

- structure exhibit superior osteoconduction in vivo. *J Biomed Mater Res.* 2002;59:110–7.
15. Suzuki M, Kim T, Tamai H, Fujiyoshi T, Moriya H. Giant geode treated with calcium phosphate cement in a rheumatoid knee. *J Rheumatol.* 2005;32:1846–8.
  16. Bromley M, Woolley DE. Chondroclasts and osteoclasts at subchondral sites of erosion in the rheumatoid joint. *Arthritis Rheum.* 1984;27:968–75.
  17. Gravallesse EM, Harada Y, Wang JT, Gorn AH, Thornhill TS, Goldring SR. Identification of cell types responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis. *Am J Pathol.* 1998;152:943–51.
  18. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature.* 1999;402:304–9.
  19. Gravallesse EM, Manning C, Tsay A, Naito A, Pan C, Amento E, et al. Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. *Arthritis Rheum.* 2000;43:250–8.
  20. van der Heijde D, Landewe R, Klareskog L, Rodriguez-Valverde V, Settas L, Pedersen R, et al. Presentation and analysis of data on radiographic outcome in clinical trials: experience from the TEMPO study. *Arthritis Rheum.* 2005;52:49–60.
  21. Genovese MC, Bathon JM, Fleischmann RM, Moreland LW, Martin RW, Whitmore JB, et al. Long-term safety, efficacy, and radiographic outcome with etanercept treatment in patients with early rheumatoid arthritis. *J Rheumatol.* 2005;32:1232–42.
  22. Schett G. Erosive arthritis. *Arthritis Res Ther.* 2007;9(Suppl 1):S2.

# Equivalent osteoblastic differentiation function of human mesenchymal stem cells from rheumatoid arthritis in comparison with osteoarthritis

Daiki Morimoto<sup>1</sup>, Shoko Kuroda<sup>2</sup>, Takuji Kizawa<sup>1</sup>, Koji Nomura<sup>1</sup>, Chikahisa Higuchi<sup>1</sup>, Hideki Yoshikawa<sup>1</sup> and Tetsuya Tomita<sup>1</sup>

**Objective.** To evaluate the osteoblastic differentiation of human mesenchymal stem cells (hMSCs) in patients with RA.

**Methods.** Heparinized bone marrow aspirate was obtained from patients with OA and RA. Mononuclear cells were cultured for 2 weeks and a colony-forming assay was performed. The phenotype of cells was analysed by flow cytometry. Passage 2 cells were cultured with  $\beta$ -glycerolphosphate (bGP) in the control group and bGP, ascorbic acid and dexamethasone in the differentiation group. After 2 weeks, ALP staining and activity were performed. After 3 weeks, Alizarin Red S assay was performed. Total RNA was extracted from cells cultured for 2 and 3 weeks. Gene expression of bone formation factor was examined by real-time PCR.

**Results.** The phenotype of cells was identical in both OA and RA and the content was thought to be hMSCs. The results of ALP activity and Alizarin Red S assay showed higher levels in the differentiation group for both OA and RA samples compared with the control group. The results of a colony-forming assay were identical in both OA and RA samples. Gene expression in the differentiation group was higher than in the control group in both OA and RA samples. There was no significant difference between OA and RA samples in all experiments.

**Conclusion.** The function of osteoblastic differentiation of hMSCs is similar between OA and RA.

**KEY WORDS:** Rheumatoid arthritis, Human mesenchymal stem cells, Osteoblast, Bone formation.

## Introduction

RA is a chronic inflammatory disease that shows diffuse symmetrical polyarthritis [1]. In RA, progressive joint destruction and general osteoporosis are the characteristic symptoms [2, 3] and patients with RA have a higher risk of fracture [4–6]. Although the aetiology of RA has not been clearly elucidated, it is demonstrated that inflammatory cytokines such as IL-1 and TNF- $\alpha$  and the activation of osteoclasts play an important role in RA [7–10]. In normal bone mineral tissue, bone metabolism is preserved between bone resorption and formation [11–13]. In RA, bone resorption is accelerated and unbalanced with bone formation [14] and although the mechanism of bone resorption by osteoclasts has been evaluated, bone formation has been rarely evaluated, and it remains unclear whether bone formation is accelerated, not changed or attenuated. In previous studies, bone metabolism in patients with RA was evaluated by measuring the biomarkers of serum and urine, and the results suggested that bone resorption was accelerated and bone formation was attenuated in RA [15, 16]. However, in these studies, bone formation was evaluated indirectly.

Bone formation is mainly controlled by the function of osteoblasts. Osteoblasts are differentiated from human mesenchymal stem cells (hMSCs) [17] and it is suggested that hMSCs differentiate into osteoblastic cells followed by the stages of production of bone matrix protein and mineralization [18].

In this study, we obtained hMSCs from patients with OA and RA and evaluated the capability of osteoblastic differentiation of hMSCs.

## Patients and methods

### Patients

All patients in the present study comprised women. In patients with OA, 26 samples were examined and mean patient age was

73.2 years (range 60–83 years); in patients with RA, 20 samples were examined and mean patient age was 58.9 years (range 46–81 years). Patients with RA who had been previously treated with biologics were excluded. Clinical and demographic features of patients with RA are shown in Table 1.

All patients with RA satisfied the 1987 ACR revised diagnostic criteria [19]. All patients with OA were assessed with radiographic images according to the Kellgren and Lawrence classification [20]. All patients with OA satisfied grade III or IV. The written informed consent from all patients was obtained according to the Declaration of Helsinki. This study was conducted under the approval of Osaka University Hospital Ethics Committee.

### Cell culture

Heparinized bone marrow (BM) aspirate was obtained from iliac BM in surgery under general anaesthesia. Heparinized BM aspirates were plated in tissue culture flasks in culture medium [ $\alpha$  MEM,

TABLE 1. Clinical and demographic data of patients with RA and OA

	RA patients (n=20)	OA patients (n=26)
Age, mean $\pm$ s.d. (range), years	58.9 $\pm$ 7.7 (46–81)	73.2 $\pm$ 4.4 (46–81)
Duration of RA, mean (range), years	14.3 (8.8–34.0)	–
RF, mean $\pm$ s.d., IU/ml	139.5 $\pm$ 122.9	–
MMP-3, mean $\pm$ s.d., ng/ml	234.5 $\pm$ 187.1	–
CRP, mean $\pm$ s.d., mg/dl	1.84 $\pm$ 1.73	–
DAS28, mean $\pm$ s.d., score	4.14 $\pm$ 1.03	–
High severity in DAS28 (>5.1), n (%)	5 (25.0)	–
Moderate severity in DAS28 (3.2–5.1), n (%)	13 (65.0)	–
Low severity in DAS28 (<3.2), n (%)	2 (10)	–
Glucocorticoid use, n (%)	15 (75.0)	–
Dosage of glucocorticoid, mean (range), mg/day	5.93 (4–10)	–
DMARD use (including MTX), n (%)	17 (85.0)	–
MTX use, n (%)	11 (55.0)	–
Other DMARD use, n (%)	9 (45.0)	–
Dosage of MTX, mean (range), mg/week	5.64 (2–8)	–

<sup>1</sup>Department of Orthopedics and <sup>2</sup>Department of Clinical Gene Therapy, Osaka University Graduate School of Medicine, Osaka, Japan.

Submitted 17 July 2008; revised version accepted 5 February 2009.

Correspondence to: Tetsuya Tomita, Department of Orthopedics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, 565-0871, Japan. E-mail: tomita@ort.med.osaka-u.ac.jp

Invitrogen, Carlsbad, CA, USA; 15% fetal bovine serum (FBS), Hyclone Laboratories, Logan, UT, USA; 100 U/ml penicillin and 100 µg/ml streptomycin, Invitrogen]. Cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, and medium was changed every other day. In the control group, cells were incubated in culture medium with 10 mM β-glycerophosphate (bGp) (Calbiochem, San Diego, CA, USA). In the differentiation group, cells were incubated in the culture medium with 10 mM bGp, 100 nM dexamethasone (dex) (Sigma, St Louis, MO, USA) and 82 µg/ml ascorbic acid (Wako Pure Chemicals, Osaka, Japan).

Passage 1 adherent cells were used for flow cytometry. Passage 2 adherent cells were used for ALP staining and activity, Alizarin Red S assay and real-time PCR.

#### Flow cytometry

Cells were detached with 0.25% trypsin/2 mM EDTA (Invitrogen), washed with FACS buffer [phosphate-buffered saline (PBS), 3% FBS, 0.1% NaN<sub>3</sub>], and resuspended in 50 µl FACS buffer. The cells were incubated with 10 µl antibody at 4°C in the dark. After 1 h, the cells were washed with FACS buffer and resuspended in 500 µl FACS buffer. FITC-coupled antibodies against CD14, CD34 (Becton Dickinson, San Jose, CA, USA) and CD105 (R&D Systems, Minneapolis, MN, USA) and phycoerythrin (PE)-coupled antibodies against CD29 and CD45 (Becton Dickinson) were used for analysis. As an isotype control, FITC- or PE-coupled non-specific mouse IgG (Becton Dickinson) was substituted for primary antibody.

In each analysis, cell fluorescence was evaluated using a FACS-Calibur instrument (Becton Dickinson) and data were analysed using Cellquest software (Becton Dickinson). Results were expressed as mean percentage of total positive cells.

#### Colony-forming assay

Mononuclear cells (MNCs) were separated by Ficoll-Paque Plus (Amersham Biosciences, Stockholm, Sweden) density gradient centrifugation from heparinized BM aspirates. MNCs from OA and RA (*n*=8, respectively) were recovered from the buffy coat and washed twice with ice-cold PBS. MNCs were plated at a density of  $1 \times 10^6$  cells/dish in 10-cm culture dishes and incubated in culture medium. Medium was changed every other day. After 2 weeks, ALP staining was performed as described below and these colony-forming units (CFU-ALPs) were counted as ALP-positive colonies. The plates were subsequently stained with crystal violet (Wako Pure Chemicals) and the fibroblasts CFU (CFU-F) were counted as crystal violet-positive colonies. The number of colonies larger than 5 mm was counted in CFU-F and CFU-ALP samples. The ratio of CFU-ALP to CFU-F was calculated in each sample.

#### ALP staining and activity

Cells were plated at a density of  $1 \times 10^4$  cells/well in 24-well plates. Cells were divided into the control and the differentiation groups (OA, *n*=13; RA, *n*=10). After 2 weeks, for ALP staining, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at 4°C. Cells were washed with 0.056 M 2-amino-2-methylpropanol (AMP; Sigma) buffer and stained by adding substrate solution (0.5 mg naphthol AS-MX phosphate and 0.5 mg Fast Red Violet LB salt/ml 0.056 M AMP buffer), and the reaction was stopped with TE buffer. For ALP activity, cells were washed with PBS and incubated with NP-40 lysis buffer (0.2% NP-40, 50 mM Tris-HCl, 1 mM MgCl<sub>2</sub>, pH 8.0) for 5 min. Cells were harvested and sonicated. ALP activity was then measured with a commercial Alkaline Phosphatase B test kit (Wako Pure Chemicals) according to the manufacturer's protocol. In all samples, measurements were performed at least in triplicate and data were calibrated by DNA quantity using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA).

#### Alizarin Red S staining and mineralization assay

Cells were incubated by the same method as used for measuring ALP activity (OA, *n*=8; RA, *n*=7). After 3 weeks, cells were washed and fixed with 4% paraformaldehyde for 10 min at 4°C. Cells were washed with PBS and stained with 1% Alizarin Red S solution for 1 h. Then cells were washed with distilled water and incubated with 100 mM cetyl pyridium chloride with shaking on a plate rotator [21]. The extracted supernatant was collected and the optical density was measured at a wavelength of 570 nm against a reference wavelength of 620 nm. In each sample, measurements were performed at least in triplicate and data were calibrated by DNA quantity using a DNeasy Tissue Kit.

#### RNA extraction

Cells were plated at a density of  $2.5 \times 10^5$  cells/dish in 10-cm culture dishes and incubated in culture medium with 10 mM bGp, 100 nM dex and 82 µg/ml ascorbic acid (OA, *n*=8; RA, *n*=7). After 2 and 3 weeks, total RNA was extracted by TRIzol reagent (Invitrogen) according to the manufacturer's protocol.

#### Real time RT-PCR analysis

The cDNA was synthesized from 1 µg of total RNA with a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). Taqman quantitative real-time PCR amplification was performed in triplicate in 384-well optical plates on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A threshold cycle (Ct value) was obtained from each amplification curve using software (Applied Biosystems). Data were expressed as fold changes using the ΔΔCt method as described in the manufacturer's guidelines (Applied Biosystems). A ΔCt value was first calculated by subtracting the Ct values for 18S ribosomal RNA from the Ct values from each sample. A ΔΔCt value was calculated by subtracting the ΔCt value of the lowest samples from each group. Fold changes were determined by raising two to the ΔΔCt power. Taqman Gene Expression Assays for pre-made Taqman probe and primer sets (Applied Biosystems) were used for real-time PCR [Primers: ALP, Hs00758162\_m1; bone sialoprotein (BSP), Hs00173720\_m1; type 1A collagen (COL1A), Hs00164004\_m1; osteocalcin (OC), Hs01587813\_g1; osteopontin (OPN), Hs00167093\_m1; Runt-related transcriptional factor 2 (Runx2), Hs00231692\_m1].

#### Statistical analysis

Data were expressed as mean ± s.d. The Mann-Whitney U-test was used for statistical analysis. A *P*-value <0.05 was considered to be statistically significant.

## Results

#### Flow cytometry

To clarify whether the content of cells was identical between OA and RA and whether the cells were hMSCs, the phenotype of cells was analysed by flow cytometry.

The results of CD14, CD34 and CD45 were almost completely negative (Fig. 1A–C) and the results of CD29 and 105 were positive in both OA and RA samples (Fig. 1D and E). Mean percentage in CD14, CD34 and CD45 was low (CD14:  $1.43 \pm 0.40\%$  in OA and  $1.1 \pm 0.21\%$  in RA; CD34:  $0.74 \pm 0.09\%$  in OA and  $0.89 \pm 0.03\%$  in RA; CD45:  $1.08 \pm 0.28\%$  in OA and  $1.15 \pm 0.16\%$  in RA) and mean percentage in CD29 and CD105 was high (CD29:  $95.36 \pm 1.40\%$  in OA and  $97.56 \pm 1.95\%$  in RA; CD105:  $96.83 \pm 4.79\%$  in OA and  $96.01 \pm 6.51\%$  in RA) (Fig. 1F).

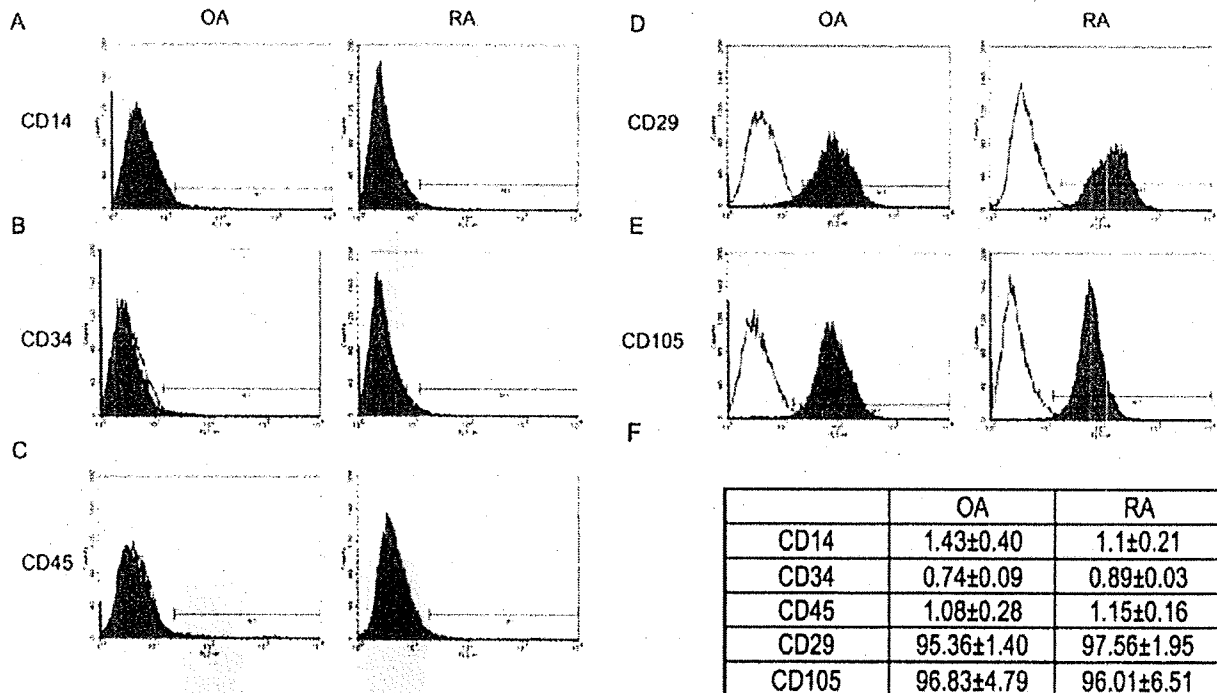


FIG. 1. The phenotype of cells in OA and RA. The phenotype of passage 1 cells was analysed by flow cytometry in OA and RA. The results of haematopoietic markers CD14, CD34 and CD45 had almost completely negative expression (A, B, C) and the results of the representative hMSCs markers CD29 and CD105 had positive expression (D, E). The mean percentage of all positive cells from three independent experiments is shown (F).

#### Colony-forming assay

To examine whether the colony-forming ability of MNCs showed a difference between OA and RA, a colony-forming assay was performed. In CFU-F, the number of positive colonies was  $52.1 \pm 30.5$  in OA and  $44.2 \pm 20.8$  in RA ( $P=0.713$ ). In CFU-ALP, the number of positive colonies was  $39.4 \pm 24.3$  in OA and  $30.6 \pm 13.4$  in RA ( $P=0.400$ ) (Fig. 2A). The ratio of CFU-ALP to CFU-F was  $75.1 \pm 16.2\%$  in OA and  $74.9 \pm 21.9\%$  in RA ( $P=0.793$ ) (Fig. 2B). There was no significant difference in CFU-F and CFU-ALP and the ratio of CFU-ALP to CFU-F between OA and RA.

#### ALP staining and activity

To examine how hMSCs are differentiated into osteoblastic cells and produce bone matrix protein, ALP staining and activity were performed. In ALP staining, cells were strongly positive in the differentiation group (Fig. 3A). In ALP activity, mean value was  $100.2 \pm 54.7$  IU/l/ $\mu$ g in OA and  $72.6 \pm 32.3$  IU/l/ $\mu$ g in RA in the control group and  $266.8 \pm 177.8$  IU/l/ $\mu$ g in OA and  $307.3 \pm 188.1$  IU/l/ $\mu$ g in RA in the differentiation group (Fig. 3B). There was no significant difference between OA and RA ( $P=0.137$  in the control group and  $P=0.664$  in the differentiation group).

#### Alizarin Red S staining and mineralization assay

Alizarin Red S assay expresses calcium deposition of cells and indicates the extent of mineralization. In Alizarin Red S staining, cells were rarely positive in the control group and were strongly positive in the differentiation group (Fig. 4A).

In the Alizarin Red S mineralization assay, the mean value is  $0.044 \pm 0.023$  U/ $\mu$ g in OA and  $0.059 \pm 0.06$  U/ $\mu$ g in RA in the control group and  $0.388 \pm 0.212$  U/ $\mu$ g in OA and  $0.227 \pm 0.165$  U/ $\mu$ g in RA in the differentiation group. There was no significant difference between OA and RA ( $P=0.817$  in the control,  $P=0.094$  in differentiation) (Fig. 4B).

#### Real-time RT-PCR

To examine the gene expression of cells at the stages of production of bone matrix protein and mineralization in the pathway differentiating hMSCs into osteoblasts, real-time PCR was performed. Gene expression of ALP, BSP, COL1A, OPN, OC and Runx2 mRNA was evaluated by real-time PCR (Fig. 5). Gene expression of ALP, OPN and COL1A at 2 and 3 weeks showed no significant difference between OA and RA. At 3 weeks, gene expression of BSP, OC and Runx2 was significantly higher than at 2 weeks but showed no significant difference between OA and RA. In OA, gene expression of Runx2 at 3 weeks was significantly higher than that at 2 weeks. In RA, gene expressions of BSP, OC and Runx2 at 3 weeks were significantly higher than at 2 weeks.

#### Discussion

In OA and RA, the haematopoietic cell surface antigens such as CD14, CD34 and CD45 were almost completely negatively expressed and the representative hMSC cell surface antigens such as CD29 and CD105 were positively expressed in flow cytometry analysis. These results indicate that the content of cells was identical in OA and RA and the phenotype of cells is thought to be hMSCs [22-24].

In a previous study, it was suggested that the absolute number of MNCs in iliac BM was increased in patients with RA and the ratio of each MNC fraction in iliac BM showed no significant difference between patients with RA compared with the non-RA controls [25]. In this study, the result of colony-forming assay showed no significant difference between OA and RA. This result suggests that the content of MNCs and the ratio of cells in RA, which are differentiated into osteoblastic cells, were not different from those in OA and this is consistent with the result of the previous study.

ALP is an early marker of osteoblast differentiation. COL1A and OPN play an important role for bone formation as bone

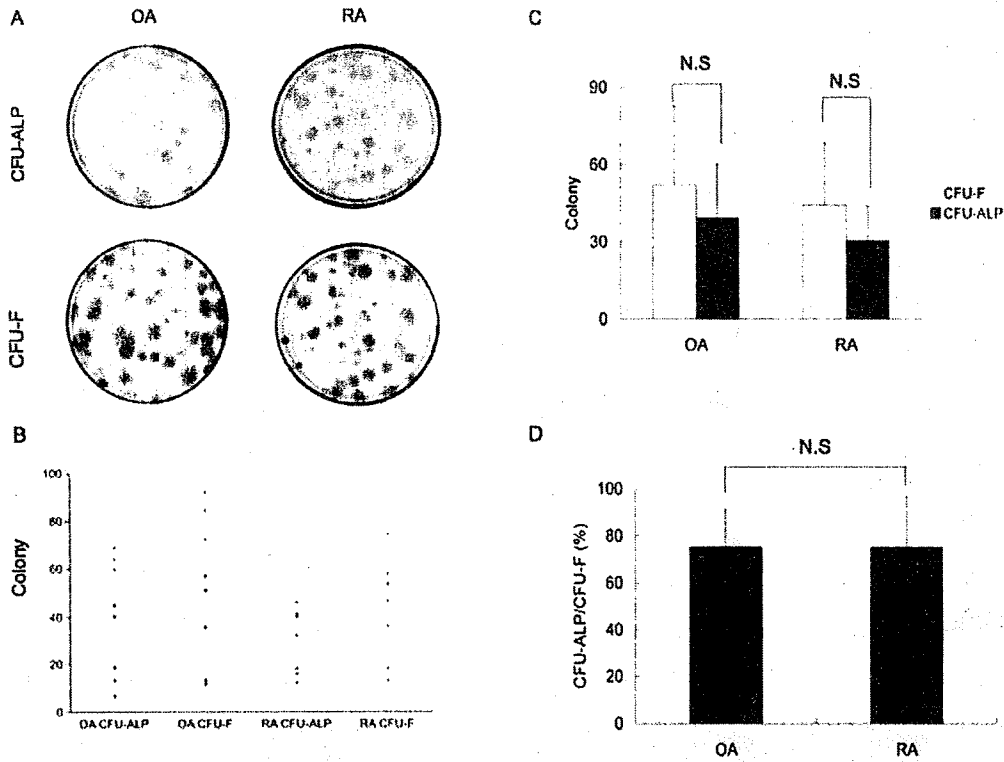


Fig. 2. CFU assay. Mononuclear cells were incubated in culture medium for 2 weeks. In CFU-F, colonies were stained with crystal violet and in CFU-ALP colonies were stained with ALP. The number of colonies with the diameter >5mm was counted. ALP staining and subsequent crystal violet staining in OA and RA are shown (A). The result of CFU-ALP indicated that ALP activity was recognized both in OA and RA. The number of colonies of CFU-F and CFU-ALP in OA and RA was displayed with single dots (B) and columns with s.d.-values (C). The number of colonies of CFU-F and CFU-ALP showed no significant difference between OA and RA. The ratio of CFU-ALP to CFU-F showed no significant difference between OA and RA (D).

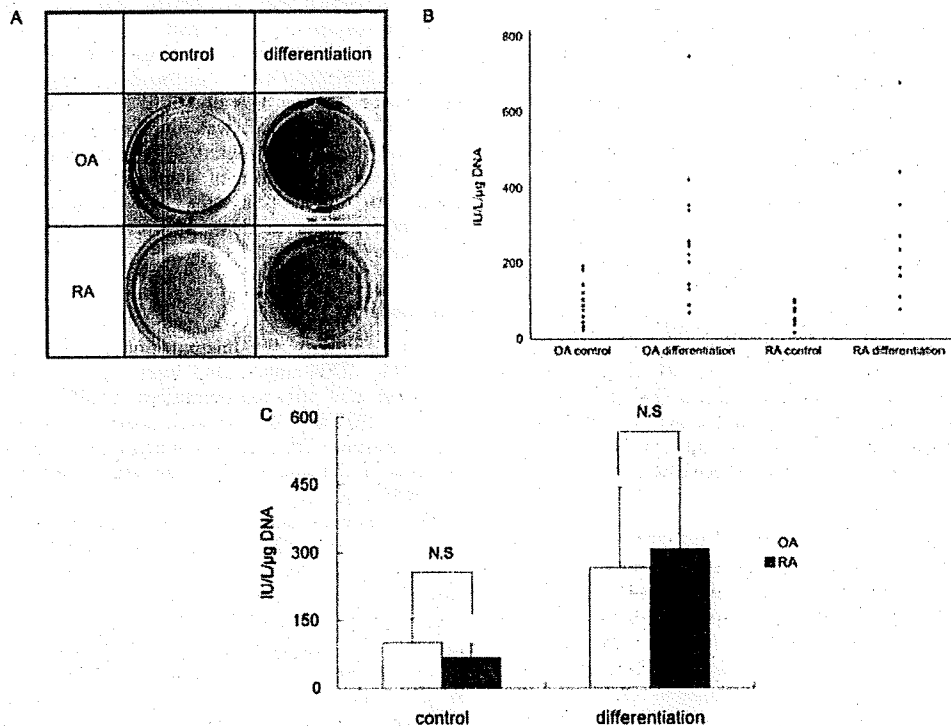


Fig. 3. ALP staining and activity. After incubation for 2 weeks, ALP staining was performed (A) and ALP activity was measured by the pNp method. The result of ALP activity in the control and differentiation group both in OA and RA was displayed with single dots (B) and with columns and s.d.-values (C). ALP activity increased significantly in the differentiation group compared with the activity in the control group in both OA and RA. There was no significant difference between OA and RA in the control and differentiation groups. All samples were performed in triplicate and data were calibrated by DNA quantity.

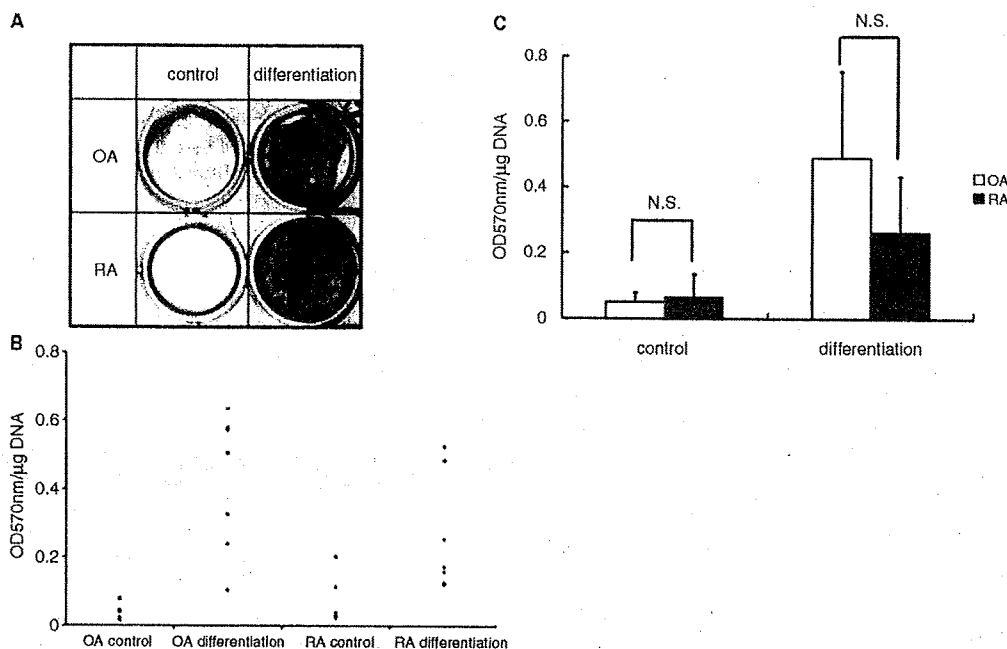


Fig. 4. Alizarin Red S staining and quantitative measuring of mineralization. After incubation for 3 weeks, Alizarin Red S staining was performed (A). Quantitative measuring of mineralization was measured by optical density of extracted supernatant at the wavelength of 570 nm. The result of Alizarin Red S mineralization assay in the control and differentiation group both in OA and RA was displayed with single dots (B) and with columns and s.d.-values (C). The result of the Alizarin Red S mineralization assay increased significantly in the differentiation group compared with that in the control group both in OA and RA. There were no significant differences in the control and differentiation groups between OA and RA. All samples were performed in triplicate and the data were calibrated by DNA quantity.

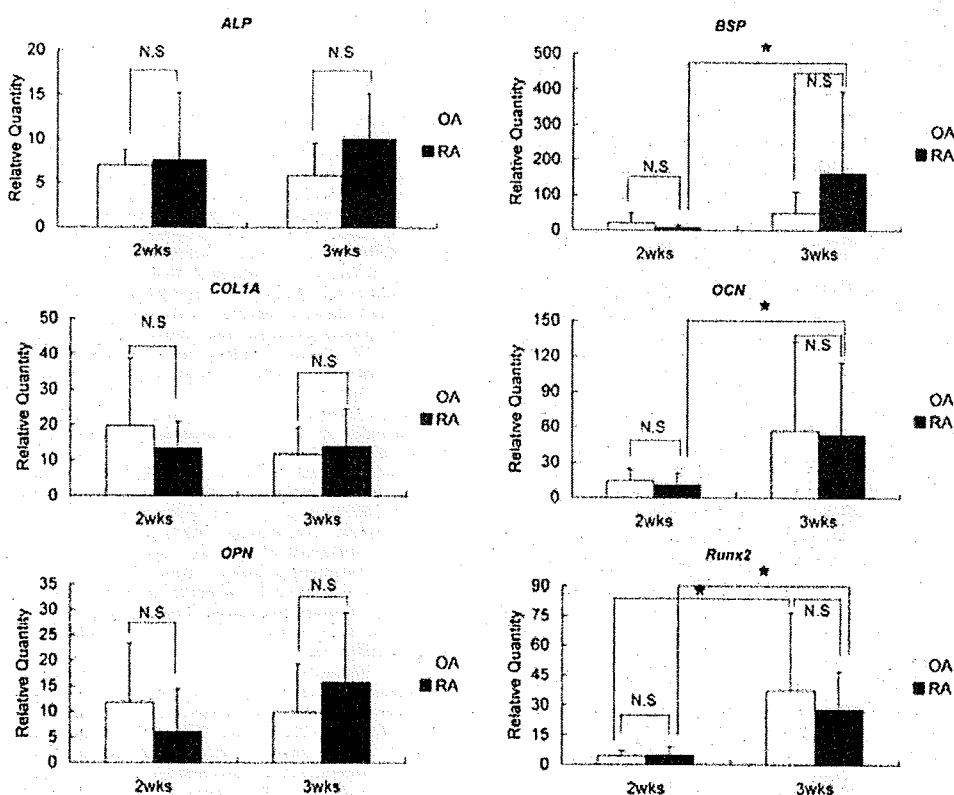


Fig. 5. Real-time PCR analysis. Cells were cultured with bGP, ascorbic acid and dex for 2 and 3 weeks. Real-time PCR was performed and analysed with the  $\Delta\Delta Ct$  method. The r18s RNA was used as an endogenous control. Gene expressions of ALP, BSP, COL1A, OC, OPN and Runx2 were evaluated. Gene expressions of ALP, COL1A and osteopontin were recognized in both OA and RA at 2 and 3 weeks. In RA, gene expressions of BSP, OC and Runx2 at 3 weeks were significantly higher than those at 2 weeks, and in OA, gene expression of Runx2 at 3 weeks was significantly higher than at 2 weeks. At 2 and 3 weeks, there were no significant differences in gene expressions between OA and RA. All samples were performed in triplicate. Relative quantity data were expressed as mean  $\pm$  s.d. (\* $P < 0.05$ ).

matrix protein. The results of ALP activity and gene expressions of ALP, COL1A and OPN showed no significant difference between OA and RA.

Hydroxyl apatite calcium is formed by a stimulus secreted by osteoblastic cells and mineralization of bone tissue is promoted. BSP and OC are late markers of osteoblast differentiation at the mineralization stage and Alizarin Red S staining expresses the deposition of calcium in cells. In RA samples, gene expressions of BSP and OC at 3 weeks were significantly higher than those at 2 weeks. These results of Alizarin Red S and gene expressions of BSP and OC indicated that hMSCs from patients with RA could be prompted to react by the stimulus and that the function of mineralization of hMSCs in RA was maintained and was almost identical with that in OA.

It was demonstrated that Runx2 is essential for osteoblastic differentiation and promotes gene expression of BSP, OPN and OC, which play roles for differentiating hMSCs into osteoblastic cells [26, 27]. Gene expression of Runx2 showed no significant difference between OA and RA. In both OA and RA, gene expression of Runx2 at 3 weeks was significantly higher than those at 2 weeks. These results of this study demonstrate that the function of osteoblastic differentiation of hMSCs shows no significant difference at the stages of production of bone matrix protein and mineralization between OA and RA. It was demonstrated that TNF- $\alpha$  suppressed osteoblast differentiation by suppressing the function of Runx2 *in vitro* [28, 29]. In this study, gene expression in RA was not significantly different from that in OA. The effect of inflammatory cytokines remained unclear and it needs to be further evaluated.

In the present study, there were several limitations. The mean age of patients with OA was higher than that of patients with RA and these groups could not be age matched. We obtained most samples at total knee arthroplasty, which may explain the age difference. We obtained hMSCs from patients with OA and RA in surgery and we could not obtain hMSCs from healthy donors. Therefore, we used hMSCs from patients with OA as a control.

RA is a heterogeneous disease and the backgrounds of patients with RA vary. It was demonstrated that patients with RA are characteristic for general osteoporosis and periarticular bone fragility. Although the main cause of general osteoporosis in RA still remains unclear, it was suggested that the disease activity, immobility and disease duration were associated with reduced bone mass in previous studies [30–32]. In this study, mean DAS28 score was 4.14 and mean CRP was 1.84 mg/dl. These results suggest that the disease activity of patients with RA in this study was moderate and this is because mean duration of disease is relatively longer and treated by appropriate medication.

Most patients with RA in this study have taken steroids. It was suggested that the daily oral and cumulative doses of steroid influenced general osteoporosis in RA [3].

In this study, the mean duration of disease was 14.3 years, which is a relatively longer period, and the mean oral steroid dose was 5.93 mg, which seems to be relatively low. The function of osteoblastic differentiation in RA seems not to be attenuated and the effect of steroid in RA remains unclear. It was demonstrated that relatively low oral dose of MTX did not influence BMD in patients with RA [33, 34]. In this study, mean oral MTX dose was 5.64 mg/week and orally taken MTX seemed not to affect osteoblastic differentiation. These results were consistent with previous results.

The function of osteoblastic cells in patients with OA and RA was previously evaluated [35]. In this previous study, osteoblastic cells were taken from periarticular bone and the cellular activity and senescence were evaluated. In the present study, we obtained hMSCs from iliac BM and the function of differentiation of hMSCs in patients with RA was evaluated. The previous study suggested the functional potential of MSCs derived from OA and RA especially in cartilage tissue engineering and the possibility of therapeutic application [24]. The present study is the first report to

evaluate the function of osteoblastic differentiation of hMSCs from patients with RA.

Although samples from patients with RA were different individually and the results varied widely, the results of this study suggested that hMSCs from patients with RA can be prompted to react by the stimulus of osteoblastic differentiation and maintain the function of osteoblastic differentiation.

## Conclusion

In conclusion, we evaluated the function of osteoblastic differentiation of hMSCs at the stages of production of bone matrix protein and mineralization in the present study. The function of osteoblastic differentiation showed no significant difference between OA and RA.

### Rheumatology key messages

- hMSCs from patients with RA maintain the function of osteoblastic differentiation.
- The osteoblastic differentiation function of hMSCs derived from RA is equivalent to hMSCs derived from OA.

## Acknowledgements

**Funding:** This work was supported by a grant from Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government, and a Grant-in-Aid from Organization for Pharmaceutical Safety and Research.

**Disclosure statement:** The authors have declared no conflicts of interest.

## References

- 1 Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. *Cell* 1996;85:307–10.
- 2 Gough AK, Lilley J, Eyre S, Holder RL, Emery P. Generalised bone loss in patients with early rheumatoid arthritis. *Lancet* 1994;344:23–7.
- 3 Kroot EJ, Nieuwenhuizen MG, de Waal Malefijt MC, van Riel PL, Pasker-de Jong PC, Laan RF. Change in bone mineral density in patients with rheumatoid arthritis during the first decade of the disease. *Arthritis Rheum* 2001;44:1254–60.
- 4 Cooper C, Coupland C, Mitchell M. Rheumatoid arthritis, corticosteroid therapy and hip fracture. *Ann Rheum Dis* 1995;54:49–52.
- 5 Hirayama T, Danks L, Sabokbar A, Athanasou NA. Osteoclast formation and activity in the pathogenesis of osteoporosis in rheumatoid arthritis. *Rheumatology* 2002;41:1232–9.
- 6 van Staa TP, Geusens P, Bijlsma JW, Leufkens HG, Cooper C. Clinical assessment of the long-term risk of fracture in patients with rheumatoid arthritis. *Arthritis Rheum* 2006;54:3104–12.
- 7 Romas E, Gillespie MT, Martin TJ. Involvement of receptor activator of NF $\kappa$ B ligand and tumor necrosis factor- $\alpha$  in bone destruction in rheumatoid arthritis. *Bone* 2002;30:340–6.
- 8 Udagawa N, Kotake S, Kamatani N, Takahashi N, Suda T. The molecular mechanism of osteoclastogenesis in rheumatoid arthritis. *Arthritis Res* 2002;4:281–9.
- 9 Goldring SR. Pathogenesis of bone and cartilage destruction in rheumatoid arthritis. *Rheumatology* 2003;42(Suppl. 2):ii11–6.
- 10 Strand V, Kavanaugh AF. The role of interleukin-1 in bone resorption in rheumatoid arthritis. *Rheumatology* 2004;43(Suppl. 3):iii10–6.
- 11 Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R. Toward a molecular understanding of skeletal development. *Cell* 1995;80:371–8.
- 12 Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature* 2003;423:337–42.
- 13 Zaidi M. Skeletal remodeling in health and disease. *Nat Med* 2007;13:791–801.
- 14 Compston JE, Vedi S, Croucher PJ, Garrahan NJ, O'Sullivan MM. Bone turnover in non-steroid treated rheumatoid arthritis. *Ann Rheum Dis* 1994;53:163–6.
- 15 Hall GM, Spector TD, Delmas PD. Markers of bone metabolism in postmenopausal women with rheumatoid arthritis. Effects of corticosteroids and hormone replacement therapy. *Arthritis Rheum* 1995;38:902–6.
- 16 Garnero P, Jovenne P, Buchs N, Delmas PD, Miossec P. Uncoupling of bone metabolism in rheumatoid arthritis patients with or without joint destruction: assessment with serum type I collagen breakdown products. *Bone* 1999;24:381–5.
- 17 Pittenger MF, Mackay AM, Beck SC *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143–7.
- 18 Komori T. Regulation of osteoblast differentiation by transcription factors. *J Cell Biochem* 2006;99:1233–9.

- 19 Arnett FC, Edworthy SM, Bloch DA *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315-24.
- 20 Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. *Ann Rheum Dis* 1957;16:494-502.
- 21 Gregory CA, Gunn WG, Peister A, Prockop DJ. An alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem* 2004;329:77-84.
- 22 Conget PA, Minguell JJ. Phenotypic and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol* 1999;181:67-73.
- 23 Lee RH, Kim B, Choi I *et al.* Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell Physiol Biochem* 2004;14:311-24.
- 24 Chen FH, Tuan RS. Mesenchymal stem cells in arthritic diseases. *Arthritis Res Ther* 2008;10:223.
- 25 Tomita T, Kashiwagi N, Shimaoka Y *et al.* Phenotypic characteristics of bone marrow cells in patients with rheumatoid arthritis. *J Rheum* 1994;21:1608-14.
- 26 Komori T, Yagi H, Nomura S *et al.* Targeted disruption of *cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 1997;89:755-64.
- 27 Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. *Osf2/cbfa1*: a transcriptional activator of osteoblast differentiation. *Cell* 1997;89:747-54.
- 28 Gilbert L, He X, Farmer P *et al.* Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology* 2000;141:3956-64.
- 29 Gilbert L, He X, Farmer P *et al.* Expression of the osteoblast differentiation factor *Runx2* (*cbfa1/aml3/pebp2alpha a*) is inhibited by tumor necrosis factor-alpha. *J Biol Chem* 2002;277:2695-701.
- 30 Laan RF, Buijs WC, Verbeek AL *et al.* Bone mineral density in patients with recent onset rheumatoid arthritis: influence of disease activity and functional capacity. *Ann Rheum Dis* 1993;52:21-6.
- 31 Haugeberg G, Uhlig T, Falch JA, Halse JI, Kvien TK. Bone mineral density and frequency of osteoporosis in female patients with rheumatoid arthritis: results from 394 patients in the Oslo county rheumatoid arthritis register. *Arthritis Rheum* 2000;43:522-30.
- 32 Lodder MC, de Jong Z, Kostense PJ *et al.* Bone mineral density in patients with rheumatoid arthritis: relation between disease severity and low bone mineral density. *Ann Rheum Dis* 2004;63:1576-80.
- 33 Minaur NJ, Kounali D, Vedi S, Compston JE, Beresford JN, Bhalla AK. Methotrexate in the treatment of rheumatoid arthritis. II. In vivo effects on bone mineral density. *Rheumatology* 2002;41:741-9.
- 34 di Munno O, Mazzantini M, Sinigaglia L *et al.* Effect of low dose methotrexate on bone density in women with rheumatoid arthritis: results from a multicenter cross-sectional study. *J Rheumatol* 2004;31:1305-9.
- 35 Yudoh K, Matsuno H, Osada R, Nakazawa F, Katayama R, Kimura T. Decreased cellular activity and replicative capacity of osteoblastic cells isolated from the peri-articular bone of rheumatoid arthritis patients compared with osteoarthritis patients. *Arthritis Rheum* 2000;43:2178-88.



## Specifically Modified Osteopontin in Rheumatoid Arthritis Fibroblast-like Synoviocytes Supports Interaction With B Cells and Enhances Production of Interleukin-6

Yasuhiro Take,<sup>1</sup> Ken Nakata,<sup>1</sup> Jun Hashimoto,<sup>1</sup> Hideki Tsuboi,<sup>2</sup> Norihiro Nishimoto,<sup>3</sup> Takahiro Ochi,<sup>4</sup> and Hideki Yoshikawa<sup>1</sup>

**Objective.** Osteopontin (OPN) is expressed by fibroblast-like synoviocytes (FLS) in rheumatoid arthritis (RA), but its pathologic role is still obscure. The present study was undertaken to analyze the role of OPN in RA by focusing on its effects on cell-cell interactions between FLS and B lymphocytes.

**Methods.** FLS obtained from 10 patients with RA and 10 non-RA subjects and a B lymphocyte cell line were studied. The characteristics of OPN expression by FLS were analyzed by Western blotting, immunoprecipitation, and immunofluorescence studies. In cocultures of FLS and B lymphocytes, the effects of OPN on adhesion of B lymphocytes to FLS and the consequent production of interleukin-6 (IL-6) were analyzed in experiments involving overexpression and knockdown of OPN and inhibitory studies with an OPN-blocking antibody. In vivo, the expression of OPN in RA synovium was examined by immunohistochemistry.

**Results.** A specifically modified 75-kd form of OPN was predominantly expressed in RA FLS, and this was associated with expression of >200-kd thrombin-cleaved OPN that was crosslinked with fibronectin and localized on the surface of the FLS. In FLS-B lymphocyte cocultures, 75-kd OPN-positive FLS produced a

significantly higher amount of IL-6 than did 75-kd OPN-negative FLS. When the FLS were separated from B lymphocytes or cultured alone, the production of IL-6 was low and was not significantly different between these 2 culture conditions. Moreover, OPN overexpression enhanced production of IL-6 in 75-kd OPN-positive FLS-B lymphocyte cocultures. Addition of the OPN-blocking antibody inhibited the adhesion of B lymphocytes to FLS. Immunohistochemical analyses revealed that localization of IL-6-positive cells coincided with the sites at which OPN and B lymphocytes were colocalized.

**Conclusion.** Specifically modified 75-kd OPN was expressed by RA FLS. This form of OPN affected FLS-B lymphocyte interactions by supporting the adhesion of B lymphocytes to FLS and enhancing the production of IL-6.

In patients with rheumatoid arthritis (RA), the synovium of the inflamed joints is the site of a chronic inflammatory reaction, in which leukocytes and macrophages infiltrate, synoviocytes proliferate, and several proinflammatory cytokines (especially, interleukin-6 [IL-6] and tumor necrosis factor  $\alpha$ ), autoantibodies, and immune complexes are produced (1). In this situation, infiltrating leukocytes and proliferating synoviocytes are thought to interact with each other. Accordingly, several in vitro studies have focused on the interaction between fibroblast-like synoviocytes (FLS) and leukocytes by studying the findings in cocultures of these cells (2–7). Coculture of FLS obtained from RA synovium with B lymphocytes causes adhesion of B lymphocytes to FLS, and consequently this interaction supports the survival of B lymphocytes and also enhances the production of cytokines and immunoglobulin (8–12). The former mechanism is dependent on vascular cell adhesion mol-

Supported by Health and Labour Sciences of Japan research grant 200500732A.

<sup>1</sup>Yasuhiro Take, MD, Ken Nakata, MD, PhD, Jun Hashimoto, MD, PhD, Hideki Yoshikawa, MD, PhD: Osaka University, Suita-city, Osaka, Japan; <sup>2</sup>Hideki Tsuboi, MD, PhD: Osaka Rosai Hospital, Sakai-city, Osaka, Japan; <sup>3</sup>Norihiro Nishimoto, MD, PhD: Wakayama Medical University, Wakayama-city, Wakayama, Japan; <sup>4</sup>Takahiro Ochi, MD, PhD: Osaka Police Hospital, Osaka-city, Osaka, Japan.

Address correspondence and reprint requests to Ken Nakata, MD, PhD, Department of Orthopaedics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita-city, Osaka 565-0871, Japan. E-mail: ken-nakata@umin.ac.jp.

Submitted for publication February 22, 2009; accepted in revised form August 31, 2009.

ecule 1 (VCAM-1) and very late activation antigen 4 (VLA-4) (10), whereas the latter is yet to be determined.

Osteopontin (OPN) is abundant in the bone matrix, where it acts as a bridge between hydroxyapatite and osteoclasts to support bone resorption (13–15). It is also secreted by T lymphocytes and assists in the maturation of B lymphocytes and the migration of macrophages (16–18). These diverse functions are partly explained by the several functional domains of OPN, including integrin-binding, calcium-binding, and heparin-binding sites. Several studies have revealed that the function of OPN depends on posttranslational modifications (19–24). These modifications, which vary between different OPN-expressing cells, consist of phosphorylation at dozens of serine and threonine residues, along with glycosylation (25), sialylation (21), transglutamination (26), and cleavage (27,28). However, the association between the details of posttranslational modification and the function of OPN is still poorly understood.

As for the relationship between OPN and arthritis, OPN-null mice are protected against inflammatory joint destruction in collagen-induced arthritis (29). A blocking antibody directed against the thrombin-cleaved neoepitope of OPN, which cooperates with several integrin receptors as a ligand (30,31), also shows a curative effect on induced arthritis in mice and monkeys (32,33). Indeed, in patients with RA, OPN, especially in the thrombin-cleaved form, is strongly detected in the synovium and synovial fluid of inflamed joints (34–36). In vitro studies on the function of OPN in arthritis have revealed that OPN stimulates the production of several proinflammatory cytokines by mononuclear cells in patients with RA (37), and also that monocytes obtained from mice with induced arthritis show increased migration toward thrombin-cleaved OPN (32). However, these studies were performed using recombinant OPN, which lacks various posttranslational modifications, and thus the posttranslational modification-dependent functions were not taken into account.

Considering these results and the fact that OPN is also strongly expressed in FLS from the RA synovium (35), we hypothesized that RA FLS express a unique form of OPN that acts to stimulate production of cytokines by creating a bridge between FLS and B lymphocytes in cocultures of these cells. The purpose of this study was to analyze the role of OPN in the development of RA, by focusing on the interaction between FLS and B lymphocytes.

## PATIENTS AND METHODS

**Patients and cell culture.** After obtaining the patients' informed consent and securing Institutional Review Board approval, synovial tissue samples were collected from 11 patients with RA, 8 patients with osteoarthritis of the knee, and 2 patients with medial meniscus degeneration who underwent meniscectomy and synovectomy. All of the patients with RA fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) revised criteria for RA (38). FLS were isolated by enzymatic digestion of the synovial tissue from 10 patients with RA and 10 non-RA subjects, as described previously (9). The FLS were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Gibco BRL). Cells from passages 4–10 were used for these experiments. A human B cell line, MC/car, was purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium (Gibco BRL) containing 10% FBS and 1% penicillin/streptomycin.

For coculture experiments,  $4 \times 10^4$  FLS were plated in 12-well plates and, on the following day,  $3.3 \times 10^5$  B lymphocytes were added to each well. To prevent cell–cell contact between FLS and B lymphocytes, a Millicell culture insert with 0.4- $\mu$ m pores (Millipore, Billerica, MA) was used. After incubation for 48 hours, enzyme immunoassay (EIA) was performed to measure the concentrations of IL-6 and OPN in the culture supernatant using a homogeneous time-resolved fluorescence human IL-6 assay kit (CIS Bio International, Saclay, France) and a human OPN assay kit (IBL Japan, Gunma, Japan).

**Protein collection, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting.** Total cell lysates of FLS were collected by placing the cells in lysis buffer (10 $\times$  Cell Lysis Buffer; New England Biolabs, Beverly, MA), using  $4 \times 10^4$  FLS plated in 12-well plates and grown in normal growth medium; some of the FLS had undergone transfection with small interfering RNA (siRNA) or lentiviral infection. We did not add any other reagent to the lysis buffer, which consisted of Tris HCl buffer with detergent, protease inhibitor (leupeptin), and phosphatase inhibitors (sodium pyrophosphate,  $\beta$ -glycerophosphate, and sodium orthovanadate). After 1 freeze–thaw cycle, samples were centrifuged and the supernatant was collected. An aliquot was obtained for protein quantification with the bicinchoninic acid assay, and the remaining supernatant was boiled in SDS sample buffer. As a control, recombinant human OPN was used.

Surface protein was collected from  $1.2 \times 10^6$  FLS using a Cell Surface Protein isolation kit (Pierce, Rockford, IL). Equal amounts of each sample were separated by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane (Hybond-P; Amersham, Piscataway, NJ), and blocked with 5% bovine serum albumin (BSA)/Tris buffered saline containing 0.1% Tween 20 (TBST). The membrane was then incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-human OPN antibody (O-17; IBL Japan) or mouse anti-OPN N-Half antibody (34E3; IBL Japan) (each at 2  $\mu$ g/ml), which detects the thrombin-cleaved neoepitope YGLR, mouse anti- $\beta$ -actin antibody (AC-15; Sigma, St. Louis,

MO) at 1:10,000, or rabbit antifibronectin antibody (H-300; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200. Specificity of the 34E3 antibody was confirmed by Western blotting of thrombin-treated recombinant OPN. After reaction with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and enhanced chemiluminescence Western blotting detection reagents (all from Amersham), images of the membrane were captured using a FAS-1000 Lumino Imaging Analyzer (Toyobo, Osaka, Japan).

**Immunoprecipitation analysis.** Total cell lysates were prepared for immunoprecipitation using RA FLS from a single patient (RA sample 4 in Figure 1A). When the cells had reached 90% confluence, they were collected in a 10-cm dish, and after centrifugation, the resulting supernatant was pre-cleared with ImmunoPure Immobilized Protein A Plus (Pierce), divided into 3 centrifuge tubes, and incubated overnight at 4°C with protein A-Sepharose bound with 10 µg of the anti-OPN antibody (O-17), the antifibronectin antibody, or normal rabbit IgG (Santa Cruz Biotechnology) as a negative control. On the following day, the samples were washed and eluted with SDS sample buffer, and then analyzed by Western blotting.

**Immunofluorescence analysis.** FLS cultured in Chamber Slides (Nunc, Rochester, NY) were fixed in 4% paraformaldehyde, permeabilized with TBST, blocked with 5% BSA/TBST, and incubated overnight at 4°C with the anti-OPN antibody (O-17) or normal rabbit IgG (Santa Cruz Biotechnology) (each at 2 µg/ml). After the samples were reacted with Alexa Fluor 488-conjugated anti-rabbit IgG at 1:500 (Molecular Probes, Eugene, OR), the fluorescence intensity of the samples was examined under an epifluorescence microscope (Nikon Eclipse 90i; Nikon, Tokyo, Japan).

**Transfection with siRNA.** Commercially available, pre-designed double-stranded RNA (nontargeting siRNA [siCONTROL Pool #1] and siRNA for human secreted phosphoprotein 1 [OPN] and IL-6 [siGENOME SMARTpool siRNA]; Dharmacon, Lafayette, CO) were used for the RNA interference experiments. One microliter of X-TremeGENE reagent (Roche Diagnostic, Penzberg, Germany) and 20 pmoles of the siRNA from either pool were diluted in serum-free medium, and the mixture was then incubated for 15 minutes and added to  $4 \times 10^4$  FLS in a 12-well plate. Two days after transfection, messenger RNA (mRNA) was collected and reverse-transcribed to first-strand complementary DNA (cDNA) using a high-capacity cDNA reverse transcription kit. Real-time reverse transcription-polymerase chain reaction (PCR) was then performed to evaluate the OPN-knockdown efficacy, using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). Three days after transfection, the cocultures were started.

**B lymphocyte adhesion assay.** Adhesion of B lymphocytes to FLS was assessed as described previously (11), with some modifications. Briefly,  $6.6 \times 10^3$  FLS were plated into a 96-well plate. After 3 days, the cells were incubated at 37°C for 2 hours with a polyclonal OPN-neutralizing antibody or normal goat IgG (both from R&D Systems, Minneapolis, MN) at 10 µg/ml. Subsequently,  $3.3 \times 10^5$  B lymphocytes were added and incubated at 37°C for another 2 hours. B lymphocytes that did not adhere firmly to the FLS were removed by vigorous washing. Three separate fields were viewed under an inverted

microscope at a magnification of 100× per well to count the adherent B lymphocytes, with results expressed quantitatively as the mean number of adherent B lymphocytes.

**Transglutaminase inhibition.** FLS were plated at  $4 \times 10^4$  cells in 12-well plates, followed by incubation with cystamine sulfate (500 µM) for 2 days to inhibit transglutaminase activity. Subsequently, total cell lysates were collected for further analysis.

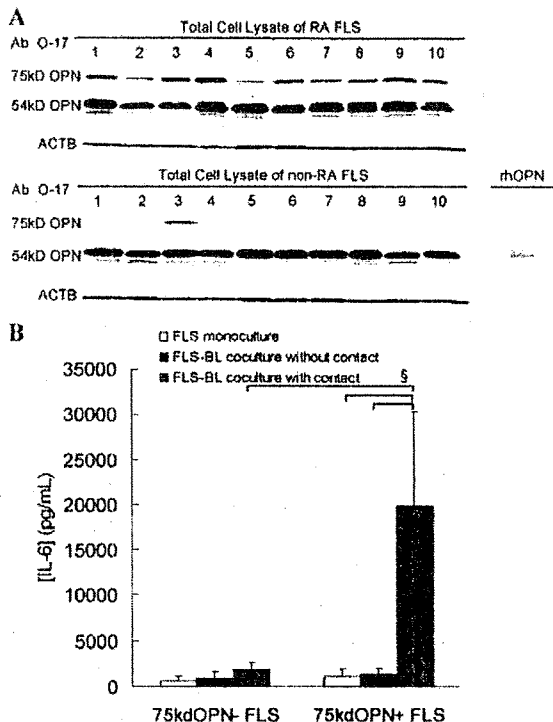
**Plasmid construction and lentiviral infection.** The full-length OPN-coding sequence was amplified by PCR from a human synovial cell cDNA library using the following primers: 5'-CCCTCGAGATGAGAATTGCAGTGATTGTC-3' (NM\_001040058, nt166-186) and 5'-CGGGATCC-TTAATGACCTCAGAAGATGCAC-3' (NM\_001040058, nt1088-1110). The PCR product was digested with *Xho* I and *Bam* HI, and then purified and ligated to pcDNA3.1 (Invitrogen, Carlsbad, CA) for sequencing. The vector was cut with *Pme* I and the OPN insert was obtained by gel extraction/purification, which was then ligated to the lentiviral expression vector pWPI. Lentivirus infection was performed on  $4 \times 10^4$  FLS in a 12-well plate, as described previously (39,40). Three days after infection, coculture was started for measurement of IL-6 production.

**Immunohistochemistry.** Specimens of synovial tissue obtained from a patient with RA were fixed in 10% formaldehyde, dehydrated, and embedded in paraffin. Sections of the tissue were cut (4 µm thick) on a microtome, and then were analyzed by immunohistochemistry, as described previously (41), using the following primary antibodies: rabbit anti-OPN antibody (O-17) or normal rabbit IgG at 2 µg/ml, mouse anti-CD79α antibody (JCB117; Nichirei, Tokyo, Japan) at 1:50, normal mouse IgG1 (R&D Systems), and goat anti-human IL-6 antibody or normal goat IgG at 2.5 µg/ml.

**Statistical analysis.** Results are expressed as the mean ± SD of at least 3 independent experiments, if not specified. For statistical comparison, 2-way factorial analysis of variance (ANOVA) (sometimes followed by a Bonferroni post hoc test) was performed using SPSS software (version 16.0; SPSS, Chicago, IL). *P* values less than 0.05 were considered significant.

## RESULTS

**Detection of specifically modified 75-kd OPN in all 10 RA FLS and 3 non-RA FLS, in conjunction with significantly higher IL-6 production in FLS-B lymphocyte cocultures.** Western blotting of the total cell lysates from FLS, using the antibody for the N-terminus of OPN (O-17), showed several bands at 75 kd and also around 54 kd (Figure 1A), which is a well-known characteristic of OPN (42). Double bands around 54 kd, each at an equal density, were detected in every FLS sample. Recombinant human OPN also migrated at around 54 kd. However, 75-kd OPN was predominantly detected in all 10 RA FLS samples (RA samples 1-10 in Figure 1A)



**Figure 1.** Detection of osteopontin (OPN) and production of interleukin-6 (IL-6) in fibroblast-like synoviocytes (FLS). **A**, Total cell lysates of rheumatoid arthritis (RA) FLS and non-RA FLS ( $n = 10$  samples each) were assessed using Western blotting with an N-terminus antibody (Ab) against OPN (O-17), in comparison with recombinant human OPN (rhOPN);  $\beta$ -actin (ACTB) served as standard. **B**, The concentration of interleukin-6 (IL-6) in the culture supernatants of FLS monocultures, FLS-B lymphocyte (BL) cocultures without contact (separated by a Millicell culture insert), and FLS-B lymphocyte cocultures with contact was evaluated by enzyme immunoassay separately in 75-kd OPN-positive FLS ( $n = 13$ ) and 75-kd OPN-negative FLS ( $n = 7$ ). Bars show the mean and SD.  $\$ = P < 0.001$  by Bonferroni *t*-test.

and in only 3 non-RA FLS samples (non-RA samples 3, 8, and 9). Thrombin-cleaved OPN was not detected around the reported molecular weight of 30 kd (43).

EIA analysis of the full-length OPN secreted in the culture supernatant showed that the concentration of OPN was 9–18 ng/ml, and this did not differ between RA and non-RA FLS (results not shown). Since the expression of 75-kd OPN was distinct, FLS were grouped into 75-kd OPN-positive and 75-kd OPN-negative FLS.

Production of IL-6 was evaluated by EIA for determination of the IL-6 concentration in the culture supernatants obtained in several culture conditions with FLS and B lymphocytes. In FLS monoculture (i.e., FLS

cultured alone), the mean  $\pm$  SD IL-6 production by the 75-kd OPN-positive FLS ( $n = 13$ ) and the 75-kd OPN-negative FLS ( $n = 7$ ) was  $1,113.3 \pm 818.9$  pg/ml and  $643.1 \pm 576.5$  pg/ml, respectively. In FLS-B lymphocyte cocultures without contact (i.e., FLS cocultured with B lymphocytes, but separated by the Millicell culture insert), the IL-6 levels were  $1,363.3 \pm 714.6$  pg/ml in 75-kd OPN-positive FLS and  $902.6 \pm 772.5$  pg/ml in 75-kd OPN-negative FLS. There were no significant differences in IL-6 production among these 4 groups.

However, in cocultures with contact (i.e., FLS and B lymphocytes cultured in contact with each other), IL-6 production was significantly elevated in 75-kd OPN-positive FLS, and showed a tendency to be elevated, although not to a significant extent, in 75-kd OPN-negative FLS. The IL-6 production by the 13 FLS positive for 75-kd OPN cocultured in contact with B lymphocytes was elevated to  $19,928.5 \pm 10,351.8$  pg/ml, which was significantly higher than that in the other 2 culture conditions ( $P < 0.001$ , by Bonferroni *t*-test) and significantly higher than that in the 7 FLS negative for 75-kd OPN that were in contact coculture with B lymphocytes ( $910.9 \pm 744.1$  pg/ml;  $P < 0.001$ , by Bonferroni *t*-test) (Figure 1B).

Transfection of the FLS with IL-6 siRNA significantly reduced the levels of IL-6 in the 75-kd OPN-positive FLS that were in contact coculture with B lymphocytes. In contrast, when B lymphocytes were cultured alone, IL-6 was not detected in the culture supernatants (results not shown).

**Production of >200-kd thrombin-cleaved OPN in the cell surface protein fraction of 75-kd OPN-positive FLS, and cell membrane distribution of OPN.** Analysis of cell surface proteins from FLS, using Western blotting with an antibody against the thrombin-cleaved neopeptide of OPN (34E3), detected the presence of >200-kd OPN in the surface fraction of 75-kd OPN-positive FLS. This band was poorly detected in 75-kd OPN-negative FLS or in the nonsurface fraction of FLS (Figure 2A). A similar result was obtained with the O-17 antibody, but the signals were much weaker (results not shown). As a loading control, Western blotting with the antifibronectin antibody probe was performed. When the anti-OPN antibody O-17 was used to analyze the 75-kd OPN-positive FLS by immunofluorescence assay, the distribution of OPN followed the cell outline, regardless of permeabilization (Figure 2B).

**Detection of >200-kd OPN in the immunoprecipitant by antifibronectin antibody, and reduction by transglutaminase inhibitor treatment.** According to a previous study, OPN appears at >200 kd when it is

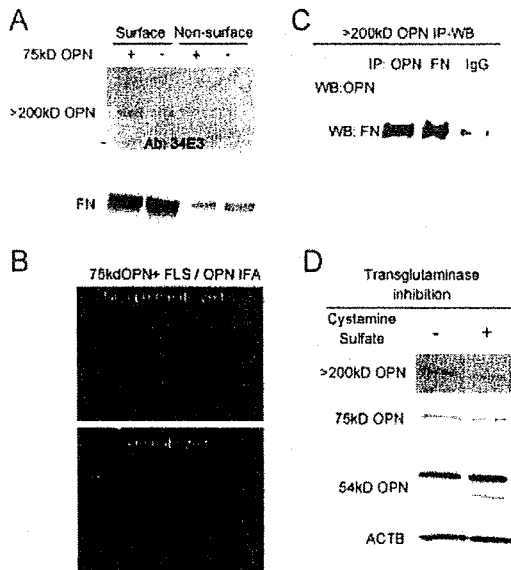


Figure 2. Characteristics of the 75-kd OPN. A, Isolation of cell surface proteins was followed by Western blotting with or without the antibody (Ab) directed against the thrombin-cleaved neopeptide of OPN (34E3). The >200-kd form of OPN was detected in the surface protein fraction of 75-kd OPN-positive FLS. Fibronectin (FN) expression was used as the loading control. B, Immunofluorescence assay (IFA) was used to assess the expression pattern of OPN on 75-kd OPN-positive FLS stained with OPN antibody O-17, with results showing a distribution of OPN corresponding to the cell outline, regardless of permeabilization. C, When >200-kd OPN was further examined by immunoprecipitation-Western blotting (IP-WB), the >200-kd band was detected in the immunoprecipitant of the OPN antibody (O-17) blotted with the FN antibody, and vice versa. Normal rabbit IgG was used as the negative control. D, The OPN-positive FLS were treated with or without a transglutaminase inhibitor, cystamine sulfate, and assessed by Western blotting. Expression of >200-kd OPN was reduced by treatment with cystamine sulfate, whereas the expression levels of 75-kd and 54-kd OPN were not altered. See Figure 1 for other definitions.

covalently crosslinked to fibronectin by transglutamination through 2 widely conserved glutamine residues at its N-terminus (26). Therefore, immunoprecipitation of total cell lysates from 75-kd OPN-positive FLS with the anti-OPN or antifibronectin antibody was performed, and this was followed by Western blotting with these antibodies. The >200-kd band was detected when the immunoprecipitant obtained with the antifibronectin antibody was probed by the anti-OPN antibody, and vice versa (Figure 2C). A transglutaminase inhibitor, cystamine sulfate, reduced the expression of >200-kd OPN when the inhibitor was added to the FLS cultures, but did not alter the levels of 75-kd or 54-kd OPN (Figure 2D).

**Enhanced IL-6 production by OPN overexpression in 75-kd OPN-positive FLS, but not in 75-kd OPN-negative FLS, in cocultures with B lymphocytes.** To elucidate the role of OPN in FLS-B lymphocyte cocultures, gain of function experiments were performed by inducing overexpression of OPN with a lentiviral vector. Western blotting of the cells after lentiviral infection showed that all 75-kd OPN-positive FLS showed up-regulation of the 75-kd, >200-kd, and 54-kd OPN bands, along with an extra band at 50 kd, whereas the 75-kd and >200-kd bands were not up-regulated in any of the 75-kd OPN-negative FLS (Figure 3A).

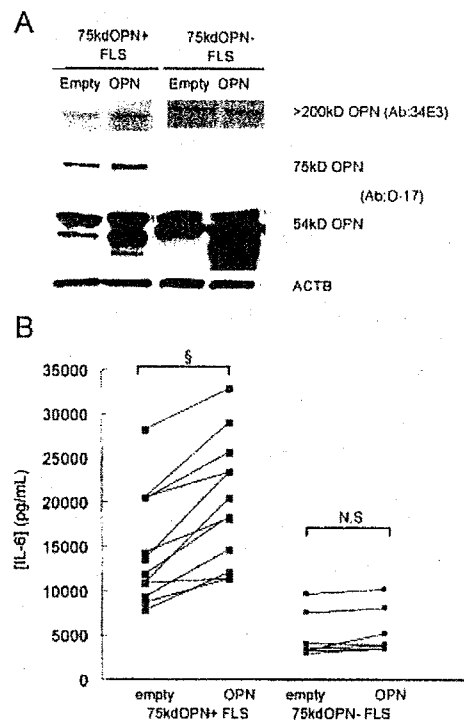
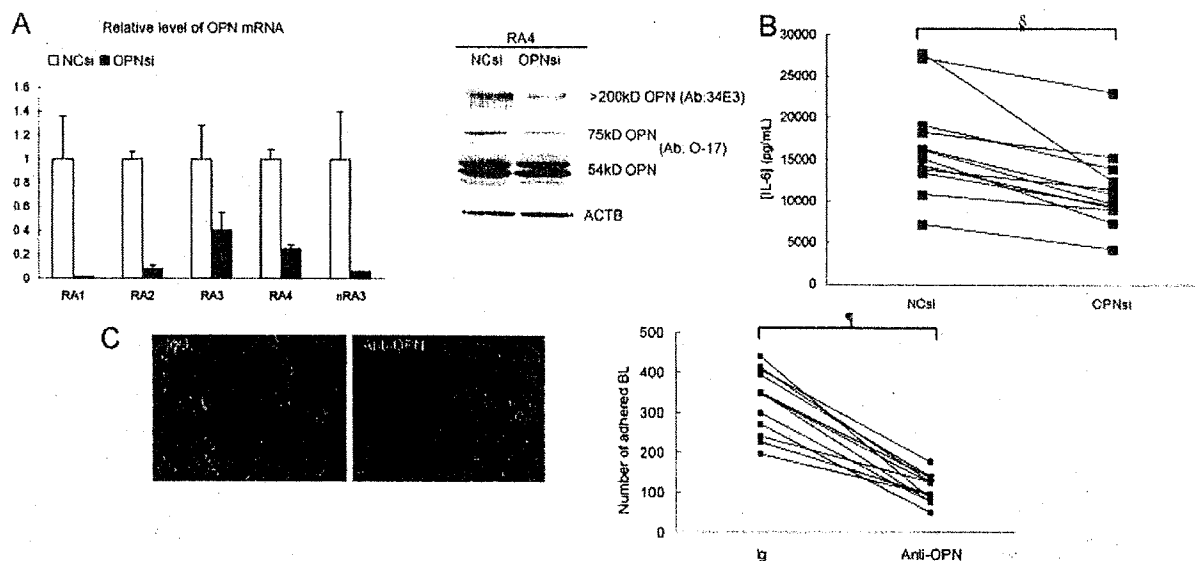


Figure 3. Gain of function experiments, targeting OPN by lentiviral infection with an overexpression vector. A, The 75-kd OPN-positive and 75-kd OPN-negative FLS were subjected to lentiviral infection with an empty vector or OPN-overexpressing vector and then assessed by Western blotting. The 54-kd form of OPN, with an extra lower band, showed increased expression in both types of FLS, while >200-kd OPN and 75-kd OPN were increased only in 75-kd OPN-positive FLS. B, After lentiviral infection of the FLS and then coculture in contact with B lymphocytes, the IL-6 concentration in the culture supernatant was evaluated. IL-6 levels were significantly increased by OPN overexpression in 75-kd OPN-positive FLS, whereas the levels were not altered in 75-kd OPN-negative FLS. Solid squares show individual FLS samples. § =  $P < 0.001$  by 2-factorial analysis of variance. NS = not significant (see Figure 1 for other definitions).



**Figure 4.** Loss of function experiments, targeting OPN by small interfering RNA (siRNA) (A and B) and a neutralizing antibody (C). **A**, OPN knockdown in 75-kD OPN-positive FLS by transfection of OPN siRNA (OPNsi) was evaluated by real-time reverse transcription-polymerase chain reaction (left) and by Western blotting (right). Bars show the mean and SD OPN mRNA levels in triplicate experiments with each of 4 RA FLS samples and 1 non-RA (nRA) FLS sample. NCsi = negative control siRNA. **B**, FLS transfected with negative control siRNA or OPN siRNA were cocultured in contact with B lymphocytes and the IL-6 concentration in the supernatant was evaluated. The IL-6 level was significantly reduced by OPN knockdown.  $\$ = P < 0.001$  by 2-factorial analysis of variance. **C**, FLS treated with an OPN-neutralizing antibody or class-matched normal IgG were cocultured in contact with B lymphocytes. After removal of nonadherent B lymphocytes by vigorous washing, adherent B lymphocytes were counted in 3 separate fields per well, as viewed under an inverted microscope (left; original magnification  $\times 100$ ). Small round cells are B lymphocytes. The number of adherent B lymphocytes was expressed as the mean (right). Solid squares show individual FLS samples.  $\# = P < 0.001$  by 2-factorial analysis of variance. See Figure 1 for other definitions.

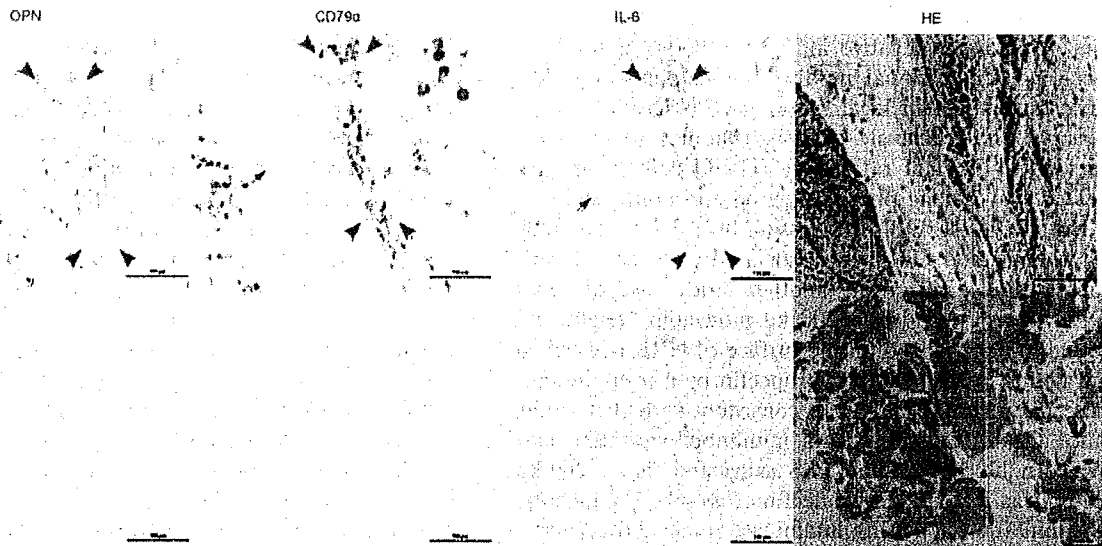
In addition, all 75-kD OPN-positive FLS showed a significant increase in IL-6 production after OPN overexpression followed by coculture in contact with B lymphocytes, when compared with cells without overexpression ( $P < 0.001$  by 2-factorial ANOVA) (Figure 3B). In 75-kD OPN-negative FLS, however, IL-6 production was not altered by overexpression of OPN, which was consistent with the lack of a 75-kD band and the unaltered  $>200$ -kD band on Western blotting.

**Significant reduction in IL-6 levels following loss of function of OPN in 75-kD OPN-positive FLS, and inhibition of adhesion of B lymphocytes on FLS in cocultures with B lymphocytes.** Loss of function experiments were performed using siRNA transfection and an OPN-neutralizing antibody. Transfection of OPN siRNA successfully knocked down OPN expression at the mRNA level as well as at the protein level in all of the 75-kD OPN-positive FLS (Figure 4A). After subsequent coculture of the FLS in contact with B lymphocytes, there was significantly lower IL-6 production by OPN-knocked down FLS compared with that by FLS

transfected with nontargeting siRNA ( $P < 0.001$  by 2-factorial ANOVA) (Figure 4B).

Adhesion of B lymphocytes to FLS occurred in FLS-B lymphocyte cocultures, as has been reported previously (9,12), but was inhibited by the blocking antibody directed against OPN. The number of B lymphocytes adherent to FLS was significantly reduced by the anti-OPN antibody in all 75-kD OPN-positive FLS ( $P < 0.001$  by 2-factorial ANOVA) (Figure 4C).

**Detection of IL-6-positive cells in the sublining region of RA synovium, at the site of OPN-B lymphocyte colocalization.** Finally, to assess the distribution of OPN, IL-6, and B lymphocytes in the RA synovium, immunohistochemical analyses were carried out using anti-IL-6, anti-OPN, and anti-pan B lymphocyte antigen CD79 $\alpha$  antibodies. One of the synovial villi located in the knee joint from an RA patient was studied. As reported previously, OPN was distributed in the fibroblastic cells and the matrix of the synovial lining and sublining regions (34,35). Clusters of B lymphocytes were found in the deeper layers of the synovium as well as in the



**Figure 5.** Immunohistochemical analysis of rheumatoid arthritis (RA) synovium. One of the synovial villi in the inflamed joint of a patient with RA was evaluated by hematoxylin and eosin (H&E) staining. The H&E-stained image in the upper right panel is a higher-magnification view of the boxed area in the lower right panel. The RA synovium samples in the other 3 upper panels were stained with the antibody against osteopontin (anti-OPN) (O-17), anti-pan B cell antigen CD79 $\alpha$ , and anti-interleukin-6 (anti-IL-6) antibodies. The corresponding panels below were stained with class-matched normal IgG as a negative control. The appearance of IL-6-positive cells coincided with the site at which OPN and B lymphocytes colocalized (arrowheads), whereas IL-6 was not detected in areas where only OPN-positive cells were distributed or at sites far from clusters of B lymphocytes (arrows).

sublining region, and some of these B lymphocyte clusters were surrounded by OPN-positive cells. IL-6-positive cells were scattered in the sublining region, as has been reported previously (44).

Of note, some of the areas of distribution of IL-6-positive cells coincided with sites at which OPN and B lymphocytes colocalized (indicated by arrowheads in Figure 5). However, IL-6 was not detected in areas where only OPN-positive cells were distributed or at sites far from clusters of B lymphocytes (indicated by arrows in Figure 5).

### DISCUSSION

The present study revealed a new role of OPN in the RA synovium, using primary cultured FLS derived from RA patients to analyze the function of FLS-expressed native OPN. A specifically modified 75-kd OPN was predominantly expressed by RA FLS and was associated with significantly elevated IL-6 production in FLS-B lymphocyte cocultures. The 75-kd OPN formed a >200-kd OPN/fibronectin-crosslinked molecule via transglutamination, which was detected on the synovial cell surface, resulting in exposure of its thrombin-cleaved neopeptide. This >200-kd cell surface OPN

affected the interactions between FLS and B lymphocytes by supporting adhesion of B lymphocytes to FLS in FLS-B lymphocyte cocultures, and consequently IL-6 production was enhanced. This FLS-B lymphocyte interaction also appeared to occur *in vivo*.

OPN by itself weighs 37 kd, as determined previously using mass spectrometry (25), but due to its various posttranslational modifications, the migration of full-length OPN, detected on SDS-PAGE, differs among cell types within ~45–80 kd (42). Accordingly, our results from Western blotting of OPN showed multiple bands with different molecular weights. In particular, a single band at 75 kd that was detected predominantly in RA FLS suggested that this specifically modified 75-kd OPN was worth investigating to determine the function of OPN in RA.

In fact, the localization of OPN was different between 75-kd OPN-negative and 75-kd OPN-positive FLS. The existence of >200-kd OPN in the cell surface fraction of 75-kd OPN-positive FLS on Western blotting, and the staining pattern of OPN on immunofluorescence assay of these FLS, showing the cell outline distribution, suggested that OPN was associated with the cell surface side of the plasma membrane. These distinct

findings were considered attributable to 75-kd OPN, since they were negative in 75-kd OPN-negative FLS. Such posttranslational modification-dependent differences in subcellular localization of OPN have also been observed in previously published studies of OPN expressed by normal rat kidney (NRK) cells, which revealed that among phosphorylated and nonphosphorylated forms of OPN expressed by NRK cells, only phosphorylated OPN was associated with the cell surface (19,23). Moreover, another study revealed that OPN was detected at >200 kd molecular weight and found to be localized on the surface of NRK cells when covalently crosslinked to fibronectin by a transglutaminase (26). Our results were consistent with that study, since the observations from immunoprecipitation and Western blotting of the FLS suggested that >200-kd OPN was an OPN/fibronectin-crosslinked molecule, and inhibition of a transglutaminase reduced the expression of >200-kd OPN. Considering these facts together, >200-kd OPN could be considered an OPN/fibronectin-covalently crosslinked molecule synthesized by a transglutaminase from 75-kd OPN.

In contrast, >200-kd cell surface OPN was poorly detected in 75-kd OPN-negative FLS. Moreover, overexpression of OPN by 75-kd OPN-negative FLS only increased the expression of 54-kd OPN, and did not induce expression of 75-kd OPN nor did it alter the expression of >200-kd OPN, suggesting that there might be an unknown enzyme in 75-kd OPN-positive FLS that performs the specific modifications of 75-kd OPN necessary for it to associate with the cell surface and to form the >200-kd OPN/fibronectin-crosslinked molecule.

To assess the relevance of 75-kd OPN in RA, we performed cocultures of FLS and B lymphocytes. Previous studies have revealed that FLS-B lymphocyte coculture allows the adhesion of B lymphocytes to FLS, which induces cell-cell interactions between FLS and B lymphocytes and, consequently, increases the production of several cytokines, including IL-6 (9,12), but the mechanism has not been elucidated. We focused on IL-6 production in FLS-B lymphocyte cocultures and found that increased IL-6 production was associated with the existence of 75-kd OPN, which suggested that 75-kd OPN together with cell-cell interactions between FLS and B lymphocytes can enhance IL-6 production. We also revealed that among FLS and B lymphocytes, FLS were the dominant cell type for IL-6 production in coculture, as was shown in experiments involving knockdown of IL-6 in FLS.

To examine whether 75-kd OPN could enhance IL-6 production, we performed overexpression and

knockdown of OPN in FLS, which demonstrated that IL-6 production was increased and decreased in accordance with the increase and decrease of 75-kd OPN and >200-kd OPN, respectively. Among these 2 OPN forms with different molecular weights, >200-kd OPN appeared to enhance IL-6 production, since the transglutaminase inhibitor that reduced the expression of only the >200-kd OPN significantly suppressed IL-6 production in 75-kd OPN-positive FLS-B lymphocyte cocultures (results not shown).

We then analyzed how 75-kd OPN or its crosslinked form, >200-kd OPN, enhanced IL-6 production in FLS-B lymphocyte cocultures. The >200-kd OPN on the cell surface exposed its thrombin-cleaved neopeptide, SVVYGLR (27), a ligand for integrin  $\alpha 9 \beta 1$ , also known as VLA-4, integrin  $\alpha 9 \beta 1$ , and integrin  $\alpha 4 \beta 7$  (30). Considering the fact that VLA-4 is expressed by B lymphocytes and supports adhesion of B lymphocytes to FLS in FLS-B lymphocyte coculture (12), >200-kd OPN was indicated as the ligand for VLA-4, which acts as an adhesion molecule. This idea was supported by the results from our B lymphocyte adhesion assay with a blocking antibody against OPN, and by the fact that the transglutaminase inhibitor also suppressed adhesion of B lymphocytes to FLS (results not shown). Meanwhile,

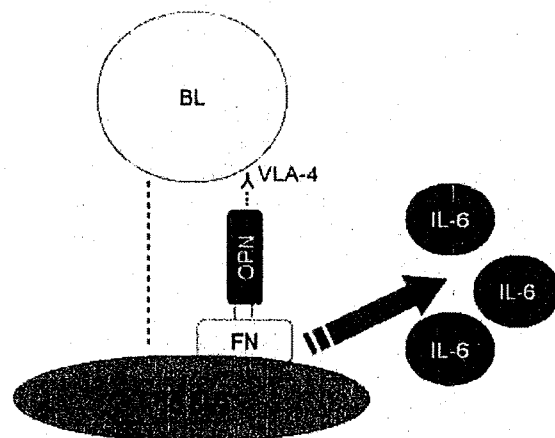


Figure 6. Diagram of the *in vitro* findings, showing cell-cell interactions between fibroblast-like synoviocytes (FLS) and B lymphocytes (BL). RA FLS were characterized by the expression of 75-kd OPN, from which a transglutaminase synthesized the >200-kd OPN/fibronectin (FN)-crosslinked molecule that was localized on the synovial cell surface in its thrombin-cleaved form. This cell surface OPN mediated cell-cell interactions between OPN and B lymphocytes by supporting adhesion of B lymphocytes to FLS through very late activation antigen 4 (VLA-4) and enhanced IL-6 production. An unknown link between FLS and B lymphocytes is also shown. See Figure 5 for other definitions.



integrin  $\alpha 4\beta 7$ , which was also expressed by B lymphocytes, did not mediate such adhesion (12).

VCAM-1, which was expressed by FLS and also supports B lymphocyte adhesion, possibly through VLA-4 (11), was detected in equal amounts among RA and non-RA FLS (results not shown). Therefore, we postulated that integrin  $\alpha 4\beta 7$  and VCAM-1 were not involved in the adhesion of B lymphocytes to FLS or subsequent IL-6 production, and that such adhesion was mediated by >200-kd OPN and VLA-4, which would further initiate the cell-cell interaction between FLS and B lymphocytes, leading to IL-6 production.

Our findings suggested that, in response to these FLS-B lymphocyte interactions, FLS boosted their IL-6 production. Similar studies on cell-cell interactions between FLS and T lymphocytes have shown that lymphocyte function-associated antigen 1, intercellular adhesion molecule 2, and the ezrin/Akt pathway are involved in their interaction, which also enhances IL-6 production (2). Thus, this pathway may also be involved in FLS-B lymphocyte interactions, although further investigation is needed to fully elucidate the mechanism.

In summary, the findings from these *in vitro* experiments showed that RA FLS are characterized by the expression of 75-kd OPN. This was the substrate of a transglutaminase that formed the >200-kd OPN/fibronectin-crosslinked molecule, a molecule localized on the surface of FLS in its thrombin-cleaved form. This surface OPN mediated cell-cell interactions between FLS and B lymphocytes, and enhanced IL-6 production (Figure 6). Moreover, such FLS-B lymphocyte interactions, or interactions between FLS-expressing OPN and B lymphocytes stimulating IL-6 production, appeared to take place *in vivo*, as shown by immunohistochemistry. Taking into account the previously reported findings on the pathogenic significance of IL-6 (45), B lymphocytes (46,47), VLA-4 (48), and the thrombin-cleaved neopeptide of OPN (32,33) in RA, the present study revealed a novel role of OPN in RA.

Regarding the weaknesses of this study, we need to mention the discrepancy in the findings between RA FLS and 75-kd OPN-positive FLS. Since 75-kd OPN was detected in all 10 RA FLS, but also in 3 non-RA FLS, it was not specific to RA FLS. There is no doubt that 75-kd OPN enhanced IL-6 production in FLS-B lymphocyte cocultures and appeared to aggravate chronic inflammation *in vivo*. However, it should not be considered the cause of RA; rather, we could postulate that it is one of several molecules with induced expression in arthritis and is involved in the chronic progression of arthritis by stimulating IL-6 production. This is

supported by the observation that, in the joint tissue of a non-RA donor, the FLS expressed amounts of 75-kd OPN comparable with those in RA FLS, and showed severe synovitis at the time of surgery.

We also have to note that the mechanism involved in the modification of 75-kd OPN has not been defined, and the responsible transglutaminase has not been identified. Further investigations in this area would be required for better understanding of the pathology of RA. Nevertheless, our results show that a specifically modified 75-kd form of OPN was expressed by RA FLS. This form of OPN affected cell-cell interactions between FLS and B lymphocytes by supporting the adhesion of B lymphocytes to FLS. As a result, IL-6 production was enhanced in FLS-B lymphocyte cocultures.

#### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Nakata had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Nakata, Ochi, Yoshikawa.

**Acquisition of data.** Take, Nakata, Nishimoto.

**Analysis and interpretation of data.** Take, Nakata, Hashimoto, Tsuboi, Nishimoto.

#### REFERENCES

1. Huber LC, Distler O, Tarner I, Gay RE, Gay S, Pap T. Synovial fibroblasts: key players in rheumatoid arthritis. *Rheumatology (Oxford)* 2006;45:669-75.
2. Singh K, Colmegna I, He X, Weyand CM, Goronzy JJ. Synovio-cyte stimulation by the LFA-1-intercellular adhesion molecule-2-ezrin-Akt pathway in rheumatoid arthritis. *J Immunol* 2008;180:1971-8.
3. Tran CN, Lundy SK, White PT, Endres JL, Motyl CD, Gupta R, et al. Molecular interactions between T cells and fibroblast-like synoviocytes: role of membrane tumor necrosis factor- $\alpha$  on cytokine-activated T cells. *Am J Pathol* 2007;171:1588-98.
4. Vallejo AN, Yang H, Klimiuk PA, Weyand CM, Goronzy JJ. Synovio-cyte-mediated expansion of inflammatory T cells in rheumatoid synovitis is dependent on CD47-thrombospondin 1 interaction. *J Immunol* 2003;171:1732-40.
5. Chomarat P, Risoan MC, Pin JJ, Banchereau J, Miossec P. Contribution of IL-1, CD14, and CD13 in the increased IL-6 production induced by *in vitro* monocyte-synovio-cyte interactions. *J Immunol* 1995;155:3645-52.
6. Bombara MP, Webb DL, Conrad P, Marlor CW, Sarr T, Ranges GE, et al. Cell contact between T cells and synovial fibroblasts causes induction of adhesion molecules and cytokines. *J Leukoc Biol* 1993;54:399-406.
7. Blue ML, Conrad P, Webb DL, Sarr T, Macaro M. Interacting monocytes and synovio-cytes induce adhesion molecules by a cytokine-regulated process. *Lymphokine Cytokine Res* 1993;12:213-8.
8. Shimaoka Y, Attrep JF, Hirano T, Ishihara K, Suzuki R, Toyosaki T, et al. Nurse-like cells from bone marrow and synovium of patients with rheumatoid arthritis promote survival and enhance function of human B cells. *J Clin Invest* 1998;102:606-18.

9. Takeuchi E, Tomita T, Toyosaki-Maeda T, Kaneko M, Takano H, Hashimoto H, et al. Establishment and characterization of nurse cell-like stromal cell lines from synovial tissues of patients with rheumatoid arthritis. *Arthritis Rheum* 1999;42:221-8.
10. Hayashida K, Shimaoka Y, Ochi T, Lipsky PE. Rheumatoid arthritis synovial stromal cells inhibit apoptosis and up-regulate Bcl-xL expression by B cells in a CD49/CD29-CD106-dependent mechanism. *J Immunol* 2000;164:1110-6.
11. Burger JA, Zvaifler NJ, Tsukada N, Firestein GS, Kipps TJ. Fibroblast-like synoviocytes support B-cell pseudoemperipolesis via a stromal cell-derived factor-1- and CD106 (VCAM-1)-dependent mechanism. *J Clin Invest* 2001;107:305-15.
12. Takeuchi E, Tanaka T, Umemoto E, Tomita T, Shi K, Takahi K, et al. VLA-4-dependent and -independent pathways in cell contact-induced proinflammatory cytokine production by synovial nurse-like cells from rheumatoid arthritis patients. *Arthritis Res* 2002;4:R10-7.
13. Tani-Ishii N, Tsunoda A, Umemoto T. Osteopontin antisense deoxyoligonucleotides inhibit bone resorption by mouse osteoclasts in vitro. *J Periodontol Res* 1997;32:480-6.
14. Dodds RA, Connor JR, James IE, Rykaczewski EL, Appelbaum E, Dul E, et al. Human osteoclasts, not osteoblasts, deposit osteopontin onto resorption surfaces: an in vitro and ex vivo study of remodeling bone. *J Bone Miner Res* 1995;10:1666-80.
15. Horton MA, Nesbit MA, Helfrich MH. Interaction of osteopontin with osteoclast integrins. *Ann N Y Acad Sci* 1995;760:190-200.
16. Lampe MA, Patarca R, Iregui MV, Cantor H. Polyclonal B cell activation by the Eta-1 cytokine and the development of systemic autoimmune disease. *J Immunol* 1991;147:2902-6.
17. Patarca R, Freeman GJ, Singh RP, Wei FY, Durfee T, Blattner F, et al. Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene: definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection. *J Exp Med* 1989;170:145-61.
18. Patarca R, Wei FY, Singh P, Morasso MI, Cantor H. Dysregulated expression of the T cell cytokine Eta-1 in CD4-8- lymphocytes during the development of murine autoimmune disease. *J Exp Med* 1990;172:1177-83.
19. Nemir M, DeVouge MW, Mukherjee BB. Normal rat kidney cells secrete both phosphorylated and nonphosphorylated forms of osteopontin showing different physiological properties. *J Biol Chem* 1989;264:18202-8.
20. Weber GF, Zawadeh S, Hikita S, Kumar VA, Cantor H, Ashkar S. Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. *J Leukoc Biol* 2002;72:752-61.
21. Shanmugam V, Chackalamparampil I, Kundu GC, Mukherjee AB, Mukherjee BB. Altered sialylation of osteopontin prevents its receptor-mediated binding on the surface of oncogenically transformed tsB77 cells. *Biochemistry* 1997;36:5729-38.
22. Christensen B, Kazanek CC, Petersen TE, Rittling SR, Denhardt DT, Sorensen ES. Cell type-specific post-translational modifications of mouse osteopontin are associated with different adhesive properties. *J Biol Chem* 2007;282:19463-72.
23. Singh K, DeVouge MW, Mukherjee BB. Physiological properties and differential glycosylation of phosphorylated and nonphosphorylated forms of osteopontin secreted by normal rat kidney cells. *J Biol Chem* 1990;265:18696-701.
24. He B, Mirza M, Weber GF. An osteopontin splice variant induces anchorage independence in human breast cancer cells. *Oncogene* 2006;25:2192-202.
25. Keykhosravi M, Doherty-Kirby A, Zhang C, Brewer D, Goldberg HA, Hunter GK, et al. Comprehensive identification of post-translational modifications of rat bone osteopontin by mass spectrometry. *Biochemistry* 2005;44:6990-7003.
26. Beninati S, Senger DR, Cordella-Miele E, Mukherjee AB, Chackalamparampil I, Shanmugam V, et al. Osteopontin: its transglutaminase-catalyzed posttranslational modifications and cross-linking to fibronectin. *J Biochem* 1994;115:675-82.
27. Senger DR, Perruzzi CA, Papadopoulos-Sergiou A, Van de Water L. Adhesive properties of osteopontin: regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. *Mol Biol Cell* 1994;5:565-74.
28. Agnihotri R, Crawford HC, Haro H, Matrisian LM, Havrda MC, Liaw L. Osteopontin, a novel substrate for matrix metalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin). *J Biol Chem* 2001;276:28261-7.
29. Yumoto K, Ishijima M, Rittling SR, Tsuji K, Tsuchiya Y, Kon S, et al. Osteopontin deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice. *Proc Natl Acad Sci U S A* 2002;99:4556-61.
30. Bayless KJ, Meininger GA, Scholtz JM, Davis GE. Osteopontin is a ligand for the  $\alpha 4 \beta 1$  integrin. *J Cell Sci* 1998;111:1165-74.
31. Yokosaki Y, Matsuura N, Sasaki T, Murakami I, Schneider H, Higashiyama S, et al. The integrin  $\alpha(9)\beta(1)$  binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. *J Biol Chem* 1999;274:36328-34.
32. Yamamoto N, Sakai F, Kon S, Morimoto J, Kimura C, Yamazaki H, et al. Essential role of the cryptic epitope SLAYGLR within osteopontin in a murine model of rheumatoid arthritis. *J Clin Invest* 2003;112:181-8.
33. Yamamoto N, Nakashima T, Torikai M, Naruse T, Morimoto J, Kon S, et al. Successful treatment of collagen-induced arthritis in non-human primates by chimeric anti-osteopontin antibody. *Int Immunopharmacol* 2007;7:1460-70.
34. Petrow PK, Hummel KM, Schedel J, Franz JK, Klein CL, Muller-Ladner U, et al. Expression of osteopontin messenger RNA and protein in rheumatoid arthritis: effects of osteopontin on the release of collagenase 1 from articular chondrocytes and synovial fibroblasts. *Arthritis Rheum* 2000;43:1597-605.
35. Ohshima S, Yamaguchi N, Nishioka K, Mima T, Ishii T, Umeshita-Sasai M, et al. Enhanced local production of osteopontin in rheumatoid joints. *J Rheumatol* 2002;29:2061-7.
36. Hasegawa M, Nakoshi Y, Iino T, Sudo A, Segawa T, Maeda M, et al. Thrombin-cleaved osteopontin in synovial fluid of subjects with rheumatoid arthritis. *J Rheumatol* 2009;36:240-5.
37. Xu G, Nie H, Li N, Zheng W, Zhang D, Feng G, et al. Role of osteopontin in amplification and perpetuation of rheumatoid synovitis. *J Clin Invest* 2005;115:1060-7.
38. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315-24.
39. Wiznerowicz M, Trono D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J Virol* 2003;77:8957-61.
40. Kita K, Kimura T, Nakamura N, Yoshikawa H, Nakano T. PI3K/Akt signaling as a key regulatory pathway for chondrocyte terminal differentiation. *Genes Cells* 2008;13:839-50.
41. Tsuboi H, Udagawa N, Hashimoto J, Yoshikawa H, Takahashi N, Ochi T. Nurse-like cells from patients with rheumatoid arthritis support the survival of osteoclast precursors via macrophage colony-stimulating factor production. *Arthritis Rheum* 2005;52:3819-28.
42. Kon S, Yokosaki Y, Maeda M, Segawa T, Horikoshi Y, Tsukagoshi H, et al. Mapping of functional epitopes of osteopontin by monoclonal antibodies raised against defined internal sequences. *J Cell Biochem* 2002;84:420-32.
43. Senger DR, Brown LF, Perruzzi CA, Papadopoulos-Sergiou A, Van de Water L. Osteopontin at the tumor/host interface: func-

- tional regulation by thrombin-cleavage and consequences for cell adhesion. *Ann N Y Acad Sci* 1995;760:83-100.
44. Hata H, Sakaguchi N, Yoshitomi H, Iwakura Y, Sekikawa K, Azuma Y, et al. Distinct contribution of IL-6, TNF- $\alpha$ , IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J Clin Invest* 2004;114:582-8.
45. Nishimoto N, Kishimoto T. Interleukin 6: from bench to bedside. *Nat Clin Pract Rheumatol* 2006;2:619-26.
46. Svensson L, Jirholt J, Holmdahl R, Jansson L. B cell-deficient mice do not develop type II collagen-induced arthritis (CIA). *Clin Exp Immunol* 1998;111:521-6.
47. Dorner T, Lipsky PE. B-cell targeting: a novel approach to immune intervention today and tomorrow. *Expert Opin Biol Ther* 2007;7:1287-99.
48. Raychaudhuri A, Chou M, Weetall M, Jeng AY. Blockade of integrin VLA-4 prevents inflammation and matrix metalloproteinase expression in a murine model of accelerated collagen-induced arthritis. *Inflammation* 2003;27:107-13.



ELSEVIER

Contents lists available at ScienceDirect

Bone

journal homepage: [www.elsevier.com/locate/bone](http://www.elsevier.com/locate/bone)

## High oxygen tension prolongs the survival of osteoclast precursors via macrophage colony-stimulating factor

Naomi Yamasaki, Hideki Tsuboi, Makoto Hirao, Akihide Nampei, Hideki Yoshikawa, Jun Hashimoto\*

Department of Orthopedics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

### ARTICLE INFO

#### Article history:

Received 20 May 2008

Revised 9 September 2008

Accepted 17 September 2008

Available online 11 October 2008

Edited by: J. Aubin

#### Keywords:

Destructive bone lesions

Hyperoxia

Macrophage colony-stimulating factor

Prolonged survival

Osteoclast precursor

### ABSTRACT

The oxygen tension affects the function, differentiation, and transformation of various cells, including bone cells. In pathological conditions such as rheumatoid arthritis (RA), rapidly destructive arthropathy, and primary or metastatic tumors, severe bone destruction or osteolysis occurs. Abundant blood vessels are often observed around these destructive lesions. At such sites, we have confirmed the increased production of reactive oxygen species (ROS) induced by a high oxygen tension and/or oxidative stress, as well as numerous osteoclasts detectable by immunohistochemistry. These findings suggest that osteoclasts are influenced by the high oxygen tension in pathological bone lesions because the zone around blood vessels has a relatively high oxygen tension. In this study, we investigated the effects of oxygen tension on osteoclastogenesis by culturing human CD14-positive cells (osteoclast precursors) with or without osteoblast-like supporting cells (Saos-4/3 cells) under a normal oxygen tension (20% O<sub>2</sub>) or a high oxygen tension (40% O<sub>2</sub>). A high oxygen tension markedly prolonged the duration of osteoclast precursor formation in the presence of supporting cells, and also markedly and persistently increased the production of macrophage colony stimulating factor (M-CSF) by supporting cells. Furthermore, we found an increase of cells expressing M-CSF and cells positive for tartrate-resistant acid phosphatase (TRAP) in hypervascular destructive bone lesions of RA patients where ROS were also abundant.

© 2008 Elsevier Inc. All rights reserved.

### Introduction

An increase in the number and activity of locally recruited osteoclasts is involved in various pathological states [1–3], including inflammatory arthritides such as rheumatoid arthritis (RA) and rapidly destructive arthropathy, as well as osteolytic bone tumors.

It has been demonstrated by both histological and clinical studies that these destructive bone lesions are accompanied by abundant neovascularization [4,5]. Rooney and Koizumi have shown that marked proliferation of blood vessels and the presence of neovascularization are typical histological features of RA based on the histological Rooney score [6–8]. Clinical studies using dynamic MRI have also demonstrated the enhancement of destructive bone lesions in patients with RA or bone tumors, which means that these lesions are highly vascular [9,10], and it has been suggested that the microvessel density is associated with the extent of bone destruction [3]. It has also been reported that multinucleated osteoclasts show a marked increase around regions of angiogenesis [11]. Furthermore, a recent study of the perivascular environment using a hypoxic probe demonstrated that the area around vessels is not hypoxic although hypoxia occurs further away from vessels [44]. The difference of oxygen tension between the

perivascular area and the more distant region raises the question of whether a relatively high oxygen tension is associated with the recruitment of osteoclasts to sites of angiogenesis.

Recent studies have shown that the oxygen tension regulates the formation and differentiation of various bone cells, including chondrocytes, osteoblasts, osteocytes, and osteoclasts [12–15]. Such reports indicate the important influence of oxygen tension on cell biology. According to Arnett et al., hypoxia reduces the lifespan of osteoclasts, but increases their proliferation and resorption [15], while Fukuoka et al. reported that hypoxia promotes osteoclast differentiation via insulin growth factor 2 (IGF2) *in vitro* [14].

These reports raise the question of whether a high oxygen tension in hypervascular regions affect osteoclastogenesis. In this study, we investigated the influence of oxygen tension on osteoclastogenesis by culturing CD14+ cells alone or with human osteoblast-like cells.

### Materials and methods

#### Chemicals

Recombinant human macrophage colony-stimulating factor (M-CSF) (Leukoprol) was obtained from Kyowa Hakko (Tokyo, Japan). Recombinant soluble receptor activator of nuclear factor- $\kappa$ B (RANKL) and osteoprotegerin (OPG) were purchased from PeproTech (London, UK). A neutralizing antibody for human M-CSF was obtained

\* Corresponding author. Fax: +81 6 6879 3559.

E-mail address: [junha@ort.med.osaka-u.ac.jp](mailto:junha@ort.med.osaka-u.ac.jp) (J. Hashimoto).