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Immunological Study on Circulating Murine Osteoprotegerin/Osteoclastogenesis Inhibitory Factor (OPG/OCIF): Possible Role of OPG/OCIF in the Prevention of Osteoporosis in Pregnancy

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Osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF) is a soluble member of the tumor necrosis factor receptor family and plays a crucial role in the negative regulation of osteoclastic bone resorption. We have immunized OPG/OCIF knockout mice with murine rOPG/rOCIF and established a panel of hybridomas producing monoclonal antibodies (mAbs) to murine rOPG/rOCIF. Utilizing the mAbs, we developed enzyme-linked immunosorbent assay (ELISA) systems: one detecting both homodimeric and monomeric forms of murine OPG/OCIF and the other detecting only dimeric form of murine OPG/OCIF. With the aid of these ELISA systems we showed that OPG/OCIF is present mainly as a monomer in murine blood. The concentration of OPG/OCIF in normal mouse sera was approximately 500 pg/ml and there was no statistical difference in the serum concentration of OPG/OCIF among genders, age, and strains. Interestingly, the concentration of circulating OPG/OCIF in mouse markedly increased during pregnancy. The result indicated that circulating OPG/OCIF plays an important role in the protection of bone from excess resorption during pregnancy in mammals.

Key Words: **osteoprotegerin; osteoclastogenesis inhibitory factor; enzyme-linked immunosorbent assay; monoclonal antibody; pregnancy; serum; marker.**

Osteoclastogenesis inhibitory factor (OCIF), also called osteoprotegerin (OPG) is a secreted member of the TNF receptor (TNFR) family (1, 2). In *in vitro* studies, OPG/OCIF inhibited osteoclastogenesis by interrupting the intercellular signaling between osteoblastic stromal cells and osteoclast progenitors (3). In normal rats, systemic administration of recombinant OPG/OCIF (rOPG/rOCIF) resulted in a marked increase in bone mineral density (BMD) associated with a decrease in the number of active osteoclasts (3). Moreover, rOPG/rOCIF prevented bone loss and restored bone strength in ovariectomized rats (4). On the other hand, OPG/OCIF knockout mice exhibited severe osteoporosis due to enhanced osteoclastogenesis (5, 6).

By using OPG/OCIF as a screening probe, we molecularly cloned a ligand for OPG/OCIF (7) and termed osteoclast differentiation factor (ODF) which is also called OPG ligand (OPGL) (4), TNF-related activation-induced cytokine (TRANCE) (8), and receptor activator of NF-κB ligand (RANKL) (9). ODF is a long-sought ligand expressed on osteoclasts/stromal cells in response to osteotropic factors, and mediates an essential signal to osteoclast progenitors for their differentiation into active osteoclasts (4, 7, 10). We then demonstrated that receptor activator of NF-κB (RANK) (9) is the signaling receptor essential for ODF-mediated osteoclastogenesis (11). It is believed that ODF, RANK, and OPG/OCIF play essential roles in osteoclastogenesis and function osteoclast (12, 13).

Recently, we have established two human OPG/OCIF ELISA systems: one equally detects both homodimeric and monomeric forms of OPG/OCIF (total OPG/OCIF ELISA) and the other is specific for homodimer (dimer-specific OPG/OCIF ELISA). Utilizing these ELISA systems, we have characterized the nature of OPG/OCIF molecules in human serum and synovial fluid. The concentration of circulating OPG/OCIF increased with age in both healthy men and women, and was positively correlated with osteoporotic bone loss (14). Serum OPG/OCIF decreased signifi-

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cantly after short-term administration of glucocorticoids suggesting the involvement of OPG/OCIF in osteoporosis developed via glucocorticoid-induced enhancement of bone resorption (15). On the other hand, OPG/OCIF concentration in synovial fluid was significantly lower in patients with rheumatoid arthritis (RA) than in those with other arthopathies (16). The concentration of OPG/OCIF correlated with severity of knee osteoarthritis (OA) (17). An OPG/OCIF ELISA system with high sensitivity will provide a useful tool for the study of the pathogenesis of bone disorders because OPG/OCIF is a useful marker of abnormal bone resorption in such as osteoporosis, hypercalcemia in malignancy, hyperparathyroidism, Paget's disease, and rheumatoid arthritis.

It is known that the body calcium is metabolized greatly in women during pregnancy. Because the fetus requires a large quantity of calcium, the mother must adapt to meet the calcium demands of the fetus. It is known that intestinal calcium absorption increases during pregnancy (18, 19). But this required may not be sufficient to provide all the fetal calcium requirements and some of the calcium might be obtained from the skeleton. Actually, measurement of several bone markers during pregnancy revealed that there is a high bone-turnover state in the late pregnancy (20). Even though the mothers are placed under such bone absorption stresses, osteoporotic fracture is a rare occurrence during pregnancy. We found it necessary to determine OPG/OCIF level during pregnancy to elucidate the mechanism involved in the bone turnover in this period.

Several models such as ovariectomized (21), immobilized (22), and collagen induced RA models (23, 24) have been established for the experimental studies of bone diseases in mice. The popularity of the mouse models owes largely to its utility in genomic studies such as mapping of quantitative trait loci (QTL) and transgenic and knockout studies, and some knockout and transgenic mice with skeletal anomalies have been reported (2, 5, 6, 25–33). Measurement of OPG/OCIF in these mice will provide some insights into the cause of skeletal anomalies found also in humans.

We have immunized OPG/OCIF knockout mice with murine rOPG/rOCIF and established a panel of hybridomas producing monoclonal antibodies (mAbs) to murine rOPG/rOCIF. Utilizing the mAbs, we developed two ELISA systems: one detecting both homodimeric form and monomeric form murine OPG/OCIF and the other detecting only dimeric form OPG/OCIF. With these assay systems, we assessed the OPG/OCIF level in pregnancy.

MATERIALS AND METHODS

Materials. Chinese hamster ovary (CHO) cells transfected with murine OPG/OCIF expression vector were cultured in EX-301 medium (JRH). Homodimeric form murine rOPG/rOCIF was purified from the conditioned media by successive chromatography on Hi-Trap Heparin (Amersham-Pharmacia, Uppsala, Sweden) and Hi-Trap SP (Amersham-Pharmacia) columns. The monomeric form murine rOPG/rOCIF was purified from the conditioned media by successive chromatography on Hi-Trap Heparin, Hi-Trap SP, and Hi-Trap Heparin columns. Production of OPG/OCIF knockout mice was reported previously (5). A murine myeloma cell line, P3X63Ag.8.653, and a murine osteoblastic cell line, ST2, were obtained from American Type Culture Collection (Rockville, MD) and Riken Cell Bank (Ibaraki, Japan), respectively. Normal mouse serum was obtained from Cedarlane Laboratories (Canada). Tetramethylbenzidine (TMB) soluble reagent, a substrate for peroxidase (POD), and TMB stop buffer were purchased from ScyTek (Logan, UT).

Preparation of mAbs to murine OPG/OCIF. Monoclonal antibodies were prepared as described previously (14) except for the use of OPG/OCIF knockout mice in place of normal mice. Briefly, OPG/OCIF knockout mice were immunized intraperitoneally with a mixture (1:1) of homodimeric form and monomeric form murine rOPG/rOCIF emulsified with an equal volume of complete Freund's adjuvant (Difco, Detroit, MI) (10 μg protein/200 μl volume/mouse) three times with 1-week intervals. Four days after the final immunization, each animal received an intravenous administration of 10 μg rOPG/rOCIF in 100 μl PBS. Splenocytes from the immunized animals were fused to P3X63Ag.8.653 mouse myeloma cells (ATCC CRL1580) according to the published method (14, 34, 35). Hybridomas secreting mAbs to murine rOPG/rOCIF were screened with an ELISA system based on the binding to homodimeric form murine rOPG/rOCIF as follows. Fifty microliter of purified homodimeric form murine rOPG/rOCIF solution (200 ng/ml in 0.1 M sodium bicarbonate pH 9.6) was added to each well of 96-well plates (MaxiSorp, Nalge Nunc, Roskild, Denmark) and the plates were incubated at 4°C overnight. The solution was removed by flicking the plates and the wells were blocked with a blocking solution [50% BlockAce (Snow Brand Milk Products, Tokyo, Japan) in water, 200 μl/well] for 2 h at room temperature. After washing three times with a washing buffer (10 mM NaPi buffer containing 0.15 M NaCl and 0.1% polysorbate 20, pH 7.0), 40 μl of calf serum and 10 μl of the conditioned medium of each hybridoma were added to the wells. The plates were incubated for 2 h at room temperature and the wells were washed five times with the washing buffer. To detect monoclonal antibodies bound to the wells, 50 μl of POD-labeled antibody to mouse IgG (KPL, Gaithersburg, MD) diluted 5000-fold with PBS containing 25% BlockAce was added to each well and the plates were incubated for 2 h at room temperature. The wells were washed six times with PBS-P and 100 μl of TMB substrate reagent was added to each well. The plates were further incubated for 1 to 5 min at room temperature. TMB stop buffer (100 μl) was added to each well and absorbance at 450 nm of the wells was measured with a Microplate Reader NJ-2000 (Nippon InterMed, Tokyo, Japan).

The hybridomas were cloned at least twice by the procedure of limiting dilution. Cloned hybridomas (1×10^7 cells) were cultured in Dulbecco's modified Eagle medium (DMEM) for 3 days at 37°C under an atmosphere of 5% CO_2 and 100% humidity. The mAbs were purified from the conditioned media of the hybridomas by Hi-Trap Protein G column according to the manufacturer's protocol (Amersham-Pharmacia, Uppsala, Sweden).

The subclass, the dissociation constant (K_d), and the ability to neutralize murine OPG/OCIF activity of each mAb were determined as previously described (14).

Horseradish peroxidase (HRP) was conjugated to purified mAbs with an EZ-Link Maleimide Activated HRP kit (Pierce) according to the manufacturer's instruction.

Development of ELISA systems for murine OPG/OCIF. An ELISA that equally detects both the monomeric form and homodimeric form murine OPG/OCIF was constructed. An anti-murine OPG/OCIF mAb designated MI-13 was diluted with 0.1 M sodium bicarbonate (pH 9.6) solution to a concentration of 10 μg/ml and the 100 μl

aliquot was added to each well of 96-well plates. After incubation at 4°C overnight, the capture solution was removed by flicking the plates and the wells were blocked with the blocking solution (300 μl/well) for 2 h at room temperature. The wells were then washed three times with the washing buffer. One hundred microliters of rOPG/rOCIF standard and a series of test samples, prepared by serially diluting the original sample with first buffer [0.2 M Tris–HCl (pH 7.4) containing 40% BlockAce, 0.1% polysorbate 20], was added to the wells of the 96-well plates and the plates were incubated for 2 h at room temperature. The wells were then washed six times with the washing buffer and 100 μl of POD-labeled anti-OPG/OCIF mAb designated MI-4, which has been diluted 1000-fold with second buffer [0.1 M Tris–HCl (pH 7.4) containing 25% BlockAce, and 0.1% polysorbate 20], was added to each well. After incubation for an additional 2 h at room temperature, the wells of the plates were washed six times with the washing buffer and 100 μl of TMB substrate reagent was added to each well. The plates were further incubated for 30 min at room temperature. TMB stop buffer (100 μl) was added to each well, and absorbance at 450 nm of the wells was measured with a Microplate Reader.

An ELISA that specifically detects only the homodimeric form murine OPG/OCIF was constructed as described above with some modifications. First, mAb against human OPG/OCIF designated OI-1 (14) was used in place of MI-13 as a solid-phase capture antibody. Second, 0.2 M Tris–HCl (pH 7.4) containing 40% BlockAce, 0.1% polysorbate 20, 0.5%Chaps, 5mM Benzamidine, and 10mM EDTA was used as 1st buffer in place of 0.2 M Tris–HCl (pH 7.4) containing 40% BlockAce and 0.1% polysorbate 20. Third, a POD-labeled mAb designated MI-9 was used in place of POD-labeled MI-4.

Mouse serum. C3H/He (C3H), C57BL/6 (B6), BALB/c, and ICR mice (6 weeks and 6 months old, respectively) were purchased from Charles River Japan Inc. (Yokohama, Japan). The mice were allowed to acclimate to the new environment for at least 7 days before euthanasia for collection of serum. ICR mice were mated and noon of the day on which the copulation plug was found was designated day 0.5 of gestation. The pregnant ICR mice were sacrificed on days 0.5, 5.5, 8.5, 11.5, and 17.5 of gestation, and 2 and 3 days after parturition to collect whole blood. The whole blood collected from each mouse was allowed to clot at room temperature, and centrifuged. The sera were stored at −30°C until use.

RESULTS

Preparation and Characterization of mAbs to OPG/OCIF

Spleens were excised from the three immunized mice and splenocytes from each spleen were fused to P3X63Ag.8.653 myeloma cells. After fourteen days culture from the fusion, the culture supernatants were analyzed for the presence of mAbs to murine rOPG/rOCIF. The conditioned media from approximately 100 wells were found to be positive in the solid-phase ELISA and 11 clones that found to be highly reactive in the ELISA were further cloned. Of those established, only MI-12 was classified as IgG2b isotype, and all the others were classified as IgG1 isotype (Table 1). The K_d values for these mAbs for the homodimeric form and the monomeric form mouse OCIF/OPG ranged from 0.005 to 0.114 nM, and from 0.374 to 36.2 nM, respectively (Table 1). OI-1, a mAb to human rOPG/OCIF, also cross-reacted with murine homodimeric rOPG/rOCIF. Four mAbs, MI-2, -4, -10, and -12 were capable of neutralizing murine OPG/OCIF activity by more than 80% (Table 1). Especially, 500 ng/ml of MI-12 almost completely neutralized the biological activity of 30 ng/ml OPG/OCIF, the level sufficient to inhibit murine osteoclastogenesis. OI-1 was not capable of neutralizing murine OPG/OCIF activity whereas it neutralized human OPG/OCIF activity.

TABLE 1

Characterization of mAbs Generated against Murine rOPG/rOCIF

mAb	K_d (nM) For monomer	K_d (nM) For dimer	Neutralization activity For monomer	Neutralization activity For dimer	Isotype
MI-1	0.447	0.066	++	++	IgG1
MI-2	4.03	0.03	+++	+++	IgG1
MI-4	0.463	0.031	+++	+++	IgG1
MI-5	0.688	0.114	++	++	IgG1
MI-6	1.94	0.038	++	++	IgG1
MI-8	0.374	0.005	++	++	IgG1
MI-9	4.59	0.055	++	+++	IgG1
MI-10	0.685	0.039	+++	+++	IgG1
MI-11	1.71	0.027	−	++	IgG1
MI-12	3.825	0.064	+++	+++	IgG2b
MI-13	36.2	0.071	++	++	IgG1
OI-1[a]	N.D.	0.026	−	+	IgG1

Note. The K_d values for monomeric and dimeric forms of murine rOPG/rOCIF were determined independently. Neutralization activity was determined on the basis of the ability of each mAb to counteract the inhibitory effect of murine OPG/OCIF inhibiting the formation of osteoclasts *in vitro,* and was arbitrarily scored as: −, <5%; +, 5 to 30%; ++, 30 to 80%; +++, >80%.

[a] OI-1 is a mAb to human rOPG/rOCIF which also cross-react with murine rOPG/rOCIF.

Development of ELISA Systems for Murine OPG/OCIF

We screened 11 mAbs for the best combinations to direct murine OPG/OCIF and succeeded in constructing two different types of ELISA systems. A system for "total" murine OPG/OCIF, which detects both the homodimeric and the monomeric forms of OPG/OCIF, was constructed with MI-13 as a capture antibody and MI-4 as a POD-labeled antibody (Fig. 1, upper panel). The other system that specifically detects the homodimeric form murine OPG/OCIF was constructed with OI-1 as a capture antibody and MI-9 as a POD-labeled antibody (Fig. 1, lower panel). The standard curves of both ELISA systems were found to be linear in the range of 31 to 500 pg/ml.

To confirm whether both ELISA systems detect OPG/OCIF in the serum, we added a various concentrations of homodimeric or monomeric OPG/OCIF (31.5–500 pg/ml) to normal mouse serum, and analyzed the resulting samples with these two ELISA systems. The ELISA for total murine OPG/OCIF detected mo-

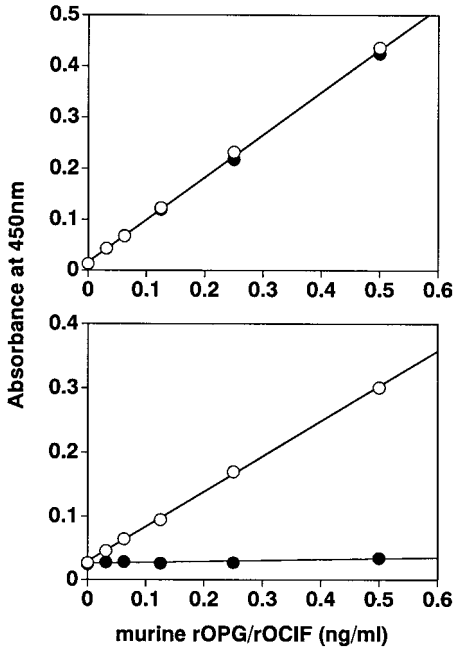

FIG. 1. Standard curves of ELISA systems for total (upper panel) and homodimeric form (lower panel) murine OPG/OCIF. Homodimeric form (○) and monomeric form (●) murine rOPG/rOCIF dissolved in the assay buffer were serially diluted to obtain concentrations in the range of 31.25 to 500 pg/ml. The resulting samples were subjected to the ELISA for total OPG/OCIF with mAbs MI-13 (capture antibody) and MI-4 (POD-labeled antibody), and to the homodimer-specific ELISA with mAbs OI-1 (capture antibody) and MI-9 (POD-labeled antibody).

nomeric form murine rOPG/rOCIF at concentrations between 31 and 500 pg/ml with recoveries of 87.4 to 109.4% in the presence of 25% normal mouse serum (Fig. 2, upper panel). Even the presence of 50% mouse serum in the assay buffer did not alter the standard curve for the homodimer-specific ELISA (Fig. 2, lower panel). Similar results were obtained with sera from several other strains of mice (data not shown). These data indicate that OPG/OCIF exists in murine serum mainly as monomeric form and that the ELISA for total murine OPG/OCIF is suitable for the measurement of OPG/OCIF in murine serum. When sera from ICR and B6 mice were diluted 2- to 8-fold with the assay buffer and analyzed with the ELISA system for total murine OPG/OCIF, a linear response was seen over the tested dilution range (Fig. 3A). When sera from pregnant mice (day 8.5) were diluted 8- to 256-fold with the assay buffer and analyzed with the two ELISA systems, a linear response was seen over the tested dilution range in either system (Fig 3B).

Serum Concentrations of OPG/OCIF in Normal and Pregnant Mice

No statistical difference in the concentration of OPG/OCIF in normal mouse serum was observed among different genders, age, and strains (Fig. 4). The level of OPG/OCIF in normal mouse serum was about 500 pg/ml, but the concentration in individual animal ranged from 200 to 1300 pg/ml.

The concentration of circulating OPG/OCIF in pregnant mice markedly increased by day 8.5 to 11.5 (Fig. 5). The OPG/OCIF level in the female mice at day 11.5 of pregnancy was 10- to 15-fold higher than that in nonpregnant mice. The OPG/OCIF level rapidly decreased and reached to almost normal after parturition. Homodimeric form of murine OPG/OCIF occupied approximately 50% of the total OPG/OCIF in the serum at day 8.5 of pregnancy. It is noteworthy that homodimeric form murine OPG/OCIF was detected in the sera collected at periods between day 8.5 and 17.5 of pregnancy.

DISCUSSION

OPG/OCIF plays essential roles in osteoclastogenesis and osteoclast function elicited through ODF-

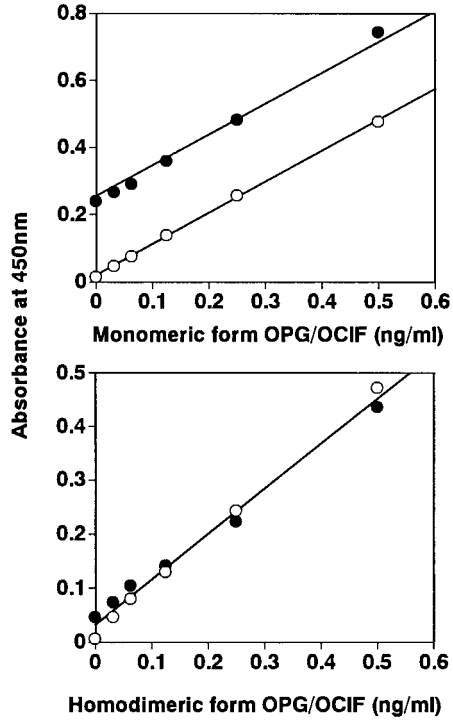

FIG. 2. Determination of the proportions of homodimeric form and monomeric form OPG/OCIF in normal mouse serum. Monomeric form murine rOPG/rOCIF was dissolved in the assay buffer at a final concentration of 500 pg/ml in the absence (○) or presence (●) of 25% mouse serum. Each solution was then serially diluted with the respective buffer to yield rOPG/rOCIF concentrations of 31.25 to 500 pg/ml and the resulting samples were subjected to the ELISA for total OPG/OCIF (upper panel). The homodimeric form murine rOPG/rOCIF was dissolved at a final concentration of 500 pg/ml in the assay buffer in the absence (○) or presence (●) of 50% mouse serum. Each solution was then serially diluted with the respective buffer to yield murine rOPG/rOCIF concentrations of 31.25 to 500 pg/ml. The resulting samples were subjected to the ELISA for the homodimer-specific ELISA (lower panel).

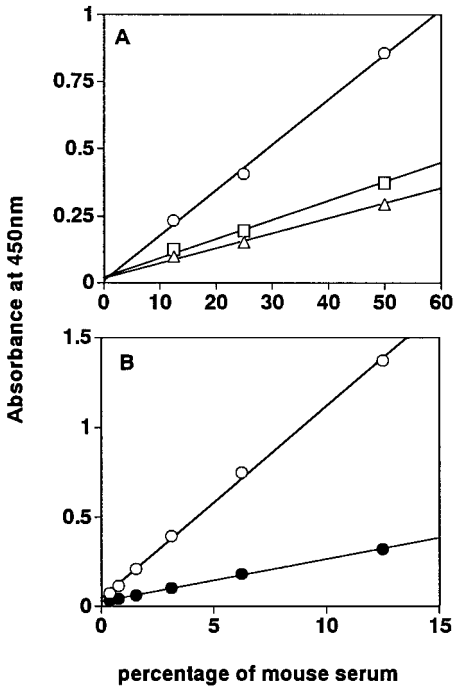

FIG. 3. Dilution profile of endogenous OPG/OCIF. (A) A commercial normal mouse serum (○) and serum samples from ICR mice (□) and B6 mice (△) were diluted 2- to 8- fold with the assay buffer. The serially diluted samples were subjected to the ELISA for total OPG/OCIF. (B) The serum from a pregnant ICR mouse (day 11.5 of pregnancy) were diluted 8- to 256-fold with the assay buffer. The serially diluted samples were subjected to the ELISA for total OPG/OCIF (○) or the homodimer-specific ELISA (●).

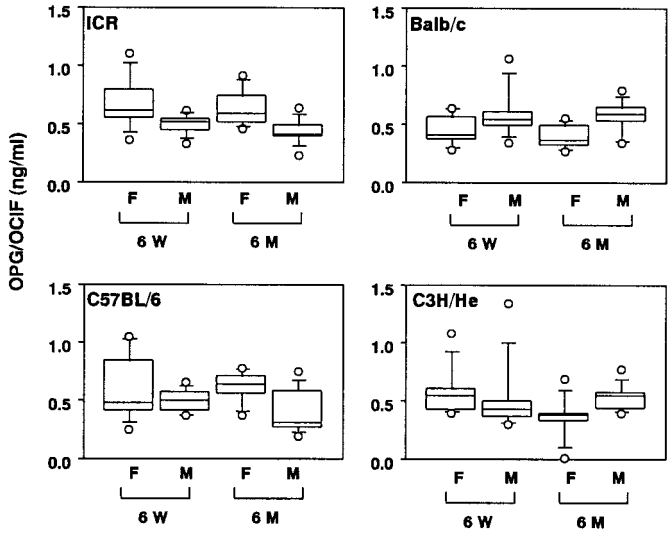

FIG. 4. Concentration of OPG/OCIF in the sera from young (6 weeks old) and old (6 months old) mice. The concentration of total murine OPG/OCIF in the sera was determined by total OPG/OCIF ELISA. The number of samples in each group was 9 or 10. Short horizontal lines indicate 10th and 90th percentiles and long horizontal lines indicated 25th, 50th, and 75th percentiles. Values outside 10th and 90th percentiles are denoted by open circles (○).

RANK signaling (12, 13). Recently, we have established human OPG/OCIF ELISA and reported the nature of OPG/OCIF molecules in human serum and synovial fluid. The sensitive ELISA for OPG/OCIF will provide a useful diagnostic tool and serve elucidating the mechanism involved in bone metabolism. Because of the importance of mice as an animal for the studies of skeletal phenotypes, we started establishing ELISA for mouse OPG/OCIF.

To establish ELISA for mouse OPG/OCIF, we first screened a panel of 33 anti-human OPG/OCIF mAbs to find out the mAbs that bind not only to human OPG/OCIF but also to murine OPG/OCIF. In this screening, we obtained OI-1, a mAb with high affinity to both human and murine OPG/OCIF. To establish hybridomas producing mAb to murine OPG/OCIF, we utilized knockout mice because we were afraid that immunization of rat with murine OPG/OCIF would be unsuccessful, because the amino acid sequence of murine OPG/OCIF is highly homologous to that of rat OPG/OCIF (95% identity) (2, 36). We immunized OPG/OCIF knockout mice with murine OPG/OCIF and established 12 hybridoma lines. With these mAbs we successfully developed two mouse OPG/OCIF ELISA systems: one detecting both dimeric and monomeric forms of murine OPG/OCIF, and the other detecting only homodimeric form OPG/OCIF. Using these two systems we characterized the circulating OPG/OCIF in mice. In normal mice, OPG/OCIF is present mainly as a monomer (Fig. 2) as previously demonstrated for OPG/OCIF in human serum (14). It is known that the half-life of human monomeric form OPG/OCIF in α-phase was significantly longer than that of the homodimer. Homodimeric form murine OPG/OCIF may also tend to redistribute from the circulation to tissues more rapidly than the monomer.

Mice have been used in studies of age-related bone loss for a long time. Mice attain a peak bone density in

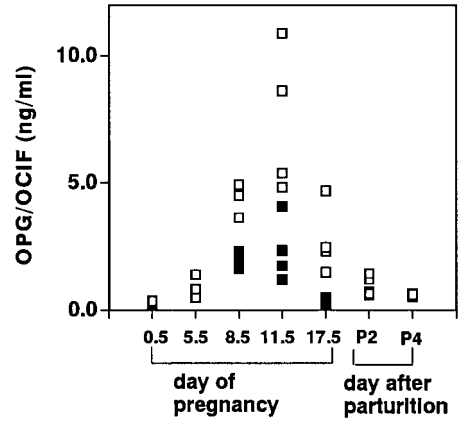

FIG. 5. Concentration of OPG/OCIF in the sera from pregnant mice. The sera from pregnant ICR mice were subjected to the ELISA for total OPG/OCIF (□) or the homodimer-specific ELISA (■).

4–6 months of age, and senescence changes in the skeleton start around 1 year of age developing osteopenia with age (21, 37). Previous studies have shown that C3H/HeJ mice have higher peak bone density and higher concentrations of some bone formation markers (IGF-1, osteocalcin, and bone ALP) than C57BL/6J mice (38–41). Whereas there was no statistical difference in the concentration of circulating murine OPG/OCIF among genders, age, and strains (Fig. 4). The mean concentration of OPG/OCIF in normal mouse serum was about 500 pg/ml but the concentration in individual animal ranged from 200 to 1300 ng/ml.

On the other hand, the concentration of circulating OPG/OCIF in pregnant female mice markedly increased during the period from day 8.5 to day 11.5 of pregnancy (Fig. 5). Surprisingly, homodimeric form murine OPG/OCIF was detected in the serum during the period between day 8.5 and day 17.5 of pregnancy. This is the first report that demonstrates the presence of homodimeric form OPG/OCIF in humors such as serum and synovial fluid. It is likely that an extremely enhanced production of OPG/OCIF surpasses proteolytic conversion of homodimeric form to monomeric one resulting in the presence of homodimeric form in the serum. Previously, we reported that OPG/OCIF gene was strongly expressed in decidua, a maternal tissue surrounding embryos, after day 5.5 of pregnancy (36). This result suggested that during pregnancy OPG/OCIF is produced by decidua. Previous studies reported that OPG/OCIF gene expression and protein production in the osteoblastic cells were stimulated by estrogen (42, 43). Because serum estrogen markedly increases in pregnancy, these facts suggest such possibility that the estrogen-induced OPG/OCIF production by desidua is responsible for the increase of OPG/OCIF in the serum during pregnancy.

Fetuses require a large quantity of calcium provided from the mother in the late pregnancy. Increased level of OPG/OCIF in the late pregnancy may be necessary to restrain calcium flow from the mother to the fetuses. It is reasonable to assume that the maternal body controls the production of OPG/OCIF to meet the calcium demands of the growing fetuses. Although estrogen level is maintained in late pregnancy, the OPG/OCIF level decreases rapidly after day 11.5. Probably, factors other than estrogen is also involved in the swift suppression of OPG/OCIF production. Pregnant women are thought to be protected from osteoporotic bone resorption although they supply large amount of calcium to their fetuses. Increased level of OPG/OCIF in the early pregnancy may play an important role in the protection of maternal bone in pregnancy.

In conclusion, we developed sensitive mouse OPG/OCIF ELISA systems. With these ELISA systems, we have characterized OPG/OCIF in mouse serum. These ELISA systems will be useful tools in the investigation of the mechanisms regulating bone metabolism.

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