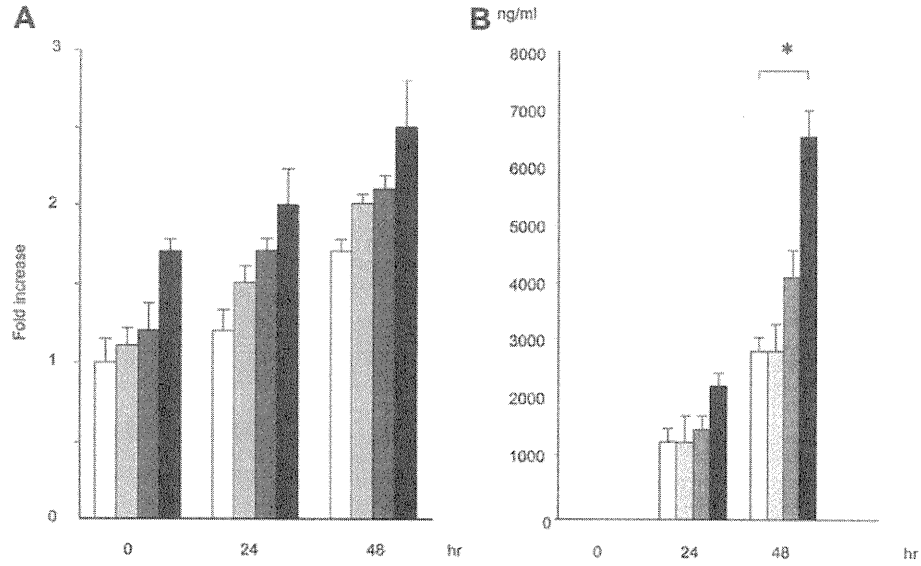

Fig. 4. A, The expression of COL1α mRNA was quantified using real-time quantitative PCR. B, The production of procollagen type I was measured by an ELISA after treatment with recombinant activin A at various concentrations: ■ 10 ng/ml, ▩ 2 ng/ml, ▨ 0.5 ng/ml, and □ PBS. Data were obtained from triplicate experiments. The mean values are shown in a bar graph with standard errors. *: $P < 0.01$.

and SSc fibroblast cell lines [28]. Therefore, the explanation of SSc etiology using only the TGF-β signaling pathway is insufficient, as many undetermined factors are likely involved in the development of SSc. ACVRIB/ALK4 is a receptor for the TGF-β superfamily of signaling ligands. However, the relationship between the activin A pathway and fibrosis in patients with SSc has not been thoroughly investigated. Our observations and several previous reports that describe activin A-induced fibrosis in various organs provide the possibility of an activin A contribution to the development of SSc.

As described in Fig. 3a, activin A-ACVRIB/ALK stimulation strikingly activated Smad pathway. However, faint phosphorylation of Smad is still detected even signaling pathway was blocked by neutralizing antibody to ALK. Smad pathway is utilized by not only activin A-ACVRIB/ALK but also TGF-β–TβRII stimulation. This observation raised the possibility that phosphorylation of Smad is partially achieved by indirect effect such as TGF-β, and suggested the presence of autocrine like secretion of TGF-β by Activin A-ACVRIB/ALK activation.

Interestingly, activin demonstrates both pro- and anti- inflammatory effects. Synovial concentrations of activin A are elevated in patients with rheumatoid arthritis but not in those with osteoarthritis [29]. Activin A accelerates the proliferation of fibroblast-like synoviocytes, and several reports suggest that activin A induces pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor (TNF) α that may contribute to fibrosis development [30,31]. In contrast, IL-1 β, TNFα, and TGF-β activate fibroblast-like synoviocytes to secrete activin A, suggesting a significant role for activin as a positive regulator of the inflammatory cytokine feedback loop [30]. Activin also affects anti-inflammatory action involving inflammatory cytokine production [29,32]. These dual roles of activin in inflammatory tissue are tightly regulated, and understanding how its functions are linked to fibrosis and inflammation requires further evaluation.

The development of fibrosis in SSc patients involves many factors in addition to cytokine signaling. We have previously reported that nitric oxide (NO) production is markedly increased in early-stage diffuse cutaneous SSc patients with active fibrosing alveolitis and that constitutive inducible nitric oxide synthase (iNOS) expression in SSc fibroblasts may contribute to increased NO production [33]. Nitric oxide (NO) production via iNOS was observed in response to activin A, strongly suggesting a link between NO and activin A-dependent fibrosis in patients with SSc [31].

We also previously reported that aberrant angiotensin II (Ang II) production may be involved in tissue fibrosis through the excessive production of extracellular matrix components in SSc dermal fibroblasts [34]. Ang II increases the binding capacity of TGF-β and upregulates the expression of the TGF-β type I receptor (TβRI/ALK5), which may counteract the Ang II-promoted growth of vascular smooth muscle cells [35]. Abnormal Ang II production in SSc fibroblasts could explain the increased expression of ACVRIB/ALK4 in these pathological cells.

Fibroblast-specific constitutively active TβRI/ALK5 transgenic mice share the remarkable fibrotic phenotype observed in SSc patients [36]. Keratin 14 promoter-driven activin A transgenic mice show abnormalities in their skin in which fatty tissue is replaced by connective tissue, and a severe thickening of the epidermis is seen [13]. These observations are similar to those resulting from connective tissue fibroblasts in SSc patients.

Activin A also participates in the wound repair process [37]. However, the hyperactivated cytokine network in SSc skin induces cell damage in connective tissue instead of promoting healing. It is speculated that a recycling wound repair process is occurring in SSc skin continuously, secondary to persistent connective tissue damage. It is hypothesized that development of SSc is a final feature of this recycled regeneration, proliferation, and death in connective tissue. Activation of the activin A pathway may provide an uncontrollable wound repair process in pathological SSc skin, or it may provide a controlled, recycled wound repair process as seen in normal skin.

No selective therapy for SSc has been established until now. Inhibition of TβRI/ALK5 using the selective inhibitor SD208 or SB41352 reduced the fibrotic marker expression with SSc-derived fibroblasts [38,39]. Using several methods, we observed that inhibition of the activin A pathway attenuated collagen production in cultured fibroblasts, especially in SSc-derived fibroblasts. Follistatin, which binds activin with high affinity and blocks activin signaling,

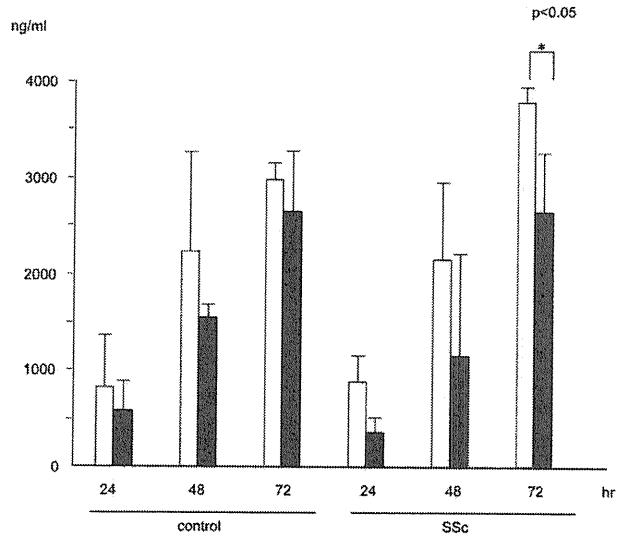

Fig. 5. Production of procollagen type I was attenuated by SB431542 treatment. Cells were pretreated with □ DMSO, ■ SB431542 (final 5 μM) for 3 h. Then cells were stimulated with PBS or rActivin A at a concentration of 10 ng/ml. Production of procollagen type I was measured at the indicated time points. Data were obtained from triplicate experiments. The mean values are shown in a bar graph with standard errors. *: $P < 0.05$.

could be a candidate for biomodulation therapy because the administration of recombinant follistatin has been shown to attenuate belomycin-induced lung fibrosis [14] and CCl_4-induced liver fibrosis in vivo [40]. Neutralizing antibodies for the activin A receptors are another candidate for biomodulation therapy for SSc, and recent advances in antibody-mediated therapy encourage the development of therapeutic trials. Several ALK inhibitors have been developed for cancer therapy, and clinical trials are underway. These compounds could also be potential therapeutic agents for SSc.

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References

[1] Pannu J, Gardner H, Shearstone JR, Smith E, Trojanowska M. Increased levels of transforming growth factor beta receptor type I and up-regulation of matrix gene program: a model of scleroderma. Arthritis Rheum 2006;54(9):3011–21.
[2] Kubo M, Ihn H, Yamane K, Tamaki K. Up-regulated expression of transforming growth factor beta receptors in dermal fibroblasts in skin sections from patients with localized scleroderma. Arthritis Rheum 2001;44(3):731–4.
[3] Kubo M, Ihn H, Yamane K, Tamaki K. Upregulated expression of transforming growth factor-beta receptors in dermal fibroblasts of skin sections from patients with systemic sclerosis. J Rheumatol 2002;29(12):2558–64.
[4] Kubo M, Ihn H, Yamane K, Tamaki K. The expression levels and the differential expression of transforming growth factor-beta receptors in dermatofibroma and dermatofibrosarcoma protuberans. Br J Dermatol 2006;154(5):919–25.
[5] Pannu J, Nakerakanti S, Smith E, ten Dijke P, Trojanowska M. Transforming growth factor-beta receptor type I-dependent fibrogenic gene program is mediated via activation of Smad1 and ERK1/2 pathways. J Biol Chem 2007;282 (14):10405–13.
[6] Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest 2007;117(3):557–67.
[7] Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. N Engl J Med 2000;342(18):1350–8.
[8] Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. Two major Smad pathways in TGF-beta superfamily signalling. Genes Cells 2002;7(12):1191–204.
[9] Ihn H. Autocrine TGF-beta signaling in the pathogenesis of systemic sclerosis. J Dermatol Sci 2008;49(2):103–13.
[10] Werner S, Alzheimer C. Roles of activin in tissue repair, fibrosis, and inflammatory disease. Cytokine Growth Factor Rev 2006;17(3):157–71.
[11] Hubner G, Alzheimer C, Werner S. Activin: a novel player in tissue repair processes. Histol Histopathol 1999;14(1):295–304.
[12] Hayashi Y, Maeshima K, Goto F, Kojima I. Activin A as a critical mediator of capillary formation: interaction with the fibroblast growth factor action. Endocr J 2007;54(2):311–8.
[13] Munz B, Smola H, Engelhardt F, Bleuel K, Brauchle M, Lein I, et al. Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. Embo J 1999;18(19):5205–15.
[14] Aoki F, Kurabayashi M, Hasegawa Y, Kojima I. Attenuation of bleomycin-induced pulmonary fibrosis by follistatin. Am J Respir Crit Care Med 2005;172 (6):713–20.
[15] Matsuse T, Ikegami A, Ohga E, Hosoi T, Oka T, Kida K, et al. Expression of immunoreactive activin A protein in remodeling lesions associated with interstitial pulmonary fibrosis. Am J Pathol 1996;148(3):707–13.
[16] Ohga E, Matsuse T, Teramoto S, Katayama H, Nagase T, Fukuchi Y, et al. Effects of activin A on proliferation and differentiation of human lung fibroblasts. Biochem Biophys Res Commun 1996;228(2):391–6.
[17] Ohga E, Matsuse T, Teramoto S, Ouchi Y. Activin receptors are expressed on human lung fibroblast and activin A facilitates fibroblast-mediated collagen gel contraction. Life Sci 2000;66(17):1603–13.
[18] Wada W, Kuwano H, Hasegawa Y, Kojima I. The dependence of transforming growth factor-beta-induced collagen production on autocrine factor activin A in hepatic stellate cells. Endocrinology 2004;145(6):2753–9.
[19] Gold EJ, Francis RJ, Zimmermann A, Mellor SL, Cranfield M, Risbridger GP, et al. Changes in activin and activin receptor subunit expression in rat liver during the development of CCl4-induced cirrhosis. Mol Cell Endocrinol 2003;201 (1–2):143–53.
[20] Ohnishi N, Miyata T, Ohnishi H, Yasuda H, Tamada K, Ueda N, et al. Activin A is an autocrine activator of rat pancreatic stellate cells: potential therapeutic role of follistatin for pancreatic fibrosis. Gut 2003;52(10):1487–93.
[21] Yamashita S, Maeshima A, Kojima I, Nojima Y. Activin A is a potent activator of renal interstitial fibroblasts. J Am Soc Nephrol 2004;15(1):91–101.
[22] Gaedeke J, Boehler T, Budde K, Neumayer HH, Peters H. Glomerular activin A overexpression is linked to fibrosis in anti-Thy1 glomerulonephritis. Nephrol Dial Transplant 2005;20(2):319–28.
[23] Gressner OA, Lahme B, Siluschek M, Rehbein K, Weiskirchen R, Gressner AM. Intracrine signalling of activin A in hepatocytes upregulates connective tissue growth factor (CTGF/CCN2) expression. Liver Int 2008;28(9):1207–16.
[24] Zhou X, Tan FK, Xiong M, Arnett FC, Feghali-Bostwick CA. Monozygotic twins clinically discordant for scleroderma show concordance for fibroblast gene expression profiles. Arthritis Rheum 2005;52(10):3305–14.
[25] Kawakami T, Ihn H, Xu W, Smith E, LeRoy C, Trojanowska M. Increased expression of TGF-beta receptors by scleroderma fibroblasts: evidence for contribution of autocrine TGF-beta signaling to scleroderma phenotype. J Invest Dermatol 1998;110(1):47–51.
[26] Needleman BW, Choi J, Burrows-Mezu A, Fontana JA. Secretion and binding of transforming growth factor beta by scleroderma and normal dermal fibroblasts. Arthritis Rheum 1990;33(5):650–6.
[27] Yamane K, Ihn H, Kubo M, Tamaki K. Increased transcriptional activities of transforming growth factor beta receptors in scleroderma fibroblasts. Arthritis Rheum 2002;46(9):2421–8.
[28] Ihn H, Yamane K, Kubo M, Tamaki K. Blockade of endogenous transforming growth factor beta signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts: association with increased expression of transforming growth factor beta receptors. Arthritis Rheum 2001;44(2):474–80.
[29] Yu EW, Dolter KE, Shao LE, Yu J. Suppression of IL-6 biological activities by activin A and implications for inflammatory arthropathies. Clin Exp Immunol 1998;112(1):126–32.
[30] Ota F, Maeshima A, Yamashita S, Ikeuchi H, Kaneko Y, Kuroiwa T, et al. Activin A induces cell proliferation of fibroblast-like synoviocytes in rheumatoid arthritis. Arthritis Rheum 2003;48(9):2442–9.
[31] Nusing RM, Barsig J. Induction of prostanoid, nitric oxide, and cytokine formation in rat bone marrow derived macrophages by activin A. Br J Pharmacol 1999;127(4):919–26.
[32] Smith C, Yndestad A, Halvorsen B, Ueland T, Waehre T, Otterdal K, et al. Potential anti-inflammatory role of activin A in acute coronary syndromes. J Am Coll Cardiol 2004;44(2):369–75.
[33] Takagi K, Kawaguchi Y, Hara M, Sugiura T, Harigai M, Kamatani N. Serum nitric oxide (NO) levels in systemic sclerosis patients: correlation between NO levels and clinical features. Clin Exp Immunol 2003;134(3):538–44.
[34] Kawaguchi Y, Takagi K, Hara M, Fukasawa C, Sugiura T, Nishimagi E, et al. Angiotensin II in the lesional skin of systemic sclerosis patients contributes to tissue fibrosis via angiotensin II type 1 receptors. Arthritis Rheum 2004;50 (1):216–26.
[35] Fukuda N, Hu WY, Kubo A, Kishioka H, Satoh C, Soma M, et al. Angiotensin II upregulates transforming growth factor-beta type I receptor on rat vascular smooth muscle cells. Am J Hypertens 2000;13(2):191–8.
[36] Sonnylal S, Denton CP, Zheng B, Keene DR, He R, Adams HP, et al. Postnatal induction of transforming growth factor beta signaling in fibroblasts of mice recapitulates clinical, histologic, and biochemical features of scleroderma. Arthritis Rheum 2007;56(1):334–44.
[37] Hubner G, Hu Q, Smola H, Werner S. Strong induction of activin expression after injury suggests an important role of activin in wound repair. Dev Biol 1996;173(2):490–8.

[38] Mori Y, Ishida W, Bhattacharyya S, Li Y, Platanias LC, Varga J. Selective inhibition of activin receptor-like kinase 5 signaling blocks profibrotic transforming growth factor beta responses in skin fibroblasts. Arthritis Rheum 2004;50(12):4008–21.
[39] Chen Y, Shi-wen X, Eastwood M, Black CM, Denton CP, Leask A, et al. Contribution of activin receptor-like kinase 5 (transforming growth factor beta receptor type I) signaling to the fibrotic phenotype of scleroderma fibroblasts. Arthritis Rheum 2006;54(4):1309–16.
[40] Patella S, Phillips DJ, Tchongue J, de Kretser DM, Sievert W. Follistatin attenuates early liver fibrosis: effects on hepatic stellate cell activation and hepatocyte apoptosis. Am J Physiol Gastrointest Liver Physiol 2006;290(1):G137–44.

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Original article

Anti-NR2A antibody as a predictor for neuropsychiatric systemic lupus erythematosus

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Abstract

Objective. The aim of this study is to establish a detection method for anti-*N*-methyl-D-aspartate receptor subunit 2A (NR2A) antibody and to evaluate the relationship between anti-NR2A antibody and various organ involvement in SLE.

Methods. Serum anti-NR2A antibody was measured by ELISA using a peptide with a core of either DWEYS or DWDYS as autoantigen. Additionally, clinical characteristics were compared between 27 anti-NR2A antibody-positive (*P* group) and 80 antibody-negative (*N* group) SLE patients using DWDYS peptide.

Results. The optical density (OD) values of anti-NR2A antibody using DWDYS and DWEYS peptides correlated significantly ($r = 0.94$, $P < 0.0001$). The median OD value was significantly higher ($P < 0.0001$) with DWDYS. Additionally, the SLEDAI was significantly higher ($P = 0.023$) in the *P* group. The frequency of neuropsychiatric SLE (NPSLE) was significantly higher ($P = 0.0002$) in the *P* group, although the frequencies of serositis and nephritis were not statistically significant. Significant correlations were found between anti-NR2A antibody and leucocyte count ($r_s = -0.31$, $P = 0.001$) and haemoglobin ($r_s = -0.42$, $P < 0.0001$), although no correlation was found between anti-NR2A antibody and the titre of anti-dsDNA antibody. NPSLE was the most significant independent variable ($P = 0.0008$) associated with anti-NR2A antibody positivity, as estimated by multiple linear regression analysis.

Conclusion. Serum anti-NR2A antibody can be associated with the complication of NPSLE and may indicate the involvement of non-nervous tissue. The use of peptides that include DWDYS is preferable to detect anti-NR2A antibody in ELISA.

Key words: Systemic lupus erythematosus, *N*-methyl-D-aspartate receptor, Neuropsychiatric involvement, Autoantibody.

CLINICAL SCIENCE

Introduction

N-methyl-D-aspartate receptors (NMDARs) are ligand-gated ion channels with crucial roles in synaptic transmission and CNS plasticity. The receptors are heteromers of NMDAR subunit 1 (NR1), which binds glycine, and NMDAR subunit 2 [NR2 (A, B, C or D)], which binds glutamate [1]. NMDAR dysfunction is implicated in multiple brain disorders, including stroke, chronic neurodegeneration, epilepsy and schizophrenia [2–5]. Additionally, anti-NMDAR antibody-associated encephalitis has been reported recently [6–8]. NMDARs are also located in non-neuronal tissues, such as bone, skin, pancreas and megakaryocytes [9–11]. Glutamate signalling via NMDAR functions in both non-nervous and nervous tissues.

SLE is a multi-system inflammatory disorder characterized by the presence of autoantibodies directed against DNA. Anti-DNA antibodies cross-react with NR2 and damage neuronal cells via an apoptotic pathway [12]. However, not all anti-DNA antibodies are able to cross-react with NR2 completely. The frequency of anti-NR2 antibody positivity has been reported to be ~30% in patients with SLE [13]. Although anti-NR2 antibody in

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