5'-TAATCTGGACTGCTTGTGGC-3'). Briefly, cDNA was obtained from 100 ng of total RNA extracted from each cell line using the Trizol reagent (Life Technologies, Rockville, MD, USA). Then, PCR was performed using the above OPN-specific primers as follows: denaturing for 1 min at 94°C, annealing for 1 min at 57°C, extension for 2 min at 72°C with 30 cycles. The cDNA of OPN was used as a positive control. The primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (the sense primer 5'-AATTACCACAACCCCTACAAAC-3'; the antisense primer 5'-CAACTCTGCAACATCTTCCTC-3') were used as an internal control. The PCR products were separated by 2% agarose gel electrophoresis.

Western blotting analysis for OPN. We examined spontaneous OPN production by Western blotting analysis in various B-cell malignant cell lines as described earlier. Briefly, each cell line was cultured for 3 d *in vitro*, and the culture supernatant was collected. Samples of 20 µl of the culture supernatant from each cell line were separated using 4–20% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for 4 h. After the separation, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA) overnight at 4°C. The membrane was incubated with blocking buffer (10% skimmed milk in 0·1% Tween 20 containing PBS) for 1 h. After washing, the membrane was incubated with rabbit anti-human OPN antibody, OPN2, generated by our laboratory (Kon *et al*, 2000), overnight at 4°C. After washing, the membrane was incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, USA) for 1 h at room temperature. After washing, the membrane was developed with Renaissance reagent (NEN Life Science Products, Boston, MA, USA) and exposed to film overnight. The recombinant OPN was used as a positive control.

Enzyme-linked immunosorbent assay (ELISA) for OPN. We measured OPN concentrations in plasma from 30 patients with MM using a sandwich ELISA kit for human OPN (Immuno-Biological Laboratories, Gunma, Japan), which was originally established by our laboratory (Kon *et al*, 2000). In this sandwich ELISA system, two distinct anti-human OPN antibodies, OPN1 and OPN3, were used. OPN1 is a rabbit polyclonal anti-human OPN antibody and was used as the coating antibody. OPN3 is a mouse monoclonal anti-human OPN antibody and was used as the detecting antibody. As a control, plasma from 21 patients with MGUS and 30 healthy volunteers was used. We also measured OPN concentrations in the culture supernatant of BMCs from 6 MM (three overt and three smouldering MM) patients and two MGUS patients. Briefly, freshly prepared BMCs (2 × 10⁶/ml) were cultured for 2 d in Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal calf serum (FCS), and then the culture supernatant was collected and subjected to the ELISA for OPN described earlier.

Statistical analysis. Values were expressed as means ± standard error of the mean (SEM). The Mann–Whitney U-test was used to assess significance. P-values less than 0·05 were considered significant.

RESULTS

BMCs from the patients with MM actively produce OPN
To examine whether myeloma cells produce OPN, OPN expression was assessed in BMCs freshly prepared from three patients with overt MM by immunocytochemistry. As shown in Fig 1, abundant OPN was detected as a brown stain in the BMCs from all patients with overt MM, showing typical myeloma cell morphology. However, as shown in Fig 2, no OPN was detected in BMCs from the patients with other haematological diseases, including MGUS. To confirm that BMCs from the patients with MM produce OPN, we measured OPN concentrations in the culture supernatant. As a result, freshly prepared BMCs from overt MM patients produced more OPN than those from either smouldering MM or MGUS patients (Table I). These findings suggest that myeloma cells actively produce OPN *in vivo*.

Myeloma cell lines express and produce OPN
Next, we examined both the expression and the production of OPN in various B-cell malignant cell lines by RT-PCR and Western blotting analysis respectively. As a result of RT-PCR analysis (Fig 3A) in two myeloma cell lines, RPMI 8226 and U266, we detected an obvious band. One B-cell line, Daudi, derived from a patient with Burkitt's lymphoma also showed a weak OPN band. However, no other B-cell malignant cell lines tested in this study showed any OPN band. In Western blotting analysis (Fig 3B), we observed OPN bands only in RPMI 8226, but not in any other B-cell malignant cell lines. This suggests that the myeloma cell lines, RPMI 8226 and U266, spontaneously produce OPN. In addition, it is suggested that Daudi, a lymphoblastic cell line derived from Burkitt's lymphoma, also produces small amounts of OPN.

Plasma OPN levels are elevated in the patients with MM
Next, we measured plasma OPN levels in patients with MM (*n* = 30) and MGUS (*n* = 21) and the healthy volunteers (*n* = 30) by ELISA. The OPN plasma levels of the patients with MM were found to be significantly higher than those of the patients with MGUS and the healthy volunteers (Fig 4).

Plasma OPN levels (mean ± SEM) were 1053 ± 177 ng/ml for MM patients, 355 ± 46 ng/ml for MGUS patients and 309 ± 34 ng/ml for healthy controls (MM versus MGUS or healthy controls P <0·05).

Plasma OPN levels correlate with the progression of MM
To clarify whether the OPN levels correlated with the progression of MM, plasma OPN levels were compared among patients with MM grouped into three clinical stages, stage I (smouldering MM; *n* = 6), stage III (overt MM, inactive; *n* = 12) and stage III (overt MM, active; *n* = 12) according to the Durie–Salmon staging system (Durie & Salmon, 1975). The plasma OPN levels in the patients with overt MM (either inactive or active) were found to be significantly higher than those of the patients with smouldering MM (Table II). Moreover, the plasma OPN levels in the patients with overt MM (active) were
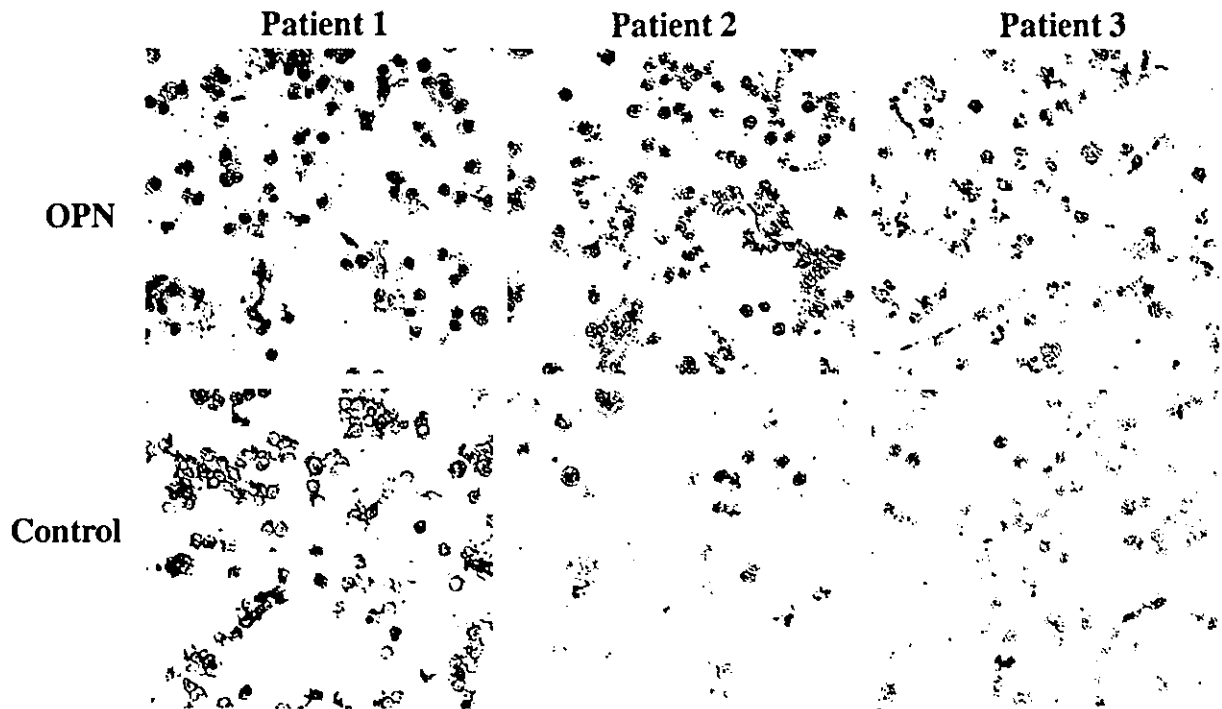
Fig 1. Immunocytochemistry for OPN in BMCs from patients with overt MM. We examined OPN expression in BMCs freshly prepared from three patients with overt MM by immunocytochemistry using the avidin–biotin complex method as described in Patients and methods. Most BMCs, which showed a typical myeloma cell morphology, stained positively for a mouse IgG monoclonal anti-human OPN antibody, 4C1, visible as brown stains (top). However, no staining was observed with the control antibody, irrelevant mouse IgG (bottom). Original magnification ×100. Patients were distinct from those shown in Table I.
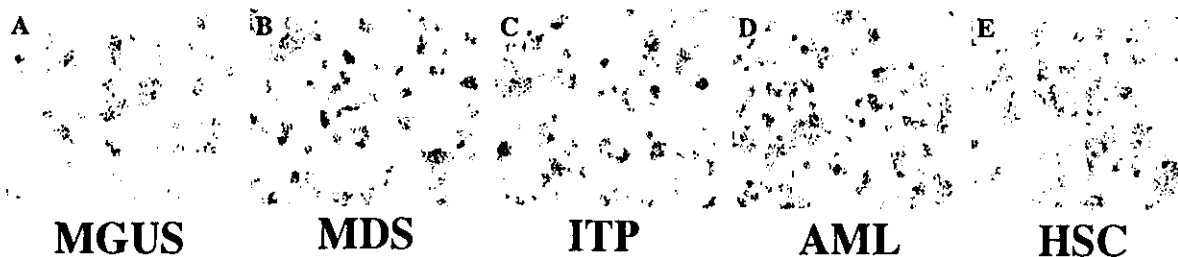

Fig 2. Immunocytochemistry for OPN in BMCs from patients with different haematological diseases. We examined OPN expression in BMCs freshly prepared from five patients with different haematological diseases by immunocytochemistry using a mouse IgG monoclonal anti-human OPN antibody, 4C1, as described in Patients and methods. None of the BMCs from the patients with MGUS (A), myelodysplastic syndrome (MDS; B), idiopathic thrombocytopenic purpura (ITP; C), acute myelocytic leukaemia (AML; D) or hereditary spherocytosis (HSC; E) showed positive staining for OPN. Original magnification ×100.

significantly higher than those of the patients with overt MM (inactive).

Plasma OPN levels correlate with the bone disease of MM
First, to clarify whether OPN levels correlated with the bone disease of MM, plasma OPN levels were compared between patients with MM grouped into two subpopulations. One was a group of patients who never manifested bone pain. The other was a group of patients who manifested obvious bone pain. The plasma OPN levels in the patients who

manifested obvious bone pain were found to be significantly higher than those in the patients who never manifested bone pain (Table II).

Secondly, plasma OPN levels were also compared between patients with MM grouped into two subpopulations. One was a group of patients who did not show any bone-resorbing lesions by magnetic resonance imaging (MRI). The other was a group of patients who showed obvious bone-resorbing lesions by MRI. The plasma OPN levels in the patients who showed obvious bone-resorbing

Table I. OPN concentrations of the culture supernatant of BMCs.

Patient	Disease	OPN (ng/ml)
1	MM (overt)	315·6
2	MM (overt)	162·7
3	MM (overt)	80·5
4	MM (smouldering)	48·3
5	MM (smouldering)	16·1
6	MM (smouldering)	49·8
7	MGUS	67·3
8	MGUS	56·1

OPN concentrations were measured in the culture supernatant of BMCs from 6 MM (three overt MM and three smouldering MM) and two MGUS patients. Freshly prepared BMCs (2 × 10/ml) were cultured for 2 d in RPMI 1640 with 10% FCS, and the culture supernatant was collected and subjected to an ELISA for OPN as described in Patients and Methods.

lesions were found to be significantly higher than those in patients who showed no lesions (Table II).

Thus, the plasma OPN levels in the patients with MM correlate significantly with bone disease in MM.

DISCUSSION

In this study, we first investigated the relationship between OPN in MM and demonstrated several new findings, suggesting the involvement of OPN in bone destruction of MM.

First, in the immunocytochemical study, we demonstrated that BMCs from patients with overt MM were positive for

OPN, whereas OPN was not detected in BMCs from patients with any other haematological diseases, including MGUS. Moreover, we also demonstrated that cultured BMCs freshly prepared from the patients with overt MM spontaneously produced more OPN than those from patients with either smouldering MM or MGUS. In addition, we demonstrated that two myeloma cell lines, RPMI 8226 and U266, spontaneously expressed OPN mRNA using RT-PCR, and that abundant OPN was detected in the culture supernatant of RPMI 8226 by Western blotting. These results suggest that myeloma cells produce OPN *in vivo*.

Previous histological studies have indicated that locally acting factors produced by myeloma cells play a crucial role in osteoclastic bone resorption seen in patients with MM (Mundy *et al*. 1974; Bataille *et al*. 1992). To date, these OAFs have not been clearly identified, although their activity is thought to be mediated by several cytokines, including IL-1β (Cozzolino *et al*, 1989), lymphotoxin (TNFβ) (Bertolini *et al*, 1986; Garrett *et al*, 1987) and IL-6 (Bataille *et al*, 1989; Bataille & Klein, 1991). An interesting report has been published regarding the association of proinflammatory cytokines with OPN expression (Matsumoto *et al*, 1998). In this report, using the subtraction cloning method, the induction of NF-IL6, a CCAAT/enhancer-binding protein family of transcription factors, was shown to enhance OPN gene expression. As NF-IL6 is one of the key molecules on the signalling pathway of proinflammatory cytokines, such as IL-1 and IL-6, OPN may be induced in part by such proinflammatory cytokines.

On the other hand, the mechanism for osteoclastic bone resorption has been clarified at the molecular level in studies using knockout mice (Soriano *et al*, 1991; Grigoriadis *et al*, 1994; Tondravi *et al*, 1997). The attachment of activated osteoclasts to the bone surface is a critical step in
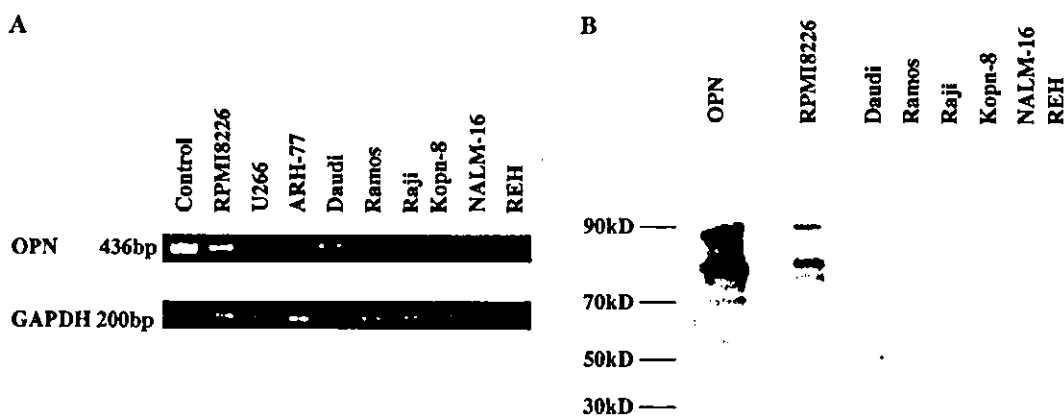

Fig 3. RT-PCR and Western blotting analysis for OPN in various B-cell malignant cell lines. We examined both expression and production of OPN in various B-cell malignant cell lines (RPMI 8226, U266, ARH77: myeloma cell lines; Daudi, Ramos, Raji: lymphoblastic B-cell lines from Burkitt's lymphoma; Kopn-8: a pre-B-cell line from human leukaemia; NALM-16, REH: prepre-B-cell lines from human leukaemia) by RT-PCR and Western blotting analysis as described in Patients and methods. The characteristics of the B-cell precursor cell lines used in this study are described in Matsuo & Drexler (1998). (A) RT-PCR analysis. RPMI 8226 showed a strong band. U266 and Daudi also showed a weak band compared with that of RPMI 8226. However, an OPN band was not detected with any of the other B-cell lines tested in this study. (B) Western blotting analysis. We observed OPN bands only in RPMI 8226, but not in any other B-cell malignant cell lines.
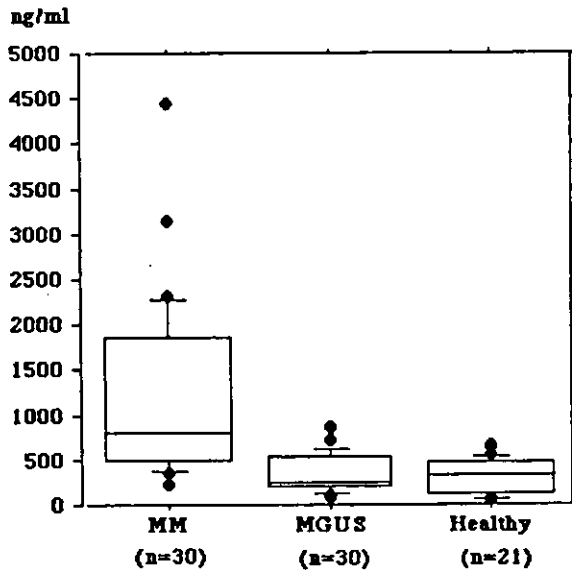
ng/ml

Fig 4. Plasma OPN levels in MM patients, MGUS patients and healthy volunteers. We measured the plasma OPN levels in the patients with MM (n = 30) and MGUS (n = 21) and the healthy volunteers (n = 30) by ELISA as described in Patients and methods. The OPN plasma levels of the patients with MM were significantly higher than those of the patients with MGUS ($P < 0.05$) and the healthy volunteers ($P < 0.05$). The upper and lower boundaries of the box indicate the 75th and 25th percentiles, respectively, and the line in the box represents the median. Whiskers are drawn to the nearest value that was not beyond a standard span from the quartiles; points beyond (outliers) are drawn individually, where the standard span 1·5 × (interquartile range).

osteoclastic bone resorption, and this step is mediated by ligation of OPN with $\alpha v \beta 3$ integrin (Reinholt et al, 1990). Moreover, the blockade of this step inhibits osteoclastic bone resorption (Crippes et al, 1996; McHugh et al, 2000), suggesting that OPN is a key factor in the final process of osteoclastic bone resorption. However, the role of OPN in the other process of osteoclastogenesis has not been fully understood. Recent investigations strongly suggest the importance of the receptor activator of NF-κB ligand (RANKL)/receptor activator of NF-κB (RANK)/osteoprotegerin (OPG) system in some osteoclastic bone-resorbing diseases, including MM (Croucher et al, 2001), as well as normal osteoclast development. Although there have been few studies conducted in this area, there is one interesting report suggesting association of OPN with the RANKL/RANK/OPG system (Ihara et al, 2001). In this report, the enhancement of Ca release from the bones by parathyroid hormone (PTH) was not observed in OPN-deficient mice. Moreover, the soluble RANKL with macrophage colony-stimulating factor did not enhance Ca release from the bones in OPN-deficient mice. As the enhancement of Ca release from the bones by PTH is mediated by RANKL, there is a possible association of OPN with the RANKL/RANK/OPG system in the bone resorption process. In addition, there is a report that human myeloma cells produce PTH-related protein (PTH-rP) (Otsuki et al, 2001). Taken

Table II. Plasma OPN levels in patients with MM at different clinical status.

Patients with MM	Plasma OPN levels (ng/ml)
Stages and disease activity of MM	
Stage I (smouldering MM) (n = 6)	383 ± 43
Stage III (overt MM, inactive) (n = 12)	816 ± 134*
Stage III (overt MM, active) (n = 12)	1991 ± 406†
Bone disease of MM	
Bone pain (−) (n = 17)	776 ± 160
Bone pain (+) (n = 13)	1822 ± 299‡
Bone-resorbing lesions (−) (n = 9)	486 ± 60
Bone-resorbing lesions (+) (n = 21)	1498 ± 222§

The plasma OPN levels were compared in MM patients who differed with respect to clinical status (stages and disease activity, findings of bone diseases). The patients with MM are grouped into three clinical stages (stage 1, stage III/inactive, stage III/active), according to the Durie–Salmon staging system (Durie and Salmon, 1975). The patients are also divided into two groups according to the findings for bone diseases, such as the manifestation of bone pain or detection of bone-resorbing lesions by MRI. The plasma OPN levels are presented as the mean ± SEM.
*Stage III/inactive versus stage I, $P < 0.05$.
†Stage III/active versus stage I, stage III/inactive, $P < 0.01$.
‡Bone pain (+) versus bone pain (−), $P < 0.05$.
§Bone-resorbing lesions (+) versus bone-resorbing lesions (−), $P < 0.01$.

together, these suggest that the osteoclastogenesis through RANKL and PTH-rP in MM may be associated with OPN.

On the other hand, we and other investigators reported recently that OPN plays a significant role in other osteoclastic bone-resorbing pathological states (or diseases), including rheumatoid arthritis (Petrow et al, 2000; Ohshima et al, 2002a,b; Yumoto et al, 2002) and post-menopausal osteoporosis (Yoshitake et al, 1999). Therefore, the present finding that myeloma cells produce abundant OPN in vivo suggests that the enhanced osteoclastic bone resorption in MM might be associated with OPN produced by myeloma cells adjacent to the bone-resorbing lesions, although further experiments are required to show direct evidence.

The Western blotting and RT-PCR studies revealed that not only myeloma cell lines but also a B-cell line, Daudi, derived from Burkitt's lymphoma, produce small amounts of OPN. Considering the finding that increased osteoclastic activity was observed adjacent to tumour cell masses in Burkitt's lymphoma (Adatia, 1968), OPN may also play roles in bone destruction in Burkitt's lymphoma.

Secondly, we demonstrated that the plasma OPN levels of patients with MM were significantly higher than those of patients with MGUS or smouldering MM, or the healthy volunteers by the ELISA study. The diagnosis of MM is usually made by a classic triad, such as marrow plasmacytosis (> 10%), lytic bone lesions and a serum and/or urine M component. The diagnosis of MM, especially the

differential diagnosis between MGUS or smouldering MM, is important, because an unsupported diagnosis of this disease in patients with MGUS or smouldering MM leads to unnecessary treatment and needless complications and, conversely, prompt diagnosis and treatment of MM prevent morbidity and mortality. To date, however, no single test distinguishes MM from MGUS or smouldering MM, although some ancillary tests, such as the plasma cell labelling index (PCLI), plasma cell counts in BM and a radiographic bone survey, are helpful. The present finding that the plasma OPN levels of MM patients were significantly higher than those of patients with MGUS or smouldering MM, or of the healthy volunteers, may be useful for the diagnosis of MM, particularly discrimination of MM from either MGUS or smouldering MM.

Thirdly, we also demonstrated that the plasma OPN levels of the patients with MM correlated with both the progression (or stages) and bone destruction of the disease. Although the Durie–Salmon staging system is a useful means of assessing tumour burden, the prognostic significance of staging has been poorly reproducible, partly because of subjectivity used in the description of bone lesions. The present findings suggest that the plasma OPN levels may be a useful biomarker for both assessment of bone destruction and prognosis of MM, although further investigations are necessary.

In conclusion, the present findings suggest that OPN may be involved in osteoclastic bone resorption in MM, and that plasma OPN levels may be a useful biomarker not only for the differential diagnosis from MGUS but also for the assessment of bone destruction (or prognosis) in MM.

ACKNOWLEDGMENTS

REFERENCES

Adatia, A.K. (1968) Dental tissues and Burkitt's tumor. *Oral Surgery*, 25, 221–234.

Bataille, R. & Klein, B. (1991) The bone resorbing activity of interleukin-6. *Journal of Bone and Mineral Research*, 9, 1143–1146.

Bataille, R., Jourdan, M., Zhang, X.G. & Klein, B. (1989) Serum levels of interleukin-6, a potent myeloma growth factor, as a reflection of disease severity in plasma cell dyscrasias. *Journal of Clinical Investigation*, 84, 2008–2011.

Bataille, R., Chappard, D. & Klein, B. (1992) Mechanisms of bone lesions in multiple myeloma. *Hematology/Oncology Clinics of North America*, 6, 285–295.

Bertolini, D.R., Nedwin, G.E., Bringam, T.S., Smith, D.D. & Mundy, G.R. (1986) Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumor necrosis factors. *Nature*, 319, 516–518.

Billadeau, D., Van Ness, B., Kimlinger, T., Kyle, R.A., Therneau, T.M., Greipp, P.R. & Witzig, T.E. (1996) Clonal circulating cells are common in plasma cell proliferative disorders: a comparison of monoclonal gammopathy of undetermined significance,

smoldering multiple myeloma, and active myeloma. *Blood*, 88, 289–296.

Cozzolino, F., Torcia, M., Aldinucci, D., Rubartelli, A., Miliani, A., Shaw, A.R., Lansdorp, P.M. & DiGuglielmo, R. (1989) Production of interleukin-1 by bone marrow cells. *Blood*, 74, 380–387.

Crippes, B.A., Engleman, V.W., Settle, S.L., Delarco, J., Ornberg, R.L., Helfrich, M.H., Horton, M.A. & Nickols, G.A. (1996) Antibody to β3 integrin inhibits osteoclast-mediated bone resorption in the thyroparathyroidectomized rat. *Endocrinology*, 137, 918–924.

Croucher, P.I., Shipman, C.M., Lippitt, J. & Perry, M. (2001) Osteoprotegerin inhibits the development of osteolytic bone disease in multiple myeloma. *Blood*, 98, 3534–3540.

Denhardt, D.T. & Gou, X. (1993) Osteopontin: a protein with diverse functions. *FASEB Journal*, 7, 1475–1482.

Durie, B.G. & Salmon, S.E. (1975) A clinical staging system for multiple myeloma. Correlation of measured myeloma cell mass with presenting clinical features, response to treatment, and survival. *Cancer*, 36, 842–854.

Durie, B., Salmon, S.E. & Mundy, G.R. (1981) Relation of osteoclast activating factor production to extent of bone disease in multiple myeloma. *British Journal of Haematology*, 47, 21–30.

Garrett, I.R., Durie, B.G., Nedwin, G.E., Gillespie, A., Bringman, T., Sabatini, M., Bertolini, D.R. & Mundy, G.R. (1987) Production of lymphotoxin, a bone-resorbing cytokine, by cultured human myeloma cells. *New England Journal of Medicine*, 317, 526–532.

Grigoriadis, A.E., Wang, Z.Q., Cecchini, M.G., Hofstetter, W., Felix, R., Fleisch, H.A. & Wagner, E.F. (1994) C-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science*, 266, 443–448.

Ihara, H., Denhardt, D.T., Furuya, K., Yamashita, T., Muguruma, Y., Tsuji, K., Hruska, K.A., Higashio, K., Enomoto, S., Nifuji, A., Rittling, S.R. & Noda, M. (2001) Parathyroid hormone-induced bone resorption does not occur in the absence of osteopontin. *Journal of Biological Chemistry*, 276, 13065–13071.

Kon, S., Maeda, M., Segawa, T., Hagiwara, Y., Horikoshi, Y., Chikuma, S., Tanaka, K., Rashid, M.H., Inobe, M., Chanbers, A.F. & Uede, T. (2000) Antibodies to different peptides in osteopontin reveal complexities in the variants secreted forms. *Journal of Cell Biochemistry*, 77, 487–498.

Kon, S., Yokosaki, Y., Maeda, M., Segawa, T., Horikoshi, Y., Tsukagoshi, H., Rashid, M.H., Morimoto, J., Inobe, M., Shijubo, N., Chambers, A.F. & Uede, T. (2002) Mapping of functional epitopes of osteopontin by monoclonal antibodies raised against defined internal sequences. *Journal of Cell Biochemistry*, 84, 420–432.

Kyle, R.A. (1992) Diagnostic criteria of multiple myeloma. *Hematology/Oncology Clinics of North America*, 6, 347–358.

Kyle, R.A. & Lust, J.A. (1989) Monoclonal gammopathies of undetermined significance. *Seminars in Hematology*, 26, 176–200.

McHugh, K.P., Hodivala-Dilke, K., Zheng, M.H., Namba, N., Law, J., Novack, D., Feng, X., Ross, F.P., Hynes, R.O. & Teitelbaum, S.L. (2000) Mice lacking beta 3 integrins are osteosclerotic because of dysfunctional osteoclasts. *Journal of Clinical Investigation*, 105, 433–440.

Matsumoto, M., Sakao, Y. & Akira, S. (1998) Inducible expression of nuclear factor IL-6 increase endogenous gene expression of macrophage inflammatory protein-α, osteopontin, and CD14 in a monocytic leukemia cell line. *International Immunology*, 10, 1825–1835.

Matsuo, Y. & Drexler, H.G. (1998) Establishment and characterization of human B cell precursor-leukemia cell lines. *Leukemia Research*, 22, 567–579.

Miyauchi, A., Alvarez, J., Greenfield, E.M., Teti, A., Grano, M., Colucci, S., Zambonin-Zallone, A., Ross, F.P., Teitelbaum, S.L. & Cheresh, D. (1991) Recognition of osteopontin and related peptides by αvβ3 integrin stimulate cell signals in osteoclasts. *Journal of Biological Chemistry*, **266**, 20369–20374.

Mundy, G.R., Raisz, L.G., Cooper, R.A., Schechter, G.P. & Salmon, S.E. (1974) Evidence for the secretion of an osteoclast stimulating factor in myeloma. *New England Journal of Medicine*, **29**, 1041–1046.

Ohshima, S., Kobayashi, H., Yamaguchi, N., Nishioka, K., Umeshita-Sasai, M., Mima, T., Nomura, S., Kon, S., Inobe, M., Uede, T. & Saeki, Y. (2002a) Expression of osteopontin at sites of bone erosion in a murine experimental arthritis model of collagen-induced arthritis: possible involvement of osteopontin in bone destruction in arthritis. *Arthritis and Rheumatism*, **46**, 1094–1101.

Ohshima, S., Yamaguchi, N., Nishioka, K., Mima, T., Ishii, T., Umeshita-Sasai, M., Kobayashi, H., Shimizu, M., Katada, Y., Wakitani, S., Murata, N., Nomura, S., Matsuno, H., Katayama, R., Kon, S., Inobe, M., Uede, T., Kawase, I. & Saeki, Y. (2002b) Enhanced local production of osteopontin in rheumatoid joints. *Journal of Rheumatology*, **29**, 2061–2067.

Oldberg, A., Franzen, A. & Heinegard, D. (1986) Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell binding sequence. *Proceedings of the National Academy of Science of the USA*, **83**, 8819–8823.

Otsuki, T., Yamada, O., Kurebayashi, J., Sakaguchi, H., Yata, K., Uno, M., Oka, T., Yawata, Y. & Ueki, A. (2001) Expression and in vitro modification of parathyroid hormone-related protein (PTH-rP) and PTH/PTH-rP receptor in human myeloma cells. *Leukemia and Lymphoma*, **41**, 397–409.

Petrow, P.K., Hummel, K.M., Schedel, J., Franz, J.K., Klein, C.L., Muller-Ladner, U., Kriegsmann, J., Chang, P.L., Prince, C.W., Gay, R.E. & Gay, S. (2000) Expression of osteopontin messenger RNA and protein in rheumatoid arthritis. *Arthritis and Rheumatism*, **43**, 1597–1605.

Prince, C.W., Oosawa, T., Butler, W.T., Tomana, M., Bhown, M. & Schrohenloher, R.E. (1987) Isolation, characterization, and biosynthesis of a phosphorylated glycoprotein from rat bone. *Journal of Biological Chemistry*, **262**, 2900–2907.

Reinholt, F.P., Hultenby, K., Oldberg, A. & Heinegard, D. (1990) Osteopontin – a possible anchor of osteoclast to bone. *Proceedings of the National Academy of Science of the USA*, **87**, 4473–4475.

Roodman, G.D. (1997) Mechanisms of bone lesion in multiple myeloma and lymphoma. *Cancer*, **80** (Suppl.), 1557–1563.

Soriano, P., Montgomery, C., Geske, R. & Bradley, A. (1991) Targeted disruption of the C-src proto-oncogene leads to osteopetrosis in mice. *Cell*, **64**, 693–702.

Tondravi, M.M., McKercher, S.R., Anderson, K., Erdmann, J.M., Quiroz, M., Maki, R. & Teitelbaum, S.L. (1997) Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature*, **386**, 81–84.

Yoshitake, H., Rittling, S.R., Denhardt, D.T. & Noda, M. (1999) Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption. *Proceedings of the National Academy of Science of the USA*, **96**, 81560–81560.

Yumoto, K., Ishigami, M., Rittling, S.R., Tsuji, K., Tsuchiya, Y., Kon, S., Nifuji, A., Uede, T., Denhardt, D.T. & Noda, M. (2002) Osteopontin deficiency protects joints against destruction in anti-type II collagen antibody induced arthritis in mice. *Proceedings of the National Academy of Science of the USA*, **99**, 4556–4561.

CONCISE COMMUNICATIONS

Mice with osteopontin deletion remain predisposed to collagen-induced arthritis

Osteopontin (OPN), also known as Eta-1 (early T lymphocyte activation protein 1), is a secreted phosphoglycoprotein that has a wide range of functions. Other than collagen, OPN is the major extracellular matrix protein in bone, and it has been shown to act as an activator in osteoclasts (1). OPN also works as a Th1 cytokine (2,3). Rheumatoid arthritis (RA) is a typical bone resorption inflammatory disease, the pathogenesis of which is thought to stem from Th1 hyperactivation (4). Moreover, we have demonstrated that expression of OPN is enhanced in arthritic joints of both RA patients (5) and mice with collagen-induced arthritis (CIA) (6). In addition, it has been reported that OPN$^{-/-}$ mice are resistant to experimental autoimmune encephalomyelitis (EAE) (3) and to anti–type II collagen (CII) antibody–induced arthritis (CAIA) (7). However, a conflicting report suggests that EAE, CIA, and CAIA are fully inducible without OPN (8), and therefore, the role of OPN in the above inflammatory diseases remains controversial. In order to investigate the role of OPN in the pathogenesis of RA, we examined its clinical and immunologic effects on CIA, a murine model for RA, using OPN$^{-/-}$ mice.

OPN$^{-/-}$ mice were generated (9) and were backcrossed with DBA/1J mice (Nippon Charles River, Kanagawa, Japan) for 6 generations to introduce CIA susceptibility. After backcrossing, the loss of OPN messenger RNA in the OPN$^{-/-}$ mice was confirmed by reverse transcriptase–polymerase chain reaction. Additionally, the absence of OPN protein in the serum of OPN$^{-/-}$ mice was confirmed by enzyme-linked immunosorbent assay (ELISA). CIA was induced by established methods, as previously described (6). Briefly, 30 male mice (17 OPN$^{+/+}$ and 13 OPN$^{-/-}$ littermates between 6 and 10 weeks of age) were immunized by intradermal injection of 100 μg of bovine CII (MCK, Tokyo, Japan), emulsified with Freund's complete adjuvant (Difco, Detroit, MI). On day 21, a booster injection was given using the same method.

Each week during a followup period of 13 weeks after the first immunization 3 independent observers assessed the mice for signs of arthritis. Severity of arthritis was graded on a 1–4 scale as follows: 0 = normal; 1 = swelling and/or redness in 1 joint; 2 = swelling and/or redness in >1 joint; 3 = swelling and/or redness in the entire paw; 4 = deformity and/or ankylosis. Each paw was graded, and the mean scores of the 4 paws were summed, such that the maximum possible score per mouse was 16. In both groups of mice, signs of arthritis began to appear ~3 weeks after the first immunization, and the final incidence rates reached 100%. Arthritis scores did not differ significantly between the OPN$^{-/-}$ and OPN$^{+/+}$ mice ($P > 0.1$) (Figure 1A).

The mice were killed 13 weeks after the first immunization. Anteroposterior radiographs of all 4 limbs were obtained with a cabinet soft x-ray apparatus (MX-20; Faxitron, Wheeling, IL). Radiologic changes were evaluated by 3 independent judges under blinded conditions and were graded from 0 to 3 as follows: 0 = normal; 1 = slight erosion; 2 = bone

resorption; 3 = joint destruction. The mean scores of the 4 paws were then summed, such that the maximum possible score per mouse was 12. Radiologic scores did not differ significantly between the 2 groups (mean ± SD 9.3 ± 2.1 in the OPN$^{-/-}$ mice, 8.3 ± 1.5 in the OPN$^{+/+}$ mice; $P = 0.13$).

Serum samples from 10 OPN$^{-/-}$ and 10 OPN$^{+/+}$ mice were obtained every 2 weeks from week 0 (before immunization) to week 12 after the first immunization. The serum levels of total IgG and of specific antibodies to CII (total IgG and subclasses IgG1 and IgG2a) were measured by ELISA as previously described (10), with minor modifications. Ninety-six–well plates were coated with anti-mouse IgG (Caltag, Burlingame, CA) or bovine CII antigen solution (2 μg/ml). Nonspecific binding was blocked with phosphate buffered saline containing 1% bovine serum albumin. Serially diluted serum samples were incubated for 2 hours at room temperature. Alkaline phosphatase–conjugated horse anti-mouse IgG heavy and light chain (Vector, Burlingame, CA) or alkaline phosphatase–conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) was then added, followed by incubation for 2 hours at room temperature. Color development of p-nitrophenyl phosphate (Sigma, St. Louis, MO) was monitored at 405 nm with an ImmunoReader NJ-2000 (Nihon InterMed, Tokyo, Japan). To establish a standard curve, serial dilutions of sera from OPN$^{+/+}$ mice with CIA were added to each plate. The standard was defined as 100 units, and antibody titers of samples were estimated relative to the standard curve.

The levels of total IgG did not show significant changes during the course of the study (data not shown). Levels of anti-CII antibodies became elevated beginning at week 4 and reached a peak between weeks 6 and 8 after the first immunization. The differences between the levels of anti-CII antibodies (subtypes IgG, IgG1, and IgG2a) in OPN$^{+/+}$ and OPN$^{-/-}$ mice during this period were not statistically significant (Figure 1B).

In the present study, we did not discern any significant differences in either the incidence or the severity of CIA induced in OPN$^{-/-}$ and OPN$^{+/+}$ mice. Hence, we observed no effects of OPN deletion on CIA, as suggested by Blom et al (8) and in contrast to the findings reported by Yumoto et al (7), using another arthritis model, the CAIA model. There are several conceivable reasons for this contradiction. First, it may be due to differences in pathogenesis between CAIA and CIA, regarding their immunologic backgrounds. CAIA occurs independently of activation of lymphocytes, and anti-CII antibodies directly induce only acute inflammation. In fact, no infiltration of activated lymphocytes is observed in the arthritic joints of animals with CAIA (11). In contrast, CIA is a well-known model of chronic arthritis that is dependent on both humoral and cellular immunity specific for CII, and is especially dependent on Th1 activation (4,10). In this study, there were no notable differences in the serum levels of anti-CII IgG antibodies (total IgG and subclasses IgG1 and IgG2a) between the 2 groups of mice, which suggests that the Th1/Th2 balance in CIA is not changed by OPN deletion. Therefore, deletion of the OPN gene may not affect lymphocyte function in CIA,
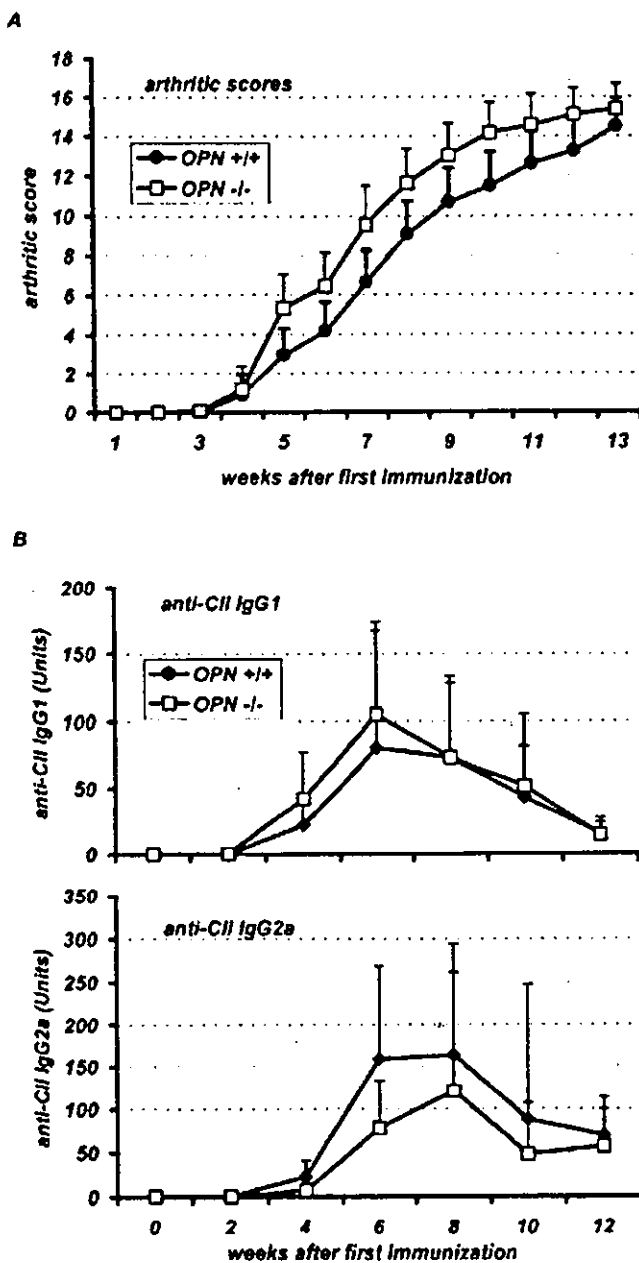
A

arthritic scores

weeks after first immunization

B

anti-CII IgG1

anti-CII IgG2a

weeks after first immunization

Figure 1. A, Arthritis scores in osteopontin-deleted (OPN$^{-/-}$) and OPN$^{+/+}$ mice. Male OPN$^{+/+}$ mice (n = 17) and their male OPN$^{-/-}$ littermates (n = 13) were immunized with type II collagen (CII) in complete Freund's adjuvant. Mean scores estimated by 3 observers for each paw were summed. Values are the mean and SD. The mean arthritis score did not differ significantly between the OPN$^{-/-}$ and OPN$^{+/+}$ mice at any time point. B, Changes in serum anti-CII antibody levels. Serum samples were obtained from OPN$^{+/+}$ mice (n = 10) and OPN$^{-/-}$ mice (n = 10) every 2 weeks from before the first immunization (0 weeks) to 12 weeks after the first immunization. Serum levels of total IgG and IgG anti-CII (data not shown) and of IgG1 and IgG2a anti-CII were measured by enzyme-linked immunosorbent assay. Values are the mean and SD. Mean levels did not differ significantly between the OPN$^{-/-}$ and OPN$^{+/+}$ mice at any time point.

allowing arthritis in OPN$^{-/-}$ mice to develop and reach the same severity as in OPN$^{+/+}$ mice.

Second, the differences may depend on the murine genetic backgrounds. DBA/1J mice, used in the present study, are highly susceptible to CIA. Blom et al induced CIA in a strain with the B10Q allele, which is also susceptible to CIA (8), whereas Yumoto and colleagues used mice of the C57BL6/129 background (7), which are normally resistant to CIA. As shown in studies of CIA with addition of interleukin-1β (IL-1β) and of IL-1 receptor antagonist–deficient mice (12,13), susceptibility to cytokines differs among strains. The role of OPN deletion could be determined by genetic backgrounds.

Third, the lack of an effect of OPN deletion may be due to compensation or substitution of the OPN gene function by 1 or more other gene(s): in knockout mice, it is understood that the influence of the deleted gene can sometimes be compensated or substituted for by other similar genes. Although surrogates for OPN have not been described, other bone matrix proteins or cytokines might fulfill this function.

In conclusion, OPN is not indispensable in the induction of CIA. However, considering the limitations in studies using knockout mice and previous reports on OPN as a cytokine and a factor in osteoclast activation (1,2,9), the role of OPN in RA and other human inflammatory diseases remains open to dispute.

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1. Rittling SR. Denhardt DT. Osteopontin function in pathology: lessons from osteopontin-deficient mice. Exp Nephrol 1999;7: 103–13.
2. Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, et al. Eta-1 (osteopontin): an early

component of type-1 (cell-mediated) immunity. Science 2000;287: 860–4.

3. Chabas D, Baranzini SE, Mitchell D, Bernard CCA, Rittling SR, Denhardt DT, et al. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. Science 2001;294:1731–5.

4. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Annu Rev Immunol 1996;14:397–440.

5. Ohshima S, Yamaguchi N, Nishioka K, Mima T, Ishii T, Umeshita-Sasai M, et al. Enhanced local production of osteopontin (OPN) in rheumatoid joints. J Rheumatol 2002;29:2061–7.

6. Ohshima S, Kobayashi H, Yamaguchi N, Nishioka K, Umeshita-Sasai M, Mima T, et al. Expression of osteopontin at sites of bone erosion in a murine experimental arthritis model of collagen-induced arthritis: possible involvement of osteopontin in bone destruction in arthritis. Arthritis Rheum 2002;46:1094–101.

7. 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.

8. Blom T, Franzen A, Heinegard D, Holmdahl R. Comment on "The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease." Science 2003;299:1845.

9. Liaw L, Birk DE, Ballas CB, Whitsitt JS, Davidson JM, Hogan BLM. Altered wound healing in mice lacking a functional osteopontin gene (sppl). J Clin Invest 1998;101:1468–78.

10. Tada Y, Ho A, Koh DR, Mak TW. Collagen-induced arthritis in CD4- or CD8-deficient mice: CD8+ T cells play a role in initiation and regulate recovery phase of collagen-induced arthritis. J Immunol 1996;156:4520–6.

11. Kagari T, Doi H, Shimozato T. The importance of IL-1β and TNF-α, and noninvolvement of IL-6, in the development of monoclonal antibody-induced arthritis. J Immunol 2002;169: 1459–66.

12. Hom JT, Cole H, Estridge T, Gliszcynski VL. Interleukin-1 enhances the development of type II collagen-induced arthritis only in susceptible and not in resistant mice. Clin Immunol Immunopathol, 1992;62:56–65.

13. Horai R, Saijo S, Tanioka H, Nakae S, Sudo K, Okahara A, et al. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. J Exp Med, 2000;191:313–20.

2 # Osteopontin as a positive regulator in the osteoclastogenesis of arthritis

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15 **Abstract**

16 We examined the role of osteopontin (OPN) in the osteoclastogenesis of arthritis using collagen-induced arthritis (CIA). Cells
17 from arthritic joints of wild-type (OPN +/+) mice spontaneously developed bone-resorbing osteoclast-like cells (OCLs). The cul-
18 tured cells showed an enhanced expression of receptor activator of nuclear factor κB ligand (RANKL) and a decreased expression of
19 osteoprotegerin (OPG). The addition of OPG reduced the number of OCLs, indicating that the osteoclastogenesis depends on the
20 RANK/RANKL/OPG system. The cells also produced OPN abundantly and anti-OPN neutralizing antibodies suppressed the
21 development of OCLs. Moreover, the addition of OPN increased the expression of RANKL and augmented differentiation of OCLs
22 from OPN-deficient (OPN −/−) cells. OPN, like the combination of 1α,25-dihydroxyvitamin D_3 and dexamethasone, also enhanced
23 the RANKL expression and decreased OPG expression in a stromal cell line, ST2. These results suggest that OPN acts as a positive
24 regulator in the osteoclastogenesis of arthritis through the RANK/RANKL/OPG system.
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26 *Keywords:* Osteopontin; RANKL; Osteoprotegerin; Collagen-induced arthritis; Stromal cell; Osteoclast

27 Rheumatoid arthritis (RA) is a major systemic au-
28 toimmune disease. Although its pathogenesis is un-
29 known, RA is characterized by chronic inflammation in
30 multiple joints, which develops into erosion of marginal
31 bone and cartilage, juxta-articular bone loss, and a
32 general reduction in bone mass [1]. It is widely accepted
33 that inflammatory cells, especially lymphocytes and
34 macrophages, are crucial players in the pathogenesis of
35 RA, and that cytokines, such as tumor necrosis factor α
36 (TNF-α), interleukin 1 (IL-1), and IL-6, are also in-
37 volved [2]. In addition, recent findings have shown that
38 osteoclasts play a key role in joint destruction and os-
39 teoporosis in RA [3–7].

40 Osteoclasts are bone-resorbing multinucleated cells
41 derived from macrophage–monocyte lineage progeni-
42 tors [8]. In RA patients and in animal models of ar-
43 thritis, increased numbers of osteoclasts have been
44 detected in pannus and in inflammatory regions of ar-
45 thritic joints [5–7]. Differentiation of osteoclasts is reg-
46 ulated by receptor activator of nuclear factor κB ligand
47 (RANKL), its receptor RANK, and osteoprotegerin
48 (OPG), which is a non-signaling decoy receptor for
49 RANKL. The ligand and receptors have recently been
50 identified as new members of the TNF ligand-receptor
51 family [9–12]. RANKL is essential for osteoclastogene-
52 sis and is also important in the development of lymph
53 nodes and in the activation of dendritic cells [10,11]. A
54 possible relationship between the RANK/RANKL/
55 OPG system and RA has recently received considerable

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56 attention. RANKL is expressed in cultured synovial
57 cells of RA patients [13], and an increase in messenger
58 RNA (mRNA) transcripts of RANKL has been de-
59 tected in synovial lymphocytes of RA patients and in
60 murine arthritis models [14]. RANK, along with
61 RANKL, is also reported to have enhanced expression
62 in arthritic mice [3,4,15]. According to other reports,
63 RANKL-deficient mice are resistant to arthritis [16] and
64 OPG prevents arthritis in animal models [14,17,18].

65   On the other hand, other than collagen, OPN is the
66 major extracellular bone matrix protein, and it is believed
67 to contribute to the balance between osteoblasts and os-
68 teoclasts in bone metabolism [19,20]. OPN is also able to
69 regulate certain inflammatory reactions as a T helper 1
70 (Th1) cytokine [21]. RA is a typical bone-resorbing in-
71 flammatory disease, the pathogenesis of which is thought
72 to stem from Th1 hyperactivation. We have previously
73 reported that OPN increases in arthritic joints of both RA
74 patients and collagen-induced arthritis (CIA) mice
75 [22,23]. In addition, it has been reported that OPN-defi-
76 cient (OPN −/−) mice resist postmenopausal osteoporosis
77 [24], disuse bone atrophy [25], and anti-type II collagen
78 antibody-transfer induced arthritis (CAIA) [26]. From
79 the prospective of suppressing osteoclasts and regulating
80 inflammation, osteopontin (OPN) is a possible thera-
81 peutic target in RA patients.

82   In this study, we examined the role of OPN in the
83 osteoclastogenesis of arthritis using an in vitro osteo-
84 clast differential model of CIA [6].

85 Materials and methods

86   *Mice.* The animal experiments in this study were performed ac-
87 cording to the guidelines of the Animal Experimentation Committee of
88 Osaka University. OPN −/− mice were generated elsewhere [27]. We
89 backcrossed these mice with DBA/1J mice (Nippon Charles River,
90 Kanagawa, Japan) for six generations to introduce CIA susceptibility.
91 After the sixth backcrossing, loss of OPN mRNA was confirmed in
92 OPN −/− mice by reverse transcriptase-polymerase chain reaction
93 (RT-PCR). Additionally, the absence of OPN protein in the serum of
94 OPN −/− mice was demonstrated using an enzyme-linked immuno-
95 sorbent assay (ELISA) as described below. Six-week-old OPN −/−
96 mice and their wild-type (OPN +/+) littermates were used. The mice
97 were bred in filter-top cages under standard pathogen-free conditions.

98   *Cell line.* A murine bone marrow stromal cell line, ST2, was ob-
99 tained from RIKEN Cell Bank (Ibaragi, Japan). ST2 cells were
100 maintained in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) containing
101 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT)
102 with 2×10^5 U/ml penicillin (PC) and 200 µg/ml streptomycin (SM) as
103 previously described [28].

104   *Induction of CIA.* CIA was induced by established methods, as
105 previously described [29]. Briefly, 18 male mice (10 OPN +/+ and 8
106 OPN −/−) were immunized by intradermal injection of 100 µg bovine
107 collagen type II (MCK, Tokyo, Japan) in 0.1 M acetic acid, emulsified
108 with an equal proportion of Freund's complete adjuvant (Difco, De-
109 troit, MI). After a 3-week period, the mice were boosted using the same
110 method. Six littermate OPN +/+ male mice without immunization
111 were used as controls. At one time, two to four mice from each group
112 were used and these experiments were repeated three times.

113   *Preparation of cells from CIA mice.* Cells from arthritic joints of
114 CIA mice were prepared as previously described [6]. Briefly, 35 days
115 after the first immunization, the immunized mice were anesthetized
116 with pentobarbital and sacrificed. After removal of skin, entirely
117 swollen arthritic paws were minced up including bone. Normal paws of
118 the non-immunized mice were used as controls. The minced tissues
119 were digested with 500 U/ml dispase (Godo Shusei, Tokyo, Japan) and
120 1 mg/ml collagenase S1 (Nitta Gelatine, Osaka, Japan) for 6 h at 37 °C
121 in complete medium (10% FCS in α-minimal essential medium (α
122 MEM; Nacalai Tesque, Kyoto, Japan), with 2×10^5 U/ml PC and
123 200 µg/ml SM). After the enzymatic reaction, the dispersed cells were
124 harvested. By this method, $1–5 \times 10^6$ live cells were obtained per
125 mouse.

126   *Cell culture.* The harvested cells were diluted to 1.5×10^5/ml and
127 cultured in 100 µl of complete medium in 96-well plastic plates in oc-
128 tuplicate at 37 °C in a humidified 5% CO_2 incubator from day 0 to day
129 7. The same experiments were repeated three times. Every 2 to 3 days
130 the culture medium was totally replaced. On day 7, the cells were fixed
131 in citrate acetone fixative and stained for tartrate-resistant acid phos-
132 phatase (TRAP) with a staining kit (Sigma, St. Louis, MO). TRAP-
133 positive osteoclast-like cells (OCLs) per well, defined as dark red cells
134 with 3 or more nuclei, were counted under a microscope. In order to
135 determine the capacity of generated OCLs to resorb bone, the har-
136 vested cells from arthritic joints were cultured on BD BioCoat Oste-
137 ologic (BD Biosciences, San Jose, CA). After a 10-day cultivation
138 period, the cells were removed by washing with hypochlorous acid,
139 according to the manufacturer's protocol. Pits were determined as
140 bright spots on slides using a microscope (Axioscop 2, Zeiss, Baden-
141 Wuerttemberg, Germany).

142   *Measurement of OPN produced by the cultured cells.* The concen-
143 trations of OPN in the culture supernatants of OPN +/+ cells were
144 measured using an OPN-ELISA kit (IBL; Gunma, Japan). The su-
145 pernatants were prepared and stored at −80 °C until used. Color re-
146 action was detected by an Immunoreader NJ-2300 (Nihon InterMed,
147 Tokyo, Japan).

148   *Treatment of the cells with OPG or anti-OPN neutralizing antibod-
149 ies.* Recombinant human OPG (PeproTech, London, England), poly-
150 clonal rabbit anti-OPN neutralizing antibodies, control normal rabbit
151 IgG (Sigma, St. Louis, MO), or phosphate buffered saline (PBS) was
152 applied to the culture medium of OPN +/+ cells at the following
153 concentration ranges: OPG; 1.5–100 ng/ml, anti-OPN antibodies; 10–
154 300 µg/ml, and control IgG; 300 µg/ml. The cells were cultured in oc-
155 tuplicate for 7 days. On day 7, TRAP staining was performed. TRAP-
156 positive OCLs were counted in a similar manner to that described
157 above. The anti-OPN neutralizing antibodies were generated in our
158 laboratory [30].

159   *Quantitative RT-PCR for RANKL and OPG in the cultured cells.*
160 Quantitative real-time PCR was performed using the ABI Prism 7700
161 Sequence Detection System (Applied Biosystems, Foster City, CA) to
162 measure the levels of mRNA for RANKL and OPG. As an internal
163 standard, β-actin was used. Briefly, using an RNeasy kit (Qiagen,
164 Hilden, Germany) with DNase treatment, RNA was extracted from
165 the harvested cells incubated in complete medium with or without
166 control antibody, anti-OPN neutralizing antibodies, or PBS for 24 h.
167 One milligram of total RNA was transcribed to synthesize cDNA
168 using SuperScript II reverse transcriptase and poly(T) primer (Invit-
169 rogen, Carlsbad, CA). Primers and TaqMan probes for real-time PCR
170 were designed using primer design software.

171   The sequences of the primers and probes were as follows: β-actin,
172 forward (5′-GCTCTGGCTCCTAGCCACCAT), reverse (5′-CTGCTT
173 GCTGATCCACATCTG), and probe (5′-AAGATCATTGCTCCTC
174 CTGAGCGCAA); RANKL, forward (5′-GCTCCGAGCTGGTGA
175 AGAAAT), reverse (5′-CCCAAAGTACGTCGCATCTTG), and
176 probe (5′-ATTCAGGTGTCCAACCCTTCCCTGCT); and OPG, for-
177 ward (5′-ATCTCGGCCACTCGAACCT), reverse (5′-CTGCTCGCT
178 CGATTTGCA), and probe (5′-CTTCTTGCCTTGATGGAGAGCCT
179 GCCT).

180　All PCRs were performed in triplicate and repeated three times.
181　Quantitation was achieved using the comparative threshold cycle (C_t)
182　method according to the manufacturer's protocol.
183　　*Treatment of the cells from OPN –/– mice with OPN.* Recombinant
184　murine OPN was added to the culture medium of OPN –/– cells at a
185　concentration range of 2.5-30 µg/ml. The cells were cultured in octu-
186　plicate for 7 days. On day 7, TRAP staining was performed. TRAP-
187　positive OCLs were counted in a similar manner to that described
188　above. The recombinant mouse OPN was generated in our laboratory
189　[30].
190　　Quantitative real-time PCR was performed to measure the levels of
191　mRNA for RANKL and OPG in the harvested OPN –/– cells incu-
192　bated with or without OPN as described above.
193　　*Murine stromal cell line, ST2, was treated with OPN, 1α,25-di-*
194　*hydroxyvitamin D₃ and/or dexamethasone.* ST2 cells were incubated for
195　24 h in complete medium with or without 30 µg/ml of recombinant
196　OPN, 10^{-8} M 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), and/or
197　10^{-7} M of dexamethasone (Dex; Sigma, St. Louis, MO). Similarly, the
198　levels of mRNA for RANKL and OPG from the cells of each group
199　were quantified.
200　　*Statistical analysis.* Values are given as means ± SD. Significant
201　differences were determined using an unpaired Student's *t* test. A *p*
202　value <0.05 was considered to be significant.

203　## Results

204　### Cells isolated from arthritic joints developed activated
205　### OCLs

206　　Cells from entirely swollen arthritic paws were har-
207　vested after the enzymatic reaction and cultured for 7
208　days. Cells from normal paws of non-immunized mice
209　were used as controls. On day 7, TRAP staining was
210　performed.
211　　The cells from arthritic joints differentiated into
212　TRAP-positive OCLs, while the cells from non-immu-
213　nized mice did not as previously reported [6] (Figs. 1A and
214　B). The numbers of OCLs from the arthritic joints and
215　from the normal joints were 96.9 ± 21.7/well and 0/well,
216　respectively. The bone resorption activity of the generated
217　OCLs was confirmed by a pit formation assay (Fig. 1C).

218　*Osteoclastogenesis of arthritic joints was dependent on the*
219　*RANK/RANKL/OPG system*

220　　After 24-h cultivation, mRNA was extracted from the
221　harvested OPN +/+ cells. Quantitative real-time PCR
222　was performed to measure the RANKL and OPG
223　mRNA levels. In the cells from arthritic joints, RANKL
224　mRNA levels increased, while OPG mRNA levels de-
225　creased compared to those in the cells from normal
226　joints (Fig. 2, *p* < 0.01). In addition, to confirm that
227　OPG inhibits the differentiation of osteoclasts, we ap-
228　plied OPG at a concentration range of 1.5–100 ng/ml to
229　the culture medium of the arthritic cells and incubated
230　them for 7 days. Application of OPG significantly re-
231　duced the numbers of OCLs in a concentration-depen-
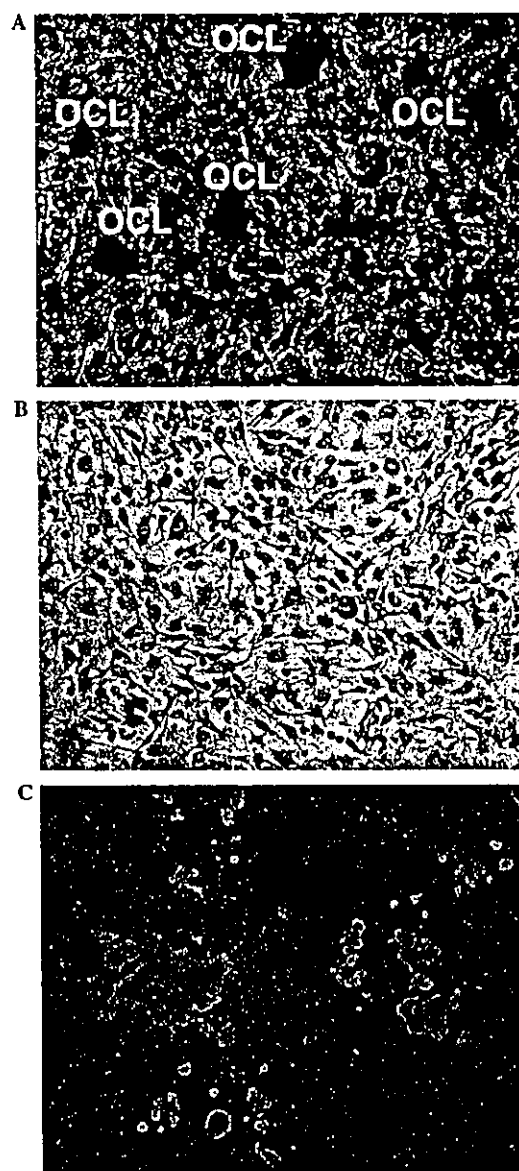232　dent manner (Fig. 3, *p* < 0.01).

Fig. 1. Cells from the joints of OPN +/+ mice were harvested and incubated in complete medium (1.5×10^4 cells/0.1 ml/well) for 7 (A,B) or 10 days (C). To evaluate the development of OCLs, TRAP staining of the cultured cells was performed as described in the Materials and methods. Cells from the arthritic joints of immunized mice (i.e., CIA mice) developed into OCLs, but those from the normal joints of non-immunized mice failed to. (A) Cells from the arthritic joints of CIA mice. (B) Cells from the normal joints of mice without immunization. (C) Pits formed by OCLs derived from cells of arthritic mice. The 'OCL' labels identify TRAP-positive osteoclast-like cells. (Original magnification; 100×.)

Cells isolated from arthritic joints produced OPN　　233

　　The levels of OPN in the culture supernatants of the 234
cells from arthritic joints increased during incubation, in 235
contrast to the cells from normal joints. As for the ar- 236
thritic cells, OPN levels were 1.50 ± 0.43 at day 3 versus 237
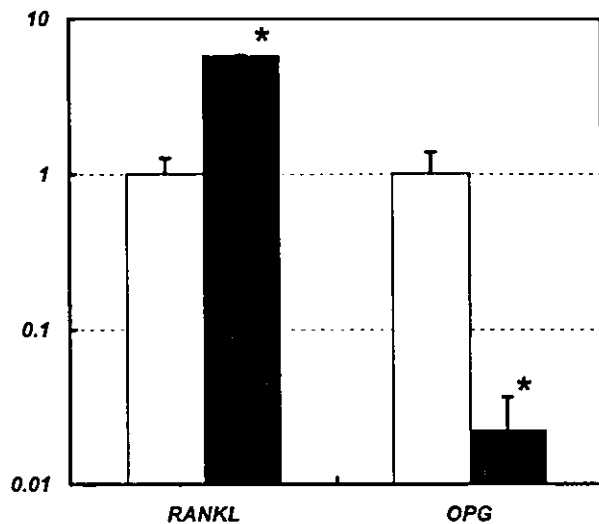7.35 ± 1.84 at day 6, *p* = 0.0008 (Fig. 4). These results 238

Fig. 2. By real-time PCR, mRNA for RANKL and OPG in the cells from joints of OPN +/+ mice was quantified. Quantitation was achieved from the C_t ratios of the mRNA either for RANKL or OPG to that for β-actin, an internal standard. The values were compared to the levels of mRNA from cells of the non-immunized normal joints. In the cells from the arthritic joints, expression of RANKL mRNA was enhanced while that of OPG mRNA decreased. White bar; in cells from the normal joints, and black bar; in cells from the arthritic joints. $*p < 0.01$.
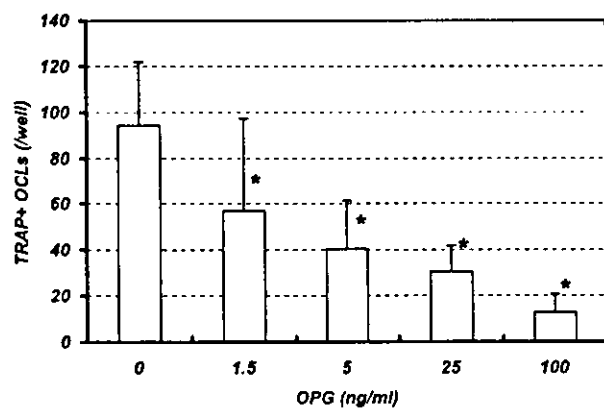

Fig. 3. Numbers of OCLs in the presence or absence of OPG. Cells from arthritic joints of OPN +/+ mice were harvested and incubated in complete medium (1.5×10^4 cells/0.1 ml/well) in octuplicate for 7 days with or without OPG applied to the culture medium. After TRAP staining, TRAP-positive multinucleated cells in each well of a 96-well culture plate were counted. Adding OPG prevents the development of OCLs in a concentration-dependent manner. $*p < 0.01$.
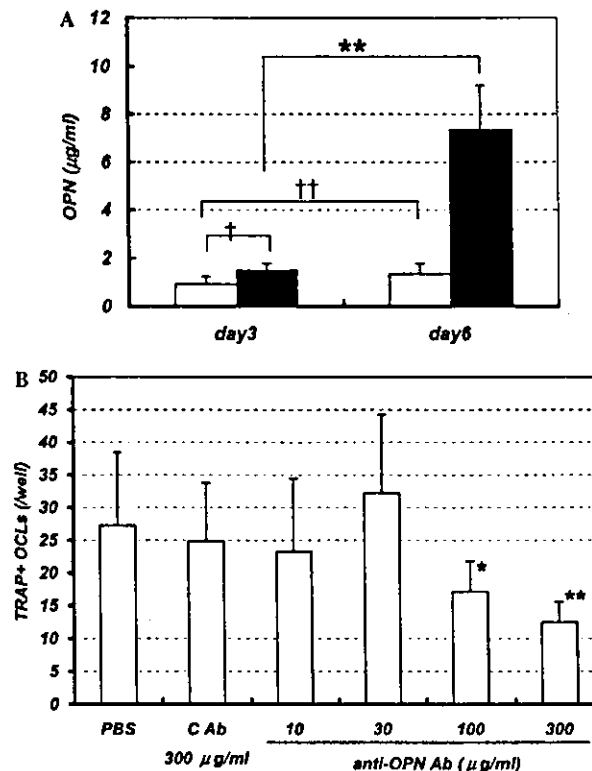

Fig. 4. (A) The concentration of OPN in the culture supernatant of OPN +/+ cells. Each measurement was performed three times in quadruplicate by ELISA. The levels of OPN secreted from cells from arthritic joints of CIA mice increased during incubation. White bar; the values of normal joints from non-immunized mice, black bar; the values of arthritic joints from CIA mice. [1]Day 3 OPN levels in the supernatant of cells from non-immunized versus immunized mice, $p > 0.05$. [11] OPN levels in the supernatant of cells from non-immunized mice, day 3 versus day 6, $p > 0.05$. *OPN levels in the supernatant of cells from immunized mice, day 3 versus day 6, $p = 0.0008$. (B) Numbers of OCLs in the presence or absence of anti-OPN neutralizing antibodies. Cells from arthritic joints of OPN +/+ mice were harvested and incubated in complete medium (1.5×10^4 cells/0.1 ml/well) in octuplicate for 7 days with polyclonal anti-OPN neutralizing antibodies, control antibody, or PBS applied to the culture medium. After TRAP staining, TRAP-positive multinucleated cells in each well of a 96-well culture plate were counted. Anti-OPN antibodies prevent development of OCLs from OPN +/+ cells. $*p < 0.05$ and $**p < 0.01$.

239   are consistent with our previous report that the serum
240   concentration of OPN is enhanced in CIA mice during
241   development of arthritis and that activated osteoclasts
242   in bone erosive lesion express OPN [22].

243   *Neutralization of OPN suppressed osteoclastogenesis*

244   In order to investigate whether OPN is involved in
245   the differentiation of osteoclasts, we added anti-OPN

neutralizing antibodies to the culture medium of ar- 246
thritic cells and incubated them for 7 days. The 247
numbers of OCLs decreased in a concentration-de- 248
pendent manner in the presence of the anti-OPN an- 249
tibodies, whereas the control antibody had no effect 250
(Fig. 4B). 251

Addition of OPN enhanced the osteoclastogenesis of 252
OPN –/– cells   *253*

We examined the effect of recombinant OPN on OPN 254
–/– cells. We added OPN to the culture medium of ar- *fi*
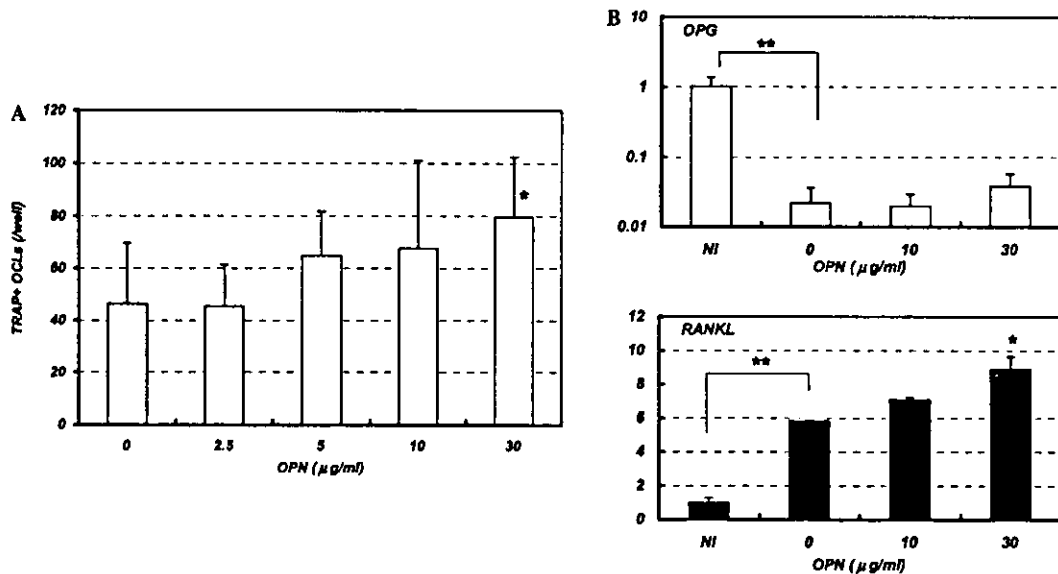thritic OPN –/– cells and incubated them for 7 days. In 256

Fig. 5. (A) Numbers of OCLs in the presence or absence of recombinant OPN in OPN –/– cells. Cells from arthritic joints of OPN –/– mice were harvested and incubated in complete medium (1.5×10^4 cells/0.1 ml/well) in octuplicate for 7 days with OPN applied to the culture medium. After TRAP staining, TRAP-positive multinucleated cells in each well of a 96-well culture plate were counted. OPN enhances the development of OCLs while their development from OPN –/– cells. $**p < 0.01$. (B) Quantitation of mRNA for RANKL and OPG of OPN –/– cells in the presence of OPN. We investigated whether OPN has a role in regulating expression of mRNA for RANKL and OPG. Using real-time PCR, mRNA from OPN –/– cells was quantified. Quantitation was achieved from the C_t ratios of the mRNA for RANKL and OPG to that for β-actin, an internal standard. The values were compared to the levels of mRNA from cells of the non-immunized normal joints. Upper panel: addition of OPN to OPN –/– cells did not reduce the expression of OPG mRNA Bottom panel: Addition of OPN to OPN –/– cells slightly enhanced the expression of RANKL mRNA. NI means values of cells from non-immunized mice without reagents. $**p < 0.01$. *Addition of 0 μg/ml OPN versus 30 μg/ml, $p < 0.05$.

257 the presence of 30 μg/ml OPN, the number of developed
258 OCLs was significantly increased (Fig. 5A).
259   To demonstrate whether OPN is involved in the en-
260 hanced expression of RANKL in arthritis, the levels of
261 mRNA for RANKL and OPG were quantified in OPN
262 –/– cells incubated with or without OPN. As is the case
263 with OPN +/+ mice shown in Fig. 2, in OPN –/– mice
264 the expression of RANKL mRNA was enhanced and
265 that of OPG mRNA was reduced in arthritic joints
266 compared to non-immunized normal joints (NI). Addi-
267 tion of 30 μg/ml OPN to OPN –/– cells increased the
268 expression of RANKL mRNA, although not signifi-
269 cantly affecting that of OPG mRNA (Fig. 5B).

270 *OPN enhances the RANKL expression and suppresses the*
271 *OPG expression on stromal cells*

272   Because the harvested cells from joints were a heter-
273 ogeneous mixture of cells derived from multiple sources,
274 such as stromal cells, osteoclast precursors, and lym-
275 phocytes, etc., there are limitations in evaluating mRNA
276 expression. To clarify the effect of OPN on the expres-
277 sion of RANKL and OPG, we used a murine stromal
278 cell line, ST2. As positive control, we used $1,25(OH)_2D_3$
279 and Dex, which are well-known agents that induce
280 stromal cells to express RANKL and reduce their ex-
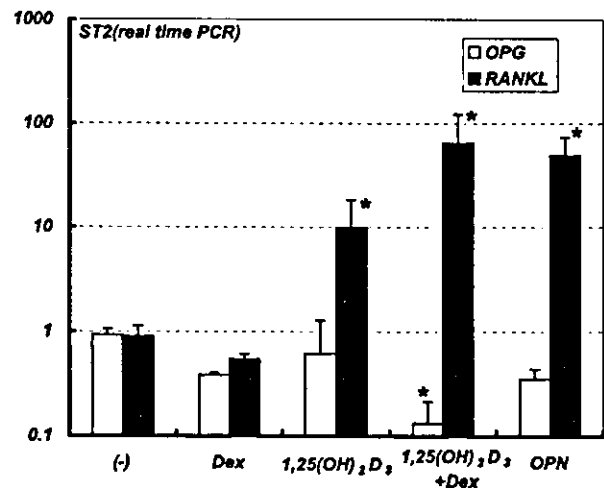281 pression of OPG [3,4,8,12,19]. While the combination of

Fig. 6. Quantitation of mRNA for RANKL and OPG of a stromal cell line, ST2, in the presence of several reagents. We investigated whether OPN has a role in regulating expression of mRNA for RANKL and OPG by comparison with other reagents. ST2 cells were incubated with or without OPN, $1,25(OH)_2D_3$, and/or Dex. After 24-h cultivation, mRNA was extracted from ST2 cells and the mRNA levels of both RANKL and OPG were quantified by real-time PCR. OPN and $1,25(OH)_2D_3$ increased the level of RANKL mRNA and reduced the level of OPG mRNA. The values were compared to the levels of mRNA from ST2 without any reagent. OPN, $1,25(OH)_2D_3$, and Dex represent 30 μg/ml of recombinant OPN, 10^{-8} M of $1,25(OH)_2D_3$, and 10^{-7} M of Dex, each. $*p < 0.01$.

282 1,25(OH)$_2$D$_3$ and Dex most effectively induced RANKL
283 mRNA and reduced OPG mRNA, OPN also increased
284 the expression of RANKL mRNA over 10-fold (Fig. 6).

285 Discussion

286   In this study, we used an in vitro osteoclast differen-
287 tiation model of CIA as previously reported [6]. In this
288 model, we confirmed that OCLs are inducible without
289 additional agents or exogenous cytokines and that the
290 generated OCLs have the ability to resorb hydroxyap-
291 atite. On the grounds that the cells from non-arthritic
292 joints fail to differentiate into OCLs, we speculate that
293 the factors induced or enhanced during the progression
294 of arthritis are crucial. As expected from previous re-
295 ports [3,13,15–17], the expression of RANKL mRNA
296 was enhanced and that of OPG mRNA was reduced in
297 arthritic joints. In addition, in vitro osteoclastogenesis
298 from arthritic joints was inhibited by OPG in a con-
299 centration-dependent manner. Putting these observa-
300 tions together, we conclude that the RANK/RANKL/
301 OPG system is the major contributor to the activation of
302 osteoclasts in CIA.

303   On the other hand, we have previously reported that
304 the expression of OPN is enhanced locally in arthritic
305 joints [22], and in this study, we confirmed that cells
306 from arthritic joints secrete OPN. Hence, we investi-
307 gated if OPN could augment osteoclastogenesis. Al-
308 though in vivo we did not discern any significant
309 differences in either the incidence or severity of CIA
310 induced in OPN –/– and OPN +/+ mice [31], in vitro
311 application of the anti-OPN neutralizing antibodies to
312 OPN +/+ cells prevented the development of OCLs in a
313 concentration-dependent fashion. Moreover, the addi-
314 tion of OPN to OPN –/– cells increased the number of
315 OCLs as a function of OPN concentration. Because of
316 the facilitatory effect of OPN on the generation of
317 OCLs, and the inhibitory effect of anti-OPN neutralizing
318 antibodies akin to that of OPG, we suspected that OPN
319 might play a role in regulating the RANK/RANKL/
320 OPG system in arthritis. Real-time quantitative PCR
321 showed that the presence of OPN increases the level of
322 RANKL mRNA in OPN –/– cells. Besides, adding
323 OPN to the culture medium of a stromal cell line, ST2,
324 which is a typical cellular source of RANKL, enhanced
325 expression of RANKL mRNA much more clearly.
326 These results suggest that OPN affected stromal cells in
327 arthritic joints and enhanced the expression of RANKL.
328 This effect of OPN can contribute to activation of os-
329 teoclasts that ultimately results in the joint destruction
330 seen in arthritis.

331   It has been reported that OPN activates osteoclasts
332 through integrin-αvβ3 by regulating cell adhesion and
333 controlling cytoskeleton [32]. In addition, several stud-
334 ies, including ours, have already suggested that OPN

335 plays an important role in the bone destruction of ar-
336 thritis by promoting osteoclastogenesis through acting
337 on osteoclast precursor cells stimulated by RANKL
338 [22,23,26,30]. This study raises the possibility that OPN
339 could act not only on the osteoclast precursor cells but
340 also on the stromal cells and directly induced RANKL
341 and suppress OPG, although to confirm this further
342 experiments are needed.

343   OPN –/– mice have been reported to be resistant to
344 experimental autoimmune encephalomyelitis (EAE) [33]
345 and CAIA [26]. However, a conflicting report suggests
346 that EAE, CIA, and CAIA are fully inducible without
347 OPN in strain with B10Q allele, which is susceptible to
348 CIA [34]. And we have reported that CIA can be fully
349 induced with DBA/1J OPN –/– mice, which is highly
350 susceptible to CIA [31]. As previous reports of IL-1β
351 and IL-1 receptor antagonist, susceptibility to cytokines
352 differs among strains. The efficacy of OPN deletion can
353 be decided by genetic backgrounds. Additionally, in
354 knockout mice, it is understood that the influence of the
355 deleted gene can sometimes be compensated or substi-
356 tuted for by other similar genes.

357   OPN may not be an essential factor in the develop-
358 ment of osteoclasts, considering that the effects of OPN
359 deletion were not clear on CIA in vivo. However, inhi-
360 bition of OPN may be an effective treatment for bone
361 destruction, not only from the prospective of inhibiting
362 the activation of osteoclasts, but also in prevention of
363 osteoclast differentiation.

References   364

[1] E.D. Harris Jr., Rheumatoid arthritis. Pathophysiology and   365
    implications for therapy, N. Engl. J. Med. 322 (1990) 1277–1289. 366
[2] M. Feldmann, F.M. Brennan, R.N. Maini, Role of cytokines in   367
    rheumatoid arthritis, Annu. Rev. Immunol. 14 (1996) 397–440.   368
[3] E. Romas, M.T. Gillespie, T.J. Martin, Involvement of receptor   369
    activator of NFκB ligand and tumor necrosis factor-α in bone   370
    destruction in rheumatoid arthritis, Bone 30 (2002) 340–346.   371
[4] L.E. Theill, W.J. Boyle, J.M. Penninger, RANK-L and RANK: T   372
    cells, bone loss, and mammalian evolution, Annu. Rev. Immunol.   373
    20 (2002) 795–823.   374
[5] M. Bromley, H. Bertfield, J.M. Evanson, D.E. Woolley, Bidirec-   375
    tional erosion of cartilage in rheumatoid knee joint, Ann. Rheum. 376
    Dis. 44 (1985) 676–681.   377
[6] Y. Suzuki, F. Nishikaku, M. Nakatuka, Y. Koga, Osteoclast-like   378
    cells in murine collagen induced arthritis, J. Rheumatol. 25 (1998) 379
    1154–1160.   380
[7] T. Kuratani, K. Nagata, T. Kukita, T. Hotokebuchi, A. Nakas-   381
    ima, T. Iijima, Induction of abundant osteoclast-like multinucle- 382
    ated giant cells in adjuvant arthritic rats with accompanying   383
    disordered high bone turnover, Histol. Histopathol. 13 (1998)   384
    751–759.   385
[8] S.L. Teitelbaum, Bone resorption by osteoclasts, Science 289   386
    (2000) 1504–1508.   387
[9] D.L. Lacey, E. Timms, H.L. Tan, M.J. Kelly, C.R. Dunstan, T.   388
    Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, H. Hsu, J. 389
    Sullivan, N. Hawkins, E. Davy, C. Capparelli, A. Eli, Y.X. Qian, 390
    S. Kaufman, I. Sarosi, V. Shalhoub, G. Senaldi, J. Guo, J.   391

Delaney, W.J. Boyle, Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation, Cell 93 (1998) 165–176.

[10] B.R. Wong, J. Rho, J. Arron, E. Robinson, J. Orlinick, M. Choa, S. Kalachikov, E. Cayani, F.S. Bartlett 3rd, W.N. Frankel, S.Y. Lee, Y. Choi, TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-jun N-terminal kinase in T cells, J. Biol. Chem. 272 (1997) 25190–25194.

[11] D.M. Anderson, E. Maraskovsky, W.L. Billingsley, W.C. Dougall, M.E. Tometsko, E.R. Roux, M.C. Teepe, R.F. DuBose, D. Cosman, L. Galibert, A homologue of TNF receptor and its ligand enhance T-cell growth and dendritic-cell function, Nature 390 (1997) 175–179.

[12] H. Yasuda, N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, E. Tsuda, T. Morinaga, K. Higashio, N. Udagawa, N. Takahashi, T. Suda, Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL, Proc. Natl. Acad. Sci. USA 95 (1998) 3597–3602.

[13] E.M. Gravallese, S.R. Goldring, Cellular mechanisms and the role of cytokines in bone erosions in rheumatoid arthritis, Arthritis Rheum. 43 (2000) 2143–2151.

[14] Y.Y. Kong, H. Yoshida, I. Sarosi, H.L. Tan, E. Timms, C. Capparelli, S. Morony, A.J. Oliveria-dos-Santos, G. Van, A. Itie, W. Khoo, A. Wakeham, C.R. Dunstan, D.L. Lacey, T.W. Mak, W.J. Boyle, J.M. Penninger, OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis, Nature 397 (1999) 315–323.

[15] E. Lubberts, B. Oppwes-Walgreen, A.R. Pettit, L. Van Den Bersselaar, L.A. Joosten, S.R. Goldring, E.M. Gravallese, W.B. Van Den Berg, Increase in expression of receptor activator of nuclear factor κB at sites of bone erosion correlates with progression of inflammation in evolving collagen-induced arthritis, Arthritis Rheum. 46 (2002) 3055 3064.

[16] A.R. Petit, H. Ji, D. von Stechow, R. Muller, S.R. Goldring, Y. Choi, C. Benoist, E.M. Gravallese, TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis, Am. J. Pathol. 159 (2001) 1689–1699.

[17] K. Redlich, S. Hayer, A. Maier, C.R. Dunstan, M. Tohidast-Akrad, S. Lang, B. Turk, P. Pietschmann, W. Woloszczuk, S. Haralambous, G. Kollias, G. Steiner, J.S. Smolen, G. Schett, Tumor necrosis factor α-mediated joint destruction is inhibited by targeting osteoclasts with osteoprotegerin, Arthritis Rheum. 46 (2002) 785–792.

[18] E. Romas, N.A. Sims, D.K. Hards, M. Lindsay, J.W. Quinn, P.F. Ryan, C.R. Dunstan, T.J. Martin, M.T. Gillespie, Osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis, Am. J. Pathol. 161 (2002) 1419–1427.

[19] F.P. Reinholt, K. Hultenby, A. Oldberg, D. Heinegard, Osteopontin—a possible anchor of osteoclasts to bone, Proc. Natl. Acad. Sci. USA 87 (1990) 4473–4475.

[20] K. Merry, R. Dodds, A. Littlewood, M. Gowen, Expression of osteopontin mRNA by osteoclasts and osteoblasts in modeling adult human bone, J. Cell Sci. 104 (1993) 1013–1020.

[21] S. Ashkar, G.F. Weber, V. Panoutsakopoulou, M.E. Sanchirico, M. Jansson, S. Zawaideh, S.R. Rittling, D.T. Denhardt, M.J.

Glimcher, H. Cantor, Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity, Science 287 (2000) 860–864.

[22] S. Ohshima, H. Kobayashi, N. Yamaguchi, K. Nishioka, M. Umeshita-Sasai, T. Mima, S. Nomura, S. Kon, M. Inobe, T. Uede, Y. Saeki, Expression of osteopontin at sites of bone erosion in a murine experimental arthritis model of collagen-induced arthritis, Arthritis Rheum. 46 (2002) 1094–1101.

[23] S. Ohshima, N. Yamaguchi, K. Nishioka, T. Mima, T. Ishii, M. Umeshita-Sasai, H. Kobayashi, M. Shimizu, Y. Katada, S. Wakitani, N. Murata, S. Nomura, H. Matsuno, R. Katayama, S. Kon, M. Inobe, T. Uede, I. Kawase, Y. Saeki, Enhanced local production of osteopontin in rheumatoid joints, J. Rheumatol. 29 (2002) 2061–2067.

[24] H. Yoshitake, S.R. Rittling, D.T. Denhardt, M. Noda, Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption, Proc. Natl. Acad. Sci. USA 96 (1999) 8156–8160.

[25] M. Ishijima, S.R. Rittling, T. Yamashita, K. Tsuji, H. Kurosawa, A. Nifuji, D.T. Denhardt, M. Noda, Enhancement of osteoclastic bone resorption and suppression of osteoblastic bone formation in response to reduced mechanical stress do not occur in the absence of osteopontin, J. Exp. Med. 193 (2001) 399–404.

[26] K. Yumoto, M. Ishijima, S.R. Rittling, K. Tsuji, Y. Tsuchiya, S. Kon, A. Nifuji, T. Uede, D.T. Denhardt, M. Noda, Osteopontin deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice, Proc. Natl. Acad. Sci. USA 99 (2002) 4556–4561.

[27] L. Liaw, D.E. Birk, C.B. Ballas, J.S. Whitsitt, J.M. Davidson, B.L. Hogan, Altered wound healing in mice lacking a functional osteopontin gene (spp1), J. Clin. Invest. 101 (1998) 1468–1478.

[28] M. Ogawa, S. Nishikawa, K. Ikuta, F. Yamamura, M. Naito, K. Takahashi, S. Nishikawa, B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac, EMBO J. 7 (1988) 1337–1343.

[29] D.E. Trentham, A.S. Townes, A.H. Kang, Autoimmunity to type II collagen: an experimental model of arthritis, J. Exp. Med. 146 (1977) 857–868.

[30] N. Yamamoto, F. Sakai, S. Kon, J. Morimoto, C. Kimura, H. Yamazaki, I. Okazaki, N. Seki, T. Fujii, T. Uede, Essential role of the cryptic epitope SLAYGLR within osteopontin in a murine model of rheumatoid arthritis, J. Clin. Invest. 112 (2003) 181–188.

[31] T. Ishii, S. Ohshima, T. Ishida, I. Kawase, T. Mima, Y. Tabunoki, H. Kobayashi, M. Maeda, T. Uede, L. Liaw, N. Kinoshita, Y. Saeki, Mice with osteopontin deletion remain predisposed to collagen-induced arthritis, Arthritis Rheum. 50 (2004) 669–671.

[32] L.T. Duong, P.T. Lakkakorpi, I. Nakamura, M. Machwate, R.M. Nagy, G.A. Rodan, PYK2 in osteoclasts is an adhesion kinase, localized in the sealing zone, activated by ligation of αvβ3 integrin, and phosphorylated by src kinase, J. Clin. Invest. 102 (1998) 881–892.

[33] D. Chabas, S.E. Baranzini, D. Mitchell, C.C.A. Bernard, S.R. Rittling, D.T. Denhardt, R.A. Sobel, C. Lock, M. Karpuj, R. Pedotti, R. Heller, J.R. Oksenberg, L. Steinman, The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease, Science 294 (2001) 1731–1735.

[34] T. Blom, A. Franzen, D. Heinegurd, R. Holmdahl, Comment on the influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease, Science 299 (2003) 1845a.

Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim

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Osteoclasts (OCs) undergo rapid apoptosis without trophic factors, such as macrophage colony-stimulating factor (M-CSF). Their apoptosis was associated with a rapid and sustained increase in the pro-apoptotic BH3-only Bcl-2 family member Bim. This was caused by the reduced ubiquitylation and proteasomal degradation of Bim that is mediated by c-Cbl. Although the number of OCs was increased in the skeletal tissues of *bim–/–* mice, the mice exhibited mild osteosclerosis due to reduced bone resorption. OCs differentiated from bone marrow cells of *bim–/–* animals showed a marked prolongation of survival in the absence of M-CSF, compared with *bim+/+* OCs, but the bone-resorbing activity of *bim–/–* OCs was significantly reduced. Overexpression of a degradation-resistant lysine-free Bim mutant in *bim–/–* cells abrogated the anti-apoptotic effect of M-CSF, while wild-type Bim did not. These results demonstrate that ubiquitylation-dependent regulation of Bim levels is critical for controlling apoptosis and activation of OCs.
Keywords: apoptosis/bim/M-CSF/osteoclast/ubiquitylation

Introduction

Apoptosis is a genetically programmed process for killing unwanted cells (Kerr *et al.*, 1972). Abnormalities in apoptosis regulation can promote cancer, autoimmune disease or degenerative disorders (Thompson, 1995).

Mammals have two distinct apoptosis signaling pathways that converge upon activation of aspartate-specific cysteine proteases (caspases), which mediate cell demolition (Strasser *et al.*, 1995). One pathway is initiated by death receptors, members of the tumor necrosis factor receptor (TNF-R) family with an intracellular death domain, and propagated through FADD adaptor protein-mediated activation of caspase-8. The other pathway is regulated by pro- and anti-apoptotic Bcl-2 family members and involves mitochondrial release of cytochrome c, which causes Apaf-1 adaptor-mediated activation of caspase-9 (Gross *et al.*, 1999; Strasser *et al.*, 2000). The anti-apoptotic Bcl-2 family members include mammalian Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1 and Boo/Diva, and *Caenorhabditis elegans* CED-9, and they share similarity within three or four Bcl-2 homology (BH) domains. So far, >20 pro-apoptotic Bcl-2 family proteins have been identified in mammals. They can be divided further into two groups: multidomain members possess homology in two or three BH regions (mammalian Bax, Bak, Bok/Mtd, Bcl-xS and Bfk), whereas the BH3 domain-only proteins (mammalian Bik/Nbk, Bad, Bid, Hrk/DP5, Bim/Bod, Noxa, Bmf and Puma/Bbc3, and *C.elegans* EGL-1) share only the short BH3 region (Huang and Strasser, 2000). Genetic studies and experiments with transfected cells have shown that BH3-only proteins are essential for initiation of apoptosis (Huang and Strasser, 2000), whereas Bax/Bak-like proteins play an essential role further downstream (Cheng *et al.*, 2001; Zong *et al.*, 2001). Bax/Bak-like proteins are ubiquitously expressed, whereas BH3-only family members have a more tissue-specific distribution, indicating that the latter may play a tissue/cell-specific and death stimulus-specific role in apoptosis. The pro-apoptotic activity of BH3-only proteins is strictly regulated at both the transcriptional and post-translational level to prevent inappropriate cell killing (Huang and Strasser, 2000).

The BH3-only protein Bim was first identified as a Bcl-2-interacting protein by screening a λ phage expression library constructed from a mouse thymic lymphoma (O'Connor *et al.*, 1998). Bim is expressed in hematopoietic, epithelial, neuronal and germ cells (O'Reilly *et al.*, 2000), and alternative splicing generates various Bim isoforms, including Bim_S, Bim_L and Bim_{EL}. Experiments with knock-out mice have shown that Bim is essential for apoptosis of T lymphocytes, B lymphocytes, myeloid cells and neurons (Bouillet *et al.*, 1999, 2002; Putcha *et al.*, 2001; Whitfield *et al.*, 2001; Villunger *et al.*, 2003). Pro-apoptotic activity of Bim is regulated both transcriptionally and post-transcriptionally (Huang and Strasser, 2000). Osteoclasts (OCs) are multinucleated giant cells primarily responsible for bone resorption (Baron, 1989; Suda *et al.*, 1992; Tanaka *et al.*, 2003). They are terminally differentiated cells, and undergo rapid apoptosis in the absence of

trophic factors such as macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) (Hughes *et al.*, 1995). We demonstrate here that Bim is critical for normal OC survival and function.

Results

Rapid induction of Bim protein in cytokine-deprived OCs

To obtain murine OCs, we used a co-culture system of osteoblastic cells and bone marrow cells in the presence of 1α, 25-dihydroxyvitamin D_3 [1α, 25(OH)$_2$D$_3$] and prostaglandin E_2 (PGE$_2$), as previously reported (Takahashi *et al.*, 1988). OCs purified from co-cultures by removing osteoblastic cells by collagenase and dispase treatment were then maintained in the presence of M-CSF (10 ng/ml) for an additional 12 h. When M-CSF was removed from the culture, OCs underwent rapid cell death. Within 24 h, 70% of OCs had died and virtually all cells died out after 48 h, as previously reported (Miyazaki *et al.*, 2000). We examined whether cytokine withdrawal caused changes in the expression levels of pro- or anti-apoptotic Bcl-2 family members. Immunoblot analysis revealed a marked increase in Bim expression levels within 3 h of M-CSF removal and it remained high for at least 12 h (Figure 1A), while expression levels of Bid, Bax or Bcl-xL were not affected. Induction of Bim was reversed by M-CSF or, albeit less efficiently, by RANKL treatment for 12 h (Figure 1A). A rapid increase in Bim levels was also observed in cytokine-deprived OC precursors (data not shown). However, no significant difference in *bim* mRNA level was observed between OCs cultured in the presence or absence of M-CSF either by RT–PCR or by real-time PCR (Figure 1C), demonstrating that the changes in Bim protein levels are due to post-tranlational mechanisms.

We previously reported that the Ras/extracellular signal-regulated kinase (ERK) pathway promotes survival of OCs (Miyazaki *et al.*, 2000), while others found that the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway has an effect on this process (Wong *et al.*, 1999; Glantschnig *et al.*, 2003). To analyze whether these pathways are involved in downregulation of Bim by M-CSF, we employed adenovirus vectors encoding constitutively active MEK1 (MEK^{CA}) or Akt (myr-Akt), which contains a Src myristoylation signal that promotes association with the plasma membrane, causing constitutive activation. As shown in Figure 1B, enforced expression of MEK^{CA} reversed the induction of Bim after M-CSF removal, while myr-Akt had less effect. Treating the cells with a specific inhibitor of MEK/ERK pathways, PD98059, completely abolished the effect of M-CSF on Bim expression (Figure 1B). These results indicate that Ras/ERK signaling is a major pathway for downregulation of Bim by M-CSF in OCs.

We next examined the effects of Bim and Bcl-xL overexpression in OC apoptosis caused by cytokine withdrawal using adenovirus vectors. Less than 5% of OCs overexpressing Bim survived 12 h after M-CSF removal, whereas 20% of control virus-infected cells remained alive at this time (Figure 1D). Co-expression of Bcl-xL abrogated the pro-apoptotic effects of the Bim$_L$ adenovirus, indicating that the balance between Bim and Bcl-xL may determine the fate of OCs (Figure 1D).

Expression of Bim in skeletal tissues

To determine the expression of *bim* in skeletal tissues, we performed *in situ* hybridization analysis using a *bim$_L$* antisense probe which detects *bim$_S$*, *bim$_L$* and *bim$_{EL}$*. Strong expression of *bim* mRNA was observed in the bone trabeculae of 5-week-old male mouse metatarsal bone, which was co-localized with tartrate-resistant acid phosphatase (TRAP) enzymatic staining, i.e. with OCs (Figure 2A and B). On the other hand, *bim* transcripts were hardly detectable in osteoblasts or chondrocytes, whose localization was determined by procollagen type IA and type IIA expression, respectively (Figure 2B–D). The expression pattern of *bim* in the skeletal tissues was confirmed further by X-gal staining of mutant mice, in which a *lacZ* reporter gene was knocked into the *bim* locus by homologous recombination (Figure 2E).

Mild osteosclerosis in Bim-deficient mice is due to reduced bone turnover

We next studied the skeletal tissues of *bim–/–* mice. Histological examination and X-ray analysis revealed that *bim–/–* mice have mild osteosclerosis (Figure 3A). Histomorphometry demonstrated that *bim–/–* mice had abnormally low levels of new bone formation and turnover of the skeletal tissues (Figure 3D and E). The osteoid surface (OS/BS) and osteoblast surface (Ob.S/BS) were lower in *bim–/–* mice compared with wild-type animals (Figure 3D), and calcein double labeling revealed a marked decrease in the mineral apposition rate (MAR) in the *bim–/–* mice (Figure 3E). Although the OC number per bone perimeter (Oc.N/B.Pm) was increased in *bim–/–* mice, the percentage of the eroded surface (ES/BS) and OC surface (Oc.S/BS) was reduced (Figure 3D). It appears likely that this defect is a consequence of the abnormally small size and impaired actin ring formation of the *bim–/–* OCs (Figure 3B and C).

Elongated life span of bim–/– OCs in vivo

We next examined whether *bim–/–* OCs have a longer life span *in vivo* (Figure 3F). Five-week-old *bim+/+* and *bim–/–* mice were fed with water containing 1 mg/ml of 5′-bromo-2′-deoxyuridine (BrdU) for 1 week (labeling period). Mice were then sacrificed either on the next day (group A) or after 6 weeks (group B) of the labeling period. Immunostaining with anti-BrdU antibody demonstrated that almost similar proportions of OCs were positively stained in group A *bim+/+* and *bim–/–* mice (50 and 48%, respectively). However, the proportion of BrdU-positive OCs was markedly decreased to <5% in group B *bim+/+* mice, while that in group B *bim–/–* mice was maintained at 33%. This suggests that *bim–/–* OCs have a longer life span than *bim+/+* OCs *in vivo*.

bim–/– OCs are resistant to cytokine withdrawal-induced apoptosis but have abnormally low bone resorption activity in vitro

Bone marrow cells obtained from *bim–/–* and *bim+/+* mice were subjected to OC formation assay by co-culturing with osteoblastic cells in the presence of 1α,25(OH)$_2$D$_3$ and PGE$_2$. The number of TRAP-positive multinucleated cells formed from *bim–/–* bone marrow cells was comparable with that from *bim+/+* cells. The *bim–/–* OCs were,
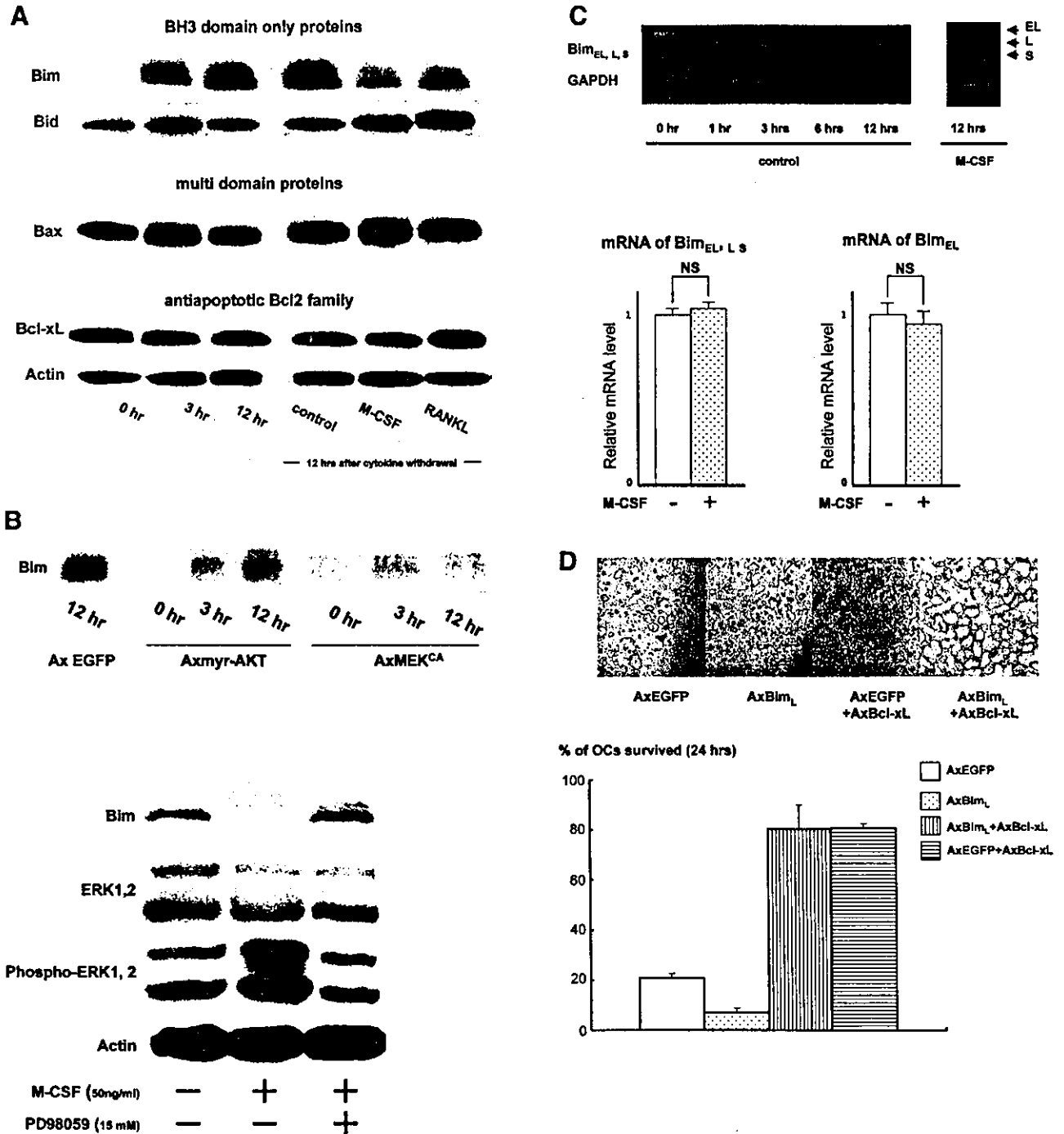
6654

Fig. 1. Regulation of Bim expression in OCs. (A) Cytokine withdrawal caused rapid upregulation of Bim levels in OCs. OCs purified from co-cultures by removing osteoblastic cells by collagenase and dispase treatment were then maintained in the presence of M-CSF (10 ng/ml) for an additional 12 h. The expression levels of Bim and other apoptosis-regulatory proteins in OCs after M-CSF removal were analyzed by western blotting using specific antibodies. Bim levels increased within 3 h, and the upregulation was sustained at least for 12 h. This upregulation of Bim level was strongly suppressed by M-CSF, and to a lesser extent by sRANKL treatment for 12 h. (B) Intracellular signaling pathways leading to Bim downregulation. Upper panel: introduction of MEK^{CA} strongly suppressed the upregulation of Bim after M-CSF removal. Overexpression of myr-Akt had less effect on Bim expression in OCs. Lower panel: treating the cells with a specific inhibitor of MEK/ERK pathways, PD98059, completely abolished the suppressive effect of M-CSF on Bim expression. (C) Transcriptional regulation of bim in OCs. No significant change in the mRNA level of three isoforms of bim, i.e. bim$_{EL}$, $_L$ and $_S$ (upper and lower left), or the bim$_{EL}$ specific mRNA level (lower right) was detected in OCs in the presence or absence of M-CSF as determined by RT-PCR (upper) or real-time PCR (lower). The y-axis indicates the relative mRNA levels. NS = not significantly different. (D) Effect of adenovirus vector-mediated overexpression of Bim$_L$ and/or Bcl-xL on OC survival. Upper panel: TRAP staining. Lower panel: percentage of OCs surviving. Overexpression of Bim$_L$ promoted apoptosis of OCs (AxBim$_L$). Not only did Bcl-xL overexpression suppress apoptosis of OCs (AxEGFP + AxBcl-xL), but co-expression of Bcl-xL together with Bim$_L$ completely abrogated the pro-apoptotic effect of Bim$_L$ (AxBim$_L$ + AxBcl-xL).
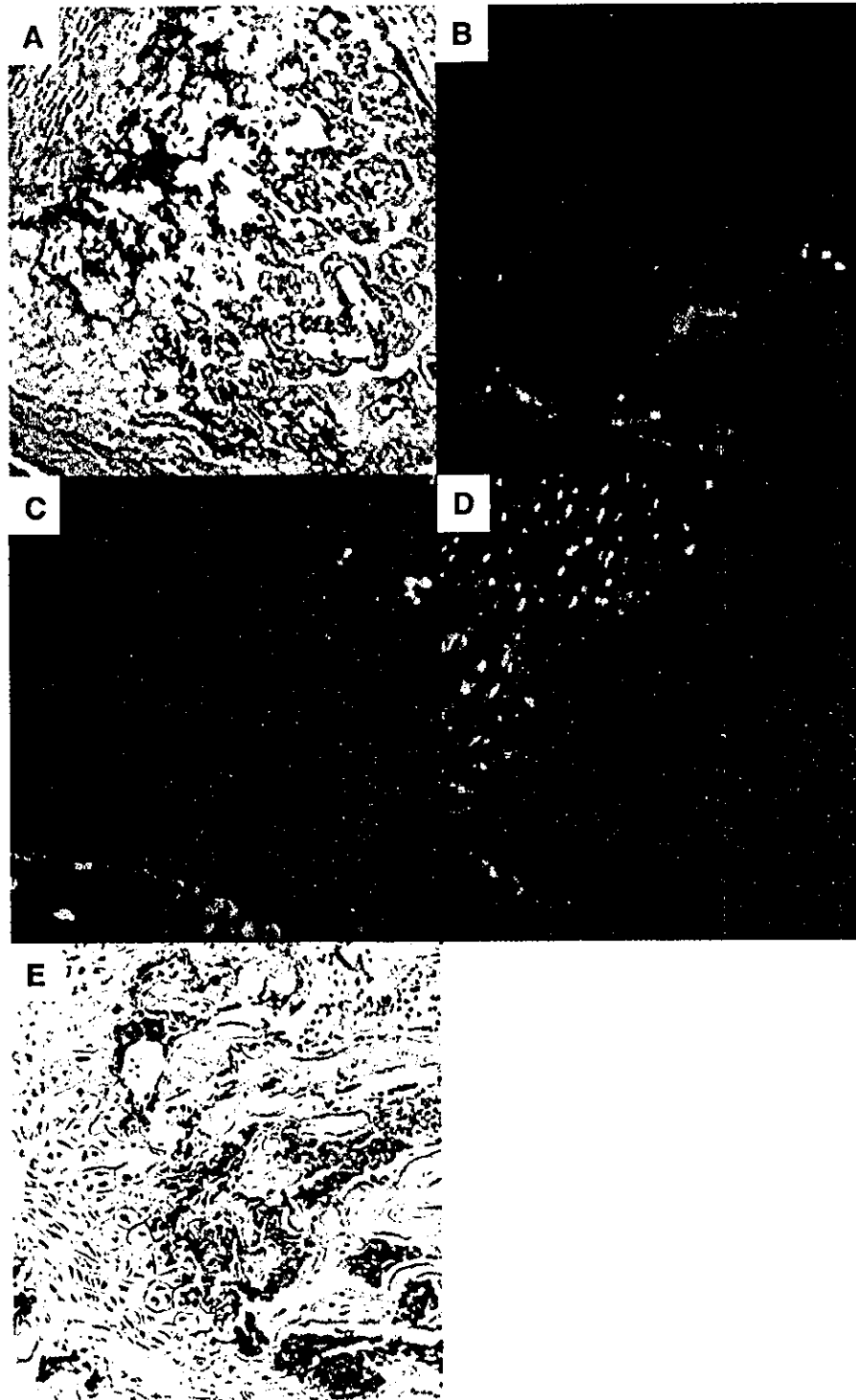
Fig. 2. *In situ* hybridization of the section of the metatarsal bones from a 5-week-old male wild-type mouse using digoxigenin-labeled mouse bim_L (B), procollagen type IA (C) and type II A (D) riboprobes, and TRAP enzymatic staining (A). The labeling was detected by anti-digoxigenin antibody and Alexa 488-labeled anti-rabbit IgG antibody. Note the co-localization of *bim* transcripts with TRAP staining (OCs) (A and B). No positive bim_L staining was co-localized with procollagen type IA staining (osteoblasts) or type IIB staining (chondrocytes). X-gal staining of the tibia from 5-week-old transgenic mice in which *lacZ* gene was introduced into the *bim* locus by homologous recombination also showed the clear positive staining in OCs but not in chondrocytes or osteoblasts (E).

however, remarkably resistant to cytokine withdrawal. Within 48 h of M-CSF depletion, all the wild-type OCs had undergone apoptosis, whereas >90% of *bim–/–* OCs remained alive (Figure 4A).

Interestingly, despite their prolonged survival, *bim–/–* OCs had less bone-resorbing activity than *bim+/+* cells, as

determined by the pit formation assay (Figure 4C). Adenovirus vector-mediated Bim_L expression in *bim–/–* OCs not only reduced their survival, but also recovered bone-resorbing activity of the cells (Figure 4B and C). These results indicate that the skeletal abnormalities in *bim–/–* mice result from impaired activity of OCs.