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Osteoblastic Differentiation Is Enhanced by Rapamycin in Rat Osteoblast-like Osteosarcoma (ROS 17/2.8) Cells

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The effects of three immunosuppressants (rapamycin, FK506 and cyclosporin A) on the proliferation and differentiation of rat osteoblasts-like osteosarcoma cell line, ROS 17/2.8 (ROS) cells were examined *in vitro*. All immunosuppressants showed a direct inhibition on the proliferation of ROS cells with different potencies. Growth inhibition by rapamycin was stronger than that by FK506 or cyclosporin A. Rapamycin caused a significant increase in alkaline phosphatase (ALP) activity and in the expression of osteopontin and osteocalcin mRNAs. FK506 caused a moderate increase in ALP activity and a decreased expression of osteopontin mRNA. Cyclosporin A caused a decrease in ALP activity and in the expression of type 1 α 1 collagen mRNA. Our study indicates that rapamycin directly acts on ROS cells and induces osteoblastic differentiation, however, the effect of FK506 and cyclosporin A is weak. Rapamycin significantly enhances the differentiation induced by 1,25(OH)$_2$-vitaminD$_3$.

The rat osteoblast-like cell line, ROS 17/2.8 (ROS) cells, was established from a rat osteosarcoma and can be matured by 1,25(OH)$_2$vitaminD$_3$ (1,25(OH)$_2$D$_3$) (1,2). ROS cell is particularly well suited for differentiation studies because specific stages of normal bone development appear to be associated with a specific pattern of expressed genes encoding bone matrix proteins. The differentiation process of preosteoblasts consists of three principle periods, proliferation, matrix development (bone maturation) and mineralization. Enhanced expression of alkaline phosphatase (ALP) and osteopontin occurs at the maturation stage of osteoblastic differentiation, and an increase in osteocalcin expression occurs at the mineralization stage (3). Concomitant with the differentiation of ROS cells is an increase in ALP activity and in the expression of several osteoblastic differentiation marker proteins, such as osteopontin and osteocalcin (4).

Rapamycin, FK506 and cyclosporin A are potent immunosuppressive agents used both experimentally and clinically for the prevention of graft rejection and the treatment of autoimmune diseases. The traditional target of these immunosuppressant is T-lymphocytes. These immunosuppressants have been reported to bind to immunophilins. Cyclosporin A binds to cyclophilin and FK506 and rapamycin bind to FK506-binding protein (FKBP). The complexes of cyclosporin A-cyclophilin and FK506-FKBP have also been shown to bind to and inhibit calcineurin, a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase. On a functional level, these complexes interfere with signal transduction in activated T-lymphocytes, and have a direct inhibitory effect on proliferation and activation. Calcineurin is a common target of the two complexes and plays a crucial role in regulating downstream effectors resulting in T-cell immunosuppression (5). Rapamycin binds to FKBP, and the rapamycin-FKBP complex binds to FKBP-rapamycin-associated protein (FRAP or RAFT). The formation of the tertiary complex inhibits the activation of p70 S6 kinase and subsequently T-cell proliferation and activation (6). Besides the action via immunophilins, these immunosuppressants have possibilities of other mechanisms via other pathways and are useful tools to dissect cell signaling pathway.

In the present study, the effect of rapamycin on proliferation and osteoblastic differentiation of ROS cells was examined and compared with the effect of FK506 and cyclosporin A. Cell proliferation, ALP activity and mRNA expression levels of type I α 1 collagen, osteopontin and osteocalcin were analyzed in the presence of these immunosuppressants.

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MATERIALS AND METHODS

Chemicals. Cyclosporin A (M_r = 1,203) and FK506 (M_r = 822) were gifts from Novartis Pharmaceuticals Co., Ltd. (Tokyo, Japan) and Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), respectively. Rapamycin (M_r = 914) was purchased from BIOMOL (Plymouth Meeting, PA), and 1,25$(OH)_2D_3$ was purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). Each drug was dissolved in ethanol and added to the culture after further dilution with the culture medium. The final ethanol concentration in the culture media was less than 0.005%.

Cell culture. ROS cells obtained from RIKEN Cell Bank (Tsukuba Science City, Japan) was maintained in Ham's F12 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) (ICN, Costa Mesa, CA) (1). Cultures were kept under 5.0% CO_2 in humidified air at 37.0°C.

Cell growth analysis. ROS cells were seeded in tissue culture plates (35-mm) at 5,000 cells/cm^2 in Ham's F12 medium supplemented with 10% FBS, and were incubated for 24 hours to allow cell attachment. The media was then replaced daily with fresh media containing various concentrations of immunosuppressants. Cells were harvested by trypsinization (0.05% trypsin, 0.02% EDTA) and were counted in a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). Thereafter, the viability of harvested cells was evaluated via the trypan blue dye exclusion test.

Measurement of ALP activity. Forty-eight hours after treatment with immunosuppressants, ALP activity of lysed ROS cells seeded was measured using ρ-nitrophenyl phosphate as a substrate, as previously described (7). Protein concentration was determined by the Protein (Bradford) assay kit (Bio-Rad, Hercules, CA), and specific activity of ALP was calculated.

cDNA probes. cDNA of type I α 1 collagen was obtained from American Type Culture Collection (Rockville, MD), and those of osteopontin and osteocalcin were generous gifts from Dr. Nomura (Department of Pathology, Osaka University Medical School, Osaka, Japan) (8). Human glyceraldehyde-3 phosphate dehydrogenase (G3PDH) cDNA was purchased from CLONTECH (Palo Alto, CA).

RNA isolation and Northern blot hybridization. Total RNA was isolated by the guanidine kit, ISOGEN (Nippon gene, Toyama, Japan) from cells grown on 100-mm plates 24 hours after treatment with immunosuppressants. Total RNA (15 μg) was separated on a 1.0% agarose gel, and transferred to a HYBOND-N nylon membrane (Amersham, Buckinghamshire, UK). Membranes were prehybridized at 42°C for 3 hours in 10% dextran sulfate in 50% formamide containing 1× Denhardt's solution. [α-^{32}P] labeled cDNA probes were prepared using a random primer DNA labeling kit (TAKARA, Shiga, Japan). The membranes were hybridized overnight at 42°C and were subsequently washed twice with 2× SSC (1× SSC: 0.15 M NaCl and 15 mM sodium citrate) containing 0.1% SDS for 15 minutes at 42°C and then twice with 0.1× SSC including 0.1% SDS for 15 minutes at 42°C. Specifically bound probe was visualized and quantified by Imaging Analyzer (Fuji Model BAS1000, Tokyo, Japan).

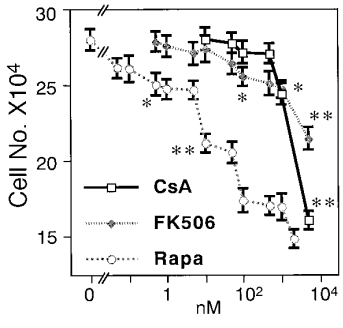

FIG. 1. Effect of immunosuppressants, cyclosporin A (CsA), FK506 and rapamycin (Rapa), on ROS cells proliferation. Results are expressed as the average number of cells of 35-mm dishes. Values represent the mean ± S.D. from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. 0 nM.

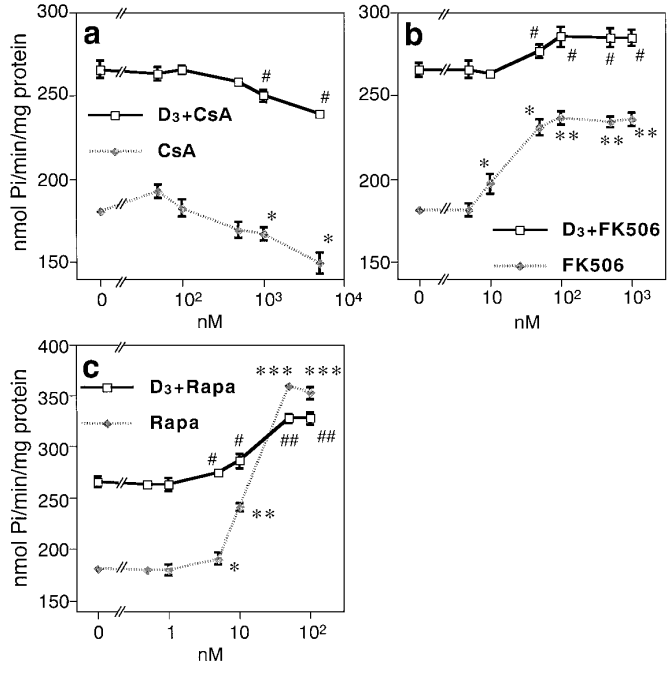

FIG. 2. Effect of immunosuppressants on ALP activity in ROS cells. (a) Cyclosporin A (CsA), (b) FK506 and (c) rapamycin (Rapa) were used with or without 1,25$(OH)_2D_3$ (D_3). Values represent the mean ± S.D. from three independent experiments. * P 0.05, ** $P <$ 0.02 and *** $P < 0.01$ vs. 0 nM. # $P < 0.05$ and ## $P < 0.02$ vs. 0 nM with D_3.

RESULTS

Effect of immunosuppressants on ROS cells proliferation. The proliferation of ROS cells was examined in the presence of each immunosuppressant (Fig. 1). The concentration of the drugs was 0 − 2 μM (rapamycin) or 0 − 5 μM (FK506 and cyclosporin A). The number of ROS cells were counted on day 3 of treatment. All immunosuppressants resulted in a dose-dependent inhibition on cell proliferation. The inhibiting effect of rapamycin on ROS cell growth was more potent than that of FK506 or cyclosporin A. More than 90% of cells were shown to be viable in all samples tested by trypan blue dye exclusion test.

Effect of immunosuppressants on ALP activity in ROS cells. ALP activity, a marker for osteoblastic phenotype, was assayed in ROS cells 48 hours after immunosuppressant treatment (Fig. 2). Rapamycin (≥ 5 nM) treatment resulted in a significant increase in ALP activity (Fig. 2c). ROS cells exposed to 10 nM 1,25$(OH)_2D_3$ for 48 hours showed a 1.5-fold increase in

ALP activity as compared to unstimulated control cells. This was consistent with the previously reported data (2). In the presence of 10 nM $1,25(OH)_2D_3$, simultaneous addition of rapamycin ($\geq$ 5 nM) also enhanced ALP activity. However at 50 or 100 nM, the effect of rapamycin in the presence of 10 nM $1,25(OH)_2D_3$ was smaller than that of rapamycin alone. Figure 2b shows that FK506 ($\geq$ 10 nM) also caused an increase in ALP activity, however the increase was smaller than that caused by rapamycin. FK506 ($\geq$ 50 nM) in the presence of $1,25(OH)_2D_3$ also induced ALP activity. In contrast, cyclosporin A ($\geq$ 1,000 nM) with or without $1,25(OH)_2D_3$ reduced ALP activity (Fig. 2a).

Effect of immunosuppressants on expression of type I α 1 collagen, osteopontin, and osteocalcin mRNAs in ROS cells. The concentrations used in this experiment (rapamycin: 50 nM, FK506: 100 nM, cyclosporin A: 1,000 nM) were decided by the reason that they inhibit ROS cell growth and affect ALP activity at these concentrations. Northern blot analysis detected mRNAs of type I α 1 collagen, osteopontin and osteocalcin (Fig. 3A). mRNA expression levels were quantified and normalized to that of G3PDH (Fig. 3B). ROS cells constitutively express mRNA for type I α 1 collagen. Independent treatment with FK506 (100 nM) or rapamycin (50 nM) did not significantly change mRNA expression levels of type I α 1 collagen, however, that with cyclosporin A (1,000 nM) reduced type I α 1 collagen mRNA expression to 63% of the control level. Ten nanomolar $1,25(OH)_2D_3$ resulted in an inhibition of type I α 1 collagen mRNA expression to 67% of that of untreated cells (Fig. 3B a). Combined treatment of each immunosuppressant with $1,25(OH)_2D_3$ did not alter the level of expression of type I α 1 collagen mRNA compared with that of $1,25(OH)_2D_3$ alone. Osteopontin and osteocalcin were only weakly expressed in untreated ROS cells (Fig. 3A). Cyclosporin A did not have any effect on osteopontin or osteocalcin mRNA expression and FK506 showed an inhibition of osteopontin mRNA expression (81% of control). Rapamycin significantly enhanced both osteopontin (1.9-fold) and osteocalcin (2.7-fold) mRNA expression. Ten nanomolar $1,25(OH)_2D_3$ resulted in a 2.5-fold and 4.7-fold induction of osteopontin and osteocalcin mRNA expression, respectively. Combined treatment of FK506 or cyclosporin A with $1,25(OH)_2D_3$ showed no further effect on osteopontin and osteocalcin mRNA expression levels. The combined treatment of rapamycin with $1,25(OH)_2D_3$ resulted in 1.8-fold and 2.7-fold enhancement of osteopontin and osteocalcin mRNA expression, respectively, over that induced by $1,25(OH)_2D_3$ alone (Figs. 3B b and c). When higher concentrations of these immunosuppresants (rapamycin: 2 μM, FK506: 5 μM, cyclosporin A: 5 μM) were applied in the media, no significant differences were detected in the expression patterns.

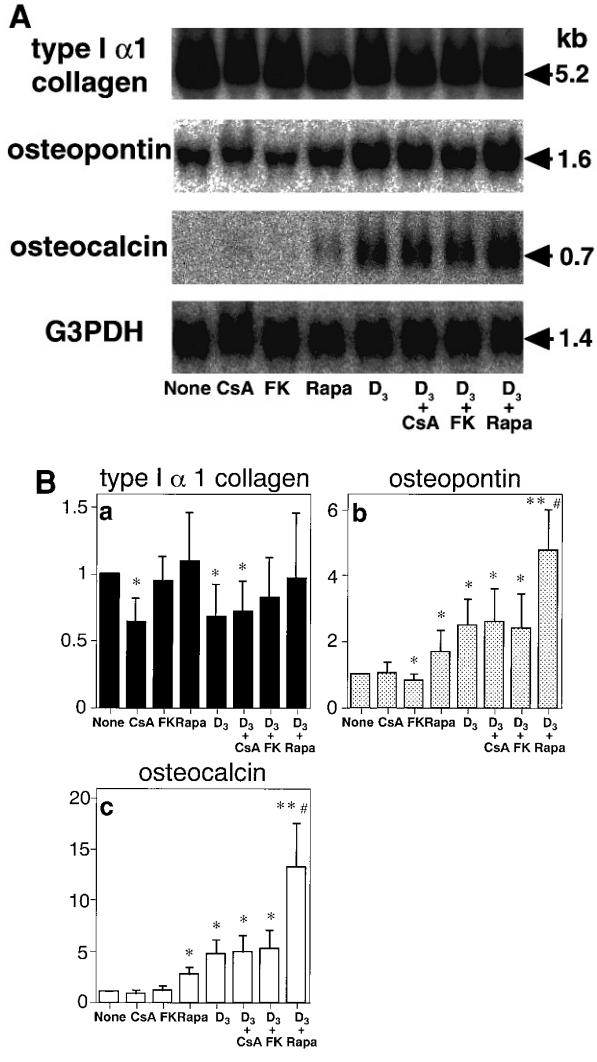

FIG. 3. Expression of type I α 1 collagen, osteopontin and osteocalcin mRNAs in ROS cells. (A): Expression of type I α 1 collagen, osteopontin and osteocalcin mRNAs was analyzed by Northern blot analysis as described in Materials and Methods. The concentration of cyclosporin A (CsA), FK506 (FK), rapamycin (Rapa) and $1,25(OH)_2D_3$ (D_3) was 1,000 nM, 100 nM, 50 nM and 10 nM, respectivly. The bottom panel shows the Northen blotting of G3PDH using the same blot. (B): Quantified ratios for Northern blots of (a) type I α 1 collagen, (b) osteopontin and (c) osteocalcin. mRNA levels were normalized by the level of G3PDH mRNA signal. Values represent the mean $\pm$ S.D. from five independent experiments. * $P < 0.05$ and ** $P < 0.02$ vs. None. # $P < 0.05$ vs. D_3.

DISCUSSION

To our knowledge, this is the first report to document the direct effects of rapamycin and FK506 on proliferation and differentiation of osteoblast-like osteosarcoma cells *in vitro*. Three principle findings were emerged from this study: [1] Treatment with each immunosuppressant had an inhibitory effect on the proliferation of ROS cells but with differing potency (rapamycin > FK506 ~ cyclosporin A). [2] ALP activity increased significantly by rapamycin and moderately by FK506, however decreased by cyclosporin A treatment. [3] Rapamycin had the most potent effect on expression of differentiation

marker proteins (osteopontin and osteocalcin), among three immunosuppressants tested.

Rapamycin, but not FK506 or cyclosporin A, significantly inhibited ROS cell proliferation at clinically effective doses for immunosuppressants (rapamycin : 11-66 nM, FK506 : 6-30 nM, cyclosporin A : 42-249 nM) (9,10). The inhibitory effect of rapamycin was dose-dependent. FK506 and cyclosporin A started to reduce cell proliferation rate significantly at concentrations which exceeded clinical doses (FK506 $\geq$ 100 nM, cyclosporin A $\geq$ 1,000 nM). With respect to cyclosporin A, our data are consistent with that reported by McCauley et al. (11).

$1,25(OH)_2D_3$ is one of the most potent biological regulators of normal bone growth and mineralization. Ten nanomolar $1,25(OH)_2D_3$ inhibited proliferation (data not shown) and induced differentiation in ROS cells *in vitro*. ALP activity and the expression of osteopontin mRNA, but not osteocalcin mRNA, were detected in untreated ROS cells and a marked increase in ALP activity and in osteopontin and osteocalcin mRNA abundance was observed in the presence of 10 nM $1,25(OH)_2D_3$. The effects of immunosuppressants administered with or without $1,25(OH)_2D_3$ on ROS cell differentiation were examined by analyzing these differentiation markers. Rapamycin, at a clinical dose, induced a significant increase in ALP activity and the expression of osteopontin and osteocalcin mRNAs. FK506 at clinically effective doses caused an increase in ALP activity whereas cyclosporin A caused a decrease. The latter was supported by McCauley et al. who reported that ALP activity was significantly reduced with micromolar levels of cyclosporin A (11). FK506 (100 nM) reduced the expression of osteopontin mRNA and cyclosporin A (1,000 nM) caused a decrease in the expression of type I α 1 collagen mRNA. These findings strongly suggest that rapamycin directly affects osteoblastic differentiation, while the effect of FK506 and cyclosporin A is weak.

In addition to suppression of T-lymphocytes activation, these drugs have also been shown to inhibit the proliferation of a variety of cells. We also have already examined the effects of immunosuppressants on a murine mesenchymal progenitor cell line, C3H10T1/2, and these drugs were shown to have an effect similar to that found in ROS cells (data not shown). Yeh et al. reported that rapamycin, but not cyclosporin A and FK506, inhibits adipogenic differentiation of 3T3-L1 cells (12). Jayaraman et al. reported that rapamycin blocked myogenic proliferation and induced differentiation into myogenic cells of BC3H1 cells (13). Their results are consistent with our results. The differential effect of three immunosuppressants on ROS cell proliferation, ALP activity and osteopontin and osteocalcin mRNA expressions could be attributed to the distinct effect on downstream pathways following formation of the complexes with immunophilins. The exact mechanism of the action of these immunosuppressants remains to be elucidated. Besides the action via immunophilins, there are possibilities of other mechanisms via other pathways. This may explain the distinct effect of FK506 and cyclosporin A, both of which share the same target proteins. Further studies will also be necessary.

Normal bone is a tissue in which the relationship between growth and differentiation must be maintained to sustain the balance between bone resorption and bone formation. FK506 and cyclosporin A have been reported to have a side effect of high-turnover osteopenia, and FK506 tends to cause severer osteopenia than cyclosporin A (14). On the other hand, the effect of rapamycin on bone metabolism has been reported to differ from that of FK506 and cyclosporin A. Rapamycin lacks an osteopenic effect, but resulted in a reduced longitudinal growth rate of long bones (15). It has not yet been determined whether the effect of these immunosuppressants on osteogenic or osteoclastic cells is direct or not. Our present study demonstrated a direct activity of these drugs on osteoblastic cells.

$1,25(OH)_2D_3$ has been used as an antitumor drug in a variety of cancers because of its potency to induce differentiation and reduce proliferation (16). Our study also indicates that $1,25(OH)_2D_3$ is a potent inducer of differentiation of immature osteosarcoma cells and reduces the proliferation rate of ROS cells. Rapamycin has already been reported to possess a potential to augment the efficacy of cisplatin on tumor cells. (17). Our study revealed that rapamycin possess as a potent and direct activity to differentiate immature ROS cells, and this effect is much stronger when rapamycin is synergistically treated with $1,25(OH)_2D_3$. Thus, the combined usage of rapamycin with $1,25(OH)_2D_3$ may augment the efficacy of $1,25(OH)_2D_3$ without causing enhancement of hypercalcemia. Further study will be need to confirm this possibility using other cancer cell line or model animals.

In conclusion, these findings strongly suggest that rapamycin directly inhibits ROS cell proliferation and induces osteoblastic maturation, while the effect of FK506 and cyclosporin A is weak.

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