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The effects of sex hormones on the expression of ODF and OPG in human gingival fibroblast and periodontal ligament cell at normal menstruation cycle and menopause.

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Periodontitis is a chronic infectious disease that leads to periodontal destruction, and is one of the major causes of tooth loss in humans. The osteoclast differentiation factor (ODF), which is also known as the receptor activator of the NF-kB ligand (RANKL), is a surface-associated ligand on bone marrow stromal cells and osteoblasts. RANKL activates its cognate receptor, RANK, on osteoclast progenitor cells, which leads to the differentiation of mononucleated precursor cells. Osteoprotegerin (OPG) is a decoy receptor that is released from stromal cells and osteoblasts to inhibit the interaction between RANKL and RANK. Although the precise mechanism of bone loss in periodontitis is unknown, the differentiation and activation of osteoclasts by OPG-ODF-RANK signaling might play the role in periodontal bone destruction. The relationship between the concentration of sex hormones and the expression of ODF and OPG was examined by treating human gingival fibroblasts and periodontal ligament cells with the normal serum concentration of estrogen or progesterone during menstruation or at menopause. The ODF/OPG relative ratio was elevated at the concentration observed during ovulation in human gingival fibroblasts and at the concentration observed between ovulation and menstruation in periodontal ligament cells treated with estrogen. However, the ratio was < 1 at all concentrations in both cells treated with progesterone. In the case of menopause simulated by estrogen depletion, the ratio was < 1 in human gingival fibroblasts but > 1 in periodontal ligament cells.

Keywords: OPG, ODF, Menstruation, Menopause, Estrogen, Progesterone, Gingival fibroblast, Periodontal ligament cell

Introduction

Periodontitis is a chronic infectious disease caused by inflammatory responses and host immune responses to a chronic infection (Clark and Loe, 1993). Many factors, such as microorganisms and the host health status, are associated with the pathogenesis of periodontitis. The destruction of the periodontal ligament and alveolar bones occur as a result of periodontitis, which leads to tooth loss (Brown *et al.*, 1990; Eklund and Burt, 1994). Although many cytokines such as PGE_2, IL-1α, IL-6 and TNF-α are associated with the activation of osteoclasts, the mechanism of bone loss in periodontitis is not completely understood (Birkedal-Hansen, 1993; Bostrom *et al.*, 1998; Galbraith *et al.*, 1997; Kong *et al.*, 1999; Tani-Ishii *et al.*, 1999).

The receptors for estrogen, progesterone and androgen were found in the gingiva but the roles of these are unclear (Vittek *et al.*, 1982a; Vittek *et al.*, 1982b). During pregnancy, a prescription of estrogen or menstruation might increase the frequency and severity of a bacterial, parasitic and viral infection (Klinger *et al.*, 1998; Raber-Durlacher *et al.*, 1994). In addition, a high concentration of estrogen might increase the sensitivity of some infections (Styrt and Sugarman, 1991). The hormonal changes occurring during adolescence increase the gingival index, and the serum concentration of

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estrogen and progesterone are related to the severity of an infection by *P. intermedia* and *P. nigrescens* (Nakagawa *et al.*, 1994). Gingivitis is exacerbated during pregnancy, puberty and menstruation. The tooth mobility and pocket depth usually increase during pregnancy with a high concentration of sex hormones (Rateitschak, 1967).

In this experiment, the local direct effects of estrogen and progesterone at the levels encountered during menstruation and menopause on the expression of OPG and ODF in the human gingival and periodontal ligament fibroblast were examined to determine the direct contributions of these hormones to the local bone metabolism of periodontal tissue.

Materials and Methods

Cells and hormones

Human gingival fibroblast and periodontal ligament fibroblast were obtained from the Department of oral biochemistry, School of Dentistry, Dankook University. Human gingival fibroblasts (HGF) were cultured using a-MEM with 10% fetal bovine serum. The 8th generation of the cells was used for all experiments. The cells were placed at 1×10^4 cells in 35 mm^2 culture dishes. The hormones were added according to the normal menstrual concentration. The six kinds of progesterone (Sigma Chemical Co., St. Louis, MO, USA) concentration and the four kinds of estrogen (Sigma Chemical Co., St. Louis, MO, USA) concentration were selected within the normal range of concentration during menstruation (Fig. 1, Table 1). The control group was maintained without hormones.

The periodontal ligament fibroblasts (PDL) were cultured under the same conditions. The 3rd generation of the cells was used for all experiments. The hormones were added at the same concentration as for the HGF.

For the menopausal simulation, each of the cells was cultured with the medium containing 1 nM or 1 μM of estrogen. After 5-day culture, the cells were maintained with serum-free media without the hormones for 48 hrs.

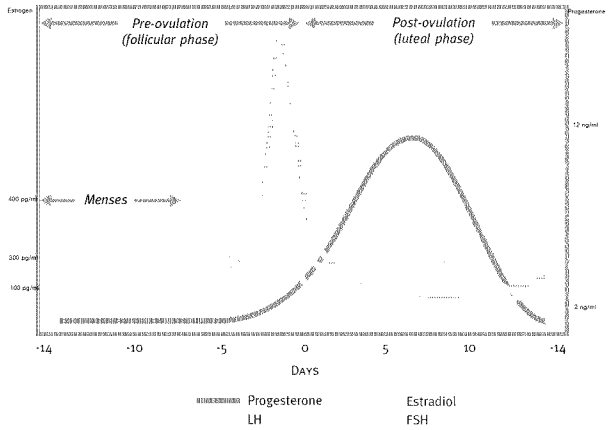

Fig. 1. Relative hormone serum levels during menstrual cycle

Extraction of the total RNA

The total RNA was extracted from the cells using TRI reagent (Molecular Research Center, Cincinnati, Ohio, USA) according to the manufacturer's instructions . Briefly, the cells were washed with 10 ml phosphate-buffered saline (PBS) and mixed with a TRI reagent solution for 10 min. Chloroform was added at the ratio of 200 μl/1 ml of TRI reagent. The supernatant was separated by centrifugation at 13,000 rpm for 15 min. The same volume of isopropanol was added to the supernatant and stored at –20°C for at least 1 hr. The precipitated total RNA pellet was recovered by centrifugation at 13,000 rpm for 15 min at 4°C. The RNA pellet was washed with 75% ethanol, air-dried and resolved in DEPC-treated DDW. The amount was determined using a spectrophotometer. The integrity was confirmed by 1.2 % agarose-formaldehyde gel.

Semi-quantitative RT-PCR

The expression of ODF by the periodontal cells was determined by RT-PCR. The total RNA, as a template was mixed with 2.5 μM random 9-mer primer, 1 mM dNTPs, 1 unit RNase inhibitor, 5 mM $MgCl_2$ and 5 units avian myeloblastosis virus reverse transcriptase XL. The AMV-RT buffer solution (TaKaRa, Otsu, Shiga, Japan) was added to make a 50 μl volume. Reverse transcription was carried out at 30°C for 10 min, 50°C for 30 min, 99°C for 5 min and 5°C for 5 min.

For the PCR, 7 μl of the cDNA was mixed with 40 pmol of each forward and reverse primer (Table 2), 2.5 mM $MgCl_2$, 5 unit Taq polymerase (Takara Ex taq), PCR reaction buffer and DDW to a final volume of 100 μl. The PCR conditions are as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 50°C for 2 min and 60°C for 1 min. The amplified product was confirmed by electrophoresis at 1 % agarose gel.

The level of ODF expression was determined by LAS-1000 plus (Fujifilms, Valhalla, NY, USA). The expression level was normalized to the level of GAPDH expression. The

Table 1. The Concentration of Estrogen and Progesterone in the Experimental Group

	Estrogen(pg/ml)		Progesterone(ng/ml)
EM1	30	PM1	0.5
EM2	60	PM2	1.0
EM3	120	PM3	2.0
EM4	240	PM4	4.0
		PM5	8.0
		PM6	16.0

Table 2. Oligonucleotides sequences used in the PCR reaction of ODF

Name	Sequence
hODF forward	5'-GGTTCCCATAAAGTGAGTCTG-3'
hODF reverse	5'-TTAAAAGCCCCAAAGTATGTT-3'
GAPDH forward	5'-TGAGAACGGGAAGCTTGTCA-3'
GAPDH reverse	5'-GGAAGGCCATGCCAGTGA-3'

relative expression of ODF was calculated using the following formula.

hOPG ELISA

The excreted hOPG from the treated cells were determined by hOPG/OCIT ELISA kit (OCT Inc., Korea) according to the manufacturer's guide. 100 μl of supernatant was added to each well of the ELISA plate and incubated at room temperature for 2 hrs. For the standard curve, the recombinant OPG protein were added at a concentration of 0, 50, 100, 250, 500, and 1000 pg/ml. After 2hr incubation, the plate was washed three times with a mixed solution of 0.05% Tween 20 and 1 X PBS. The biotinylated OPG detection antibody was diluted to 1 μl/ml with 1% BSA in PBS, and 100 μl of the diluted antibody solution was added to each well. After 2 hr incubation, the plate was washed four times with a mixed solution of 0.05% Tween 20 and 1 X PBS. 100 μl of Streptavidin-HRP was added and incubated for 30 minutes at room temperature. The plate was washed four times with a mixed solution of 0.05% Tween 20 and 1 X PBS. 100 μl of the substrate solution was added to each well and incubated for 15 minutes at room temperature. The reaction was quenched by adding 50 μl of a 1M H_3PO_4 stop solution. The absorbance at 450 nm was read using an ELISA-reader. The amount of hOPG excretion was determined by the standard curve, and the relative level of secretion was calculated by dividing the amount of hOPG excretion with that from the control group.

Results

In human gingival fibroblasts exposed to 30 pg/ml of estrogen encountered during menstruation, the level of ODF expression approximately 93% higher than the untreated control (Fig. 2). The level of OPG secretion was significant higher at 120 pg/ml and 240 pg/ml (*: $P < 0.05$) of estrogen that the control cells. The relative expression ratio of OPG and ODF(ODF/OPG) was 1.69 only at 30 pg/ml. Therefore, the local osteoclastic activity may be increased in the presence of low estrogen concentrations.

When HGF was treated with the concentration of progesterone during menstruation, the level of OPG secretion was approximately 30% higher than the control only at 0.5 ng/ml (Fig. 3). However, the level of ODF expression was lower than the control at all concentrations. In particularly, it was approximately 30% lower at 0.5 ng/ml. The relative expression ratio of OPG and ODF (ODF/OPG) was < 1 at all concentrations. The lowest value of ODF/OPG ratio was 0.23 at 0.5 ng/ml. Therefore, the local effects of progesterone on the human gingival fibroblast during menstruation may be protective by decreasing the osteoclastic activity.

When the periodontal ligament fibroblasts were treated with the concentration of estrogen during menstruation, the level of ODF expression and OPG secretion approximately 70-30% and 10-50% higher than the control (Fig. 4). In particular, the level of increase was statistically high at 30 pg/ml and 240 pg/ml (*: $P < 0.01$). The relative expression ratio of ODF and OPG increased in a dose-dependent manner. Therefore,

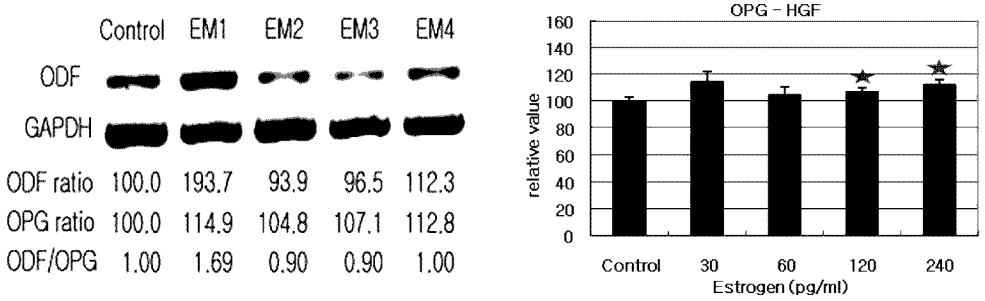

Fig. 2. Relative level of OPG and ODF expression in human gingival fibroblast treated with the serum concentration of estrogen during menstruation(*: $P < 0.05$). The relative value (ratio) was calculated as a percentage of the control (normalized to 100%).

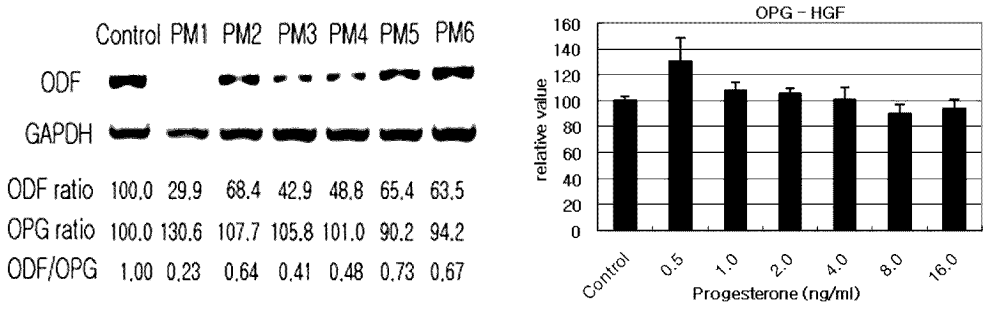

Fig. 3. Relative level of OPG and ODF expression in human gingival fibroblast treated with the serum progesterone concentration during menstruation. The relative value(ratio) was calculated as a percentage of the control(normalized to 100%).

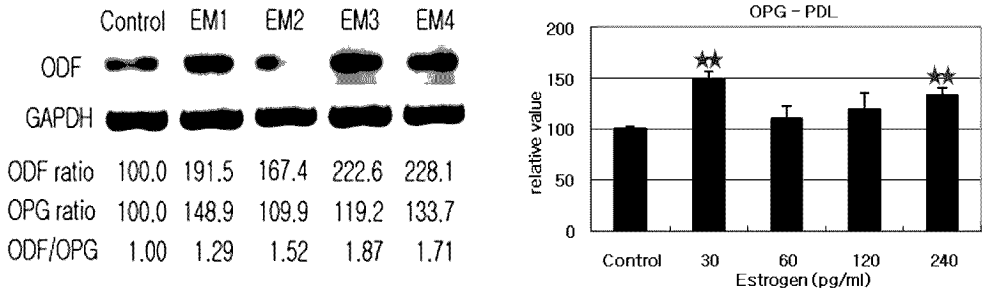

Fig. 4. Relative level of OPG and ODF expression in human periodontal ligament cells treated with the serum concentration of estrogen during menstruation (**: $P < 0.01$). The relative value (ratio) was calculated as a percentage of the control (normalized to 100%).

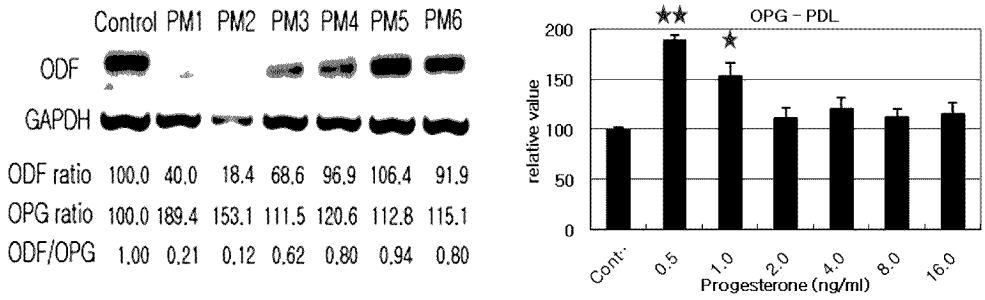

Fig. 5. Relative level of OPG and ODF expression in human periodontal ligament cell treated with the serum concentration of progesterone during menstruation (*: $P < 0.05$, **: $P < 0.01$). The relative value (ratio) was calculated as a percentage of the control (normalized to 100%).

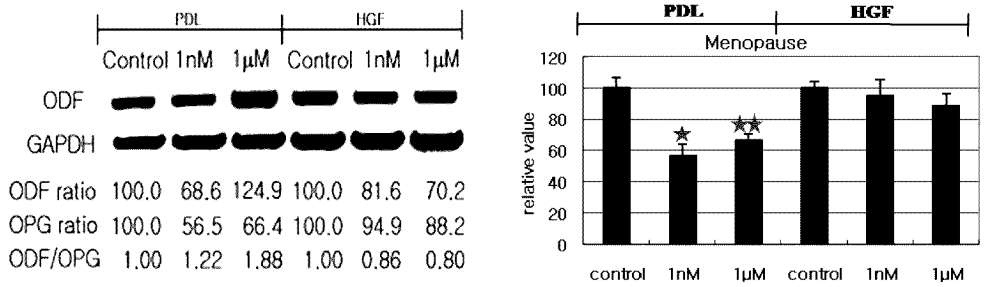

Fig. 6. Relative level of OPG and ODF expression in human periodontal ligament cells and human gingival fibroblasts treated with the simulated concentration of estrogen at menopause (*: $P < 0.05$, **: $P < 0.01$). The relative value (ratio) was calculated as a percentage of the control (normalized to 100%).

the effects of estrogen on periodontal ligament cells might result in the local activation of the osteoclastic activity.

When the periodontal ligament fibroblasts were treated with the menstrual concentration of progesterone, the level of ODF expression was apparently decreased (Fig. 5). However, the level of OPG secretion increased at 0.5 ng/ml and 1 ng/ml (*: $P < 0.01$). The relative expression ratio of ODF and OPG was < 1.0 for all concentrations. In particular, the ratio was 0.1-0.2 at low concentrations. Therefore, the effects of pro- gesterone on periodontal ligament cells might result in the local inhibition of osteoclastic activity.

When the menopausal conditions were simulated, the level of ODF expression and OPG secretion in periodontal ligament fibroblast were lower than the control but increased in a dose-dependent manner. Because the level of OPG secretion were low, the relative expression ratio of ODF and OPG (ODF/OPG) were > 1.0. However, the level of ODF expression and OPG secretion were lower in the human gingival fibroblasts, and the ODF/OPG ratio was slightly < 1.0 (Fig. 6). Therefore, periodontal ligament cells may increase the local osteoclastic activity but human gingival fibroblast cell have no effect on the local osteoclastic activity.

Discussions

Osteoprotegerin (OPG/OCIF/TR1) is a member of the TNF receptor superfamily, and a decoy receptor secreted by osteoblasts and stromal cells. It inhibits the differentiation of osteoclasts by blocking the interaction of RANK/RANKL (Kwon *et al.*, 1998; Simonet *et al.*, 1997; Tan et al., 1997; Tani-Ishii *et al.*, 1999).

The Osteoclast Differentiation Factor (ODF/RANKL/TRANCE / OPGL) is secreted by bone marrow stromal cells and osteoblasts. ODF increases the life of dendritic cells and the differentiation of osteoclasts by binding to RANK on the cell surface of osteoclast (Anderson *et al.*, 1997; Lacey *et al.*, 1998; Wong *et al.*, 1997; Yasuda *et al.*, 1998). The level of RANKL expression in the periodontium increases during tooth eruption and the orthodontic tooth movement (Fukushima *et al.*, 2003; Lossdorfer *et al.*, 2002; Oshiro *et al.*, 2002) as well as in periodontitis (Liu *et al.*, 2003; Mogi *et al.*, 2004).

Estrogen is used to prevent and treat osteoporosis on account of its inhibitory action on bone resorption. Although the precise mechanism of bone protection by estrogen is unclear, it has been suggested that estrogen inhibits the formation, activity and survival of osteoclasts (Manolagas *et al.*, 2000). In addition, estrogen stimulates the secretion of osteoprotegerin and inhibits osteoclastogenesis (Aubin *et al.*, 2000; Boyce *et al.*, 2005; Hofbauer *et al.*, 2004a; Hofbauer *et al.*, 2004b).

The OPG in GCF usually originates from osteoblasts, periodontal ligament fibroblasts and gingival fibroblasts (Tsuda *et al.*, 1997). The ratio of ODF/OPG is approximately 0.79 in healthy people and increases to > 1.0 in periodontitis patients (Liu *et al.*, 2003).

The results showed that estrogen increased the level of ODF expression in gingival fibroblasts only at low concentrations (30 pg/ml) but had no effect on OPG secretion. Therefore, the relative expression ratio (ODF/OPG) was 1.7 only at low concentrations. In periodontal ligament fibroblasts, the level of ODF expression was increased at all concentrations. The relative expression ratio (ODF/OPG) was high because the secretion of OPG was increased slightly by estrogen. This means that the local osteoclastic activity would be increased by gingival fibroblasts at low estrogen concentrations (around the menstruation) and by periodontal ligament fibroblasts at high concentrations (between ovulation and menstruation).

Progesterone decreased the level of ODF expression in gingival fibroblasts at all concentration, particularly at low concentrations but had no significant effect on OPG secretion. Therefore, the relative expression ratio (ODF/OPG) was maintained at approximately 0.5. In the PDL cells, progesterone decreased the level of ODF expression, particularly at low concentrations (70% inhibition) but increased the level of OPG secretion at all concentrations. Therefore, the relative expression ratio (ODF/OPG) was < 1.0 (0.12) at low concentration. This means that the local osteoclastic activity is decreased by progesterone between the menstruation and ovulation.

The local cumulative osteolytic effects by the two hormones might be strong between ovulation and menstruation as a result of the weak osteo-protective effect of progesterone during this period. This prediction is in accord with Machtei *et al.*, who reported that the gingival inflammation measured by the Gingival Index was high during this period (Machtei *et al.*, 2004). Therefore, the inflammation and the risk of local bone destruction might be increased by sex hormones. In addition, the secretion of OPG and the expression of ODF were lower in moderate periodontitis patients than in healthy people or patients with severe periodontitis. Therefore, the bone destruction is highly active during moderate periodontitis (Mogi *et al.*, 2004).

In *in vitro* menopause, the relative expression ratio (ODF/OPG) was 0.8 in gingival fibroblasts, which is within the normal range. However, the ratio was > 1.0 in periodontal ligament cells for the dose-dependant increase in ODF expression and decrease in OPG secretion. Tezal *et al.* reported that the bone density at menopause was related to the amount of interproximal bone loss (Tezal *et al.*, 2000). Therefore, a low bone density might be a risk factor for periodontal disease. These results show that periodontal ligament cells can play an important role in local alveolar bone loss at menopause. Loza *et al.* reported that estrogen promotes the activity of osteoblasts by inhibiting cytokine production. In menopause, the lower estrogen level promotes cytokine production, which increases the alveolar bone resorption and osteopososis (Loza *et al.*, 1996).

Because the local effect of estrogen on the alveolar bone might be mediated by periodontal ligament cells in the viewpoint of ODF/OPG ratio, further studies will be needed to clarify the clinical effects of estrogen on the alveolar bone of edentulous patients.

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