3．同一症例における関節液，滑膜組織でのhyaluronidase発現の検討

同一患者，同一関節の関節液と滑膜組織が共に採取できた12症例について，関節液中のHA分子量，関節液中のzymographyによるhyaluronidase活性，滑膜組織でのISHによるhyaluronidase陽性細胞数の相関を検討すると，Hyal-1，2ともに滑膜組織中の陽性細胞数が多い症例ほど関節液中のHA分子量が低下している傾向がみられた［Hyal-1：$R=-0.72$（$p<0.01$），Hyal-2：$R=-0.61$（$p<0.05$）］（図4A）．また，Hyal-1，2ともに滑膜組織中の陽性細胞数が多い症例ほど関節液中のhyaluronidase活性が高値を示すという正の相関が見られた［Hyal-1：$R=0.59$（$p<0.05$），Hyal-2：$R=0.52$（N.S.：not significant）］（図4B）．

IV. 考　察

今回の我々の検討では，RA患者の関節内におけるhyaluronidaseの発現や分布，活性状態と関節液中のHA分子量との負の相関が明らかになった．

淡白分解酵素の活性については，分解基質を添加したゲルを利用したzymographyにて検討している報告はいくつか存在する[14, 18-20]．hyaluronidase活性についてもzymographyを用いて検討した報告が見られるが，その方法や条件は研究者によって異なる[12-15]．今回我々は電気泳動の条件，泳動時間，ゲル基質に添加するHA分子量，サンプル量，酵素反応液の種類やpH，酵素反応時間など様々な条件下でzymographyを試みた．SDS-PAGEを用いたzymographyでは分子量の検出は可能であったが，酵素活性が失われ，バンドの検出が不鮮明であった為，本研究では分子量の測定はできないが活性の保たれるnative-PAGEを用いて検討を行った．ゲルに添加するHAは分子量190万のものから分子量2万の低分子量のものまで試みたが，高分子量のHA添加ゲルではHAの粘稠度が高すぎてゲルとの親和性が低かった為，ゲルが均一にならず泳動パターンの乱れが大きかった．一方，低分子量のHA添加ゲルではゲルとの親和性は良いがAlcian blue染色の染色性が悪く，バンドとしてのコントラストが得にくかった．今回，親和性，染色後のバンドのコントラストの双方を考慮して，最も安定した結果の得られた分子量80万のHA添加ゲルを用いてzymographyを行った．また，泳動後の酵素反応液の種類やpHの違いでもバンド検出に大きな差が認められ，特にpHに関しては3.7～4.0の酸性域では明確なバンドの検出が見られたのに対し，中性，アルカリ性域では酵素反応はほとんど見られなかった．この事は，Hyal-2が酸性下で活性を持つという性質[21]や，過去の報告[12, 13]とも矛盾しない．しかしながら，同様のzymographyでウシ血清中ではhyaluronidase活性はpH7.0でも検出されるという報告がある事や[15]，生体内ではpH 3.7という酸性域は破骨細胞周囲などの限られた部位にしか存在しない事などからも，生体内ではpH以外にも様々な条件が関与してhyaluronidaseの活性化が起こっているという事が推測される．

これまでに生体内で，特に関節域でのhyaluronidaseについての報告はまだ多くはないが，近年Williamsらは競走馬の関節液中のhyaluronidase活性について[15]，Nagayaらはヒト関節液中のhyaluronidase活性と関節炎の炎症度との比較について[14]報告している．また軟骨組織でのmRNAの証明などもいくつか報告されており[22]，線維芽細胞が軟骨細胞に分化する際にhyaluronidaseのmRNAが増加するという報告[22]があることから，軟骨細胞の分化や代謝にも重要な機能を示すことが示唆される．

RA患者の関節内では滑膜組織におけるHAの分布の減少や関節液中のHA濃度や分子量の低下がみられることがUzukiらによ

って報告されている[3,4]．上記のような報告をふまえ，本研究ではまずRA患者の関節内のHA分解の一因と思われる関節液中のhyaluronidase活性を定量化し，同一患者の関節液中HA分子量との相関をRA 46例の関節液において検討した．その結果，hyaluronidase活性の高い症例ほど関節液中のHA分子量は減少する傾向が得られた．次に，RA滑膜におけるhyaluronidaseのmRNA陽性細胞の発現や分布を見ると，炎症の高度なRA早期，活動期の多層化した滑膜表層細胞には多数の陽性細胞が見られるのに対し，滑膜細胞の多層化の目立たない線維化期のRA滑膜では陽性細胞はほとんど陰性であった．また，同一患者において滑膜組織におけるhyaluronidase陽性細胞数は関節液中のhyaluronidase活性とは正の相関，HA分子量との間には負の相関を認めている．Nagayaらによると，関節炎の炎症度が高い症例では関節液中のhyaluronidase活性も高いという報告もある[14]．この事と我々の結果から，RA患者の関節内では，炎症に伴い関節液中のhyaluronidase活性の上昇が起こり，それと共に多層化した滑膜組織からのhyaluronidaseの産生が亢進していることが示唆される．このようにRA患者の関節内HAは滑膜組織と関節液中にあるhyaluronidaseによって分解され，関節液中のHAの低分子化が生じるものと思われる．その結果として，関節液の粘弾性や潤滑作用，軟骨保護作用など物理的機能の低下が生じ，更には低分子化したHAが炎症性サイトカインの作用を亢進させ[6-8]，RAにおける関節破壊や滑膜炎の持続を助長している可能性が考えられる．その他にもHAの低分子化に関してはhyaluronidase以外にも活性酸素中間体からのフリーラジカルによる切断や[4,24]，HAS-3による低分子HAの直接の合成なども関与していると報告されている[4,25]．また近年はMioらによるhyaluronidaseの内因性阻害因子についての報告もある[26]．ヒト関節内におけるHA代謝は未だ完全には解明されておらず，関節内におけるHAの低分子化が及ぼす悪影響が明らかになっている現在，更なる研究が期待される．

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Evidence of the efficacy of radiation synovectomy with yttrium-90: comment on the article by Jahangier et al

To the Editor:

The study by Jahangier et al on the efficacy of yttrium-90 radiation synovectomy (radiosynoviorthesis [RSO]) versus intraarticular glucocortocoids (GCs) in the treatment of knee arthritis (Jahangier ZN, Jacobs JW, Lafeber FP, Moolenburgh JD, Swen WA, Bruyn GA, et al. Is radiation synovectomy for arthritis of the knee more effective than intraarticular treatment with glucocorticoids? Results of an eighteen-month, randomized, double-blind, placebo-controlled, crossover trial. Arthritis Rheum 2005;52:3391–402) is important because of the shortage of evidence-based studies on this topic. Contrary to the results and conclusions in a previous (2001) study by the same group (Jahangier ZN, Moolenburgh JD, Jacobs JW, Serdijn H, Bijlsma JW. The effect of radiation synovectomy in patients with persistent arthritis: a prospective study. Clin Exp Rheumatol 2001;19:417–24), Jahangier and colleagues drew very unfavorable conclusions regarding ^{90}Y RSO from the results of their recent study. However, there are striking differences between the 2 studies. The patients in the recent study were, on average, at a more advanced stage of disease (mean ± SD duration of synovitis 38 ± 38 months [range 6–240] in the RSO plus GC arm and 35 ± 32 months [range 2–120] in the placebo plus GC arm in the 2005 study, but 17 ± 13 months [range 1–60] in the 2001 study).

We believe the new trial does in fact show considerable evidence for the efficacy of ^{90}Y RSO. In the case of the secondary end point, duration of remission, there was a striking difference between the two randomized groups in favor of RSO: mean ± SD 27 ± 29 months in the RSO plus GC group versus 18 ± 25 months in the placebo plus GC group.

Figure 1 of this publication provides the most convincing evidence for the efficacy of RSO in this trial. The figure, which lists the mean composite change index—an index of unproven value—presents data on patients who remained in the study. Data on patients in both treatment arms, in both the randomized and the crossover portions of the study, are included. In the comparison of the 2 randomized treatment groups, the figure shows a striking difference in favor of RSO at all time points, which becomes very prominent at 12 months and 18 months. At 12 months the mean composite change index in the placebo plus GC group was ~4.9, while in the RSO plus GC group it was ~7.7, ~60% higher. A similar picture was seen at 18 months, with a mean composite change index of ~4.4 in the placebo plus GC group and ~7.7, or 75% higher, in the RSO plus GC group. The interpretation of the data in the 2 crossover groups is not as straightforward. In the group that started on placebo but was switched to ^{90}Y RSO, there was still considerable progress at 12 months and 18 months, although not as much as was seen in the randomized RSO group (mean composite change index ~6 at 12 months and ~6.7 at 18 months, considerably higher than in the randomized placebo group). In patients who started on RSO but were switched to placebo by the investigators after ≥6 months because RSO therapy did not appear successful, good improvement was seen at 12 months and 18 months, with results similar to those in the original randomized RSO group. What is not immediately evident from Figure 1 is the fact that the month 0 time point refers to the start of the placebo treatment, but is in fact also 6 months after the original ^{90}Y RSO treatment in these patients. We attribute the improvements seen in this group at 12 months and 18 months after the crossover placebo treatment (and thus 18 months and 24 months after RSO treatment) to the long-term effect of RSO in this group and not to the placebo treatment, with which one would expect only short-term improvement (up to 3 months).

In summary, we find considerable evidence for the efficacy of RSO in the trial described by Jahangier et al. Consequently, we arrive at the opposite conclusion from that drawn by the authors.

Drs. Mödder and Langer have received speaking fees (less than $10,000) from Schering AG.

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Specificity of enzyme-linked immunosorbent assay for IgG anti-NR2 glutamate receptor antibodies: comment on the concise communication by Yoshio et al

To the Editor:

We read with interest the concise communication by Yoshio et al (1) describing an association of IgG anti-NR2 glutamate receptor antibodies in cerebrospinal fluid (CSF) with neuropsychiatric systemic lupus erythematosus (NPSLE). DeGiorgio et al first demonstrated that IgG anti-NR2 glutamate receptor antibodies caused neuronal death in mice, when injected into the brain (2). They also reported the presence of these antibodies in CSF from an SLE patient with progressive cognitive decline (2). It was further demonstrated by investigators in that group that the presence of IgG anti-NR2 glutamate receptor antibodies within the brain resulted in cognitive decline in mice (3). These findings suggested that these antibodies might cause diffuse psychiatric/neurologic syndromes in human SLE. However, Yoshio and colleagues found that, compared with lupus patients without NPSLE, CSF IgG anti-NR2 glutamate receptor antibody levels were increased in lupus patients with neurologic syndromes of the central nervous system, but not in those with diffuse psychiatric/neuropsychological syndromes alone (1).

To measure IgG anti-NR2 glutamate receptor antibodies in sera and CSF, Yoshio et al used an enzyme-linked immunosorbent assay (ELISA) with the synthetic DWEYSVWLSN peptide conjugated to bovine serum albumin (BSA) as antigen (1). Our group previously demonstrated that sera from SLE patients frequently express antibodies to human serum albumin (HSA), BSA, and ovalbumin (4). Therefore, subtracting binding activity to these carrier proteins would be mandatory in order to determine specific activities of antibodies to peptides conjugated to carrier proteins, including BSA and HSA (4). However, Yoshio and colleagues did not subtract

Table 1. Measurement of IgG anti-NR2 glutamate receptor antibodies (optical density at 492 nm) using enzyme-linked immunosorbent assay*

Sample	CSF or serum	Maxisorp		Pro-Bind	
		HSA–NR2	HSA	HSA–NR2	HSA
1	CSF	0.146	0.065	0.090	0.067
2	CSF	0.223	0.068	0.147	0.124
3	CSF	0.206	0.278	0.053	0.090
4	CSF	0.428	0.521	0.050	0.060
5	CSF	0.974	0.480	0.088	0.082
6	CSF	0.205	0.323	0.224	0.280
7	CSF	0.248	0.139	0.259	0.249
8	CSF	0.044	0.135	0.101	0.139
9	CSF	0.332	0.137	0.030	0.020
10	Serum	0.628	0.686	0.094	0.116
11	Serum	0.434	0.795	0.100	0.180
12	Serum	0.272	0.093	0.050	0.044
13	Serum	0.181	0.209	0.082	0.099
14	Serum	0.333	0.339	0.057	0.221
15	Serum	0.280	0.139	0.051	0.050
16	Serum	0.878	0.464	0.616	0.522

* Cerebrospinal fluid (CSF) or serum samples from systemic lupus erythematosus patients were assayed for IgG anti-NR2 glutamate receptor antibodies by enzyme-linked immunosorbent assay as described in the text. Peroxidase-conjugated $F(ab')_2$ goat anti-human IgG was used at 1:10,000 (Maxisorp plates) and at 1:5,000 (Pro-Bind plates). HSA–NR2 = human serum albumin conjugated with synthetic DWEYSVWLSN peptide.

BSA binding activities. It appears that all sera with anti-HSA also contain antibodies to BSA (4). We therefore reexamined whether CSF and sera from SLE patients contain antibodies to HSA, using the method described by Yoshio et al.

Wells of a 96-well microtiter plate (Falcon Pro-Bind; Becton Dickinson, Lincoln Park, NJ or Nunc-immuno module F8 Maxisorp; Nunc, Roskilde, Denmark) were coated with HSA (Miles, Elkhart, IN) or HSA conjugated (at a 1:1 weight ratio) with highly purified synthetic DWEYSVWLSN peptide (purity >95%) (HSA–NR2 peptide), at 20 μg/ml in phosphate buffered saline (PBS), overnight at 4°C. The wells were blocked with Block Ace (Dainippon, Osaka, Japan) for Falcon Pro-Bind plates or with PBS containing 1% BSA (Miles) for Nunc Maxisorp plates, for 2 hours at room temperature. Before being added to the wells, serum and CSF samples were diluted 1:200 and 1:2, respectively, in PBS containing 1% BSA. After incubation at 37°C for 1 hour, bound IgG anti-NR2 glutamate receptor antibody was detected with peroxidase-conjugated $F(ab')_2$ fragments of goat anti-human IgG (Cappel, Cochranville, PA). Binding activity was expressed as optical density at 492 nm (OD_{492}) as measured in a 2-wavelength microplate photometer (MTP-450; Corona Electric, Ibaraki, Japan).

As seen in Table 1, all 16 samples exhibited positive binding to HSA–NR2 peptide in Falcon Pro-Bind plates (Yoshio and colleagues' method) as well as in Nunc Maxisorp plates. However, 8 of the 16 samples showed higher binding activity (OD_{492}) to HSA alone than to HSA–NR2, indicating that those samples would yield false-positive results for IgG anti-NR2 glutamate receptor antibodies unless nonspecific binding to HSA alone were subtracted. In addition, the levels of binding activity obtained with Falcon Pro-Bind plates were ~20–30% of those with Nunc Maxisorp plates, even though peroxidase-conjugated $F(ab')_2$ goat anti-human IgG was used at 1:5,000 in the former plates and at 1:10,000 in the latter. Therefore, it would be preferable to use Nunc Maxicorp plates. Nonetheless, these results confirm that ~50% of serum and CSF samples contain antibodies to HSA (and presumably to BSA as well, since it was previously shown that all sera positive for anti-HSA contained antibodies to BSA [4]).

These findings raise serious concern about the specificity of the ELISA used by Yoshio et al. It is highly likely that the presence of anti-BSA antibodies would have significantly influenced their results and conclusions. Therefore, their conclusion that IgG anti-NR2 glutamate receptor antibodies in CSF may cause focal neurologic damage such as seizure disorders, aseptic meningitis, and transverse myelopathy is not supported.

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Cortisol metabolism by 11β-hydroxysteroid dehydrogenase as a novel target in the treatment of inflammation- or immune-mediated bone loss: comment on the article by Makrygiannakis et al

To the Editor:

We read the report by Makrygiannakis et al (1), on the effects of intraarticular corticosteroids on bone biology regulation, with great interest, since it may have major consequences regarding the development of novel treatment concepts in rheumatoid arthritis (RA). In Makrygiannakis and colleagues' study, evaluation of RANKL, osteoprotegerin (OPG), and surface marker expression in synovium of arthritis patients or in human osteoblast-like cells showed that a decrease in inflammation is accompanied by down-modulation of markers of bone destruction. These findings offer a ration-

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Association of cerebrospinal fluid anti-ribosomal P protein antibodies with diffuse psychiatric/neuropsychological syndromes in systemic lupus erythematosus

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Abstract

We explored the relationship of antibodies to the whole ribosomal P proteins (P0, P1, and P2) in cerebrospinal fluid (CSF) with diffuse psychiatric/neuropsychological syndromes in systemic lupus erythematosus (SLE). CSF samples were obtained from 71 SLE patients (52 patients with diffuse psychiatric/neuropsychological syndromes [diffuse NP-SLE] and 19 patients with neurological syndromes or peripheral neuropathy [focal NP-SLE]) as well as from 24 patients with non-inflammatory neurological disease. Immunoglobulin G (IgG) antibodies to the C-terminal 22-amino acid ribosomal P synthetic peptide (anti-P_{C22}) and those to purified bovine ribosomal P proteins (P0, P1, and P2) (anti-whole P) were determined by enzyme-linked immunosorbent assay; affinity-purified IgG anti-P_{C22} were used as the standard. The concentrations of antibodies to epitopes other than the C-terminal 22 amino acids of ribosomal P proteins were calculated by subtracting anti-P_{C22} from anti-whole P (anti-$P_{EX.C22}$). CSF anti-whole P levels were significantly elevated in diffuse NP-SLE compared with focal NP-SLE or control patients. By contrast, there were no significant differences in CSF anti-P_{C22} levels among the three groups. Of note, CSF anti-$P_{EX.C22}$ levels were significantly elevated in diffuse NP-SLE compared with the other two groups. CSF anti-$P_{EX.C22}$ levels were not significantly correlated with CSF anti-P_{C22} levels, but with CSF antibodies against the recombinant ribosomal P0 protein lacking the C-terminal 22 amino acids (C22-depleted rP0). Moreover, levels of CSF anti-$P_{EX.C22}$ or CSF anti-C22-depleted rP0, but not CSF anti-P_{C22}, were significantly correlated with CSF anti-neuronal cell antibodies (anti-N). These results indicate that CSF IgG antibodies to the epitopes other than the C-terminal 22 amino acids of ribosomal P proteins, which might contain one of the major targets of CSF anti-N, are associated with the development of diffuse NP-SLE.

Introduction

Central nervous system (CNS) involvement is a relatively common and serious complication of systemic lupus erythematosus (SLE) [1,2]. Previous studies have demonstrated the association of serum antibodies directed against the C-terminal 22-amino acid sequences of ribosomal P protein (anti-P_{C22}) with CNS involvement in patients with SLE (neuropsychiatric SLE [NP-SLE]), especially diffuse psychiatric/neuropsychological syndromes (diffuse NP-SLE) [3-5]. However, the mechanism by which serum anti-P_{C22} leads to the development of diffuse NP-SLE has not yet been elucidated. In fact, the role of anti-P_{C22} in the cerebrospinal fluid (CSF) in the

ACR = American College of Rheumatology; anti-C22-depleted rP0 = antibodies directed against recombinant ribosomal P0 protein lacking the C-terminal 22 amino acids; anti-N = anti-neuronal cell antibodies; anti-P_{C22} = antibodies directed against the C-terminal 22-amino acid sequences of ribosomal P protein; anti-$P_{EX.C22}$ = autoantibodies directed against the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence; anti-whole P = antibodies to the whole ribosomal P proteins; C22-depleted rP0 = recombinant ribosomal P0 fusion protein lacking the C-terminal 22 amino acids; CNS = central nervous system; CSF = cerebrospinal fluid; ELISA = enzyme-linked immunosorbent assay; HSA = human serum albumin; IgG = immunoglobulin G; IL-6 = interleukin-6; NMDA = *N*-methyl-d-aspartate; non-CNS SLE = systemic lupus erythematosus without neuropsychiatric manifestations; NP-SLE = neuropsychiatric systemic lupus erythematosus; OD_{492} = optical density at 492 nm; PBS = phosphate-buffered saline; SLE = systemic lupus erythematosus.

pathogenesis with diffuse NP-SLE or even their presence in the CSF remains uncertain. Thus, Golombek and colleagues [6] detected the presence of CSF anti-P_{C22} in all four of the patients with lupus psychosis in their studies, whereas others did not [3,4,7].

On the other hand, autoantibodies, which react with the neuronal cell lines or brain tissue, have been reported in the sera of patients with NP-SLE [8-10]. However, they have been shown to be present in SLE patients with no clinical evidence of CNS involvement [10]. In fact, in a cross-sectional study of SLE patients, no significant association was found between serum lymphocyte/brain cross-reacting antibodies and NP-SLE (present in 32% of cases with NP-SLE and 23% of those without NP-SLE) [10]. Of note, using a radioimmunoassay with the SK-N-SH neuroblastoma cell as a target, Bluestein and colleagues [11] demonstrated that immunoglobulin G (IgG) anti-neuronal cell antibodies (anti-N) were present in much higher concentrations in the CSF from patients with active NP-SLE than in the CSF from SLE patients without active CNS involvement. Using a cell enzyme-linked immunosorbent assay (ELISA) with SK-N-MC neuroblastoma cell lines fixed with paraformaldehyde, we also confirmed that CSF IgG anti-N levels were significantly elevated in patients with diffuse NP-SLE compared with those in SLE patients without diffuse NP-SLE [7]. However, the fine epitopes to which CSF anti-N were directed have not yet been delineated.

The presence of the immunodominant C-terminal epitope of ribosomal P proteins was demonstrated to be present on the surface of human neuroblastoma cells [12]. However, CSF anti-P_{C22} could be detected in only a fraction of patients with diffuse NP-SLE, whereas almost all the patients with diffuse NP-SLE expressed CSF anti-N [7]. Of note, previous studies also demonstrated the presence of a 38-kDa protein that is closely related to, or identical with, ribosomal P0 protein in purified human plasma membranes [12]. In addition, it has been shown that autoantibodies directed against the ribosomal P proteins are not only directed against the common C-terminal 22 amino acids, but against the N-terminal sequence of the ribosomal P2 or P1 proteins [13]. In fact, recent studies have revealed that measurement of CSF IgG anti-ribosomal P protein antibodies with Western blotting using purified ribosomes, containing whole ribosomal P0, P1, and P2 proteins, was more sensitive [14]. Because ribosomal P0 protein contains epitopes other than the C-terminal 22 amino acids, it is possible that CSF from patients with diffuse NP-SLE contains antibodies to such epitopes. The current studies, therefore, were carried out to compare the CSF levels of antibodies to the whole ribosomal P proteins (anti-whole P) in patients with diffuse NP-SLE and in patients with focal NP-SLE or non-SLE non-inflammatory neurological disorders.

Materials and methods

Patients and samples

One hundred and three patients with SLE were included in the present study. All patients fulfilled the American College of Rheumatology (ACR) 1982 revised criteria for the classification of SLE [15]. Of the 103 patients with SLE, 52 showed diffuse psychiatric/neurological syndromes (diffuse NP-SLE) according to the 1999 ACR definition of NP-SLE [16], 19 patients showed CNS manifestations other than diffuse NP-SLE (focal NP-SLE), and 32 patients showed no CNS manifestations (non-CNS SLE). Ten of the 52 patients with diffuse NP-SLE also presented seizures. Because of the difficulties in confirming the neurological diagnosis and in assigning the cause to SLE, we defined NP-SLE as (a) the presence of neuropsychiatric manifestations and (b) the elevation of CSF Ig indices [17,18] and/or the elevation of CSF interleukin-6 (IL-6) levels [19]. Thus, the 52 patients all showed increased CSF Ig indices and/or CSF IL-6 in the present study. In addition, 24 patients with non-SLE non-inflammatory neurological diseases (9 cerebrovascular diseases, 8 cervical spondylosis, 4 degenerative diseases, 2 diabetic neuropathy, and 1 epilepsy) were studied as a control. The 127 patients all gave informed consent, and the study was approved by the institutional ethical committee of Teikyo University School of Medicine (Tokyo). The detail and demographic features of the 127 patients are shown in Table 1. CSF specimens were obtained by a lumbar puncture when the patients showed active disease. These samples were kept frozen at -20°C until assayed. All assays were performed without knowledge of the diagnosis or clinical presentations.

Human anti-P_{C22} sera and affinity purification of anti-P_{C22}

IgG fractions were purified from the anti-P_{C22}-positive sera of SLE patients by means of a protein G-Sepharose 4FF column (Amersham Pharmacia Biotech, now part of GE Healthcare, Little Chalfont, Buckinghamshire, UK). Anti-P_{C22} were purified from the IgG fractions of SLE sera by means of an *N*-hydroxysuccinimide-activated Sepharose HP column (GE Healthcare) coupled with synthetic ribosomal P peptide-human serum albumin (HSA) conjugates as previously described [20]. Anti-P_{C22} thus purified reacted strongly with ribosomal P peptide-HSA conjugates, but not with HSA alone in an ELISA. It was also confirmed on Western blot analysis that purified anti-P_{C22} reacted with native ribosomal P proteins (P0, P1, and P2) (data not shown).

Measurement of autoantibodies to ribosomal P proteins

Antibodies for the C-terminal 22-amino acid ribosomal P synthetic peptide (anti-P_{C22}) in sera and CSF and those for purified whole ribosomal P proteins (anti-whole P) in CSF were determined by specific ELISA using the highly purified synthetic C-terminal 22-amino acid ribosomal P peptide conjugated to HSA as an antigen as previously described [5] and highly purified bovine ribosomal P proteins (P0, P1, and P2) (purity of more than 90%) (Arotec Diagnostics Limited, Wel-

Table 1

Profiles of the patients studied

Diagnosis	Number of patients	Gender (male/female)	Age in years (mean ± SD)
SLE	103		
Diffuse NP-SLE	52	4/48	37.8 ± 14.2
Acute confusional state	20		
Anxiety disorder	3		
Cognitive dysfunction	10[a]		
Mood disorder	12[b]		
Psychosis	7		
Focal NP-SLE	19	2/17	39.0 ± 14.8
Cerebrovascular disease	6		
Headache	2		
Movement disorder	1		
Seizure disorder	6		
Polyneuropathy	4		
Non-CNS SLE	32	3/29	42.7 ± 13.9
Non-SLE control	24	22/2	48.0 ± 13.7

[a]One patient also presented mood disorder. [b]One patient also presented cognitive dysfunction. Non-CNS SLE, systemic lupus erythematosus without neuropsychiatric manifestations; NP-SLE, neuropsychiatric systemic lupus erythematosus; SD, standard deviation; SLE, systemic lupus erythematosus.

lington, New Zealand). Antibodies for the epitope representing regions of the ribosomal P proteins other than P_{C22} were similarly determined by ELISA using recombinant ribosomal P0 fusion protein lacking the C-terminal 22 amino acids (C22-depleted rP0) as previously described [21].

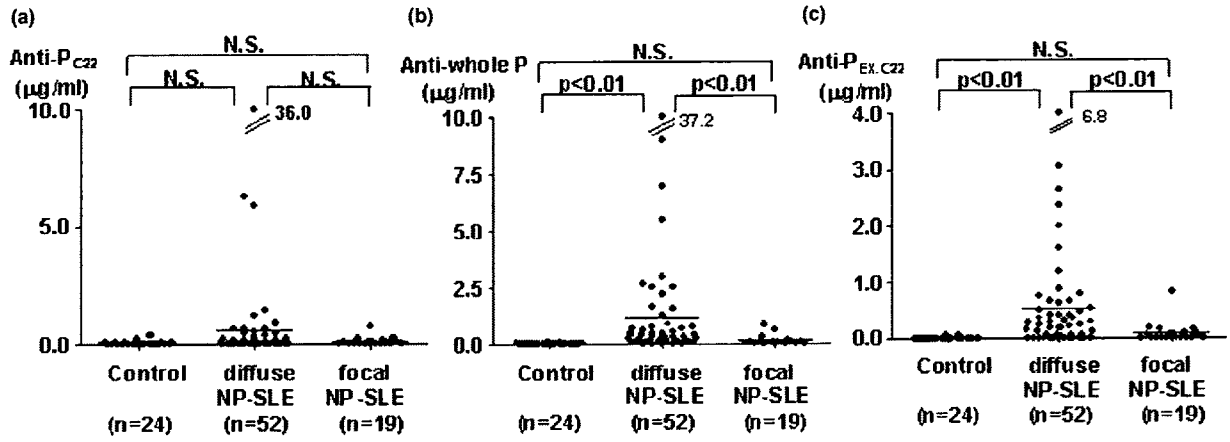
Briefly, wells of a 96-well microtiter plate were coated with ribosomal P peptide-HSA conjugates at 15 μg/ml or highly purified bovine ribosomal P proteins at 1.0 μg/ml in phosphate-buffered saline (PBS) (pH 7.2) or C22-depleted rP0 at 5 μg/ml in 6 M urea/10 mM Tris-HCl (pH 7.5) with 2 mM 2-mercaptoethanol (coating buffer) at 4°C overnight. Each well was then overcoated with Block Ace (Dainippon Pharmaceutical, Osaka, Japan), diluted 1:4 with PBS. Prior to being added to the antigen-coated wells, serum and CSF samples were usually diluted 1:200 and 1:2, respectively, in PBS containing 1% bovine serum albumin (Miles, now part of Bayer Corp., Emeryville, CA, USA). Bound antibody was detected with peroxidase-conjugated F $(ab')_2$ fragments of goat anti-human IgG (MP Biochemicals, Solon, OH, USA). After incubation with substrate solution containing 60 mg of *o*-phenylenediamine and 10 μl of 30% H_2O_2 in 100 ml of 0.05 M citrate phosphate buffer (pH 4.8) at 37°C for 30 minutes, the reaction was stopped by addition of 5 N H_2SO_4, and the absorbance (optical density) at 492 nm (OD_{492}) was read with a two-wavelength microplate photometer (MTP-120; Corona Electric Co., Ltd., Ibaraki, Japan). Determinations of OD_{492} were normalized to affinity-purified anti-P_{C22} such that anti-P_{C22} and anti-whole P activity might be converted to micrograms per milliliter of IgG. Antibodies directed against C22-depleted rP0 (anti-C22-depleted rP0) were expressed by arbitrary unit designation using a standard serum.

Non-specific binding activities to HSA for anti-P_{C22} or those to wells with PBS alone or coating buffer alone for anti-whole P or anti-C22-depleted rP0 were also determined in reference to the standard curves for binding activities to ribosomal P peptide (P_{C22})-HSA conjugates, highly purified ribosomal P proteins, or C22-depleted rP0. The specific anti-P_{C22}, anti-whole P, or anti-C22-depleted rP0 activities were thus determined by subtracting the values for the non-specific binding activity from those for binding activity to P_{C22}-HSA conjugates or to highly purified ribosomal P proteins or C22-depleted rP0. The intra-assay and interassay variances (coefficient of variation values) for anti-whole P were 13.8% and 15.7%, respectively, and those for anti-P_{C22} were previously described [7].

Measurement of anti-N

Anti-N in the CSF samples were determined by a cell ELISA using human neuroblastoma cell line SK-N-MC as previously

Figure 1



Cerebrospinal fluid antibodies to various components of ribosomal P proteins. CSF antibodies to the C-terminal 22-amino acid sequence of ribosomal P protein (anti-P_{C22}), highly purified ribosomal P proteins (anti-whole P), and epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$). Anti-P_{C22} **(a)**, anti-whole P **(b)**, and anti-$P_{EX.C22}$ **(c)** in CSF from patients with non-inflammatory neurological diseases (Control), with diffuse neuropsychiatric systemic lupus erythematosus (NP-SLE), or with focal NP-SLE were compared. Horizontal lines indicate the mean values. Statistical analysis was performed by Kruskal-Wallis test with multiple comparisons (Scheffé's method). CSF, cerebrospinal fluid; N.S., not significant.

described [7]. Briefly, SK-N-MC cells were seeded at a density of 5×10^4 per well in wells of a flat-bottomed 96-well tissue culture plate (no. 3596; Costar, now part of Corning Life Sciences, Acton, MA, USA) for 48 hours, after which the cells were fixed with 1% paraformaldehyde in PBS for 5 minutes at 37°C. After three washes with PBS containing 0.05% Tween 20, 50 μl of the appropriately diluted samples or various concentrations of standard sera were added and the plates were incubated for 1 hour at 37°C. Bound IgG anti-N were detected with peroxidase-conjugated $F(ab')_2$ fragments of goat anti-human IgG as previously described [7]. Determination of OD_{492} was normalized to standard sera for anti-N obtained from patients with diffuse NP-SLE such that anti-N activity might be converted to an arbitrary unit scale. The concentration of anti-N that produced half of the maximal absorbance at 492 nm, given by the saturating concentration of anti-N in the cell ELISA plate, was arbitrarily defined as 1 U/ml [7].

Statistical analysis

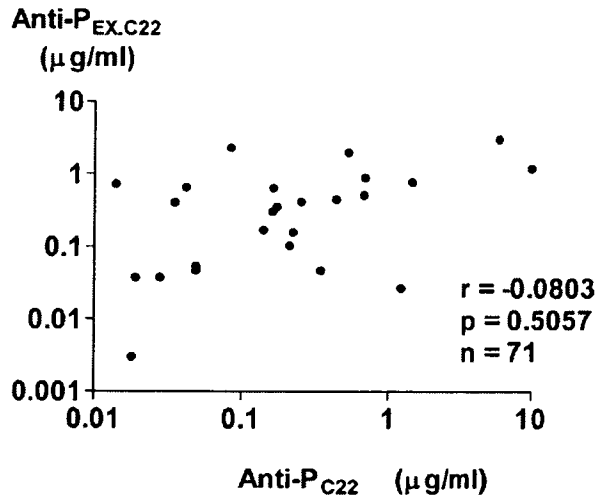
Differences in CSF anti-P_{C22}, anti-whole P, anti-$P_{EX.C22}$, and anti-C22-depleted rP0 among various groups were analyzed by Kruskal-Wallis test with multiple comparison (Scheffe's method). The correlation of anti-P_{C22} levels with anti-$P_{EX.C22}$ or anti-C22-depleted rP0 levels and the correlation of anti-N levels with anti-P_{C22}, anti-$P_{EX.C22}$, or anti-C22-depleted rP0 levels were evaluated by Spearman rank correlation test. Differences in serum anti-P_{C22}, anti-whole P, and anti-$P_{EX.C22}$ levels between non-CNS SLE and NP-SLE were analyzed by Welch's *t* test.

Results

Initial experiments examined CSF anti-P_{C22} levels in the three groups of patients. Although anti-P_{C22} levels in CSF appeared to be higher in diffuse NP-SLE, there were no significant differences in their levels among the three groups, including diffuse NP-SLE, focal NP-SLE, and non-inflammatory neurological control (Figure 1a). The results therefore confirm the previous observation that CSF anti-P_{C22} might not be prevalent in diffuse NP-SLE. By contrast, anti-whole P levels in CSF from patients with diffuse NP-SLE were significantly elevated compared with those from patients with focal NP-SLE or with non-inflammatory neurological diseases (Figure 1b). In addition, it should be noted that CSF anti-whole P levels were significantly higher than CSF anti-P_{C22} levels in 67 patients with diffuse NP-SLE and focal NP-SLE ($P < 0.0001$ as evaluated by Wilcoxon signed rank test). These results suggest that in addition to anti-P_{C22}, CSF from patients with NP-SLE might contain autoantibodies that recognize ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence.

To explore in detail the prevalence of the autoantibodies directed against the ribosomal P protein, epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$) were calculated by subtracting anti-P_{C22} from anti-whole P. As can be seen in Figure 1c, anti-$P_{EX.C22}$ levels in CSF from patients with diffuse NP-SLE were significantly elevated compared with those from patients with focal NP-SLE or with non-inflammatory neurological diseases. As shown in Figure 2, there was no significant correlation between CSF anti-P_{C22} and CSF anti-$P_{EX.C22}$ levels, obviating the possibility that CSF anti-$P_{EX.C22}$ activities might result from contamination of CSF anti-P_{C22} in patients with SLE. These results indicate that autoantibodies directed against ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence are strongly associated with the development of diffuse NP-SLE. Moreover, the data indicate that the expression of such

Figure 2



Correlation between autoantibodies to various components of ribosomal P proteins. The correlation between antibodies to the C-terminal 22-amino acid sequence of ribosomal P protein (anti-P_{C22}) and those to the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$) in cerebrospinal fluid from patients with systemic lupus erythematosus (SLE), including 52 patients with diffuse neuropsychiatric SLE (NP-SLE) and 19 patients with focal NP-SLE, was analyzed. Statistical analysis was performed by Spearman rank correlation test.

autoantibodies in CSF is not related to the presence of anti-P_{C22} in CSF.

To confirm the presence of autoantibodies to ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence, IgG antibodies to recombinant ribosomal P0 protein lacking the C-terminal 22 amino acids (C22-depleted rP0) were examined in CSF from 65 SLE patients with neuropsychiatric manifestations. Affinity-purified anti-P_{C22} reacted with ribosomal P peptide-HSA conjugates, but not with C22-depleted rP0, confirming the lack of the C-terminal 22-amino acid sequence in the C22-depleted rP0 (Figure 3). As shown in Figure 4, CSF anti-C22-depleted rP0 levels were significantly correlated with CSF anti-$P_{EX.C22}$ levels in these 65 patients. In addition, anti-C22-depleted rP0 levels in CSF from patients with diffuse NP-SLE were significantly elevated compared with those from patients with focal NP-SLE or with non-inflammatory neurological diseases (Figure 5). Accordingly, the frequency of positive expression of anti-C22-depleted rP0 in CSF from patients with diffuse NP-SLE was higher than that in CSF from patients with focal NP-SLE or with non-inflammatory neurological diseases (Table 2). These results confirm the presence of autoantibodies to ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence.

We next examined whether CSF anti-whole P might account for anti-N activities in CSF from patients with NP-SLE. As shown in Table 3, levels of CSF anti-whole P and anti-PC22 as well as CSF anti-N were decreased when CSF was incubated with paraformaldehyde-fixed SK-N-MC cells for 120 minutes at room temperature, confirming that CSF anti-whole P or anti-PC22 are constituents of CSF anti-N. However, as shown in Figure 6a, CSF anti-N levels were not significantly correlated with CSF anti-PC22 levels in SLE patients, including those with diffuse NP-SLE and focal NP-SLE. By contrast, CSF anti-N levels were significantly correlated with CSF anti-PEX.C22 or CSF anti-C22-depleted rP0 levels (Figure 6b,c

Finally, we examined serum levels of anti-P_{C22}, anti-whole P, and anti-$P_{EX.C22}$ in patients with non-CNS SLE or with NP-SLE. The values of anti-P_{C22}, anti-whole P, and anti-$P_{EX.C22}$ in 24 patients with non-SLE non-inflammatory neurological diseases were 2.44 ± 2.92 μg/ml, 4.92 ± 6.51 μg/ml, and 3.41 ± 6.06 μg/ml (mean ± standard deviation), respectively. As shown in Figure 7, serum anti-P_{C22} as well as anti-whole P levels in NP-SLE were significantly elevated compared with those in non-CNS SLE, which is consistent with previous studies [3-5]. Serum anti-P_{C22} and anti-whole P levels appeared to be higher in diffuse NP-SLE than those in focal NP-SLE, although

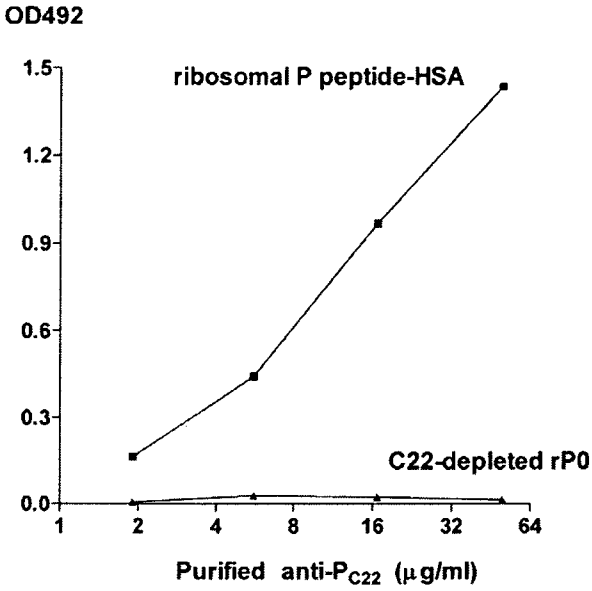
Table 2

Summary of the frequency of positive expression of antibodies to various ribosomal P protein components in cerebrospinal fluid[a]

	Percentage positive[b]		
	Control	Diffuse NP-SLE	Focal NP-SLE
Anti-P_{C22}	4.2% (1/24)	23.1% (12/52)	5.3% (1/19)
Anti-whole P	0% (0/24)	78.8% (41/52)	31.6% (6/19)
Anti-$P_{EX.C22}$	4.2% (1/24)	65.4% (34/52)	26.3% (5/19)
Anti-C22-depleted rP0	5.3% (1/19)	44.7% (21/47)	5.6% (1/18)

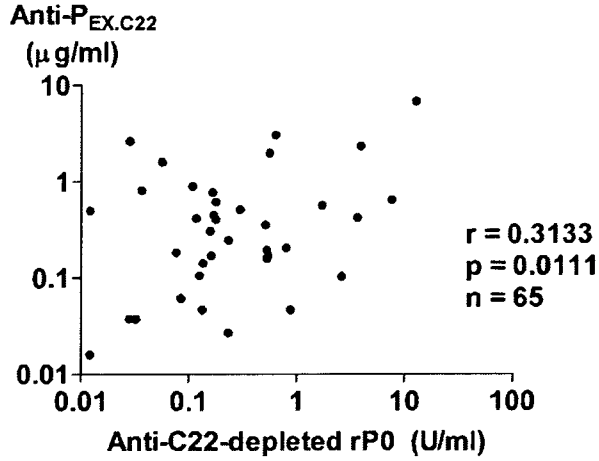
[a]Antibodies to the C-terminal 22-amino acid sequence of ribosomal P protein (anti-P_{C22}), to highly purified ribosomal P proteins (anti-whole P), to the epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$), and to recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence (anti-C22-depleted rP0) in cerebrospinal fluid from patients with non-inflammatory neurological diseases (Control), with diffuse neuropsychiatric systemic lupus erythematosus (NP-SLE), or with focal NP-SLE were compared. [b]Cutoff values were set as the mean + 3 standard deviations of the values in control group. Values in parenthesis mean (numbers of patients with positive results/total patient numbers) in each group.

Figure 3



Differential reactivity of purified antibodies to the C-terminal 22 amino acids of ribosomal P protein. Differential reactivity of purified antibodies to the C-terminal 22-amino acid sequence of ribosomal P protein (anti-P_{C22}) with ribosomal P peptide-human serum albumin (HSA) conjugates and with recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence (C22-depleted rP0). Purified anti-P_{C22} react with ribosomal P peptide-HSA conjugates, but not with C22-depleted rP0 on enzyme-linked immunosorbent assay plates. OD492 (optical density at 492 nm) values that are subtracted by non-specific binding activities are plotted.

Figure 4



Correlation between autoantibodies to various components of ribosomal P proteins. The correlation between antibodies to recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence (anti-C22-depleted rP0) and those to the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$) in cerebrospinal fluid patients, including 47 patients with diffuse neuropsychiatric systemic lupus erythematosus (NP-SLE) and 18 patients with focal NP-SLE, was analyzed. Statistical analysis was performed by Spearman rank correlation test.

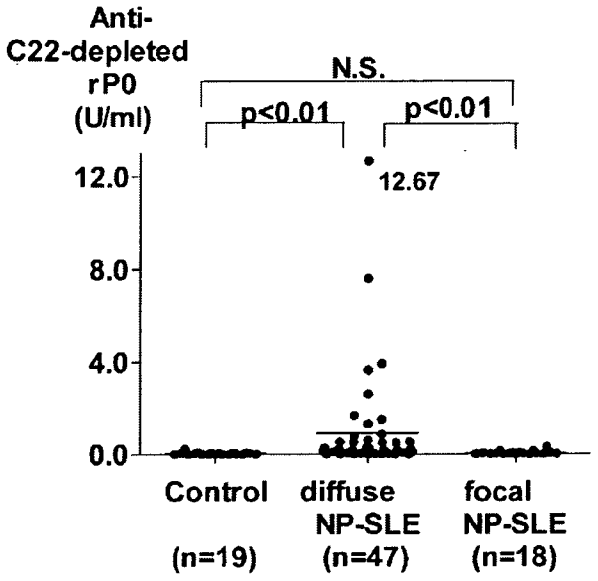
there were no statistical significances by Kruskal-Wallis test with multiple comparisons. Of note, there were no significant differences in serum anti-$P_{EX.C22}$ levels between non-CNS SLE and NP-SLE. These results suggest that in contrast with the CSF results, serum anti-P_{C22}, but not serum anti-P). The data therefore suggest that C22-depleted rP0 might contain one of the major targets, against which CSF anti-N are directed.$_{EX.C22}$, are associated with NP-SLE, especially diffuse NP-SLE.

Discussion

A number of studies have suggested that CSF anti-N play an important role in the pathogenesis of diffuse NP-SLE [7,11]. However, the epitopes to which CSF anti-N are directed have not been delineated. Of note, previous studies have demonstrated that epitopes antigenically related to ribosomal P proteins are present on the surface of SK-N-MC neuroblastoma cells [12]. Although anti-P_{C22} have been shown to be major autoantibodies to ribosomal P proteins [3,4,22], the frequency of their detection in CSF from patients with diffuse NP-SLE was not high enough to ensure their involvement in the pathogenesis of this disease [3,4,7]. Therefore, it was suggested that anti-P_{C22} might not be a major constituent of anti-N in CSF from patients with diffuse NP-SLE. Consistently, the data in the current studies indicated that CSF anti-P_{C22} levels were not significantly elevated in patients with diffuse NP-SLE compared with those in patients with focal NP-SLE or with non-inflammatory neurological diseases. However, it was still possible that CSF autoantibodies directed to ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence were more prevalent. Thus, the results in the current studies have also demonstrated that levels of CSF anti-whole P as well as CSF anti-$P_{EX.C22}$ were significantly higher in patients with diffuse NP-SLE than in patients with focal NP-SLE or non-inflammatory neurological diseases. The data therefore indicate that CSF antibodies to ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence are associated with diffuse NP-SLE.

To confirm the presence of antibodies for the epitopes representing regions of the ribosomal P proteins other than the C-terminal 22-amino acid sequence, antibodies to recombinant ribosomal P0 protein lacking the C-terminal 22 amino acids (C22-depleted rP0) [21] were evaluated. The results clearly demonstrate that CSF anti-C22-depleted rP0 levels were significantly correlated with CSF anti-$P_{EX.C22}$ levels. In addition, levels of CSF anti-C22-depleted rP0 as well as CSF anti-$P_{EX.C22}$ were significantly elevated in diffuse NP-SLE. The data therefore confirm that CSF antibodies to ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence play a role in the pathogenesis of diffuse NP-SLE, but further studies are required to identify the fine epitopes.

Figure 5



Cerebrospinal fluid antibodies to recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence. Antibodies to recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence (anti-C22-depleted rP0) (U/ml) in cerebrospinal fluid from patients with non-inflammatory neurological diseases (Control), with diffuse neuropsychiatric systemic lupus erythematosus (NP-SLE), or with focal NP-SLE were compared. Horizontal lines indicate the mean values. Statistical analysis was performed by Kruskal-Wallis test with multiple comparisons (Scheffé's method). N.S., not significant.

In has been demonstrated that purified human plasma membranes contain a 38-kDa protein that is closely related or identical to ribosomal P0 proteins [12]. Therefore, it was suggested that autoantibodies to ribosomal P proteins, especially those directed to epitopes other than the C-terminal 22-amino acid sequence, might be involved (at least in part) in CSF anti-N activities. In fact, levels of CSF anti-$P_{EX.C22}$ as well as CSF anti-P_{C22} or CSF anti-whole P were decreased after incubation of CSF with paraformaldehyde-fixed SK-N-MC cells, confirming that CSF anti-$P_{EX.C22}$ as well as anti-P_{C22} are constituents of CSF anti-N. However, CSF anti-P_{C22} levels were not significantly correlated with CSF anti-N levels in the present study. By contrast, CSF anti-$P_{EX.C22}$ or CSF anti-C22-depleted rP0 levels were significantly correlated with CSF anti-N levels. These results indicate that ribosomal P0 proteins contain one of the major targets of CSF anti-N in their portions other than the C-terminal 22-amino acid sequence. Of note, recent studies have demonstrated that autoantibodies directed against the *N*-methyl-d-aspartate (NMDA) receptor mediated apoptotic death of neurons *in vivo* and *in vitro* in murine systems [23]. Of note, anti-NMDA receptor antibodies were also detected in CSF from a single patient with SLE [22]. It is therefore likely that anti-NMDA receptor antibodies might also be involved in CSF anti-N activities and thus play a pivotal role in the pathogenesis of diffuse NP-SLE. Further studies with a large number of patients are required to confirm the involvement of anti-NMDA receptor antibodies in diffuse NP-SLE and to explore its relationship with anti-N.

A number of studies have indicated that serum anti-ribosomal P protein antibodies, including anti-P_{C22} or anti-whole P, are frequently observed in patients with NP-SLE [3-5,24]. Consistently, the results in the current studies have also disclosed that levels of serum anti-P_{C22} as well as serum anti-whole P are significantly higher in NP-SLE than those in non-CNS SLE. Of

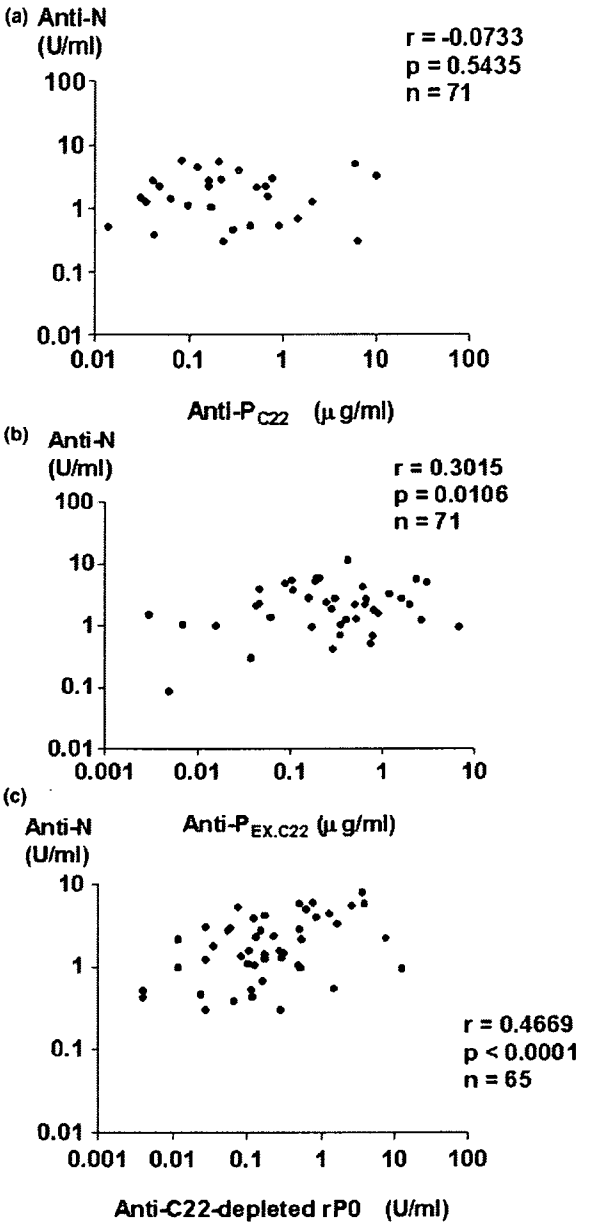
Table 3

Absorption of CSF autoantibodies to various components of ribosomal P proteins by neuronal cells

Patient	Autoantibodies	Without absorption	With absorption
1	Anti-whole P (μg/ml)	7.243	1.435
	Anti-P_{C22} (μg/ml)	3.456	1.019
	Anti-$P_{EX.C22}$ (μg/ml)	3.787	0.416
	Anti-N (U/ml)	4.083	1.950
2	Anti-whole P (μg/ml)	0.140	0.050
	Anti-P_{C22} (μg/ml)	0.070	0.042
	Anti-$P_{EX.C22}$ (μg/ml)	0.070	0.008
	Anti-N (U/ml)	0.789	0.588

Cerebrospinal fluid (CSF) samples (50 μl/well) were incubated in wells of a 96-well flat-bottomed microtiter plate with or without confluent SK-N-MC cells fixed with 1% paraformaldehyde at room temperature for 2 hours. After the incubation, CSF samples were recovered and were examined for anti-whole P, anti-P_{C22}, anti-$P_{EX.C22}$, and anti-N as described in Materials and methods. Anti-N, anti-neuronal cell antibodies; anti-P_{C22}, antibodies directed against the C-terminal 22-amino acid sequences of ribosomal P protein; anti-$P_{EX.C22}$, autoantibodies directed against the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence; anti-whole P, antibodies to the whole ribosomal P proteins.

Figure 6



Correlation between autoantibodies to ribosomal P proteins and anti-neuronal cell antibodies. The correlation of antibodies to the C-terminal 22-amino acid sequence of ribosomal P proteins (anti-P_{C22}) **(a)**, those to the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$) **(b)**, or those to recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence (anti-C22-depleted rP0) **(c)** with anti-neuronal cell antibodies (anti-N) in cerebrospinal fluid from systemic lupus erythematosus (SLE) patients, including 52 patients **(a,b)** or 47 patients **(c)** with diffuse neuropsychiatric SLE (NP-SLE) and 19 patients **(a,b)** or 18 patients **(c)** with focal NP-SLE, was analyzed. Statistical analysis was performed by Spearman rank correlation test.

note, serum anti-$P_{EX.C22}$ levels were not significantly elevated in NP-SLE compared with those in non-CNS SLE. These findings contrast sharply with the results of CSF studies. Thus, in CSF, anti-$P_{EX.C22}$, but not anti-P_{C22}, were significantly associated with diffuse NP-SLE, whereas in serum, anti-P_{C22}, but not anti-$P_{EX.C22}$, were associated with NP-SLE.

The mechanism by which anti-whole P cause neuronal damage remains unclear. We previously reported that the expression of IL-6 mRNA in neurons was upregulated in the brain of an SLE patient who died of active diffuse NP-SLE [25]. Of note, we recently disclosed that anti-P_{C22} upregulate the expression of mRNAs for IL-6 and tumor necrosis factor-alpha in human peripheral blood monocytes [20]. It should be pointed out that anti-$P_{EX.C22}$ as well as anti-P_{C22} might be able to bind the ribosomal P protein on neuronal cells [12]. Taken together, these results suggest that anti-whole P or anti-$P_{EX.C22}$ might also upregulate the expression of IL-6 mRNA in neurons and thus result in the alteration of their functions. Further studies to explore the targets and the effects on their functions of anti-P_{C22} and anti-$P_{EX.C22}$ (or anti-P_{AA9}) would improve our understanding of the pathogenesis of NP-SLE.

In summary, the current studies have demonstrated that the expression of autoantibodies directed against the epitopes of ribosomal P proteins other than the C-terminal 22-amino acid sequence is increased in CSF from patients with diffuse NP-SLE. The presence of such autoantibodies might account for CSF anti-N activities, although there might be other antibodies that bind to neuronal cells, such as anti-NMDA receptor antibodies. Further studies to explore the whole spectrum of epitopes of neurons to which autoantibodies are directed as well as the mechanism by which such autoantibodies cause damage to neurons are needed for a complete understanding of the pathogenesis of diffuse NP-SLE.

Conclusion

The present study has disclosed that CSF IgG antibodies to the epitopes of ribosomal P0 proteins other than the C-terminal 22 amino acids are associated with the development of diffuse NP-SLE as one of the major CSF anti-N components.

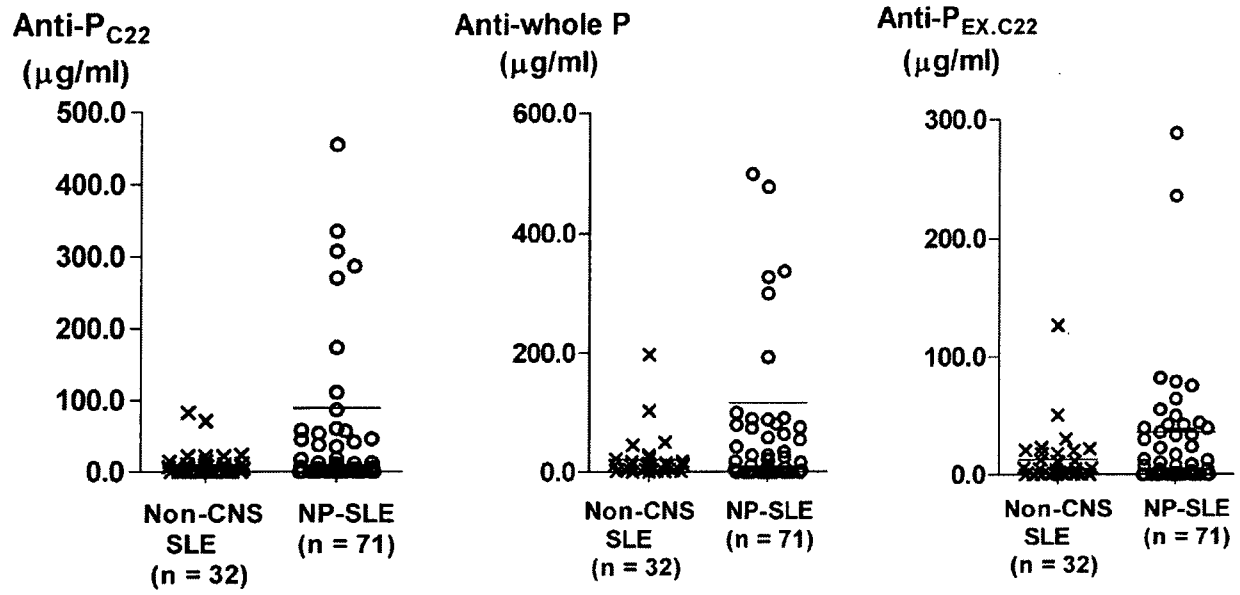
Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SH designed the study and participated in experimental procedures, collection, analysis, and interpretation of data and manuscript preparation. YA and MT contributed to the collection and analysis of data. TY helped to prepare C22-depleted rP0 and to develop ELISA for anti-C22-depleted rP0. All authors read and approved the final text before submission of the manuscript.

Figure 7



Serum autoantibodies to various components of ribosomal P proteins. Anti-P_{C22}, anti-whole P, and anti-$P_{EX.C22}$ in sera from SLE patients without neuropsychiatric manifestations (non-CNS SLE) (cross), with diffuse NP-SLE (open circle), or with focal NP-SLE (closed circle) were compared. Horizontal lines indicate the mean values. Statistical analysis between non-CNS SLE versus NP-SLE (focal + diffuse) was performed by Welch's *t* test. Anti-P_{C22}, antibodies directed against the C-terminal 22-amino acid sequences of ribosomal P protein; anti-$P_{EX.C22}$, autoantibodies directed against the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence; anti-whole P, antibodies to the whole ribosomal P proteins; non-CNS SLE, systemic lupus erythematosus without neuropsychiatric manifestations; NP-SLE, neuropsychiatric systemic lupus erythematosus; SLE, systemic lupus erythematosus.

Acknowledgements

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Antiinflammatory Mediator Lipoxin A4 and Its Receptor in Synovitis of Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To evaluate the role of an antiinflammatory lipid mediator, lipoxin A_4 (LXA_4), in inflammatory arthritis, we measured the level of LXA_4 in synovial fluid and lipoxin A_4 receptor (ALX) expression in synovial tissues obtained from patients with rheumatoid arthritis (RA) and osteoarthritis (OA).

Methods. Levels of LXA_4 and its analog (15-epi-LXA_4) in synovial fluid from 30 patients with RA and 15 patients with OA were measured by a specific ELISA. Reverse transcription-polymerase chain reaction (RT-PCR), real-time quantitative PCR, and *in situ* hybridization were performed to detect mRNA for ALX and 15-LOX, and LXA_4 synthetase, in synovial tissues from 20 patients with RA and 10 patients with OA.

Results. Both LXA_4 and 15-epi-LXA_4 showed significantly higher levels in RA synovial fluid (10.34 ± 14.12 ng/ml for LXA_4) than OA synovial fluid (0.66 ± 0.77 ng/ml for LXA_4). Logarithmic concentration of LXA_4 was significantly correlated with that of leukotriene B_4 and prostaglandin E_2 in RA and OA synovial fluids. Expressions of ALX and 15-LOX mRNA were stronger in RA synovium than OA synovium. Expression of mRNA for interleukin 13 (IL-13), which induces 15-LOX, was significantly stronger in RA synovium than OA synovium.

Conclusion. ALX is an important target of LXA_4 in synovial tissues of patients with RA. 15-LOX induced by IL-13 might regulate the production of LXA_4 to have an antiinflammatory effect against proinflammatory lipid mediators in inflamed joints. These findings could lead to the development of new therapy for inflammatory arthritis such as RA. (First Release Oct 1 2007; J Rheumatol 2007;34:2144–53)

Key Indexing Terms:
RHEUMATOID ARTHRITIS LIPOXIN A_4 LIPOXIN INFLAMMATION

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Lipoxin A_4 (LXA_4) and its recently identified carbon-15 epimeric form, aspirin-triggered LXA_4 (15-epi-LXA_4), potently inhibit neutrophil activity and appear to serve as an endogenous "stop signal" that regulates excessive leukocyte trafficking and promotes resolution of inflammation[1]. LXA_4 is synthesized by 5-lipoxygenase (LOX) and 12-LOX, or by 5-LOX and 15-LOX, via cell–cell interactions, while 15-epi-LXA_4 is produced by 5-LOX and acetylated prostaglandin H synthase-II after treatment with aspirin. Both 15-epi-LXA_4 and LXA_4 modulate leukocyte responses by interacting with the lipoxin A_4 receptor (ALX), which is a specific G-protein-coupled receptor[2]. ALX was first identified in retinoic acid-differentiated HL-60 cells and then was cloned in mice, showing a high affinity to its endogenous lipid ligands (LXA_4 and 15-epi-LXA_4) as well as their stable bioactive analogs[3]. These compounds inhibit acute inflammation and reperfusion injury in both human cell models and murine models[4-6].

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by inflammatory polyarthritis and is associated with higher levels of proinflammatory arachidonic acid metabolites, such as leukotriene B_4 (LTB_4)[7] and prostaglandin E_2 (PGE_2)[8], in the synovial fluid than in that of patients with

osteoarthritis (OA). 5-LOX is expressed in synoviocytes[9] and the synovial lining layer[10] of patients with RA. Neutrophils in the synovial fluid[11] and peripheral blood[12] of patients with RA generate more LTB_4 via upregulated 5-LOX than the same cells from healthy individuals. PGE_2 induces 15-LOX in human neutrophils[4], while 15-LOX was reported to be upregulated by interleukin 4 (IL-4)[13] and IL-13[14] in human monocytes. 15-LOX is a key enzyme of LXA_4 synthesis in inflammatory sites[4]. An earlier study showed that polymorphonuclear cells from patients with RA can release more lipoxins than those from healthy individuals[15] and another exhibited the production of LXA_4 and 15-epi-LXA_4 by OA cartilage explants and ALX on OA chondrocytes[16,17]. These studies suggest the relationship between the lipoxin system and the pathogenesis of RA and OA.

We investigated LXA_4 in synovial fluid, as well as the differences of ALX distribution and expression in synovial tissues between patients with RA and OA. Factors that may regulate the lipoxygenase pathway were also analyzed to assess the role of LXA_4 and its analogs in the pathogenesis of RA.

MATERIALS AND METHODS

Reagents. A mouse anti-5-LOX monoclonal antibody was purchased from Research Diagnostic Inc. (Flanders, NJ, USA). The anti-CD3 antibody, anti-CD4 antibody, anti-CD8 antibody, and anti-CD68 antibody, and anti-von Willebrand factor (vWF) antibody were all purchased from Dako (Carpinteria, CA, USA). A DIG-High Prime DNA Labeling and Detection Kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany) and a Vectorstain ABC kit came from Vector Laboratories Inc. (Burlingame, CA, USA). Finally, 3,3'-diaminobenzidine (DAB) was obtained from Dojindo (Kumamoto, Japan) and a real-time quantitative polymerase chain reaction (PCR) kit (qPCR Mastermix for Sybr Green I) was purchased from Eurogentec (Seraing, Belgium).

Patients and samples. Synovial fluid specimens were obtained by arthrocentesis of the knee joint in 30 patients with RA and 15 with OA who consulted our institution (Table 1). Synovial tissue samples were obtained from 20 patients with RA and 10 with OA during orthopedic procedures for treatment at our institution. All patients with RA fulfilled the criteria of the American College of Rheumatolgy[18,19]. OA was diagnosed according to clinical and radiological criteria. All patients gave informed consent for the use of their samples in research. In both groups, the clinical characteristics of the patients (sex, age, C-reactive protein, and therapy) were consistent with the diagnosis.

Table 1. Clinical features of 45 patients with RA or OA used in the analysis of synovial fluid.

Feature	Diagnostic Group RA	OA
Patients, n	30	15
Female:male	22:8	10:5
Age, yrs	57.23 ± 13.30	66.07 ± 12.20
CRP, mg/dl	3.037 ± 1.889	0.2256 ± 0.2308
Medication		
PSL, n (%)	27 (90)	0 (0)
PSL, mean ± SD (mg/day)	4.724 ± 2.658	0 ± 0
NSAID, n (%)	25 (83)	6 (40)
Aspirin, n (%)	4 (13)	1 (7)
Statin, n (%)	0 (0)	1 (7)

CRP: C-reactive protein; PSL: prednisolone; Statin: HMG-CoA reductase inhibitor; NSAID: nonsteroidal antiinflammatory drugs.

Samples were fixed in 4% paraformaldehyde within 6 h of resection and were embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) after cooling in liquid nitrogen. Synovial fluid cells were obtained from the synovial fluid samples of patients with RA, and the percentages of neutrophils and mononuclear cells were evaluated by examination after Wright-Giemsa staining.

Measurement of LXA_4, LTB_4, and PGE_2 in synovial fluid. Synovial fluid samples were collected in polypropylene tubes and centrifuged at 1800 g for 10 min at 4°C. Cell-free supernatants of the fluid were stored at –70°C until use. LXA_4, LTB_4, and PGE_2 were separated from the supernatant by passage through octadecylsilyl silica columns (Sep-Pak C18, Waters Corp., Milford, MA, USA), followed by elution with methyl formate for LTB_4 and LXA_4, or with ethyl acetate containing 1% methanol for PGE_2. After evaporation to dryness, the residue was resuspended in the extraction buffer of each ELISA kit. The LXA_4 level in synovial fluid was determined with an ELISA kit (Neogen Corp., Lexington, KY, USA) according to the manufacturer's instructions, which was specific for LXA_4 and showed little cross-reactivity [LXA_4 100%, lipoxin B_4 1.0%, 15-hydroxyeicosatetraenoic acid (HETE) 0.1%, 5-HETE < 0.1%, and 12-HETE < 0.1%]. LTB_4 and PGE_2 were also measured in the same samples of synovial fluid with specific ELISA kits (LTB_4, Neogen Corp.; PGE_2, Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturers' instructions.

Assessment of human LXA_4 receptor (ALX), 15-LOX, and IL-13 mRNA expression in synovial tissue by reverse transcription-PCR (RT-PCR) and real-time quantitative PCR. Total RNA was isolated from tissues or synovial fluid cells using the guanidium thiocyanate/phenol/chloroform method (Isogen Reagent Kit; Nippon Gene Co. Ltd., Toyama, Japan), and cDNA was synthesized from 2 μg of total RNA using RAV2 reverse transcriptase and Oligo (dT) primers (Takara Shuzo Co. Ltd., Shiga, Japan), as described[20].

The RT-PCR primers for human ALX (GenBank accession no. U81501) were 5'-CTG GCC CTG GCT GAC TTT TCT TT-3' (sense: 219–241 bp) and 5'-GCC ACC TTC AGC CTC TCC TCA-3' (antisense: 581–601 bp), and the PCR product obtained with these primers was 383 bp in size[21]. The primers for human 5-LOX (GeneBank accession no. J03571) were 5'-CCG GCA CTG ACG ACT ACA TCT A-3' (sense: 81–102 bp) and 5'-CAC GGG GGT AAA TCC TTG TGG-3' (antisense: 514–533 bp), and the PCR product had a size of 453 bp[9]. The primers for human 15-LOX (GenBank accession no. M23892) were 5'-TGG CCG ACC TCG CTA TCA AAG ACT-3' (sense: 548–571 bp) and 5'-TGG GGG ATC CGT AGG CAA GAA AAG-3' (antisense: 991–1014 bp), and the PCR product had a size of 467 bp[22]. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession no. M33197) was used as the internal control, with 2 primers (5'-CAT CAT CTC TGC CCC CTC TG-3' and 5'-CCT GCT TCA CCA CCT TCT TG-3') yielding an expected PCR product of 437 bp[23].

Real-time quantitative PCR was performed to compare the expression of ALX, 15-LOX, IL-4, and IL-13 mRNA in the synovial tissue samples obtained from patients with RA and OA. cDNA from 20 patients with RA and 10 with OA was subjected to real-time quantitative PCR with the following primers: 5'-AAC CCC ATG CTT TAC GTC TTT GTG-3' (sense: 912–935 bp) and 5'-ATT GGC AGC CGT GTC ATT AGT TG-3' (antisense: 1012–1034 bp) for human ALX[21], yielding a product of 123 bp; and 5'-ACC AGC CCC AGC AAG AGC ACA AG-3' (sense: 1081–1103 bp) and 5'-TTC AAG GGG TCT ACA TGG CAA CTG-3' (antisense: 1180–1203 bp) primers for human GAPDH (the control) yielding a product of 123 bp[23]. The primers for human 15-LOX were 5'-CCG GAT TTT CTG GTG TGG TTC-3' (sense: 615–634 bp) and 5'-ACT AGG CGA GCA GGA AGG TGA-3' (antisense: 738–758 bp), and the PCR product had a size of 144 bp[22]. The primers for human IL-4 (GenBank accession no. BC066278) were 5'-TCT GTG CAC CGA GTT GAC C-3' (sense: 203–221 bp) and 5'-ACC CAG GCA GCG AGT GT-3' (antisense: 322–338 bp), and the product had a size of 136 bp[24]. The primers for human IL-13 (GenBank accession no. NM_002188) were 5'-GGC CCT GAG CTC GGT GGA C-3' (sense: 679–697 bp) and 5'-CTA CAC

CCC TCC CCT GCC CTA-3' (antisense: 715–735 bp), with the product having a size of 57 bp[25].

Real-time PCR was done with a real-time quantitative PCR kit (qPCR Mastermix for Sybr Green I, Eurogentec) according to the manufacturer's protocol. Detection was performed by identifying the fluorescence of SYBR Green fluorescent dye (Eurogentec). Amplification was performed according to the standard protocol recommended by the manufacturer (2 min at 50°C, 10 min at 95°C, 40 cycles of 95°C for 15 s each, and annealing for 1 min at 56.9°C for ALX, 56.7°C for 15-LOX, 54.6°C for IL-4, and 55.5°C for IL-13). All samples were measured in duplicate. Analysis was carried out with an ABI Prism 7700 Sequence Detection System (Applied Biosystems Japan Ltd., Tokyo, Japan), and the calculated cycle threshold values (Ct) were exported to Microsoft Excel. For comparison between the sample groups, relative mRNA levels were subsequently normalized against values found in the patients with OA, which were defined as the baseline (reference value = 1).

In situ hybridization of ALX and 15-LOX in synovial tissue. PCR products of ALX and 15-LOX were purified from agarose gels using a StrataPrep PCR purification kit (Stratagene Cloning Systems, La Jolla, CA, USA) and were cloned using a Qiagen PCR cloning kit (Qiagen, Tokyo, Japan). Then the cDNA of ALX and 15-LOX were sequenced using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems).

Cryosections of synovium were mounted on silane-coated glass slides and fixed with 4 % (w/v) paraformaldehyde. DIG-labeled antisense riboprobes for human ALX and 15-LOX were prepared by *in vitro* transcription of the pDrive Cloning Vector (Qiagen), which contained human ALX and 15-LOX cDNA[26], and a sense riboprobe prepared in the same way. The synovial sections were treated with 10 μg/ml proteinase K and hybridized with the labeled riboprobes in hybridization solution (Novagen, Madison, WI, USA) for 18 h at 50°C in moistened plastic boxes. After hybridization, the sections were treated with 20 μg/ml RNase A. After extensive washing, the binding of each probe was visualized with an alkaline-phosphate conjugated anti-DIG antibody in 5-bromo-4-chloro-3 indolyl-phosphate and 4-nitroblue tetrazolium chloride solution (Roche Diagnostics GmbH). Then the slides were counterstained with hematoxylin before examination.

Immunohistochemistry for 5-LOX and surface markers. Immunoperoxidase staining was done using a Vectorstain kit according to the manufacturer's protocol[27]. Sections prepared from frozen samples were incubated in methanol containing 3% (v/v) H_2O_2 for 20 min to enhance endogenous peroxidase activity. Then the sections were preincubated in 0.3% (v/v) bovine serum albumin (Sigma-Aldrich Japan K.K., Tokyo, Japan) in phosphate-buffered saline (PBS) for 1 h, followed by incubation with diluted goat serum for 20 min. Subsequently, incubation was done in a humidified chamber for 1 h with an anti-5-LOX antibody (1:100; Research Diagnostics Inc.), anti-CD68 antibody, anti-CD3 antibody, anti-CD4 antibody, anti-CD8 antibody, anti-CD68 antibody, and anti-vWF antibody, or purified normal mouse IgG. After further washing with PBS, sections were incubated with biotinylated goat anti-mouse IgG (Dako) for 30 min, and washed again in PBS. Color was developed by treatment with DBA and the sections were counterstained with hematoxylin.

Statistical analysis. Results are expressed as the mean ± standard deviation. Mean values were compared by the Mann-Whitney test and $p < 0.05$ was considered to indicate a significant difference.

RESULTS

LXA_4 in synovial fluid of patients with RA and OA. The LXA_4 level in synovial fluid from affected joints of patients with RA or OA was measured by ELISA, and an increase of LXA_4 was detected in RA synovial fluid (Figure 1A). The mean concentration of LXA_4 in 30 RA synovial fluid samples was 10.34 ± 14.12 ng/ml, which was significantly greater than in the OA samples (0.66 ± 0.77 ng/ml; $p = 0.0023$). 15-epi-LXA_4 also showed a significantly higher concentration in RA synovial fluids (4.366 ± 4.376 ng/ml) than in OA synovial fluid (0.8553 ± 1.692 ng/ml; $p < 0.0001$; Figure 1B). No significant correlation was found between LXA_4 or 15-epi-LXA_4 levels and patients' clinical features such as sex, age, serum C-reactive protein level, and medications except for prednisolone. As for prednisolone, most patients with RA took it and no patient with OA did. Only 4 patients with RA took aspirin and their 15-epi-LXA_4 levels in synovial fluids were not higher than those without aspirin (3.813 ± 6.192 with aspirin vs 4.451 ± 4.190 without aspirin). In patients with OA, a tendency of high LXA_4 and 15-epi-LXA_4 levels in synovial fluids was detected in patients treated without nonsteroidal antiinflammatory drugs (NSAID; containing aspirin) than those treated with NSAID (for LXA_4, 0.2971 ± 0.1687 with NSAID vs 0.9750 ± 0.9597 without NSAID; for 15-epi-LXA_4, 0.2871 ± 0.2310 with NSAID vs 1.353 ± 2.253 without NSAID), but this difference did not reach statistical significance ($p = 0.0820$ and 0.2239, respectively). In patients with RA or OA, logarithmic concentrations of LXA_4 were significantly correlated with those of LTB_4 (Figure 1C; Pearson $r = 0.8464$, $p < 0.0001$) or PGE_2 (Figure 1D; Pearson $r = 0.7210$, $p < 0.0001$). Logarithmic concentrations of PGE_2 and LTB_4 were also significantly correlated (Figure 1E; Pearson $r = 0.7931$, $p < 0.0001$). Moreover, significant correlation between logarithmic concentrations of LXA_4 and 15-epi-LXA_4 was detected (Figure 1F; Pearson $r = 0.8119$, $p < 0.0001$).

ALX mRNA expression in synovial tissues of patients with RA or OA. We examined the expression of ALX mRNA in synovial tissues from 20 patients with RA and 10 with OA by RT-PCR and real-time quantitative PCR. Figure 2A shows that ALX mRNA signals were more strongly expressed in the synovial tissue of patients with RA compared to those with OA.

Real-time quantitative PCR revealed 10-fold higher expression of ALX mRNA in RA synovium (9.7 ± 14.48) than OA synovium (1.00 ± 1.01; $p = 0.0165$; Figure 2B).

To determine the distribution of ALX mRNA in the synovial tissues of patients with RA and OA, we performed *in situ* hybridization using DIG-labeled riboprobes. Macrophages were identified by positive staining with anti-CD68 antibody, while fibroblast-like cells were spindle-shaped cells that showed negative staining with anti-CD68 antibody and anti-CD3 antibody. Endothelial cells were identified using anti-vWF antibody. Strong signals for ALX mRNA were seen in macrophages and in a few fibroblast-like cells of the lining layer in patients with RA (Figure 3A, 3B), whereas these cells had faint signals in patients with OA (Figure 3C, 3D).

ALX mRNA expression by synovial fluid cells of patients with RA. Cells in the synovial fluid of 10 patients with RA were used to analyze ALX mRNA expression, revealing 84% ± 5% neutrophils and 16% ± 5% mononuclear cells. ALX mRNA was weakly expressed in cells from the RA synovial fluid by RT-PCR (Figure 2C).

5-LOX and 15-LOX mRNA expression in the synovial tissues of patients with RA or OA. 5-LOX and 15-LOX are the synthetases for LXA_4, and expression of their mRNA in the syn-

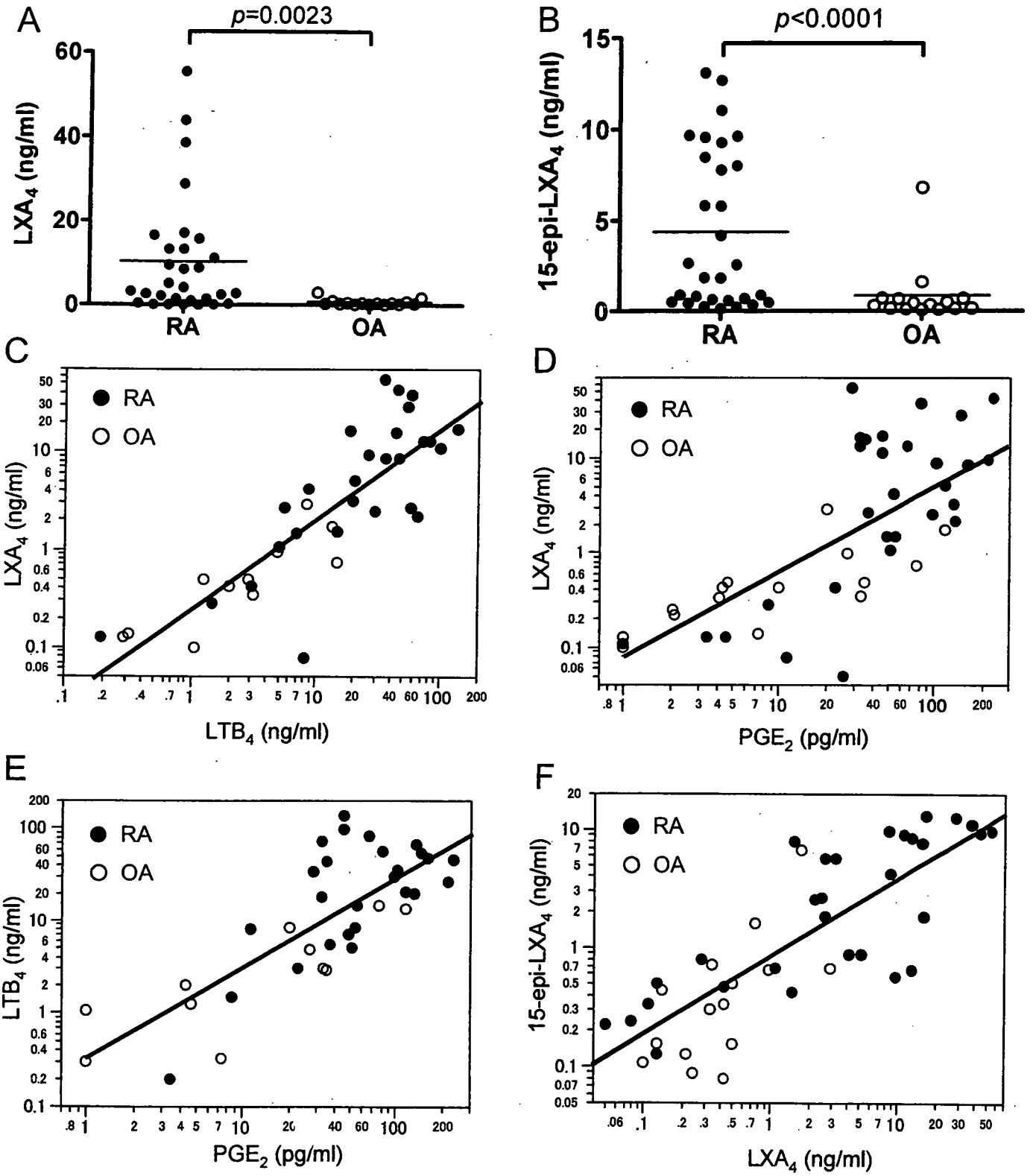

Figure 1. A. Concentration of LXA_4 in synovial fluid of patients with RA or OA. LXA_4 level was significantly higher in RA synovial fluid (10.34 ± 14.12 ng/ml) than OA synovial fluid (0.66 ± 0.77 ng/ml; p = 0.0023). B. Concentration of 15-epi-LXA_4 in RA and OA synovial fluids. 15-epi-LXA_4 level was also significantly higher in RA synovial fluid (2.60 ± 0.91 ng/ml) than in OA synovial fluid (0.94 ± 0.92 ng/ml; p < 0.0001). Results represent mean ± SD (n = 30 RA, n = 15 OA). C. Double logarithmic plot shows a significant positive correlation between concentrations of LXA_4 and LTB_4 in RA and OA synovial fluids (r = 0.8464, p < 0.0001). D. Logarithmic concentrations of PGE_2 also correlated with those of LXA_4 (r = 0.7210, p < 0.0001). E. Logarithmic concentrations of PGE_2 correlated with those of LTB_4 (r = 0.7931, p < 0.0001). F. Logarithmic concentrations of LXA_4 correlated with those of 15-epi-LXA_4 (r = 0.8119, p < 0.0001).

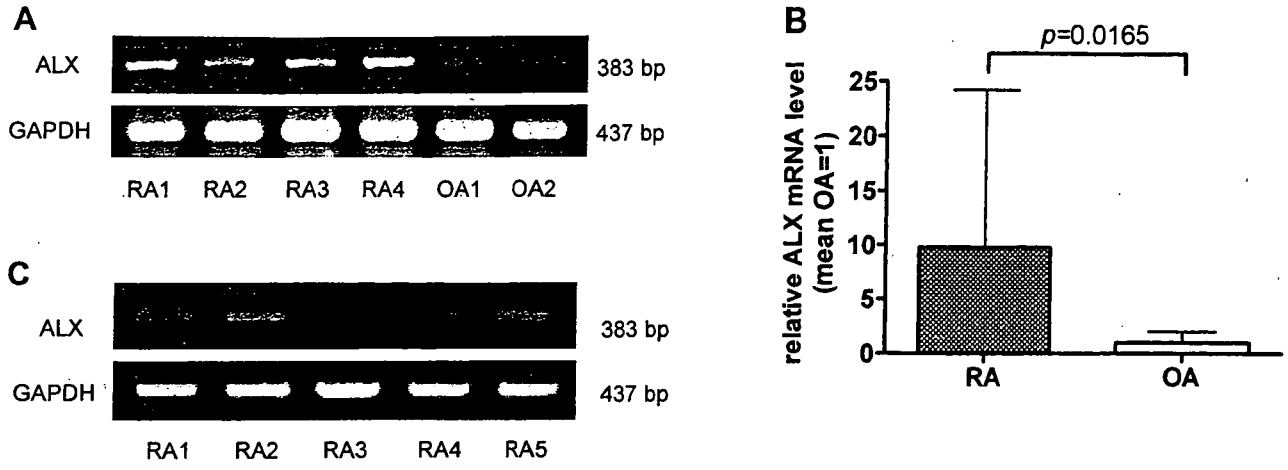

Figure 2. A. ALX mRNA expression in synovial tissues from 4 patients with RA and 2 with OA. ALX mRNA was detected by RT-PCR in all synovial samples, but its expression was relatively weak in OA tissues. B. Comparison of the expression of ALX mRNA between synovial tissues from patients with RA and OA. ALX mRNA expression was significantly stronger in RA synovium (9.74 ± 14.48) than in OA synovium (1.00 ± 1.01, $p < 0.0165$). Results represent the mean ± standard deviation (n = 20 for RA and n = 10 for OA). mRNA levels are shown relative to the mean value for OA, which was defined as 1. C. ALX mRNA expression in synovial fluid cells from 5 patients with RA. ALX mRNA expression in these cells was weak.

ovial tissues of patients with RA or OA was detected by RT-PCR. Figure 4A shows consistent strong expression of 5-LOX mRNA in RA and OA synovium. Compared with 5-LOX mRNA expression, 15-LOX mRNA expression was weaker in both RA and OA synovium. When 15-LOX mRNA expression in RA and OA synovium was compared by real-time quantitative PCR, its expression was stronger in the synovial tissues of patients with RA (3.01 ± 6.49) compared with those from patients with OA (1.00 ± 1.14), but this difference did not reach statistical significance ($p = 0.1659$; Figure 6B).

In situ hybridization of 15-LOX and immunohistochemical analysis of 5-LOX in synovial tissues of patients with RA. Localization of 5-LOX and 15-LOX in the synovial tissues of patients with RA was analyzed by *in situ* hybridization or immunohistochemistry, respectively. Intense signals for 15-LOX mRNA were detected in macrophages infiltrating RA synovium (Figure 5A, 5B). Immunostaining revealed 5-LOX expression in the synovial lining cells in samples from patients with RA (Figure 5C), as we reported[10].

Quantitative analysis of IL-4 and IL-13 mRNA in synovial tissues of patients with RA and OA. 15-LOX is known to be induced by IL-4[13] and IL-13[14] in human monocytes. When the levels of IL-4 and IL-13 mRNA in synovial tissues from patients with RA and OA were assessed by real-time quantitative PCR, the tissues obtained from patients with RA showed significantly (4-fold) higher expression of IL-4 mRNA (4.16 ± 3.85) than those from patients with OA (1.00 ± 1.10; $p = 0.0060$; Figure 6A). Analysis of IL-13 mRNA also revealed higher expression in the synovial tissues of patients with RA (5.71 ± 7.88) compared with those from patients with OA (1.00 ± 0.69), but this difference was not significant ($p = 0.1659$; Figure 6B).

DISCUSSION

In our study, we detected an increase of LXA_4 and its analog (15-epi-LXA_4) in the synovial fluid as well as increased expression of the LXA_4 receptor (ALX) in synovial tissues of patients with RA compared with those from patients with OA. LXA_4 is an antiinflammatory mediator, and logarithmic concentration of LXA_4 in synovial fluid was positively correlated with that of LTB_4 and PGE_2, which were proinflammatory mediators. Sodin-Semrl, *et al*[28] reported that functioning LXA_4 receptors were expressed by cultured synovial fibroblasts, and suppressed IL-1ß-induced synovial cell activation. In activated synovial fibroblasts, LXA_4 inhibits the synthesis of inflammatory cytokines and matrix metalloproteinases, and also stimulates tissue inhibitor of metalloproteinase-1 production *in vitro*[28]. These findings suggest that LXA_4 is involved in a negative feedback loop that opposes inflammatory cytokine-induced activation of synovial fibroblasts, although other ligands of ALX, for example annexin 1 (also called lipocortin 1) or serum amyloid A, can bind ALX and abrogate inflammation[29-31].

In vivo studies have recently demonstrated that LXA_4 significantly decreases inflammatory infiltrates and edema and has a more potent effect than equimolar concentrations of corticosteroids in mouse and guinea pig models of cutaneous inflammation[32]. Similarly, LXA_4 possesses antiinflammatory effects that may be involved in regulating pathophysiological processes related to the development of inflammatory arthritis such as RA. LXA_4 has a regulatory role in the cytokine network as demonstrated by suppression of tumor necrosis factor-α (TNF-α)-stimulated release of IL-1ß and macrophage inflammatory peptide-2, as well as superoxide production[33]. Numerous types of cells, including neutrophils, monocytes, endothelial cells, and fibroblasts, express high affinity G-protein-coupled receptors (GPCR) for LXA_4 (ALX). Recent