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ORIGINAL ARTICLE

Comparison of trimethoprim–sulfamethoxazole and aerosolized pentamidine for primary prophylaxis of *Pneumocystis jiroveci* pneumonia in immunocompromised patients with connective tissue disease

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Abstract To evaluate the efficacy of primary prophylaxis for *Pneumocystis jiroveci* pneumonia (PCP) in patients with connective tissue disease (CTD) and immunosuppression, we compared trimethoprim–sulfamethoxazole (TMP-SMZ) with aerosolized pentamidine. Forty-eight CTD patients of Kitasato University Hospital whose CD4+ lymphocyte count in the peripheral blood was less than 300 μl^{-1} were reviewed from 2002 to 2004. Twenty-seven patients received TMP–SMZ and none of them developed PCP. Among 18 patients receiving aerosolized pentamidine, three patients developed PCP. These data indicate that TMP–SMZ is better for prophylaxis than aerosolized pentamidine.

Keywords Trimethoprim–sulfamethoxazole · Pentamidine aerosol · *Pneumocystis jiroveci* pneumonia

Introduction

Pneumocystis jiroveci pneumonia (PCP) occurs in patients with impaired cellular immunity, especially debilitated premature infants, patients with primary immunodeficiency, patients on immunosuppressive therapy, and most commonly patients with human immunodeficiency virus (HIV) infection [1, 2]. Considerable progress has been made in the treatment of connective tissue disease (CTD) through the use of potent immunosuppressive agents. However, we encounter PCP quite often in this field because of the marked immunosuppression caused by corticosteroids and other immunosuppressants [3]. In patients with CTD, PCP may have a higher mortality rate than it does in patients with HIV [4].

For primary prevention of PCP in HIV patients, oral trimethoprim–sulfamethoxazole (TMP–SMZ) and aerosolized pentamidine are commonly used [1]. In patients with AIDS, TMP–SMZ has been shown to be more effective than aerosolized pentamidine at conventional doses for the prevention of primary and recurrent PCP. Recent studies have also shown that TMP–SMZ the first choice for prophylaxis in HIV patients [1, 2]. In CTD patients, we also choose TMP–SMZ for primary prophylaxis when they develop immunodeficiency. However, especially in patients with systemic lupus erythematosus (SLE) adverse effects of TMP–SMZ such as allergic reactions and renal dysfunction are not uncommon [5, 6]. In such cases, we use aerosolized pentamidine as the first-line drug.

Although several studies have examined the efficacy of these two drugs, there have been no reports of a comparison between their prophylactic effect in CTD patients. In the present study we retrospectively compared TMP–SMZ with aerosolized pentamidine for the prevention of PCP.

Materials and methods

Patients

A total of 330 CTD patients were treated at our department from January 2002 to December 2004 and received more

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than 30 mg of predonisolone daily (including steroid pulse therapy) and/or immunosuppressant therapy. Among them, 45 patients had a peripheral blood CD4+ lymphocyte count of less than 300 μl^{-1} and received prophylaxic PCP. The diagnosis of CTD was based on the criteria listed in several reports. The average age of the patients at the time hospitalization was 49.2 ± 17.5 years, and the male to female ratio was 16:29. Seventeen patients had vasculitis, 14 had SLE, seven had polymyositis/dermatomyocytis (PM/DM), three had adult Still's disease, and four had other diseases (cryoglobulinemic vasculitis). Immunosuppresants (cyclophosphamide, cyclosporine, methotrexate, azathioprine, and mizoribine) were administered to 25 patients and the mean total dose of steroids (prednisolone equivalent) was 18,118 mg (Table 1).

Occurrence of PCP was presumed when a patient developed respiratory distress and had an increased serum β-D-glucan level without any other fungal infection [7]. PC was detected in sputum or BALF by polymerase chain reaction (PCR) analysis.

Table 1 Clinical profile of the two groups

		ST group	PI group
Number of patients		27	19
Age at start of study (mean ± SD)		50.4 ± 17.1 years	48.3 ± 17.8 years
Sex (female:male)		18:9	13:6
CTD			
Vasculitis	18	13	5
SLE	14	4	10
PM/DM	7	5	2
Adult still	3	2	1
Others	4	3	1
Immunologic data			
CD4 (μl^{-1})		174	160
alb (g/dl)		3.5 ± 0.5	3.2 ± 0.5
IgG (mg/dl)		949 ± 332	1083 ± 696
Drugs			
PSL dose (mg)		11,098	27,562
Immuno suppressants		16 (59.3%)	9 (52.6%)
Complications			
IPF		13 (44.4%)	4 (21.1%)

ST group, patients receiving TMP–SXZ; PI group, patients receiving aerosolized pentamidine

CTD, Connective tissue disease; PM/DM, polymyositis/dermatomyositis; Adult Stll, adult Still's disease; others (cryoglobulinemic vasculitis); alb, serum albumin level; IgG, immunoglobulin G; PSL, predonisolne; IPF, interstitial pneumonitis

Methods

As prophylaxis for PCP, 1 tablet of TMP–SMZ was administered daily, which contained 400 mg of sulfamethoxazole and 80 mg of trimethoprim (Baktar®). Patients who could not take TMP–SMZ, such as those with renal dysfunction and/or allergies, were given aerosolized pentamidine at a dose of 300 mg every 4 weeks. Fifteen patients (33.3%) had interstitial lung disease (ILD). Results are expressed as the mean ± standard deviation. Difference between the two prophylaxis groups were assessed by using Fisher's exact test and $P < 0.05$ was considered significant (Table 2). The Mann–Whitney test was used for comparison of the CD4+ lymphocyte count between the two groups.

Results

Forty-eight patients had a CD4+ lymphocyte count in the peripheral blood of less than 300 μl^{-1} and received prophylaxis for PCP: 27 patients received TMP–SMZ and the other 18 patients were given aerosolized pentamidine.

Eighteen patients had vasculitis, including ANCA-associated vasculitis, Wegener's granulomatosis, and cryoglobulinemic vasculitis. The 14 patients with SLE tended to have lupus nephritis or drug allergies and consequently received aerosolized pentamidine. Seven patients had PM/DM and three patients had adult Still's disease.

To compare the level of immune function, we assessed the peripheral blood CD4+ cell count in the group receiving TMP–SMZ (ST group) and that receiving pentamidine (PI group). We found that CD4+ cell counts were similar in both groups (Fig. 1). In addition, the serum levels of albumin and IgG, the immunosuppressant doses, and the total dose of steroids showed no significant differences between the two groups. These data indicate that there was no difference of underlying immunity between the ST group and the PI group.

Next, we compared the incidence of PCP between the ST and PI groups (Table 2). In the PI group, three out of 18

Table 2 Comparison of TMP–SMZ and aerosolized pentamidine for primary prophylaxis of *Pneumocystis jiroveci* pneumonia (PCP)

	ST group	PI group
PCP (+)	0 (0%)	4 (21%)
PCP (−)	27 (100%)	15 (79%)

P value: $P = 0.0238$. Comparison of the incidence of *Pneumocystis jiroveci* pneumonia (PCP) between the ST group (TMP–SMZ) and the PI group (aerosolized pentamidine). In the PI group, 4 of 19 patients (21%) developed PCP and the primary prophylaxis rate was 79%. No patient developed PCP in the ST group and difference between the two groups was significant ($P = 0.023$, Fisher's exact test)

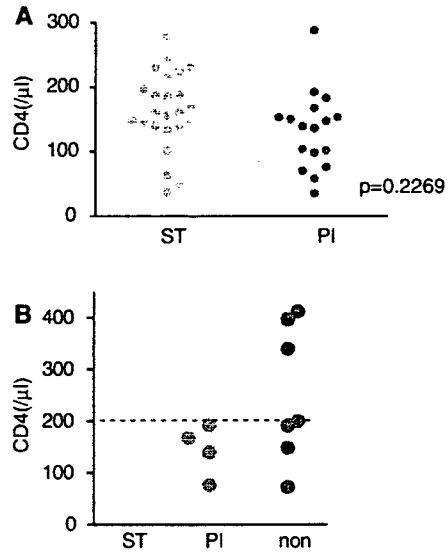

Fig. 1 **a** Comparison of the CD4+ lymphocyte count in the ST and PI groups (ST vs. PI). Evaluation of CD4 counts to compared the base immunity between two groups. No significant difference was found. **b** Comparison of the CD4+ lymphocyte count in the patients with PCP (ST vs. PI: groups and PI group vs. no prophylaxis). We examined the CD4+ lymphocyte count at the onset of PCP. Among eight patients who did not receive prophylaxis for PCP, 5 had a CD4+ lymphocyte count of 200 μl^{-1} or less and we confirmed that a low CD4+ lymphocyte count is one of the most important factors leading to PCP. Daily TMP–SMZ was useful for prophylaxis, while pentamidine in four cases

patients (17%) developed PCP and the effective prophylaxis rate was 83%. In contrast, no patient developed PCP in the ST group and the effective prophylaxis rate was 100%. TMP–SMZ was significantly more effective than aerosolized pentamidine as prophylaxis for PCP.

Details of the three patients who developed PCP during aerosolized pentamidine prophylaxis are shown in Table 3. To assess their immune function, we evaluated the CD4+ cell count, the albumin and IgG levels, and the existence of ILD compared with the patients who did not develop PCP in the PI group. No significant differences were found (Fig. 1a, b).

Table 3 Clinical features of four patients who developed PCP during aerosolized pentamidine prophylaxis

	Age	Diseases	Sex	CD4	alb	IgG	ILD
1.	74	Clioglobulinemia	M	76	2.8	2,952	–
2.	63	MPA	M	197	3.1	518	+
3.	62	STILL	F	192	2.9	1,087	–
4.	63	MPA	F	139	2.5	1,185	+

MPA Microscopic polyangiitis, *STLL* adult Still's disease, *M* male, *F* female, *CD4* CD4+ lymphocyte count in the peripheral blood at the onset of PCP, *Alb* serum albumin concentration, *IgG* serum immunoglobulin G concentration, *ILD* interstitial lung disease

Adverse reactions occurred in six patients receiving TMP–STZ, including hemolytic disorder in two, thrombocytopenia in one, leucopenia in one, skin rash in two, liver dysfunction in one, and renal dysfunction in one. All adverse reactions were mild and continuation of treatment was possible. None of the patients showed adverse reactions to aezolized pentamidine.

Discussion

We evaluated the prophylactic effect of TMP–SMZ against PCP in patients whose CD4+ lymphocyte count was less than 300 μl^{-1}. Among CTD patients who were admitted to our rheumatology department from 2002 to 2004, 11 patients had PCP confirmed by positive PCR analysis of sputum or BALF. Review of the clinical records showed that eight out of 11 patients had not received any prophylaxis, while the other three patients all received aerosolized pentamidine as prophylaxis for PCP. Thus, no patient administered TMP–SMZ developed PCP in our department and this was the most useful prophylactic agent for patients with CTD. On the other hand, aerosolized pentamidine prophylaxis failed to prevent the occurrence of PCP in three cases.

Another group also reported on the use of TMP–SMX for PCP prophylaxis in patients older than 50 years receiving high-dose steroid or immunosuppressant therapy and none of them developed PCP during about 5 years.

We examined the CD4+ lymphocyte count in the 11 patients with PCP. Among the eight patients who did not receive any prophylaxis, five patients had a CD4+ lymphocyte count of 200 μl^{-1} or less. Li et al. also reported that the CD4+ lymphocyte count was less than 250 μl^{-1} in all 7 patients with CTD who developed. These data indicate that a decrease of the CD4+ lymphocyte count is one of the risk factors for PCP. Setting a higher limit for the CD4+ lymphocyte count and starting aerosolized pentamidine earlier might have reduced the PCP rate among the patients who could not use TMP–SMZ. It was previously reported that the serum IgG and albumin levels were low at the onset of PCP and that the serum IgG level was decreased significantly in CTD patients. However, these levels did not change in our patients at the onset of PCP.

In patients with HIV and a CD4+ lymphocyte count <200 μl^{-1}, TMP–SMZ once daily is more effective as primary and secondary prophylaxis against PCP than aeroslized pentamidine once a month. Other reports also indicate that aeroslized pentamidine therapy often fails as prophylaxis for PCP in patients with HIV. However adverse drug reactions are more frequent with TMP–SMZ. Aerosolized pentamidine has a low incidence of systemic adverse reactions because the drug is delivered directly to the lungs.

However, adverse reactions to TMP–SMZ were mild and recovered after temporary withdrawal of therapy, so its continuation was possible in our CTD patients.

This study has the limitations of being retrospective and not double blind. However, patients with immunosuppression need prophylaxis for PCP, so we could not design a double-blind study that included patients who did not take any prophylactic agent on ethical grounds.

In conclusion, immunosuppressive therapy is widely used these days and PCP is becoming a serious problem in CTD patients. We concluded that TMP–SMZ is superior for prophylaxis of PCP compared with aerosolized pentamidine and we recommend the use of TMP–SMZ for patients on strong immunosuppressive regimens.

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Intracellular IL-1α-binding proteins contribute to biological functions of endogenous IL-1α in systemic sclerosis fibroblasts

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The aberrant production of precursor IL-1α (pre-IL-1α) in skin fibroblasts that are derived from systemic sclerosis (SSc) is associated with the induction of IL-6 and procollagen, which contributes to the fibrosis of SSc. However, little is understood about how intracellular pre-IL-1α regulates the expression of the other molecules in fibroblasts. We report here that pre-IL-1α can form a complex with IL-1α-binding proteins that is translocated into the nuclei of fibroblasts. Immunoprecipitation that used anti-human IL-1α Ab and ^{35}S-labeled nuclear extracts of fibroblasts showed three specific bands (≈31, 35, and 65 kDa). The 31-kDa molecule was identified as pre-IL-1α, and the 35- and 65-kDa molecules might be pre-IL-1α-binding proteins. A partial sequencing for the 10 aa from the N-terminals of the molecules showed 100% homology for HAX-1 (HS1-associated protein X-1) and IL-1 receptor type II (IL-1RII). Suppression of the genes of HAX-1 or IL-1RII induced the inhibitory effects of IL-1 signal transduction, including production of IL-6 and procollagen, by fibroblasts. In particular, pre-IL-1α was not translocated into the nucleus by an inhibition of HAX-1. These findings reveal that nuclear localization of pre-IL-1α depends on the binding to HAX-1 and that biological activities might be elicited by the binding to both HAX-1 and IL-1RII in SSc fibroblasts.

IL-1 receptor type II | HS1-associated protein X-1 | fibrosis | collagen | IL-6

Systemic sclerosis (SSc) is a connective tissue disease of unknown etiology that is characterized by the fibrosis of systemic organs (1). Because skin thickening manifests in most patients, researchers have analyzed the molecular and biological functions of lesional skin fibroblasts that are derived from SSc patients (2). In previous reports we demonstrated that SSc fibroblasts expressed IL-1α mRNA constitutively and that aberrant production of precursor IL-1α (pre-IL-1α) contributed to skin fibrosis in SSc (3–5). IL-1α is a multifunctional molecule that is involved in a variety of inflammatory disorders, including sepsis, arthritis, myositis, psoriasis, periodontitis, and Alzheimer's disease (6). Pre-IL-1α is synthesized as a result of the transcription and translation of the *IL1A* gene. Under some circumstances, pre-IL-1α (31 kDa) is proteolytically cleaved to yield a mature form of IL-1α (17 kDa) (7). Because the N-terminal propiece of pre-IL-1α (NTP-IL-1α) contains a nuclear localization sequence (NLS), pre-IL-1α can be translocated into the nucleus, whereas mature IL-1α can be released from cells (8).

This pathway is complicated, however. The signal transduction of IL-1α is initiated by the binding of IL-1α (precursor or mature form) to cell-surface receptors on various cells (IL-1 receptor type I and IL-1 receptor accessory protein) (9, 10). The intracellular accumulation of pre-IL-1α in skin fibroblasts suggests an alternative pathway. Only a few studies, including ours, have reported the biological effects of intracellular IL-1α in fibroblasts and endothelial cells (11–15). Although the precise pathway of signal transduction was not determined in those studies, the authors speculated that intracellular pre-IL-1α might exhibit a biological function directly and that the pathway of signal transduction might be distinct from the pathway that was mediated by binding the specific receptors. Our previous study (5) revealed that intracellular pre-IL-1α directly influenced the phenotype of SSc fibroblasts. These observations prompted us to explore the mechanism whereby intracellular pre-IL-1α exhibits its biological functions through the alternative pathway. In the present study we investigate the molecules that bind to pre-IL-1α in human fibroblasts and the effects of the IL-1α-binding proteins on nuclear localization and biological functions of IL-1α.

Results

Localization of Intracellular IL-1α in SSc Fibroblasts. Although we previously demonstrated the nuclear localization of pre-IL-1α in SSc fibroblasts, we performed immunohistochemistry on five lines of SSc fibroblasts and three lines of normal fibroblasts. We visualized the signals of intracellular IL-1α in all five SSc fibroblast lines and did not detect them in the three normal fibroblast lines. A representative result of Cy3 staining is shown in Fig. 1. The specific signals were mostly distributed in the nucleus, consistent with our previous results (5).

Immunoprecipitation (IP). To detect candidates of intracellular IL-1α-binding proteins, we used cell lysates of SSc fibroblasts and anti-IL-1α Ab to perform IP. As shown in Fig. 2, autoradiography indicated that the lengths of the specific bands were ≈31, 35, and 65 kDa. Columns 2 and 3 show representative data from IP that use cell lysates of SSc fibroblasts with anti-IL-1α Ab under a nonreducing and a reducing condition, respectively, and column 1 shows data that use cell lysates of SSc fibroblasts with rabbit IgG under a reducing condition. The 31-kDa band corresponded to the predicted pre-IL-1α in fibroblasts. Because the 35- and 65-kDa bands were also candidates of the intracellular IL-1α-binding proteins, we used a protein sequencer to partially analyze the N-terminals of these molecules. The 35-kDa molecule was homologous to HAX-1 (HS1-associated protein X-1; amino acid sequence MSLFDLFRGF), and the 65-kDa molecule was homologous to IL-1 receptor type II (IL-1RII; amino acid sequence FTLQPAAHTG). We observed no specific bands below the 30-kDa molecule (data not shown). Thus, we con-

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The authors declare no conflict of interest.

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Abbreviations: Ct, threshold cycle; HAX-1, HS1-associated protein X-1; icIL-1RA, intracellular IL-1 receptor antagonist; IL-1RII, IL-1 receptor type II; IP, immunoprecipitation; NTP-IL-1α, N-terminal propiece of pre-IL-1α; pre-IL-1α, precursor IL-1α; SSc, systemic sclerosis.

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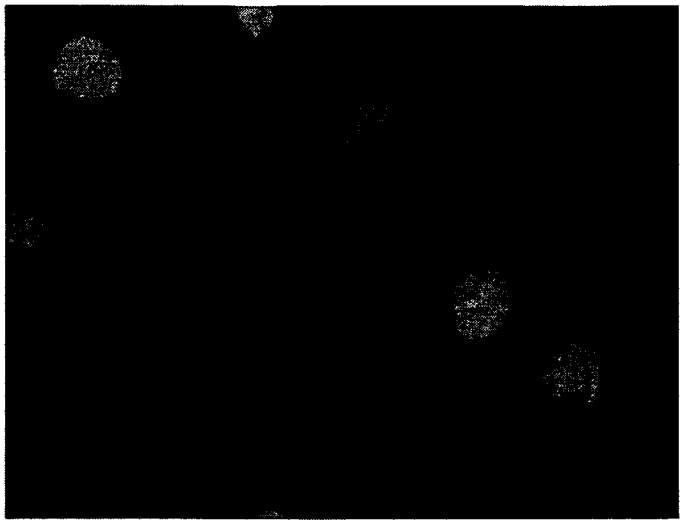

Fig. 1. Nuclear localization of IL-1α in SSc fibroblasts. SSc fibroblasts were cultured in plates on a four-chamber slide. Cells were fixed with 2% paraformaldehyde plus 0.1% Triton X-100. The primary Ab was monoclonal anti-human IL-1α Ab, which detected pre-IL-1α and mature IL-1α after incubation with Cy3-conjugated anti-mouse IgG Ab. A representative result in SSc fibroblasts was obtained with fluorescence microscopy.

cluded that intracellular IL-1α was almost pre-IL-1α (31 kDa) in SSc fibroblasts, consistent with our previous studies (3–5).

Expression of IL-1RII and HAX-1 in Fibroblasts. To confirm the expression of IL-1RII and HAX-1 in SSc and normal fibroblasts, we used RT-PCR to analyze the expression of mRNA. The cDNA that was derived from five fibroblast lines of patients with SSc contained mRNA of both IL-1RII and HAX-1, but three normal fibroblast lines contained the HAX-1 mRNA alone (Table 1). Western blotting indicated that HAX-1 was expressed in SSc and normal fibroblasts, but we detected IL-1RII in SSc fibroblasts alone (Fig. 3). Immunocytochemical studies revealed different distributions of HAX-1 and IL-1RII between SSc and normal fibroblasts (Fig. 4). HAX-1 was localized in the nuclei and cytosol of SSc fibroblasts but in only the cytosol of normal fibroblasts. IL-1RII was localized in the nuclei and cytosol of

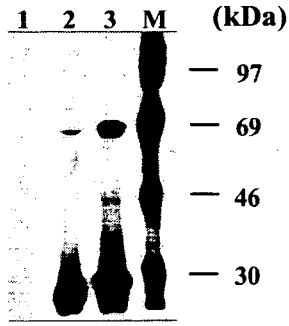

Fig. 2. Pre-IL-1α-binding proteins were detected by IP. Fibroblasts from SSc were cultured by using [^{35}S]methionine/cystein for 16 h. After a pulse, cells were harvested and sonicated to extract nuclear and cytosolic proteins. IP was performed with cell lysates and anti-human IL-1α Ab or control rabbit IgG combined with protein G-Sepharose. Immunoprecipitates were fractionated by 10% SDS/PAGE, and radiolabeled polypeptides were visualized by autoradiography. Column 1, lysate reacted with rabbit IgG fractionated under a reducing condition; column 2, lysate reacted with anti IL-1α Ab fractionated under a nonreducing condition; column 3, lysate reacted with anti IL-1α Ab fractionated under a reducing condition; column M, molecular marker.

Table 1. Expression of IL-1RII and HAX-1 mRNA in cultured fibroblasts

Subjects	IL-1RII	HAX-1
SSc		
1	17	4.3
2	15	5.2
3	29	42
4	31	26
5	25	18
HC		
1	<0.01	20
2	0.02	21
3	<0.01	48

Total RNA was extracted from cultured fibroblasts derived from five patients with SSc and three healthy controls (HC). Real-time RT-PCR was performed by using an ABI 7900HT and FAM-labeled TaqMan gene expression assay kit. GAPDH mRNA expression was used as an endogenous control.

SSc fibroblasts, and no fluorescent signal was detected in normal fibroblasts.

Binding Capacities of IL-1RII to Pre-IL-1α in Fibroblasts. To investigate the binding capacities of IL-1RII to pre-IL-1α in fibroblasts, we produced murine fibroblasts that were transfected with human pre-IL-1α, human IL-1α, or human NTP-IL-1α, which were cotransfected with human IL-1RII. We detected human IL-1RII, which was expressed by the pcDNA3 vector, in murine fibroblasts by anti-IL-1RII Ab (Fig. 5, second row). Because the three forms of human IL-1α were expressed as V5-tagged proteins in murine fibroblasts, these proteins were detected by anti-V5 Ab (Fig. 5, third row). Finally, after cell lysates from murine fibroblasts were immunoprecipitated with anti-IL-RII Ab, human IL-1α and human pre-IL-1α were detected by Western blotting with anti-V5 Ab (Fig. 5). These results indicate that intracellular IL-1RII binds to pre-IL-1α and mature IL-1α via the amino acid sequence from 113 to 271 aa.

To confirm the interaction of IL-1α with IL-1RII and HAX-1, we fused cDNA that encodes three kinds of IL-1α to the λ repressor protein (λcI) of pBT plasmid and fused each target gene of IL-1RII and HAX-1 to the N-terminal domain of RNA polymerase of pTRG plasmid. We grew double transforming cells of pre-IL-1α with IL-1RII or HAX-1 in selection LB plates and had a β-galactosidase activity (Fig. 6). The cells transformed with NTP-IL-1α were positive only when cotransformed with HAX-1, and the cells transformed with IL-1α were positive only when cotransformed with IL-1RII. Previously, HAX-1 was reported to be associated with three sites of NTP-IL-1α (16). By considering all this evidence, we present a schematic of putative pre-IL-1α complex in Fig. 7.

Functional Roles of IL-1RII and HAX-1 in the Signal Transduction of Pre-IL-1α. To further investigate the roles of IL-1RII and HAX-1 as a pre-IL-1α-binding protein, we produced SSc fibroblasts that deplete IL-1RII or HAX-1 by means of RNA interference.

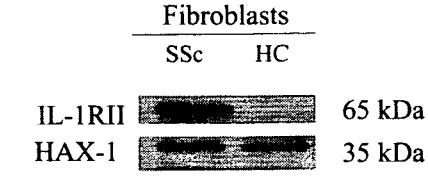

Fig. 3. Western blot analysis of IL-1RII and HAX-1 in fibroblasts. Cell lysates were prepared from fibroblasts derived from systemic sclerosis (SSc) and a healthy donor (HC).

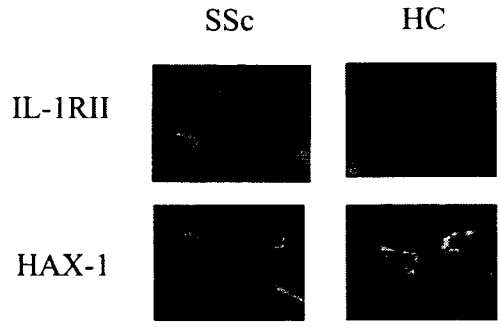

Fig. 4. Cellular distribution of IL-1RII and HAX-1 by immunofluorescence staining. Fibroblasts were fixed by 2% paraformaldehyde plus 0.1% Triton X-100 and then reacted with anti-IL-1RII or anti-HAX-1 Ab. After they were treated with FITC-conjugated anti-mouse IgG, a fluorescence image was obtained.

IL-1RII and HAX-1 proteins were suppressed in all five lines of SSc fibroblasts transfected with a small siRNA-expressing vector. A representative result of Western blotting is shown in Fig. 8. We used five lines of SSc fibroblasts to conduct the experiments, and then we scanned each band on x-ray films on a scanning densitometer. We measured the intensity of each molecule by subtracting the intensity of background from that of the band. The mean ratio of specific/random siRNA was 0.06 in IL-1RII and 0.21 in HAX-1. An inhibition of IL-1RII did not affect the nuclear localization of pre-IL-1α, but inhibiting HAX-1 caused the nuclear staining of pre-IL-1α in SSc fibroblasts to disappear (Fig. 9). We used five different lines of SSc fibroblasts to confirm this result. We previously demonstrated that aberrant production of pre-IL-1α in the nucleus contributed to IL-6 and procollagen type I production in SSc fibroblasts. To explore the effects of IL-1RII and HAX-1 on IL-6 and procollagen type I production in SSc fibroblasts, we suppressed the production of both IL-6 and procollagen type I by the knockdown of IL-1RII and HAX-1 (Fig. 10 *A* and *B*). The results indicate the mean of triplicate experiments that use five SSc fibroblasts and three normal fibroblasts.

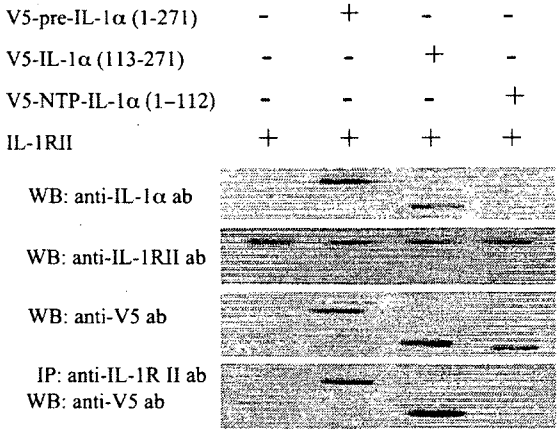

Fig. 5. A binding assay of pre-IL-1α and IL-1RII was performed with murine fibroblasts (NIH 3T3) transfected with human IL-1α and IL-1RII. The cDNA of human IL-1α (amino acids 113–271), pre-IL-1α (amino acids 1–271), and NTP-IL-1α (amino acids 1–112) were subcloned into the pcDNA4-V5 vector. The cDNA of human IL-1RII was subcloned into the pcDNA3 vector. NIH 3T3 cells were transfected with one of three kinds of pcDNA4-V5 and pcDNA3, as indicated above the panels. Cell lysates were extracted from each transfectant by sonication. The upper three panels indicate the results of Western blotting (WB) with anti-IL-1α Ab, anti-IL-1RII Ab, and anti-V5 Ab. The lowest panel indicates the results of WB with anti-V5 Ab after IP that used anti-IL-1RII Ab.

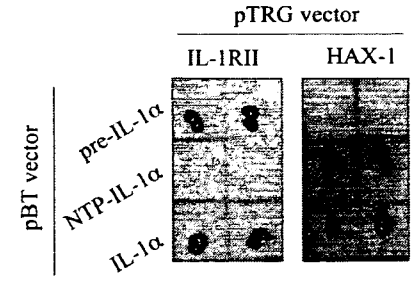

Fig. 6. A bacterial two-hybrid system was performed to confirm the interaction between pre-IL-1α and its binding proteins (IL-1RII and HAX-1). The IL-1α proteins were fused to the bacteriophage λ repressor protein by using pBT plasmid, and the target proteins (IL-1RII and HAX-1) were fused to the N-terminal domain of RNA polymerase by using pTRG plasmid. The suitable *E. coli* was transformed by the two plasmids, and LB agar plates, including tetracycline, chloramphenicol, kanamycin, and X-gal, were used to select positive clones.

Discussion

The results of the present study provide solid evidence that intracellular pre-IL-1α consists of a protein complex with IL-1RII and HAX-1 and that the formation of this complex is indispensable for pre-IL-1α-induced biological functions (IL-6 production and procollagen type I synthesis by fibroblasts). Our previous findings indicated that nuclear localization of pre-IL-1α plays a crucial role in the fibrogenic phenotype of skin fibroblasts derived from patients with SSc (3–5). The present study demonstrates the importance of the pre-IL-1α complex for the fibrogenic phenotype of SSc fibroblasts.

Early research indicated that IL-1α or pre-IL-1α is secreted from cells and exhibited an inflammatory response and immunity through the specific IL-1 receptors on the surface of targeted cells. However, intracellular pre-IL-1α has been shown to stimulate proliferation of renal fibroblasts (11) and to regulate the migration and the life span of endothelial cells (12), independent of secretion and cell-surface IL-1 receptors. Some researchers suggested that the nuclear localization sequence in the NTP-IL-1α molecule might be essential for the biological activity of intracellular IL-1α (11, 12). Recently, Buryskova *et al.* (13) observed that intracellular pre-IL-1α functionally activated transcription, interacting with histone acetyltransferase complexes. Werman *et al.* (14) reported that intracellular IL-1α is

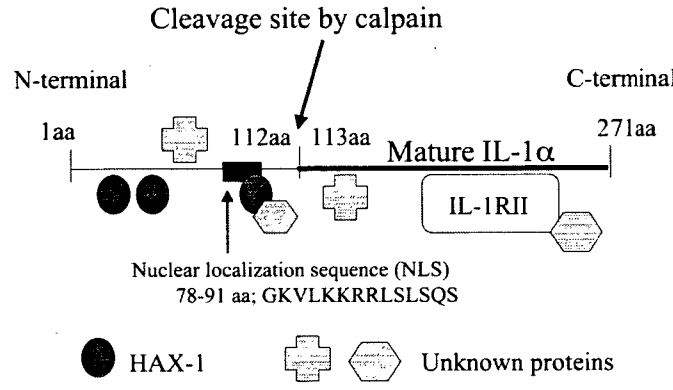

Fig. 7. A putative structural component of the pre-IL-1α complex in fibroblasts. IL-1RII binds to the C-terminal domain of pre-IL-1α, and HAX-1 binds to the N-terminal domain in fibroblasts. However, unknown proteins, aside from these two, may bind to pre-IL-1α and unknown proteins may directly bind to IL-1RII or HAX-1. The component proteins within the pre-IL-1α complex may possess the DNA- or RNA-binding motif, which may allow the pre-IL-1α complex to modulate the fibrosis and inflammation.

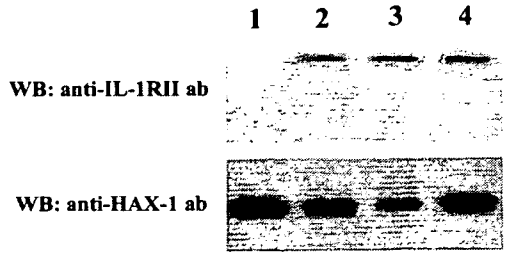

Fig. 8. Depletion of IL-1RII and HAX-1 by RNA interference. A DNA fragment that targeted the sequence in the ORFs of IL-1RII and HAX-1, and a control with a corresponding random sequence were obtained and were cloned into pSilencer 3.1H1-neo, an siRNA-expressing vector. Stable transfectants selected by G418 were cultured in DMEM plus 10% FBS for 72 h, and cell lysates were prepared by sonication. The cell lysates from various transfectants were resolved in 15% SDS/PAGE and transferred to nitrocellulose membrane. Anti-IL-1RII Ab (*Upper*) and anti-HAX-1 Ab (*Lower*) were used to perform Western blotting (WB). Lane 1, siRNA vector that contained the sequences of IL-1RII; lane 2, siRNA vector that contained random sequences for IL-1RII; lane 3, siRNA vector that contained the sequences of HAX-1; lane 4, siRNA vector that contained random sequences for HAX-1.

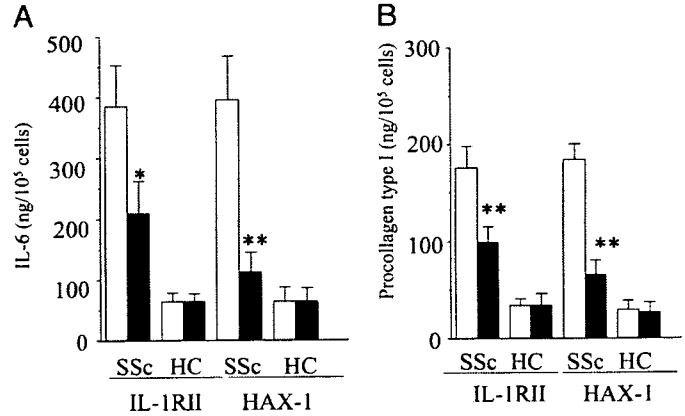

Fig. 10. IL-6 and procollagen type I C-peptide production decreases in SSc fibroblasts by the suppression of IL-1RII or HAX-1. Fibroblasts were cultured in serum-free media. After 48 h of culturing, commercial ELISA kits were used to measure IL-6 (*A*) and procollagen type I C-peptide (*B*) in culture supernatants. Open bars, random siRNA transfectants; filled bars, specific siRNA transfectants; SSc, fibroblasts derived from SSc (n = 5); HC, fibroblasts derived from healthy controls (n = 3). *, $P < 0.05$; **, $P < 0.01$ (compared with random siRNA transfectants).

involved in the transcriptional activation of several proteins. Although IL-1α was traditionally understood to exhibit biological functions such as inflammation, autoimmunity, and fibrosis through IL-1 receptors on the cell surface, the abovementioned findings and this study strongly support the theory of a nuclear site of action for IL-1α.

Another important finding is a role of IL-1RII, which binds pre-IL-1α inside human fibroblasts that are derived from SSc. McMahon *et al.* (17) and Sims *et al.* (18) reported that IL-1RII is a cell-surface receptor on B lymphocytes and neutrophils with a binding affinity for IL-1α, pre-IL-1α, and IL-1β, but it is not capable of the signal transduction of IL-1 because of the lack of the endoplasmic domain. Our current results revealed that IL-1RII combined with pre-IL-1α plays a crucial role in the biological features of pre-IL-1α within SSc fibroblasts. We also found the differential expression of IL-1RII between SSc (n = 5) and normal fibroblasts (n = 3) at the cellular mRNA and protein levels. Constitutive expression of IL-1RII, as well as pre-IL-1α, may be an important phenotype of SSc fibroblasts, although the mechanisms whereby intracellular IL-1RII was highly expressed in SSc fibroblasts remain to be clarified.

Suzuki *et al.* (19) first identified the HAX-1 protein by screening the proteins that interact with HS1 (hematopoietic lineage cell-specific protein 1). HS1 is B cell-signaling protein and is one of the major substrates of the Src and Syk/Zap-70 kinases (20). The HS1 protein mainly exists in the cytoplasm and

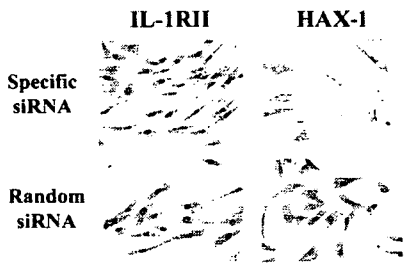

Fig. 9. Cell distribution of pre-IL-1α in SSc fibroblasts depleting IL-1RII or HAX-1. We obtained a DNA fragment targeting the sequence of IL-1RII or HAX-1, which was cloned into pSilencer 3.1 H1-neo, an siRNA-expressing vector. As a control, a scramble DNA fragment was generated that had the same number of nucleotides but did not display sequence identity with IL-1RII and HAX-1 (random siRNA). These vectors were transfected into SSc fibroblasts, and intracellular pre-IL-1α was detected by using immunocytochemistry of DAB staining.

nucleus, and, when the molecule is associated with HAX-1, it moves to the mitochondrial membrane. HAX-1 also interacts with pre-IL-1α in human chondrocytes, although the biological properties for the complex of HAX-1 and pre-IL-1α have not been fully elucidated (16). The HAX-1 protein appears to be expressed ubiquitously in various normal tissues and to constitute the domain that is responsible for binding to the pre-IL-1α, HS1, cortactin, PKD2, EBNA-LP, Bcl-2, and HIV1 Vpr proteins (21–23). The fact that HAX-1 interacts with a variety of structurally unrelated proteins suggests an essential function for HAX-1 that involves intracellular signaling and shuttling of various intracellular molecules. Our observations indicate the importance of HAX-1 for the nuclear localization of pre-IL-1α in fibroblasts. Posttranslational modifications such as phosphorylation and myristoylation of NTP-IL-1α are well recognized mechanisms that are involved in the transport of pre-IL-1α to the nucleus (24, 25). Notably, myristoylation occurs on lysine residues 82 and 83 of pre-IL-1α, located in the nuclear localization sequence (NLS). HAX-1 was associated with three segments of NTP-IL-1α, including the NLS segment (16), which suggests that the binding of HAX-1 with the NLS (KVLKKRR) of pre-IL-1α might facilitate the nuclear localization of the pre-IL-1α complex in fibroblasts. Taken together, the findings strengthen the conclusion that proteins associated with HAX-1 can shuttle between nuclear and cytoplasmic compartments.

A previous investigation looking for a nuclear target of pre-IL-1α revealed the interaction between pre-IL-1α and necdin by a yeast two-hybrid system (26). Necdin is a 47-kDa protein that functions as a cell-growth suppressor in a manner similar to that of the retinoblastoma tumor suppressor protein, Rb (27, 28). In our study, IP showed a faint band (≈47 kDa) that was subjected to N-terminal amino acid sequence analysis. However, we could not identify the molecule because of the small amount of peptide. Although we did not confirm that necdin was one of the intracellular pre-IL-1α-binding proteins, we did detect the expression of necdin in SSc and normal fibroblasts (data not shown). Moreover, the suppression of necdin with an RNAi method did not affect IL-6 and procollagen type I production in SSc fibroblasts (data not shown), which is inconsistent with the results of previous studies. This discrepancy may be explained, in part, by the different cell types used in each experiment (fibroblasts versus Saos-2 osteosarcoma cells).

Recent reports by Higgins *et al.* (29) and Kanangat *et al.* (30) demonstrated the biological functions of intracellular IL-1 receptor antagonist (icIL-1RA) in SSc fibroblasts. They indicated that icIL-1RA was overexpressed in SSc fibroblasts and that icIL-1RA was involved in the fibrogenic phenotype of SSc fibroblasts. Although we did not examine the expression of icIL-1RA in this study, icIL-1RA may have bound to intracellular IL-1RII that consisted of the pre-IL-1α complex. To determine whether icIL-1RA is the fourth component of the pre-IL-1α complex in SSc fibroblasts would have a potential role in delineating the molecular events of the fibrosis in SSc that are associated with the pre-IL-1α complex.

In conclusion, our study found the formation of the pre-IL-1α complex, which consists of pre-IL-1α, IL-1RII, and HAX-1, inside SSc fibroblasts. This complex plays a crucial role in the fibrogenic phenotype of SSc fibroblasts. Because of its nuclear localization, we believe this complex acts in the nuclei of fibroblasts; however, based on a search of the National Center for Biotechnology Information conserved domain database, these proteins do not have a DNA-binding motif. We speculate that this complex is part of a larger one. A putative pre-IL-1α complex is illustrated in Fig. 7.

Materials and Methods

Cell Culture. After providing informed consent, five female patients with SSc (median age 46) and three healthy female donors (median age 42) were enrolled in this study, which met the standards of our institutional review board. All patients were classified into diffuse cutaneous SSc according to the criteria of the American Rheumatism Association (31) and the classification of LeRoy *et al.* (32). Skin fibroblast lines were obtained from biopsied skin and explanted into tissue cultures. A murine fibroblast-like cell line, NIH 3T3, was also used in this study and was obtained from the American Tissue Culture Collection. The culture media consisted of DMEM (Sigma, St. Louis, MO) with 10% FBS (Sigma) and antibiotics (penicillin and streptomycin; Invitrogen, Carlsbad, CA) or of a serum-free medium (QBSF-51; Sigma). In this experiment, cells were used in the third through the fifth passages.

Immunocytochemical Staining. Monolayer fibroblast cultures (5×10^3 cells per well) were grown for 48 h in four-chamber slides (Lab-Tek; Nalge Nunc, Tokyo, Japan). Fibroblasts were washed twice with cold PBS and fixed with 2% paraformaldehyde plus 0.1% Triton X-100 in PBS. The primary Abs used in this experiment were monoclonal anti-human IL-1α Ab (R & D Systems, Cambridge, MA), monoclonal anti-human IL-1 receptor type II Ab (R & D Systems), and monoclonal anti-HAX Ab (BD Biosciences, San Jose, CA). Cells were incubated with the primary Ab (5 μg/ml) or as controls with preimmune mouse IgG (5 μg/ml; Dako, Kyoto, Japan) for 1 h at 4°C. The primary Ab was detected by incubation with biotinylated anti-mouse IgG Ab as the secondary Ab for 30 min at room temperature and then incubated with Avidin/Biotin-HRP Complex (ABC; Vector Laboratories, Burlingame, CA). Cells were then stained by DAB-peroxidase substrate (Sigma). Hematoxylin was used for nuclear staining. The chamber slides were dried and examined by light microscopy. Alternatively, after the treatment of the first Ab, cells were incubated with FITC- or Cy3-conjugated anti-mouse IgG Ab (Sigma). The chamber slides were washed three times and then mounted in 90% glycerol-PBS that contained 0.1% paraphenylendiamine and 1% *n*-propylgalate. A fluorescence image was obtained with fluorescence microscopy (Nikon, Tokyo, Japan).

Immunoprecipitation. SSc fibroblasts were cultured in DMEM (methionine/cystein-free) that contained 5% dialyzed FBS and 100 μCi/ml [^{35}S]methionine/cystein (1 Ci = 37 GBq; Amersham Bioscience, Buckinghamshire, U.K.) for 16 h. After a pulse, cells were harvested and suspended in 3 ml of IP procedure (IPP) buffer (10 mM Tris, pH 8.0/0.5 M NaCl/0.1% Nonidet P-40/0.1 mM PMSF/1 μg/ml leupeptin) and then sonicated on ice. Nuclear and cytosolic extracts were obtained together after centrifugation and were used for IP studies. A 40-μl volume of protein G-Sepharose was preincubated with rabbit anti-human IL-1α Ab (100 ng; Genzyme, Cambridge, MA) or control rabbit IgG (100 ng; Dako) and was added to the extracts and rotated for 3 h at 4°C. Immunoprecipitates were washed three times with IPP buffer and then fractionated by 10% sodium dodecyl (lauryl) sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) with molecular weight markers-^{14}C methylated protein (Amersham Bioscience). Radiolabeled polypeptides were visualized by autoradiography.

Peptide Sequencing. Two specific bands (65 and 35 kDa) were subjected to direct peptide sequencing. For sequencing, the proteins that were separated by SDS/PAGE were electrophoretically transferred onto poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad, Richmond, CA). The PVDF membrane was stained with Coomassie brilliant blue R-250, and each band was excised and subjected to N-terminal amino acid sequence analysis (Procise 494 HT protein sequencing system; Applied Biosystems, Foster City, CA).

RT-PCR. Total RNA was extracted from cultured fibroblasts with TRIzol reagent (Invitrogen), and then 1 μg of total RNA was reverse-transcribed into cDNA with SuperScript III (Invitrogen) according to the manufacturer's instructions. Real-time RT-PCR was performed in triplicate with an ABI 7900HT system (Applied Biosystems) and a fluorescein-labeled (FAM-labeled) TaqMan gene expression assay kit (Applied Biosystems) for IL-1RII, HAX-1, and GAPDH as an endogenous control. The results were analyzed with SDS 2.1 software (Applied Biosystems). Those genes' expressions were calculated from the accurate threshold cycle (Ct), which is the PCR cycle at which an increase in fluorescein from TaqMan probes can first be detected above a baseline signal. The Ct values for GAPDH were substituted from the Ct values for IL-1RII and HAX-1 in each well to calculate ΔCt. The triplicate ΔCt values for each sample were averaged.

Construction of Expression Plasmids and Transfection. The cDNA encoding human IL-1α, pre-IL-1α, and NTP-IL-1α were all isolated by PCR and subcloned into pcDNA4-V5 (Invitrogen). The cDNA encoding human IL-1RII was isolated by PCR and subcloned into pcDNA3 (Invitrogen). For stable transfections, NIH 3T3 cells in 60-mm dishes (70% confluent) were incubated with 3 ml of Opti-MEM (Invitrogen) that contained 5 μg of DNA and 18 μl of Lipofectamine 2000 (Invitrogen). After 5 h, 3 ml of DMEM with 20% FBS was added. After 24 h, the medium was changed to DMEM with 10% FBS, followed by an additional 24 h of culture. G418 (400 μg/ml) was added to the culture medium 48 h after transfection and kept for 15 days. The G418-resistant colonies were harvested by gentle digestion with trypsin, and cells were preserved in liquid N_2 with Cellbanker (Mitsubishi Kagaku Iatron, Tokyo, Japan) until use.

Western Blotting. Confluent fibroblasts were maintained in a serum-free medium for 48 h. Cells were then trypsinized and washed with PBS. Cell lysates were prepared from fibroblasts, including PBS that contained 0.1 mM PMSF and 1 μg/ml leupeptin by sonication on ice. The cell lysates were resolved in 15% polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad). The membranes were incubated with the primary Abs for 1 h. Horseradish peroxidase-conjugated antimouse IgG Ab (Santa Cruz Biotech-

nology, Santa Cruz, CA) was applied to the membrane and incubated for 1 h. The blot was developed by the enhanced chemiluminescence system (Amersham) and exposed on x-ray film. The primary Abs used in this experiment were monoclonal anti-human IL-1α Ab (R & D Systems), monoclonal anti-human IL-1 receptor type II Ab (R & D Systems), monoclonal anti-V5 Ab (Invitrogen), and monoclonal anti-HAX Ab (BD Biosciences).

Bacterial Two-Hybrid System. Reagents and protocol were obtained from Stratagene (BacterioMatch two-hybrid system). The pre-IL-1α protein was fused briefly to the full-length bacteriophage λ repressor protein (λcI) with pBT plasmid (Stratagene), which contained the N-terminal DNA-binding domain and the C-terminal dimerization domain. The target proteins (IL-1RII and HAX-1) were fused to the N-terminal domain of the α-subunit of RNA polymerase with pTRG plasmid (Stratagene). The pre-IL-1α protein was tethered to the λ operator sequence upstream of the reporter promoter through the DNA-binding domain of λcI. When the pre-IL-1 and target proteins interact, they recruit and stabilize the binding of RNA polymerase at the promoter and activate the transcription of a reporter gene, the Amp^r gene. A second reporter gene, β-galactosidase, is expressed from the same activatable promoter, which provides an additional mechanism to validate the pre-IL-1α and target proteins' interaction. The suitable *Escherichia coli* host strain (XL1-Blue MRF' Kan) was transformed with the two plasmids. Blue colonies are positive in LB agar plates, including tetracycline, chloramphenicol, kanamycin, and X-gal.

Depletion of HAX-1 and IL-1RII by RNA Interference. The siRNA target-finder algorithm, which is available on the Ambion (Austin, TX) web site (www.ambion.com), was used to select 21 nucleotide oligomers to be tested for RNA interference. We obtained a DNA fragment targeting the sequence in the ORF and a control with a corresponding random sequence. These two DNA fragments were cloned into pSilencer 3.1 H1-neo (Ambion), an siRNA-expressing vector, according to the manufacturer's instructions. Target sequences for siRNAs of HAX-1 and IL-1RII were selected to be 5'-AACCCAAGGTTCCATAGTCCT-3' and 5'-AAGAAGAGACACGGATGTGGG-3', respectively. A random 21-nt sequence as a control was generated that had the same numbers of nucleotides but did not display sequence identity with HAX-1 and IL-1RII. Basic local alignment search tool analysis ensured that sequence identity between a random nucleotide and homosapience cDNA in the National Center for Biotechnology Information database was 15 nucleotides or fewer. The random sequences for HAX-1 and IL-1RII were 5'-AACCGCGAATCTCATAGTCCT-3' and 5'-AAGGAGAGCAGCGGATGTAAG-3', respectively. The method for stable transfections was described earlier.

Measurement of IL-6 and Procollagen Type I. Fibroblasts were cultured in 24-well culture plates with serum-free medium for 48 h, and then the supernatants were collected and preserved at -30°C until use. IL-6 and procollagen type I were measured by using commercial ELISA kits [R & D Systems and Takara Shuzo (Kyoto, Japan), respectively].

Statistical Analyses. The results of IL-6 and procollagen type I concentrations were shown as mean $\pm$ SD, and comparisons of data were performed with Student's t test. Differences were considered to be significant at $P < 0.05$.

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Longterm Effect of Intermittent Cyclical Etidronate Therapy on Corticosteroid-Induced Osteoporosis in Japanese Patients with Connective Tissue Disease: 7-Year Followup

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ABSTRACT. ***Objective.*** To determine the efficacy and safety of intermittent cyclical etidronate therapy of up to 7 years for corticosteroid-induced osteoporosis.

Methods. One hundred two Japanese patients who originally participated in a 3-year prospective randomized study were enrolled into an open-label followup study. All patients had received > 7.5 mg of prednisolone daily for at least 90 days before entry into the original study and were randomly assigned to 2 treatment arms: E, those receiving etidronate disodium (200 mg per day) for 2 weeks together with 3.0 g of calcium lactate and 0.75 μg of alphacalcidol daily; and C, controls receiving only the latter. Endpoints included changes from baseline in bone mineral density (BMD) of the lumbar spine and the rate of new vertebral fractures.

Results. The mean (± SD) lumbar spine BMD had increased by 5.9% ± 8.8% (p = 0.00007) and 2.2% ± 5.8% (p = 0.013) from baseline after 7 years in groups E and C, respectively. This improvement in BMD in group E was significantly better than in group C (p = 0.02). The frequency of new vertebral fractures was lower in group E, resulting in reduction of the risk of such new fractures by 67% at year 7 (odds ratio 3.000; 95% confidence interval, 0.604–14.90; p = 0.18). There were no severe adverse events in group E during our study.

Conclusion. Our results indicate that longterm (up to 7 years) intermittent cyclical etidronate therapy is safe and effective for prevention and treatment of corticosteroid-induced osteoporosis in patients with connective tissue diseases. (First Release Nov 15 2007; J Rheumatol 2008;35:142–6)

Key Indexing Terms:
CORTICOSTEROID-INDUCED OSTEOPOROSIS
BONE MINERAL DENSITY
BISPHOSPHONATE
CONNECTIVE TISSUE DISEASES

Longterm corticosteroid treatment in patients with connective tissue disease (CTD) causes osteoporosis as the major adverse event. Bisphosphonate therapy has proven to be effective in both prevention and treatment of corticosteroid-induced osteoporosis (CIOP)[1-4]. Guidelines for treating patients with CIOP recommend the use of bisphosphonates as a first-line drug[5]. Nitrogen-containing bisphosphonates, such as alendronate or risedronate, have proven efficacy for both prevention and treatment of CIOP. However, use of these bisphosphonates is associated with gastrointestinal adverse events[6]. We previously conducted a 3-year prospective randomized study to determine the efficacy and safety of etidronate (the first available nitrogen-free bisphosphonate) for treating CIOP[7]. Although longterm followup (7 yrs) of intermittent cyclical etidronate therapy in patients with postmenopausal osteoporosis has been reported[8], few studies are available on the longterm effects of etidronate in patients with CIOP[9]. Further, there are no reports on the continued effectiveness and safety of etidronate for CIOP in patients with CTD. For this reason, we have followed up the original 3-year prospective study for an additional 4 years to determine the longterm efficacy of intermittent cyclical etidronate for treating CIOP in patients with CTD.

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MATERIALS AND METHODS

Patients. In the original 3-year study, 102 patients with different CTD were enrolled (56 with systemic lupus erythematosus; 12 rheumatoid arthritis; 10 polymyositis/dermatomyositis; 9 vasculitis syndrome; 8 adult-onset Still disease; 5 polymyalgia rheumatica; 1 systemic sclerosis; and 1 Sjögren's syndrome). Patients' ages ranged from 21 to 73 years and they had been taking

> 7.5 mg of prednisolone (PSL) daily for at least 90 days. The results of this study have been reported elsewhere[7].

Study design. In the original 3-year prospective randomized study, all patients were randomly assigned to one of 2 investigational groups. Patients in the etidronate group (E) received 200 mg/day etidronate disodium (Didronel, Sumitomo Pharmaceuticals, Osaka, Japan) for 2 weeks, together with 3.0 g of calcium lactate and 0.75 μg of alphacalcidol (Alfarol, Chugai Pharmaceuticals, Tokyo, Japan) daily for 90 days. This cycle was repeated 28 times during the 7-year observation period. Patients were instructed to take their medication with water at bedtime. The control group (C) received only 3.0 g of calcium lactate and 0.75 μg of alphacalcidol daily for 90 days. In the followup study, the patients were to continue taking the same treatments to which they had been assigned earlier. However, a change of treatment was allowed if their doctor decided that it was necessary for treatment. All patients had provided written informed consent.

Bone mineral density (BMD) and radiological measurements. Lateral and anteroposterior lumbar and thoracic spine radiographs were taken and evaluated at Keio University Hospital at baseline and every year for 7 years. All lumbar and thoracic spine images were evaluated by experienced physicians who were blinded to treatment assignments. The diagnosis of vertebral fracture and osteoporosis was based on the criteria defined by the Japanese Society for Bone and Mineral Research in 1996[10]. A vertebral fracture was defined as: (1) The ratio of the central height (C) to the anterior height (A) of the vertebra was less than 0.8, or the ratio of C to the posterior height (P) was less than 0.8. (2) The ratio of A to P was less than 0.75. (3) A crushed vertebra was recorded when its height was reduced by more than 20% in either A, C, or P compared with the adjacent vertebrae.

Classification of BMD was based on the following criteria: Normal BMD: > 80% of the young adult mean (YAM). Osteopenia: between 70% and 80% of YAM. Osteoporosis: < 70% of YAM.

This definition of osteoporosis (i.e., < 70% of YAM) also corresponds to the osteoporosis criteria recommended by the World Health Organization [less than −2.5 standard deviation (SD) of YAM]. All BMD measurements were made by dual-energy x-ray absorptiometry using an XR-36 (Norland Medical Systems, Fort Atkinson, WI, USA). Because we had already documented changes in bone formation and bone absorption markers in the original 3-year prospective randomized study, we did not monitor biochemical markers of bone turnover in this followup study.

Statistical analysis. The baseline characteristics and homogeneity of the patients' background in an intent-to-treat population was compared between the 2 investigational groups by chi-square test, Student's t-test, and Mann-Whitney U-test, as appropriate. Regardless of whether patients were still receiving the assigned medication, all available BMD data were used to perform an intent-to-treat analysis. If the measurement of the lumbar spine BMD at 7 years was not available, the measurement obtained at the time closest to this was used in the analysis. The patients whose BMD data could not be evaluated correctly because of previous compression fractures are excluded from the analysis. The primary efficacy analysis was based on the differences between the 2 investigational groups in the percentage change of lumbar spine BMD (L2-L4) from baseline to last measurement. The percentage change of BMD from the baseline was compared by an analysis of variance model (SPSS version 14.0). The comparison of percentage change of BMD between the 2 groups was calculated by Student's t-test. Odds ratios adjusted by menopausal status stratum as a factor were calculated for differences of the incidence of vertebral fractures at 7 years between the 2 treatment groups. Significance level was set at 5% and all results expressed as mean ± SD.

RESULTS

At the beginning of this followup study, 7 patients in group E and 6 patients in group C could not be included because of death or loss to followup. There were no significant differences between groups in baseline characteristics in that subset of patients whose data could be used for an intent-to-treat analysis (43 and 45 in groups E and C whose BMD data were available, respectively; Table 1). During 7 years, the average daily dose of PSL in each year was not significantly different between the 2 groups. The number of patients taking steroid in groups E and C was also not significantly different (Table 2). During the followup study, there were no adverse events in either group. However, 2 patients in group E and one in group C died due to progression of their underlying CTD or infection during this study. Ten patients in group C began to receive bisphosphonate as well as alphacalcidol and calcium lactate because their rheumatologist decided that they would benefit from such treatment. On the other hand, only 1 patient in group E was changed from etidronate to alendronate.

After 7 years of treatment, the mean (± SD) percentage change in BMD of the lumbar spine in group E (5.9% ± 8.8%) was significant compared to baseline ($p = 0.00007$). This was also the case, albeit to a lesser extent (2.2% ± 5.8%), in group C ($p = 0.013$; Figure 1). This improvement of BMD was significantly greater in group E than in group C ($p = 0.02$).

In a separate analysis of premenopausal and postmenopausal women, both of these subgroups of group E showed an increase in the mean percentage change in BMD during their treatment course. In premenopausal women, both groups E and C had significant increases of lumbar spine BMD from baseline at 7 years ($p = 0.001$ and $p = 0.02$, respectively). These increases were significantly higher in group E than C in a subgroup of premenopausal women (6.7% ± 9.1% vs 2.3% ± 4.5%; $p = 0.04$). Although the postmenopausal subgroup of group E showed an increase in BMD of the lumbar spine, this failed to achieve significance (2.8% ± 8.0%; $p = 0.23$). BMD of this subgroup in group C remained at baseline (−0.03% ± 7.60%; $p = 0.99$). There were also no significant differences between the 2 groups in this respect.

Analysis of the subgroups based on the baseline BMD revealed that the osteoporosis + osteopenia subgroup in group E showed a significant increase of the lumbar spine BMD from baseline at 7 years ($p = 0.0009$). This increase was significantly greater in group E than in group C at 7 years (7.8% ± 9.5% vs 2.0% ± 6.3%; $p = 0.04$). Both E and C groups of the normal BMD subgroup showed significant increases in lumbar spine BMD at year 7 (3.9% ± 7.7%, $p = 0.03$ and 2.4% ± 5.6%, $p = 0.03$, respectively). Again, there were no significant differences between the 2 groups ($p = 0.40$).

The mean percentage change in lumbar spine BMD in group E improved from baseline by approximately 5% over the 7 years (Figure 2). Although group C showed no decrease of BMD from baseline, the increase in group E was significantly greater than in group C at 7 years ($p = 0.02$).

Six patients in group C had a total of 11 new vertebral fractures during the followup period (Table 3), whereas only 2 patients in group E had a total of 3 new fractures. At year 7, cyclic etidronate therapy had reduced the risk of new vertebral fracture by 67% [odds ratio (OR) 3.00; 95% confidence inter-

Table 1. Baseline characteristics at the beginning of the followup study (values are means ± SD).

Characteristics	Group E, n = 46	Group C, n = 45	p
Men, n(%)	6 (13)	8 (18)	NS
Premenopausal women, n (%)	25 (54)	24 (53)	NS
Postmenopausal women, n (%)	15 (33)	13 (29)	NS
Mean age, yrs			
Men	53 ± 17	43 ± 16	NS
Women	43 ± 14	43 ± 13	NS
Total corticosteroid dose, mg			
Men	2,577 ± 1245	1,705 ± 497	NS
Premenopausal women	2,497 ± 1816	1,852 + 506	NS
Postmenopausal women	2,143 ± 918	1,880 ± 931	NS
Lumbar spine BMD, g/cm^2			
Men	0.95 ± 0.17	0.90 ± 0.18	NS
Premenopausal women	0.86 ± 0.18	0.93 ± 0.14	NS
Postmenopausal women	0.78 ± 0.15	0.79 ± 0.14	NS

Group E: etidronate; Group C: control. BMD: bone mineral density; NS: not significant; SD: standard deviation.

Table 2. Average daily dose of PSL and ratio of patients still receiving corticosteroids (years after start of the prospective study).

Year		1	2	3	4	5	6	7
Average daily dose of PSL (mg/day, mean ± SD)	Group E	10.7 ± 3.8	9.4 ± 5.4	8.3 ± 3.8	7.7 ± 3.1	8.1 ± 4.0	9.3 ± 5.1	8.5 ± 4.4
	Group C	9.7 ± 3.7	8.1 ± 2.8	8.0 ± 3.3	7.8 ± 4.1	7.7 ± 4.1	8.0 ± 4.6	7.7 ± 3.4
	p value	NS	NS	NS	NS	NS	NS	NS
Ratio of patients receiving PSL (%)	Group E	100	100	100	98	95	92	92
	Group C	100	100	100	98	98	98	95
	p value	NS	NS	NS	NS	NS	NS	NS

Group E: etidronate; Group C: control. PSL: prednisolone; NS: not significant.

val 0.604–14.90; p = 0.18). The adjusted OR by menopausal status was calculated as 2.05.

There were no adverse events in group C, but 2 occurred in group E during the 3-year prospective study. However, there were no adverse events in either group during the followup period. Although serum calcium monitoring had not been performed systematically, where measured, no hypercalcemia was found in either group. None of the patients had gastrointestinal symptoms severe enough to discontinue the etidronate throughout the entire 7 years of followup in group E.

DISCUSSION

Our study demonstrated that longterm intermittent cyclical etidronate therapy increased the BMD of the lumbar spine in Japanese patients with CIOP. No significant reduction in risk of vertebral fractures at 7 years was achieved. However, this might be due to small sample size; future studies on larger numbers of patients will be required to draw a definitive conclusion on this point.

It has been reported before that etidronate increases BMD in CIOP[11-15], but there is only one report on longterm observation indicating efficacy of continuing etidronate therapy for more than 5 years in patients with asthma receiving oral and/or inhaled corticosteroids[9]. The longterm efficacy of intermittent etidronate therapy for CIOP in patients with CTD has to our knowledge never been evaluated. Ours is thus the first comprehensive study on the efficacy of longterm cyclical etidronate therapy of up to 7 years for CIOP in patients with CTD. Previous studies showed that alendronate or risedronate, which are both nitrogen-containing bisphosphonates, can maintain continuing increases in BMD, as well as effect a reduction of the fracture rate[4,16-19]. Our study is consistent with these findings with respect to maintaining BMD.

In our study, both groups C and E showed higher increases in lumbar spine BMD at years 6 and 7. In group C, it might be due to the fact that 9 patients added bisphosphonates besides activated vitamin D_3 during the followup study. Indeed, the mean percentage changes in patients who added bisphosphonates were higher compared with those patients who did not at year 7, although the differences were not statistically significant. In contrast, only 1 patient changed from etidronate to alendronate in group E. However, this patient and 2 patients who discontinued the corticosteroids during the followup study showed considerable increases in lumbar

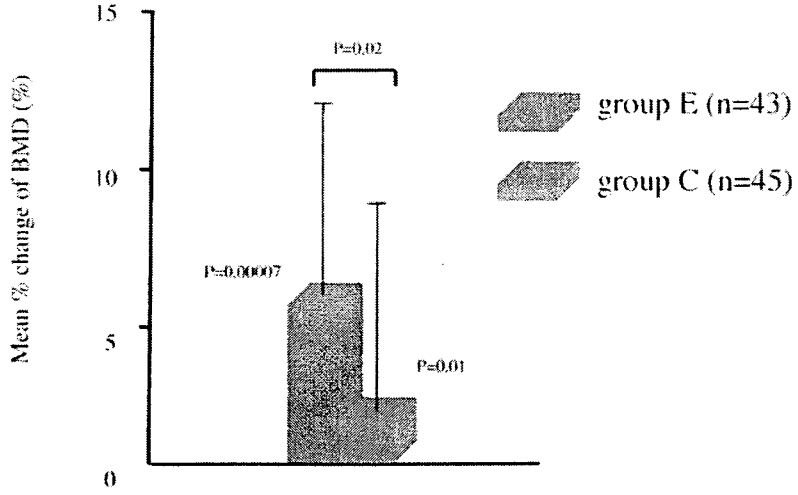

Figure 1. The mean (± SD) percentage change in bone mineral density (BMD) of the lumbar spine between baseline (0 yr) and 7 years of groups E and C. In all patients, the mean (± SD) percentage change in BMD of the lumbar spine increased 5.9% ± 8.8% (p = 0.00007) from baseline at 7 years in group E and 2.2% ± 5.8% (p = 0.013) in group C. The improvement of BMD in group E was significantly higher than in group C at 7 years (p = 0.02).

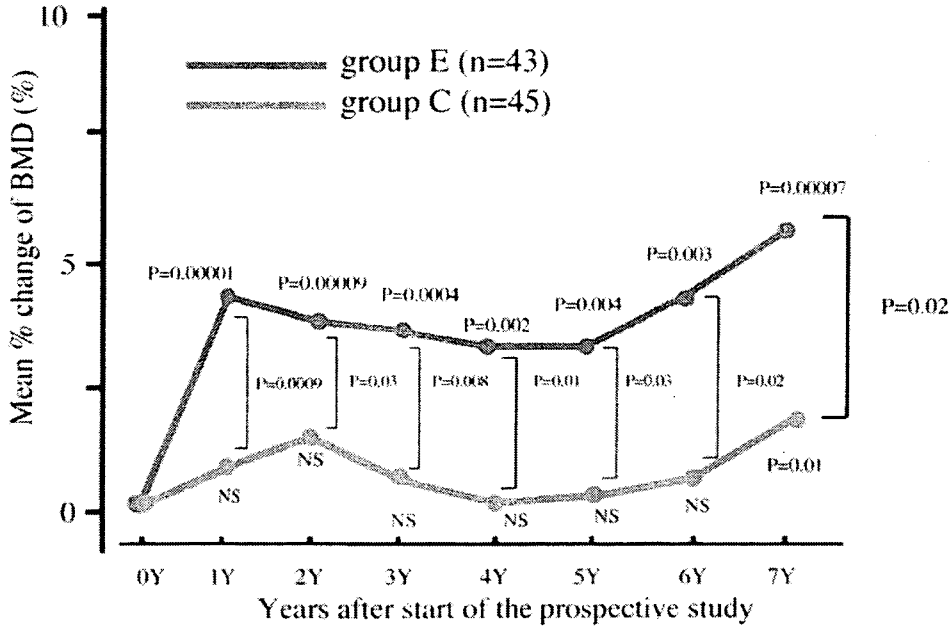

Figure 2. The mean percentage change in lumbar spine BMD in group E improved from baseline by approximately 5% over the 7-year study.

spine BMD at years 6 or 7. These increases in lumbar spine BMD might have contributed to the increase in the mean percentage change of lumbar spine BMD to some degree. As far as we know, there were no patients who took other medications. Persistent prescriptions for etidronate and activated vitamin D_3 have been confirmed in the medical records. Therefore, we think that other medications or compliance with treatment would not affect the BMD data in either group.

The ability of activated vitamin D_3 to prevent the loss of BMD caused by corticosteroids has been reported[20]. In our study, the control group (receiving activated vitamin D_3) slightly increased their lumbar spine BMD and maintained this for 7 years. However, as there was no placebo control in our study, we could not compare the rate of vertebral fracture in the group receiving activated vitamin D_3 to that of the placebo group.

Table 3. Incidence of vertebral fractures at 7 years in group E (etidronate) and C (control).

	Group E	Group C
Male	0/4	1/6
Female		
Premenopausal	1/24	1/23
Postmenopausal	1/10	4/13
Total	2/38	6/42
Total vertebral fracture	3	11

A randomized, double-blinded, multicenter study showed almost the same efficacy of either 200 mg or 400 mg of cyclical intermittent etidronate therapy in Japanese patients with involutional osteoporosis[21]. The current approved dose of etidronate for osteoporosis in Japan is 200 mg daily. When the dose of 200 mg of etidronate was ineffective or the patients had severe osteoporosis, 400 mg was used. Our results here indicated that 200 mg of etidronate was sufficiently effective to prevent or treat Japanese patients with CIOP. This might be due to racial differences or the difference in the dose per unit body weight between Japanese and Caucasian patients. Because our study did not include large numbers of patients, further observations will be needed to confirm this hypothesis.

Regarding adverse events, it was notable that severe side effects were not seen throughout the 7 years of the study. No gastrointestinal disorders, bone necrosis, or disturbance of bone formation was recorded. Only 2 patients had an adverse event of any kind in group E during the 3-year prospective study. No other patients dropped out of the study because of drug intolerance or discomfort. Treatment compliance was good and we can conclude that intermittent cyclical etidronate therapy is very well tolerated. Moreover, in view of its cost-effectiveness[22], cyclical intermittent etidronate therapy should be considered as a routine treatment option for CIOP, especially in patients with previous or current gastrointestinal disorders.

Intermittent cyclical etidronate therapy significantly increased BMD of the lumbar spine and maintained it over 7 years in patients with CIOP. There was a tendency towards a reduction in the incidence of vertebral fractures at 7 years in etidronate-treated patients compared to activated vitamin D_3-treated patients, but this did not achieve statistical significance. Longterm intermittent cyclical etidronate therapy is a safe, well tolerated, and effective therapy for the prevention and treatment of CIOP in Japanese patients with CTD.

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Decreased CD161$^+$CD8$^+$ T cells in the peripheral blood of patients suffering from rheumatic diseases

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Objectives. **Although it has been reported that the numbers of both CD4$^-$CD8$^-$ and CD4$^+$ natural killer T (NKT) cells are selectively decreased in the peripheral blood of patients with rheumatic diseases, there have been no reports concerning a novel subpopulation of CD8$^+$ NKT cells. To examine whether CD161$^+$CD8$^+$ T cells, which are closely related to CD8$^+$ NKT cells, are also decreased in patients with rheumatic diseases, we have investigated the expression of CD161, together with that of CD28, CD25 and CD62L, on T cells in the peripheral blood of these patients.**

Methods. **The rheumatic diseases evaluated in this study were systemic lupus erythematosus (SLE) ($n = 54$), mixed connective-tissue disease (MCTD) ($n = 15$), systemic sclerosis (SSc) ($n = 14$), polymyositis/dermatomyositis (PM/DM) ($n = 13$) and rheumatoid arthritis (RA) ($n = 24$). Healthy donors were examined as controls ($n = 18$). The expression of CD161, CD28, CD25 and CD62L on T cells was analysed by flow cytometry.**

Results. **Both the frequency of CD161 expression on CD8$^+$ cells and the absolute number of CD161$^+$CD8$^+$ cells were significantly decreased in patients with SLE, MCTD, SSc and PM/DM. Only the absolute number of CD161$^+$CD8$^+$ T cells was significantly decreased in RA. CD161 expression on CD28$^-$CD8$^+$ T cells was significantly decreased in SLE, MCTD and SSc. The absolute number of CD161$^+$CD8$^+$CD62L$^-$ T cells was significantly decreased in SLE, MCTD and SSc.**

Conclusions. **Both the frequency and the absolute number of CD161$^+$CD8$^+$ T cells were decreased in the peripheral blood of patients suffering from SLE, MCTD, SSc and PM/DM. This result suggests that there is also an abnormality of NKT cells in the CD8$^+$ population.**

KEY WORDS: CD161$^+$CD8$^+$ T cells, CD8$^+$ NKT cells, Rheumatic diseases.

Introduction

Autoimmune diseases are characterized by the reaction of lymphocytes against auto-antigens and by the production of auto-antibodies. Although the etiologies of autoimmune diseases are not clearly defined, most models for the pathogenesis of these disorders include the activation of both T and B cells including self-reactive clones. Immunological tolerance in healthy individuals regulates this activation of lymphocytes and prevents the onset of autoimmune diseases. Various mechanisms are involved in this tolerance, with an important role for regulatory T cells that suppress self-antigen-reactive cells.

Natural killer T (NKT) cells have been identified as a subpopulation of CD4$^-$CD8$^-$ (double negative, DN) or CD4$^+$ T cells, and express both specific α/β T cell receptors (TCRs) and NK markers in humans [1] and mice [2]. In humans, NKT cells have the invariant TCR Vα24JαQ gene, which is recognized by CD1d [3]. The majority of human NKT cells express TCR Vα24Jα18Vβ11 [4] and the CD161 (NKR-P1A) molecule on their surface as an NK marker. Human NKT cells are believed to have regulatory effects on immune tolerance or autoimmunity [1], and in fact the number of both CD4$^-$CD8$^-$ and CD4$^+$ NKT cells is selectively decreased in the peripheral blood of patients with systemic lupus erythematosus (SLE), systemic sclerosis (SSc), rheumatoid arthritis (RA) and Sjögren's syndrome (SS) [4].

CD161 is also expressed on CD8$^+$ T cells [5, 6]. The TCRs of CD161$^+$CD8$^+$ T cells have Vα24Jα36, Vα24Jα45 or Vα24Jα37 [7], which are different from those of CD4$^-$CD8$^-$ or CD4$^+$ NKT cells. A recent study has further demonstrated a novel subpopulation of human Vα24$^-$ CD8$^+$ NKT cells, a majority of which express the CD161 molecule and recognize the CD1d molecules [8]. Moreover, it has been demonstrated that CD8$^+$ NKT cells have a regulatory function of inhibiting the proliferation of antigen-specific activated T cells in humans [9]. Thus, these CD161$^+$CD8$^+$ T cells may be a different population or a subpopulation of the conventional CD4$^-$CD8$^-$ or CD4$^+$ NKT cells, and have a regulatory function. However, there have been no reports concerning CD161$^+$CD8$^+$ T cells or CD8$^+$ NKT cells in rheumatic diseases, and the function of the CD161 molecule is still unclear.

In the present study, we investigated the expression of CD161 on peripheral blood T cells from patients with various rheumatic diseases to examine whether CD161$^+$CD8$^+$ T cells are also decreased. This is the first report to document a decrease of CD161$^+$CD8$^+$ T cells in the peripheral blood of patients suffering from SLE, mixed connective-tissue disease (MCTD), SSc and polymyositis/dermatomyositis (PM/DM). We also investigated CD161 expression on CD28$^-$CD8$^+$ T cells and CD4$^-$CD25$^+$ T cells, and the expression of the CD62L molecule on CD161$^+$CD8$^+$ T cells to help clarify how the CD161 molecule

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or $CD161^+CD8^+$ T cells might function in the peripheral blood of these patients.

Patients and methods

Patients

The study population comprised 120 patients (18 men and 102 women), and each patient was followed at the Department of Rheumatology and Internal Medicine, Juntendo University School of Medicine, from October 1999 to February 2004. The demographic features of the patients and healthy donors are shown in Table 1. SLE, SSc and RA were diagnosed according to the American College of Rheumatology criteria [10–12]. MCTD was diagnosed according to Kasukawa's criteria [13], and PM/DM was diagnosed according to Bohan's criteria [14]. The PM/DM group did not include patients with amyopathic DM. The control group that included the healthy donors did not match the age of some of the patient groups, because of the predominant age at onset of each disease. The treatments that the patients received are shown in Table 2. Eight patients with SLE, four with MCTD, seven with SSc, one with PM/DM and two with RA did not receive any therapy, which means that these patients were not treated with steroids, immunosuppressants or plasmapheresis.

TABLE 1. Demographic features of the patients included in this study

Groups	(*n*)	Males/females	Age (years) Mean ± S.D.	Median
SLE	54	7/47	33.4 ± 12.2	31
GN	33	4/29		
NPSLE	4	0/4		
TH	9	1/8		
APS	3	1/2		
Others*	5	1/4		
MCTD	15	1/14	41.3 ± 17.9	39
SSc	14	2/12	49.2 ± 15.0	57
PM/DM	13	4/9	50.0 ± 14.2	53
RA	24	4/20	59.0 ± 14.0	62
HD	18	9/9	30.7 ± 3.8	31

SLE, systemic lupus erythematosus; MCTD, mixed connective-tissue disease; SSc, systemic sclerosis; PM/DM, polymyositis/dermatomyositis; RA, rheumatoid arthritis; HD, healthy donors; GN, glomerulonephritis; NPSLE, neuropsychiatric systemic lupus erythematosus; TH, thrombocytopaenia; APS, anti-phospholipid syndrome.

*Others consisted of one patient with lupus colitis, one patient with serositis, one patient with vasculitis and two patients without complications.

For example, among the eight patients with SLE, two were treated with anti-allergic drugs, one with an antihistaminic, one with diclofenac sodium, one with an oral cephem, and three were fully drug-free before analysis. After analysis, all of these eight SLE patients received prednisolone as soon as possible. Among them, two patients received not only prednisolone but also 500 mg/month cyclophosphamide pulse therapy, and another patient received not only prednisolone but also methylprednisolone pulse therapy. Another two patients with SLE were treated by plasmapheresis once a week and peripheral blood samples from these patients were obtained just before the procedure. All the patients and healthy donors were fully informed and gave their consent to participate.

Clinical assessment

Disease activity of SLE patients was assessed in terms of the SLE Disease Activity Index (SLEDAI) score [15], the level of CH50 (normal range 25–54 units), and anti-DNA antibodies (normal range <6 IU/ml). Other disease activities were assessed by the level of C-reactive protein (CRP, normal range <0.3 mg/dl) in SSc, PM/DM and RA patients and by the level of creatinine kinase (CK) (normal range 9–93 IU/l) in PM/DM patients.

Monoclonal antibodies (mAbs)

The following monoclonal antibodies (mAbs) were used in this study: fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (RPA-T4), anti-CD161 (DX12), phycoerythrin (PE)-conjugated anti-CD8 (RPA-T8), anti-CD25 (M-A 251), anti-CD28 (CD28.2), anti-CD62L (Dreg 56), antigen-presenting cell (APC)-conjugated anti-CD3 (HIT 3a), biotin-conjugated anti-CD8 and anti-CD4, and purified anti-CD161 (DX12, mouse IgG1) (a blocking antibody) and isotype-matched control mAb (mouse IgG1). All mAbs were obtained from PharMingen (San Diego, CA, USA). Alexa 488-conjugated streptavidin was obtained from Molecular Probes (Eugene, OR, USA).

Cell preparation

Peripheral venous blood of patients and healthy donors was diluted 1:2 with phosphate-buffered saline (PBS) (Dulbecco, Nissui, Japan), and the peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation with lymphocyte separation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). After two washings with PBS, the PBMC count was determined with a blood cell counter, and the cell suspension was adjusted to a concentration of 1×10^6 cells/ml in PBS.

Staining and flow cytometric analysis

The PBMCs (1×10^6 cells) were incubated with FITC-conjugated anti-CD161 mAb, PE-conjugated anti-CD25, anti-CD28 or

TABLE 2. Treatments of the patients included in this study

Groups	None	Prednisolone (mg/day) 5–30	31–60	61–80	CyA	CYC	D-PC	AZA	MZB	SASP	MTX	BUC	Gold	PP
SLE	8	18	24	3	0	0	0	1	1	0	0	0	0	2
MCTD	4	8	3	0	0	1	0	0	0	0	0	0	0	0
SSc	7	4	1	0	1	0	1	1	0	0	0	0	0	0
PM/DM	1	4	6	1	1	0	1	0	0	0	0	0	0	0
RA	2	17	0	0	0	0	3	0	2	5	5	3	1	0

SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease; SSc, systemic sclerosis; PM/DM, polymyositis/dermatomyositis; RA, rheumatoid arthritis; CyA, cyclosporine A (50 mg/day); CYC, oral cyclophosphamide (50 mg/day); D-PC, D-penicillamine (100–200 mg/day); AZA, azathioprine (50 mg/day); MZB, mizoribine (150 mg/day); SASP, salazosulfapyridine (500–1000 mg/day); MTX, methotrexate (4–6 mg/week); BUC, bucillamine (50–200 mg/day); Gold, injected gold sodium thiomalate (20 mg/week); PP, plasmapheresis (once/week); None, patients received neither steroids, immunosuppressives nor plasmapheresis.

anti-CD62L mAb, APC-conjugated anti-CD3 mAb, and biotin-conjugated anti-CD8 or anti-CD4 mAb on ice for 30 min. After two washings with PBS, Alexa 488-conjugated streptavidin was added and incubation was continued for a further 30 min. After two additional washes, the samples were fixed with 0.5% paraformaldehyde in PBS. Flow cytometric analysis was performed with an FACScaliber (Becton Dickinson, San Jose, CA, USA) and data were processed with the CellQuest program (Becton Dickinson).

Effect of anti-CD161 mAb cocultured with pokeweed mitogen (PWM) and quantification of IgG by ELISA

The PBMCs from healthy donors (1×10^5 cells/well) were cultured with 0.1 μg of PWM (Sigma, St Louis, MO) in 96-well round-bottom plates in 0.2 ml of culture medium for 7 days at 37°C in a humidified atmosphere with 5% CO_2. Various amounts of purified anti-CD161 mAb (a blocking antibody) (0, 0.5 and 10 μg/ml) or isotype-matched control mAb (mouse IgG1) (0, 0.5 and 10 μg/ml) were added at the beginning of the experiments. The culture supernatants were harvested and added to goat anti-human IgG (Bethyl, Mongomery TX)-coated 96-well flat-bottom ELISA plates. After discarding the supernatants and washing with Tween-20 PBS, the bound human IgG was detected with horseradish peroxidase (HRP)-labelled goat anti-human IgG at a dilution of 1:100 000 followed by the addition of tetramethylbenzidine (Bio-Rad Laboratories, Hercules, CA), and the amount of IgG present was assessed by spectrophotometric analysis at 450 nm.

Statistical analysis

Data are expressed as median and mean ± S.D. Data were analysed using a statistical software package (StatView 5.0; SAS Institute Inc., USA), the Mann–Whitney U-test and Spearman's rank correlation. Differences at $P < 0.05$ were considered to be statistically significant.

Results

Representative patterns of CD161 expression by flow cytometric analysis

Figure 1 demonstrates the representative patterns of CD161 expression on T cells from one healthy donor and patients with SLE and RA by flow cytometric analysis. Since CD161 was expressed mainly on NK cells, PBMCs were gated with APC-conjugated anti-CD3 mAb, and then three-colour analysis was performed. The population of $CD161^+CD8^+$ cells was decreased in patients with both SLE and RA in comparison with the healthy donors. The numbers indicate the percentage of each population.

Decreased frequency and absolute number of $CD161^+CD8^+$ T cells in patients with SLE, MCTD, SSc and PM/DM

We then investigated the expression of CD161 on T cells from patients with various rheumatic diseases and healthy donors. Both the frequency and the absolute number of $CD161^+CD8^+$ T cells were significantly decreased in patients with SLE (both $P < 0.001$), MCTD ($P < 0.01$ and $P < 0.001$), SSc ($P < 0.05$ and $P < 0.001$) and PM/DM ($P < 0.05$ and $P < 0.001$), when compared with the healthy donors (Fig. 2). In patients with RA, the absolute number of $CD161^+CD8^+$ T cells was significantly decreased ($P < 0.001$), although the frequency of $CD161^+CD8^+$ T cells was not (Fig. 2). Furthermore, this reduction was correlated with the decrease of $CD8^+$ T cells in patients with RA (data not shown). On the other hand, the absolute number of $CD161^+CD4^+$ T cells

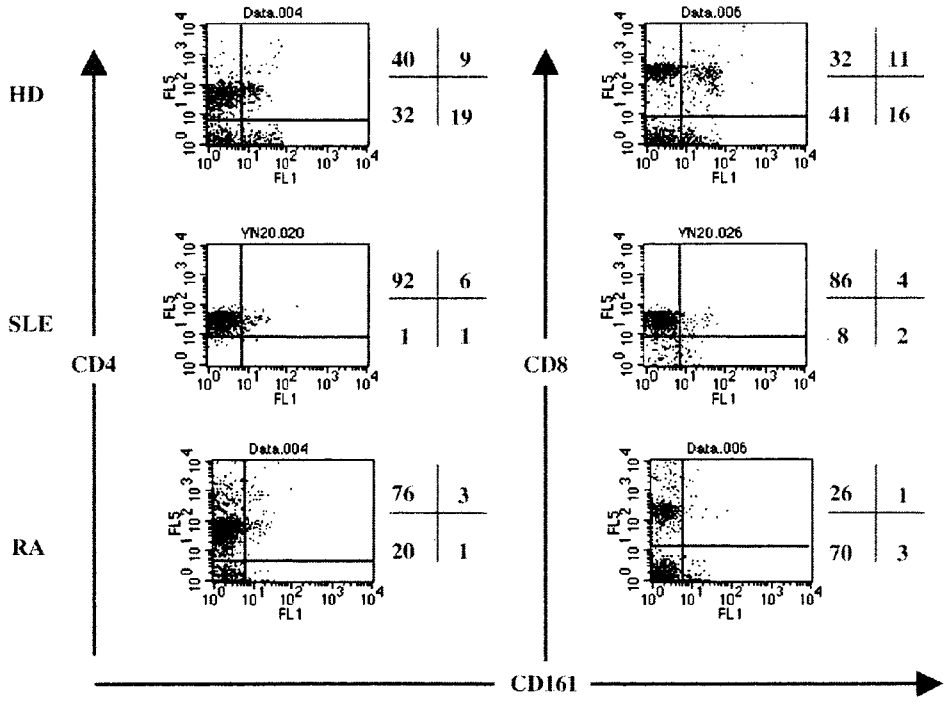

FIG. 1. Representative patterns of CD161 expression on T cells in peripheral blood from one healthy donor and patients with SLE and RA by flow cytometric analysis. PBMCs were gated with APC-conjugated anti-CD3 mAb, and then a three-colour analysis was performed. The population of $CD161^+CD8^+$ cells was decreased in patients with both SLE and RA in comparison with the healthy donors. The numbers indicate the percentage of each population. HD, healthy donors; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis.

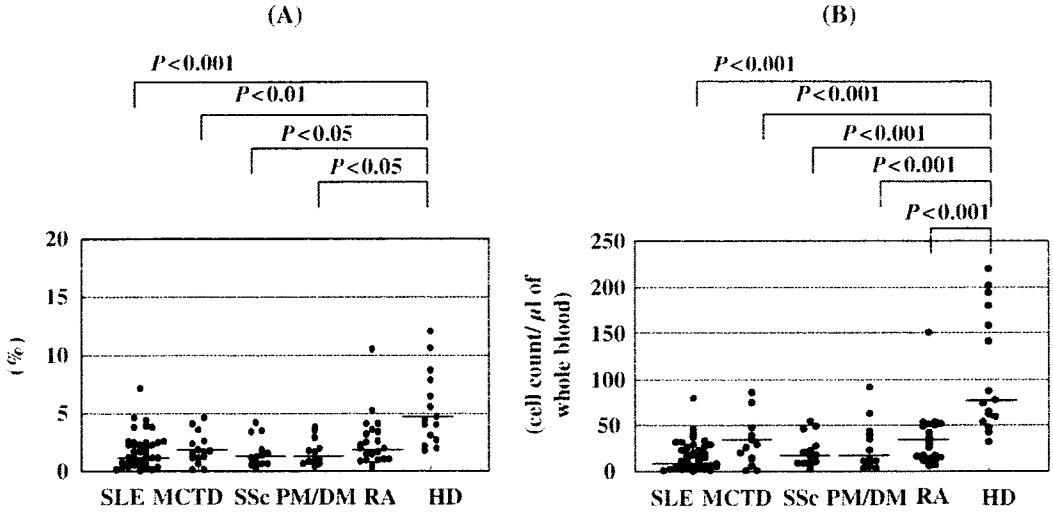

FIG. 2. (A) Frequency of $CD3^+CD8^+CD161^+$ T cells in the peripheral blood of patients suffering from rheumatic diseases (%). The frequency of $CD3^+CD8^+CD161^+$ T cells in the peripheral blood was significantly decreased in patients with SLE, MCTD, SSc and PM/DM. The median (range) of the frequency of these cells was 1.2 (0.04–7.1) in patients with SLE, 1.6 (0.1–4.6) in patients with MCTD, 1.2 (0.2–4.2) in patients with SSc, 0.9 (0.43–3.8) in patients with PM/DM, 1.8 (0.27–10.5) in patients with RA, and 4.6 (1.7–12.0) in the healthy donors. Horizontal bars indicate the median. (SLE, $n=51$; MCTD, $n=15$; SSc, $n=14$; PM/DM, $n=13$; RA, $n=24$; HD, $n=16$). (B) Absolute number of $CD3^+CD8^+CD161^+$ T cells in the peripheral blood of patients suffering from rheumatic diseases (cell count/μl of whole blood). The absolute number of these cells in the peripheral blood was significantly decreased in patients with SLE, MCTD, SSc, PM/DM and RA. The median (range) of the absolute number of these cells was 12 (0–80) in patients with SLE, 29 (1–86) in patients with MCTD, 17.5 (2–55) in patients with SSc, 13 (3–92) in patients with PM/DM, 28 (6–151) in patients with RA and 76 (32–220) in the healthy donors. Horizontal bars indicate the median. (SLE, $n=51$; MCTD, $n=15$; SSc, $n=14$; PM/DM, $n=13$; RA, $n=24$; HD, $n=16$). SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease; SSc, systemic sclerosis; PM/DM, polymyositis/dermatomyositis; RA, rheumatoid arthritis; HD, healthy donors.

was significantly decreased only in SLE patients when compared with the healthy donors ($P<0.001$), although the frequency of $CD161^+CD4^+$ T cells was not decreased significantly (data not shown).

Lack of significant correlation between the frequency or absolute number of $CD161^+$ T cells and age or sex

Next, since an influence of age was expected, we investigated the correlation between the frequency or absolute number of $CD161^+$ T cells and age in each patient group and in the healthy donor group. However, no significant correlations could be demonstrated (data not shown). In addition, to exclude any influence of sex, we compared the frequency or absolute number of $CD161^+$ T cells between males and females in each patient group and in the healthy donor group, but no significant differences were evident.

No influence of treatment on the frequency or absolute number of $CD161^+$ T cells

To exclude the influence of treatment, we compared the frequency and absolute number of $CD161^+$ T cells between the patients given treatment and those who did not receive any steroid, immunosuppressant or plasmapheresis in each rheumatic disease group, but no significant differences were evident. In addition, we investigated the correlation between the frequency or absolute number of $CD161^+$ T cells and the dose of steroid in each patient group, but again no significant correlation could be demonstrated (data not shown). Moreover, we found no influence of any kind of immunosuppressant. On the other hand, although we compared the frequency or absolute number of these cells between the patients with SLE who received plasmapheresis and those who did not, no differences were evident. We also compared the absolute number of these cells between the inflow and outflow circuits during plasmapheresis in SLE patients, but found no differences. Finally, we determined whether there was any correlation between the frequency or absolute number of these cells and treatment in the eight patients with SLE who did not receive any steroid, immunosuppressant or plasmapheresis, and found no significant differences between the pre- and post-treatment results (data not shown).

Lack of significant correlation between $CD161^+$ T cells and clinical parameters

Next, we investigated the correlation between the frequency or absolute number of $CD161^+$ T cells and the clinical parameters CH50, anti-DNA antibody and SLEDAI in SLE patients, but no significant correlations could be demonstrated (data not shown). We also investigated the correlation between the levels of CRP in RA patients, or the levels of CRP or CK in PM/DM patients, or CRP in SSc patients, and the frequency or absolute number of $CD161^+$ T cells in these patients, but again were unable to demonstrate any correlation (data not shown).

Decreased frequency of CD161 expression on $CD28^-CD8^+$ T cells in patients with SLE, MCTD and SSc

A recent study [16] has revealed that human suppressor T cells are derived from an oligoclonal population of $CD8^+$ $CD28^-$ T cells. Therefore, we examined the relationship between CD161 expression and these cells. First, we examined both the frequency and the absolute number of $CD28^-CD8^+$ T cells in the patient groups, but there were no significant differences in comparison with the healthy donors (data not shown). Then, we analysed the frequency of CD161 expression on $CD28^-CD8^+$ T cells. The frequency of CD161 expression on $CD28^-CD8^+$ T cells was significantly decreased in patients with SLE, MCTD and SSc when compared

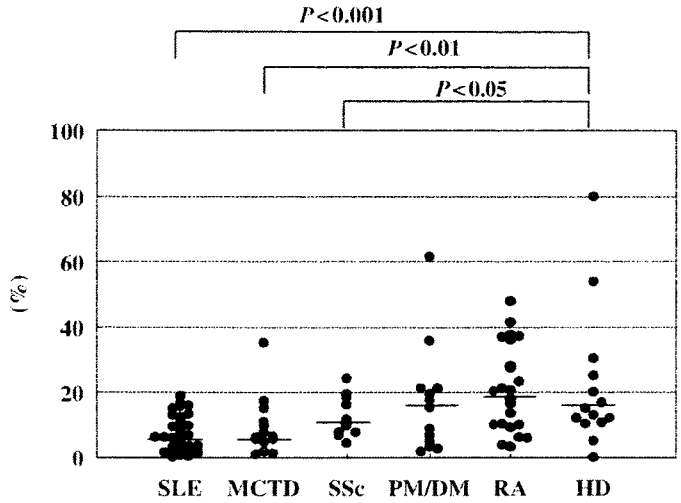

FIG. 3. Frequency of CD161 expression on CD3$^+$CD8$^+$CD28$^-$ T cells in the peripheral blood of patients suffering from rheumatic diseases (%). The frequency of CD161 expression on CD3$^+$CD8$^+$CD28$^-$ T cells in the peripheral blood was significantly decreased in patients with SLE, MCTD and SSc. The median (range) of the frequency of CD161 expression on these cells was 6.7 (0–18.9) in patients with SLE, 6.2 (1.0–35.3) in patients with MCTD, 10.8 (4.5–24.4) in patients with SSc, 15.5 (1.7–61.5) in patients with PM/DM, 19.3 (3.4–47.9) in patients with RA and 14.0 (0–80.0) in the healthy donors. Horizontal bars indicate the median. SLE, $n=41$; MCTD, $n=15$; SSc, $n=12$; PM/DM, $n=13$; RA, $n=24$; HD, $n=14$.

with the healthy donors (Fig. 3). Thus, the population of CD28$^-$CD8$^+$ T cells contained a decreased proportion of CD161$^+$CD8$^+$ T cells. Moreover, we compared the frequency of CD161 expression on CD28$^-$CD8$^+$ T cells between patients with and without steroid, immunosuppressant or plasmapheresis treatment in each group of rheumatic diseases, but there were no significant differences. Thus, no other influence of treatments was evident.

Lack of difference in the frequency of CD161 expression on CD4$^+$CD25$^+$ T cells between each of the patient groups and the healthy donors

It has been reported that a proportion of CD4$^+$CD25$^+$ T cells expressing Fox p3 exert regulatory effects [17, 18]. To examine the function of the CD161 molecule, we investigated its relationship with CD4$^+$CD25$^+$ T cells. Neither the frequency nor the absolute number of CD4$^+$CD25$^-$ T cells differed significantly between each of the patient groups and the healthy donors (data not shown). Next, we examined CD161 expression on CD4$^+$CD25$^+$ T cells in healthy donors, and found that the frequency of CD161 expression was significantly higher than that on CD4$^+$CD25$^-$ T cells (data not shown). Finally, we analysed the frequency of CD161 expression on CD4$^+$CD25$^+$ T cells in each of the patient groups, but found no significant differences from the healthy donors (data not shown).

Lack of enhanced production of IgG upon treatment with purified anti-CD161 mAb

To investigate whether the CD161 molecule has an inhibitory function, we examined the production of IgG by PBMCs from healthy donors by treatment with purified anti-CD161 mAb (a blocking antibody) (0, 0.5 and 10 μg/ml) and co-culture with PWM. However, no enhanced production of IgG was observed upon treatment with purified anti-CD161 mAb when compared with isotype-matched control mAb (data not shown).

Lack of significant difference in the frequency of CD62L expression on CD8$^+$ T cells between patients with rheumatic diseases and healthy donors

CD62L (L-selectin) molecules are constitutively expressed by most leucocytes (lymphocytes, neutrophils, monocytes and NK cells), and cooperate with other selectins and integrins in supporting leucocyte 'rolling' along the inflamed vascular endothelium prior to adhesion and transmigration. This molecule is cleaved from the cell surface after activation of the cells [19], and its expression is related to cell migration into inflammatory tissue. Therefore, to examine the possibility that CD161$^+$CD8$^+$ T cells may migrate into inflammatory tissue and become decreased in peripheral venous blood, we investigated CD62L expression on CD8$^+$ T cells. However, there was no significant difference in the frequency of CD62L expression on CD8$^+$ T cells between patients with rheumatic diseases and healthy donors (data not shown). In addition, age, sex or treatment had no influence on CD62L expression.

Increased frequency of CD62L expression on CD161$^+$CD8$^+$ T cells in patients with SLE and SSc

We investigated the frequency of CD62L expression on CD161$^+$CD8$^+$ T cells and the absolute numbers of both CD161$^+$CD8$^+$CD62L$^+$ and CD161$^+$CD8$^+$CD62L$^-$ T cells. The mean $\pm$ S.D. and median (range) of the frequency of CD62L expression on CD161$^+$CD8$^+$ T cells were 55.1 $\pm$ 20.4 and 49.2 (15.1–92.4) in patients with SLE, 51.1 $\pm$ 23.9 and 51.3 (22.1–91.5) in patients with MCTD, 54.0 $\pm$ 22.1 and 50.1 (35.3–80.6) in patients with SSc, 52.1 $\pm$ 25.4 and 56.2 (18.3–92.5) in patients with PM/DM, 52.7 $\pm$ 24.2 and 58.0 (11.9–81.1) in patients with RA, and 34.4 $\pm$ 13.5 and 35.0 (13.8–60.0) in the healthy donors, respectively. The mean $\pm$ S.D. and median (range) of the frequency of CD62L expression on CD161$^-$ CD8$^+$ T cells were 73.8 $\pm$ 18.8 and 77.0 (35.6–98.8) in patients with SLE, 72.8 $\pm$ 21.3 and 72.2 (40.6–94.8) in patients with MCTD, 82.9 $\pm$ 3.7 and 82.1 (79.7–87.7) in patients with SSc, 74.8 $\pm$ 19.8 and 72.5 (39.1–97.9) in patients with PM/DM, 67.9 $\pm$ 25.1 and 74.7 (19.5–93.0) in patients with RA, and 83.0 $\pm$ 9.9 and 84.9 (61.9–99.8) in the healthy donors, respectively. As shown in Fig. 4A, the frequency of CD62L expression on CD161$^+$CD8$^+$ T cells was significantly decreased in SLE ($P<0.01$), MCTD ($P<0.05$), SSc ($P<0.05$), and the healthy donors ($P<0.001$) when compared with that on CD161$^-$CD8$^+$ T cells. However, the frequency of CD62L expression on CD161$^+$CD8$^+$ T cells was significantly increased in SLE ($P<0.01$) and SSc ($P<0.01$), when compared with the healthy donors.

Decreases in the absolute numbers of CD161$^+$CD8$^+$CD62L$^-$ T cells in patients with SLE, MCTD and SSc, and in absolute numbers of CD161$^+$CD8$^+$CD62L$^+$ T cells in patients with SLE

The absolute numbers of CD161$^+$CD8$^+$CD62L$^-$ T cells were significantly decreased in patients with SLE, MCTD and SSc (Fig. 4B), and the absolute numbers of CD161$^+$CD8$^+$CD62L$^+$ T cells were significantly decreased only in patients with SLE (Fig. 4C). Although we compared the absolute numbers of CD161$^+$CD8$^+$CD62L$^-$ T cells between the patients with and without treatment for each group of rheumatic diseases, no significant differences were found. Moreover, we compared the frequency of CD161 expression on these cells between patients given low-dose steroids and those given high-dose steroids, but there were no evident differences.