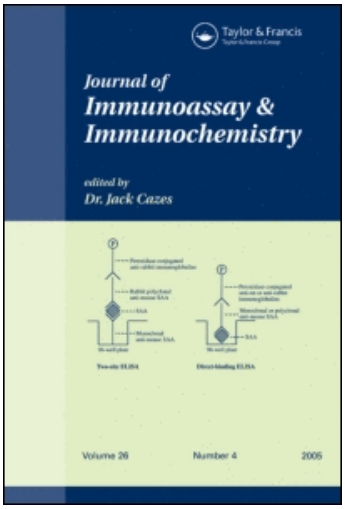

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Osteoclast Differentiation Factor in Human Osteosarcoma Cell Line

Kyoko Kinpara[a]; Makio Mogi[a]; Masatoshi Kuzushima[a]; Akifumi Togari[a]
[a] Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, Nagoya, (JAPAN)

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OSTEOCLAST DIFFERENTIATION FACTOR IN HUMAN OSTEOSARCOMA CELL LINE

Kyoko Kinpara, Makio Mogi*, Masatoshi Kuzushima and Akifumi Togari
Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, Nagoya 464-8650 (JAPAN)
e-mail: makio@dpc.aichi-gakuin.ac.jp

ABSTRACT

A sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for human osteoclast differentiation factor (ODF/RANKL/OPGL/TRANCE) utilizing a polyclonal antibody that recognizes both human soluble ODF and mouse ODF in combination with a osteoclasogenesis inhibitory factor (OCIF/OPG) was developed. We can quantify the ODF level in not only human ODF (detection limit: 0.05 ng/ml), but also mouse ODF by virtue of cross-reactivity. Employing this assay system, we demonstrated that ODF is constitutively present as a membrane-bound form in both the human osteosarcoma cell lines, MG-63, HOS and SaOS-2, and the mouse osteoblastic cell line MC3T3-E1.

KEY WORDS : ODF, OCIF, ELISA, Sarcoma, MC3T3-E1.

INTRODUCTION

Accumulating evidence indicates that osteoclast differentiation factor (ODF), also known as the receptor activator of NF-κB ligand (RANKL), is the final extracellular mediator that causes osteoclast precursors to differentiate into mature osteoclasts (1, 2, 3, 4, 5), and that ODF expression in bone marrow stromal and osteoblastic cells is upregulated by many bone-resorbing factors such as $1,25(OH)_2D_3$, prostaglandin E_2, parathyroid hormone and interleukin-11 (1, 6). In contrast, osteoclastogenesis-inhibitory factor (OCIF) also known as osteoprotegerin (OPG) inhibits osteoclast development (7, 8), because it is an endogenous decoy receptor for ODF to block its actions (1, 2). Thus, divergent mechanisms for bone resorption may converge as the regulation of ODF activity. The primary sites of synthesis of ODF have been demonstrated to be osteoblasts (1). Soluble ODF is derived from membrane-anchored ODF, and each ODF is biologically active via their ability to interact with the ODF receptor, RANK and exert a variety of biological activities both *in vivo* and *in vitro*. Although RT-PCR and Northern blot analysis for ODF were performed in several studies (1, 3, 4, 5, 9, 10), there is no study on the protein level of ODF due to unavailability of an ELISA. To clarify the role of ODF in the mammalian body, a specific, quantitative and highly sensitive assay for ODF is now indispensable. However, when we attempted to develop an ELISA for the detection of human ODF, using commercially available different polyclonal IgG, we could not find a suitable set for the capture and the detection of the antibody.

In view of these limitations, we developed a new two-site sandwich ELISA for human ODF employing polyclonal anti-ODF IgG and OPG as immobilized capture components. This yields a quantitative assay

of high accuracy and good reproducibility. Furthermore, this assay can also be utilized to quantify mouse ODF.

MATERIALS AND METHODS

Fully bioactive recombinant human ODF and anti-human ODF (rabbit) were obtained from the Pepro Tech. EC Ltd., (London, England). Recombinant human OPG/Fc chimera was from R&D Systems. Inc. (Minneapolis, MN, USA). Tetrametylbenzidine/H_2O_2 substrate (Kirkegaard & Perry Lab Inc. MD, USA) and anti-rabbit IgG conjugated with peroxidase (Bio-Rad, CA, USA) was obtained. ELISA for OPG was obtained from CosmoBio (Tokyo, Japan). Phenylmethylsulfonylfluoride (PMSF), Triton-X-100 and other reagents used were all commercially obtained.

ELISA procedure

The immunoreagents were essentially similar to those described previously (11, 12, 13). Briefly, 50 µl/well of the capture OPG/Fc (200 ng/ml or 1 µg/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. The sample or standard (ODF) in an appropriate diluent were incubated at 37°C with vigorous shaking in a final volume of 50 µl of buffer-1. After 1 h the reaction medium was removed by aspiration, each well was washed three times with 1 ml of chilled washing buffer (0.05% Tween 20 in PBS), and then 50 µl of anti-ODF

IgG (100 ng/ml of buffer-1) was added to each well and incubated for 1 hr at 37°C. After washing, 50 µl/well of anti-rabbit IgG conjugated with peroxidase (Bio-Rad, 1/1000 of original solution) was added to each well and incubated for 1 hr at 37°C. The bound enzyme protein was assayed with tetramethyl-benzidine/H_2O_2 as a substrate, and stopped by 50 µl/well of 1 M phosphoric acid. The measurement was conducted at 450 nm by Biolumin 960 (Molecular Dynamics, USA). Value were presented as the mean ± SEM of 4-6 cultures/group. Each experiment was repeated three times. Differences between controls and experiment treatment groups were determined by paired Students *t*-test.

Cell culture.

MC3T3-E1 cells, established from newborn mouse calvaria, are a clonal osteogenic cell line whose cells can differentiate into osteoblasts, and are frequently used as a model to study the characterization of osteoblasts *in vitro* (14, 15). The human osteosarcoma cell line SaOS-2 was obtained from RIKEN cell bank (Tsukuba, Japan), and HOS and MG-63 from the American Type Culture Collection (Rockville, MA, USA). SaOS-2 cells in McCoy's 5A, HOS and MG-63 cells in Eagle's MEM were cultured in plastic dishes. The cell culture for the human osteosarcoma cell line MG-63, SaOS-2 and HOS followed a standard protocol. Each cell was plated in a 10-cm dish at 1 x 10^6/dish, and used when the cells were subconfluent. All samples (cells and conditioned medium) were stored at - 80 °C until assayed. Cell samples were homogenized, lysed in lysis buffer (0.1 % Triton-X-100 and 0.1 mM PMSF in 50 mM sodium phosphate buffer, pH 7.5), and centrifuged at 14,000 x *g* for 10 min to remove insoluble

materials. Lysed-protein content was determined by protein dye binding, with BSA employed as a standard, by use of a DC-Bio-Rad protein-assay kit (16).

RESULTS

Standardization of ELISA

Calibration curves based on the use of known concentrations of human ODF are shown in Figure 1. The working range of the assay was between 0.1 and 10 ng/ml for human ODF. The detection limit of the assay (2 x blank) was 0.05 ng/ml for human ODF. The samples with low or high ODF concentration were analyzed to assess precision (inter- or intra-assay variation) (Table 1). The coefficients of variation (CV) for the interassay ranged from 2.7 to 10.0%. All CVs for the intraassay were less than 11%. In addition, the recoveries of human ODF added to plasma were 90.7-108.7%, when ODF at three different levels (0.15, 0.75, and 4 ng/ml) was added to three plasma samples (20 μl) containing 0.28-0.85 ng/ml of ODF (Table 2).

According to the manufacture's information, there is 86% homology between human and mouse OPG at the protein level, and human OPG can also bind to mouse ODF. When the MC3T3-E1 extract was assigned for immunoreactive ODF protein using the present ELISA, logit transformation of the displacement data generated lines with slopes that were not significantly different from the slope of the line generated by authentic human ODF, indicating the presence of immunoreactive ODF protein in the mouse osteoblastic cells (Figure 1), and this assay could also detect mouse ODF due to the cross-reactivity of the antibodies. The CV of intra- or interassay variation was less than 12.4%.

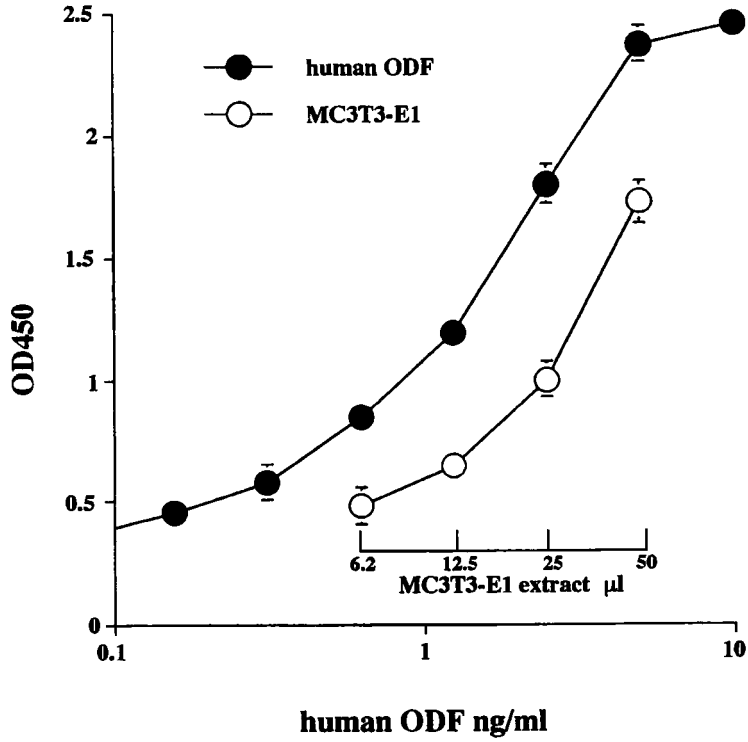

Fig. 1. Standard curves of purified ODF generated with the ELISA. Human ODF or mouse MC3T3-E1 extract was incubated, and data are expressed as the mean ± SEM of six.

Effect of free OPG and Triton-X-100 on the ELISA

The requirement of OPG/Fc chimera as an immobilized capture protein has a disadvantage, that is, the assay might be interfered by endogenous free OPG. We confirmed that human osteosarcoma cells continuously produced OPG (0.1 to 10 ng/ml), as shown in Table 3. As shown in Figure 2, relatively high concentrations of free OPG (10 and 100 ng/ml) interfered with the assay using immobilized OPG (200 ng/ml). However, the high concentration of immobilized OPG (1 μg/ml) overcame this problem in the presence of free OPG (10 ng/ml).

TABLE 1

Intra- and interassay variability of
ODF ELISA system for human ODF

Sample	ODF (ng/ml)	Coefficient of Variation (CV %)
Interassay		
1	0.20 ± 0.02	10.0
2	1.00 ± 0.08	8.0
3	4.11 ± 0.11	2.7
Intraassay		
1	0.19 ± 0.02	10.5
2	0.99 ± 0.09	9.1
3	4.08 ± 0.28	6.9

Mean ± S.E.M. n = 6

High concentrations of Triton-X-100 also disturbed the assay as shown in Figure 3. The dilution with buffer-1 (final concentration: 0.025% of Triton-X-100) showed good recovery, therefore, we applied these diluted lysis-samples to the current ELISA.

Determination of sODF in human and mouse samples

Due to unavailability of an ELISA system for ODF, previous assays for ODF were conducted by RT-PCR or Northern blot analysis. All samples (the extract from Triton-treated cells and conditioned medium) could be tested directly in this ELISA. Although human sarcoma cells

TABLE 2

Recovery test of ODF in the ELISA system using human plasma

Added		Calculated (ng/ml)	Found (ng/ml) (% recovery)
Sample 1	0	-	0.28
	0.15	0.43	0.40 (93.0)
	0.75	1.03	1.10 (93.6)
	4	4.28	4.40 (97.3)
Sample 2	0	-	0.50
	0.15	0.65	0.59 (90.7)
	0.75	1.25	1.15 (108.7)
	4	4.50	4.40 (102.3)
Sample 3	0	-	0.85
	0.15	1	0.93 (93.0)
	0.75	1.60	1.75 (91.4)
	4	4.85	4.80 (101.0)

TABLE 3

ODF and OPG concentrations in the conditioned medium of the human osteosarcoma cell line

Human osteosarcoma	ODF (ng/ml)	OPG (ng/ml)	OPG (total ng)
MG-63 (6)	< 0.05	3.21 ± 0.40	12.84 ± 1.61
Hos (6)	< 0.05	0.45 ± 0.02	1.79 ± 0.10
SaOS-2 (6)	< 0.05	0.97 ± 0.05	3.87 ± 0.21

Mean ± S.E.M. Number of cases are shown in parentheses.
Conditioned medium of the human osteosarcoma cell line were obtained under the 0.1%-FCS condition for 24 hr.

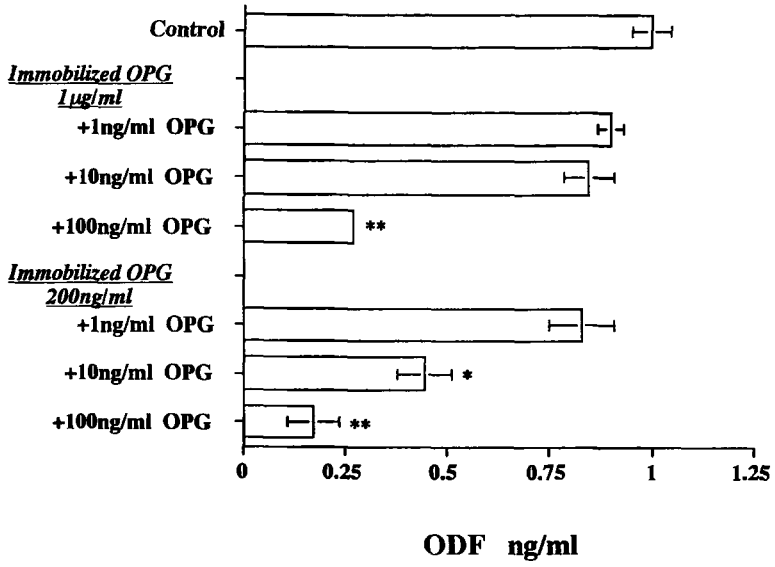

Fig.2. Interference by exogenous OPG in the ODF ELISA. Data are expressed as the mean ± SEM of six. $*P < 0.05$; $**P < 0.01$ (compared with control).

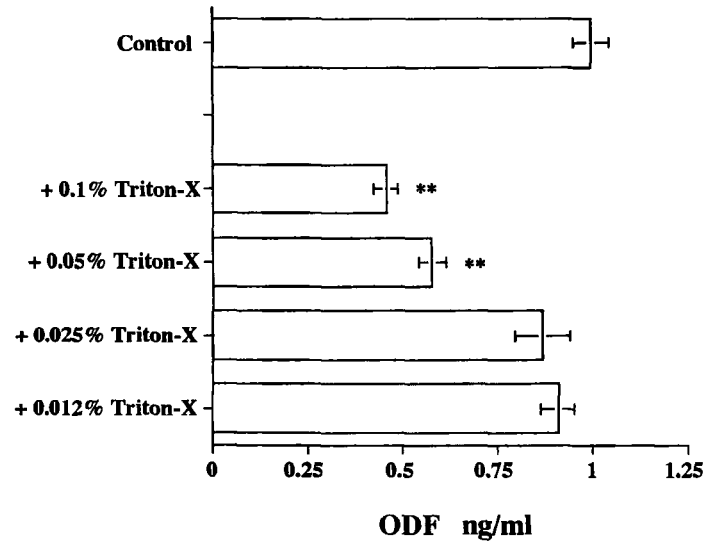

Fig.3 Interference by Triton-X-100 in the ODF ELISA. Data are expressed as the mean ± SEM of six. $**P < 0.01$ (compared with control).

TABLE 4

Determination of ODF and OPG in human and mouse samples treated with Triton X-100

Sample	ODF (ng/mg protein)	ODF (total ng)	OPG (ng/mg)	OPG (total ng)
Human osteosarcoma cell line				
MG-63 (6)	0.13 ± 0.01	0.12 ± 0.01	1.15 ± 0.20	1.03 ± 0.15
Hos (6)	0.17 ± 0.01	0.20 ± 0.01	0.30 ± 0.02	0.36 ± 0.01
SaOS-2 (6)	0.13 ± 0.04	0.07 ± 0.02	0.64 ± 0.06	0.35 ± 0.02
MC3T3-E1 (6)	0.10 ± 0.01	0.13 ± 0.01	-	-

Mean ± S.E.M. Number of cases are shown in parentheses.
The cells of the human osteosarcoma cell line were obtained under the 0.1%-FCS condition for 24 hr, and then solubilized by Triton-X-100 (0.1%). Commercially available ELISA for human OPG could not measure OPG content in MC3T3-E1 cells.

did not produce detectable levels of ODF in the conditioned medium, it could measure the relatively low level of Triton-solubilized ODF in the control human sarcoma cell lines, MG-63, HOS and SaOS-2 cells (Table 2 and Table 3). The calculated levels of immunoreactive ODF protein in MC3T3-E1 are also shown in Table 4. When we subjected the Triton-extracts from human osteosarcoma to SDS-PAGE and Western blotting using anti-ODF IgG, the samples gave a high-density

20-kDa band, which corresponded to the same relative molecular mass as authentic human ODF (data not shown). These findings indicate that ODF is constitutively present as a membrane-bound form in human osteosarcoma and mouse MC3T3-E1 cells.

DISCUSSION

This ELISA offers three main advantages: First, it is the first quantitative assay for ODF protein. The careful choice of antibodies combined with OPG as an immobilized capture made it possible to determine 0.05 ng/ml of ODF in human samples. Secondly, we used OPG as an immobilized capture protein. Although a different commercially available anti-ODF antibody was not successful in the development of ELISA, the biologically active form ODF could be detected when OPG was substituted for anti-ODF antibody. Finally, this ELISA is applicable to determine the concentration of mouse ODF. The present findings suggest that the binding site recognized by the primary-capture OPG, was identical between human and mouse ODF. The most striking finding was that ODF itself was constitutively present in both human osteosarcoma and the mouse osteoblastic cell line MC3T3-E1. The main source of endogenous ODF was the osteoblastic cells as shown by RT-PCR or Northern blot analysis (1, 3, 4, 5, 9, 10), and the present findings confirmed that ODF protein was produced in these cells. We detected a membrane-bound form of ODF but not ODF in all conditioned media (Table 3 and 4), suggesting that osteoblastic cells did not produce the soluble form of ODF at a detectable level. Although lymphocyte-origin ODF was mainly present as a soluble form (17), the current findings suggested that cell-

to-cell interaction between osteoblasts/stromal cells and osteoclast progenitors was essential for the osteoclast-like cell formation (18). Taken together, this ELISA could become a powerful tool for investigating the relationship between ODF and various diseases such as rheumatoid arthritis and postmenopausal osteoporosis, or analyzing the control mechanisms of ODF production from osteoblastic cells.

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