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Deflazacort Increases Osteoclast Formation in Mouse Bone Marrow Culture and the Ratio of *RANKL/OPG* mRNA Expression in Marrow Stromal Cells

Information on precise effects of deflazacort on bone cell function, especially osteoclasts, is quite limited. Therefore, the present study was undertaken to test effects of deflazacort on osteoclast-like cell formation in mouse bone marrow cultures and on the regulation of osteoprotegerin (*OPG*) and its ligand (*RANKL*) mRNA expressions by RT-PCR in the ST2 marrow stromal cells. TRAP-positive mononuclear cells increased after the treatment of deflazacort at 10^{-9} to 10^{-7} M alone for 6 days in a dose-dependent manner. Number of TRAP-positive multinucleated cells (MNCs) increased significantly with combined treatment of deflazacort at 10^{-7} M and 1,25-$(OH)_2D_3$ at 10^{-9} M compared to that of cultures treated with 1,25-$(OH)_2D_3$ alone ($p<0.05$). Exposure to deflazacort at 10^{-7} M in the presence of 1,25-$(OH)_2D_3$ at 10^{-9} M in the last 3-day culture had greater stimulatory effect on osteoclast-like cell formation than that of the first 3-day culture did. Deflazacort at 10^{-10}-10^{-6} M downregulated OPG and upregulated RANKL in mRNA levels in a dose-dependent manner. These observations suggest that deflazacort stimulate osteoclast precursor in the absence of 1,25-$(OH)_2D_3$ and enhance differentiation of osteoclasts in the presence of 1,25-$(OH)_2D_3$. These effects are, in part, thought to be mediated by the regulation of the expression of OPG and RANKL mRNA in marrow stromal cells.

Key Words : *Glucocorticoids; Osteoclasts; OPG; RANKL*

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INTRODUCTION

Treatment with pharmacologic doses of glucocorticoids frequently results in significant osteoporosis (1). Patients on high-dose glucocorticoid therapy are susceptible to bone loss and vertebral fractures within weeks to months of initiation of therapy (2). It is generally accepted that glucocorticoids decrease bone formation and increase bone resorption in vitro as well as in vivo (3-5). In contrast to the consistent finding of reduced osteoblastic activity, the exact mechanism responsible for the effects of glucocorticoids on bone resorption is less clear. It may involve both direct effects on osteoclasts (4) as well as indirect effects mediated through osteoblasts (6).

Despite these side effects, glucocorticoids are necessary for the treatment of a variety of medical diseases. Deflazacort is a synthetic glucocorticoid with antiinflammatory and antiimmune properties similar to those reported for prednisone and has been reported to be relatively bone sparing (7). Although long-term clinical trials comparing deflazacort with prednisone are not available, an initial study suggests that trabecular bone loss is less with deflazacort compared to prednisone (8). Deflazacort and other glucocorticoids have similar inhibitory actions on aspects of bone formation in cultures of intact calvariae and osteoblast-enriched cells. Information on precise effects of deflazacort on bone cell function, especially osteoclasts, is quite limited. Therefore, the present study was undertaken to compare the effect of deflazacort and dexamethasone on aspects of osteoclast formation in mouse marrow culture. Recently osteoprotegerin (*OPG*) and its ligand (*RANKL*) were cloned and demonstrated to be critical regulators of osteoclastogenesis (9, 10). OPG is a novel receptor that blocks osteoclast formation. Either in a cell membrane-bound or in a soluble form, RANKL stimulates osteoclastogenesis and osteoclastic bone resorption. OPG and RANKL are expressed by marrow stromal cells, osteoblast-like cell lines and primary cultures of osteoblasts derived from mouse calvaria. Recent studies demonstrated that *OPG* and *RANKL* gene expressions in osteoblastic cells are regulated by various calciotropic hormones and cytokines (11). Furthermore, Hofbauer et al. has identified that glucocorticoids promote osteoclastogenesis by inhibiting OPG and concurrently stimulating RANKL production by osteoblastic lineage cells, thereby enhancing bone resorption (12).

In order to find out differences of deflazacort in terms of

bone effects, we have investigated the effects of deflazacort on osteoclast-like cell formation in mouse bone marrow cultures, and on *OPG* and *RANKL* mRNA expression in ST2 cells.

MATERIALS AND METHODS

Bone marrow culture system

Six- to 9-week-old male ICR mice were sacrificed and bone marrow was obtained as described by Takahashi et al. (13). Briefly, tibiae and femora were dissected free of adherent soft tissues, both epiphyses were cut off with scissors, and the marrow cavities were vigorously flushed out with α-minimum essential medium (α-MEM, Gibco BRL, Grand Island, NY, U.S.A.) using a syringe. Freshly isolated whole bone marrow cells were cultured in α-MEM containing 10% fetal calf serum (FCS, Gibco BRL) at 1.5×10^6 cells/mL in 48-well plates (Nunc, Denmark). Cultures were fed every 3 days by replacing half of the media. 10^{-9} M of 1,25-$(OH)_2D_3$ (Calbiochem, U.S.A.) and various concentrations of deflazacort (Gruppo Lepetit, Italy) were added at the beginning of the culture and at each media change. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After various periods of culture, cells were fixed with citrate-acetone-formaldehyde fixative, and stained for TRAP using an acid phosphatase kit (Sigma). TRAP-positive cells containing three or more nuclei were scored as osteoclasts microscopically. The results were expressed as the mean ± SEM for four cultures.

Semiquantitative RT-PCR

Osteoclast formation-supporting stromal cell line, ST2 cells (RIKEN cell bank, Tsukuba, Japan) were cultured in 6-well plates containing α-MEM supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. When the effect of deflazacort was to be tested, ST2 cells were serum-starved in α-MEM containing 0.5% FCS for a period of 24 hr, followed by the addition of deflazacort at final concentrations ranging from 10^{-10} to 10^{-6} M. Total RNA was extracted from the ST2 cells with Tri-reagent (Molecular Research Center, Cincinnati, OH, U.S.A.) and then used for reverse transcriptase PCR (RT-PCR) assay. PCR reactions were done using gene-specific PCR primers and *Taq* polymerase (SR producs, U.K.) in a thermal cycler (Perkin-Elmer Corp., Norwalk, CT, U.S.A.). Specific primer sets were designed from published cDNA sequences: murine *OPG* (sense: 5′-AACCCC AGAGCGAAACAC-3′; antisense: 5′-AAGAAGGCCTCT TCACAC-3′), murine *RANKL* (sense: 5′-GGTCGGGCA ATTCTGAATT-3′; antisense: 5′-GGGGAATTACAAAG TGCACCAG-3′), *β-actin* (sense: 5′-GTGGGCCGCCCT AGGCACCAG-3′; antisense: 5′-CACTTTGATGTCACG CACGATTTC-3′). The amplified samples were run on a 1.5-2.0% agarose gel, stained with ethidium bromide, and photographed under UV illumination. Optical density was determined using a digital image processing and analysis program (Vilber Lourmate, France). Ratios of *OPG* or *RANKL*/*β-actin* PCR product density were determined for semiquantitation of mRNA expression. The conditions for the PCR reaction for mouse *OPG* cDNA of 219 bp was 95°C for 50 sec, 54°C for 20 sec, and 72°C for 20 sec for 35 cycles. The conditions for PCR analysis of *RANKL* were the same as for *OPG* except an annealing temperature of 62°C. PCR was performed for 27 cycles for *β-actin*.

Statistical analysis

Statistical analysis was performed using Student's t-test to evaluate differences between the sample of interest and its respective control. For analysis of dose response, multiple measurement ANOVA was used. A p value of <0.05 was considered significant.

RESULTS

Effects of glucocorticoids on the formation of osteoclast-like cells

TRAP-positive multinucleated cells (MNCs) did not increase in cultures treated with deflazacort alone, whereas TRAP-positive mononuclear cells increased (data not shown).

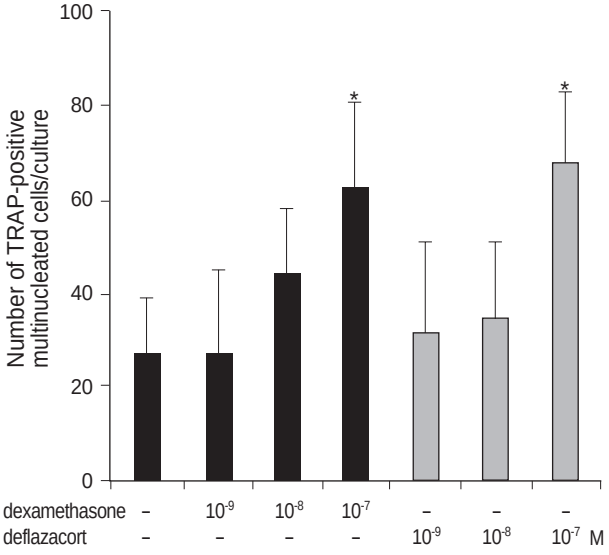

Fig. 1. Effects of increased concentration of glucocorticoids on the formation of TRAP-positive multinucleated cells. TRAP-positive cells were counted after mouse marrow culture in the presence of 1,25-$(OH)_2D_3$, 10^{-9} M for 6 days. Data are expressed as the mean ± SEM for four cultures (*: $p<0.05$).

Fig. 1 shows the effects of increased concentration of glucocorticoids on the formation of TRAP-positive MNCs in the presence of 1,25-$(OH)_2D_3$ (10^{-9} M) for 6 days. The addition of dexamethasone or deflazacort to the cultures resulted in enhancement of TRAP-positive MNC formation induced by 1,25-$(OH)_2D_3$. A significant increase was observed at the concentration of 10^{-7}M dexamethasone or deflazacort ($p<0.05$). To characterize the time course for the effects of glucocorticoids on osteoclast formation, mouse bone marrow cultures were treated with dexamethasone or deflazacort for varying time. The number of TRAP-positive MNCs by 10^{-9} M 1,25-$(OH)_2D_3$ and 10^{-7} M glucocorticoids reached a nearly maximal level with treatment of dexamethasone or deflazacort in the last 3 days of culture (Fig. 2).

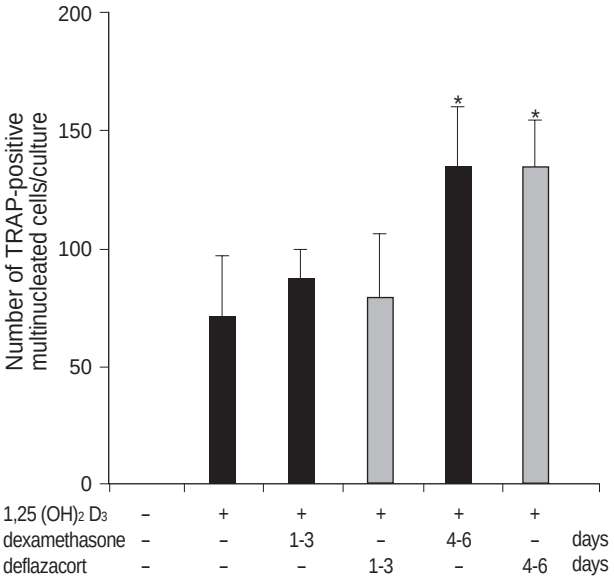

Fig. 2. Effects of varying treatment time with glucocorticoids on the TRAP-positive multinucleated cells in mouse bone marrow cultures in the presence of 1,25-$(OH)_2D_3$, 10^{-9} M for 6 days. During the indicated times, cells were cultured with 10^{-7} M of glucocorticoids (*: $p<0.05$).

Regulation of *OPG* and *RANKL* mRNA expression by deflazacort in ST2 cell

OPG, *RANKL*, and *β-actin* mRNA expression levels were assessed by RT-PCR in ST2 cell cultures that were treated with or without deflazacort for 8 hr. The amounts of *OPG* and *RANKL* mRNA were compared with the amount of β-actin mRNA. As shown in Fig. 3, the level of *OPG* mRNA expression decreased significantly after treatment with deflazacort at and above the concentration of 10^{-7} M. On the contrary, stimulation with deflazacort for 8 hr increased the *RANKL* mRNA levels in a dose-dependent manner (Fig. 4).

DISCUSSION

Deflazacort has a similar effect on osteoclast formation com-

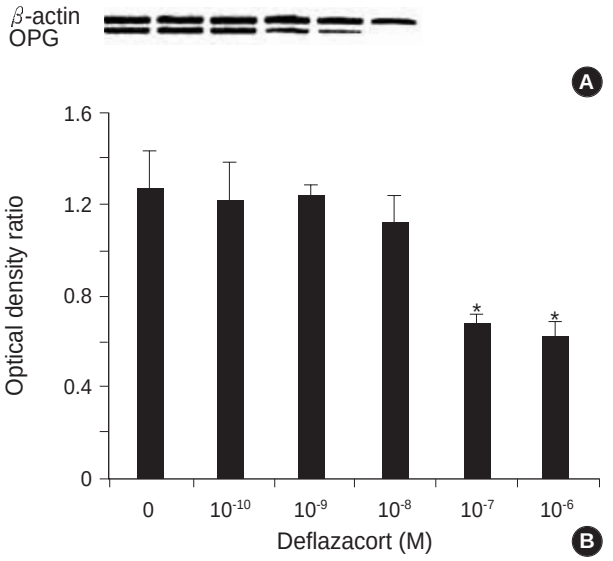

Fig. 3. mRNA expression of *OPG* and *β-actin* assessed by RT-PCR in ST2 cells. ST2 cells were precultured and then stimulated, with or without deflazacort, for 8 hr. (A) Photographs of the PCR analysis from a representative experiment. (B) Mean optical density ratio values for OPG, normalized to β-actin of triplicate independent experiments, to document the consistency of the results (*: $p<0.01$).

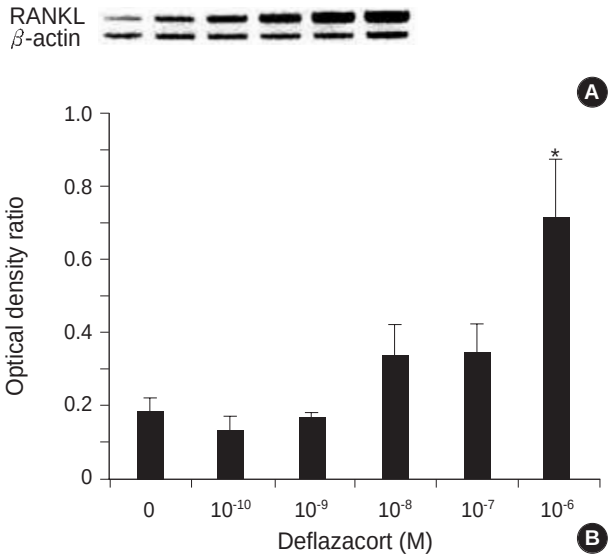

Fig. 4. mRNA expression of *RANKL* and *β-actin* assessed by RT-PCR in ST2 cells. ST2 cells were precultured and then stimulated, with or without deflazacort, for 8 hr. (A) Photographs of the PCR analysis from a representative experiment. (B) Mean optical density ratio values for *RANKL*, normalized to β-actin of triplicate independent experiments, to document the consistency of the results (*: $p<0.01$).

pared with those of dexamethasone (14). No differences in osteoclast formation of deflazacort and dexamethasone were detected in this study (Fig. 1). The number of TRAP-positive mononuclear cells was also increased by treatment with deflazacort alone, suggesting that deflazacort may induce proliferation or differentiation of osteoclast precursors in mouse bone marrow cultures. Time-course effect for TRAP-positive MNC formation showed that deflazacort had its greatest stimulatory effects on osteoclast formation during the later stages of the cultures, the period of differentiation, rather than during the proliferation phase of mouse bone marrow cultures (Fig. 2). Although it is possible that deflazacort stimulated osteoclast-like cell formation by directly acting on hematopoietic cells, based on our results, it is more likely that deflazacort mediates its effects through stromal cells in enhancing osteoclast formation in mouse bone marrow cultures. Most of the TRAP-positive mononuclear cells and MNCs were found adjacent to colonies of osteoblast-like cells stained for alkaline phosphatase. Alkaline phosphatase positive cells appeared almost on day 3 (13).

The balance between osteoblast and osteoclast functions is regulated systemically by a variety of hormones and locally by the production of paracrine factors by osteoblasts or bone marrow stromal cells that regulate osteoclast function (15, 16). The development of active osteoclasts in vitro requires intimate contact between osteoblastic stromal cells and osteoclast precursors (17). RANKL, receptor activator of NF-kappa B (RANK), and OPG have recently been identified as agonist, receptor, and decoy receptor for osteoclastogenesis. Factors that stimulate bone resorption increase RANKL expression and, with some exceptions, decrease OPG expression (11, 18). In the present study, although we did not evaluate protein expressions, deflazacort treatment on mouse marrow stromal cells inhibited the production of *OPG* and increased *RANKL* in mRNA levels (Fig. 3, 4). No differences on *OPG* and *RANKL* mRNA regulation of deflazacort and dexamethasone were detected in this semiquantitative RT-PCR (data not shown). This differential regulation of OPG and RANKL by deflazacort would favor the formation of osteoclasts. These findings are in line with a study that showed dexamethasone promotes osteoclastogenesis by inhibiting OPG and stimulating RANKL production by osteoblastic lineage cells (12). We can not explain the bone-sparing effect of deflazacort in terms of osteoclast formation based on our study. Glucocorticoids, including deflazacort, affect multiple parameters of mineral metabolism (19-21). All these studies assumed that the potency of prednisone relative to deflazacort was 1.2. Subsequent re-examination of the relative potencies of these two glucocorticoids has found that the true relative potency is 1.4-1.8 (22). This study suggest that deflazacort have been overestimated in the past, and demonstrated no bone sparing effect when used in a therapeutically equivalent dose.

In conclusion, as in other glucocorticoids, deflazacort also has stimulatory effects on osteoclast formation, and displays similar differential regulation of the expression of genes responsible for osteoclastogenesis in marrow stromal cells.

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