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Cyclosporin A and Its Non-Immunosuppressive Derivative Exhibit a Differential Effect on Cell-Mediated Mineralization in Culture

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Abstract Chronic immunosuppressive treatment with cyclosporin A (CsA) is associated with decreased bone density. However, in culture, CsA inhibits osteoclast differentiation and bone resorption. This raises the question as to whether CsA also affects osteoblast function. Immunophilin, one of the CsA-binding cyclophilins that is implicated in the immunosuppressive action of CsA via calcineurin, is a peptidyl prolyl cis-trans isomerase (PPI). CsA also binds a mitochondrial membrane PPI which is implicated in controlling permeability transition pores. Therefore, in the present study we tested the effect of CsA on cell mediated mineralization in parallel with mitochondrial rhodamine retention as an indicator of mitochondrial membrane potential. Rat marrow stromal cells were grown in dexamethasone (DEX) medium to stimulate mineralization in culture, and CsA was added to various cultures using different treatment schedules. Low dose (0.1 μM) CsA inhibited mineralization, compared to controls, when present in the cultures during days 3–11 of DEX stimulation. Contrarily, high dose CsA (1.0 μM) resisted the inhibitory effect of the low dose. SDZ 220–384 (SDZ), a non-immunosuppressive derivative of CsA which is known, like CsA, to bind to mitochondrial cyclophilin but does not inhibit calcineurin, was also tested. Both high and low doses of SDZ decreased mineralization when present in the cultures from day 3 or from day 0. The similar effect of the low CsA dose and SDZ on mineralization is in accord with their ability to block permeability transition pores. The differential effect, on day 21 mineralization, between high CsA dose and SDZ took place in parallel to their opposing effects on mitochondrial membrane potential. On days 4–8, mitochondrial rhodamine retention was higher under CsA than under SDZ. Under these conditions there was no significant difference between the effects of these drugs on cell proliferation measured on day 11; there was a minor decrease in specific alkaline phosphatase activity by SDZ, too small to explain the extent of mineralization inhibition by SDZ. These results suggest that permeability transition pores might be involved in controlling mineralization. Unlike SDZ, CsA exhibits an additional effect on the mitochondrial membrane potential and on mineralization when applied at a high dose on day 3. Therefore identifying the additional activity of high dose CsA, which is missing in SDZ, may be beneficial. Such activity is expected to resist changes in rhodamine retention and decreased mineralization induced by SDZ, and yet enable preservation of immunosuppressive activity of CsA. J. Cell. Biochem. 64:209–216.

Key words: cyclosporin A; cell-mediated; mineralization; marrow-stroma; mitochondria; membrane potential

Cyclosporin A (CsA), frequently used as an immunosuppressive agent to prevent transplant rejection, may be associated with osteopenia in clinical practice [Katz and Epstein, 1992] and in experimental animals [Movsowitz et al., 1988]. Paradoxically, in bone culture experiments, CsA inhibits hormone-induced bone resorption [Stewart et al., 1986; Klaushofer et al., 1987] probably by inhibition of osteoclast differentiation [Orcel et al., 1991]. These discrepant opposing effects of CsA suggested that the systemic CsA-suppressed T cell function is responsible for osteopenia in vivo, but experiments using nude rats did not exclude other mechanisms for CsA effects on bone [Buchinsky et al., 1995]. CsA was found to inhibit ionomycin-induced calcium influx via plasma membranes into lymphocytes [Matyus et al., 1986]. On the other hand it has increased calcium influx into hepatocytes [Nicchitta et al., 1985]. CsA also inhibits calcium dependent pores of the inner mitochondrial membrane in heart cells [Crompton et al., 1988]. For these reasons and based on

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the background presented below it is worthwhile to search for CsA effects on the osteoblastic aspects of bone turnover, and perhaps the role of calcium in cell signaling and in apatite deposition. CsA should be expected to affect mineralization by interfering with processes of energy metabolism in osteoprogenitor cells which may control mineralization.

Mineralization of the growth plate cartilage is linked both to anaerobic metabolism [Shapiro et al., 1982; Haselgrove et al., 1993] and to calcium flux from the mitochondria [Lehninger, 1970; Brighton and Hunt, 1978]. In hypertrophic condrocytes [Posner, 1978] and in soft tissues [Lehninger et al., 1978a] the mitochondria accumlates amorphous calcium phosphate by a respiration dependent mechanism. Calcium efflux from the mitochondria in general takes place through different channels [Lehninger et al., 1978b] and by different mechanims [Richter, 1992] than its influx. It has also been shown that endochondral mineralization depends on calcium accumulation through the plasma membrane, in maturing chondrocytes [Wuthier, 1993], via nifedipine and verapamil-sensitive channels [Zimmerman et al., 1994]. Although calcium traffic across organelle and plasma membranes has a signaling function in all tissues the question how, and whether at all, this calcium traffic also contributes directly to mineralization of calcified tissues, remains unsettled. Additional observations indicate that mitochondria may influence mineralization. Dexamethasone (DEX) recruits osteoprogenitor cells (OPC) among marrow stromal cells [Bellows et al., 1987; Leboy et al., 1991; Kamalia et al., 1992] and induces cell-mediated mineralization. Mitochondrial membrane potential diminishes on day 3 of DEX stimulation similarly to unstimulated cells. However it is still higher in DEX stimulated cells especially on days 7–8 in which a surge in rhodamine retention takes place [Klein et al., 1993c]. Inhibition of succinate dehydrogenase (SDH) in DEX-stimulated stromal cells (especially on day 3 of DEX stimulation) further selects OPC and also increases mineralization [Klein et al., 1993a, 1996]. Contrarily, levamisole (independently of its effect on alkaline phosphatase) diminishes mineralization in DEX-stimulated stromal cells [Klein et al., 1993b]. The antagonizing effects of levamisole and SDH on mineralization are associated and in accord with antagonizing effects on mitochondrial membrane potential [Klein et al., 1996]. Therefore, modulation of the membrane potential is either causing or responding to inducers or inhibitors of mineralization. CsA inhibits mitochondrial permeability transition pores [Petronilli et al., 1994] in addition to the inhibition of calcineurin [Liu, 1993]. CsA retards permeability transition by inhibiting a mitochondrial cyclophilin which acts as a peptidyl prolyl cis-trans isomerase [reviewed by Galat and Metcalfe, 1995], or as a chaperone. Both CsA and its non-immunosuppressive derivative SDZ-220 384 (SDZ) bind the mitochondrial cyclophilin [Petronilli et al., 1994], but SDZ does not inhibit the phosphatase activity of calcineurin [Schreier et al., 1993]. In the present study we examined in culture the effect of both drugs, CsA and SDZ, looking for differential effects on cell-mediated mineralization and on the mitochondrial membrane potential.

MATERIALS AND METHODS

Reagents

ALP kit 104 LL, dexamethasone, ascorbate, β-glycerophosphate arsenazo III, and rhodamine 123 were purchased from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Grand Island Biological Company (Grand Island, NY). Cyclosporin A and SDZ 220–384 are from Sandoz (Basle, Switzerland).

Stromal Cell Culture

Marrow cell suspensions from femurs and tibiae of female Sabra rats, weighing 60–80 g, were seeded in 25 cm^2 flasks, 10^8 cells/flask. Stromal cells were obtained as described by Maniatopoulos et al. [1988], removing the nonadherent hematopoietic cells during the first 10 days of culture. Remaining adherent stromal cells were propagated in the same maintenance medium consisting of Dulbecco modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum and antibiotics in 9% CO_2 to induce an anaerobic atmosphere. For experimental cultures, stromal cells were removed a week later by trypsinization and plated in 96 well microtiter plates, 5,000 cells/well, and grown in osteoprogenitor cell (OPC) stimulation medium (DEX medium). This consisted of maintenance (ordinary) medium containing 10^{-8} M dexamethasone, 50 μg/ml ascorbate, and 10 mM β glycerophosphate. The medium was changed on day 7 and subsequently every 4 days.

Measurement of In Vitro Precipitated Calcium

After 3 weeks in culture, OPC stimulation medium as opposed to ordinary medium induces cell-mediated mineralization detected by alizarin staining above and around cell foci [Klein et al., 1993a]. The features of this mineral by X-ray diffraction and X-ray dispersion are characteristic of apatite, similar to those shown by Maniatopoulos et al. [1988]. To quantitate these precipitates plates were washed twice with TBS and incubated in 0.5 N HCl overnight. Appropriately diluted samples were measured by the metallochromic method with Arsenazo III as described elsewhere [Klein et al., 1995]. The precipitates were expressed as µg Ca/well and presented also as indices of experimental to control ratios.

Rhodamine Retention Measurement

Rhodamine 123 (Rho) retention was used to indicate mitochondrial membrane potential [Chen, 1989]. Rho stock solution concentrated twentyfold in distilled water was added at a final concentration of 0.1 µg/ml. After a 2 h incubation the Rho-containing medium was removed. The cells were washed twice with TBS (50 mM Tris, pH 7.6, 150 mM NaCl) and continued their cultivation until Rho retention measurement on the following day. Cultures were prepared for Rho retention measurement immediately after removal of the growth medium. Cells were washed with TBS and incubated for 30 min at 37°C with 150 µl of 0.2% Triton-X 100, dissolving membranes to release intracellular Rho. Rho fluorescence was measured in a Perkin Elmer (Buckinghamshire, England) LS-5B luminescence spectrometer, by exitation at 505 nm and emission at 540 nm. The Rho content of cell extract pools of 4 wells each were calculated as arbitrary fluorescence units per cell count pools of the respective 4 wells in the same plate. The result of each sample consists of the mean ± SE of 5 such pools.

Quantitative Cell Staining

The cells were counted by measuring optical density of methylene blue bound under alkaline pH to carboxylic groups within the entire cell, using the method of Goldman and Bar-Shavit [1979]. Cells were fixed in 0.5% glutaraldehyde for 30 min, rinsed with D H_2O, and air dried overnight. Borate buffer (0.1 M boric acid brought to pH 8.5 with NaOH) 0.2 ml/well, was added to the cells for 2 min and rinsed with tap water. Cells were then incubated in 0.1 ml of 1% MB in borate buffer for 60 min at room temperature, rinsed with water, and air dried. The MB was then eluted from the stained cells by incubation with 0.2 ml of 0.1 N HCl at 37°C for 60 min. optical density (O.D.) of the eluted MB was measured at 620 nm by a multichannel optical densitometer; 1.0 U is equivalent to 5×10^4 stromal cells.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was measured in situ in microtiter plates. Day 11 of dexamethasone stimulation was set for ALP assay and cell count [Klein et al., 1993a]. Growth medium was removed and cells were washed twice in situ with 0.2 ml TBS (50 mM Tris, 150 mM NaCl, pH 7.6). ALP substrate, pNPP (p-nitrophenyl phosphate) in TBS, 1.33 mg/ml was dispensed 0.2 ml/well. Plates were placed in the tissue culture incubator for 90 min and optical density of the hydrolysed pNPP was measured in a multichannel optical densitometer at 405 nm wave length. ALP specific activity was expressed as nMol/time/cell.

Statistical Analysis

Each experimental sample contained 20 replicas adjacent to 20 replicas of its own control sample. The significance of results induced by cyclosporins was analysed against the private controls of each sample using the Wilcoxon paired-sample test for n = 9–20 replicas. This choice was based on previous observations regarding dependence of the control replicas on the experimental replicas within each sample [see Figure 3A in Klein et al., 1994] and on the inability to demonstrate a normal distribution within each 20 replicas. The replicas were paired according to their position in the culture plate.

RESULTS

Temporal Dissection of CsA Effect on Day 21 Mineralization

DEX-stimulated stromal cells were exposed to a low (0.1 µM) and a high (1.0 µM) concentration of CsA, throughout various periods during osteogenic differentiation. Figure 1A shows the mineralization levels (compared to untreated controls) resulting from different schedules of exposure to CsA, and expressed as µg calcium/

well on day 21. The response of the cultures to CsA varied according to the exposure period along differentiation and according to the drug concentration. The high and low dose CsA treatments represent two separate experiments that are comparable in the context of their own paired-controls illustrated by the treated/untreated indices in Figure 2. The period between day 3 to 11 of DEX stimulation was critical for decreasing day 21 mineralization by the low dose CsA. In contrast, the high dose abrogated the inhibitory effect of the low dose on day 21 mineralization, and in several time intervals it has increased mineralization, albeit not significantly. This suggested the existence of a biphasic response to CsA and also that CsA may affect mineralization by at least two different mechanisms. Of particular interest was the antagonistic effect on mineralization exhibited under exposure during days 3–11 or 4–7, in which the high dose resisted a decrease in mineralization below that of the controls whereas the low dose decreased mineralization significantly. Figure 1B shows the day 21 cell counts under various CsA treatment schedules, expressing cell count/well compared with untreated controls. A reciprocal trend between the cell counts (Fig. 1B) and mineralization (Fig. 1A) is demonstrated only for low dose CsA treatment period of days 3–11, regarding comparison between treated and untreated controls.

At this point it seemed that for the treatment period of days 3–11 the opposing effects on cell proliferation resulted in opposing effects on differentiation (this was later disproved, Fig. 4) and on mineralization. The rest of the CsA treatment schedules did not show this reciprocal pattern, between proliferation and differentiation, within the treated and respective controls. The two cultures treated with low dose CsA using protocols in which treatment was continued beyond day 11 up to day 21, resulted in excessively high proliferation. This increased proliferation did not change the mineralization level relative to controls, which may indicate that excessive proliferation is associated with resistance to inhibition of the mineralization only by the low dose CsA, which is well illustrated by the normalized results in Figure 1A vs. B.

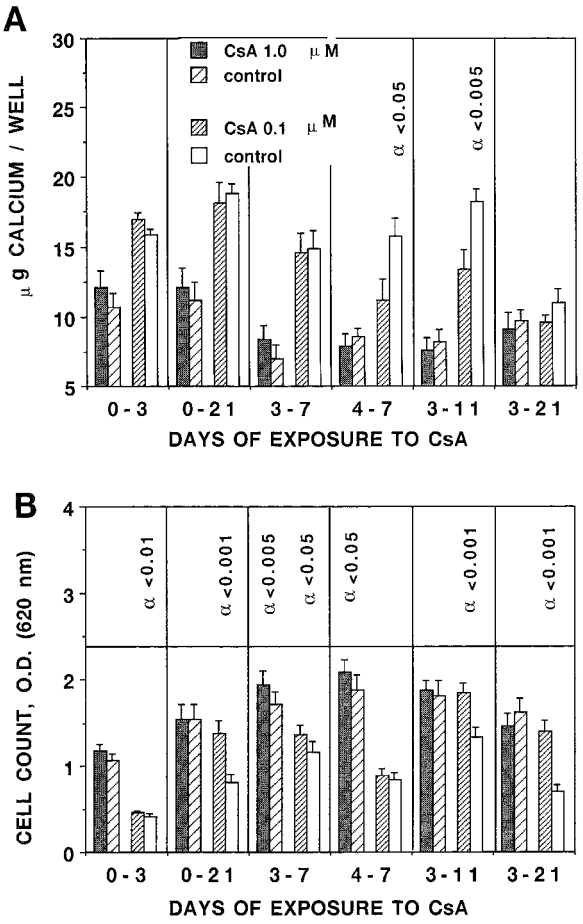

Fig. 1. Effect of CsA on cell mediated mineralization. CsA, at two concentrations, was added to different stromal cell cultures on different days post DEX stimulation. The high and low concentrations represent two separate experiments. Media were changed on days 0, 7, and 14, and for protocols 0–3 and 3–11, also on day 3 and 11, respectively. **A:** Mineralization was measured on day 21 as μg calcium/well. **B:** Day 21 cell counts were performed by methylene blue staining. Each pair of bars represents the mean ± SEM result of treated culture to its own untreated control of DEX stimulated culture, n = 20. The significance of differences between experimental and controls is indicated.

SDZ Effect on Mineralization

SDZ, the non-immunosuppressive derivative of CsA, was added to the cultures on days 0, 3, or 7 of DEX stimulation and maintained until day 21. Figure 3 shows the mineralization values, relative to controls, under the effect of this CsA derivative. SDZ inhibited mineralization when applied on day 0 or on day 3, at both concentrations. However, when added on day 7 the low SDZ dose lost its inhibitory effect; only the high dose slightly, but significantly, inhibited mineralization. Thus SDZ tended to decrease mineralization maximally when applied from day 3, contrary to the high dose of CsA which did not change mineralization. The low dose CsA applied on day 3 or 4 resulted in low

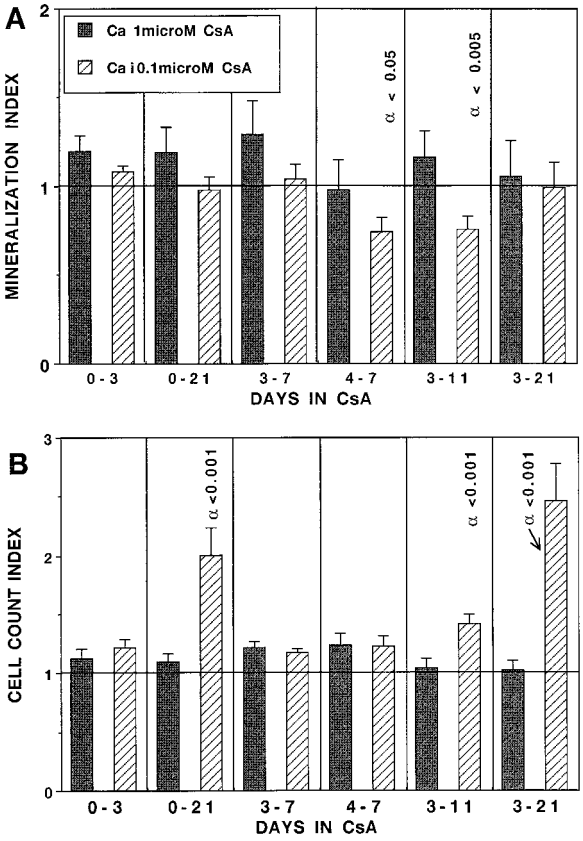

Fig. 2. Normalized values of CsA-induced cell mediated mineralization. Results expressed as indices derived from ratios between CsA treated and untreated cultures (see Fig. 1 legend for experimental conditions). Horizontal line indicates the level of zero response.

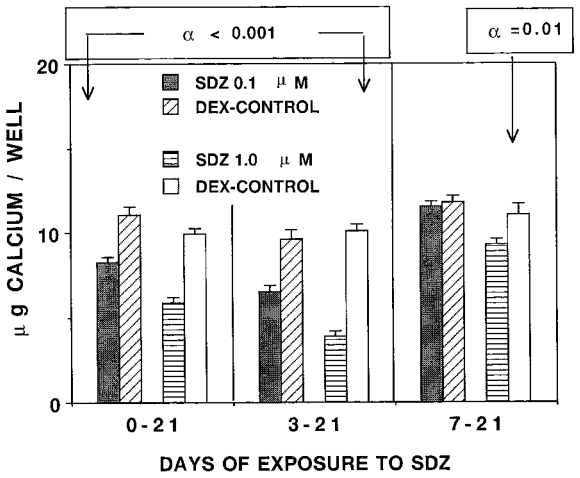

Fig. 3. Effect of SDZ on cell-mediated mineralization. SDZ was added and maintained in the cultures during the indicated days. Media were changed on days 0, 7, and 14. Mineralization values of SDZ-treated cultures are exhibited each next to its own untreated control, n = 20.

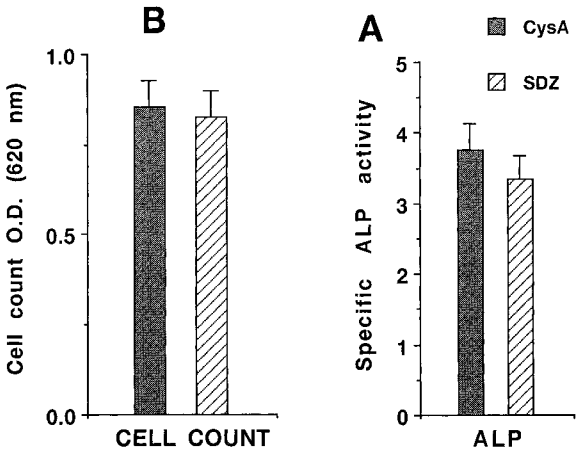

Fig. 4. Effect of cyclosporins on ALP and on cell counts. Cyclosporins, 1.0 μM, were added to the cultures on day 3 of DEX stimulation. Media were changed on days 0 and 7. ALP activity and cell counts were measured on day 11, and were expressed as specific ALP activity (nMol/90 min/5 × 10^4 cells), and as O.D. of eluted stain (1 O.D. U = 5 × 10^4 cells), n = 20. The small difference in specific ALP activity between CsA and SDZ treated cultures is significant ($\alpha < 0.05$).

mineralization compared with controls (Figs. 1A, 2A), and the high dose CsA showed no significant difference from the controls. It follows that at high dose, CsA antagonizes the SDZ effect on mineralization. If the common characteristics of CsA and SDZ are responsible for diminished mineralization, their manifestation is presumably revealed by application of a low dose of these drugs between days 3–11 of DEX stimulation, for CsA and days 3–21 for SDZ. On the other hand, the opposing effects on mineralization that resulted from application of the drugs at high dose on day 3 are probably due to another property of CsA not shared with its derivative, SDZ.

Effect of Cyclosporins on ALP Activity and Cell Count

The opposite trend of mineralization, resulting from application of CsA and SDZ started on day 3, is independent of ALP activity and of cell proliferation. This is indicated by Figure 4 which does not show a significant difference between the effect of these drugs on day 11 cell counts and only a slightly higher specific ALP activity under CsA. The absolute ALP activity/well did not change significantly (not shown). Therefore, the mechanism by which high dose CsA increases mineralization is probably a biochemical effect on mineralization rather than a change in OPC differentiation. Similarly, SDZ de-

creased mineralization by a direct biochemical effect rather than by selection of cells without ALP activity or by killing of OPC.

Cyclosporins Effect on Rhodamine Retention

The opposite effects of CsA and SDZ on mineralization induced upon their application on day 3 were accompanied by an opposing trend of mitochondrial Rho retention (Fig. 5). CsA increased, whereas SDZ decreased Rho retention significantly mainly on day 4. This difference in Rho retention was abolished from days 8 to 11, when the mean Rho retention values turned over in favor of SDZ-treated cultures. Pooling the Rho retention results of days 4 to 8 showed significantly higher Rho retention values under CsA than under SDZ ($\alpha < 0.05$). On the other hand, the Rho retention values pooled from days 8 to 11 did not show a significant difference between CsA to SDZ treated cultures.

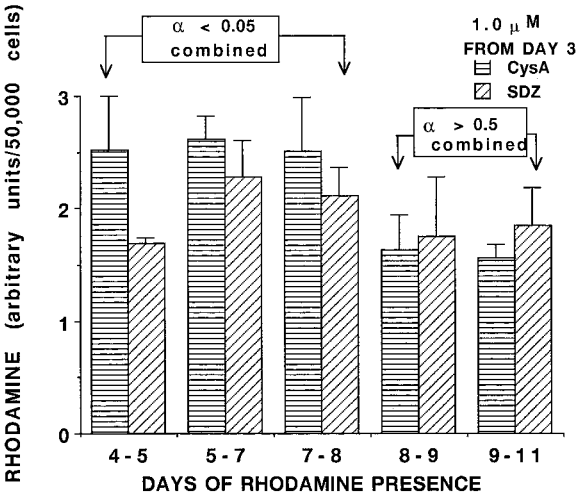

Fig. 5. Differential effect of cyclosporins on Rho retention. Cyclosporins, 1.0 μM, were added to the cultures on day 3 of DEX stimulation. Subsequently, on days 4, 5, 7, 8, and 9 Rho was added to separate cultures, to a final concentration of 1 μg/ml. Twenty-four hours later the cultures were washed twice with TBS and Rho was extracted. Cells, treated similarly, in parallel cultures were counted by MB staining. Cell extracts from groups, each of 4 wells, were pooled, diluted 1:2 in distilled water, and their fluorescence measured. Results are expressed as arbitrary fluorescence units per cell count of the respective parallel wells. Results of CsA treated cultures, with Rho retention higher than in SDZ cultures (of days 4, 5, and 7, n = 15), were pooled for analysis of statistical significance. Results of cultures measured from days 8 and 9, the time points at which the mean Rho retention trend was reversed, were also pooled for analysis of significance (n = 10).

DISCUSSION

SDZ, at 1.0 and 0.1 μM, decreased mineralization when applied to the cultures from day 0 or 3 of DEX stimulation; 0.1 μM CsA applied on days 3–11 had a similar effect. This is consistent with both agents having equal capacity in blocking mitochondrial permeability transition pores (MPTP) [Petronilli et al., 1994]. It is also in agreement with the mitochondria playing a role in mineralization.

Antagonistic effects were observed when high vis-a-vis low CsA concentrations were applied to the cultures on days 3–11, resisting inhibition and inhibiting mineralization, respectively. This difference in mineralization is probably due to diverse or antagonistic mechanisms whose activation is dose and time dependent. The response to SDZ was less time dependent, and showed similarity to the effect of low dose CsA. SDZ does not display time or dose dependent antagonistic effects on mineralization, suggesting that SDZ has less mechanistic options than CsA for affecting mineralization. Therefore, the effect of SDZ on mineralization is less complex than that of CsA. A possible inhibitory effect of SDZ on calcium uptake by the OPC early in the culture period cannot be excluded.

The low dose CsA has a mitogenic effect on cells upon continuous exposure beyond day 11 up to day 21. Our data are not sufficient to determine whether this late mitogenic response has an effect on mineralization. However, it should be noticed that the main direct effect of these drugs on mineralization takes place early after DEX stimulation, indicating that the cyclosporins do not interfere with the extracellular calcium phosphate deposition, at least not directly.

A phenomenon connected to mitochondria seen in these cultures is the decrease in inner membrane potential (MtMP) after day 3, based on a decrease in Rho retention [Klein et al., 1993c]. Since permeability transition pores are voltage-dependent and tend to open upon membrane depolarization [Petronilli et al., 1994], the decreased MtMP, from day 3, provides higher probability for permeability transition (pore opening). Permeability transition pores are also responsive to increased cytosolic calcium, which opens the pores for molecules <1,500 daltons. Oxidative stress can cause pore opening, which is associated with binding of the mitochondrial cyclophilin (CyP) to the inner

membrane matrix and the causing of mitochondrial swelling [Connern and Halestrap, 1994], which, in turn, is prevented by CsA binding to CyP. The high MtMP on days 4–5, resulting from day 3 treatment with high dose CsA, was probably unrelated to the delay in direct blocking of permeability transition, as this capability is shared by low dose CsA and by SDZ [Petronilli et al., 1994] and yet these agents showed an opposing effect on MtMP. CsA at high dose must have an additional effect on MtMP, opposing and overriding its own low dose effect. Whether SDZ and CsA display a differential dose dependent effect on CyP conformation or on permeability transition pores is yet to be tested. The differential phosphatase inhibitory effect of CsA on calcineurin, which is not achieved by SDZ, although both bind the calcineurin-associated immunophilin with a similar dissociation constant [Schreier et al., 1993], indicates that binding is necessary but insufficient for T-cell immunosuppression. This statement can be extended (for the present work) to the differential effect of these agents on MtMP. The differential effect on signal transduction relevant in T-cells (calcineurin inhibition) [Liu, 1993; Schreier et al., 1993] may be relevant for the mineralization and MtMP modulation in DEX stimulated stromal cells. The differential effect resulting from CsA and SDZ application from day 3 is probably not due to a general influence on matrix maturation since one of its markers, ALP activity, and cell counts did not differ sufficiently under a high dose of both agents to justify the observed differences in mineralization.

The opposing effects of high dose CsA and SDZ on mineralization and on MtMP are reminiscent of results obtained recently under treatment with levamisole and malonate [Klein et al., 1996], which suggested that modulation of the inner membrane potential is associated with modulation of mineralization.

CsA application at low dose, on days 3–11 or 4–7 in the present study, clearly shows its potential to inhibit the osteogenic part of bone turnover, at least its mineralization component. This activity might provide a feedback to osteoclasts, which could explain the findings of Stewart et al. [1986] and Orcel et al. [1991] who showed that CsA inhibited osteoclast differentiation and bone resorption. According to our results, further development of immunosuppressive cyclosporins should consider CsA analogues, which inhibit the calcineurin activity and yet have a minimal effect on mitochondrial cyclophilin and on mineralization.

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