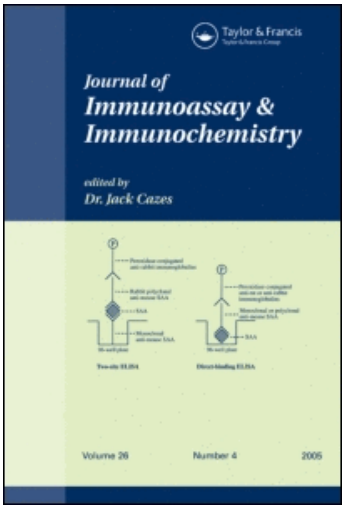

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

ELISA FOR RANKL-OPG COMPLEX IN MOUSE SERA

Makio Mogi[a]; Ayami Kondo[a]
[a] Department of Medicinal Biochemistry, School of Pharmacy, Aichi-Gakuin University, Nagoya, Japan

Online publication date: 23 March 2010

To cite this Article Mogi, Makio and Kondo, Ayami(2010) 'ELISA FOR RANKL-OPG COMPLEX IN MOUSE SERA', Journal of Immunoassay and Immunochemistry, 31: 2, 103 — 110
To link to this Article: DOI: 10.1080/15321811003617305
URL: http://dx.doi.org/10.1080/15321811003617305

Journal of Immunoassay and Immunochemistry, 31:103–110, 2010

ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321811003617305

Taylor & Francis
Taylor & Francis Group

ELISA FOR RANKL-OPG COMPLEX IN MOUSE SERA

Makio Mogi and Ayami Kondo

Department of Medicinal Biochemistry, School of Pharmacy, Aichi-Gakuin University, Nagoya, Japan

□ *A sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for the mouse RANKL-OPG complex was developed by utilizing a polyclonal antibody that recognizes mouse soluble RANKL as an immobilized capture component and mouse OPG IgG labeled with peroxidase. We could quantify the RANKL-OPG complex level (detection limit: 1 pmol/L). Employing this assay system, we demonstrated that the RANKL-OPG complex was constitutively present in the serum of OPG +/− mice, but not in that of OPG −/− or wild-type C57BL/6J mice.*

Keywords ELISA, OPG, RANKL

INTRODUCTION

Recent studies have identified many of the molecules, especially cytokines, required for osteoclast formation. In particular, osteoclast formation from precursor cells, as well as osteoclast activation, requires the 45-kDa receptor activator of NF-kB ligand (RANKL).[1] *In vivo* treatment of mice with RANKL activates osteoclasts, promotes bone loss, and causes severe hypercalcemia.[2] Recent evidence indicates that a soluble form of RANKL is secreted by activated T-lymphocytes and osteoblasts.[3,4] More importantly, RANKL secreted by activated T-cells promotes joint inflammation and bone and cartilage destruction in rheumatoid arthritis, a chronic inflammatory disease.[4]

Osteoprotegerin (OPG: 55 kDa), a glycoprotein of the TNF receptor superfamily secreted by osteoblasts, is a decoy receptor for RANKL.[5] When OPG is present to bind to RANKL, the cell-to-cell signaling between marrow stromal cells and osteoclast precursors is inhibited; and, thus, osteoclasts are not formed.[2–6] Thus, RANKL and OPG expressed by

Address correspondence to Makio Mogi, Department of Medicinal Biochemistry, School of Pharmacy, Aichi-Gakuin University, Nagoya 464-8650, Japan. E-mail: makio@dpc.aichi-gakuin.ac.jp

bone-associated cells play important roles during osteoclast formation, by balancing induction and inhibition.[7]

A recent study using animal models suggests the involvement of RANKL and OPG in the pathogenesis of periodontal disease.[8] In addition to the increase in the level of RANKL protein in the inflamed synovium of rheumatoid arthritis patients,[9] we demonstrated earlier that OPG concentrations in the synovial fluid were lower in patients with rheumatoid arthritis (as compared with patients with other forms of arthritis), which resulted in an increased local and systemic RANKL:OPG ratio.[10] We also demonstrated an elevation of RANKL and a decrease in OPG in gingival crevicular fluid of patients with periodontal disease[11,12] and in synovia of patients with tempomandibular joint disorder,[13] suggesting that RANKL and OPG are important factors involved in bone and joint destruction in humans, and that OPG has a protective role in bone-destructive diseases.

Whereas there is no direct proof, it is reasonable that the majority of RANKL is present as the RANKL-OPG complex in body fluid due to the large amount of OPG *in vivo*. Further, it is likely that the determination of RANKL by commercially available ELISA is interfered with due to a large amount of OPG in the samples. Although RT-PCR, Northern blot analysis, and ELISA for RANKL were performed in several studies,[4,14–16] there is no study on the protein level of RANKL-OPG complex due to the unavailability of an ELISA. For clarification of the role of RANKL in the mammalian body, a specific, quantitative, and highly sensitive assay for the RANKL-OPG complex is indispensable.

In view of these limitations, we developed a new two-site sandwich ELISA for mouse RANKL-OPG complex employing polyclonal anti-OPG IgG labeled with peroxidase and anti-RANKL IgG as the immobilized capture component. This ELISA showed high accuracy and good reproducibility.

EXPERIMENTAL

Animals and Drugs

The sera from male OPG −/−, OPG +/− (heterozygote), and WT mice (C57BL/6J: Japan Clea Co.) were kindly donated by Prof. N. Udagawa of the Department of Oral Biochemistry, Matsumoto Dental University (Nagano, Japan). Other chemicals and reagents were of analytical grade. Fully bioactive recombinant mouse RANKL and anti-mouse RANKL IgG (goat) were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Recombinant mouse OPG and anti-mouse OPG IgG (goat) labeled with horseradish peroxidase were also from R&D Systems. Inc. Tetramethylbenzidine/H_2O_2 substrate was from Kirkegaard & Perry Lab Inc. (MD, USA).

ELISA Procedure

The immunoreagents were essentially similar to those described previously.[17–19] Briefly, 100 μL/well of the capture anti-mouse RANKL IgG (500 ng/mL of PBS) was transferred to an ELISA plate (Nunc, Denmark, 96 wells, flat bottom) and incubated overnight at 4°C; then each well was aspirated, and the plate blocked by adding 250 μL of PBS containing 1% bovine serum albumin (BSA, buffer-1) to each well. Incubation was carried out at room temperature for a minimum of 1 hr. Next, each well was washed with buffer (0.05% Tween 20 in PBS), and the sample or standard (RANKL and OPG mixture) in an appropriate diluent was then added final volume of 100 μL of buffer-1 and the plate was incubated at 37°C with vigorous shaking for 1 hr. Then, the reaction medium was removed by aspiration; and each well was washed 3 times with 1 mL of chilled washing buffer, after which 100 μL of anti-mouse OPG IgG labeled with peroxidase (100 ng/mL of buffer-1) was added to each well. Incubation was then carried out for 1 hr at 37°C. After washing, the bound enzyme protein was assayed with tetramethyl-benzidine/H_2O_2 as a substrate (100 μL/well), and the enzymic reaction was stopped by adding 100 μL of 1 M phosphoric acid to each well. The measurement was made at 450 nm with a Biotrak-II plate reader (Amersham Bioscience, USA). Values were presented as the mean ± SEM of 6 samples/group. Each experiment was repeated 3 times. Commercially available ELISA for RANKL and OPG (R&D Systems, Inc., Minneapolis, MN, USA) were conducted by following the manufacturer's standard protocol. Differences between controls and experiment treatment groups were determined by using paired Student's *t*-test.

RESULTS

Evaluation for the Complex of RANKL and OPG

The complex of RANKL and OPG was determined by the quantitative sandwich ELISA system using microplates, pre-coated with anti-RANKL polyclonal antibodies, and peroxidase-linked anti-OPG polyclonal antibodies. Quantitative analysis of the complex formed between RANKL and OPG showed that RANKL bound to OPG at an equivalent molarity (Fig. 1) and that pre-incubation of RANKL and OPG for 30 min at 25°C was sufficient time to form the complex, which was stable up to 6 hr (data not shown).

Standardization of ELISA for RANKL-OPG Complex

Calibration curves based on the use of known concentrations of mouse RANKL-OPG complex are shown in Fig. 2. The working range of the assay

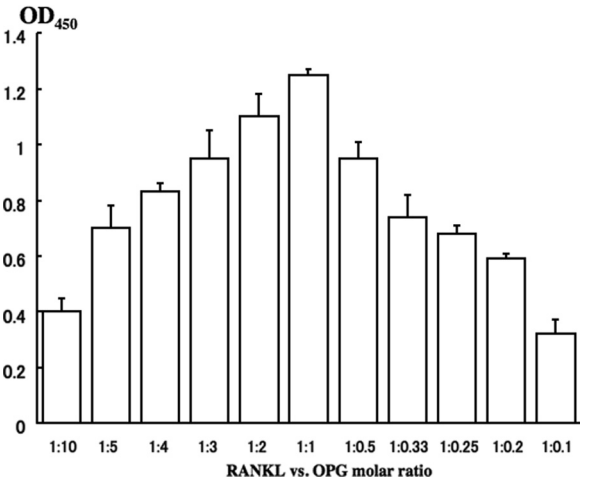

FIGURE 1 Evaluation of maximum binding ratio for RANKL/OPG by current ELISA. Data are expressed as the mean ± SD of six wells. After pre-incubation of RANKL and OPG for 30 min at 25°C, the samples were subjected to the ELISA analysis.

was between 1.56 and 100 pmol/L for the mouse RANKL-OPG complex. The detection limit of the assay (2 × blank) was 1 pmol/L for the mouse RANKL-OPG complex. The samples with low or high complex concentrations were analyzed to assess precision (inter- or intra-assay variation, Table 1). The coefficient of variation (CV) for the interassay ranged from 5.1 to 13.6%. All CVs for the intraassays were 15.8% or less.

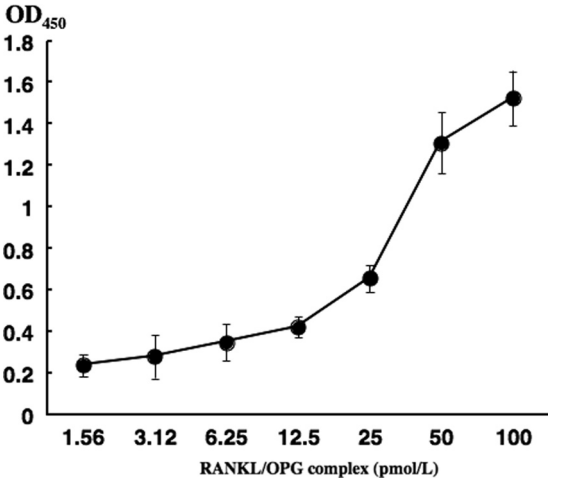

FIGURE 2 Standard curve of purified RANKL-OPG complex generated with the ELISA. Mouse standard complexes were assayed, and the data are expressed as the mean ± SD of six wells.

TABLE 1 Intra- and Inter-assay Variability of ELISA System for Mouse RANKL-OPG Complex

Sample	RANKL-OPG (pmol/L)	Coefficient of Variation (CV %)
Inter-assay		
1	4.4 ± 0.6	13.6
2	25.0 ± 2.1	8.4
3	102 ± 5.2	5.1
Intra-assay		
1	3.8 ± 0.6	15.8
2	18.8 ± 1.7	9.0
3	97.2 ± 8.6	8.8

Mean ± S.E.M. n = 6.

RANKL: 45 kDa, 4.5 ng/ml = 4.5 μg/L = 100 pmol/L.

OPG: 55 kDa, 5.5 ng/ml = 5.5 μg/L = 100 pmol/L.

Therefore, RANKL/OPG complex (100 pmol/L) = 4.5 ng/ml of RANKL mixed with 5.5 ng/ml of OPG. The mixture was incubated at 25°C for 30 min, and then the amount of complex was measured by the current ELISA.

It is likely that the determination of RANKL by commercially available ELISA is interfered with by the large amount of OPG in the samples. In fact, the determination of RANKL in samples with a commercial ELISA could not be evaluated accurately in the presence of a large amount of OPG (Table 2). In contrast, the addition of OPG did not affect the determination of the RANKL-OPG complex level in the samples determined by the current ELISA (Table 3).

We hypothesize that the use of anti-OPG IgG as an immobilized capture antibody would be disadvantageous, since the assay might be interfered by large amount of endogenous free OPG. In fact, when we configured the ELISA system with anti-OPG IgG as the immobilized capture antibody and anti-RANKL IgG labeled with peroxidase for detection, we could not determine RANKL-OPG complexes in the samples in the presence of a large amount of OPG (data not shown).

TABLE 2 Recovery Test of RANKL in the Commercially Available ELISA System for RANKL Using PBS with a the Large Amount of OPG

OPG Added (pmol/L)	RANKL (4 pmol/L)	RANKL (20 pmol/L)
0	4.05 ± 0.5 (100)	20.2 ± 1.5 (100)
6	3.76 ± 0.62 (93)	19.6 ± 0.7 (97)
20	2.26 ± 0.52 (55)**	17.6 ± 1.1 (87)*
60	0.84 ± 0.15 (21)**	10.3 ± 2.2 (51)**

Mean ± S.E.M. n = 6.

*$p < 0.05$, **$p < 0.01$ (compared with 0 OPG added).

TABLE 3 Recovery Test of RANKL-OPG Complex in Current ELISA System for RANKL-OPG Using PBS with a Large Amount of OPG

OPG Added (pmol/L)	RANKL-OPG (20 pmol/L)	RANKL-OPG (100 pmol/L)
0	19.5 ± 2.0 (100)	100.6 ± 10.4 (100)
20	19.2 ± 2.4 (98)	100.0 ± 7.2 (99)
60	18.8 ± 2.7 (96)	99.3 ± 11.6 (99)
200	18.1 ± 2.8 (93)	97.8 ± 9.5 (97)

Mean ± S.E.M. n = 6.

TABLE 4 Determination of RANKL, OPG, and RANKL-OPG Complex in Mouse Serum

Mouse	RANKL	OPG pmol/L	RANKL-OPG
Wild (4)	1.8 ± 0.1	31.4 ± 5.1	ND
OPG +/− (4)	8.7 ± 0.3	11.3 ± 1.1	7.4 ± 0.8
OPG −/− (4)	54.0 ± 1.4	ND	ND

Mean ± S.E.M. Number of cases is shown in parentheses.
ND: non-detectable.

Determination of Mouse RANKL-OPG Complex in Mouse Samples

All samples (sera from male OPG −/−, OPG +/− (heterozygote), and WT mice (C57BL/6J)) could be tested directly with this ELISA. Mouse knockout mouse for OPG (OPG −/−) did not produce detectable levels of OPG or RANKL-OPG complex in the samples (Table 4). Interestingly, only the OPG +/− (heterozygote) mouse produced detectable levels of RANKL, OPG, and RANKL-OPG complex in its serum. These findings indicate that the RANKL-OPG complex is constitutively present in the serum of OPG +/− (heterozygote) mice.

DISCUSSION

Four main points may be highlighted regarding our new ELISA: First, it is the first quantitative assay for RANKL-OPG complex protein. The careful choice of antibodies made it possible to determine 1 pmol/L of complex in mouse samples (Fig. 2). Secondly, we used anti-RANKL IgG as the immobilized capture antibody, because anti-OPG antibody as the immobilized capture antibody combined with anti-RANKL IgG labeled with peroxidase was not successful in the development of our ELISA. Thirdly, we confirmed that commercially available ELISA for RANKL was negatively affected by a large

amount of OPG, whereas the addition of OPG did not affect the accurate determination of RANKL-OPG complexes in the samples (Tables 2 and 3). Finally, the most striking finding was that the RANKL-OPG complex was constitutively present in the serum from OPG+/− mice (Table 4). Serum concentrations of RANKL in OPG+/− mice were similar to those of the RANKL-OPG complex, suggesting that most of the RANKL detected in the serum of OPG+/− mice had formed a complex with OPG. The main source of endogenous RANKL and OPG are osteoblastic cells and T-lymphocytes as shown by RT-PCR or Northern blot analysis.[4,14–16] Since lymphocyte-origin RANKL is mainly present as a soluble form and there is a huge amount of OPG present in plasma, it is reasonable that most RANKL would be present as the RANKL-OPG complex form in plasma. It remains to be elucidated whether RANKL-OPG complexes are involved in the pathophysiology of bone-destructive diseases. Further, we could not detect the RANKL-OPG complex in the serum of wild-type C57BL/6J mice. As one of reason, it is possible that current ELISA could not measure the complex due to lower concentration in the sample.

In conclusion, this ELISA could become a powerful tool for investigating the relationship between RANKL-OPG complex and various diseases such as rheumatoid arthritis and postmenopausal osteoporosis, or analyzing the control mechanisms of RANKL production from osteoblastic cells and T-lymphocytes in animal models.

ACKNOWLEDGMENT

This study was partly supported by a grant-in-aid from the Ministry of Education.

REFERENCES

1. Suda, T.; Takahashi, N.; Udagawa, N.; Jimi, E.; Gillespie, M.T.; Martin, T.J. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr. Rev. **1999**, *20* (3), 345–357.
2. Lacey, D.L.; Timms, E.; Tan, H.L. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell **1998**, *93* (2), 165–176.
3. Wong, B.R.; Josien, R.; Lee, S.Y. TRANCE (tumor necrosis factor (TNF)-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. J. Exp. Med. **1997**, *186*, 2075–2080.
4. Kong, Y.Y.; Feige, U.; Sarosi, I. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature **1999**, *402*, 304–309.
5. Simonet, W.S.; Lacey, D.L.; Dunstan, C.R. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell **1997**, *89* (2), 309–319.
6. Yasuda, H.; Shima, N.; Nakagawa, N. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc. Natl. Acad. Sci. USA **1998**, *95*, 3597–3602.

7. Hofbauer, L.C.; Heufelder, A.E. Role of receptor activator of nuclear factor-kappa B ligand and osteoprotegerin in bone cell biology. J. Mol. Med. **2001**, *79* (5–6), 243–253.
8. Teng, Y.-T.A.; Nguyen, H.; Gao, X.; Kong, Y.-Y.; Gorczynski, R.M.; Singh, B. Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection. J. Clin. Invest. **2000**, *106*, R59–R67.
9. Romas, E.; Gillespie, M.T.; Martin, T.J. Involvement of receptor activator of NFkappa B ligand and tumor necrosis factor-alpha in bone destruction in rheumatoid arthritis. Bone **2002**, *30* (2), 340–346.
10. Kotake, S.; Udagawa, N.; Hakoda, M.; Mogi, M.; Yano, K.; Tsuda, E. Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. Arthritis. Rheum. **2001**, *44* (5), 1003–1012.
11. Mogi, M.; Otogoto, J.; Ota, N.; Togari, A. Differential expression of RANKL and osteoprotegerin in gingival crevicular fluid of patients with Periodontitis. J. Dent. Res. **2004**, *83* (2), 166–169.
12. Mogi, M.; Otogoto, J. Expression of cathepsin-K in gingival crevicular fluid of patients with Periodontitis. Arch. Oral Biol. **2007**, *52*, 894–898.
13. Wakita, T.; Mogi, M.; Kurita, K.; Kuzushima, M.; Togari, A. Increase in RANKL:OPG ratio in synovial of patients with TMJ disorder. J. Dent. Res. **2006**, *85* (7), 627–632.
14. Burgess, T.L.; Qian, Y.; Kaufman, S. The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. J. Cell Biol. **1999**, *145* (3), 527–538.
15. Fuller, K.; Wong, B.; Fox, S.; Choi, Y.; Chambers, T.J. TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. J. Exp. Med. **1998**, *188* (5), 997–1001.
16. Kotake, S.; Udagawa, N.; Takahashi, N. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J. Clin. Invest. **1999**, *103*, 1345–1352.
17. Kinpara, K.; Mogi, M.; Kuzushima, M.; Togari, A. Osteoclast differentiation factor in human osteosarcoma cell line. J. Immunoassay **2000**, *21* (4), 327–340.
18. Mogi, M.; Harada, M.; Kondo, T. Interleukin-1β, interleukin-6, epidermal growth factor; and transforming growth factor-α are elevated in the brain from parkinsonian patients. Neurosci. Lett. **1994**, *180*, 147–150.
19. Mogi, M.; Inagaki, H.; Kojima, K.; Minami, M.; Harada, M. Transforming growth factor-α in human submandibular gland and saliva. J. Immunoassay **1995**, *16* (4), 379–394.