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Cyclosporin A promotes mineralization by human cementoblastoma-derived cells in culture

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Objective: The immunosuppressive drug cyclosporin A has been shown to induce cementum deposition *in vivo* in experimental animals. Using cementoblastoma-derived cells, we have studied whether this drug will be useful to study cementum mineralization and differentiation *in vitro*.

Methods: Human cementoblastoma cells and gingival fibroblasts (controls) were cultured and treated with 0.5, 1.0 and 5.0 μg/ml of cyclosporin A. Cell proliferation was evaluated by MTT (tetrazolium) assay and cell number, and cell viability was assessed by trypan blue dye exclusion. Induction of mineralization was evaluated by alizarin red S staining to detect mineralized nodules and by reverse transcription–polymerase chain reaction (RT–PCR) to assess the expression of bone differentiation markers alkaline phosphatase, osteocalcin, bone sialoprotein and core-binding factor a1 (Cbfa1).

Results: Cyclosporin A at 5.0 μg/ml concentration reduced significantly the increase in the number of cementoblastoma cells. A dose-dependent increase in the number of mineralized nodules occurred in cultures of cementoblastoma-derived cells treated with cyclosporin A, and RT–PCR analyses showed significantly higher levels of expression of alkaline phosphatase, bone sialoprotein, type I collagen, matrix metalloproteinase-1, osteocalcin, osteopontin, and Cbfa1. Human gingival fibroblast proliferation and cell number were not affected. Mineralized nodules were not detected in gingival fibroblasts and bone specific proteins were not expressed.

Conclusions: Presence of cyclosporin A during 14-day culture period appears to suppress the proliferation of cementoblastoma cells and induce the formation mineralized-like tissue by these cells.

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Cyclosporin A is a potent immunosuppressive agent used to prevent organ transplant rejection. Chronic cyclosporin A treatment might be responsible for high-turnover osteopenia (1) and is associated with significant side-effects, including nephropathy, hypertension, hepatotoxicity, neurotoxicity and gingival overgrowth (2–5). The direct effect of cyclosporin A on bone turnover is indicated by increased osteoblastic activity (6, 7), and *in vitro* reports have shown that cyclosporin A has a protective effect on bone metabolism (8, 9), probably by inhibition of osteoclast differentiation (10). Paradoxically, it has been shown that cyclosporin A inhibits mineralization *in vitro* on dexamethasone-stimulated marrow stromal cell cultures and that it could affect mineralization by interfering with processes of energy metabolism in osteoprogenitor cells and controlling mineralization (11). However, it has been postulated that cyclosporin A does not interfere with the extracellular

calcium phosphate deposition. The cyclosporin A also induces corticomedullary intratubular mineralization, and this could be a consequence of impaired tubular calcium uptake that may saturate intratubular calcium concentration (12).

The study by Ayanoglou *et al.* (13) revealed that cyclosporin A promotes osteodentin formation in rat molars and that it might increase the activity of odontoblasts by modifying its phenotype or by increasing the number of cells, or by both mechanisms. Ayanoglou (14) also found that oral administration of cyclosporin A in Sprague-Dawley rats promoted the formation of mineralized islets inside the gingival connective tissue in the proximity of root surfaces and voluminous deposits of new cementum covering the root areas, and postulated that cyclosporin A stimulates paravascular cementoblast progenitor cells.

We have studied whether the cyclosporin A has the potential to induce cementum differentiation in culture because this model could be a useful to study cementum formation *in vitro*. We have used cells obtained from a human cementoblastoma for this purpose and we examined the effect of cyclosporin A on cell proliferation, cytotoxicity and mineralization, and on the expression of alkaline phosphatase, osteocalcin, bone sialoprotein, core-binding factor a1 (Cbfa1) and other cementum matrix proteins.

Materials and methods

Cell culture

Human cementoblastoma cells and human gingival fibroblasts were derived through the conventional explant technique and were characterized as previously described (15–17). The cells were cultured in 75-cm^2 cell culture flasks containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 μg/ml ascorbic acid, and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin, Sigma Chemical Co., St Louis, MO, USA). Cells between the second and seventh passages were used.

Cyclosporin A effect on cell proliferation and cytotoxicity

Cytotoxic effect and effect on proliferation were evaluated by the colorimetric MTT (tetrazolium) [3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The MTT analysis is dependent on the reduction of the tetrazolium salt MTT by the mitochondrial dehydrogenase of viable cells to form a blue formazan product. Cementoblastoma cells were plated at 1×10^4 density in a 200-μl volume in 96-well plates (Costar, Cambridge, MA, USA). Cells were treated for 5 days with varying concentrations of cyclosporin A (Sigma) in 0.005% ethanol, replacing the medium with fresh medium and cyclosporin A daily. Because dental plaque may serve as a reservoir for cyclosporin A, we chose cyclosporin A concentrations ranging from one to 20 times as much as blood levels in patients (29). The dosages of cyclosporin A selected were similar to the range of blood values found in patients administered cyclosporin A and those used for *in vitro* studies of cyclosporin A effect on bone resorption and osteoclast function (8, 10, 11). Control cultures were treated with vehicle, 0.005% ethanol. At the end of the treatment intervals, 10 μl of MTT solution (5 mg/ml: Boehringer Mannheim, Indianapolis, IN, USA) in phosphate-buffered saline was added to the wells and incubated at 37°C for 4 h. After the MTT incubation, 100 μl lysing buffer (20% sodium dodecyl sulfate, 50% dimethyl formamide pH 4.7) was added to each well and incubated overnight at 37°C. The resultant solution was read in a microplate reader at 570 nm. The optical density reflects the number of living cells present in the culture. To determine if cyclosporin A affected cell number, 2×10^4 cells were plated into 24-well culture plates, incubated overnight in 10% fetal bovine serum and exposed to cyclosporin A from the following day (day 0). Cells were harvested by trypsinization and counted in a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL, USA). Viability was evaluated via trypan blue dye exclusion. Experiments were performed in triplicate and repeated twice.

Mineralization assay

Human cementoblastoma cells and human gingival fibroblasts (2×10^4 cells) were plated in 24-well plates (Costar) and incubated with 10% fetal bovine serum alone (controls) or with 0.5, 1.0 and 5.0 μg/ml cyclosporin A (experimental) for 3, 7, 10 and 14 days. Culture media were replaced daily and cells were fixed with 70% ethanol and air-dried at indicated times. Cultures were tested for calcium precipitation by staining with 2% Alizarin Red S (Aldrich Chemical Company Inc., Milwaukee, WI, USA) for 5 min, and plates were examined for number of nodules (18, 19). The number of calcifying foci per well was counted macroscopically on a 5-mm grid. Experiments were performed in triplicate and repeated twice.

Reverse transcription–polymerase chain reaction (RT-PCR)

Cells were plated in six-well plates at 5×10^4 density and exposed to 10% fetal bovine serum or 10% fetal bovine serum plus 1.0 μg/ml cyclosporin A (this concentration was optimum for promoting mineralization in cementoblastoma cultures) and incubated for 0, 3 and 14 days. Total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as described previously (20). cDNA was synthesized using Superscript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 1.0 μg of total RNA and oligo dT primer according to the manufacturer's protocol. The reaction mixture (20 μl) contained 1 × reverse transcriptase buffer, 500 μM each of dCTP, dGTP, dATP and dTTP, 20 U of RNAse inhibitor and 100 U of M-MLV enzyme, and incubation was for 1 h at 37°C followed by 99°C for 5 min. Aliquots of 2 μl of the cDNA were amplified with 10 pmol each of 5′ and 3′ primers, 1 × PCR buffer, dCTP, dGTP, dATP and dTTP each at 0.4 mM, 1.5 mM $MgCl_2$ and 0.5 U Taq polymerase (Promega, Madison, WI, USA). Amplication was for 30 cycles in a thermal cycler (MJ Research, Watertown, MA, USA).

Previously published primer sequences and reaction conditions were used for alkaline phosphatase, osteocalcin, osteopontin, Cbfa1 and type I collagen (21), bone sialoprotein (22), matrix metalloproteinase-1 (MMP-1) (23), and GAPDH. GAPDH primers were purchased from Stratagene (La Jolla, CA, USA). Reaction products were separated and visualized after electrophoresis in 2% agarose gels containing 0.5 μg/ml ethidium bromide. Identity of PCR products was confirmed by sequencing the products. They were quantified by measuring band density using an Electrophoresis Documentation and Analysis System (EDAS) 290 (Eastman Kodak, Rochester, NY, USA), and normalized for GAPDH. The PCR reactions were performed at least three times.

Statistical analysis

Experiments were performed in triplicate. Each value represents the mean ± SE. The significance of differences was determined using a corrected Bonferroni *p*-value. Differences with *p*-values < 0.05 were considered significant.

Results

Cyclosporin A effect on cell proliferation

Cyclosporin A inhibited the proliferation of the human cementoblastoma cells during the first and second days of culture as revealed by the MTT assay, and the inhibition was 15, 16 and 21% and 26, 21 and 21% at 0.5, 1.0 and 5.0 μg/ml, respectively. However, no significant differences were observed on days 3–5 (Fig. 1A). Cyclosporin A significantly reduced cell numbers when compared to controls only at concentration of 5.0 μg/ml on days 3–5 of treatment (Fig. 1B). To determine if this was due to cytotoxicity, we performed trypan blue dye exclusion, and the results showed that the viability of cementoblastoma-derived cells treated with cyclosporin A was greater than 92%. Human gingival fibroblast proliferation was not affected by cyclosporin A significantly at all concentrations used (Figs 1C and D).

Effect of cyclosporin A on mineralization

Staining of control and experimental cultures with Alizarin Red S showed a

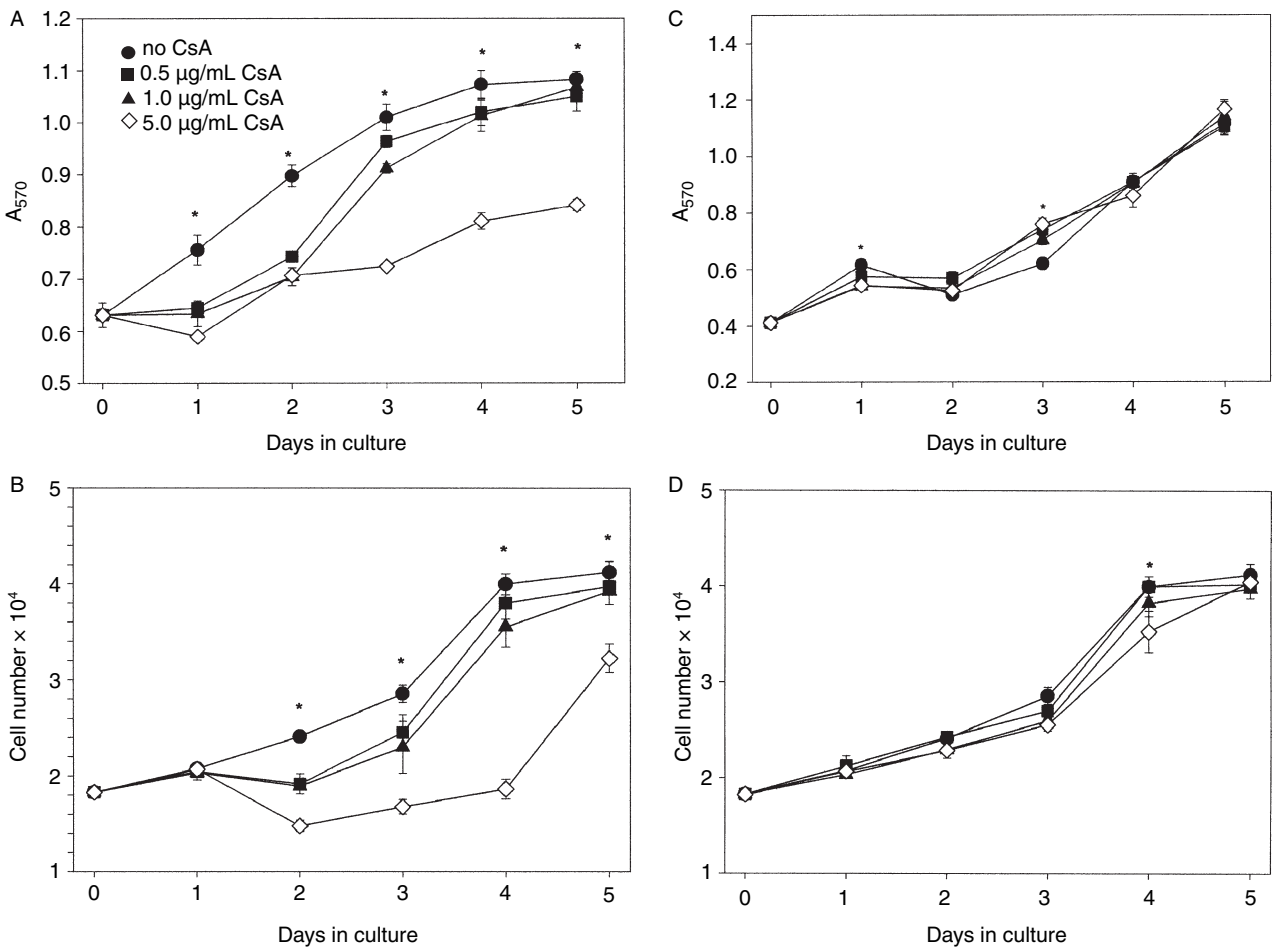

Fig. 1. Effect of cyclosporin A (CsA) on proliferation of human cementoblastoma-derived cells and human gingival fibroblasts. (A) Optical density at 570 nm of lyzed cementoblastoma cells from the MTT colorimetric tetrazolium reduction assay. (B) Cell number, cementoblastoma cells. (C) A_{570}, gingival fibroblasts. (D) Cell number, gingival fibroblasts. Means ± SD are shown. Asterisks indicate statistical significance ($p < 0.05$).

dose-dependent increase of nodules in experimental cultures of cementoblastoma-derived cultures and this occurred even though the cultures did not contain dexamethasone in the medium (Fig. 2A). In cells treated with 0.5 and 1.0 μg/ml of cyclosporin A, the number of nodules increased by 143 and 189% at 7 days, 168 and 200% at 10 days, and 231 and 224% at 14 days, respectively, relative to cultures without cyclosporin A (Fig. 2C). These increases were statistically significant. Interestingly, the number of nodules was relatively less at 5.0 μg/ml cyclosporin A; after 7, 10 and 14 days mineral nodule formation was up by 21%, 65% and 133%, respectively (Fig. 2C). No nodules were observed in human gingival fibroblast cultures treated with cyclosporin A at all concentrations (Fig. 2B).

RT-PCR analysis

The results of electrophoresis of RT–PCR products obtained from

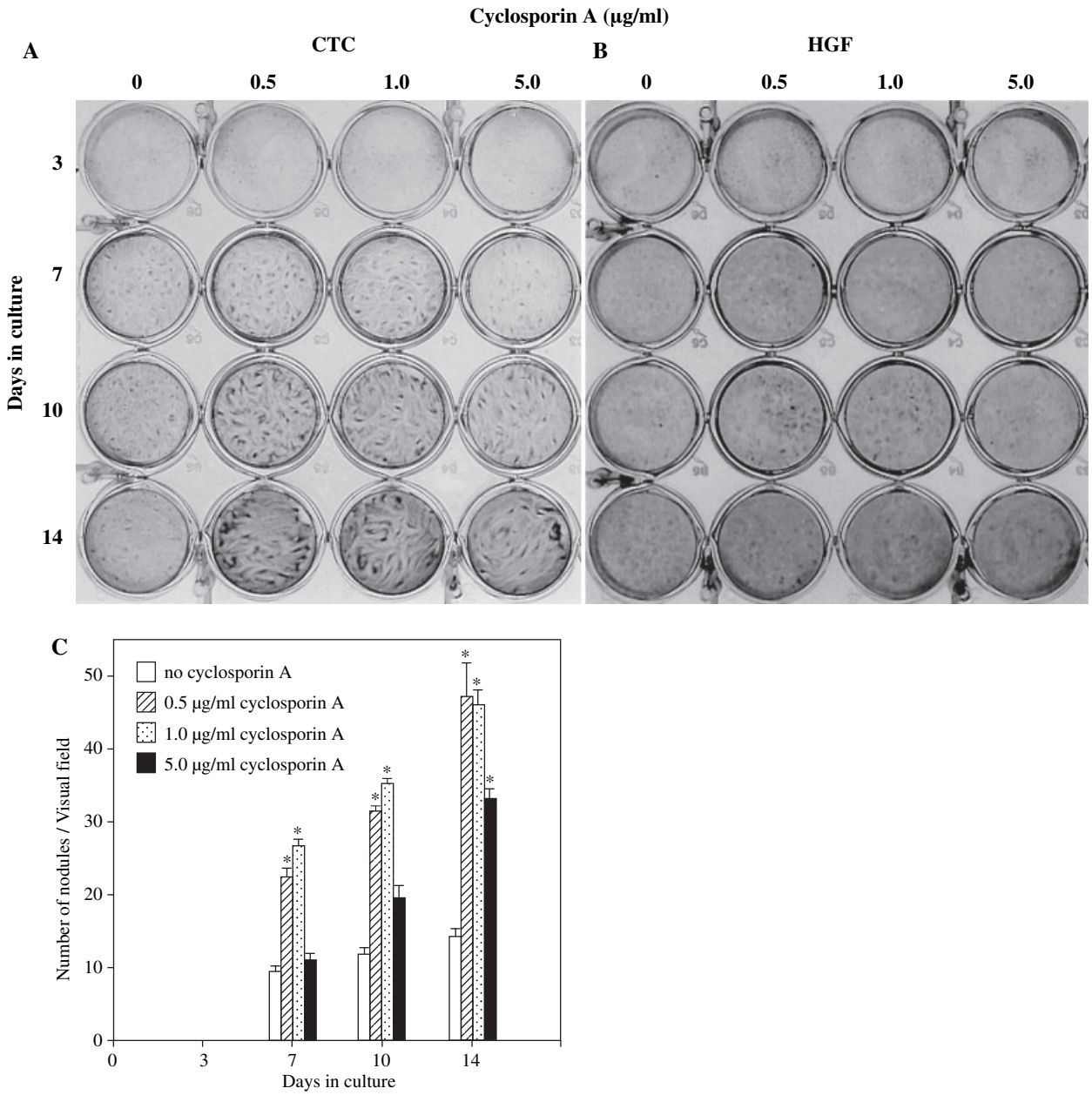

Fig. 2. Alizarin red stained cultures of (A) human cementoblastoma-derived cells (CTC) and (B) human gingival fibroblasts (HGF), treated without and with cyclosporin A at 0.5, 1.0 and 5.0 μg/ml for 3, 7, 10 and 14 days. (C) Number of calcifying nodules per well as counted macroscopically on a 5-mm grid on human cementoblastoma-derived cells treated with cyclosporin A at 0.5, 1.0 and 5.0 μg/ml for 3, 7, 10 and 14 days. The human gingival fibroblasts did not form nodules at all cyclosporin A concentrations. Asterisks indicate statistical significance compared to control ($p < 0.05$).

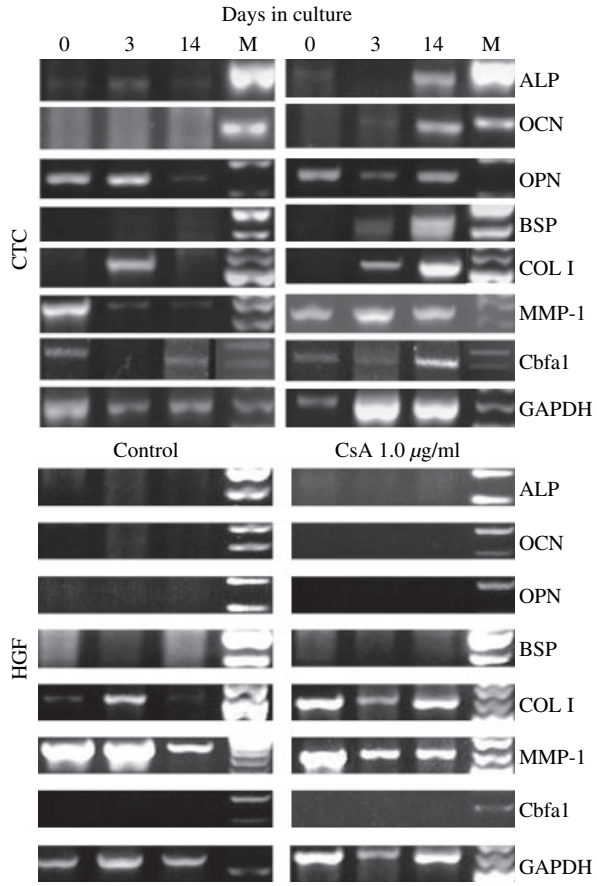

Fig. 3. Representative agarose gels containing reverse transcription–polymerase chain reaction products from cementoblastoma-derived cells (CTC) and human gingival fibroblasts (HGF) treated without and with cyclosporin A (CsA, 1.0 μg/ml) for 0, 3 and 14 days. ALP, alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; BSP, bone sialoprotein; COLI, type I collagen; MMP-1, matrix metalloproteinase-1; Cbfa1, core binding factor-a1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. M, DNA ladder.

cells incubated with and without cyclosporin A are presented in Fig. 3. The expression of osteopontin, type I collagen and MMP-1 varied in the cementoblastoma cells; however, significantly higher levels of expression was detected for alkaline phosphatase, bone sialoprotein, osteocalcin and Cbfa1 in the presence of cyclosporin A relative to minus cyclosporin A controls (Fig. 3, top panel). The differences were prominent and statistically significant after 14 days of culture (Fig. 4). Interestingly, MMP-1 showed higher levels of mRNA expression at 3 and 14 days relative to the controls (Figs 3 and 4). In contrast to the cementoblastoma cells, human gingival fibroblasts expressed only type I collagen and MMP-1 (Fig. 3, bottom panel); in the presence of cyclosporin A MMP-1 expression was less after 7 days and similar to controls after 14 days in these cells, and type I collagen was higher after 14 days.

Discussion

To our knowledge this is the first report documenting the effect of cyclosporin A on proliferation, differentiation and mineralization of cementoblasts-like cells *in vitro*. Our findings show that, although the magnitude varied at different concentrations, cyclosporin A had an inhibitory effect on the

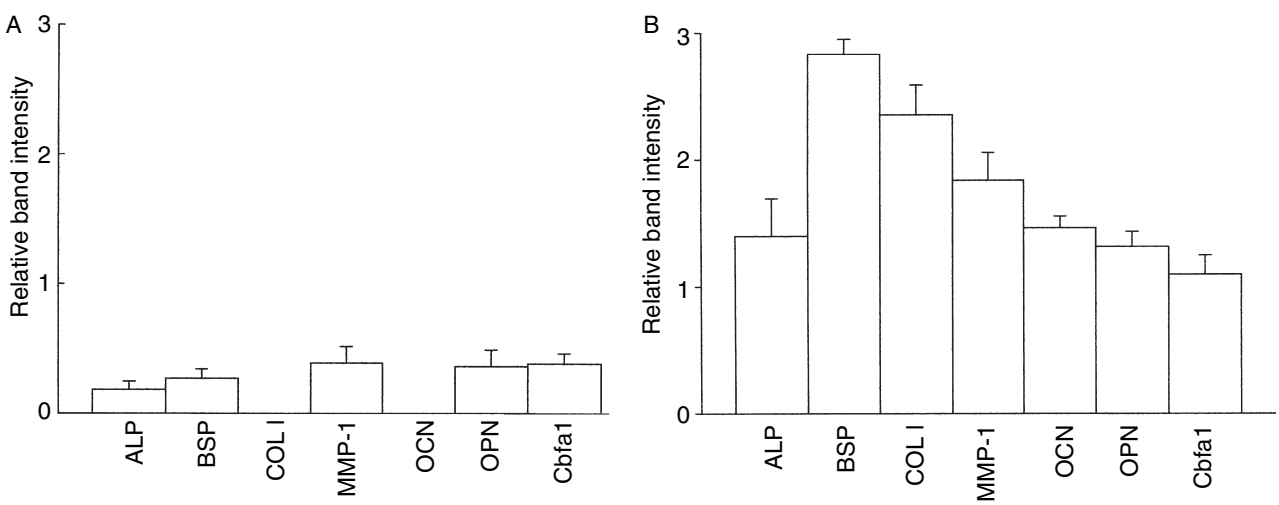

Fig. 4. Graphical analysis of reverse transcription–polymerase chain reaction product band intensities relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), illustrating expression of cementum matrix proteins by cementoblastoma cells after 14 days of culture. Cyclosporin A concentration was 1.0 μg/ml. (A) Without cyclosporin A; (B) with cyclosporin A. ALP, alkaline phosphatase; BSP, bone sialoprotein; COLI, type I collagen; MMP-1, matrix metalloproteinase-1; OCN, osteocalcin; OPN, osteopontin; Cbfa1, core binding factor-α1.

proliferation of cementoblastoma cells, and the effect was prominent at 5.0 μg/ml concentration. However, the proliferation of gingival fibroblasts was not affected; although this is not consistent with increased cell number in patients manifesting gingival overgrowth, others have also reported decrease, as well as increase or no effect, in mitogenic effect (24–28). This could be a result of different cell subpopulations responding differently to the drug and clonal selection promoted by cyclosporin A. This possibility is supported by the observations that cyclosporin A induced phenotypic modifications in gingival fibroblasts that resulted in differentiation of cementoblast-like cells (13), and in the formation of osteodentin spurs deposited on dentin (29). We believe that cyclosporin A induced suppression of proliferation of cementoblastoma cells, but not gingival fibroblasts, due to its promoting differentiation and mineral nodule formation by the cementoblastoma cells. It is also possible that, as the nodules become calcified, cells die and cell number decreases. The findings by McCauley *et al*. (30) also indicate that cyclosporin A inhibits proliferation, mitogenesis, alkaline phosphatase activity and cell attachment of rat osteoblasts.

The cyclosporin A stimulated mineralization by cementoblastoma-derived cell cultures at all concentrations of cyclosporin A used. RT–PCR studies revealed that in the presence of cyclosporin A these cells express higher levels of mRNAs for mineralized tissue-related molecules when compared to untreated controls. The increased expression of bone sialoprotein, alkaline phosphatase, osteocalcin osteopontin and Cbfa1 mRNA expression is significant at 14 days. The strong expression of osteocalcin, bone sialoprotein and alkaline phosphatase clearly indicates that cyclosporin A has the potential to induce differentiation of cementoblastoma cells. We did not examine the production of cementum-attachment protein, a cementum specific protein, by these cells because it is produced in the absence of cyclosporin A (31) and DNA probes are not yet available.

Cyclosporin A has been shown to have a direct effect on bone turnover and to increase osteoblastic activities. In addition, a direct inhibitory effect of cyclosporin A on bone resorption has also been reported both *in vivo* and *in vitro* (32). Increased bone formation has also been observed in experimental animals using low cyclosporin A doses, and a direct inhibitory effect of cyclosporin A on bone resorption has been reported *in vivo* and *in vitro* (33). Together, these observations indicate that cyclosporin A alters extracellular matrix synthesis and degradation and could increase the cementoid mineralized matrix available for mineralization. Our results on cementoblastoma cells are especially noteworthy because cyclosporin A effect occurred in the absence of the synthetic glucocoticoid dexamethasone, which induces osteoblastic phenotypic markers in immature osteoblasts and less-committed cells (34). However, cell culture data analysis should be cautiously extrapolated to the *in vivo* situation due to the effect of cell selection and the *in vivo* complex system would be more representative of the interactions between cyclosporin A and the heterogeneous cell populations in the periodontium. In spite of the limits of this study, our results indicate that cyclosporin A may be useful to promote cementum formation during periodontal regeneration, and that the culture system using putative cementoblastic cells is an excellent *in vitro* model to study the molecular and cellular mechanism involved in cementogenesis.

Acknowledgements

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