UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Cirurgia de Cabeça e Pescoço

Eventos moleculares associados ao aumento gengival induzido por ciclosporina A são atenuados pela superexpressão de Smad7

Tese de Doutorado apresentada à Faculdade de Odontologia de Piracicaba da UNICAMP para obtenção do Título de Doutor em Estomatopatologia na Área de Estomatologia.

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UMA POESIA, UMA FILOSOFIA

Shunji Nishimura

"É a flor que desbrocha

Em um instante qualquer

Não importa se alguém olha

Ou se não existe um olhar sequer

E no silêncio das horas

Canta um pássaro contente

Sem se importar se alguém ouve

Ou se ninguém está presente."

Resumo

O aumento gengival induzido por ciclosporina A (CsA) é causado por um acúmulo excessivo de matriz extracelular, resultado da ação do fator de crescimento transformante-beta 1 (TGF-β1) sobre a atividade dos fibroblastos gengivais. O presente estudo investigou in vitro os efeitos da superexpressão de Smad7, um inibidor da sinalização de TGF-β1, nos eventos biológicos associados ao acúmulo de matriz extracelular induzido por CsA. Para verificar os efeitos inibitórios de Smad7, nós superexpressamos Smad7 em fibroblastos de gengiva normal (GN) de uma maneira estável. Células superexpressando Smad7 e células controle foram incubadas com CsA e a síntese de colágeno tipo I, produção e atividade de metaloproteinase de matriz 2 (MMP-2) e proliferação celular foram analisadas por ELISA, zimografia, curva de crescimento, método de incorporação de bromodeoxiuridina (BrdU) e análise do ciclo celular. Os efeitos de CsA na viabilidade celular e apoptose dos fibroblastos de GN também foram avaliados. Análises de western blot e imunofluorescência para fosforo-Smad2 foram realizados para verificar a ativação da cascata de sinalização de TGF-β1. Apesar do tratamento com CsA estimular a produção de TGF-β1 tanto nas células controle quanto nos fibroblastos superexpressando Smad7, a via de sinalização de TGF-β1 foi marcantemente inibida nas células superexpressando Smad7, como revelado pelos níveis reduzidos de fosforilação de Smad2. Nas células superexpressando Smad7, os efeitos de CsA na proliferação, síntese de colageno tipo I e produção e atividade de MMP-2 foram significativamente bloqueados. A superexpressão de Smad7 bloqueou a proliferação dos fibroblastos gengivais induzida por CsA via regulação de p27. CsA e a superexpressão de Smad7 não induziram morte celular. Os resultados deste estudo confirmaram que a expressão

de TGF-β1 é correlacionada com os eventos moleculares associados ao aumento gengival induzido por CsA, e sugerem que a superexpressão de Smad7 é efetiva no bloqueio de tais eventos, incluindo proliferação, síntese de colágeno tipo I e atividade de MMP-2.

Palavras-chave: Crescimento gengival, Ciclosporina A, Fator de crescimento transformante-beta 1, Smad7, Colágeno tipo 1, Metaloproteinase de matriz 2.

Abstract

Cyclosporin 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-beta1 (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. To assess the inhibitory effects of Smad7, we stably overexpressed Smad7 in fibroblasts from normal gingiva (NG). Smad7 overexpressing cells and controls were incubated with CsA and type I collagen synthesis, matrix metalloproteinase 2 (MMP-2) production and activity, and cellular proliferation were evaluated by ELISA, zymography, growth curve, BrdU-incorporation assay and cell cycle analysis. CsA effects on cell viability and apoptosis of NG fibroblasts were also evaluated. Western blot and immunofluorescence for phospho-Smad2 were performed to measure the activation of TGF-β1 signaling. Although the treatment with CsA stimulated TGF-β1 production in both control and Smad7 overexpressing fibroblasts, its signaling was marked inhibited in Smad7 overexpressing cells as revealed by low levels of Smad2 phosphorylation. In Smad7 overexpressed cells, the effects of CsA on proliferation, synthesis of type I collagen and MMP-2 production and activity were significantly blocked. Smad7 overexpression blocked CsA-induced fibroblast proliferation via p27 regulation. Neither CsA nor Smad7 overexpression induced cell death. 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 the blockage of those events, including proliferation, type I collagen synthesis and MMP-2 activity.

Keywords: Gingival overgrowth, Cyclosporin A, Transforming growth factor-beta1, Smad7, Type I collagen, Matrix metalloproteinase 2.

Sumário

1. *Introdução*

A ciclosporina A (CsA) é uma droga imunossupressora potente usada com sucesso na prevenção da rejeição de transplantes renais e no tratamento de algumas doenças de origem imunológica (Amor *et al*., 2010). Estruturalmente a CsA é um undecapeptídeo cíclico, neutro, lipofílico, tendo sido originalmente isolada do extrato de fungos *Tolypocladium inflatum* por Borel e colaboradores (Borel *et al*., 1976). Embora tenha sido observado apenas um limitado efeito antifúngico, foi constatada uma potente capacidade imunossupressora. Em 1978, CsA foi testada com sucesso na prevenção da rejeição de transplantes renais (Calne *et al*., 1978) e, em 1979, observaram uma eficácia deste fármaco no tratamento da psoríase (Mueller & Hermann, 1979). A formulação original da CsA (Sandimmune; Novartis, East Hanover, NJ) foi aprovada pela FDA (United States Food and Drug Administration) para o uso na prevenção da rejeição de transplantes em 1983. Nos últimos anos a CsA vem também sendo utilizada com bons resultados no tratamento de doenças de caráter auto-imune, como a psoríase, artrite reumatóide, doença de Crohn, entre outras (Gerards *et al*,. 2003; McDonald *et al*., 2005; Pedraz *et al*., 2006; Pereira *et al*., 2006).

Atualmente, o fármaco é comercializado pela Novartis para administração oral nas formas de cápsula (25, 50 e 100 mg/cápsula) ou solução (100 mg/ml), e para administração injetável (50 mg/ml, 100 mg/ml e 250 mg/5 ml) com os nomes comerciais de Sandimmun Neoral e Sandimmun. No Brasil tem-se a opção do correspondente genérico com boa capacidade imunossupressora. Após a administração, CsA é absorvida principalmente no intestino delgado e o pico de concentração plasmática ocorre de 3 a 4 horas após o uso da droga com vida média de 17-40 horas. A metabolização acontece no fígado, sendo os metabólitos, na sua grande maioria, excretados via fezes com apenas 10% sendo excretado na urina (Ho *et al.,* 1996). Os níveis séricos de CsA recomendados para o sucesso na imunossupressão de pacientes transplantados variam entre 150 e 250 ng/ml nos

primeiros 90 dias de tratamento, com redução para 100-150 ng/ml nos períodos posteriores.

O mecanismo de ação da CsA se dá através de uma inibição reversível da resposta imunológica mediada por linfócitos T $CD4^+$, bloqueando a síntese da interleucina-2 (IL-2) e do interferon gama (IFNγ), elementos necessários para ativação de macrófagos e monócitos. Em menor intensidade, a CsA é capaz de suprimir a resposta imune humoral, pois afeta a proliferação e maturação de células B. Além disso, a inibição da produção de IL-2 limita a amplificação clonal e a ativação de linfócitos T auxiliares ($CD4^+$) e citotóxicos ($CD8^+$), além de interferir na produção de citocinas, como IL-3, IL-4, IL-5 e IL-6, do fator necrotizante tumoral (TNF) e do fator estimulador de colônias de macrófacos (Ho *et al.*, 1996). A ação supressora da CsA depende da ligação com seu receptor citoplásmático ciclofilina, levando a formação de um complexo heterodimérico com calmodulina (serina/treonina fosfatase dependente de cálcio). Este complexo inibe a atividade de fosfatase da calcineurina, bloqueando a capacidade da calmodulina de regular a expressão gênica de citocinas, principalmente do fator nuclear de células T ativado (NFAT) (Fruman *et al.,* 1992, Kahan, 1999).

O uso da CsA está associado ao desenvolvimento de inúmeros efeitos colaterais como nefrotoxicidade, hepatotoxicidade, hipertensão arterial, síndrome urêmica hemolítica, hipercolesterolemia, hirsutismo, diabetes, entre outras (Garcia *et al.*, 2004; Melnikov *et al.*, 2011), sendo o efeito colateral de maior interesse para a área odontológica o aumento gengival.

O aumento gengival induzido pela CsA é clinicamente caracterizado por um crescimento lento, nodular, indolor e pode variar desde uma leve mudança no contorno do tecido das papilas interdentais até a cobertura completa dos dentes, interferindo no mecanismo de oclusão, mastigação e fala, além de ocasionar problemas estéticos (Seymour, 2006). Este aumento gengival ocorre, em média, após 3 meses de tratamento; porém, existem relatos de desenvolvimentos após 1 mês do início da administração (Vescosi *et al.*, 2005; Wright *et al.,* 2005). A

incidência do aumento gengival nos usuários de CsA varia amplamente entre os diversos estudos existente (13% a 85%) (Ramalho *et al*., 2003), mas um estudo sistemático e altamente controlado demonstrou uma incidência média entre 25 e 30% (Boltchi *et al*., 1999). Esta variação na incidência e a gravidade do aumento gengival parecem estar relacionadas com a dosagem e tempo de utilização, níveis de higienização e inflamação, além de características genéticas do paciente (Vescosi *et al*., 2005). Outra característica que parece contribuir para esta grande variação na incidência do aumento gengival é o tipo de órgão transplantado. Por exemplo, a incidência é alta nos transplantados de órgãos sólidos como rins, enquanto fica em torno de 2% em casos de transplantes de medula óssea. Em relação à idade dos pacientes, crianças e adolescentes são mais acometidos pelo aumento gengival quando comparados com adultos, sugerindo uma possível relação do uso da CsA com hormônios sexuais e metabolismo dos fibroblastos gengivais (Seymour *et al*. 2000; Wright *et al*., 2005).

Histologicamente, os aumentos gengivais induzidos pela CsA são caracterizados por um acúmulo excessivo de fibras colágenas no tecido conjuntivo, permeada por um grande número de vasos sanguíneos e células inflamatórias, sendo que o epitélio é do tipo pavimentoso estratificado paraqueratinizado, com graus variados de hiperplasia e formação de longas criptas que infiltram o tecido conjuntivo subjacente (Spolidorio *et al*., 2001). A principal célula inflamatória é o plasmócito, com um número pequeno de linfócitos que se localizam, principalmente, próximos ao epitélio juncional. Áreas focais de degeneração mixomatosa do tecido conjuntivo têm sido observadas em regiões subjacentes ao tecido epitelial (Mariani *et al*., 2004).

O tratamento e a prevenção do aumento gengival induzido por drogas permanecem insatisfatórios, sendo que a redução na dose da CsA pode ser considerada eficaz do ponto de vista odontológico, mas nem sempre é possível na prática médica devido à possibilidade da perda do enxerto. Então, para o paciente transplantado há pouca chance de diminuir ou suspender a droga, o que faz com

que as cirurgias gengivais repetidas continuem sendo a principal opção de tratamento (Ramalho *et al*., 2003). As técnicas cirúrgicas que tem sido utilizadas incluem a gengivectomia com bisel invertido ou não, associada à gengivoplastia, retalho com posicionamento apical, eletrocauterização e laserterapia (Pilloni *et al*., 1998; Ilgenli *et al*., 1999; Guelmann *et al*., 2003), porém a recorrência é constante. Outra possibilidade de tratamento seria a substituição da CsA por outra droga, como é o caso da droga Tacrolimus que reduz para à metade a incidência de aumento gengival quando comparada ao uso da CsA (de Oliveira Costa *et al*., 2006), o que muitas vezes não é possível devido ao elevado custo do tratamento. Alguns estudos avaliaram a utilização de antibióticos de uso sistêmico como, por exemplo, o metronidazol e a azitromicina na tentativa de reduzir a gravidade e a freqüência de recorrências do aumento gengival; porém, na literatura ainda não há consitência para indicação do uso dessas drogas, sendo necesários mais estudos para se propor uma terapia adequada (Gomez *et al.,* 1997; Seymour, 2006; Kwun *et al*., 2003; Clementini *et al.,* 2008).

Os mecanismos biológicos envolvidos no desenvolvimento do aumento gengival induzido por CsA ainda não estão totalmente esclarecidos; porém, alguns estudos sugerem um estímulo direto nos fibroblastos do tecido conjuntivo gengival (Cotrim *et al*., 2003; Bostrom *et al*., 2005). Algumas características envolvidas neste processo, como a influência na proliferação celular, indução de apoptose, atividade fagocitária, produção e atividade de metaloproteinases de matriz (MMPs) e seus inibidores teciduais (TIMPs) e análise da produção de colágenos em culturas celulares de fibroblastos tratadas com CsA, têm sido estudadas.

Com relação ao estímulo proliferativo, um estudo demonstrou que o tratamento de fibroblastos gengivais com níveis séricos de CsA de 100 a 200 ng/ml ocasionou um aumento da proliferação celular, porém concentrações elevadas da droga levaram a uma inibição da proliferação destas células (Cotrim *et al.,* 2003). Yoshida e colaboradores (2005) observaram um aumento entre 23 e 25% da proliferação de células isoladas do tecido gengival de ratos quando submetidas ao

tratamento com CsA durante 3 dias comparadas com o grupo controle. Outros estudos confirmaram esta capacidade da CsA de estimular a síntese de DNA e a proliferação celular, mesmo na presença de lipopolissacarídeos bacterianos, que inibem a proliferação de fibroblastos gengivais (Bartold,1989; Chae *et al.,* 2006). Recentemente, Andrukhov e colaboradores (2010) demonstraram que tratamentos com 0,1 a 0,01 µg/ml de CsA ocasionaram um estímulo na proliferação de células do ligamento periodontal, que foi acompanhado por um aumento da viabilidade celular. Estes autores também observaram que níveis elevados da CsA (1 µg/ml) não exerceram estes mesmos efeitos (Andrukhov *et al.,* 2010). Para elucidar os mecanismos envolvidos na indução da proliferação celular por CsA, Andrés e colaboradores (2001) estudaram os efeitos da CsA nas proteínas envolvidas no controle e progressão do ciclo celular em culturas celulares de hepatócitos. Estes autores observaram um aumento nos níveis de ciclina D1 e ciclina E (reguladores de progressão da fase G1 do ciclo celular), de PCNA (antígeno nuclear de proliferação celular) e do fator nuclear kappa B (fator nuclear envolvido na transcrição de ciclina D1) após o tratamento destas células com CsA. Corroborando com estes resultados, recentemente um estudo demonstrou in vivo que a expressão imunohistoquímica de ciclina D1, de quinase dependente de ciclina 4 (CDK4) e da proteína retinoblastoma (Rb) foram maiores nos tecidos provenientes de aumentos gengivais induzidos por CsA comparados com tecido gengival normal (Chiang *et al.,* 2011). In vitro este mesmo estudo demonstrou um aumento da expressão de CDK4, ciclina D1 e de Rb fosforilado em fibroblastos gengivais tratados com CsA (Chiang *et al.,* 2011).

O acúmulo de tecido conjuntivo fibroso, em particular colágeno, é uma característica encontrada com freqüência no aumento gengival induzido pela CsA. Estudos demonstraram que este acúmulo é resultado de um aumento da produção de colágeno em conjunto com uma inibição da sua degradação. Um estudo realizado in vivo, utilizando técnicas de imunohistoquímica em tecidos gengivais de ratos tratados com CsA, observou um aumento dos feixes de fibras de colágeno tipo I, acompanhado de uma diminuição da expressão das colagenases nestes

tecidos (Kataoka *et al.,* 2000). Bolzani e colaboradores (2000) demonstraram uma diminuição da atividade de MMP-1 e MMP-2 em tecidos gengivais de ratos tratados com CsA. Estes autores também observaram uma diminuição na atividade gelatinolítica de MMP-2 e na produção de MMP-1 e MMP-3 em culturas celulares humanas de gengiva normal tratadas com CsA. Corroborando com este estudo, Cotrim e colaboradores (2002) observaram uma inibição da expressão de MMP-1 e MMP-2 em culturas celulares de fibroblastos tratadas com CsA. Hyland e colaboradores (2003) observaram que o tratamento de fibroblastos gengivais com CsA nas concentrações de 0-2000 ng/ml ocasionou uma diminuição significativa dos níveis tanto do mRNA quanto dos níveis protéicos de MMP-1 em uma maneira dose-dependente. Este mesmo tratamento ocasionou uma diminuição da atividade colagenolítica nos meios de cultura dos fibroblastos. Concomitantemente, estes autores observaram uma diminuição significativa da expressão de MMP-1 em tecidos provenientes de aumentos gengivais induzido por CsA comparado com tecidos normais. Gagliano e colaboradores (2004) demonstraram um estímulo na produção de colágeno tipo I por fibroblastos gengivais quando do tratamento com CsA. Estes autores também observaram uma diminuição na produção de MMP-1 pelos fibroblastos gengivais após o tratamento com CsA. Sukkar e colaboradores (2007) observaram que fibroblastos provenientes de tecidos de aumento gengival induzido por CsA apresentavam uma redução significativa dos níveis de MMP-1 comparado com fibroblastos provenientes de tecido normal. Chiu e colaboradores (2009), utilizando tecidos gengivais de ratos tratados com 30 mg/kg/dia de CsA, observaram uma diminuição significativa da expressão do mRNA e da produção protéica de MMP de membrana tipo 1 (MT1-MMP) nestes tecidos comparados com tecidos gengivais dos animais do grupo controle. Estes autores também observaram uma diminuição significativa dos níveis protéicos de MMP-2 nos tecidos gengivais de ratos tratados com CsA. Além disso, in vitro o tratamento de fibroblastos gengivais com CsA ocasionou uma diminuição significativa da atividade de MMP-2. Porém, alguns estudos demonstraram que o acúmulo de colágeno nos aumentos gengivais induzidos por CsA não se deve somente ao aumento na sua

produção ou degradação através das MMPs, mas, também, por uma inibição do processo fagocitário mediado por fibroblastos. Kataoka e colaboradores (2000), em um estudo realizado in vivo, demonstraram uma inibição da atividade fagocitária de fibroblastos em tecidos gengivais de ratos submetidos ao tratamento com CsA, sendo que esta inibição foi acompanhada de um acúmulo excessivo de tecido conjuntivo fibroso. Em concordância com estes resultados, Arora e colaboradores (2001), em um estudo in vitro, observaram uma diminuição da atividade fagocitária de fibroblastos gengivais quando submetidas ao tratamento com CsA.

Em oposição ao efeito inibitório que a CsA exerce sobre a síntese de IL-2, estudos demonstram um estímulo na produção de TGF-β1, efeito que pode estar relacionado ao aparecimento dos principais efeitos colaterais associados com uso da droga. Por exemplo, TGF-β1 é considerado o responsável pelo desenvolvimento da nefropatia alográfica crônica, principal causa de insucesso tardio de transplantes renais (Campistol *et al*., 2001). Além disso, foi demonstrado que a CsA eleva os níveis de produção de TGF-β1, favorecendo a indução e promoção de tumores malignos (Hojo *et al*., 1999; Tanaka *et al*., 2002). Estudos in vitro demonstraram que CsA é capaz de estimular a síntese de TGF-β1 em linfócitos T, células proximais tubulares e em fibroblastos túbulo-intersticiais (Shin *et al.,* 1998; Waiser *et al.,* 2002). Waiser e colaboradores (2002) demonstraram que CsA ocasiona um aumento da expressão de TGF-β1 em culturas de células mesenquimais renais de ratos em uma maneira dose e tempo dependentes. Um estudo, realizado com ratos e coelhos, demonstrou um aumento da expressão de TGF-β1 nos compartimentos túbulo-intersticial e vascular quando sob tratamento com CsA. Cotrim e colaboradores (2002) utilizando culturas celulares de fibroblastos gengivais demonstraram que o tratamento destas células com CsA, em concentrações similares aos níveis séricos de pacientes que fazem uso da droga, ocasionou um estímulo na expressão e produção de TGF-β1; este por sua vez, de uma maneira autócrina, inibiu significativamente a produção e atividade das MMPs, sugerindo ser TGF-β1 um regulador chave dos mecanismos bioquímicos associados ao desenvolvimento do aumento gengival induzido por CsA. Estes mesmos autores,

em 2003, demonstraram que a indução da proliferação de fibroblastos gengivais por CsA ocorre via indução de TGF-β1, pois após realizarem o bloqueio deste fator, os efeitos de CsA na proliferação celular foi significantemente reduzido (Cotrim *et al.,* 2003). Spolidorio e colaboradores (2005) demonstraram um aumento significativo dos níveis de TGF-β1 na saliva de ratos submetidos ao tratamento com CsA.

TGF-β1 é uma citocina multifuncional pertencente à família TGF-β de polipeptídeos. As ações de TGF-β1 são mediadas por interações heteroméricas com receptores serina/treonina quinase tipo I e II (Goumans *et al.*, 2002). O início da sinalização necessita da ligação de TGF-β1 com os receptores transmembrânicos; isso ativa os mensageiros citoplasmáticos desta cascata, incluindo as proteínas Smads. Smad2 e Smad3 são ativados por TGF-β1; Smad4 atua como cofator, enquanto Smad6 e Smad7 possuem uma atividade inibitória na cascata de ativação de TGF-β (Derynck & Zhang, 2003). Especificamente, a ativação de TGF-β1 ocorre através da fosforilação de Smad2 ou Smad3 que, então, se associam a Smad4 e são translocados para o interior do núcleo. No núcleo estes complexos atuam diretamente como fatores de transcrição, iniciando a ativação de genes específicos, ou indiretamente, associando-se a proteínas de ligação do DNA (Moustakas *et al.*, 2001; Mehra & Wrana, 2002; Massague & Gomis, 2006). Smad6 e Smad7 irão atuar impedindo a fosforilação de Smad2 ou Smad3, bloqueando a ativação desta cascata e conseqüentemente os efeitos de TGF-β1.

Métodos de bloqueio da síntese de TGF-β1 vêm sendo testados como uma forma de tratamento de doenças causadas por fibroses intersticiais (Gressner *et al.*, 2002). Em um estudo recente, Sobral e colaboradores (2007) observaram que o tratamento de fibroblastos gengivais com IFNγ inibiu significativamente a produção de colágeno tipo I induzida por TGF-β1. Além disso, este estudo demonstrou que miofibroblastos provenientes de tecido gengival de pacientes com fibromatose gengival hereditária (FGH) também tiveram sua produção de colágeno tipo I inibida

após o tratamento com IFNγ. Estes autores demonstraram que IFNγ inibiu a ativação da cascata de TGF-β1, principalmente pelo estímulo da expressão de Smad7, sugerindo ser esta via responsável pelos efeitos na produção de colágeno e conseqüentemente no acúmulo de tecido conjuntivo. Em um estudo subseqüente, estes mesmos autores demonstraram que a superexpressão de Smad7 em células de FGH provocou uma inibição dos efeitos de TGF-β1, observadas através de uma inibição da produção de colágeno tipo I (Sobral *et al.*, 2011).

Diante dos argumentos a cima, o objetivo do nosso estudo foi avaliar se a superexpressão de Smad7 em fibroblastos gengivais pode ser capaz de bloquear os efeitos da CsA sobre os eventos moleculares associados ao aumento gengival induzido pela CsA.

2. *Proposição*

A proposição deste estudo foi caracterizar os efeitos da neutralização da atividade de TGF-β1, via superexpressão de Smad7, na proliferação, viabilidade, apoptose, síntese de colágeno tipo I e produção e atividade de MMP-2 em fibroblastos de gengiva normal tratados com CsA.

3. *Manuscrito*

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Molecular events associated with cyclosporin A-induced gingival overgrowth are attenuated by Smad7 overexpression

Running title: Smad7 effects in CsA-induced gingival overgrowth.

Key words: gingival overgrowth; cyclosporin A; transforming growth factor beta1; Smad7; type I collagen; matrix metalloproteinase 2; proliferation.

Abstract

Background and Objective: Cyclosporin 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-beta1 (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.

Methods: The effects of Smad7 were assessed by stable overexpression of Smad7 in fibroblasts from normal gingiva (NG). Smad7 overexpressing cells and controls were incubated with CsA and type I collagen synthesis, matrix metalloproteinase 2 (MMP-2) production and activity, and cellular proliferation were evaluated by ELISA, zymography, growth curve, BrdU-incorporation assay and cell cycle analysis. CsA effects on cell viability and apoptosis of NG fibroblasts were also evaluated. Western blot and immunofluorescence for phospho-Smad2 (pSmad2) 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 pSmad2. In Smad7 overexpressed cells, the effects of CsA on proliferation, synthesis of type I collagen and MMP-2 production and activity 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 the blockage of those events, including proliferation, type I collagen synthesis and MMP-2 activity.

Introduction

CsA is a potent immunosuppressive drug with beneficial effects on prevention of transplant rejection and in the treatment of several immunorelated 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 opposite, CsA induces the synthesis of TGF-β in various cell types with autocrine functions (3, 4). Unfortunately the use of CsA is associated with significant side 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 demonstrated that CsA alters the homeostatic equilibrium between synthesis and degradation of extracellular matrix molecules, particularly inducing type I collagen production and reducing MMP levels (6). In addition, CsA has been reported to modify proliferation and to decrease apoptosis of gingival fibroblasts (7, 8, 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 in an autocrine fashion inhibits MMP-2 release by gingival fibroblasts (11). Further study has demonstrated that CsA significantly stimulates fibroblast proliferation in a dose-dependent manner, and the neutralization of TGF-β1 production inhibits CsA effects on proliferation, demonstrating an autocrine stimulatory effect of TGF-β1 in CsA-treated NG fibroblast proliferation (12).

TGF-β1 is a growth factor involved in many cellular processes, including proliferation, differentiation, migration and cell death (13). TGF-β1 signaling involves the activation of a cytoplasmic downstream pathway composed mainly by Smad proteins. The binding of TGF-β1 to its transmembrane receptors resulted in

phosphorylation of Smad2, which partner with Smad4 and translocate 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 inhibit 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 non-inflamed and hyperplastic gingival of one male with 24 years. Cells were maintained in Dulbecco's modified Eagle's media (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO_2 air atmosphere. NG-Smad7 stable 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 0.1% FBS media containing 200 ng/ml of CsA (Sandimmun-Sandoz, São Paulo, Brazil).

Western blot analysis

Cells were washed with cold 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 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, Hercules, CA, USA). Fifty micrograms of total protein per sample was resolved in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and transferred onto nitrocellulose membranes. The membranes were blocked overnight with 10% non-fat 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 a 8-well culture chamber slide and incubated at 37ºC 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 non-specific binding, the cells were blocked with 3% BSA 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 Labs, Burlingame, CA, USA) at 1:250. Cells were mounted with a fluorescent

mounting media containing DAPI (Vectashield, Vector Labs) and examined under a photomicroscope equipped with epifluorescence (Leica Microsystems, Wetzlar, Germany). To generate fluorescent labeled images, cells were excited at 480/40 nm with a 527/30band pass filter. Cells untreated with primary antibodies were used as negative controls.

ELISA

Production of TGF-β1, type I collagen and MMP-2 were determined by ELISA after the methods of Sobral *et al.* (20) and Sobral *et al.* (23). The values were expressed as production/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, Hercules, CA, USA). The intensities of the negative bands were determined using a GS-700 imaging densitometer (Bio Rad, Hercules, CA, USA). 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 by chelating Zn^{++} ions of their catalytic domain, were utilized. Following immunoprecipitation, conditioned media was incubated with 2 µg of purified antibody against MMP-2 (The Binding Site, Birmingham, England), and immunocomplexes were pulled down with protein A-Sepharose (Sigma-Aldrich) for 1 h at 4ºC. After washing the Sepharose beads 3 times with 1 ml of cold PBS containing 0.5% Tween 20 (Sigma-Aldrich), the immunoprepicitated proteins were eluted in 50 µl of non-reducing sample buffer and analyzed by gelatin zymography.

BrdU-incorporation assay

Cells were plated in 8-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 media was replaced by media with or without CsA. Proliferation rates were determined 24 h after treatment by measuring BrdU incorporation into DNA with an immunohistochemical analysis kit (GE Healthcare, Vienna, Austria). BrdU-labeling index, expressed as the percentage of cells labeled with BrdU, was determined by counting 500 cells in 2 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/well in DMEM containing 10% FBS. After 24 h, the cells were washed with PBS and cultured in serum-free media for an additional 24 h. Following serum starvation, cells were cultured with media with or without CsA and counted on days 3, 5, 7, 9 and 11.Fresh culture media was added every other day.

Cell cycle analysis

Propidium iodide (PI) was used to assess cell cycle. For these studies a hypotonic citrate solution containing PI (Sigma-Aldrich) was added to ~$1x10^6$ 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-FITC 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-FITC at 1:500 and PI. 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-FITC positive and PI 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/well) were added in triplicate to wells of 24-wells culture plate and cultured at 37ºC for 24 h. Before adding the CsA, cells were cultured for 24 h in serum-free media. After treatment for 24 h, cell viability was determined using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) assay (25).

Statistical analysis

The data represent the mean ± 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

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, as compared to parental and control-transfected NG cells (NG-Control), was verified by western blot (Fig. 1). Endogenous Smad7 was detectable in 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. CsA significantly induced the production of TGF-β1 by NG cells, and its

effects were not modified by Smad7 overexpression (Fig. 2). TGF-β1 levels were ~5-10 times greater in supernatants of CsA-treated NG cells compared to untreated cell supernatants (Fig. 2). No differences on TGF-β1 production were found among the cell lines treadted with CsA ($p=0.15$). Since 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. CsA 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 in NG-Smad7 cells after treatment with 200 ng/ml of CsA.

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

Previous investigations of the molecular events that lead to CsA-induced gingival overgrowth pointed to the important role of TGF-β1. Thus, the influence of Smad7 overexpression in NG cells under CsA treatment was in vitro examined on cellular events related to gingival overgrowth. As shown in Figure 4, Smad7 overexpressing cells exhibited a strongly reduced capacity to produce type I collagen after CsA treatment, as compared to both untransfected and NG-Control cells. CsA-treated NG and NG-Control cells demonstrated a ~5-fold 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. Supernates 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. Gelatin

zymography performed with culture supernates confirmed that CsA effects 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 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 gelatinolitic band was confirmed as MMP-2 by immunoprecipitation assay and by inhibition with 1,10-phenanthroline (Fig. 6C).

Smad7 overexpression inhibits CsA-induced gingival fibroblasts proliferation

To determine whether Smad7 overexpression blocks CsA-induced fibroblast proliferation, we have employed growth curve, BrdU incorporation assay and cell cycle analysis. CsA effects in the growth of NG, NG-Control and NG-Smad7 cells are depicted on Table 1. CsA demonstrated a direct effect in the proliferation of NG and NG-Control fibroblasts, but not in NG-Smad7 cells. From day 5 for NG-Control cells and day 7 for NG cells, the differences in number of cells were significantly higher 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 controls 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 (Supplementary Figure 1 and 2).

Since our results provided evidences that CsA induces an increase in the percentage of cells in S phase of cell cycle, we performed western blot analysis for

p27, an important regulator of G_1/S transition. As depicted in Figure 9, CsA treatment severely reduced p27 levels of NG and NG-Control cells, whereas overexpression of Smad7 prevented its degradation.

Discussion

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

CsA-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, 28, 29). There are several studies examining the in vitro events related to CsA-induced gingival enlargement and these results are inconsistent, demonstrating for example both increases and decreases in collagen synthesis (30, 31, 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, 11, 12). In the present study, we extended these findings and further demonstrated that Smad7 overexpression blocks TGF-β1 signaling activation via decrease in Smad2 phosphorylation, reducing CsA effects in 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. production and activity of MMP-2, which is inhibited by CsA, were increased 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 and collaborators (33) that CsA induces the expression of TGF-β, a large number of studies demonstrated that most of the side effects related to CsA treatment are associated with this growth factor (3, 34, 35, 36). In particular, Hojo *et al.* (34) showed that CsA induces cancer progression in different cell lines, including invasiveness of non-transformed cells, via secretion of TGF-β1. TGF-β1 is considered 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 why chronic 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 showed 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 in sites exhibiting gingival overgrowth than in healthy sites (42). The pro-fibrotic 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 nucleus. Previous reports have demonstrated that overexpression of Smad7 generated by gene transfer prevented renal injury in CsA-treated animals (43). Similarly, the treatment with cartilage oligomeric matrix protein-angiopoietin-1 (COMP-Ang1) protein 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 TGF-β1 biological activity by Smad7 overexpression may have a therapeutic potential by reduce fibrosis, however, since TGF-β1 is expressed in a wide variety of human tissues where control important molecular events, side effects are expected. Altogether those 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 attenuate this process. Interestingly, a number of studies have showed that high levels of TGF-β1 are related to the pathogenesis of the gingival overgrowths induced by nifedipine and phenytoin (45, 46, 47), sustaining further investigation of TGF-β1 signaling and Smad7 overexpression effects in those drug-induced gingival overgrowth.

Although the pro-proliferative effects of CsA are recognized for more than decades, the first comprehensive study on the levels of proteins involved in the control and progression of the cell cycle in gingiva fibroblasts was recently published (48). In this study, the authors described in vivo that the immunohistochemical expression of cyclin D1, CDK4 and retinoblastoma protein (Rb1) 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 Rb1 after treatment with CsA. Similar results were observed in 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 exposition. In agreement to our results, p27 expression was downregulated in human Jurkat lymphoblastic CD4+ T cells after CsA treatment (50). Recently, Park and colleagues (51) demonstrated that fasudil, an inhibititor of Rho/Rho-kinase (ROCK) 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 PCNA-positive cells in association with downregulation of p27, whereas fasudil reduced CsA-mediated increase of PCNA-positive cells with p27 upregulation (51). p27 expression 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 neutralizing TGF-β1 signaling, resulting in attenuation in vitro 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 is important to confirm our results.

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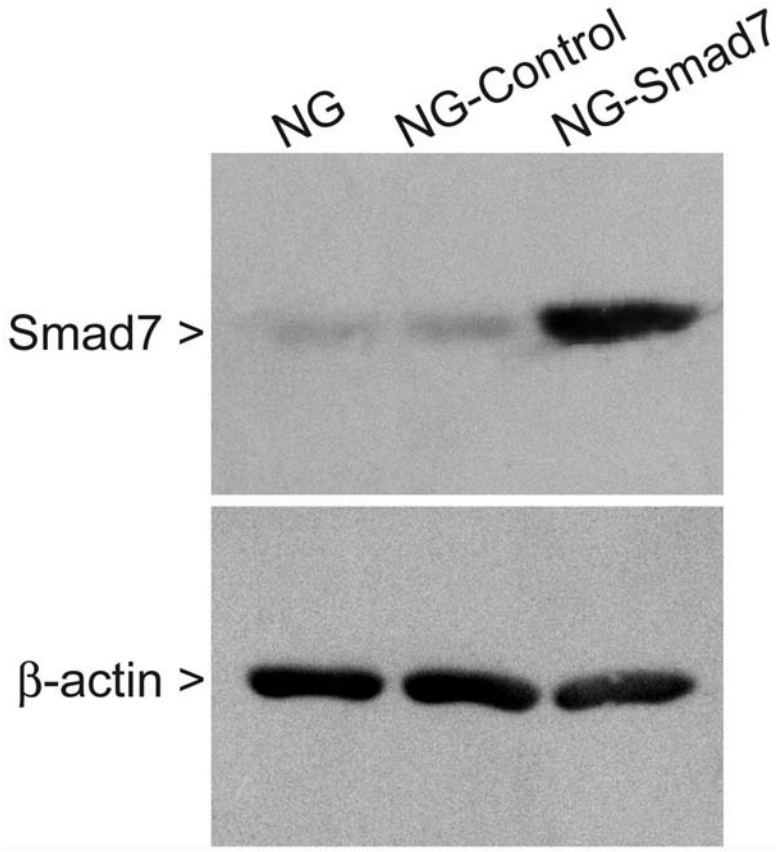


Figure 1. Characterization of NG fibroblasts overexpressing Smad7. Representative western blot analysis of Smad7 in NG, NG-Control and NG-Smad7 transfectants, revealing very low levels of Smad7 in control cells in opposite to increased levels in NG-Smad7 cells.

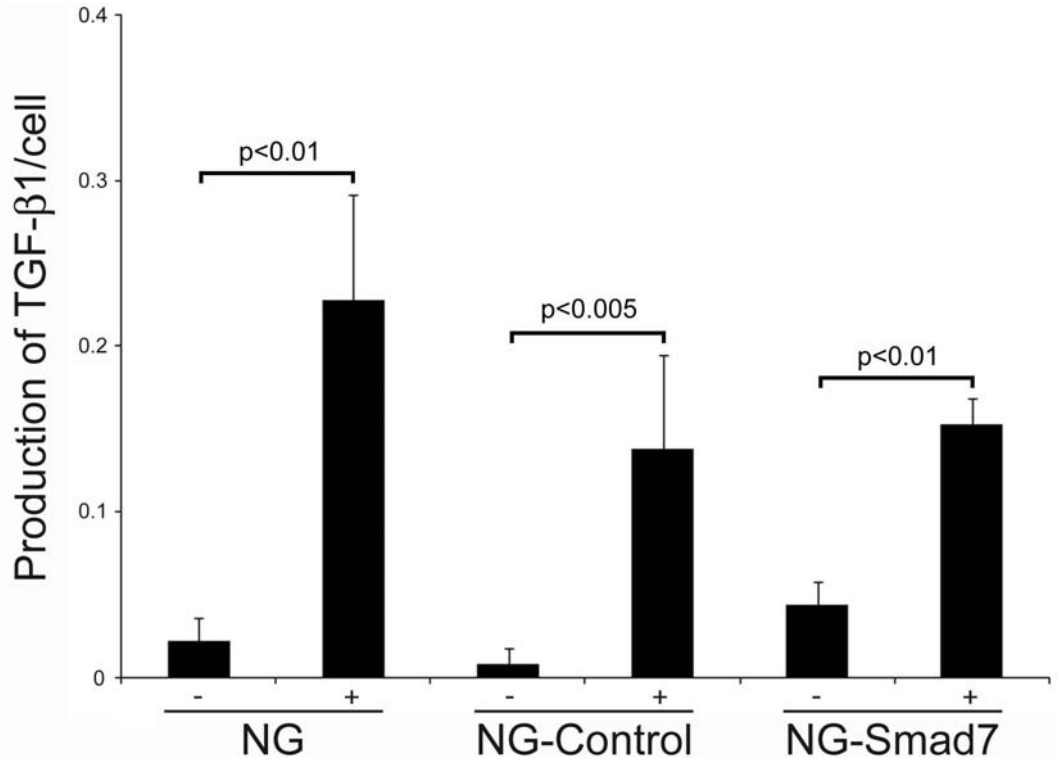


Figure 2. Effect of CsA on production of TGF-β1 by NG fibroblasts. TGF-β1 in culture supernates was quantified by ELISA. Data are the mean ± SD and represent 3 experiments in triplicate for each cell line. The amount of TGF-β1 produced by NG, NG-Control and NG-Smad7 fibroblasts was significantly stimulated by CsA.

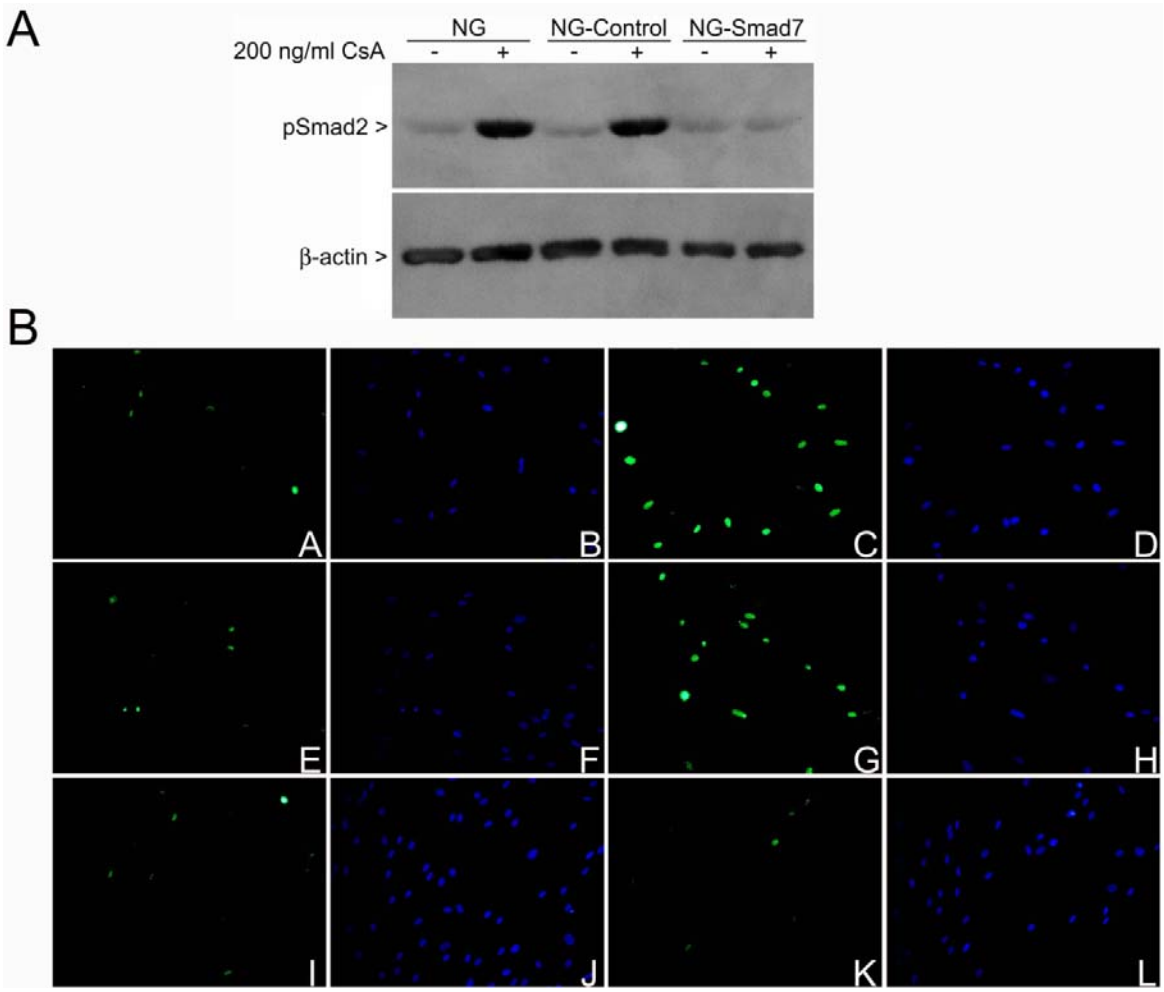


Figure 3. Phosphorylation and nuclear localization of Smad2 is attenuated in NG-Smad7 cells after treatment with CsA. (A) Representative western blot analysis of phosphorylated Smad2 (pSmad2) in NG, NG-Control and NG-Smad7 cells treated with 200 ng/ml of CsA. (B) Immunofluorescence analysis demonstrated that CsA induced pSmad2 nuclear localization in both NG and NG-Control cells, but overexpression of Smad7 markedly blocked it. A-D: NG cells; E-H: NG-Control cells; I-L: NG-Smad7 cells. Unstimulated cells are in A, E and I, and CsA-treated cells are in C, G and K (original magnification x100).

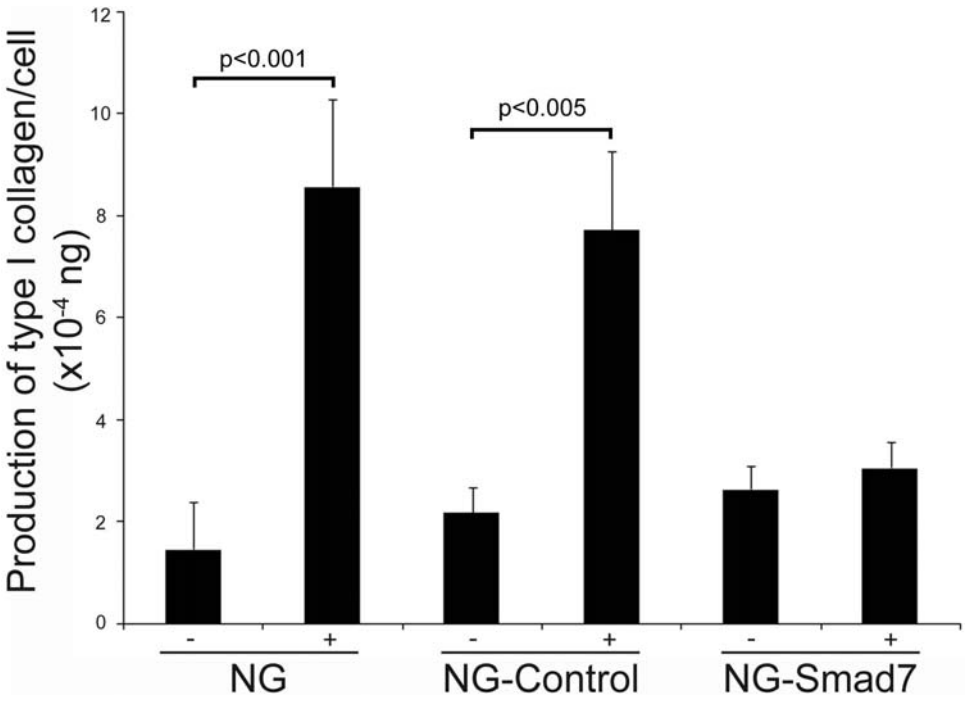


Figure 4. Overexpression of Smad7 blocks the effects of CsA on production of type I collagen by NG fibroblasts. ELISA analysis revealed that overexpression of Smad7 significantly blocked CsA stimulatory effects on type I collagen production.

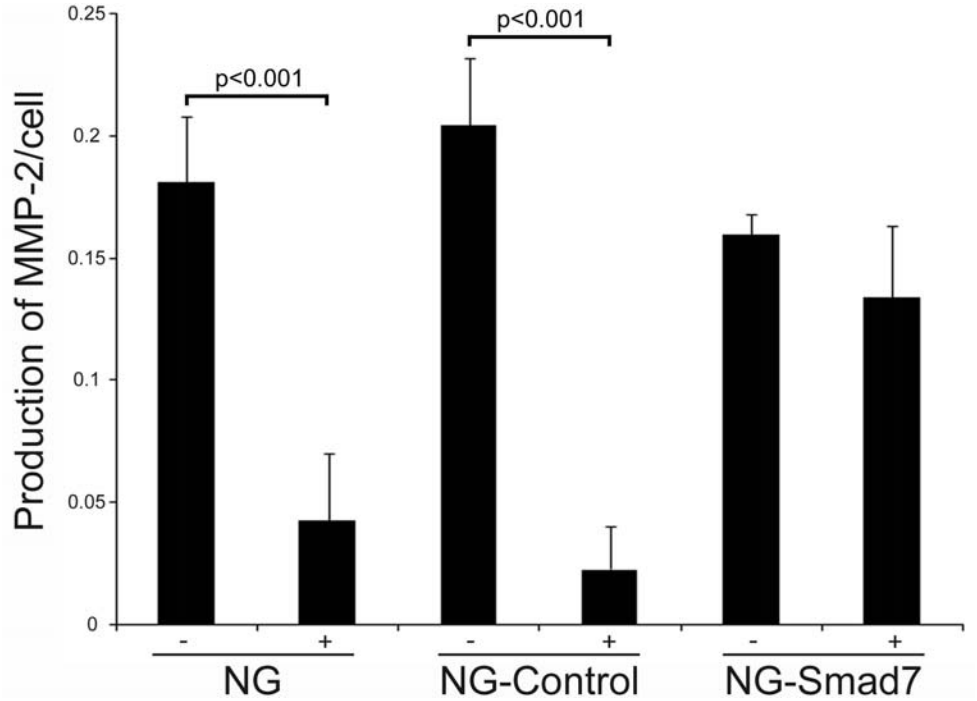


Figure 5. Smad7 overexpression attenuated CsA-induced downregulation of MMP-2 production. Data are expressed as production of protein/cell. The amount of MMP-2 synthesized by NG and NG-Control was significantly reduced by CsA treatment. Similar effect was not observed in NG-Smad7 fibroblasts.

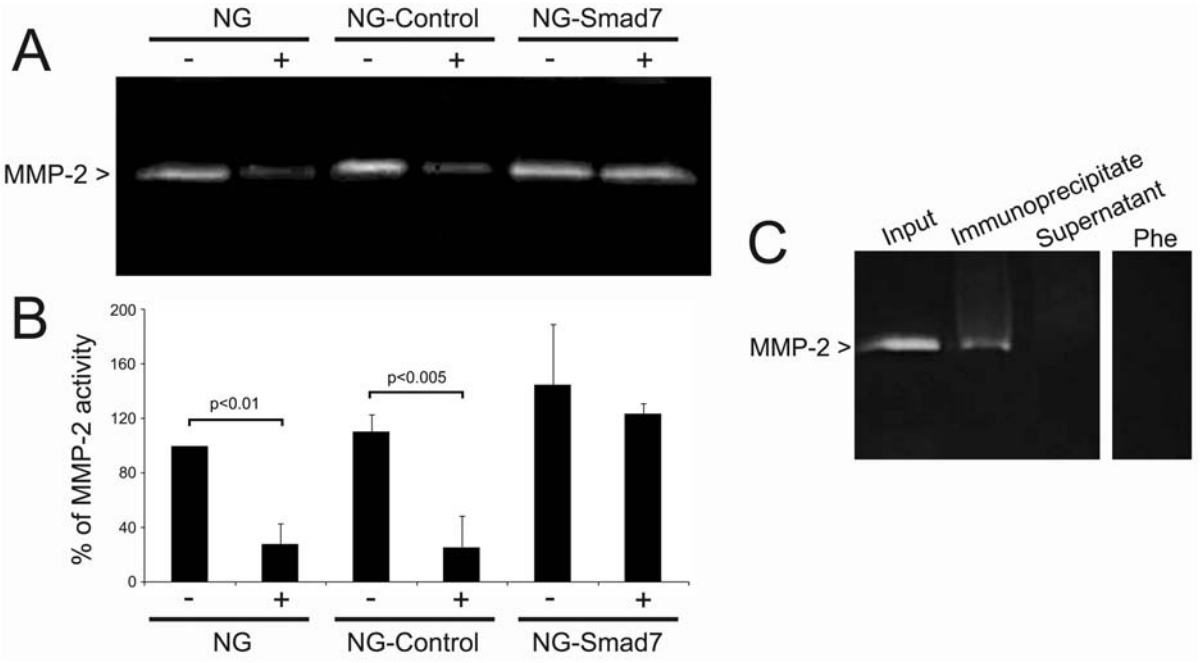


Figure 6. Effect of CsA on activity of MMP-2 is inhibited by Smad7 overexpression. (A) Zymographic analysis of the culture supernates of fibroblast cells detected a gelatinolytic enzyme at ~70 kDa. (C) This enzyme was characterized as MMP-2 by immunoprecipitation and complete inhibition of activity with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

Table 1. Overexpression of Smad7 blocks CsA induction of fibroblast proliferation. Data (number of cell x10^4) represent mean ± SD of one representative experiment performed in triplicate.

	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

*p<0.05; **p<0.01; ***p<0.001.

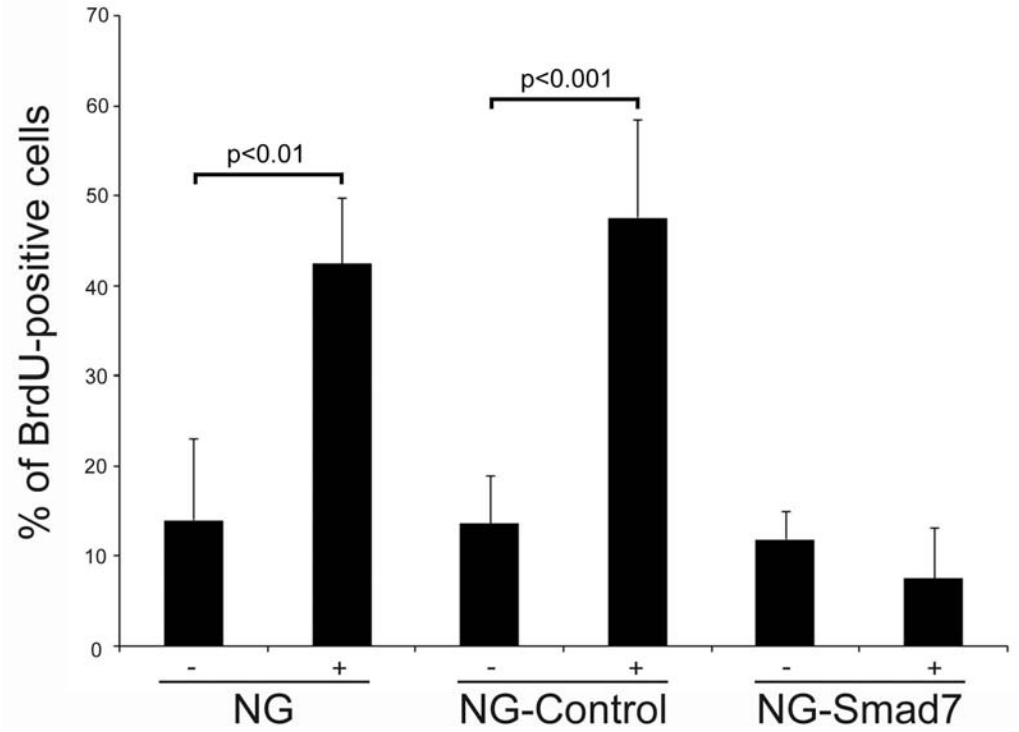


Figure 7. Overexpression of Smad7 inhibits the proliferative effect of CsA. Serum-starved cells were cultured in presence of 200 ng/ml of CsA for 24 h. The data correspond to the mean percentage of positive cells of 2 independent experiments. Addition of 200 ng/ml of CsA significantly stimulates the proliferation rate of NG and NG-Control fibroblasts, whereas in Smad7 overexpressing cells this effect was not observed.

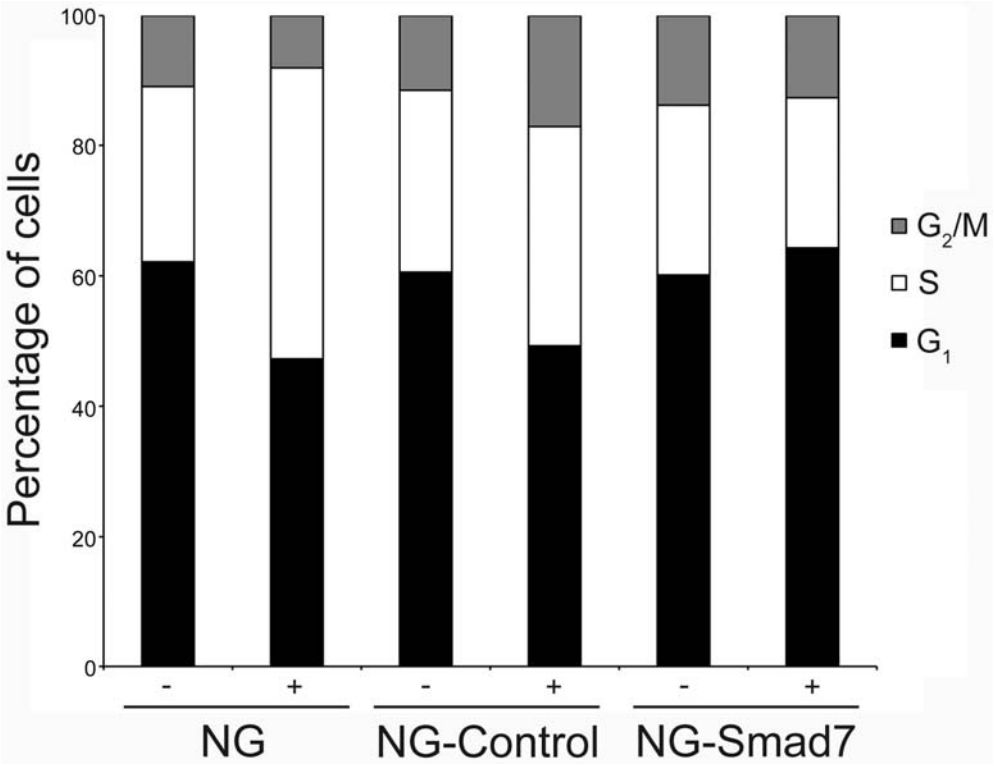


Figure 8. High levels of Smad7 blocks CsA induction of the cell cycle. Cell cycle analysis by flow cytometry showed that the incubation of NG and NG-Control cells with 200 ng/ml of CsA for 24 enhances the S and G_2/M population, whereas in NG-Smad7 cells the retention in G_1 phase is observed.

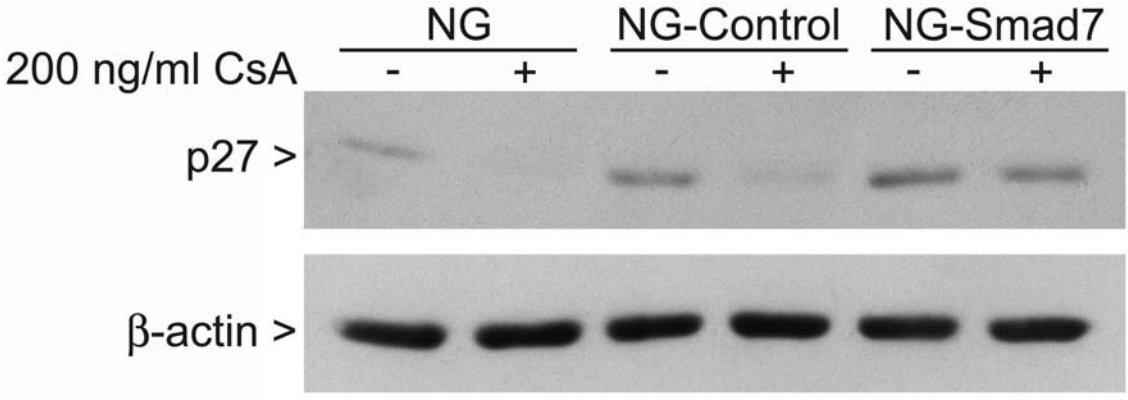
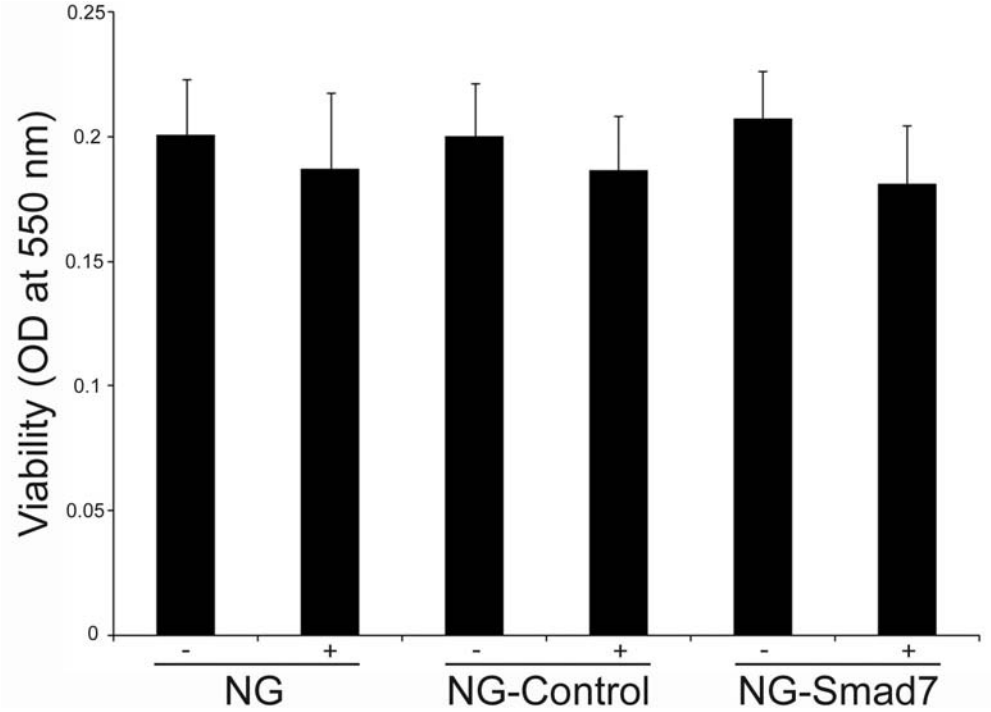
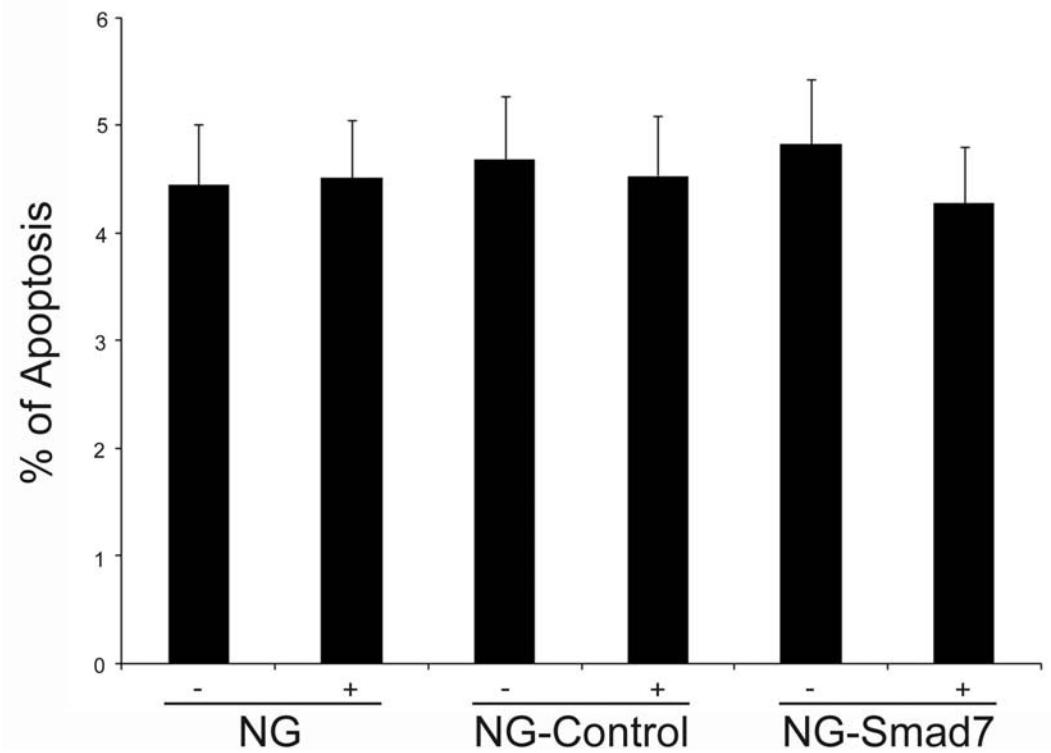


Figure 9. Effects of CsA in the expression of p27. The expression of p27 was decreased with CsA treatment in both NG and NG-Control cells, however, the downregulation was abolished by overexpression of Smad7.



Supplementary Figure 1. Effect of CsA on cytotoxicity of NG fibroblasts. NG, NG-Control and NG-Smad7 cells were incubated for 24 h with 200 ng/ml of CsA, followed by MTT colormetric assay. Results are expressed in absorbance at 550 nm and represent the mean ± SD of 3 independent experiments. CsA had no cytotoxic effects to NG fibroblasts.



Supplementary Figure 2. Effects of CsA on apoptosis of NG fibroblasts. Cells were treated with CsA for 24 h, washed in PBS, incubated with annexin V-FITC and PI, and analyzed by flow cytometry. A low number of apoptotic cells was found in both control and Smad7 overexpressing cells, and no significant differences from CsA-treated cells were observed.

4. *Conclusões*

O presente estudo demonstrou que

1. A superexpressão de Smad7 inibiu a ativação da cascata de sinalização de TGF-β1 induzida por CsA via inibição da fosforilação de Smad2.

2. A superexpressão de Smad7 inibiu os efeitos de CsA na síntese de colágeno tipo I e na produção e atividade de MMP-2.

3. A superexpressão de Smad7 inibiu a proliferação de fibroblastos gengivais induzida por CsA via regulação de p27.

Em conclusão, os resultados deste estudo suportam a hipótese que a superexpressão de Smad7 pode ser clinicamente efetiva na prevenção e tratamento de aumentos gengivais de pacientes em tratamento com CsA e garantem novos estudos utilizando modelos animais de aumento gengival.

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Anexo. Certificado Comitê de Ética

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Participação dos miofibroblastos no aumento gengival induzido por ciclosporina, na fibromatose gengival hereditária e nos carcinomas espinocelulares orais"**, protocolo nº 141/2006, dos pesquisadores Ricardo Della Coletta, Fernanda Aseredo, Joseli Assem Bersaneti, Lays Martin Sobral, Michele Gassen Kellermann e Patrick Franz Montan, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 30/05/2011.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Myofibroblasts participation in gingival overgrowth induced by cyclosporine, hereditary gingival fibromatosis and oral spinocellular carcinoma"**, register number 141/2006, of Ricardo Della Coletta, Fernanda Aseredo, Joseli Assem Bersaneti, Lays Martin Sobral, Michele Gassen Kellermann and Patrick Franz Montan, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 05/30/2011.

Profa. Dra. Lívia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.