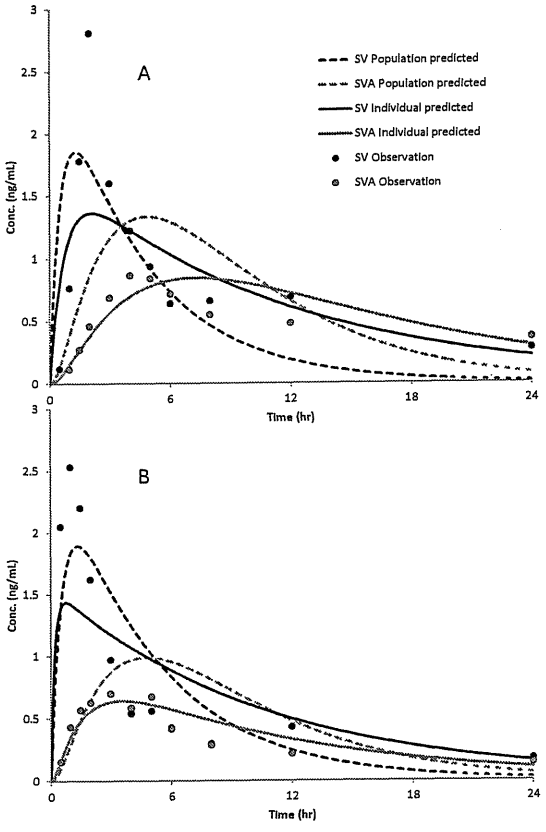


図 1 脂肪の影響を示す代表的被験者の血中濃度推移
A: 脂肪 7.38 kg, *SLCO1B1 c. 521* T/T, 中国人.
B: 脂肪 28.04 kg, *SLCO1B1 c. 521* T/C, 白人.

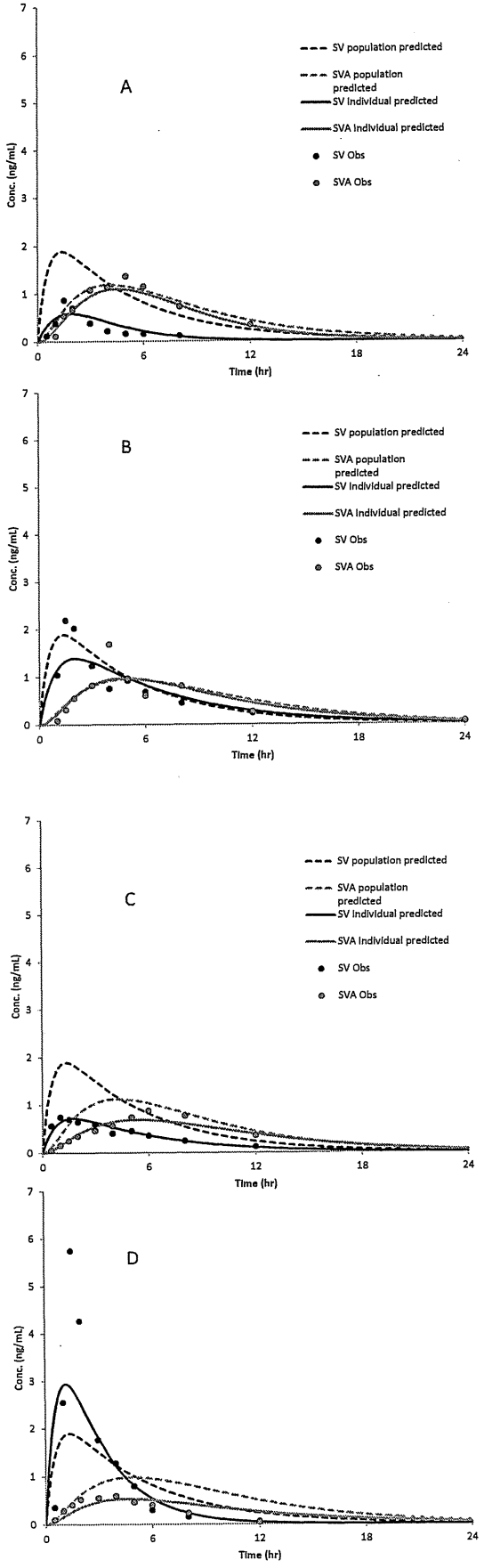


図 2 民族の影響を示す代表的被験者の血中濃度推移
A: 脂肪 12.34 kg, *SLCO1B1 c. 521* T/T, 日本人.
B: 脂肪 12.89 kg, *SLCO1B1 c. 521* T/T, 中国人.
C: 脂肪 12.62 kg, *SLCO1B1 c. 521* T/T, 韓国人.
D: 脂肪 12.83 kg, *SLCO1B1 c. 521* T/T, 白人.

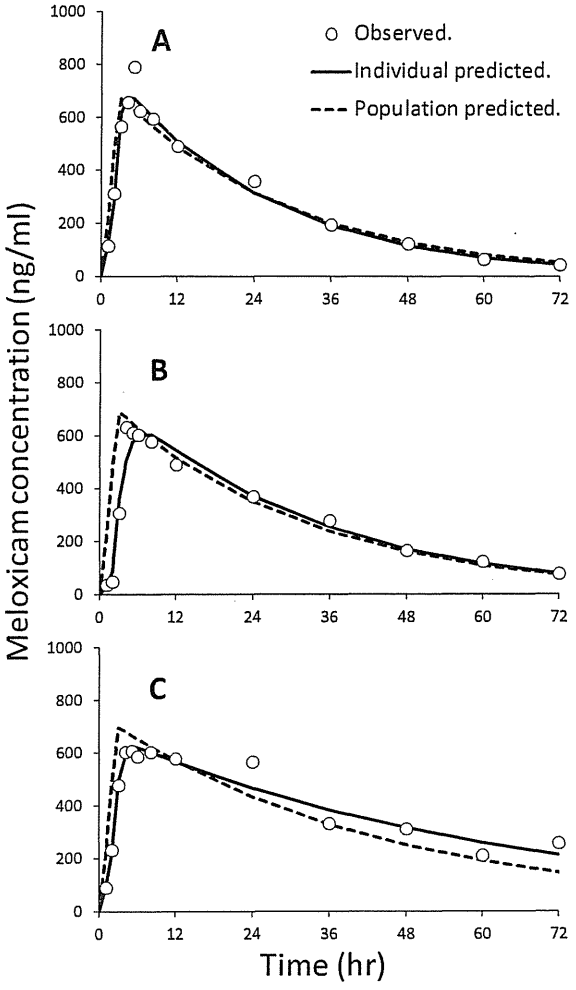


図3 *CYP2C9*遺伝子多型の影響を示す代表的被験者の血中濃度推移
A: *CYP2C9*1/*1*, 除脂肪体重 55.4 kg.
B: *CYP2C9*1/*2*, 除脂肪体重 54.8 kg.
C: *CYP2C9*1/*3*, 除脂肪体重 54.9 kg.

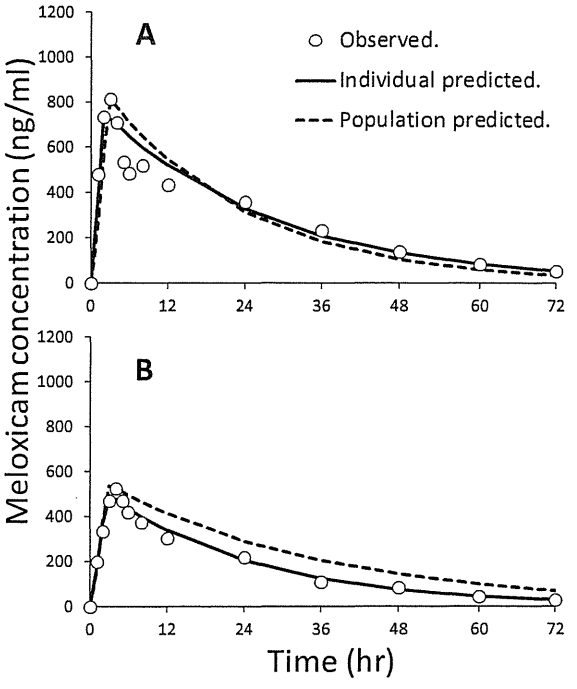


図 4 除脂肪体重の影響を示す代表的被験者の血中濃度推移
A: *CYP2C9*1/*1*, 除脂肪体重 43.6 kg.
B: *CYP2C9*1/*1*, 除脂肪体重 75.2 kg.

平成24年度　厚生労働科学研究費補助金（地球規模保健課題推進研究事業）
研究分担報告書

# 遺伝子発現を場にした薬物代謝の民族差に関する基盤研究

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**研究要旨：**
我が国を含む東アジア諸民族を国際共同治験に組み込む治験システムの構築において、医薬品の応答性における民族的要因を補完することはきわめて重要と思われる。近年、核内レセプターの分子生物学的研究基盤を背景にその作用を選択的に制御する創薬が活発に展開されている。本研究は、核内レセプターの中でも臨床薬理学的に重要なグルココルチコイドレセプターGR と PXR に焦点をあて、それらの周辺分子の同定、エピジェネティック制御機構の解明、を通じて、これらのリガンドとなる薬剤の薬効と副作用の民族差、人種差を解明する分子基盤を確立することを目的とする。
GR に関しては、核内タンパク HEXIM1 が GR にリガンド非依存性に結合し、その機能を抑制することがわかった。HEXIM1 は 7SK RNA を介さず、タンパク-タンパク相互作用によって GR 機能を負に制御する。今後、HEXIM1 の遺伝子レベルの多型解析から GR を標的とした薬剤の効果に関する民族差に迫る。PXR はある種の薬物依存性に CYP3A4 などの薬物代謝酵素の発現を遺伝子レベルで制御する。これまで PXR 自体の民族的差は軽微とされていた。今回、質量分析法などによって新たな PXR 結合タンパク候補を複数単離し、現在その同定を試みている。今後、GR、PXR による遺伝子発現制御のエピジェネティック制御機構をさらに明確にすることによって、これらのレセプターを標的とする医薬の薬効･副作用の民族差･人種差に関わる分子基盤を解明しうると考える。

## A.　研究目的：

医薬品の応答性における民族的要因を補完することによって、我が国を含む東アジア諸民族を国際共同治験に組み込む治験システムの構築が可能になり、日本および東アジア諸国を含めた国際共同治験が効率的に実施できるものと考えられる。そこで、薬効を規定する薬効受容体の遺伝子変異と薬効の発現について基礎的に検討し、薬効に民族差を生じる原因の一部を解明する方法を確立することは急務と言える。

核内レセプターはリガンド依存性転写因子として多彩な生理的役割を果たしている。従来、グルココルチコイドに対する応答性の人種差、民族差のメカニズムはGRの遺伝子多型、SNIPsに関する研究から解析されてきたが、いまだにその分子基盤は脆弱であり、臨床薬理学的応用可能な成果に乏しい。近年、GRを含む核内レセプターによる遺伝子発現制御は組織あるいは遺伝子選択的なエピジェネティック制御を受ける実態が明らかとなり、GRと標的DNAを含むその周辺分子の解析からグルココルチコイド応答性の理解が進展している。しかし、かかる研究をグルココルチコイド応答性の民族差や人種差に結びつけた研究はない。一方、pregnane X receptor (PXR) は胆汁酸などの内因性生理活性物質や薬剤をリガンドとし、それらの吸収・分配・代謝・排泄を担う臓器である肝臓と小腸に高発現している特異な核内レセプターである。その代表的標的遺伝子には、CYP3A4、CYP7αなどがある。これまで、PXRの遺伝子多型、SNIPsと薬物の有効性、副作用の民族差、人種差に関して臨床薬理学的重要性から多くの研究がなされてきた。しかし、PXRのエピジェネティック制御機構には不明な点が多く、その周辺分子の解析も立ち後れている。そこで、われわれは、核内レセプターの中でも臨床薬理学的に重要なこれらの二つの分子に焦点をあて、転写共役因子を含むそれらの周辺分子の同定、エピジェネティック制御機構の解明、を通じて、これらのリガンドとなる薬剤の薬効と副作用の民族差、人種差を解明する分子基盤を確立することを目的とする。

## B.　研究方法：

1）GR、PXR 機能をエピジェネティックに制御する分子の同定とその機能解析

すでに構築した Flag タグ付き GR、PXR 発現系を bait として GR､PXR 結合タンパク質を質量分析法によって網羅的に同定する。各々に関して、発現プラスミドを作成し、GR、PXR 応答性遺伝子発現に与える影響をトランスフェクション法を用いたレポーターアッセイで検討する。

2） 同定した周辺分子の個体における機能解析
各々の核内レセプターに関して、同定したエピジェネティック制御分子のアデノウイルス発現系構築、遺伝子改変動物作成などによって個体における役割の解析を行う。

（倫理面での配慮）
組み替えDNA実験、動物実験などは法令、施設基準などを遵守して実施した。

C. 研究結果：

Flag-GR、GST-HEXIM1を用いた解析などから、核内タンパクHEXIM1がGRにリガンド非依存性に結合することを見いだした。HEXIM1は脳、肺、肝臓、心筋などにおいて高発現していたが、心筋においては胎生期の右心系圧が高い時期に発現しており、出生後は速やかに消失することがわかった。HEXIM1はnon-coding RNAと通常結合しているが、GRと複合体を形成する際には7SK RNAは関与していなかった。HEXIM1発現アデノウイルスを用いた検討などから、GRとHEXIM1は直接のタンパク-タンパク相互作用によって結合することがわかった。Cre-loxPシステムを用いて臓器特異的HEXIM1トランスジェニックマウス作出系を樹立した。すでに、aMHC-Creマウスとの交配によって心筋特異的HEXIM1トランスジェニックマウスの作成に成功した。かかるマウスは外観上は野生型と区別できない。また、通常の飼育下では寿命も同等であった。今後、グルココルチコイド応答の個体差に関するHEXIM1の意義をかかる動物モデルを用いてさらに究明する。

PXRの抗体・ワクチンセンターファクターとして、すでにSmall heterodimer partner (SHP/NC0B2)、nuclear receptor corepressor 2 (NCoR2/SMRT)、steroid receptor coactivators 1 (SRC1/NCOA1)と2 (SRC2/GRIP1)、nuclear receptor interacting protein 1 (NRIP1/RIP140)、peroxisome proliferator-activated receptor-gamma coactivator (PGC-1)、Forkhead transcription factor FKHR (FOXO1)が知られている。われわれの質量分析法による検討から、やはりこれらのタンパク質がPXRに結合することを確認した。一方、これら以外にも多数のPXR結合タンパク候補を単離した。中でも、既知のタンパク質であるCBP/p300はPXRに直接結合しCYP3A4などの標的遺伝子発現を正に制御することがわかった。なお、今後、分子量60kDa、140 kDaのタンパク質に関しても詳細に究明する。

D. 健康危険情報：
とくになし。

E. 研究発表：


1. Yoshikawa N, Shimizu N, Maruyama T, Sano M, Matsuhashi T, Fukuda K, Kataoka M, Satoh T, Ojima H, Sawai T, Morimoto C, Kuribara A, Hosono O, Tanaka H. Cardiomyocyte-specific overexpression of HEXIM1 prevents right ventricular hypertrophy in hypoxia-induced pulmonary hypertension in mice. **PLoS One**. 2012;7(12):e52522. doi: 10.1371/journal.pone.0052522.
2. Kondo S, Iwata S, Yamada T, Inoue Y, Ichihara H, Kichikawa Y, Katayose T, Souta-Kuribara A, Yamazaki H, Hosono O, Kawasaki H, Tanaka H, Hayashi Y, Sakamoto M, Kamiya K, Dang NH, and Morimoto C. Impact of the integrin signaling adaptor protein NEDD9 on prognosis and metastatic behavior of human lung cancer. **Clin. Cancer Res.**, 2012;18(22):6326-38. doi: 10.1158/1078-0432.CCR-11-2162.


F. 知的財産権の出願・登録状況：
なし。

G. 添付資料
なし。

# Ⅳ. 研究業績

# Changes of Serum Soluble Receptor Activator for Nuclear Factor-κB Ligand after Glucocorticoid Therapy Reflect Regulation of Its Expression by Osteoblasts

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**Context:** Osteoporosis is a serious complication of systemic glucocorticoid therapy. The role of serum soluble receptor activator for nuclear factor-κB ligand (RANKL) in glucocorticoid-induced osteoporosis remains unclear.

**Objective:** The objective of the study was to clarify the influence of serum soluble RANKL on the osteoprotegerin (OPG)/RANKL/receptor activator for nuclear factor-κB system in patients with systemic autoimmune diseases receiving glucocorticoid therapy.

**Patients and Methods:** Sixty patients (40 women) with systemic autoimmune diseases who received initial glucocorticoid therapy with prednisolone (30–60 mg/d) plus bisphosphonate therapy were prospectively enrolled. Serum soluble RANKL and OPG levels were measured at 0, 1, 2, 3, and 4 wk after starting glucocorticoid therapy. The effects of dexamethasone on production of RANKL and OPG mRNA and protein by cultured normal human osteoblasts were evaluated by RT-PCR and ELISA, respectively.

**Results:** The mean serum soluble RANKL level of the patients was unchanged by glucocorticoid therapy. Because the distribution of serum soluble RANKL was bimodal, the patients were stratified into two groups. Serum soluble RANKL decreased significantly in the higher soluble RANKL group (≥0.16 pmol/liter), whereas it increased significantly in the lower soluble RANKL group. The mean serum OPG level of the patients decreased significantly. Bone mineral density increased in the higher soluble RANKL group after starting glucocorticoid therapy, whereas it decreased in the lower soluble RANKL group. In cultures of unstimulated human osteoblasts, RANKL mRNA expression was increased and OPG mRNA was decreased by dexamethasone. Up-regulation of RANKL and OPG mRNA by IL-6 was suppressed by dexamethasone.

**Conclusion:** Serum soluble RANKL might be a useful marker of bone remodeling in patients with systemic autoimmune diseases receiving glucocorticoid therapy. ***(J Clin Endocrinol Metab* 97: E1909–E1917, 2012)**

Glucocorticoids are widely used to treat a variety of diseases, including systemic autoimmune diseases. Although glucocorticoids can improve the outcome for patients with these diseases, various side effects of long-term treatment, such as osteoporosis, have become an important problem (1–4). Receptor activator for nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) have been identified as secreted cytokines that play an important role in regulating bone resorption by osteoclasts (5–8). Glucocorticoids decrease bone density through

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Abbreviations: AOSD, Adult-onset Still's disease; BAP, bone alkaline phosphatase; BMD, bone mineral density; BMI, body mass index; CRP, C-reactive protein; DM, dermatomyositis; NHOst, normal human osteoblast; NTX, N-telopeptide crosslinked of type I collagen; OC, osteocalcin; 25-OHD, 25-hydroxyvitamin D; OPG, osteoprotegerin; PINP, procollagen type I N propeptide; PM, polymyositis; RANKL, receptor activator for nuclear factor-κB ligand; SLE, systemic lupus erythematosus; sRANKL, soluble RANKL; TRACP-5b, tartrate-resistant acid phosphatase isoform 5b; ucOC, undercarboxylated OC.

multiple mechanisms, including inhibition of sex steroid hormones, inhibition of gastrointestinal absorption and renal reabsorption of calcium, promotion of parathyroid hormone secretion, and inhibition of bone formation by suppressing osteoblasts together with stimulation of bone resorption via effects on osteoclasts mediated by changes in the production of RANKL and OPG (1–4). The OPG/RANKL/receptor activator for nuclear factor-κB system is one of the important mechanisms involved in various kind of osteoporosis (9–11). In general, glucocorticoids stimulate RANKL production (12–15) and inhibit OPG production (12–16) *in vitro*, thereby enhancing bone resorption. However, the pathophysiological role of serum soluble RANKL (sRANKL) *in vivo* has not been clarified yet. In the present study, we prospectively measured serum sRANKL and OPG levels before and after the initiation of glucocorticoid therapy in patients with systemic autoimmune diseases. We also performed *in vitro* studies to assess the effects of glucocorticoids on primary cultured human osteoblasts.

## Patients and Methods

### Patients and study protocol

Patients and healthy controls were recruited at Toho University Omori Hospital and the Research Center for Clinical Pharmacology of Kitasato University, respectively. This study was approved by the Ethics Committee at Toho University Omori Medical Center (approval no. 21-61) and at Research Center for Clinical Pharmacology of Kitasato University (approval no. 06104). This was a prospective observational study that involved 60 patients with systemic autoimmune diseases, including 21 patients with systemic lupus erythematosus (SLE), 15 patients with polymyositis (PM)/dermatomyositis (DM), 19 patients with vasculitis syndrome, and five patients with adult-onset Still's disease (AOSD). Patients who were starting treatment with prednisolone at doses from 30 to 60 mg daily [mean daily dose: 45.2 ± 1.9 mg (SEM)] based on our standard therapeutic regimen were eligible for this study. All patients gave written informed consent before enrollment. None of them had received any treatment for their diseases at the time of enrollment.

In guidelines on the management and treatment of glucocorticoid-induced osteoporosis of the Japanese Society for Bone and Mineral Research, a bisphosphonate was recommended as a first-line drug for prevention of glucocorticoid-induced osteoporosis (17). We then adopted administration of a bisphosphonate (alendronate 35 mg/wk or risedronate 17.5 mg/wk) as a supplemental drug for our regimen of high-dose glucocorticoid therapy. This regimen was used for all patients in this study. None of the patients received supplemental calcium and vitamin D during this study.

Fasting morning blood samples were collected prospectively just before the patients started treatment and after 1, 2, 3, and 4 wk of glucocorticoid therapy. Serum samples were immediately frozen at −80 C until the measurement of markers of bone metabolism. The healthy control subjects were matched for sex, menopausal status, and age (±5 yr). Serum samples were immediately frozen at −80 C until assay.

### Serum biochemical markers

As inflammation markers, serum levels of C-reactive protein (CRP; Sekisui Medical, Tokyo, Japan) and IL-6 (Fujirebio Inc., Tokyo, Japan) were measured by the latex-enhanced nephelometric method and enzyme immunoassay, respectively. Serum 25-hydroxyvitamin D (25-OHD; Diasorin Inc., Stillwater, MN) was measured by RIA. Serum levels of soluble RANKL (sRANKL; Biomedica, Vienna, Austria) and OPG (Biomedica) were determined by ELISA. As bone formation markers, serum levels of osteocalcin (OC; Mitsubishi Kagaku Bioclinical Laboratories, Tokyo, Japan) and procollagen type I N-terminal peptide (PINP; Orion Diagnostica, Espoo, Finland) were determined by immunoradiometric assay. The serum level of undercarboxylated OC (ucOC; Sanko Junyaku Co., Ltd., Tokyo, Japan) was measured by an electrochemiluminescence immunoassay and bone alkaline phosphatase (BAP; Quidel, San Diego, CA) was measured by an enzyme immunoassay. As bone resorption markers, serum levels of the N-telopeptide crosslinked of type I collagen (NTX; Inverness, Princeton, NJ) and tartrate-resistant acid phosphatase isoform 5b (TRACP-5b; DS Pharma Biomedical Co., Ltd., Tokyo, Japan) were measured by enzyme immunoassay.

### Measurement of bone mineral density (BMD)

Before starting glucocorticoid therapy, the BMD of the lumbar spine (L2–4) was measured by dual-energy X-ray absorptiometry using Discovery A (Hologic, Waltham, MA), being automatically calculated from the bone area (square centimeters) and bone mineral content (grams) and was expressed in grams per square centimeters. BMD was measured again after 15 ± 4 months of glucocorticoid treatment and the percent change from baseline was calculated.

### Cell culture

Normal human osteoblasts (NHOst) were obtained from Lonza Inc. (Williamsport, PA) and were maintained at 37 C in osteoblast growth medium (Lonza) supplemented with 10% fetal bovine serum, 50 μg/ml gentamycin sulfate, 2.5 μg/ml amphotericin-B, and 50 mM L-ascorbic acid under a humidified atmosphere of 5% $CO_2$ in air. NHOst were resuspended in 5 ml of osteoblast growth medium supplemented with 1% (vol/vol) fetal bovine serum at $1.0 \times 10^5$ cells/ml and then cultured for 24 h in 35-mm dishes.

### Measurement of RANKL and OPG production

To evaluate the effect of dexamethasone on RANKL and OPG production, NHOst were incubated with or without 100 ng/ml IL-6 (R&D Systems Inc., Minneapolis, MN) plus 100 ng/ml soluble IL-6 receptor (sIL-6R; R&D Systems) for 24 h and then were coincubated with dexamethasone (0, 10, 100, or 1000 nM). Culture supernatants were collected, centrifuged, and stored at −80 C for subsequent analysis. RANKL and OPG concentrations in the culture supernatants were measured in duplicate by ELISA (Biomedica) according to the instructions of the manufacturer.

**TABLE 1.** Demographics and clinical data of the study population at baseline

| | Patients (n = 60) | Healthy controls (n = 60) |
|---|---|---|
| Age (yr) | 55.2 ± 2.7 | 55.2 ± 1.5 |
| Number of males/females | 20/40 | 20/40 |
| Postmenopausal women (%) | 23 (57.5) | 23 (57.5) |
| BMI (kg/m$^2$) | 21.7 ± 0.6 | 22.1 ± 2.6 |
| BMD (g/cm$^2$) | 0.96 ± 0.03 | 0.92 ± 0.02 |
| Serum markers | | |
| CRP (mg/dl) | 4.11 ± 0.61[a] | 0.05 ± 0.00 |
| | 2.15 [0.30–7.05][b] | 0.05 [0.04–0.07] |
| IL-6 (pg/ml) | 34.4 ± 5.7[a] | 2.2 ± 0.2 |
| | 17.2 [8.7–37.4][b] | 1.8 [1.4–2.5] |
| 25-OHD (ng/ml) | 16.8 ± 1.1[a] | 26.2 ± 0.9 |
| | 16.0 [10.0–21.0][b] | 26.0 [22.0–32.0] |
| OC (ng/ml) | 3.30 ± 0.49[a] | 6.18 ± 0.31 |
| | 2.35 [1.35–3.68][b] | 5.70 [4.35–7.68] |
| ucOC (ng/ml) | 1.56 ± 0.20[a] | 3.84 ± 0.28 |
| | 1.01 [0.55–2.31][b] | 3.48 [2.31–4.86] |
| BAP (U/liter) | 16.0 ± 0.8 | 20.8 ± 1.0 |
| | 14.3 [11.5–19.3] | 19.4 [14.3–24.4] |
| PINP ($\mu$g/liter) | 44.5 ± 3.4 | 54.7 ± 2.6 |
| | 37.6 [27.1–51.9] | 55.2 [39.8–70.0] |
| NTX (nmolBCE/liter) | 15.1 ± 0.6 | 14.6 ± 0.9 |
| | 14.7 [11.9–16.7] | 13.6 [11.3–17.6] |
| TRACP-5b (mU/dl) | 198.7 ± 12.6 | 182.7 ± 10.8 |
| | 183 [136–227] | 178 [121–244] |

Data are mean ± SEM and median [25th to 75th percentile range].

[a] $P < 0.05$ for the patients compared with healthy controls by Student's $t$ test.

[b] $P < 0.05$ for the patients compared with healthy controls by Mann-Whitney $U$ test.

### Assessment of RANKL and OPG gene expression

NHOst were cultured under various conditions in medium containing 1% (vol/vol) fetal bovine serum, and RNA was extracted by using an RNeasy minikit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed with a SuperScript first-strand synthesis system (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions, using 1 $\mu$g total cellular RNA as the template. Equal amounts of each reverse-transcribed product were amplified by PCR with HotStarTaq polymerase (QIAGEN). The sequences of the primers were as follows: RANKL, sense 5′-GCCAGTGGGAGATGTTAG-3′ and antisense 5′-TTAGCTGCAAGTTTTCCC-3′; OPG, sense 5′-GCTAACCTCACCTTCGAG-3′, and antisense 5′-TGATTGGACCTGGTTACC-3′; $\beta$-actin (endogenous control), sense 5′-CCTCGCCTTTGCCGATCC-3′, and antisense 5′-GGATCT TCATGAGGTAGTCAGTC-3′. The RANKL to $\beta$-actin and OPG to $\beta$-actin ratios were determined for semiquantification of mRNA expression. PCR for amplification of RANKL cDNA (486 bp) involved 40 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec, whereas 30 cycles under the same conditions were used to amplify OPG cDNA (324 bp). PCR for $\beta$-actin cDNA (626 bp) was performed with 28 cycles of 95 C for 30 sec, 56 C for 30 sec, and 72 C for 30 sec. The amplified cDNA fragments were resolved by electrophoresis on 2% (wt/vol) agarose gel and were detected under UV light using an LAS-3000 (Fujifilm Corp., Tokyo, Japan) after staining of the gel with ethidium bromide.

### Statistical analysis

Statistical analysis was performed with Prism version 5.0 software (GraphPad Software, San Diego, CA). Numerical data were expressed as both the mean ± SEM and the median with the interquartile range. An assessment of the changes during glucocorticoid treatment was performed with a Friedman's test followed by a Dunnett's multiple comparison test. To compare two groups, the Mann-Whitney $U$ test or Student's $t$ test was applied for numerical data and the $\chi^2$ test (or Fisher's exact test) was used for categorical data. Multiple comparisons were performed by the Kruskal-Wallis test. The level of significance was set at $P < 0.05$.

## Results

### Profile of the subjects

Table 1 shows the demographic and clinical data of the patients and healthy controls. There were no significant differences of body mass index (BMI) and BMD between the patients and the matched controls. As shown in Table 1, serum CRP and IL-6 levels were significantly increased, whereas the serum 25-OHD level was significantly decreased in the patients with systemic autoimmune diseases before glucocorticoid therapy when compared with those in the healthy controls. Nine of 60 patients were withdrawn during 15 months due to death (n = 5) or hospital transfer (n = 4). Fifty-one patients continued to receive prednisolone at 15 months except for a patient with AOSD. The mean daily and cumulative doses of prednisolone during 15 months were 9.5 ± 4.0 (SEM) and 9296 ± 372 mg, respectively. There were no significant

differences in the mean initial, daily, and cumulative doses of prednisolone between the higher and lower sRANKL groups (Table 2).

### Serum sRANKL, OPG, and inflammatory markers

As shown in Fig. 1A, the serum sRANKL level of the patients with systemic autoimmune diseases before glucocorticoid therapy was significantly higher than that of the healthy controls. The mean serum sRANKL level of the patients was unchanged by glucocorticoid therapy (Fig. 1B). There was no significant difference in serum sRANKL level [median (25th to 75th percentile range)] [SLE, 0.034 (0.000–0.186), PM/DM, 0.003 (0.000–0.085), vasculitis syndrome, 0.001 (0.000–0.178), and AOSD, 0.229 (0.094–0.366) pmol/liter, Kruskal-Wallis test, $P = 0.2378$] among these systemic autoimmune diseases. As shown in Fig. 1C, the serum OPG level of the patients before glucocorticoid therapy was significantly higher than that of the healthy controls, whereas the mean serum OPG level of the patients decreased significantly after starting glucocorticoid therapy (Fig. 1D). There was no significant difference in serum OPG level [SLE, 5.23 (3.90–6.50), PM/DM, 4.59 (4.38–5.61), vasculitis syndrome, 5.83 (4.48–9.31), and AOSD, 5.30 (3.80–3.73) pmol/liter, $P = 0.2413$] among these systemic autoimmune diseases.

As shown in Fig. 1 (E and F), the mean serum CRP and IL-6 levels of the patients were significantly decreased by glucocorticoid therapy. There were significant differences in the serum levels of CRP [SLE, 1.4 (0.2–2.9), PM/DM, 0.3 (0.1–1.5), vasculitis syndrome, 7.2 (3.1–12.2), and AOSD, 7.9 (0.6–14.4) mg/dl, $P < 0.0001$] and IL-6 [SLE, 12.0 (7.3–21.6), PM/DM, 11.0 (6.4–17.4), vasculitis syndrome, 46.1 (18.0–121.0), and AOSD, 26.2 (18.5–147.1) pg/ml, $P < 0.0005$] among these diseases at baseline.

**TABLE 2.** Demographics and clinical data of higher and lower sRANKL groups

| | Higher sRANKL group (n = 20) | Lower sRANKL group (n = 40) |
|---|---|---|
| Age (yr) | 53.2 ± 4.2 | 56.2 ± 2.7 |
| Number of males/females | 6/14 | 14/26 |
| Postmenopausal women (%) | 7 (50.0) | 16 (61.5) |
| BMI (kg/m$^2$) | 20.6 ± 0.7 | 22.3 ± 0.7 |
| BMD (g/cm$^2$) | 0.96 ± 0.04 | 0.95 ± 0.03 |
| Systemic autoimmune disease | | |
| SLE | 8/21 (38%) | 13/21 (62%) |
| PM/DM | 1/15 (7%) | 14/15 (93%) |
| Vasculitis syndrome | 7/19 (37%) | 12/19 (63%) |
| AOSD | 4/5 (80%) | 1/5 (20%) |
| Serum markers | | |
| CRP (mg/dl) | 5.62 ± 1.03[a] | 3.91 ± 0.93 |
| | 5.10 [2.20–7.75][b] | 1.55 [0.23–4.15] |
| IL-6 (pg/ml) | 43.2 ± 12.2 | 31.1 ± 6.5 |
| | 24.5 [9.9–66.1] | 15.8 [7.3–3.4] |
| sRANKL (pmol/liter) | 0.434 ± 0.099[a] | 0.018 ± 0.005 |
| | 0.251 [0.178–0.479][b] | 0.000 [0.000–0.023] |
| OPG (pmol/liter) | 6.63 ± 0.56 | 5.62 ± 0.34 |
| | 5.95 [4.52–8.21] | 5.15 [4.16–6.83] |
| 25-OHD (ng/ml) | 15.2 ± 1.4 | 17.8 ± 1.4 |
| | 14.5 [10.0–17.8] | 17.0 [10.5–22.0] |
| OC (ng/ml) | 1.93 ± 0.34 | 2.56 ± 0.23 |
| | 1.6 [1.0–2.8] | 2.3 [1.5–3.7] |
| ucOC (ng/ml) | 1.24 ± 0.36 | 1.66 ± 0.23 |
| | 0.73 [0.41–1.35] | 1.18 [0.59–2.49] |
| BAP (U/liter) | 14.6 ± 1.4 | 16.7 ± 1.0 |
| | 12.9 [10.7–14.6] | 15.7 [12.8–20.1] |
| PINP ($\mu$g/liter) | 39.4 ± 4.1 | 47.1 ± 4.6 |
| | 30.6 [25.4–51.7] | 39.2 [29.9–57.0] |
| NTX (nmolBCE/liter) | 14.4 ± 1.0 | 15.4 ± 0.7 |
| | 14.5 [11.4–16.1] | 15.0 [12.0–17.3] |
| TRACP-5b (mU/dl) | 190.9 ± 24.2 | 201.5 ± 13.7 |
| | 181 [103–220] | 185 [137–238] |
| Initial glucocorticoid dose (mg/d) | 45.0 ± 2.0 | 45.8 ± 1.6 |
| Mean glucocorticoid dose (mg/d) at 15 months | 9.0 ± 0.9 | 9.7 ± 0.7 |
| Cumulative glucocorticoid dose (mg) | 9210 ± 566 | 9319 ± 449 |

Data are mean ± SEM and median [25th to 75th percentile range].

[a] $P < 0.05$ for higher sRANKL group compared with lower sRANKL group by Student's *t* test.

[b] $P < 0.05$ for higher sRANKL group compared with lower sRANKL group by Mann-Whitney *U* test.

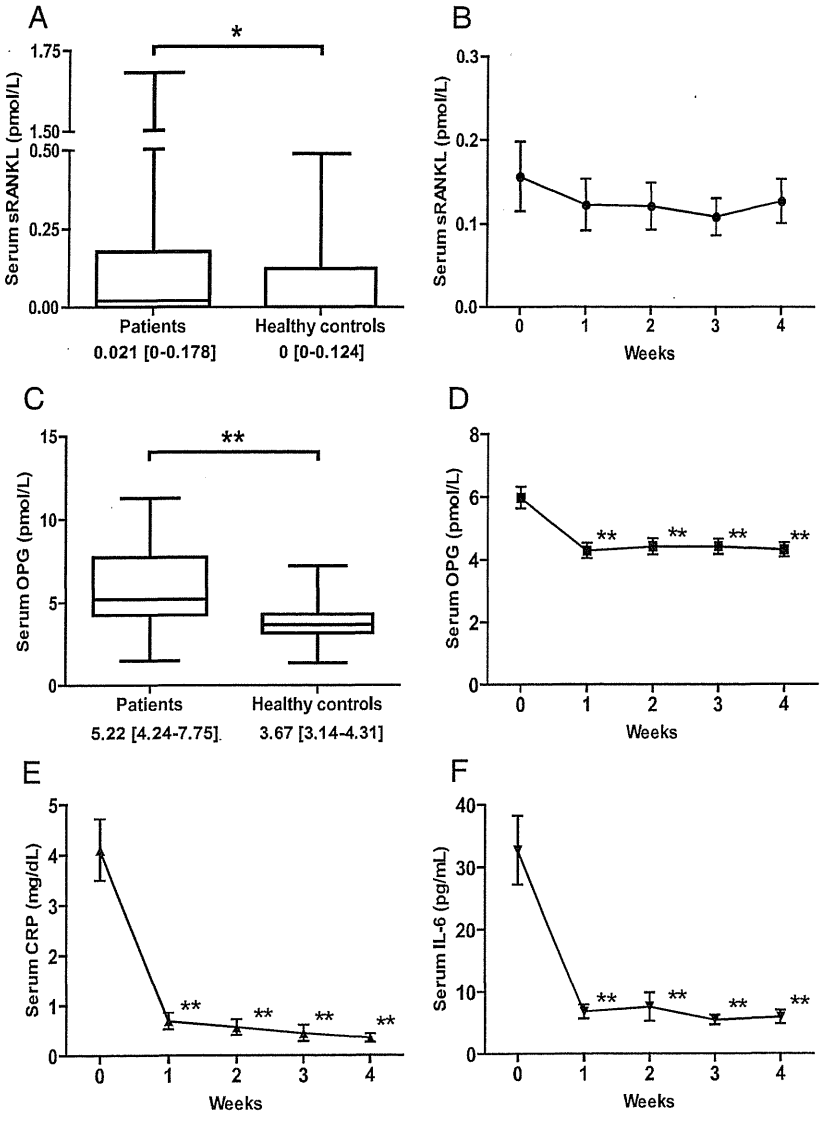


**FIG. 1.** Serum levels of sRANKL (A and B), OPG (C and D), CRP (E), and IL-6 (F) in the patients and healthy controls. Data are expressed as the median with 25th to 75th percentiles in *brackets* (A and C). *, $P < 0.05$; **, $P < 0.0001$ by the Mann-Whitney *U* test. Changes of serum sRANKL (B), OPG (D), CRP (E), and IL-6 (F) during glucocorticoid therapy are shown. Data are expressed as the mean ± SEM (B, D, E, and F). **, $P < 0.0001$ by Friedman's test followed by Dunnett's multiple comparison test.

## Serum sRANKL stratification

Because we found that the distribution of serum sRANKL was bimodal, the patients were stratified into two groups. A cutoff level for the definition of the higher serum sRANKL group was 0.16 pmol/liter. With this definition, the number of the higher sRANKL group was 20 patients, whereas that of the lower sRANKL group was 40 patients, respectively. Table 2 compares the demographic and clinical data of the higher and lower sRANKL groups. There were no significant differences of sex, menopausal status, age, BMI, BMD, serum IL-6, serum 25-OHD, serum OPG, bone formation markers (OC, ucOC, BAP, and PINP), and bone resorption markers (NTX and TRACP-5b) between the higher and lower sRANKL groups. Baseline serum CRP levels were significantly elevated in the higher sRANKL group compared with those in the lower sRANKL group (Table 2). The percentage of patients with SLE and PM/DM in the higher sRANKL group tended to be lower than those patients with vasculitis syndrome and AOSD.

As shown in Fig. 2A, the mean serum sRANKL level of the higher sRANKL group was significantly decreased by glucocorticoid therapy. In contrast, the mean serum sRANKL level of the lower sRANKL group showed a significant increase after the start of glucocorticoid therapy (Fig. 2B).

## Bone turnover markers

As shown in Table 1, the baseline serum OC and ucOC levels of the patients were significantly lower than those of the healthy controls. However, the baseline levels of the other bone formation markers (BAP and PINP) and bone resorption markers (NTX and TRACP-5b) were not significantly different between the patients and healthy controls.

## Bone mineral density

There was no significant difference of baseline in BMD between the higher and lower sRANKL groups (Table 2). As shown in Fig. 3, BMD [median (25th to 75th percentile range)] increased from baseline by 0.93% (−1.23–5.51%) with glucocorticoid therapy in the higher sRANKL group, whereas it decreased by −2.03% (−5.99 to 0.38%) in the lower sRANKL group. The difference in the change of BMD between the two groups was statistically significant ($P < 0.05$). There were no statistically significant differences in the baseline BMD (mean ± SEM, SLE, 1.02 ± 0.05; PM/DM, 0.91 ± 0.04; vasculitis syndrome, 0.94 ± 0.04; AOSD, 0.94 ± 0.18 g/cm$^2$, $P = 0.3744$) and in the changes of BMD [median (25th to 75th percentile range)] [SLE, −2.77 (−6.05 to −0.26), PM/DM, −1.91 (−13.02 to 4.83), vasculitis syndrome, 1.00 (−3.21 to 5.51), and AOSD, −0.82% (−2.09 to 0.45%), $P = 0.2580$] among systemic autoimmune diseases.

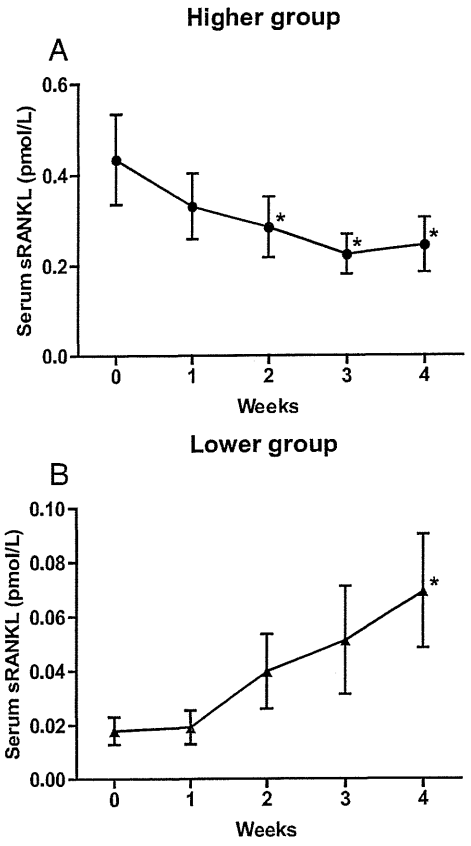


**FIG. 2.** Changes of serum sRANKL in the higher (A) and lower (B) sRANKL groups during glucocorticoid therapy. Data are expressed as the mean ± SEM. *, $P < 0.05$ by Friedman's test followed by Dunnett's multiple comparison test.

### RANKL and OPG mRNA expression

We also investigated the effect of dexamethasone on RANKL and OPG mRNA expression *in vitro*. As shown in Fig. 4 (A and B), RANKL mRNA expression was increased by addition of dexamethasone to cultures of unstimulated NHOst. RANKL mRNA expression was also increased by incubation of these cells with IL-6 plus soluble IL-6 receptor, whereas IL-6-stimulated up-regulation of RANKL mRNA expression was suppressed by the addition of dexamethasone.

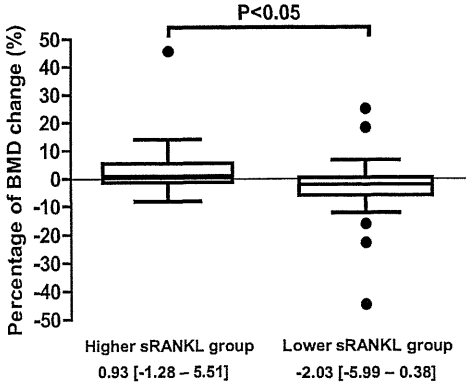


**FIG. 3.** Changes of BMD due to glucocorticoid therapy in the higher and lower sRANKL groups. Data are expressed as the median with the 25th to 75th percentiles in *brackets*. $P < 0.05$ by the Mann-Whitney *U* test.

As shown in Fig. 4 (A and C), OPG mRNA expression was decreased by addition of dexamethasone to unstimulated NHOst, whereas it was increased by incubation with IL-6 plus soluble IL-6 receptor. The IL-6-stimulated up-regulation of OPG mRNA expression was attenuated by dexamethasone.

### RANKL and OPG protein expression

We attempted to measure RANKL protein levels in NHOst culture supernatants by ELISA, but RANKL was not detectable with the assay kit. As shown in Fig. 4D, the OPG protein level in the culture medium of unstimulated NHOst was decreased by incubation with dexamethasone. Secretion of OPG protein was significantly increased by IL-6 plus soluble IL-6 receptor, whereas IL-6-stimulated increase of OPG protein secretion was suppressed by addition of dexamethasone.

## Discussion

In the present study, the mean serum sRANKL level of all 60 patients with systemic autoimmune diseases did not change after the initiation of glucocorticoid therapy. This finding is similar to that reported by von Tirpitz *et al.* (18), who did not detect any significant change of serum sRANKL after 1–12 wk of glucocorticoid therapy in 27 patients with Crohn's disease. In both their subjects and ours, the distribution of baseline serum sRANKL levels was skewed and the range was wide. Unlike von Tirpitz *et al.*, we also carried out a more detailed investigation after dividing the patients into two groups with higher and lower baseline serum sRANKL levels. This revealed a difference in the response to glucocorticoid therapy, with the serum sRANKL level decreasing in the higher sRANKL group and increasing in the lower sRANKL group.

At present, it is not clear whether sRANKL has effects on bone metabolism that are similar to the actions of RANKL *in vitro*. sRANKL is a truncated ectodomain that is released from the cell-bound form by enzymatic cleavage and is a soluble molecule similar to that produced by recombinant technology or that secreted by activated T cells (19). Injection of sRANKL causes a rapid increase of serum calcium levels due to both activation of osteoclasts and enhanced generation of these cells (20). Therefore, it is possible that the changes of serum sRANKL detected in this study had a clinically important influence on bone metabolism.

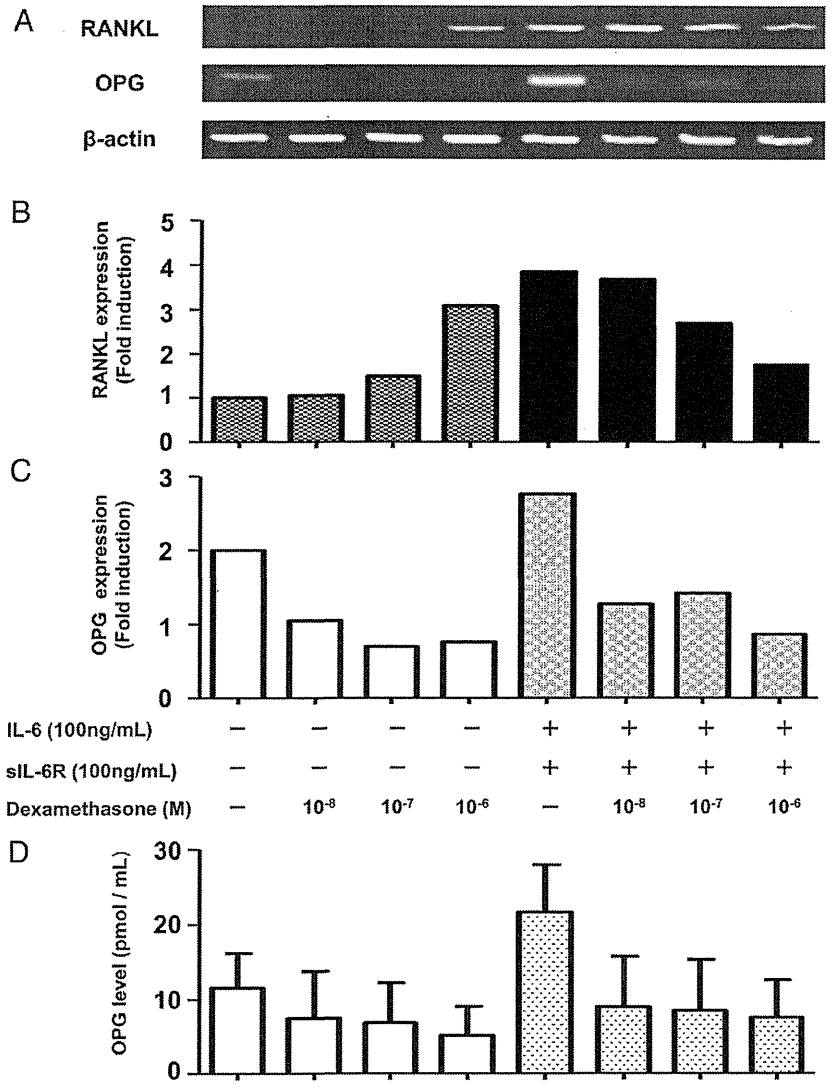


**FIG. 4.** Effect of dexamethasone and IL-6 on RANKL and OPG expression by NHOst. RANKL, OPG, and β-actin mRNA were detected in NHOst by RT-PCR (A). RANKL mRNA expression was normalized by that of β-actin mRNA (B). OPG mRNA expression was normalized by that of β-actin mRNA (C). Detection of OPG protein was by ELISA (D).

Our study showed that BMD increased after the start of glucocorticoid therapy in the higher sRANKL group, whereas it decreased in the lower sRANKL group. From this result and the differing behavior of serum sRANKL in the two groups, the risk of osteoporosis related to glucocorticoid therapy seems to be greater in the lower sRANKL group. Our findings also suggest that the risk of osteoporosis might be predicted by measuring the serum sRANKL before glucocorticoid therapy. All patients in this study received a bisphosphonate. Mechanism of action of bisphosphonate is considered to improve bone resorption by the suppression of osteoclast. All patients should have a favorable result on increasing BMD; however, the changes of BMD were different from each other. We then suggested that the differences in the changes of BMD during glucocorticoid therapy were caused at least in part by the changes of serum sRANKL.

CRP was also significantly higher in the higher sRANKL group compared with that in the lower sRANKL group. It is known that CRP tends to be low in patients with SLE (21) and PM/DM (22), regardless of disease activity. Because patients with SLE or PM/DM dominated the lower sRANKL group, the difference of CRP between the two sRANKL groups might have been due to a difference in the mix of systemic autoimmune diseases. In contrast, serum IL-6 level had a trend to be increased, but the difference was not statistically significant between the two sRANKL groups. It is well established that the elevated serum IL-6 levels in systemic autoimmune diseases may be more useful index for active disease than CRP (23). We showed that no statistically significant difference was observed in the serum sRANKL level among these diseases. The mechanisms of serum sRANKL production and regulation remained to be studied.

Our *in vitro* study demonstrated dual regulation of RANKL mRNA expression by dexamethasone in NHOst with or without IL-6 stimulation. In general, glucocorticoids enhance RANKL mRNA expression by osteoblast-like cancer cell lines (12–15). Regarding the mechanism involved in the increase of RANKL mRNA promoted by dexamethasone, Kondo *et al.* (15) reported that the expression of RANKL mRNA was up-regulated through the glucocorticoid-responsive element half-site or the activator protein-1 site. However, there have been no previous reports about the suppressive effect of dexamethasone on cytokine-stimulated osteoblasts that we detected in this study. We found that RANKL mRNA expression was up-regulated by adding IL-6 to cultures of osteoblasts, which was similar to the report of O'Brien *et al.* (24). We also found that dexamethasone suppressed this up-regulation of RANKL mRNA by IL-6, and these *in vitro* findings corresponded to the results of our clinical study.

The mean serum OPG level of our patients with systemic autoimmune diseases showed a significant decrease after they started glucocorticoid therapy. Similarly, it has been reported that serum OPG levels are significantly de-

creased by glucocorticoid therapy in patients with Crohn's disease (18) or chronic glomerulonephritis (25). Moreover, our *in vitro* studies showed that the expression of OPG mRNA by cultured NHOst was decreased after adding dexamethasone, irrespective of whether the cells received IL-6 stimulation. Because a decrease of OPG promotes osteoclast differentiation and activation, our experimental and clinical data concerning OPG may help to explain the mechanism of glucocorticoid-induced osteoporosis.

In the present study, the serum 25-OHD level of the patients with systemic autoimmune diseases before glucocorticoid therapy was significantly lower than that of the healthy controls. Cutolo (26) reported that serum 25-OHD level was decreased in patients with rheumatoid arthritis and SLE when compared with that in healthy controls. It was suggested that low serum levels of 25-OHD might be partially related to prolonged daily darkness, different genetic background, and nutritional factors in these patients.

We found that serum OC and ucOC levels were already low before the initiation of glucocorticoid therapy in our patients with active systemic autoimmune diseases. Kim *et al.* (27) reported that the serum OC level tended to decrease in healthy subjects who were immobilized for 14 d, so it might be possible that the relative immobility of patients with active disease contributes to their lower serum OC and ucOC levels.

In conclusion, the OPG/RANKL/RANK system seems to have an important role in the mechanism of glucocorticoid-induced osteoporosis, and sRANKL might be a useful marker of bone remodeling in patients with systemic autoimmune disease receiving glucocorticoid therapy.

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Disclosure Summary: All of the authors have no conflicts of interest to declare.

## References

1. Longo DL2012 Osteoporosis: glucocorticoid-induced osteoporosis. In: Fauci A, Kasper D, Hauser S, Jameson J, Loscalzo J, eds. Harrison's principles of internal medicine. 18th ed. New York: McGraw-Hill Medical; 3135
2. Buttgereit F, Straub RH, Wehling M, Burmester GR 2004 Glucocorticoids in the treatment of rheumatic diseases: an update on the mechanisms of action. Arthritis Rheum 50:3408–3417
3. den Uyl D, Bultink IE, Lems WF 2011 Glucocorticoid-induced osteoporosis. Clin Exp Rheumatol 29:S93–S98
4. Maricic M 2011 Update on glucocorticoid-induced osteoporosis. Rheum Dis Clin North Am 37:415–431, vi
5. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ 1999 Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 20:345–357
6. Theill LE, Boyle WJ, Penninger JM 2002 RANK-L and RANK: T cells, bone loss, and mammalian evolution. Annu Rev Immunol 20: 795–823
7. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L 1997 A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 390:175–179
8. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T 1998 Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602
9. Hofbauer LC, Kühne CA, Viereck V 2004 The OPG/RANKL/RANK system in metabolic bone diseases. J Musculoskelet Neuronal Interact 4:268–275
10. Rogers A, Eastell R 2005 Circulating osteoprotegerin and receptor activator for nuclear factor κB ligand: clinical utility in metabolic bone disease assessment. J Clin Endocrinol Metab 90:6323–6331
11. Vega D, Maalouf NM, Sakhaee K 2007 CLINICAL review #: the role of receptor activator of nuclear factor-kappaκB (RANK)/RANK ligand/osteoprotegerin: clinical implications. J Clin Endocrinol Metab 92:4514–4521
12. Hofbauer LC, Gori F, Riggs BL, Lacey DL, Dunstan CR, Spelsberg TC, Khosla S 1999 Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. Endocrinology 140:4382–4389
13. Chung H, Kang YS, Hwang CS, Moon IK, Yim CH, Choi KH, Han KO, Jang HC, Yoon HK, Han IK 2001 Deflazacort increases osteoclast formation in mouse bone marrow culture and the ratio of RANKL/OPG mRNA expression in marrow stromal cells. J Korean Med Sci 16:769–773
14. Humphrey EL, Williams JH, Davie MW, Marshall MJ2006 Effects of dissociated glucocorticoids on OPG and RANKL in osteoblastic cells. Bone 38:652–661
15. Kondo T, Kitazawa R, Yamaguchi A, Kitazawa S 2008 Dexamethasone promotes osteoclastogenesis by inhibiting osteoprotegerin through multiple levels. J Cell Biochem 103:335–345
16. Vidal NO, Brändström H, Jonsson KB, Ohlsson C 1998 Osteoprotegerin mRNA is expressed in primary human osteoblast-like cells: down-regulation by glucocorticoids. J Endocrinol 159:191–195
17. Nawata H, Soen S, Takayanagi R, Tanaka I, Takaoka K, Fukunaga M, Matsumoto T, Suzuki Y, Tanaka H, Fujiwara S, Miki T, Sagawa A, Nishizawa Y, Seino Y; Subcommittee to Study Diagnostic Criteria for Glucocorticoid-Induced Osteoporosis 2005 Guidelines on the management and treatment of glucocorticoid-induced osteoporosis of the Japanese Society for Bone and Mineral Research (2004). J Bone Miner Metab 23:105–109
18. von Tirpitz C, Epp S, Klaus J, Mason R, Hawa G, Brinskelle-Schmal N, Hofbauer LC, Adler G, Kratzer W, Reinshagen M 2003 Effect of

systemic glucocorticoid therapy on bone metabolism and the osteoprotegerin system in patients with active Crohn's disease. Eur J Gastroenterol Hepatol 15:1165–1170
19. **Findlay DM, Atkins GJ** 2011 Relationship between serum RANKL and RANKL in bone. Osteoporos Int 22:2597–2602
20. **Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ** 1998 Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176
21. **Bertouch JV, Roberts-Thompson PJ, Feng PH, Bradley J** 1983 C-reactive protein and serological indices of disease activity in systemic lupus erythematosus. Ann Rheum Dis 42:655–658
22. **Gabay C, Gay-Croisier F, Roux-Lombard P, Meyer O, Maineti C, Guerne PA, Vischer T, Dayer JM** 1994 Elevated serum levels of interleukin-1 receptor antagonist in polymyositis/dermatomyositis. A biologic marker of disease activity with a possible role in the lack of acute-phase protein response. Arthritis Rheum 37:1744–1751
23. **Stuart RA, Littlewood AJ, Maddison PJ, Hall ND** 1995 Elevated serum interleukin-6 levels associated with active disease in systemic connective tissue disorders. Clin Exp Rheumatol 13:17–22
24. **O'Brien CA, Gubrij I, Lin SC, Saylors RL, Manolagas SC** 1999 STAT3 activation in stromal/osteoblastic cells is required for induction of the receptor activator of NF-κB ligand and stimulation of osteoclastogenesis by gp130-utilizing cytokines or interleukin-1 but not 1,25-dihydroxyvitamin D3 or parathyroid hormone. J Biol Chem 274:19301–19308
25. **Sasaki N, Kusano E, Ando Y, Nemoto J, Iimura O, Ito C, Takeda S, Yano K, Tsuda E, Asano Y** 2002 Changes in osteoprotegerin and markers of bone metabolism during glucocorticoid treatment in patients with chronic glomerulonephritis. Bone 30:853–858
26. **Cutolo M** 2009 Vitamin D and autoimmune rheumatic diseases. Rheumatology (Oxford) 48:210–212
27. **Kim H, Iwasaki K, Miyake T, Shiozawa T, Nozaki S, Yajima K** 2003 Changes in bone turnover markers during 14-day 6 degrees head-down bed rest. J Bone Miner Metab 21:311–315

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Inflammation Research

ORIGINAL RESEARCH PAPER

# Tacrolimus down-regulates chemokine expressions on rheumatoid synovial fibroblasts: screening by a DNA microarray

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**Abstract**
*Objective* Although the effects of tacrolimus on T cells are well-known, direct effects on rheumatoid synovial fibroblasts (RSF) remain unclear. We studied the effects of tacrolimus on RSF by a DNA microarray analysis.
*Materials and methods* Tacrolimus and interleukin (IL)-1$\beta$ were added to cultured RSF. Total RNA was prepared from the cells and the gene expression profile was analyzed by a DNA microarray screening system. mRNA expressions influenced by tacrolimus in the screening system were confirmed by real-time PCR. The effects of tacrolimus on nuclear translocation of nuclear factor-$\kappa$B (NF-$\kappa$B) were also examined.
*Results* The mRNA expressions of CCL3, CCL4, and CXCL8 were up-regulated by IL-1$\beta$ and down-regulated by tacrolimus. The levels of these IL-1$\beta$-induced chemokines in culture supernatant were decreased by a therapeutic concentration of tacrolimus. Tumor necrosis factor-$\alpha$ as well as IL-1$\beta$ induced these chemokines, while tacrolimus inhibited their production and mRNA expression. Chemotaxis of polymorphonuclear cells in response to IL-1$\beta$ was also inhibited by tacrolimus. Nuclear translocation of p50 and p65 NF-$\kappa$B in response to IL-1$\beta$ was decreased by tacrolimus.
*Conclusion* IL-1$\beta$-induced chemokine expressions were down-regulated by tacrolimus, suggesting that tacrolimus exerts its anti-inflammatory effect partly through inhibiting chemokine production by RSF.

**Keywords** Tacrolimus · Chemokines · Rheumatoid arthritis · Synovial fibroblasts · DNA microarray

## Introduction

Tacrolimus, a calcineurin inhibitor, is an immunosuppressant that was isolated from *Streptomyces tsukubaensis* [1]. Its main action is the inhibition of calcineurin in T lymphocytes. Calcineurin is a heterodimeric phosphatase consisting of catalytic subunit A and regulatory subunit B. Tacrolimus inhibits the phosphatase activity of calcineurin after forming a complex with intracellular receptor FK506-binding proteins (FKBPs) that results in the inhibition of a transcription factor, nuclear factor of activated T-cells (NFAT), which is involved in the production of cytokines such as interleukin (IL)-2, -3, or -4, tumor necrosis factor-$\alpha$ (TNF$\alpha$), and interferon-$\gamma$ [1, 2]. In clinical practice, tacrolimus has become the most important drug for patients undergoing transplantation [3]. Clinical trials have demonstrated that tacrolimus is also effective for the treatment of rheumatoid arthritis (RA) [4–6], and it was approved as an anti-rheumatic drug in Japan in 2005.

RA is a chronic, inflammatory autoimmune disease of unknown cause. Although there are a variety of systemic

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manifestations, RA is particularly characterized by proliferating synovitis in multiple joints. Migration of leukocytes into synovial tissue is one of the important pathological changes in the rheumatoid joint, where the infiltration and activation of leukocytes results in progressive destruction of cartilage and bone. This inflammatory process is mediated via cytokines that are produced by activated leukocytes and other cells in rheumatoid synovial tissue. Such cytokines, especially TNFα, IL-6, and IL-1, have attracted attention as targets for the treatment of RA [7]. When recruitment of leukocytes into synovial tissues occurs, chemokines play a pivotal role, so a chemokine-targeting strategy could also be useful for RA [8].

As mentioned above, the effects of tacrolimus on T cells are well characterized, but its direct effect on rheumatoid synovial fibroblasts (RSF) remains unclear. In this study, we assessed the effects of tacrolimus on chemokine production by RSF using a DNA microarray.

## Materials and methods

### Reagents

Tacrolimus was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). Recombinant human IL-1β and TNFα were purchased from BD Biosciences (CA, USA). Stock solutions were dissolved in phosphate-buffered saline (PBS) or ethanol when appropriate. The final concentration of ethanol in the experiments was always <0.1 % and control wells contained an equivalent concentration of the vehicle.

### Cell culture

Rheumatoid synovial cells were prepared as described previously [9] from synovial tissues obtained during total knee replacement or hip replacement from patients who fulfilled the 1987 revised criteria for RA [10]. The study protocol was approved by the ethics committee of Toho University (approval number: 19021), and all patients gave written consent to the use of their tissues for this research. Synovial tissue was digested for 2 h with 0.2 % (weight/volume) bacterial collagenase (Immuno-Biological Laboratories, Gunma, Japan) and then was suspended in RPMI 1640 medium with 10 % (volume/volume) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Subsequently, the cells were incubated at 37 °C in 5 % $CO_2$ for several days, and nonadherent cells were removed. The fibroblast-like adherent cells were defined as rheumatoid synovial cells and were used within two passages. Among the adherent cells, neither T cells (CD3+) nor monocyte/macrophages (CD14+) were detected by two-color immunofluorescence and flow cytometry.

### Gene array analysis

RSF were cultured in a 75-$cm^2$ flask (2.5 × $10^6$ cells) for 24 h, after which the cells were incubated with PBS, recombinant human IL-1β (1 ng/ml), or tacrolimus (100 nM) in RPMI 1640 medium containing 1 % FBS for 24 h. Total RNA was prepared from the cells and the gene expression profile of RSF after stimulation with IL-1β and/or tacrolimus was analyzed by Kurabo with a CodeLink inflammatory cytokines expression bioarray system (#CBHU002: GE Healthcare UK Ltd., Buckinghamshire, England). Differences greater than 1.8-fold were considered likely to reflect a significant difference in gene expression, while gene data were excluded if the results were "low quality" or "error".

### PCR

RSF were cultured in a 75-$cm^2$ flask (2.5 × $10^6$ cells) for 24 h, and then were incubated with PBS or recombinant human IL-1β (1 ng/ml) with or without tacrolimus (100 nM) in RPMI 1640 medium containing 1 % FBS for 24 h. Total RNA was extracted from the cells by using an RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). After that, first-strand complementary DNA was synthesized with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions, employing 1 μg of total RNA from the cells as a template. Genomic contamination was excluded by processing samples with the same reagents and equal amounts of RNA. Then an aliquot (1 μg) of each reverse transcription product was amplified by PCR (30 cycles) with HotStarTaq polymerase (Qiagen). The primers for FKBPs, calcineurin Aα and Aβ, and GAPDH were purchased from Sigma and their sequences were as follows: FKBP12 forward: 5′-GCCGCCATGGGAGTGCAGGTGGAAACC-3′, FKBP12 reverse: 5′-TTCCAGTTTTAGAAGCTCCAC-3′, FKBP38 forward: 5′-CCCCCAGCAGCATGGCATCG-3′, FKBP38 reverse: 5′-TGGCGGCCAGGACGAAGTCC-3′, FKBP51 forward: 5′-ACGGAGTCGGCGAGCCCCCG-3′, FKBP51 reverse: 5′-CAATGGCAGCCTGCTCCAAT-3′, FKBP52 forward: 5′-ACGAAGGCGTGCTGAAGGTC-3′, FKBP52 reverse: 5′-TGTGCTTCCTCATTGGAAAA-3′, calcineurin Aα forward: 5′-CGACAGGAAAAAAATTTGCTGGAT-3′, calcineurin Aα reverse: 5′-TTGTTTGGCTTTTCCTGTACATG-3′, calcineurin Aβ forward: 5′-AACCATGATAGAAGTAGAAGCTC-3′, calcineurin Aβ reverse: 5′-CACACACTGCTGGATAGTTATAA-3′, GAPDH forward: 5′-CCACCCATGGCAAATTCCATGGCA-3′, GAPDH reverse: 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. The lengths of the amplified fragments were 329, 738, 859, 847, 673, 572, and 598 bp, respectively.

The amplified cDNA fragments were resolved by electrophoresis on 2 % (w/v) agarose gel, and were

detected under ultraviolet light after staining of the gel with ethidium bromide. In some experiments, gene expression was measured with a TaqMan real-time PCR (Applied Biosystems) using TaqMan probe and primer pairs for IL-8 (NM_000584.2), CCL3 (NM_002983), CCL4 (NM_002984), and $\beta$-actin (NM_001101.3).

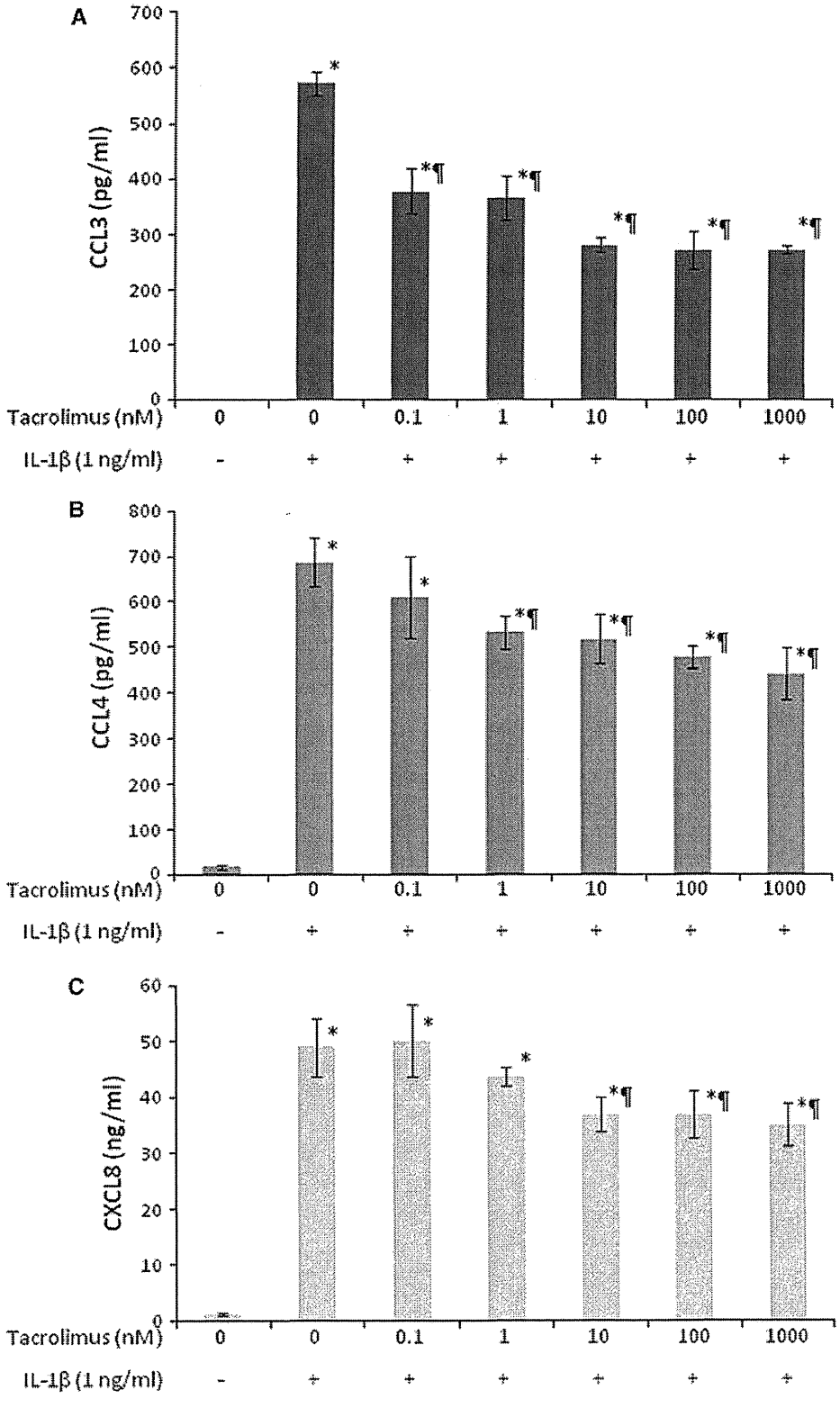


**Fig. 1** Chemokine production is inhibited by tacrolimus. Cells were incubated with IL-1$\beta$ with or without each dose of tacrolimus for 24 h. **a** CCL3, **b** CCL4, and **c** CXCL8 levels in the culture supernatant were measured by ELISA. Experiments were performed in triplicate and all values are the mean ± SD. *$P < 0.05$ versus untreated cells, ¶$P < 0.05$ versus IL-1$\beta$ stimulation

Expression was normalized for $\beta$-actin as an endogenous control.

### Evaluation of chemokine secretion

RSF were cultured for 24 h in 24-well plates at $1 \times 10^5$ cells/well. The cells were then incubated for 24 h with PBS or recombinant human IL-1$\beta$ with or without tacrolimus in RPMI 1640 medium containing 1 % FBS, after which chemokine concentrations in the culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). Each assay was done in triplicate.

### Chemotaxis assay

The study protocol for experimental use of blood samples from healthy donors was approved by the ethics committee of Toho University (approval number: 21008), and all healthy donors gave written informed consent to the use of their blood for this research. Polymorphonuclear cells (PMN) were prepared using Polymorphprep (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions. Briefly, heparinized whole blood (10 ml) was collected from healthy human donors, layered over a density gradient, and spun at 500*g* for 30 min. The PMN-rich fraction was then collected, washed, and suspended in RPMI 1640 medium containing 1 % (v/v) FBS (1-RPMI).

For measurement of PMN migration, a disposable 96-well chemotaxis chamber (ChemoTx®, Neuro Probe, MD, USA) was used according to the manufacturer's instructions, as described previously [11]. In brief, 29 µl of cell culture supernatant, the vehicle, or IL-8 (Chemicon, CA, USA) as a positive control was added to the bottom chamber, and 25 µl of cell suspension with a concentration of $1.0 \times 10^6$ PMN/ml was placed onto a filter with 5 µm pores. After incubation for 1 h at 37 °C in 5 % $CO_2$, cell migration into the lower chamber was assessed by measurement of mitochondrial NADH-dependent dehydrogenase activity with a Cell Counting Kit (Dojindo Molecular Technologies, Kumamoto, Japan) using a sulfonated tetrazolium salt (WST-1: 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt) [12]. Each measurement was done in triplicate. The ratio of directed migration to random migration (D/R ratio) [11] was calculated by dividing the mean fluorescence of PMN that migrated toward an individual chemotactic factor by the mean fluorescence of random PMN migration toward the vehicle (D/R = total fluorescence of wells containing the chemoattractant/total fluorescence of wells containing the vehicle).

### Nuclear extracts and nuclear factor-κB (NF-κB) assay

Extraction of nuclear proteins from cultured RSF was performed according to the instructions of the nuclear extract kit manufacturer (Active Motif, Tokyo, Japan). Nuclear extracts were obtained from RSF treated with PBS or recombinant human IL-1$\beta$ (1 ng/ml) with or without tacrolimus for 3 h. Nuclear translocation of active NF-κB was assessed with an NF-κB Trans-AM™ ELISA (Active Motif) according to the manufacturer's instructions. Raji nuclear extracts were used as the positive control. Two micrograms of nuclear extract was obtained per well and each measurement was done in triplicate.

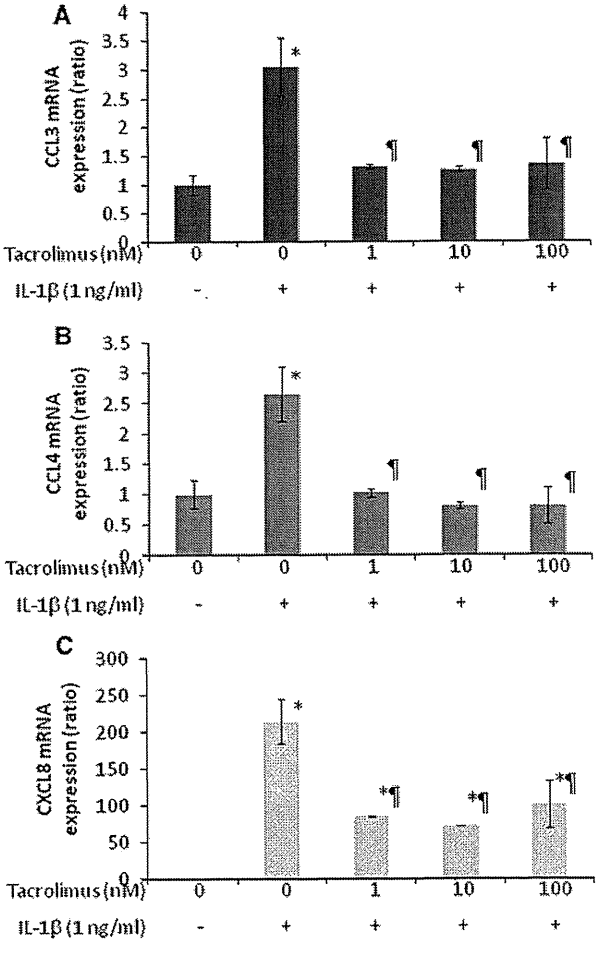


**Fig. 2** Chemokine mRNA expression is inhibited by tacrolimus. Cells were incubated with IL-1$\beta$ with or without each dose of tacrolimus for 24 h. **a** CCL3, **b** CCL4, and **c** CXCL8 mRNA expression were measured by real-time PCR. The results were normalized for $\beta$-actin expression as an endogenous control. Experiments were performed in triplicate and all values are the mean ± SD. $^*P < 0.05$ versus untreated cells, $\P P < 0.05$ versus IL-1$\beta$ stimulation

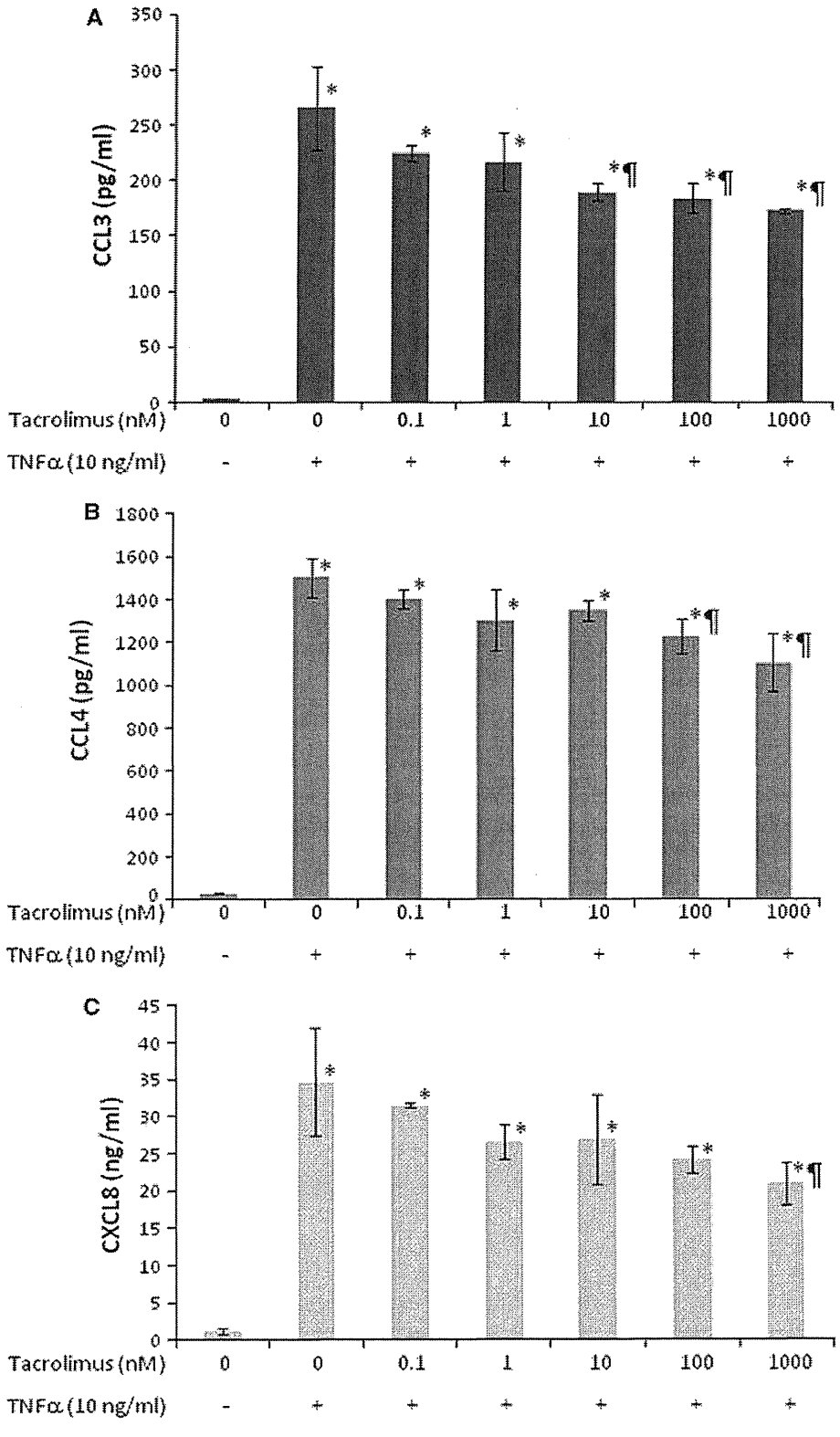


**Fig. 3** Chemokine production is inhibited by tacrolimus. Cells were incubated with TNFα with or without each dose of tacrolimus for 24 h. **a** CCL3, **b** CCL4, and **c** CXCL8 levels in culture supernatant were measured by ELISA. Experiments were performed in triplicate and all values are the mean ± SD. *$P < 0.05$ versus untreated cells, ¶$P < 0.05$ versus TNFα stimulation

Statistical analysis

All experiments were repeated as least three times using RSF obtained from three different patients. Differences between groups were assessed by Tukey's test. Statistical analysis was performed using SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA).

## Results

Screening of chemokines by microarray analysis

To identify the chemokine genes regulated by IL-1$\beta$ and tacrolimus, DNA microarray analysis was employed. Genes were considered for further analysis if they showed at least 1.8-fold up-regulation by IL-1$\beta$ and down-regulation by tacrolimus. IL-1$\beta$ induced a number of pro-inflammatory cytokines, but only three chemokines were inhibited by tacrolimus: CCL3, CCL4, and CXCL8. These were all up-regulated by IL-1$\beta$ (CCL3 by +6.02-fold, CCL4 by +3.59-fold, and CXCL8 by +150-fold) and down-regulated by tacrolimus (CCL3 by −1.82-fold, CCL4 by −2.33-fold, and CXCL8 by −2.23-fold) (Detailed in supplementary figure and table).

Inhibition of chemokine production by tacrolimus

To confirm the result of microarray analysis, ELISA was performed. Untreated cells produced low levels of chemokines and the effect of tacrolimus was not significant (data not shown). In contrast, chemokine production was strongly stimulated by IL-1$\beta$, while tacrolimus inhibited it in a dose-dependent manner (Fig. 1). A therapeutic concentration of tacrolimus (10 nM) potently inhibited the production of chemokines. Real-time PCR also showed that tacrolimus inhibited chemokine production stimulated by IL-1$\beta$ (Fig. 2). Similar results were demonstrated in experiments using TNF$\alpha$ (Figs. 3, 4).

Inhibition of chemotaxis by tacrolimus

We evaluated the chemotactic effect of supernatants of RSF that had been incubated with IL-1$\beta$ with or without tacrolimus on PMN obtained from healthy subjects. Culture supernatant of RSF incubated with IL-1$\beta$ induced significant chemotaxis of human PMN (Fig. 5), while tacrolimus inhibited chemotaxis by PMN in a dose-dependent manner. Similar results were demonstrated in experiments using TNF$\alpha$.

Intracellular signaling

Nuclear translocation of activated NF-$\kappa$B was assessed by ELISA. As shown in Fig. 6, nuclear translocations of p50 and p65 NF-$\kappa$B were increased by IL-1$\beta$. Tacrolimus treatment resulted in inhibition of the nuclear translocations of p50 and p65 induced by IL-1$\beta$. Other NF-$\kappa$B family members, namely p52, RelB, and c-Rel, were not activated by IL-1$\beta$ (data not shown).

Expression of FKBPs and calcineurin A by RSF

We performed RT-PCR to investigate whether FKBPs and calcineurin A$\alpha$ and A$\beta$ were expressed by RSF. Not only the main tacrolimus receptor, FKBP12, but also expression of FKBP38, 51, and 52 mRNA was detected in all RSF investigated (Fig. 7a). RT-PCR demonstrated that RSF expressed both calcineurin A$\alpha$ and A$\beta$, but their expression

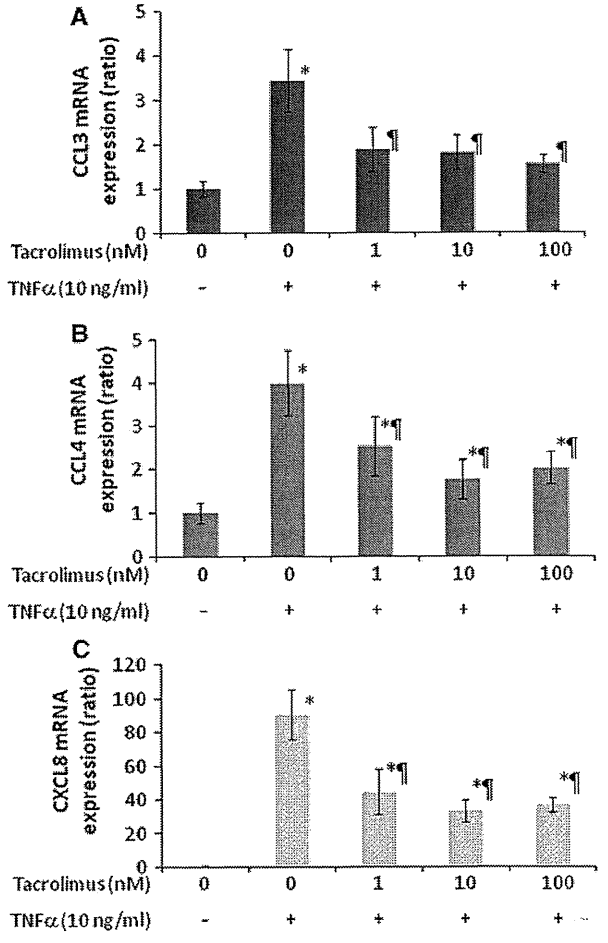


**Fig. 4** Chemokine mRNA expression is inhibited by tacrolimus. Cells were incubated with TNF$\alpha$ with or without each dose of tacrolimus for 24 h. **a** CCL3, **b** CCL4, and **c** CXCL8 mRNA expression was measured by real-time PCR. The results were normalized for $\beta$-actin as an endogenous control. Experiments were performed in triplicate and all values are the mean ± SD. *$P < 0.05$ versus untreated cells, ¶$P < 0.05$ versus TNF$\alpha$ stimulation