Cyclosporin A-induced gingival overgrowth is not associated with myofibroblast transdifferentiation

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Abstract: Cyclosporin A (CyA) induces gingival overgrowth via its stimulatory effects on expression of transforming growth factor-beta1 (TGF-β1) and collagen. It is not known whether CyA has a direct effect on gingival fibroblasts or induces its effect indirectly via stimulation of myofibroblast transdifferentiation. The present study was undertaken to examine the *in vivo* and *in vitro* effect of CyA on myofibroblast transdifferentiation. Rats were treated for 60 days with a daily subcutaneous injection of CyA, and the gingival overgrowth tissue was analyzed by immunohistochemistry. *In vitro*, fibroblasts from normal gingiva (NG) were cultured in the presence of different concentrations of CyA, and subjected to semi-quantitative reverse transcriptase-polymerase chain reaction and western blot. Although CyA treatment stimulated TGF-β1 expression by NG fibroblasts, it lacked to induce expression and production of isoform α of smooth muscle actin (α-SMA), the specific myofibroblast marker. The expression levels of connective tissue growth factor (CTGF), which has been considered a key molecule to promote the transdifferentiation of myofibroblasts via TGF-β1 activation, were unaffected by CyA. Our results demonstrate that CyA-induced gingival overgrowth is not associated with activation of myofibroblast transdifferentiation, since CyA is not capable to increase CTGF expression.

Descriptors: Gingival overgrowth; Cyclosporine; Fibroblasts; Transforming growth factor beta; Connective tissue growth factor.

Introduction

Cyclosporin A (CyA) is a cyclic endecapeptide with potent immunosuppressive properties, which has made it the drug of choice in many transplantation procedures.[1,2] Following the clinical use of CyA, a number of adverse effects were recorded. One of these was gingival overgrowth that was first reported by Calne *et al.*[2] (1979), however the term "CyA-induced gingival enlargement" was mentioned for the first time in dental literature by Rateitschak-Plus *et al.*[3] (1983). In the gingiva as well as in the kidneys, liver and heart, CyA is known to promote collagen deposition.[4] CyA induces transforming growth factor-beta1 (TGF-β1) expression by human gingival fibroblasts,[5,6] which is, in association with an elevated synthesis of collagen, an intrinsic characteristic of myofibroblasts.[7]

Myofibroblasts are characterized by expression of the specific isoform α of smooth muscle actin (α-SMA) and, when activated, synthesize el-

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evated levels of extracellular matrix proteins. Those cells are considered the main cellular type involved in extracellular matrix deposition in fibrotic diseases.[8] Transdifferentiation of myofibroblasts has been traditionally controlled by transforming growth factor-β1/connective tissue growth factor (TGF-β1/CTGF) pathway.[9] We recently demonstrated that the presence of myofibroblasts is associated with the gingival fibrotic overgrowth of patients with hereditary gingival fibromatosis (HGF).[10] Myofibroblasts were identified in HGF tissues whose cells expressed high levels of both TGF-β1 and CTGF but not in tissues characterized for cells expressing elevated levels of TGF-β1 and low levels of CTGF.[10] We also demonstrated that TGF-β1 promotes a dose- and time-dependent increase in the expression of α-SMA, whereas interferon γ (IFNγ) blocks it and prevents the fibroblast-myofibroblast switch induced by TGF-β1 on normal gingiva (NG) cultures via stimulation of Smad 7 and, subsequently, inhibition of CTGF.[11] Since gingival overgrowth induced by CyA provides an excellent model for the study of connective tissue fibrosis, CyA-treated NG fibroblasts produce abundant extracellular matrix and high levels of TGF-β1, and myofibroblasts represent a hallmark of interstitial fibrosis, the purpose of this study was to analyze the expression of α-SMA, TGF-β1 and CTGF in CyA-induced gingival overgrowth.

Material and Methods

All procedures of this study were approved by the Human Ethical Committee in Research and by the Animal Care Committee at the Piracicaba Dental School, University of Campinas.

Animals

Ten male Wistar rats (*Rattus norvegicus albinus*) received daily subcutaneous injections of 10 mg/kg body weight of CyA (Sandimmun, Novartis, São Paulo, SP, Brazil) for 60 days.[12] The control group (10 rats) received 0.9% sodium chloride (NaCl) sterile saline. After 60 days, CyA-treated animals revealed an evident gingival overgrowth involving all teeth. Gingival tissues from molar areas were collected, fixed in 10% formalin, and 3-μm paraffin sections subjected to hematoxylin and eosin (H&E) stain and immunohistochemical analysis using anti-α-SMA antibodies.

Immunohistochemical analysis

Immunohistochemical analysis was performed as previously described using the monoclonal mouse anti-α-SMA (Dako Corp., Carpenteria, CA, USA) and a streptavidin-biotin peroxidase complex method.[13]

Cell cultures and treatments

NG fibroblasts (NG1 to NG5) were derived from healthy individuals (3 males and 2 females) with a mean age of 26.41 ± 4.52 years, who were submitted to procedures of tooth extraction. At the moment of the surgery, a fragment of the normal gingival tissue was removed and used for explant cultures. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil) and antibiotics at 37°C in a 5% CO_2 air atmosphere.[14] To assess the effect of CyA on myofibroblast transdifferentiation, cells were cultured in 0.1% FBS medium containing CyA for 5 days. On the third day, fresh medium containing the same concentrations of CyA was added to the cells. We chose the concentrations of 200 ng/ml (average level of CyA in the serum of patients undergoing CyA-treatment) and 1,000 ng/ml of CyA.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA in each sample were determined by analyzing spectrophotometric absorption at 260/280 nm (Genesys 2, Spectronic Inst., Rochester, NY, USA). Two μg of total RNA per sample were used to generate cDNA. The resulting cDNAs were subsequently amplified, analyzed, and quantified as previously described.[10,11] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Linear amplification range for each gene was determined by preparing 6 similar reactions but amplified by different number of cycles (20, 25, 30, 35, 40, and 45 cycles). For all genes,

the chosen cycle was in the exponential phase of the PCR amplification. Primer sequences, PCR conditions and the amplified lengths have been described elsewhere.[10,11]

Western blot analysis

α-SMA e β-actin detections by western blot were performed after the methods of Bitu *et al*.[10] (2006). Reactions were developed using the Enhanced Chemiluminescent Western blot kit (GE Healthcare, Vienna, VI, Austria).

Statistical analysis

All experiments were performed at least thrice, and the data is presented as mean of % of stimulation taking as reference the untreated cells. Student's *t*-test (two-tailed) was used for statistical analysis, and in our comparisons $p < 0.05$ pointed out to statistical significance.

Results

Sixty days after treatment with CyA, the gingiva of the molars and incisors showed evident enlargement (data not shown). In the CyA-treated samples, the oral epithelium was hyperplasic with hyperkeratosis and deep inter-digitations into the subjacent connective tissue. The connective tissue was dense and formed by thick collagen fibers interspersed

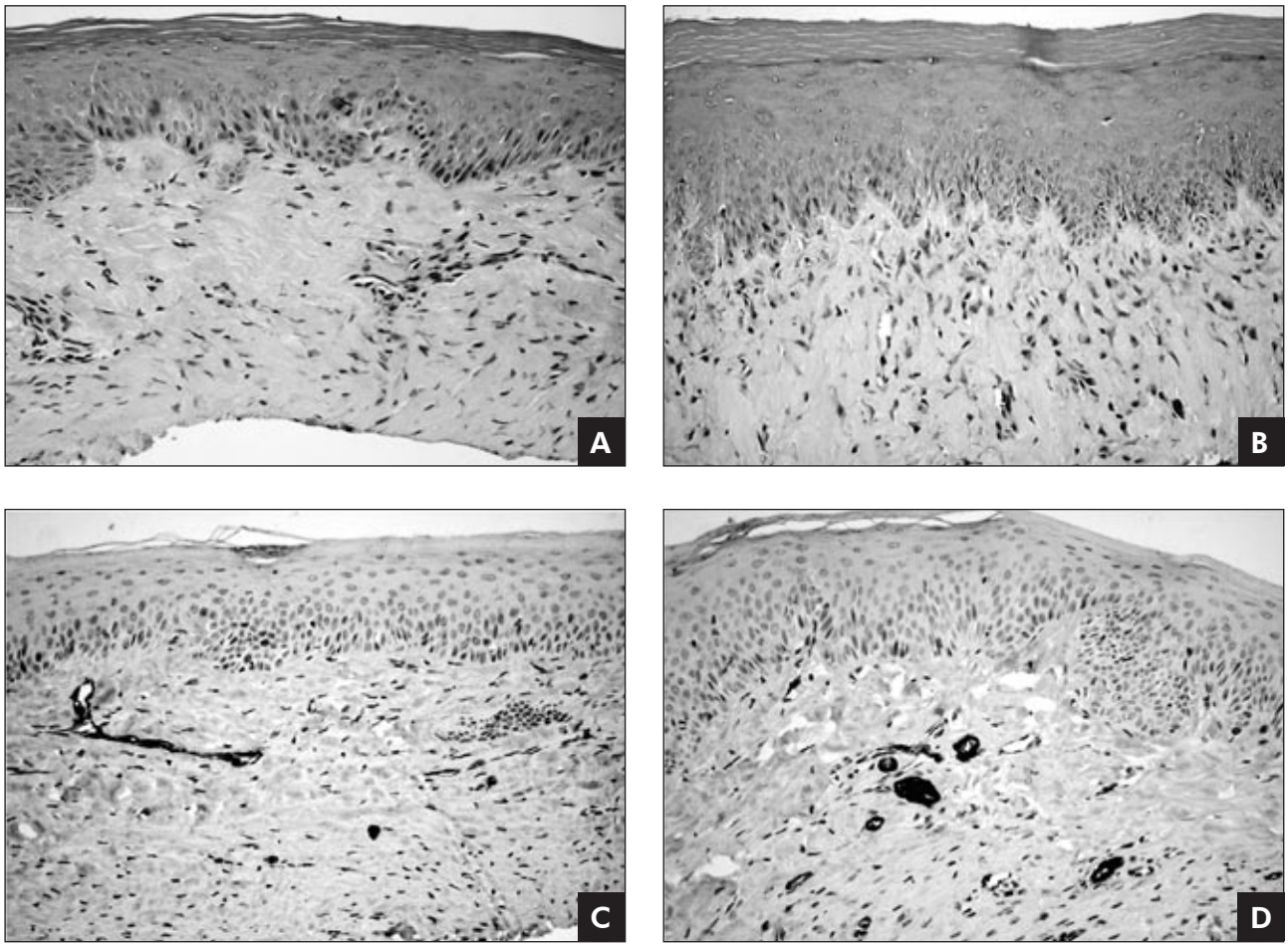
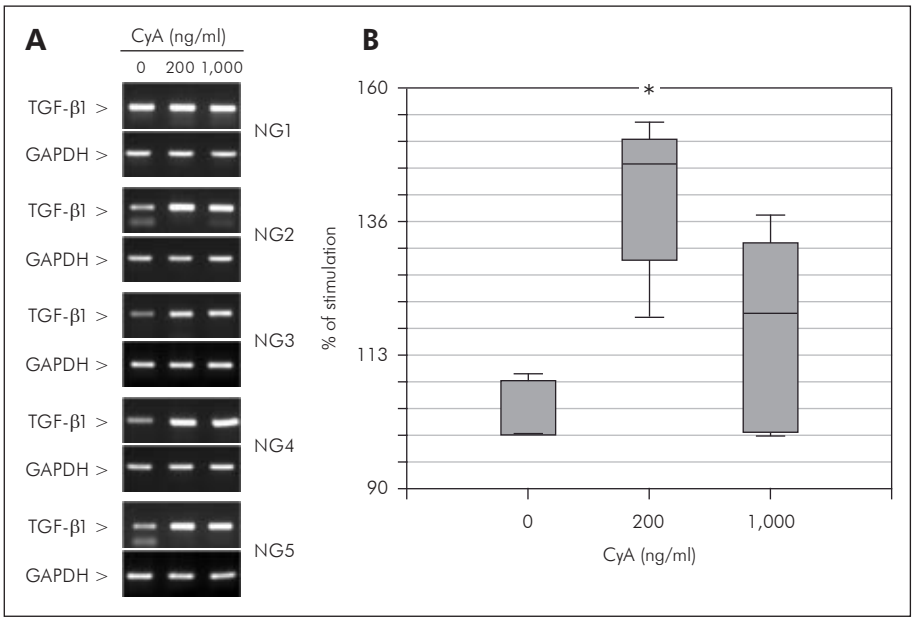


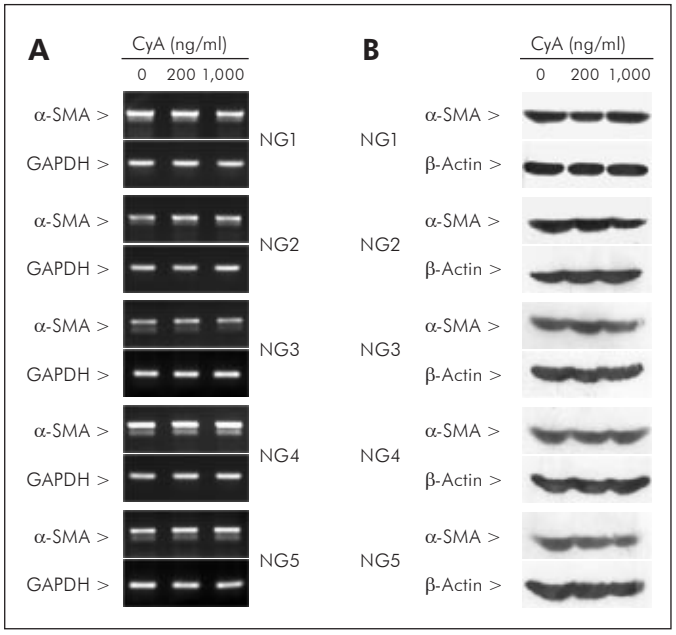
Figure 1 - Immunohistochemical detection of α-SMA in the control and CyA-treated samples. Histological features and α-SMA immunohistochemical expression of a representative sample of the control **(A and C)** and CyA-treated **(B and D)** group of this study. Histological findings of CyA-treated samples demonstrated that the epithelium displays mild hyperplasia, but the increased mass is primarily the result of a marked accumulation of dense fibrous connective tissue. All samples from both groups were negative for α-SMA-positive cells in the connective tissue, except in the smooth muscle of the blood vessel walls (internal positive control). (Original magnification x100)

with delicate vessels and fibroblasts (Figure 1). Immunohistochemical analysis revealed that all gingival tissues from both control and CyA-treated groups were negative for α-SMA-positive cells in the connective tissue, except in the smooth muscle of the blood vessel walls (internal positive control) (Figure 1C and 1D).

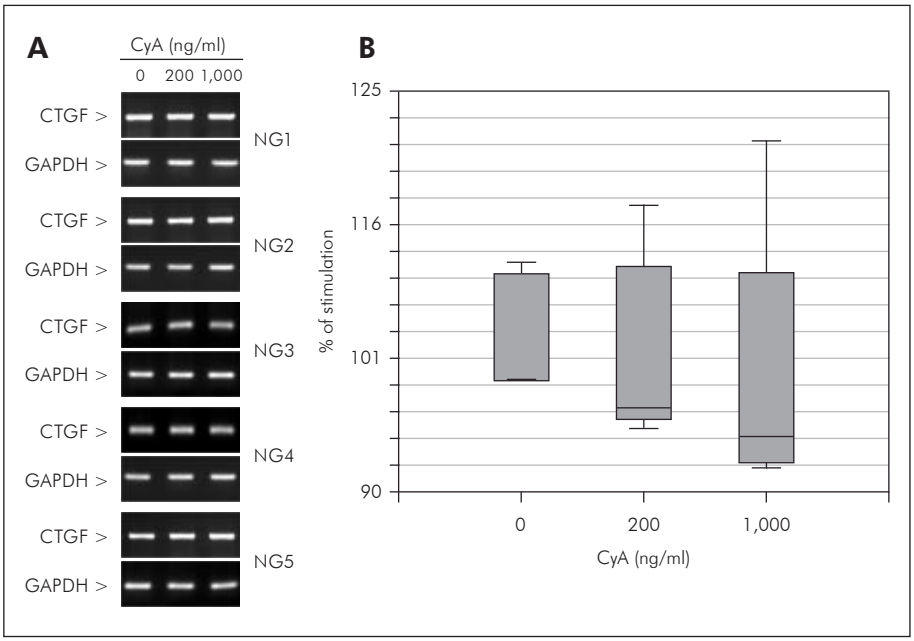
CyA stimulated TGF-β1 expression by human NG fibroblasts (Graph 1). TGF-β1 expression in NG fibroblasts treated with 200 ng/ml of CyA was significantly elevated in approximately 42% of the value observed in corresponding normal control fibroblasts ($p < 0.005$; Fig. 2B). CyA at 1,000 ng/ml induced TGF-β1 expression, but not at a significant level (Graph 1B). Although CyA at 200 ng/ml induced TGF-β1 expression, it did not alter α-SMA mRNA and protein levels (Graph 2A and 2B). Furthermore, CyA was not able to induce transdiffer-



Graph 1 - CyA induces expression of TGF-β1 in human normal gingiva (NG) fibroblasts. **(A)** CyA at 200 ng/ml induced TGF-β1 expression in all NG cell lines, with exception of the NG1 cells. **(B)** Densitometric analysis of the TGF-β1 bands demonstrated a significantly higher expression in NG fibroblasts treated with 200 ng/ml of CyA compared to control cells. *$p < 0.005$



Graph 2 - CyA does not induce transdifferentiation of NG fibroblasts to myofibroblasts. RT-PCR analysis **(A)** or western blot **(B)** to α-SMA.



Graph 3 - CTGF expression levels are unaffected by CyA. RT-PCR analysis of CTGF **(A)**. Densitometric analysis of CTGF bands normalized by GAPDH **(B)**.

entiation of NG fibroblast to myofibroblasts in concentration 5-fold higher than the therapeutically relevant dose of the drug (Graph 2).

Since TGF-β1 induces transdifferentiation of NG fibroblasts to myofibroblasts via stimulation of a CTGF-dependent pathway, we sought to determine whether CTGF expression levels could explain the absence of CyA effect on NG fibroblast transdifferentiation. RT-PCR analysis showed that CTGF expression is not stimulated by CyA treatment (Graph 3).

Discussion

The introduction of CyA has led to an improvement in the prognosis of solid organ transplantation.[1,2] Despite its beneficial effects on long term graft survival, the mortality by cardiovascular diseases and renal failure is greater among CyA-treated graft recipients than the normal population.[15,16] This is probably a consequence of the common and severe adverse effects of CyA, such as hypertension, nephrotoxicity and pathologic connective tissue accumulation.[15-17] Gingival overgrowth is other common side effect of CyA treatment, observed in 13-81% of the patients undertaking CyA.[18,19] The most prominent pathologic manifestation of the gingival overgrowth is an excessive accumulation of extracellular matrix, predominantly type I collagen. Many studies have shown increased transcriptional and translational levels of type I collagen in both tissue and fibroblast cultures derived from CyA-induced gingival overgrowth.[20-22]

A growing body of evidence has connected myofibroblasts with the pathogenesis of several fibrotic processes, since they are the main source of the increased extracellular matrix deposition.[7,8,23,24] The regulatory cytokine TGF-β1 has been traditionally considered an inducer of the myofibroblast phenotypes both *in vivo* and *in vitro*.[25] Since CyA induces gingival overgrowth in the presence of fibroblasts characterized by the synthesis of elevated levels of TGF-β1,[5,6] we evaluated the effects of CyA on transdifferentiation of gingival fibroblasts to myofibroblasts. The rat model associated with primary cultures of human gingival fibroblast treated with CyA was chosen on the basis of our previous experiments to mimic the CyA-induced gingival overgrowth seen in humans.[5,6,12] The main finding of the present study was that myofibroblast transdifferentiation is not induced by CyA, even with CyA stimulating TGF-β1 expression. Furthermore, we were able to demonstrate that CTGF expression levels were unaffected by CyA, suggesting a reason for the lack of myofibroblast transdifferentiation. Previous stud-

ies demonstrated that CTGF expression is elevated in phenytoin-induced gingival overgrowth tissues, but not in CyA or nifedipine-induced gingival overgrowth.[26] In opposite to our findings, Vaquero *et al*.[27] (1999) demonstrated the emergency of myofibroblasts in the pancreas of CyA-treated rats. Those cells were mainly distributed in association with areas of excessive connective tissue accumulation without inflammatory infiltrates. Similarly, elevated α-SMA immunostaining indicating myofibroblast transdifferentiation has been found in the kidneys of rats subjected to CyA treatment.[28] More recently, it was demonstrated that CyA promotes myofibroblast emergency via transformation of epithelial cells.[29] It was also demonstrated that this event is associated with the induction of CTGF expression by CyA, suggesting a dependency of TGF-β1/CTGF pathway. Increasing evidence supports the hypothesis that CTGF is an important downstream mediator of the profibrotic effects of TGF-β1, although both overlapping and distinct fibrogenic effects in human cells have been observed. The absence of myofibroblast transdifferentiation after CyA treatment of NG fibroblasts may be tissue and cell type-dependent, since CyA induction of myofibroblast transdifferentiation seems to be originated from epithelial-mesenchymal transition and not from transformation of fibroblast into myofibroblasts.[30] Additionally, the elevation in TGF-β1 levels alone seems to be insufficient to induce myofibroblast transdifferentiation, and it seems that elevated levels of CTGF are needed for this phenomenon. Future studies will be necessary to determine the exact role of TGF-β1/CTGF pathway in the pathogenesis of the gingival overgrowth induced by CyA.

Conclusion

We demonstrated that the extracellular matrix accumulation observed in CyA-induced gingival overgrowth is not associated with activation of myofibroblast transdifferentiation.

Acknowledgments

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