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Molecular events associated with ciclosporin A-induced gingival overgrowth are attenuated by Smad7 overexpression in fibroblasts

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Background and Objective: Ciclosporin A (CsA)-induced gingival overgrowth is attributed to an exaggerated accumulation of extracellular matrix, which is mainly due to an increased expression of transforming growth factor-β1 (TGF-β1). Herein, the *in vitro* investigation of effects of overexpression of Smad7, a TGF-β1 signaling inhibitor, in the events associated with CsA-induced extracellular matrix accumulation was performed.

Material and Methods: The effects of Smad7 were assessed by stable overexpression of Smad7 in fibroblasts from normal gingiva. Smad7-overexpressing cells and control cells were incubated with CsA, and synthesis of type I collagen, production and activity of MMP-2 and cellular proliferation were evaluated by ELISA, zymography, growth curve, bromodeoxyuridine incorporation assay and cell cycle analysis. The effects of CsA on cell viability and apoptosis of fibroblasts from normal gingiva were also evaluated. Western blot and immunofluorescence for phospho-Smad2 were performed to measure the activation of TGF-β1 signaling.

Results: Although the treatment with CsA stimulated TGF-β1 production in both control and Smad7-overexpressing fibroblasts, its signaling was markedly inhibited in Smad7-overexpressing cells, as revealed by low levels of phospho-Smad2. In Smad7-overexpressing cells, the effects of CsA on proliferation, synthesis of type I collagen and the production and activity of MMP-2 were significantly blocked. Smad7 overexpression blocked CsA-induced fibroblast proliferation via p27 regulation. Neither CsA nor Smad7 overexpression induced cell death.

Conclusion: The data presented here confirm that TGF-β1 expression is related to the molecular events associated with CsA-induced gingival overgrowth and suggest that Smad7 overexpression is effective in blocking these events, including proliferation, type I collagen synthesis and MMP-2 activity.

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Ciclosporin A (CsA) is a potent immunosuppressive drug with beneficial effects in prevention of transplant rejection and in the treatment of several immune-related conditions (1). Its immunosuppressive effect is due to the inhibition of interleukin 2 production by activated T lymphocytes through a calcineurin-dependent pathway (2). In contrast, CsA induces the synthesis of transforming growth factor-β1 (TGF-β1) in various cell types with autocrine functions (3,4). Unfortunately, the use of CsA is associated with significant adverse effects, including nephropathy, hypertension, hepatotoxicity and gingival overgrowth (5). Although the clinical effects of CsA-induced gingival enlargement are well documented, the molecular mechanisms behind this unlikely effect are not fully understood. Experimental investigations have demonstrated that CsA alters the homeostatic equilibrium between synthesis and degradation of extracellular matrix molecules and, in particular, induces type I collagen production and reduces MMP levels (6). In addition, CsA has been reported to modify proliferation and to decrease apoptosis of gingival fibroblasts (7–9).

Our previous studies revealed that CsA downregulates the production and activity of extracellular matrix degradation enzymes, such as MMP-1, MMP-2 and MMP-3, by gingival fibroblasts (10). Furthermore, we observed that CsA treatment results in induction of TGF-β1 production and secretion, which inhibits MMP-2 release by gingival fibroblasts in an autocrine fashion (11). Further study has demonstrated that CsA significantly stimulates fibroblast proliferation in a dose-dependent manner, and the neutralization of TGF-β1 production inhibits the effects of CsA on proliferation, demonstrating an autocrine stimulatory effect of TGF-β1 in CsA-treated normal gingival (NG) fibroblast proliferation (12).

Transforming growth factor-β1 is a growth factor involved in many cellular processes, including proliferation, differentiation, migration and cell death (13). Signaling by TGF-β1 involves the activation of a cytoplasmic downstream pathway composed mainly of Smad proteins. The binding of TGF-β1 to its transmembrane receptors results in phosphorylation of Smad2, which associates with Smad4 for translocation to the nucleus, where they act as transcriptional regulators of target genes (14). In addition, activation of TGF-β1 signaling also results in the expression of inhibitory Smads, which include Smad7. Smad7 is not constitutively expressed but is rapidly induced by TGF-β1 in several cell types, including fibroblasts (15). The inhibitory Smad7 decreases Smad2 phosphorylation by blocking its access to TGF-β receptors or causing degradation of TGF-β receptors via a negative feedback mechanism (16). The *in vitro* proliferative effects of TGF-β1 are accompanied by reducing expression of the cyclin-dependent kinase (CDK) inhibitor, p27 (17). In quiescent cells, p27 binds and inactivates cyclin E-CDK2, but in early G1, p27 promotes assembly and nuclear import of D-type cyclin-CDKs and with the progression throughout G1, proteosomal degradation of p27 permits cyclin E-CDK2 and cyclin A-CDK2 to stimulate the G1–S transition, which involves E2F activation (18,19). Our previous study demonstrated that Smad7 overexpression in hereditary gingival fibromatosis cells was effective in prevention of TGF-β1 effects by inhibiting type I collagen production (20). In the present study, we have explored whether Smad7 overexpression could block CsA effects on molecular events associated with CsA-induced gingival overgrowth.

Material and methods

Cell culture, plasmid and treatment

Fibroblasts from normal gingiva (NG1 cell line) were previously established (21), and were derived from noninflamed and hyperplastic gingiva of one man aged 24 years. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 100 μg/mL penicillin and 100 μg/mL streptomycin at 37°C in air containing 5% CO_2. Stable NG-Smad7 cells were generated as previously described using the pcDNA3-Smad7 plasmid (22). Control cells (NG-Control) were transfected with the vector alone. To assess the effect of CsA, in all experiments, cells were cultured in DMEM containing 0.1% FBS and 200 ng/mL of CsA (Sandimmun-Sandoz, São Paulo, Brazil).

Western blot analysis

Cells were washed with cold phosphate-buffered saline (PBS) and lysed in a detergent-free buffer (10 mM Tris–HCl pH 7.4, 5 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonylfluoride, 10 μg/mL soybean trypsin inhibitor, 1 μg/mL leupeptin and 1 μg/mL aprotinin) associated with mechanical disruption for detection of Smad7 and phospho-Smad2 (pSmad2) proteins or with a buffer containing 20 mM Tris–HCl pH 8, 137 mM NaCl, 10% sucrose, 1% Triton X-100, 10% glycerol, 2 mM EDTA and protein inhibitors for p27. After centrifugation, protein concentrations were measured using a protein assay according to the manufacturer's instructions (Bradford protein assay; Bio-Rad Laboratories, Hercules, CA, USA). Fifty micrograms of total protein per sample was resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis in reducing conditions, and transferred onto nitrocellulose membranes. The membranes were blocked overnight with 10% nonfat dry milk in PBS containing 0.1% Tween 20, rinsed in the same buffer, and incubated for 2 h with the following antibodies: anti-Smad7 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-pSmad2 (Santa Cruz), anti-p27 (BD Biosciences, Pharmigen, San Diego, CA, USA) and anti-β-actin (Sigma-Aldrich, St Louis, MO, USA). Reactions were developed using a chemiluminescent western blot system (enhanced chemiluminescent western blot kit; GE Healthcare, Vienna, Austria).

Immunofluorescence

Ten thousand cells were plated in each well of an eight-well culture chamber slide and incubated at 37°C in humidified air containing 5% CO_2 for

24 h. Following incubation, cells were washed in PBS and fixed in 70% ethanol for 15 min. To prevent nonspecific binding, the cells were blocked with 3% bovine serum albumin in PBS for 1 h. Cells were then incubated for 1 h with anti-pSmad2 antibodies diluted 1:100, followed by incubation with secondary anti-IgG conjugated with fluorescein (Vector Laboratories, Burlingame, CA, USA) at 1:250 dilution. Cells were mounted with a fluorescent mounting medium containing 4′, 6 diamidino-2-phenylindole (Vectashield; Vector Laboratories) and examined under a photomicroscope equipped with epifluorescence (Leica Microsystems, Wetzlar, Germany). To generate fluorescence-labeled images, cells were excited at 480 nm/40 nm with a 527 nm/30 nm bandpass filter. Cells untreated with primary antibodies were used as negative controls.

ELISA

Production of TGF-β1, type I collagen and MMP-2 was determined by ELISA after the methods of Sobral *et al.* (20,23). The values are expressed as production per cell.

Zymography, densitometric analysis and characterization of MMP-2

Zymographic analysis was performed as described elsewhere (24). Gelatinolytic activity was visualized after staining with Coomassie blue R-250 (Bio-Rad Laboratories). The intensities of the negative bands were determined using a GS-700 imaging densitometer (Bio-Rad Laboratories). To confirm the identity of the enzyme, immunoprecipitation assay and incubation with 1 mM of 1,10-phenanthroline (Sigma-Aldrich), a specific inhibitor of MMP activity that acts by chelating Zn^{2+} ions of their catalytic domain, was utilized. Following immunoprecipitation, conditioned medium was incubated with 2 μg of purified antibody against MMP-2 (The Binding Site, Birmingham, UK), and immunocomplexes were pulled down with protein A–Sepharose (Sigma-Aldrich) for 1 h at 4°C. After washing the Sepharose beads three times with 1 mL of cold PBS containing 0.5% Tween 20 (Sigma-Aldrich), the immunoprepicitated proteins were eluted in 50 μL of nonreducing sample buffer and analyzed by gelatin zymography.

Bromodeoxyuridine (BrdU) incorporation assay

Cells were plated in eight-well chamber slides at a density of 20,000 cells per well in 500 μL of DMEM containing 10% FBS. After 24 h, the cells were washed with PBS and cultured in serum-free medium for an additional 24 h. Following serum starvation for synchrony, the medium was replaced by medium with or without CsA. Proliferation rates were determined 24 h after treatment by measuring incorporation of BrdU into DNA with an immunohistochemical analysis kit (GE Healthcare, Vienna, Austria). The BrdU-labeling index, expressed as the percentage of cells labeled with BrdU, was determined by counting 500 cells in two independent reactions for each concentration using the Kontron 400 image analysis system (Zeiss, Echingbei Munich, Germany).

Growth curve

Cells were plated in 24-well culture plates at a density of 30,000 cells per well in DMEM containing 10% FBS. After 24 h, the cells were washed with PBS and cultured in serum-free medium for an additional 24 h. Following serum starvation, cells were cultured with medium with or without CsA and counted on days 3, 5, 7, 9 and 11. Fresh culture medium was added every other day.

Cell cycle analysis

Propidium iodide was used to assess the cell cycle. For these studies, a hypotonic citrate solution containing propidium iodide (Sigma-Aldrich) was added to approximately 1×10^6 washed cells to a concentration of 50 μg/mL. Cells were labeled for 2 h then analyzed on the FACScalibur flow cytometer equipped with an argon laser (Becton-Dickinson, San Jose, CA, USA). Fluorescence data were displayed on a four-decade-long scale, and a minimum of 10,000 events was collected on each sample. The distribution of cells in the cell cycle was analyzed using the software ModFit (Verity Software House, Topsham, ME, USA).

Apoptosis analysis

Apoptosis was determined by annexin V–fluorescein isothiocyanate labeling. Briefly, cells were harvested, washed with PBS and resuspended in the binding buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing annexin V–fluorescein isothiocyanate at 1:500 and propidium iodide. After 20 min of incubation in the dark at room temperature, cells were washed with PBS and analyzed with the FACScalibur flow cytometer. Apoptotic cells were quantified as the number of annexin V–fluorescein isothiocyanate-positive and propidium iodide-negative cells divided by the total number of cells. A minimum of 10,000 events was analyzed in each sample.

Cell viability

Cells (30,000 cells per well) were added in triplicate to wells of a 24-well culture plate and cultured at 37°C for 24 h. Before adding the CsA, cells were cultured for 24 h in serum-free medium. After treatment for 24 h, cell viability was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) assay (25).

Statistical analysis

The data represent the means ± SD, and all assays were performed at least three times. One-way analysis of variance (ANOVA) and multiple comparisons were used based on Newman–Keuls test at 5% significance ($p \leq 0.05$).

Results

Smad7 overexpression blocks CsA-induced TGF-β1 signaling pathway activation via inhibition of Smad2 phosphorylation

The NG fibroblasts were transfected with either empty pcDNA or pcDNA-Smad7 encoding Smad7, both carrying

a neomycin-resistance gene. Following selection with G418, Smad7 production, in comparison to parental and control-transfected NG cells (NG-Control), was verified by western blot (Fig. 1). Endogenous Smad7 was detectable at very low levels in both parental and NG-Control fibroblasts, whereas NG-Smad7 cells produced high levels of the protein.

To understand the role of Smad7 overexpression in TGF-β1 signaling induced by CsA, we first examined TGF-β1 production in NG fibroblasts treated with 200 ng/mL of CsA, the average level of CsA in the serum of patients undergoing treatment. Ciclosporin A significantly induced the production of TGF-β1 by NG cells, and its effects were not modified by Smad7 overexpression (Fig. 2). The TGF-β1 levels were approximately five to 10 times greater in supernatants of CsA-treated NG cells compared with untreated cell supernatants (Fig. 2). No diffences on TGF-β1 production were found among the cell lines treated with CsA ($p = 0.15$). As TGF-β1 signaling starts with activation of Smad2 in many cells lines, we examined the activation pattern of pSmad2 in the control and Smad7-overexpressing cells. Treatment with CsA induced phosphorylation of Smad2 in both NG and NG-Control cells, whereas pSmad2 was fairly low in NG-Smad7 cells in the absence or presence of CsA (Fig. 3A). To examine the localization of pSmad2 after CsA treatment, we performed immunofluorescence analysis. Ciclosporin A treatment of NG and NG-Control fibroblasts caused a marked transnuclear location of pSmad2, which was not observed in NG-Smad7 cells (Fig. 3B). Few nuclear-positive cells were found among the NG-Smad7 cells after treatment with 200 ng/mL of CsA.

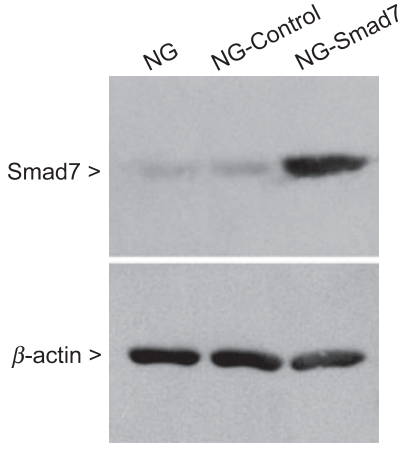

Fig. 1. Characterization of NG fibroblasts overexpressing Smad7. Representative western blot analysis of Smad7 in NG, NG-Control and NG-Smad7 transfectants, revealing very low levels of Smad7 in control cells, in contrast to the increased levels in NG-Smad7 cells.

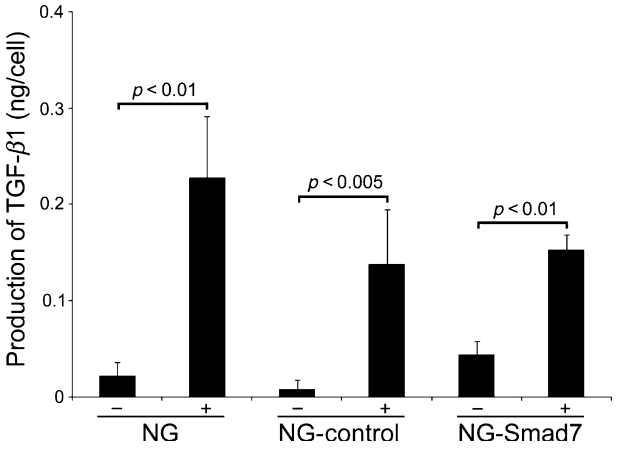

Fig. 2. Effect of ciclosporin A (CsA) on production of transforming growth factor-β1 (TGF-β1) by NG fibroblasts. Transforming growth factor-β1 in culture supernatants was quantified by ELISA. Data are the means ± SD and represent three experiments in triplicate for each cell line. The amount of TGF-β1 produced by NG, NG-Control and NG-Smad7 fibroblasts was significantly stimulated by CsA.

Smad7 overexpression inhibits effects of CsA on the synthesis of type I collagen and on MMP-2 production and activity

Previous investigations of the molecular events that lead to CsA-induced gingival overgrowth pointed to an important role of TGF-β1. Thus, the influence of Smad7 overexpression on cellular events related to gingival overgrowth in NG cells under CsA treatment was examined *in vitro*. As shown in Fig. 4, Smad7-overexpressing cells exhibited a strongly reduced capacity to produce type I collagen after CsA treatment, in comparison to both untransfected and NG-Control cells. Ciclosporin A-treated NG and NG-Control cells demonstrated an approximately fivefold greater type I collagen production than untreated cells ($p < 0.001$ for NG cells and $p < 0.005$ for NG-Control cells). Likewise, modulation of MMP-2 production and activity by CsA, as estimated by ELISA and zymography, respectively, were significantly altered by Smad7 overexpression. Supernatants collected from NG, NG-Control and NG-Smad7 fibroblasts were used to perform MMP-2 immunoassay (Fig. 5). The production of MMP-2 by NG and NG-Control cells was significantly inhibited by CsA treatment ($p < 0.001$), but CsA did not alter MMP-2 secretion by NG-Smad7 cells. Gelatin zymography performed with culture supernatants confirmed that the effects of CsA on MMP-2 gelatinolytic activities were blocked by Smad7 overexpression (Fig. 6A). Densitometric analysis demonstrated that MMP-2 activity of NG and NG-Control cells was dramatically reduced to 20–40% of that observed in the corresponding untreated cells ($p < 0.01$ for NG cells and $p < 0.005$ for NG-Control cells), whereas MMP-2 activity was quite similar in CsA-treated and untreated NG-Smad7 cells (Fig. 6B). The 70 kDa gelatinolytic band was confirmed as MMP-2 by immunoprecipitation assay and by inhibition with 1,10-phenanthroline (Fig. 6C).

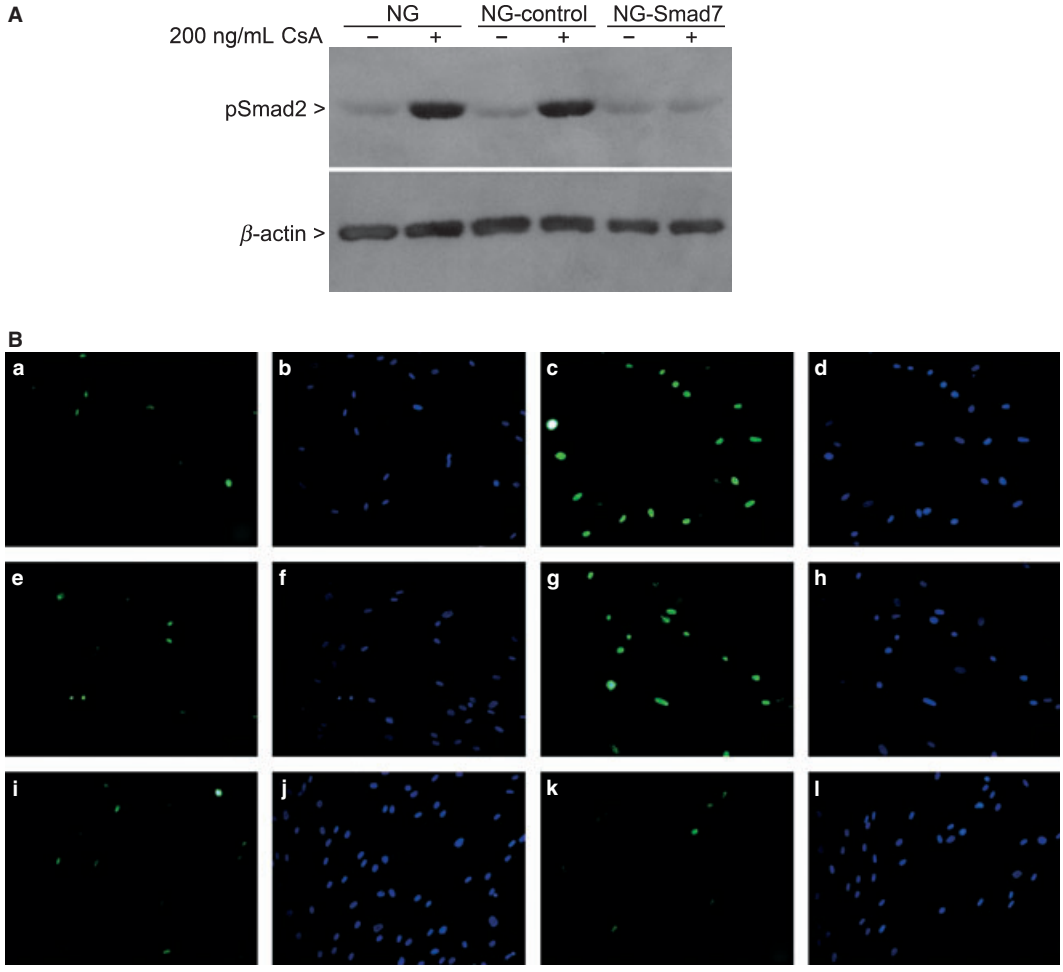

Fig. 3. Phosphorylation and nuclear localization of Smad2 is attenuated in NG-Smad7 cells after treatment with CsA. (A) Representative western blot analysis of phosphorylated Smad2 (pSmad2) in NG, NG-Control and NG-Smad7 cells treated with 200 ng/mL of CsA. (B) Immunofluorescence analysis demonstrated that CsA induced pSmad2 nuclear localization in both NG and NG-Control cells, but overexpression of Smad7 markedly blocked it. The panels show NG cells (Ba–d), NG-Control cells (Be–h) and NG-Smad7 cells (Bi–l). Unstimulated cells are shown in Ba, e and i, and CsA-treated cells are in Bc, g and k. (original magnification ×100).

Smad7 overexpression inhibits CsA-induced proliferation of gingival fibroblasts

To determine whether Smad7 overexpression blocks CsA-induced fibroblast proliferation, we have employed growth curve, BrdU incorporation assay and cell cycle analysis. The effects of CsA on the growth of NG, NG-Control and NG-Smad7 cells are shown in Table 1. Ciclosporin A demonstrated a direct effect on the proliferation of NG and NG-Control fibroblasts, but not on NG-Smad7 cells. From day 5 for NG-Control cells and day 7 for NG cells, the differences in the number of cells were significantly greater with CsA treatment. To confirm these results, proliferation of fibroblasts cultured for 24 h with 200 ng/mL of CsA was assessed by measuring BrdU incorporation into DNA. Figure 7 shows that CsA significantly increased BrdU-labeling indexes of NG and NG-Control fibroblasts in comparison with the control cultures without CsA ($p < 0.01$ for NG cells and $p < 0.001$ for NG-Control cells). Similar effects of CsA on BrdU-labeling index were not observed in Smad7-overexpressing cells. Flow cytometric DNA content analysis 24 h after release from synchronism showed a higher S and G_2/M fraction in NG and NG-Control cells under CsA treatment and a relatively low G_1 phase fraction. In contrast, NG-Smad7 cells revealed a lower S and G_2/M fraction after CsA incubation (Fig. 8). In addition, no cytotoxic effect or increased apoptosis was observed after incubation with 200 ng/mL of CsA (Figs S1 and S2).

As our results provided evidence that CsA induces an increase in the percentage of cells in the S phase of the cell cycle, we performed western blot analysis for p27, an important regulator of the G_1–S transition. As depicted in Fig. 9, CsA treatment severely reduced p27 levels of NG and NG-Control cells, whereas overexpression of Smad7 prevented p27 degradation.

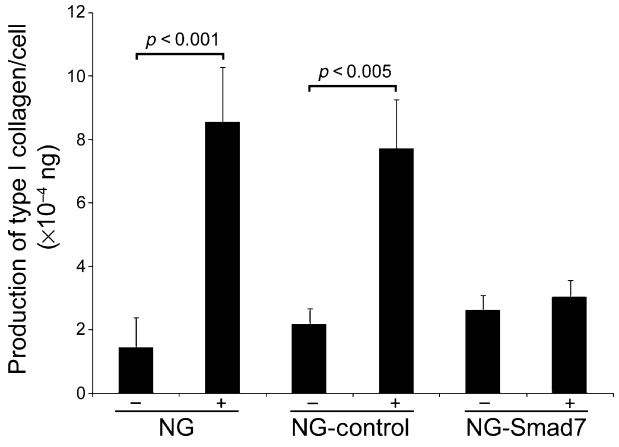

Fig. 4. Overexpression of Smad7 blocks the effects of CsA on production of type I collagen by NG fibroblasts. ELISA analysis revealed that overexpression of Smad7 significantly blocked CsA stimulatory effects on type I collagen production.

Discussion

Gingival overgrowth is the enlargement of the gingiva, characterized by an expansion and accumulation of the connective tissue with the occasional presence of an increased number of cells. The most common forms of gingival overgrowth are induced by systemic drugs, including phenytoin, an antiseizure drug, nifedipine, a calcium-channel-blocker with antihypertensive activity, and CsA. Gingival overgrowth induced by long-term administration of CsA presents in up to 80% of treated patients and is an important adverse effect with difficult treatment and management (26). Cessation or substitution of the drug is the only effective form of treatment. Surgical removal of the enlarged tissue provides functional and psychological benefits, but recurrence is to be expected. Thus, knowledge of the biochemical mechanisms involved in CsA-induced gingival overgrowth is fundamental for the development of therapeutic and prophylactic approaches.

Ciclosporin A-induced gingival overgrowth is characterized by deposition of dense collagen bundles, although some reports have documented increased synthesis of extracellular matrix, including fibronectin and glycosaminoglycans (27–29). There are several studies examining the *in vitro* events related to CsA-induced gingival enlargement, but their results are inconsistent, demonstrating, for example, both increases and decreases in collagen synthesis (30–32). Our previous investigations showed that, *in vitro*, NG fibroblasts treated with CsA are more metabolically active than untreated cells and secrete large amounts of TGF-β1, whose autocrine activity correlates with phenotypes related to collagen accumulation (10–12). In the present study, we extended these findings and further demonstrated that Smad7 overexpression blocks the activation of TGF-β1 signaling via a decrease in Smad2 phosphorylation, reducing the effects of CsA on gingival fibroblast proliferation, type I collagen synthesis and MMP-2 production and activity. Fibroblast proliferation and production of type I collagen induced by CsA were significantly reduced by overexpression of Smad7, and the inhibitory effects of CsA on MMP-2 production and activity were blocked in Smad7-overexpressing cells. Moreover, CsA treatment and overexpression of Smad7 did not affect viability and apoptosis of NG fibroblasts.

Since the first evidence reported by Shehata *et al.* (33) that CsA induces the expression of TGF-β, a large number of studies have demonstrated that most of the adverse effects related to CsA treatment are associated with this growth factor (3,34–36). In particular, Hojo *et al.* (34) showed that CsA induces cancer progression in different cell lines, including invasiveness of nontransformed cells, via secretion of TGF-β1. Transforming growth factor-β1 is considered to be a multifunctional cytokine implicated in many important biological events, including cell growth and differentiation, apoptosis, motility, adhesion, immune cell function and extracellular matrix production (13,37,38). Although these responses play a central role in physiological circumstances of tissue repair, such as embryonic development and wound healing, often this process does not properly resolve when chronic

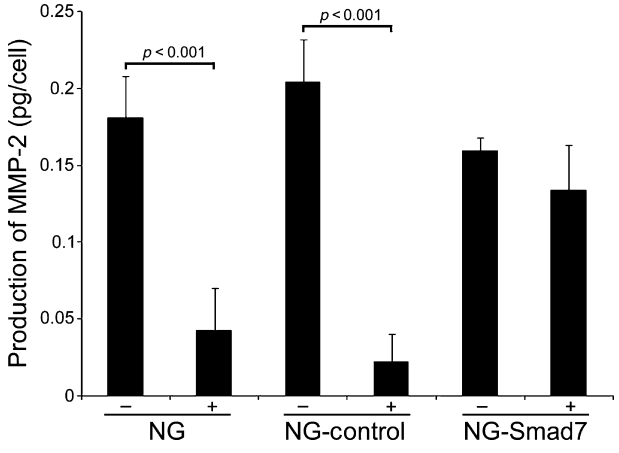

Fig. 5. Smad7 overexpression attenuates CsA-induced downregulation of MMP-2 production. Data are expressed as production of protein per cell. The amount of MMP-2 synthesized by NG and NG-Control cells was significantly reduced by CsA treatment. A similar effect was not observed in NG-Smad7 fibroblasts.

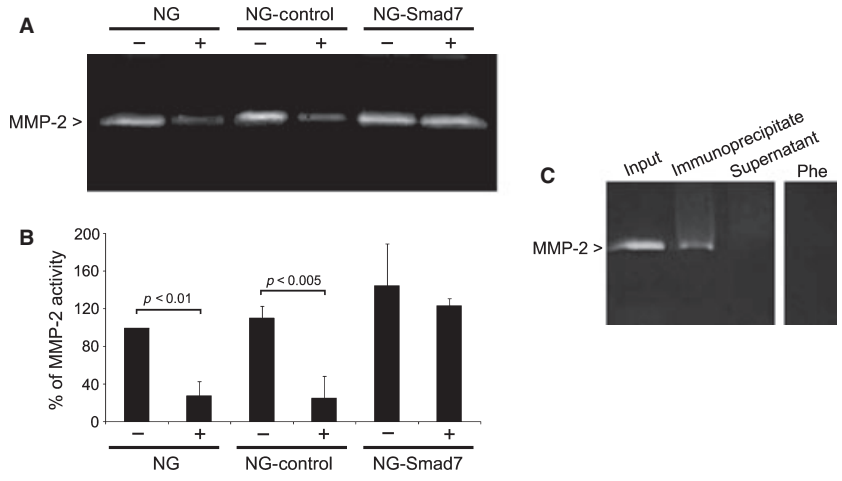

Fig. 6. The effect of CsA on the activity of MMP-2 is inhibited by Smad7 overexpression. (A) Zymographic analysis of the culture supernatants of fibroblast cells detected by a gelatinolytic enzyme at approximately 70 kDa. (B) Densitometric analysis demonstrated a significant lower MMP-2 activity in NG and NG-Control cells after CsA treatment. Ciclosporin A did not affect MMP-2 activity in NG-Smad7 fibroblasts. (C) The gelatinolytic enzyme was characterized as MMP-2 by immunoprecipitation and complete inhibition of activity with 1 mM of 1,10-phenanthroline (Phe).

pathological conditions take place. In our results, TGF-β1 production by NG fibroblasts was significantly elevated after CsA treatment. In accordance with the presented data, previous *in vitro* studies have shown that CsA regulates the transcription and secretion of TGF-β1 by gingival fibroblasts (7,8,11,12,29,39,40). In the *in vivo* model of daily subcutaneous injection of CsA, a significant induction of the immunohistochemical expression of TGF-β1 was observed in the gingival overgrowth tissues (41). Moreover, the levels of TGF-β1 were also significantly higher at sites exhibiting gingival overgrowth than at healthy sites (42). The profibrotic properties of TGF-β1 are mediated by its receptors that phosphorylate Smad2, which regulates the transcription of specific genes. Here, we observed an increase in Smad2 phosphorylation in CsA-treated cells, but no significant changes in pSmad3 levels were observed (data not shown). In cells overexpressing Smad7, CsA induced release of TGF-β1, but its autocrine signaling was blocked, as revealed by low levels of pSmad2 and lack of nuclear translocation to the nucleus. Previous reports have demonstrated that overexpression of Smad7 generated by gene transfer prevented renal injury in CsA-treated animals (43). Likewise, treatment with cartilage oligomeric matrix protein-angiopoietin-1 demonstrated a protective effect on damaged peritubular capillaries, hemodynamic alteration and inflammation in CsA-induced renal injury and fibrosis due to an increase in Smad7 levels with subsequent reduction of Smad2/3 activation (44). Thus, blockage of the biological activity of TGF-β1 by Smad7 overexpression may have a therapeutic potential by reducing fibrosis; however, as TGF-β1 is expressed in a wide variety of human tissues which control important molecular events, adverse effects are expected. Altogether, these findings confirm that TGF-β1, in an autocrine manner, mediates the molecular mechanism of CsA-induced gingival fibrosis, and that forced expression of Smad7 is capable of attenuating this process. Interestingly, a number of studies have shown that high levels of TGF-β1 are related to the pathogenesis of the gingival overgrowths induced by nifedipine and phenytoin (45–47), sustaining further investigation of TGF-β1 signaling and Smad7 overexpression effects in those drug-induced forms of gingival overgrowth.

Although the pro-proliferative effects of CsA have been recognized for several decades, the first comprehensive study on the levels of proteins involved in the control and progression of the cell cycle in gingival fibroblasts was published only recently (48). In this study, the authors described that *in vivo* the immunohistochemical expression of cyclin D1, CDK4 and retinoblastoma protein was higher in CsA-treated gingival tissues than in the control, whereas *in vitro* results with gingival fibroblasts showed increased expression of CDK4 and cyclin D1 and enhanced phosphorylation of retinoblastoma protein after treatment with CsA. Similar results were observed in

Table 1. Overexpression of Smad7 blocks ciclosporin A (CsA) induction of fibroblast proliferation

	Day 3	Day 5	Day 7	Day 9	Day 11
NG	1.28 ± 0.10	3.30 ± 0.11	5.47 ± 0.42	7.81 ± 0.89	12.14 ± 0.58
NG + CsA	1.47 ± 0.12	3.79 ± 0.40	10.19 ± 1.05**	14.0 ± 2.38***	17.42 ± 0.56*
NG-Control	2.08 ± 0.21	5.0 ± 0.22	8.13 ± 0.52	10.25 ± 0.26	12.81 ± 0.60
NG-Control + CsA	2.11 ± 0.14	6.36 ± 0.56*	10.95 ± 0.91**	18.9 ± 0.38***	18.61 ± 1.30*
NG-Smad7	1.82 ± 0.22	5.37 ± 0.84	7.0 ± 0.39	11.29 ± 0.75	12 ± 0.49
NG-Smad7 + CsA	1.89 ± 0.17	4.28 ± 0.39	8.33 ± 0.84	11.26 ± 0.48	13.53 ± 0.17

Data (number of cells × 10^4) represent the mean ± SD of one representative experiment performed in triplicate. Cells were treated with 200 ng/mL of ciclosporin A (+CsA). Abbreviations: NG, NG1 cell line; NG-Control, NG1 cells transfected with pcDNA3 vector; and NG-Smad7, NG1 cells transfected pcDNA3-Smad7 vector. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

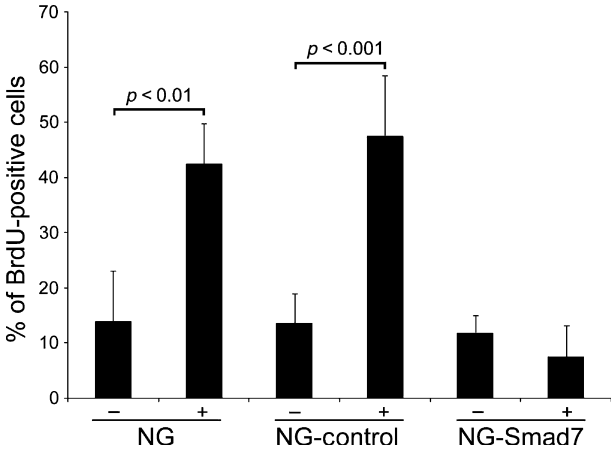

Fig. 7. Overexpression of Smad7 inhibits the proliferative effect of CsA. Serum-starved cells were cultured in presence of 200 ng/mL of CsA for 24 h. The data correspond to the mean percentage of bromodeoxyuridine (BrdU)-positive cells in two independent experiments. Addition of 200 ng/mL of CsA significantly stimulated the proliferation rate of NG and NG-Control fibroblasts, whereas in Smad7-overexpressing cells this effect was not observed.

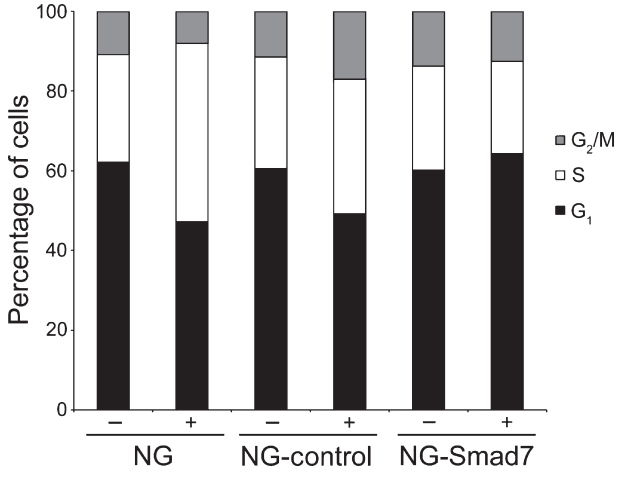

Fig. 8. High levels of Smad7 block CsA induction of the cell cycle. Cell cycle analysis by flow cytometry showed that the incubation of NG and NG-Control cells with 200 ng/mL of CsA for 24 h enhances the S and G_2/M population, whereas in NG-Smad7 cells retention in the G_1 phase is observed.

cultured hepatocytes after exposure to CsA (49). Interestingly, while CsA induced hepatocyte proliferation with invariable levels of p27, our results demonstrated that the expression of p27 was decreased after treatment with CsA, and NG transfectants overexpressing Smad7 demonstrated high levels of p27 even after CsA exposure. In agreement with our results, p27 expression was downregulated in human Jurkat lymphoblastic CD4+ T cells after CsA treatment (50). Recently, Park *et al.* (51) demonstrated that fasudil, an inhibititor of the Rho/Rho-kinase pathway, was effective in attenuating CsA-induced nephropathy. Interestingly, this inhibitory effect was accompanied by suppression of TGF-β1 expression and Smad signaling, and upregulation of p27. Indeed, the authors demonstrated that CsA increases proliferating cell nuclear antigen (PCNA)-positive cells in association with downregulation of p27, whereas fasudil reduced the CsA-

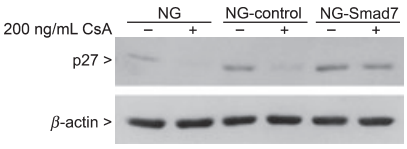

Fig. 9. Effects of CsA on the expression of p27. The expression of p27 was decreased by CsA treatment in both NG and NG-Control cells; however, the downregulation was abolished by overexpression of Smad7.

mediated increase of PCNA-positive cells with p27 upregulation (51). The expression of p27 is known to be regulated by TGF-β1 and various other stimuli independent of p53 expression, and its upregulation may reduce cell proliferation (52). Further studies are needed to investigate the participation of p27 in the CsA-induced gingival fibroblast proliferation.

In conclusion, our results demonstrate that overexpression of Smad7 is effective *in vitro* in neutralizing TGF-β1 signaling, resulting in attenuation of the biological events related to CsA-induced gingival overgrowth. *In vivo* studies with animal models and samples from patients with CsA-induced gingival overgrowth are important to confirm our results.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Effect of CsA on cytotoxicity of NG fibroblasts.

Figure S2. Effects of CsA on apoptosis of NG fibroblasts.

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