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Effects of Immunosuppressants on Receptor Activator of NF-κB Ligand and Osteoprotegerin Production by Human Osteoblastic and Coronary Artery Smooth Muscle Cells

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Osteoporosis and vasculopathy are common after organ transplantation and have been largely attributed to the use of immunosuppressants. Osteoprotegerin (OPG) is produced by osteoblastic and arterial cells, and inhibits osteoclast functions by neutralizing receptor activator of NF-κB ligand (RANKL). Because OPG-deficient mice develop osteoporosis and arterial calcification, we assessed the effects of immunosuppressants on OPG and RANKL expression by human osteoblastic and coronary artery smooth muscle cells (CASMC). Cyclosporine A, rapamycin, and FK-506 decreased OPG mRNA and protein levels in undifferentiated marrow stromal cells (by 63, 44, and 68%, respectively, $P < 0.001$). All three immunosuppressants increased RANKL mRNA levels in these cells by 60 to 210%. In contrast to these effects on marrow stromal cells, rapamycin, which may be relatively bone-sparing, increased OPG mRNA and protein production (by 120%, $P < 0.001$) in mature osteoblastic cells. Cyclosporine A also decreased OPG mRNA and protein production (by 52%, $P < 0.001$) of CASMC. In conclusion, immunosuppressants decrease OPG mRNA and protein production and increase RANKL gene expression by marrow stromal cells, and cyclosporine suppresses OPG production in CASMC. These studies thus provide a potential mechanism for immunosuppressant-induced bone loss, and the propensity of cyclosporine A to cause vascular disease.

Key Words: **cyclosporine A; osteoblasts; osteoprotegerin; rapamycin; stromal cell; smooth muscle cell.**

Rapid development of osteoporosis and vascular disease are common complications of organ transplantation (1, 2). Immunosuppressants such as cyclosporine A (CsA), rapamycin, and FK-506 have been implicated in the pathogenesis of both post-transplant osteoporosis and vascular disease (1–4). However, some studies have suggested a bone-sparing effect for rapamycin (5, 6), and a lower incidence of adverse vascular effects with rapamycin and FK-506 (7, 8). Various studies have demonstrated that CsA administration increases osteoclastic activity and bone resorption (9, 10). Moreover, CsA has been shown to stimulate vascular smooth muscle cell proliferation (11) and to induce arterial smooth muscle cell contraction (7), which may contribute to arteriosclerosis and hypertension. Although the clinical adverse effects of immunosuppressants on bone metabolism and the vascular system are well appreciated, the molecular mechanism(s) underlying them have remained unclear.

Recently, receptor activator of NF-κB (RANK) ligand (RANKL) was identified as a novel member of the tumor necrosis factor (TNF) ligand superfamily and was shown to be essential for osteoclast differentiation and activation (12–14). RANKL is produced by osteoblast/stromal cells, chondrocytes, mesenchymal cells, and activated T cells, and exists in three forms: a cell-bound form, a truncated form derived from the cellular form by post-translational processing, and a secreted form (12, 15, 16). RANKL acts by binding to its receptor, RANK, which is located on osteoclastic and dendritic cells (13, 17). The stimulatory effects of RANKL are neutralized by the decoy receptor, osteoprotegerin (OPG), which is produced by various cells, including stromal/osteoblastic cells and vascular smooth muscle cells (18). RANKL-deficient mice (14) and transgenic mice overexpressing OPG (18) develop osteopetrosis due to decreased osteoclastogenesis and bone resorption, whereas OPG-deficient mice have severe osteoporosis and arterial calcification (19), indicating a potential protective role of OPG for both bone and vascular tissue.

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Since posttransplantation osteoporosis results, in part, from increased osteoclastic bone resorption following initiation of drug therapy after transplantation, and OPG represents a potent inhibitor of osteoclast differentiation and function, we tested the hypothesis that immunosuppressants used after transplantation alter the RANKL/OPG cytokine system by human osteoblastic lineage cells. We have previously shown that glucocorticoids, agents concurrently used for immunosuppression, decrease OPG and increase RANKL production by osteoblastic lineage cells (20). The possible effects of CsA, rapamycin, or FK-506 on this system are, however, unknown. Moreover, because OPG may play a role in vascular biology, we also assessed whether immunosuppressants alter OPG production by vascular smooth muscle cells.

MATERIALS AND METHODS

Materials. Culture flasks and dishes were obtained from Corning (Corning, NY), cell culture medium and immunosuppressants were purchased from Sigma (St. Louis, MO). The random primer labeling kit (Decaprime II) was from Ambion (Austin, TX) and [α-^{32}P]-dCTP was from DuPont-NEN (Boston, MA). The human β-actin cDNA insert and ExpressHyb solution were purchased from Clontech (Palo Alto, CA).

Cell cultures. RNA and conditioned medium were obtained from the following human osteoblastic cells: (i) a conditionally immortalized human marrow stromal cell line which represents the immature bipotential osteoblast precursor phenotype and can differentiate towards the osteoblastic and adipocytic phenotype (hMS) (21); (ii) a conditionally immortalized fetal human osteoblastic cell line (hFOB) which displays the mature osteoblastic phenotype (22); and (iii) coronary artery smooth muscle cells (CASMC; lot #7F0540) which were purchased from Clonetics (Walkersville, MD).

Both conditionally immortalized cell lines, hMS and hFOB, proliferate at 33.5°C (the permissive temperature, when the temperature-sensitive mutant SV 40 large T antigen is active) and differentiate at 39.5°C (the restrictive temperature, when the SV 40 large T antigen is inactive) (21, 22). At the restrictive temperature, these cells are essentially a clonal population of normal pre-osteoblastic (hMS) and osteoblastic (hFOB) cells. The CASMC cells were grown at 37°C. All osteoblastic cells (hMS, hFOB) were maintained in phenol-free medium supplemented with 10% charcoal-stripped fetal calf serum (FCS), and were grown in serum-free medium supplemented with 0.125% (w/v) bovine serum albumin (BSA) for 3 days prior to RNA isolation. The CASMC were grown in SmGM-2 medium supplemented according to the manufacturer's instructions with human epidermal growth factor and human fibroblast growth factor (Clonetics, Walkersville, MD).

Northern blot analysis. Total RNA was isolated using the RNeasy kit and the QiaShredder from Qiagen (Hilden, Germany). Poly-A+ RNA was isolated using the PolyATract mRNA kit from Promega (Madison, WI). Five to 10 μg of total RNA or 1 μg of poly-A+ RNA were separated on a 1.5% (w/v) agarose/formaldehyde gel and then transferred to a nylon membrane (Hybond N+, Amersham, Arlington Heights, IL) by capillary blotting. The human cDNA inserts, a β-actin cDNA that hybridized to a 2.0 kb mRNA, a full-length OPG cDNA that hybridized to three mRNA species of 2.9, 4.4, and 6.6 kb, respectively (23), and a RANKL cDNA that hybridized to a mRNA species of 2.4 kb (12) were radiolabeled using a random primer DNA labeling kit. Hybridization and stringent washing were carried out as reported elsewhere (20). Band intensity was quantified by densitometry. All experiments were carried out at least three times, and representative blots are shown. Control hybridization with human β-actin cDNA verified that equal amounts of RNA were loaded.

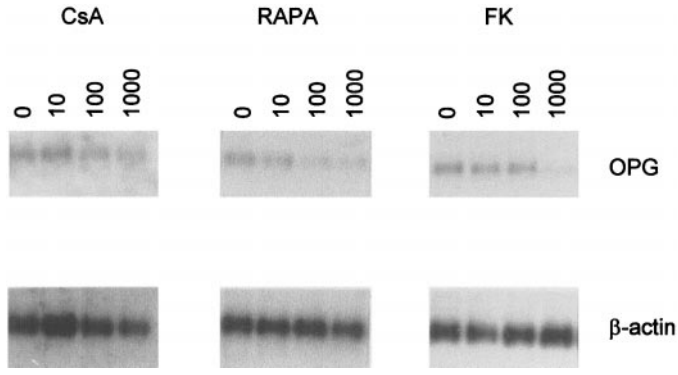

FIG. 1. Regulation of OPG mRNA steady-state levels in a human marrow stromal cell line (hMS) by immunosuppressants. Ten micrograms of total RNA was isolated from hMS cells treated for 24 h (at 39.5°C) with various concentrations of cyclosporine A (CsA), rapamycin (RAPA), or FK-506 (FK; the numbers indicate the dose of immunosuppressants in ng/ml) and analyzed by Northern blot. OPG mRNA (2.9 kb) (upper panel), β-actin (2.0 kb) (lower panel).

OPG protein measurement. Conditioned medium was harvested from cultured cells and centrifuged to remove cell debris. OPG protein concentration was determined in triplicate measurements using a sandwich ELISA as described elsewhere (20).

Statistical analysis. Unless otherwise stated, all values are expressed as the mean ± SEM. Student's paired t-test was used to evaluate differences between the sample of interest and its respective control. All tests were two-tailed. For analysis of dose response, multiple measurement ANOVA was used. A P value of <0.05 was considered significant.

RESULTS

Regulation of OPG and RANKL production by immunosuppressants in human stromal and osteoblastic cells. Because OPG mRNA steady-state levels are 8- to 10-fold higher in the conditionally immortalized hMS cell line when cultured at 39.5°C (20, 23) (when the cells begin to differentiate), the hMS cells were incubated for 48 h at 39.5°C in serum-free medium supplemented with 0.125% (w/v) BSA and then treated with either vehicle (ethanol) or various immunosuppressants (CsA, rapamycin, and FK-506) at various concentrations for 24 h at 39.5°C under serum-free conditions. CsA decreased OPG mRNA steady-state levels in the hMS cell line in a dose-dependent fashion (by 51% at a concentration of 1 μg/ml, Fig. 1). In addition, treatment of hMS cells with rapamycin and FK-506 also decreased OPG mRNA steady-state levels in a dose-dependent fashion (by 33 and 79% at a concentration of 1 μg/ml, respectively, Fig. 1). Consistent with the Northern data, treatment of hMS cells with CsA, rapamycin, and FK-506 at a concentration of 1 μg/ml decreased OPG protein secretion by 63, 44, and 68%, respectively (Table 1).

TABLE 1

Osteoprotegerin Protein Secretion by Osteoblastic Lineage Cells Treated with Immunosuppressant Drugs

	OPG protein concentration (ng/ml)
Human marrow stromal cell line (hMS)	
Control	4.87 ± 0.04
Cyclosporine A (1 μg/ml)	1.78 ± 0.03*
Rapamycin (1 μg/ml)	2.72 ± 0.03*
FK 506 (1 μg/ml)	1.55 ± 0.02*
Human fetal osteoblastic cells (hFOB)	
Control	1.59 ± 0.02
Cyclosporine A (1 μg/ml)	1.58 ± 0.03
Rapamycin (1 μg/ml)	3.50 ± 0.11*
FK 506 (1 μg/ml)	1.43 ± 0.02

Note. Cells were treated for 24 h with the immunosuppressant at 1 μg/ml, and OPG protein secretion was determined in triplicate measurements with an ELISA. The data are presented as the mean ± SEM.

* $P < 0.001$ by Student's paired t test.

By contrast, rapamycin dose-dependently increased OPG mRNA steady-state levels in the hFOB cell line (which represents the mature osteoblastic phenotype) by 128% at a concentration of 1 μg/ml (Fig. 2). Neither CsA nor FK-506 had an effect on OPG mRNA steady-state levels in hFOB cells (Fig. 2). Treatment of hFOB cells with rapamycin at a concentration of 1 μg/ml increased OPG protein secretion by 120% ($P < 0.001$), whereas CsA and FK-506 at this concentration had no effect (Table 1).

We then assessed whether immunosuppressants also regulated RANKL mRNA steady-state levels in osteoblastic cells. Compared to vehicle-treated controls, treatment with CsA, rapamycin and FK-506 increased RANKL mRNA steady-state levels in hMS cells by 60 to 210% (Fig. 3).

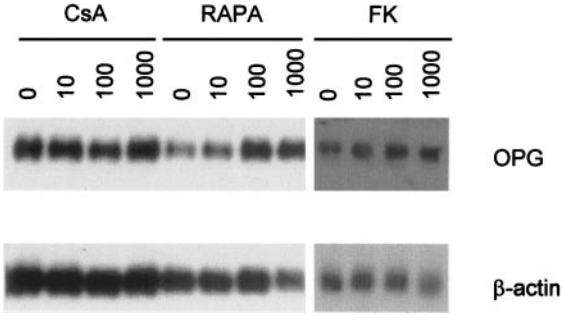

FIG. 2. Regulation of OPG mRNA steady-state levels in a fetal osteoblastic cell line (hFOB) by immunosuppressants. Ten micrograms of total RNA was isolated from hFOB cells treated for 24 h (at 39.5°C) with various concentrations of cyclosporine A, rapamycin, or FK-506 (the numbers indicate the dose of immunosuppressants in ng/ml). Northern analysis demonstrated OPG mRNA (2.9 kb) (upper panel) and β-actin (2.0 kb) levels (lower panel).

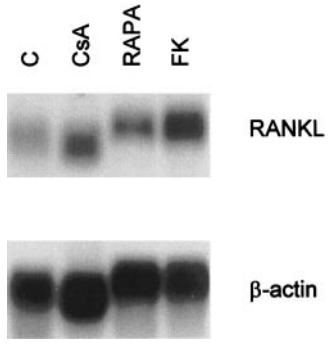

FIG. 3. Regulation of RANKL mRNA steady-state levels in human marrow stromal (hMS) cells by immunosuppressants as assessed by Northern analysis. One microgram of poly-(A+) RNA was isolated from hMS cells treated for 24 h (at 39.5°C) with either vehicle (ethanol), cyclosporine [CsA], rapamycin [RAPA], or FK-506 [FK] (each at 1 μg/ml). RANKL mRNA (2.4 kb) (upper panel), β-actin (2.0 kb) (lower panel).

Regulation of OPG expression by immunosuppressants in human coronary smooth muscle cells. Because OPG is produced by vascular cells (18) and OPG deficiency is associated with vascular calcification (19), and since immunosuppressants adversely affect vascular function (7, 11), we also assessed whether commonly used immunosuppressive drugs regulate OPG mRNA steady-state levels and protein secretion in coronary smooth muscle cells (CASMC). Treatment of CASMC with CsA at a concentration of 10 μg/ml decreased OPG mRNA steady-state levels (by 50%), while rapamycin and FK-506 had no effect (Fig. 4). Consistent with the Northern analysis, CsA also decreased OPG protein secretion by CASMC cells in a dose-dependent fashion (vehicle [normalized to 100%, mean ± SEM], 100 ± 1.3%; CsA, 1 μg/ml, 88 ± 1.0%; CsA, 10 μg/ml, 48 ± 0.8%; $P < 0.001$ by ANOVA). RANKL mRNA could not be detected in CASMC cells.

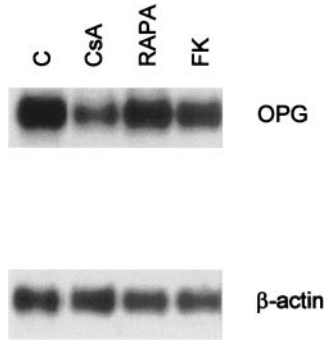

FIG. 4. Regulation of OPG mRNA steady-state levels in coronary artery smooth muscle cells (CASMC) by immunosuppressants. Five μg of total RNA were isolated from CASMC cells treated for 24 h (at 37°C) with either vehicle (V), cyclosporine A (CsA), rapamycin (RAPA), or FK-506 (FK, each at 10 μg/ml). OPG mRNA (2.9 kb) (upper panel), β-actin (2.0 kb) (lower panel).

DISCUSSION

Over the last three years, RANKL has emerged as the long-sought after osteoclast differentiation factor (24, 25). The effects of RANKL include promotion of osteoclastogenesis, stimulation of osteoclast survival, fusion, and activation as well as inhibition of osteoclast apoptosis. OPG, which neutralizes RANKL, has opposite effects, and has been shown to prevent ovariectomy-induced bone loss (18). *In vivo,* RANKL deficiency (14) and OPG overexpression (18) resulted in increased bone mass due to decreased osteoclast formation and activation, whereas OPG deficiency resulted in profound osteoporosis due to enhanced osteoclast formation and activation (19). Because immunosuppressants increase osteoclastic bone resorption (9, 10), we hypothesized that immunosuppressants may modulate the expression of RANKL and/or OPG by stromal/osteoblastic cells.

We demonstrate here that the immunosuppressants most commonly employed in the clinical setting (CsA, rapamycin, FK-506) significantly decreased OPG mRNA steady-state levels and protein secretion by osteoblast precursor cells (human marrow stromal cells) in a dose-dependent fashion. Moreover, the magnitude of inhibition by these immunosuppressants was comparable to that of glucocorticoids (20), a potent inhibitor of OPG production and an agent also used as an adjunct in immunosuppressant programs. Furthermore, we demonstrate that all of these immunosuppressants stimulated RANKL mRNA steady-state levels in marrow stromal cells, thus substantially increasing the RANKL/OPG ratio. Our data thus indicate that undifferentiated marrow stromal cells are an important target for immunosuppressants in bone and may mediate, at least in part, the adverse effects of these drugs on bone resorption. Of note, RANKL and OPG regulate interactions between T cells and dendritic cells, and also affect early differentiation of T and B lymphocytes (13, 17). Thus, regulation of RANKL and OPG production by bone marrow-residing stromal and immune cells may also be part of the mechanism for the immunosuppressive effects of these agents.

Our data may also provide some insights into the mechanism for the potential bone-sparing effects of rapamycin (5). While rapamycin decreased OPG production in undifferentiated bone marrow-derived stromal cells, in contrast to CsA and FK-506, it increased OPG production in the hFOB cells, which represent mature osteoblastic cells. Thus, the negative effect of rapamycin on undifferentiated preosteoblastic cells may be compensated for by increased production of OPG in mature osteoblasts *in vivo.* One potential mechanism whereby rapamycin increases OPG production in hFOB cells may be related to its stimulatory effect on osteoblastic cell differentiation (26) and its inhibitory effect on adipocytic cell differentiation (27). Consistent with this concept, our group has previously shown that other calcitropic factors (1,25-dihydroxyvitamin D_3, bone morphogenetic protein-2) which promote osteoblastic differentiation in hFOB cells, also increase OPG production in these cells (23), and that OPG production increases by 6-fold during osteoblastic lineage cell differentiation (28).

A potential protective role for OPG in the vascular system was suggested by its *in situ* localization to the arterial wall (18) and the development of arterial calcification in OPG-deficient mice (19). Moreover, arterial calcifications in OPG knock-out mice could be prevented by transgenic overexpression of OPG from mid-gestation through adulthood, but not by administration of OPG after birth (29). However, the regulation of OPG production in vascular cells has not been studied in detail. Since immunosuppressants have also been implicated in post-transplant vascular disease (7, 11), we hypothesized that altered OPG production by vascular cells may represent a potential paracrine mechanism for the detrimental vascular effects of immunosuppressants. We selected smooth muscle cells for our studies because they are a well established CsA target (7, 11). CsA, but not rapamycin or FK-506, inhibited OPG mRNA and protein production by these cells at non-toxic concentrations (30, 31). Of note, studies in an experimental arterial autograft model in the rat have found that CsA treatment markedly accelerated atherogenic changes, including dystrophic calcifications, in the grafts (32). By contrast, rapamycin and FK-506 appear to have a lower incidence of adverse vascular effects (7, 8). Combined with the presence of arterial calcifications in the OPG-deficient mice (19), these findings suggest the hypothesis that CsA-induced reductions in vascular OPG production may contribute to its propensity to cause vascular disease. Although RANKL is expressed by marrow stromal/osteoblastic cells and immune cells such as B and T cells, we were unable to detect RANKL mRNA in CASMC which is consistent with the findings of Min *et al.* (29). Thus, it is possible that immune cells infiltrating the arterial wall may initiate and promote medial inflammation which, over time and in the absence of sufficient concentrations of OPG, may lead to arterial calcification. Clearly, more studies are required to assess the biologic effects of RANKL, RANK and OPG on the vascular system *in vivo.*

In summary, we show that like glucocorticoids (20), commonly used immunosuppressants (CsA, rapamycin, and FK-506) inhibited the production of the secreted decoy receptor OPG and stimulated RANKL production by human bone marrow-derived stromal cells. In contrast to the other agents, rapamycin, which may be bone sparing, increased the production of OPG by mature osteoblastic cells, thus potentially attenuating its adverse effects on the marrow stromal cells. These studies therefore provide a potential mechanism for immunosuppressant-induced bone loss. Moreover, the effects of CsA on OPG production by vascular cells may also contribute to its propensity to cause vascular disease.

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