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Effect of cyclosporin A on proliferation and differentiation of human periodontal ligament cells

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Abstract

Objective. Cyclosporin A (CsA) is widely used to prevent rejection after organ transplantation. However, it also causes several side-effects, including gingival overgrowth and bone resorption. Cellular mechanisms underlying the effect of CsA on periodontal tissue remain unclear. In this study, we investigated the effect of CsA on the proliferation and expression of characteristic markers in periodontal ligament cells (PDLs). ***Material and methods.*** The proliferation and viability of PDLs were measured by direct cell counting and 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide assay, respectively. mRNA expression levels of the specific proteins alkaline phosphatase (ALP), osteocalcin (OC) and collagen type 1 (Coll-1) were quantified using real-time polymerase chain reaction. Finally, ALP activity of PDLs was investigated using a specific colorimetric assay. ***Results.*** We found that proliferation of PDLs was stimulated by 0.01–0.1 μg/ml CsA and unaffected by 1 μg/ml CsA. The viability of PDLs was increased by 0.1 μg/ml CsA and not affected by 0.01 μg/ml and 1 μg/ml CsA. Furthermore, the mRNA expression levels of ALP, OC and Coll-1 in PDLs were significantly increased upon stimulation with 0.1 μg/ml CsA for 24 h or by stimulation with 0.01 μg/ml CsA for 48 h. In contrast, significantly lower expression levels of all three proteins in PDLs were observed upon stimulation with 1 μg/ml CsA for 48 h. The ALP activity of PDLs exhibited a similar pattern of changes upon CsA stimulation. ***Conclusion.*** Our data demonstrated that CsA may influence both the proliferation and differentiation of human PDLs, which may play an important role in the homeostasis of periodontal tissue.

Key Words: *Cell culture, cyclosporin A, differentiation, periodontal ligament, proliferation*

Introduction

Cyclosporin A (CsA) immunosuppression is widely used to avoid rejection of allograft tissue [1]. However, CsA therapy causes numerous side-effects, including gingival overgrowth [2–4] and bone resorption [5]. In humans, cyclosporin therapy may predispose the individual to destruction of periodontal tissue [6]. One clinical study showed that CsA therapy in combination with Ca^{2+} channel blockers results in significantly increased periodontal pocket depth [7]. Studies using *in-vivo* animal models showed that CsA induces alveolar bone loss in rats [8,9] and ferrets [10].

Several *in-vitro* studies investigated the effect of CsA on the function of cells composing periodontal tissue. Particularly, many studies showed a stimulating effect of CsA on proliferation of human gingival fibroblasts [11–14], but some studies did not observe such an effect [15,16]. Differentiation of mouse osteoblasts was stimulated by CsA at concentrations up to 1 μM, but inhibited by higher drug concentrations [17]. CsA was also shown to inhibit proliferation and induce mineralization of human cementoblastoma cells [15].

Periodontal ligament cells (PDLs) are the main component of periodontal ligament. These cells have unique characteristics since they exhibit properties of both fibroblasts and osteoblasts (for a review, see references [18–22]). Like fibroblasts, PDLs are continuously producing extracellular matrix but, in contrast, PDLs also participate in repair and remodelling of the neighbouring alveolar bone. To date there have been no reports about the effect of CsA on the functional properties of PDLs, although such an effect may substantially change the homeostasis of periodontal tissue. Therefore, in the present study we investigated the effect of CsA on the proliferation and expression of the specific proteins alkaline

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phosphatase (ALP), osteocalcin (OC) and collagen type 1 (Coll-1) in human PDLs.

Material and methods

Cell culture

Cells were isolated from healthy patients undergoing routine extraction of their third molar teeth. Patients were informed in detail before the surgical procedures and gave their written agreement. The study protocol was approved by the Ethics Committee of the Medical University of Vienna (Protocol No. 400/2004). PDLs were isolated by scraping the ligament tissue from the teeth root surface and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum (FBS), streptomycin (50 μg/ml) and penicillin (100 U/ml) under an humidified air atmosphere containing 5% CO_2 at 37°C. The medium was changed every 3 days for 10–15 days until confluent cell monolayers were formed. Cells from passage levels 3–6 were used.

Cell proliferation and viability

In proliferation experiments, PDLs were seeded in 500 μl of DMEM with 10% FBS at an initial density of 2×10^4 cells/well in 24-well plates. After 24 h, the media in test wells were replaced by DMEM supplemented with 1% FBS and containing CsA at concentrations of 0.01, 0.1 and 1.0 μg/ml and control wells by medium without CsA. Each group contained six different wells. After 24 or 48 h of stimulation, cell proliferation was assessed by direct cell counting. In these experiments, the cells were collected by trypsinization after CsA stimulation and counted in blinded fashion using a cell counting chamber under a microscope. Cell viability was measured using 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) tests (Sigma, St Louis, MO) according to the manufacturer's instructions.

Real-time polymerase chain reaction

In the real-time polymerase chain reaction (RT-PCR) experiments, PDLs were seeded in 500 μl of DMEM with 10% FBS at an initial density of 1×10^5 cells/well in 24-well plates. After 72 h, the media in test wells were replaced by DMEM supplemented by 1% FBS and containing CsA at concentrations of 0.01, 0.1 and 1.0 μg/ml and control wells by medium without CsA. Each group contained four different wells. After either 24 or 48 h stimulation, total cellular mRNA was isolated and reverse-transcribed into cDNA using the TaqMan® Gene Expression Cells-to-CT™ kit (Ambion/Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. For each experimental group, 10^5 cells were taken for mRNA isolation and subsequent analysis. RT-PCR was performed in an Applied Biosystems Step One Plus RT-PCR system in paired reactions using Taqman® gene expression assays with the following ID numbers (all from Applied Biosystems): ALP, Hs01029144_m1; OC, Hs00609452_g1; Coll-1, Hs00164004_m1; and β-actin, Hs99999903_m1. Triplicate RT-PCRs were prepared for each sample under the following thermocycling conditions: initiation at 95°C for 10 min, then 40 cycles each consisting of denaturation at 95°C for 15 s and hybridization–elongation at 60°C for 2 min. The point at which the PCR product was first detected above a fixed threshold [termed the cycle threshold (C_t)] was determined for each sample. Changes in the expression of target gene were calculated using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = (C_t^{\text{target}} - C_t^{\beta\text{-actin}})_{\text{sample}} - (C_t^{\text{target}} - C_t^{\beta\text{-actin}})_{\text{control}}$, taking samples without CsA treatment as a control. RT-PCR experiments were repeated three times.

ALP activity

The cell number and stimulation conditions for the ALP activity assay were similar to those for RT-PCR experiments. The activity of ALP was measured using a method similar to that described in our previous studies [23,24]. Briefly, after stimulation, cells were collected by trypsinization, lysed in 200 μl of phosphate-buffered saline containing 0.2% Triton X-100 and homogenized by sonication. The total protein content in cell homogenates was determined using a commercially available kit (Micro/Macro BCA; Pierce Chemical Co., Rockford, IL). The ALP activity was assayed using the conversion of a colourless *p*-nitrophenyl phosphate to a coloured *p*-nitrophenol according to the manufacturer's protocol (Sigma). The colour changes were measured spectrophotometrically at 405 nm. The amounts of resulting products released by the cells were quantified by comparison with a standard curve and normalized to the total protein content in cell homogenates. ALP activity experiments were repeated three times.

Statistical analysis

Data are expressed as mean ± SEM. The normal distribution of all data was tested using the Kolmogorov–Smirnov test. After confirming a normal distribution, the statistical differences between CsA-stimulated groups and controls were analysed using a *t*-test. All statistical analyses were performed using the statistical program SPSS 14.0 (SPSS Inc, Chicago, IL). Differences were considered statistically significant at $P < 0.05$.

Results

Effect of CsA on proliferation and viability of PDLs

Figure 1 shows the effect of CsA on the proliferation rate of human PDLs. Significantly enhanced cell proliferation was observed upon stimulation with 0.1 μg/ml CsA for 24 and 48 h, as well as upon stimulation with 0.01 μg/ml CsA for 48 h. No significant changes in proliferation of PDLs were found upon stimulation with 1 μg/ml CsA.

The effects of various CsA concentrations (0.01–1 μg/ml) on the viability of PDLs measured using the MTT assay are shown in Figure 2. Cell viability was significantly increased upon stimulation with 0.1 μg/ml CsA for both 24 and 48 h. No significant changes in viability of PDLs were found upon stimulation with 0.01 or 1 μg/ml CsA for any period of time.

Gene expression of specific markers of PDLs

Figure 3 shows the changes in the mRNA expression levels of ALP, OC and Coll-1 in PDLs upon stimulation with various CsA concentrations. As can be seen, both CsA concentration and stimulation time determined changes in gene expression. A significant increase in the expression levels of ALP, OC and Coll-1 was observed upon stimulation with 0.01 μg/ml CsA for 48 h and with 0.1 μg/ml CsA for 24 h. The mRNA expression level of all three proteins was significantly decreased after stimulation with 1 μg/ml CsA for 48 h.

ALP activity

Figure 4 shows the effect of various CsA concentrations on the ALP activity of PDLs. Similar to the mRNA expression level, the changes in ALP activity were substantially dependent on both CsA concentration and stimulation time. After 24 h of stimulation, the ALP activity was significantly increased by CsA at concentrations of 0.01–0.1 μg/ml and significantly decreased by CsA at a concentration of 1 μg/ml. After 48 h of stimulation, the ALP activity was significantly increased by 0.01 μg/ml CsA, unaffected by 0.1 μg/ml CsA and significantly decreased by 1 μg/ml CsA.

Discussion

In the present study, we report for the first time the effect of CsA on the proliferation and expression levels of various specific markers in human PDLs. PDLs are a unique cell population exhibiting both fibroblast-like and osteoblast-like properties [25]. In our study, we investigated the effect of CsA on the expression levels of ALP, OC and Coll-1 genes, as well as on the ALP activity of PDLs. ALP is a characteristic marker enzyme of osteoblasts [26] and OC is a marker of late osteoblast differentiation [27]. Owing to expression of these osteoblast-related proteins, PDLs participate in alveolar bone homeostasis. Coll-1 is a component of extracellular matrix and plays an important role in the formation of periodontal soft tissue [28]. Thus, PDLs actively participate in the formation and remodelling of both hard and soft periodontal tissue. Data from our study showed that the effect of CsA on PDLs varies depending on drug concentration and stimulation time.

In the present study we investigated cell proliferation using direct cell counting, the method which has been widely used in many studies [29,30]. The effect of CsA on the proliferation rate of PDLs was concentration-dependent. Particularly, an increased proliferation rate of PDLs was observed upon stimulation with up to 0.1 μg/ml CsA, but not at 1 μg/ml

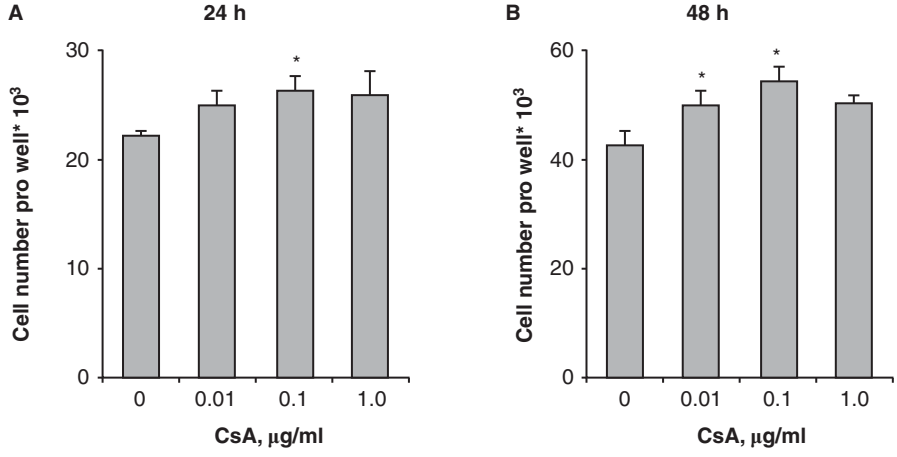

Figure 1. Effect of CsA on proliferation of PDLs after stimulation periods of (A) 24 and (B) 48 h measured by direct cell counting. Cells non-stimulated with CsA were taken as controls. Each value represents the mean ± SEM of five different experiments. *Significantly higher compared to control ($P < 0.01$).

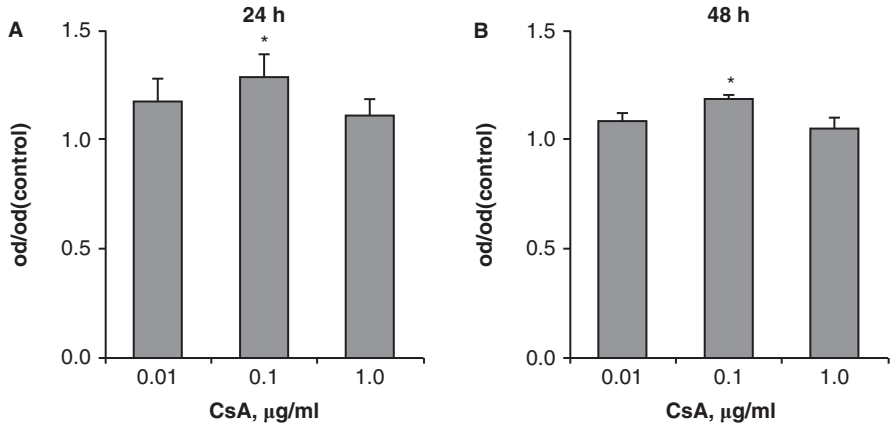

Figure 2. Effect of CsA on viability of PDLs after stimulation for (A) 24 and (B) 48 h measured using an MTT assay. Cells non-stimulated with CsA were taken as controls. Each value represents the mean ± SEM of five different MTT experiments. The optical density (OD) values of the different concentrations were normalized with the average value of the OD of control (=1). *Significantly higher OD values compared to control ($P < 0.01$).

CsA. Qualitatively similar results were obtained in a viability MTT assay, showing that viability of PDLs was significantly increased by 0.1 μg/ml CsA and unaffected by 1 μg/ml CsA. The MTT assay is based on measurements of the mitochondrial function of cells and is often taken as a indication of the proliferation of viable cells [31]. A qualitatively similar effect of CsA on cell proliferation was described previously in a study on human gingival fibroblast [13]. Particularly, a dose-dependent increase in human gingival fibroblast proliferation by CsA at concentrations up to 0.2 μg/ml and a subsequent decrease in the proliferation rate at higher CsA concentrations (0.4–0.8 μg/ml) was observed. The molecular mechanisms underlying the stimulating effects of CsA on the proliferation of human PDLs are unclear. One recent study showed that CsA stimulates production of transforming growth factor-β and interleukin-6 by human gingival fibroblasts and that these cytokines, in turn, stimulate cell proliferation [12]. However, the existence of a similar autocrine mechanism in PDLs needs to be proved in future studies.

Our study demonstrated that CsA affects the expression of characteristic markers of PDLs in both dose- and time-dependent manners. Low CsA concentrations and/or short stimulation times (e.g. 0.01 μg/ml CsA for 24 h or 0.1 μg/ml CsA for 48 h) induced a significant increase in the expression level of specific markers of PDLs. In contrast, stimulation of PDLs with a high concentration of CsA for a prolonged time (1 μg/ml CsA for 48 h) resulted in significantly decreased mRNA expression levels of all three proteins. Changes in the ALP activity of PDLs were similar to those observed for ALP mRNA. Interestingly, a study of mouse osteoblasts revealed a qualitatively similar effect of CsA on ALP activity, which was stimulated by low (<1 μM) and inhibited by high (>1 μM) CsA concentrations [17]. The

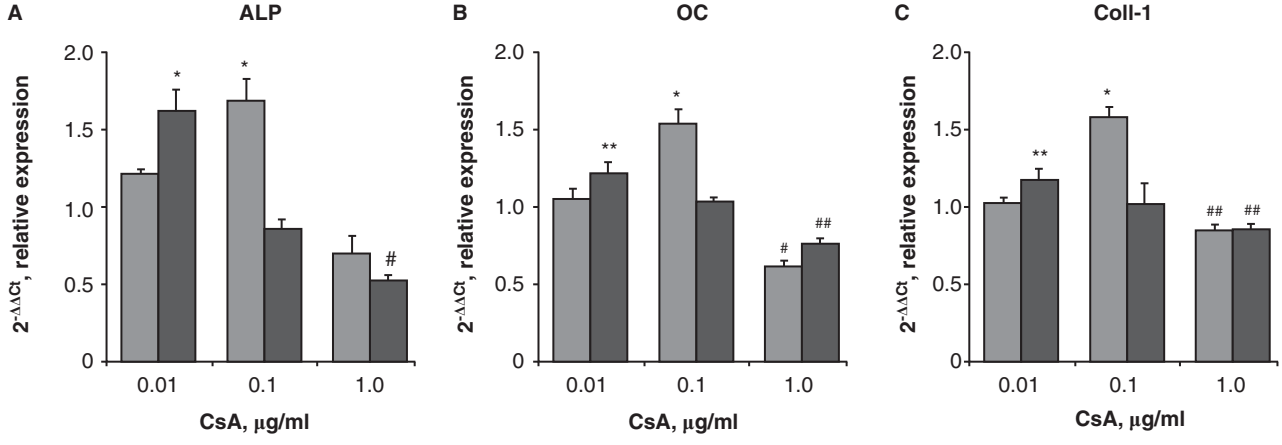

Figure 3. Relative expression levels of (A) ALP, (B) OC and (C) Coll-1 gene upon incubation with different CsA concentrations for 24 h (light grey columns) or 48 h (dark grey columns). β-actin was used as an endogenous control gene. Each value represents the mean ± SEM of three different RT-PCR experiments. *,**Significantly higher gene expression compared to control ($P < 0.01$ and $P < 0.05$, respectively). #,##Significantly lower gene expression compared to control ($P < 0.01$ and $P < 0.05$, respectively).

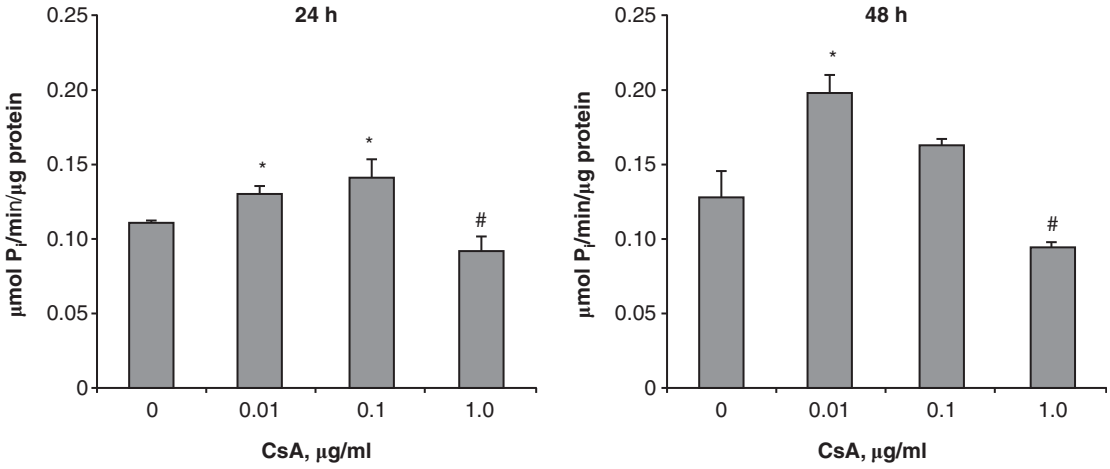

Figure 4. ALP activity of PDLs after (A) 24 or (B) 48 h of CsA stimulation. Each value represents the mean±SEM of three different experiments. *Significantly higher phosphatase activity compared to control ($P < 0.05$). #Significantly lower phosphatase activity compared to control ($P < 0.05$).

mechanisms underlying the biphasic dose-dependent nature of the effect of CsA on differentiation of PDLs are not known.

In the present study we used CsA at concentrations which are usually found in the blood of patients undergoing CsA therapy [3], and therefore our data may be physiologically relevant. However, the data of our *in-vitro* study should be cautiously extrapolated to the *in-vivo* situation because of at least two factors. First, periodontium contains heterogeneous cell populations, and therefore an integrated response to CsA needs to be considered. Second, some changes in the characteristics of PDLs may occur during *in vitro* culturing [32]. Keeping in mind these limitations of our *in-vitro* study, we can suppose that the increases in the expression levels of ALP, OC and Coll-1 in PDLs by CsA suggest that this drug at low doses promotes formation of bone and connective tissue. In contrast, according to our data, high CsA doses should induce the destruction of periodontal tissue and bone resorption. Nevertheless, the exact effects of CsA on the homeostasis of periodontal tissue need to be further investigated in clinical studies. To date, some studies reported higher values of probing depth in individuals receiving CsA therapy after organ transplantation, but these differences were not always significant [33–35]. Studies using animal models have also controversially discussed the effect of CsA on periodontium. Particularly, daily administration of 10–30 mg/kg body weight CsA in rats resulted in alveolar bone loss [8,9]. In contrast, rats treated with lower CsA doses (2 mg/kg body weight daily) exhibited an enhanced demineralized freeze-dried bone matrix-induced alveolar bone formation [36]. Thus, there are some hints indicating that the effect of CsA on homeostasis of periodontal tissue *in vivo* could depend on drug concentration, but this assumption requires further confirmation.

Summarizing, our data suggest for the first time that CsA affects functional characteristics of PDLs. In low concentrations, CsA stimulates the expression of specific markers of PDLs and these changes may have a positive effect on the formation of both soft and hard periodontal tissue. However, in high concentrations, CsA has an opposite effect, which may contribute to predisposing individuals to periodontal tissue destruction.

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