Ana Paola Pereira Cotrim

Papel do Fator de Crescimento Transformante- beta1 (TGF-ß1) na Proliferação Celular e Expressão de Metaloproteinases de Matriz e seus Inibidores Teciduais em Fibroblastos Gengivais Humanos Tratados com Ciclosporina A

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas para obtenção do Título de Doutor em Estomatopatologia, área de Patologia

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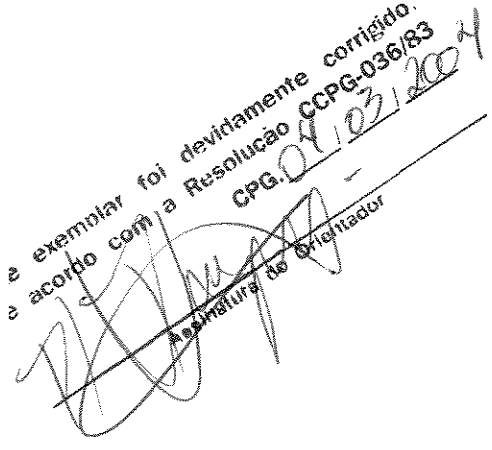

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Orientador: Prof. Dr. Ricardo Della Coletta
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4. Prof. Dr. JACKS JORGE JUNIOR _____

5. Prof. Dr. EDGARD GRANER _____

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SUMÁRIO

RESUMO

Ciclosporina A é a principal droga imunossupressora utilizada na prevenção da rejeição de transplantes. Esta droga induz inúmeros efeitos colaterais, incluindo o aumento gengival observado em 8 a 70% dos pacientes em tratamento com a droga. Embora a patogênese dos aumentos gengivais seja desconhecida, recentemente foi demonstrado que ciclosporina A induz a expressão do fator de crescimento transformante-beta1 (TGF-B1). O objetivo deste trabalho foi investigar o papel de TGF-B1 na patogênese do aumento gengival induzido por ciclosporina A, explorando um possivel efeito autócrino deste fator na proliferação celular e na expressão de metaloproteinases de matriz (MMPs) e seus inibidores teciduais (TIMPs). Para determinar o efeito de ciclosporina A na expressão e produção de TGF-B1, culturas primárias de fibroblastos gengivais humanos normais foram tratadas com concentrações crescentes de ciclorporina A por 24 h, e os níveis de expressão e produção de TGF-β1 analizados por pelo método semi-quantitativo da transcriptase reversa-reação em cadeia da polimerase (RT-PCR) e ELISA, respectivamente. Os efeitos de ciclorporina A e TGF-β1 na proliferação de fibroblastos gengivais foram analisados através de 4 ensaios de proliferação celular, incluindo ensaio de crescimento celular, análise da incorporação de bromodeoxiuridina (BrdU), quantificação da expressão imunohistoquímica do antígeno nuclear de proliferação celular (PCNA) e potencial mitótico. O efeito de ciclosporina A na expressão de MMP-1, MMP-2, TIMP-1 e TIMP-2 foram determinados por RT-PCR. Para determinar o efeito autócrino de TGF-B1 na proliferação celular e na expressão de MMPs e TIMPs, nós utilizamos oligonucleotides complementares

a região de iniciação da tradução de TGF-B1. Ciclosporina A, em níveis similares aos encontrados no sangue de pacientes em uso da droga, significantemente estimulou a expressão de produção de TGF-B1. De uma maneira similar, ciclosporina A estimulou a proliferação celular e inibiu a expresao de MMP-1 e MMP-2 por fibroblastos gengivais. Os níveis de TIMP-1 e TIMP-2 foram inalterados pelo tratamento com ciclorporina A. O tratamento com ciclosporina A em combinação com o bloqueio na produção de TGF-β1 por oligonucleotideos complementares reduziu os efeitos de ciclosporina A na proliferação celular e na expressão de MMP-1 e MMP-2 por fibroblastos gengivais. Nossos resultados demonstram que ciclosporina A induz a expressão TGF-β1 em fibroblastos gengivais, o qual de uma maneira autocrina exacerba a proliferação e reduz a expressão de MMPs em fibroblastos gengivais normais.

Abstract

Cyclosporin A (CyA) is a widely used immunosuppressant which possesses significant side effects including gingival overgrowth. The pathogenesis of this condition is not fully understood. However, recent studies showed that CyA regulates the transcription of several cytokines including transforming growth factor-beta1 (TGF-β1). The aim of this study was to analyze the potential role of TGF-ß1 in the pathogenesis of CyA-induced gingival overgrowth. We hypothesize that TGF-β1 is an important autocrine regulator of cell proliferation, synthesis of matrix metalloproteinases (MMPs) and its tissue inhibitors TIMPS in the experimental settings of CyA induced gingival overgrowth.

To test these hypothesis gingival fibroblasts (GF) from normal gingiva were incubated with increasing concentrations of CyA and the expression and production of TGF-ß1 determined by RT-PCR and ELISA. Proliferative activity of CyA-treated GF was studied by growth curve (via cell counting), BrdU incorporation, quantification of PCNA and mitotic potential. MMPs expression levels were analyzed by RT-PCR. To determine the effects of TGF-ß1 on the proliferation rate and on the expression of MMPs by GF under CyA treatment, the cells were incubated with CyA and antisense oligonucleotides (AON) against the TGF-ß1 mRNA.

CyA simultaneously stimulated TGF-ß1 expression, increased proliferation and inhibited expression of MMP-1 and MMP-2 with a slight effect on TIMP-1 and TIMP-2 expressions. Both CyA and TGF-β1 stimulated significantly the proliferation of GF in a dose-dependent manner. When TGF-ß1

was inhibited using specific antisense oligonucleotides (AON) a reduced TGF-β1 production was noticed (demonstrated by ELISA) with no significant effect on the mRNA levels. When cells were treated with AON simultaneously with CyA, the effects of CyA on proliferation and expression of MMPs were neutralized, the original proliferation rate and MMPs expression were restored.

Our investigation of the molecular events that lead to CyA-induced gingival overgrowth showed that TGF-ß1 in an autocrine fashion upregulates proliferation and downregulates expression of MMPs, which may underlie the clinical changes associated with CyA treatment.

1. INTRODUÇÃO

Aumento gengival é um termo genérico usado clinicamente para identificar uma alteração volumétrica da gengiva, frequentemente resultado do acúmulo excessivo de grandes quantidades de tecido conjuntivo fibroso. O aumento gengival pode ser iatrogênico, idiopático, inflamatório, medicamentoso ou hereditário (Takagi et al., 1991). No caso dos aumentos gengivais medicamentosos, os bloqueadores dos canais de cálcio: ciclosporina A, fenitoina e nifedipina são as drogas mais associadas.

Os aumentos gengivais estão certamente ligados a um desequilíbrio nos mecanismos biologicos locais, o que pode envolver: aumento na síntese e/ou diminuição na degradação de matriz extracelular; aumento na proliferação celular local (fibroblastos gengivais); ou provavelmente ambos, levando a um desequilíbrio generalizado no controle de síntese e degradação da matriz extracelular e na proliferação celular, o que é controlado por moléculas sinalizadoras como por exemplo o fator de crescimento transformante β1 (TGF-1). (Bartold PM 1989; Boltchi et al, 1999)

A Ciclosporina A é uma molécula cíclica e hidrófoba composta por onze aminoácidos com alta capacidade imunossupressora. Esta molécula é o produto metabólico de dois fungos, Tolypocladium inflatum e Cylindrocarpon lucidum (Borel et. Al., 1976). Embora apresente funções antifúngicas e antibacterianas, a principal indicação terapêutica de ciclosporina A esta relacionada com a função imunossupressora, por bloquear a síntese de interleucina 2 e outras citocinas em linfócitos T, sendo amplamente utilizada

na prevenção da rejeição de transplantes e no tratamento de doenças autoimunes (Kanitakis e Thivolet, 1990).

A ciclosporina A pode promover uma série de efeitos colaterais que estão relacionados principalmente com variações na absorção e metabolismo da droga e com a susceptibilidade individual. Dentre estes efeitos colaterais podemos listar: nefro, hepato e neurotoxicidade, hipertensão arterial, indução de cálculos biliares, diabetes, alteração no metabolismo ósseo e hirsutismo, além de infecções oportunistas associadas indiretamente à droga (Ota e Bradley, 1983; Goldman et. al. 1985; O'valle et. al. 1994). Para a área odontológica, os efeitos colaterais mais importantes são a hipertrofia das papilas linguais fungiformes e o aumento gengival (Silverberg et el. 1996; Marshall and Bartold, 1998). A média de incidência de aumento gengival em pacientes tratados com ciclosporina A é de 25%, com uma variabilidade de 8 `a 70% (Seymour and Heasman 1988). De modo interessante, estudos que utilizam doses e tempo de duração de tratamento maiores mostram uma incidência de 70%. Acredita-se que esta variação dependenda de susceptibilidade genética, duração e dose da droga, além das concentrações séricas e salivares (Marshall and Bartold, 1988). Um programa de remoção e controle de placa pode melhorar as condições gengivais mas não impede a evolução do aumento gengival (Seymour & Jacobs, 1992). Já a redução da dose de ciclosporina A se mostra efetiva no não desenvolvimento do aumento gengival (Daly, 1992).

Clinicamente o aumento gengival induzido por ciclosporina A se apresenta como lesões nodulares fibróticas comumente restritas a gengiva

aderida, mas podendo se estender para a gengiva livre, interferindo nos movimentos fisiológicos de oclusão, mastigação e fonação, além de problemas estéticos. Histologicamente, o aumento gengival apresenta uma mucosa recoberta por epitélio pavimentoso estratificado hiperplásico e com múltiplas e longas criptas epiteliais. No tecido conjuntivo subjacente observa-se acentuado acúmulo de fibras colágenas permeadas por fibroblastos, vasos sangüíneos e ocasionais células inflamatórias (Rostock et al, 1986; Wondimu et al, 1993).

Observações em estudos de culturas celulares, apesar de variáveis, demonstram que fibroblastos gengivais na presença de ciclosporina A apresentam um alterado comportamento proliferativo, um desequilíbrio na relação síntese e degradação de matriz extracelular e um aumento na produção de moléculas sinalizadoras e reguladoras destes processos. (Meller et al., 2002; Spolidorio et al. 2002) Existem evidências na literatura de que a indução de TGF-ß1 pelo tratamento com ciclosporina A pode ser o principal fator relacionado com os efeitos colaterais da droga (Pascual et al., 1998; Khanna et al., 1998) incluindo a hiperplasia gengival (Silverberg et al., 1996; Marshall and Bartold, 1998). Contudo, o mecanismo da participação de TGF-ß1 nos aumentos gengivais, especialmente os causados pela ciclosporina A, permanece incerto.

Foi demostrado que ciclosporina A estimula a produção de TGF-1 in vitro em linfócitos T humanos, linhagens celulares de células provenientes do túbulo proximal de camundongos e fibroblastos tubulointersticiais (Shin et al., 1998), e in vivo em rim de ratos e camundongos (Waiser J et al.. 2002). Este mesmo

aumento na expressão de TGF-β1 foi também encontrado em células renais de pacientes transplantados tratados com CyA (Campistol J.M., et al 2001). TGF-β1 tem sido considerado uma citocina fibrogenética chave na patogênese da nefropatia alográfica crônica, a principal causa de insucesso tardio de transplantes renais. Independente dos eventos que podem desencadear este processo, a nefropatia alográfica crônica está relacionada principalmente a fibrose ou acúmulo de matriz extracelular (glomerular, arteriolar e intersticial) regulada por TGF-β1 (Campistol J.M., et al 2001; Waiser J et al, 2002).

Bartold (1989), mostrou que ciclosporina A estimula a síntese de DNA e a proliferação de fibroblastos gengivais in vitro mesmo sem a adição de soro fetal bovino as culturas celulares. Neste mesmo estudo ciclosporina A foi utilizada em conjunto a lipopolissacarideos, em concentrações que normalmente inibiriam a proliferação dos fibroblastos gengivais, ainda assim a ciclosporina A reteve sua capacidade de estimular a proliferação celular. Em tecido gengival, Bolzani et al. mostraram que ciclosporina A, em concentrações similares as concentrações séricas de pacientes sob tratamento com droga, inibe significativamente a produção de MMP-1 e MMP-3, alem de diminuir a atividade gelatinolítica de MMP-2. Coletta et al (1999), demostraram que a elevada expressão de TGF-β1 em fibroblastos gengivais de pacientes com fibromatose gengival hereditária induz a redução nos níveis de expressão de MMP-1 e MMP-2, levando a um acúmulo de matriz extracelular. Hyland et al. (2003) mostraram recentemente que ciclosporina A em linhagens celulares de fibroblastos gengivais inibe significativamente os níveis de MMP-1. Em um artigo de revisão da literatura Boltchi et al (1999), relatam o aumento

na expressão de TGF-β1 e PDGF (fator de crescimento derivado de plaquetas), como os possíveis responsáveis pelo maior índice proliferativo e aumento na síntese de matriz extracelular em fibroblastos gengivais sob a influência de ciclosporina A.

TGF-β foi originalmente purificada da placenta humana, plaquetas e rim bovino (Roberts et al, 1984). A denominação fator de crescimento transformante (TGF) é devido à habilidade deste fator permitir que células normais cresçam em soft agar, uma característica exclusiva de células malignas, e por causar transformações fenotípicas em fibroblastos (Roberts et al., 1981; Tucker et al., 1983). O gene que codifica TGF-β1 esta localizado no 19q13, praticamente todos os tipos celulares apresentam receptores para TGF-β, sendo assim seu papel tanto fisiológico, quanto no desenvolvimento de patologias extenso (Flanders and Roberts, 2000). TGF-β regula muitos processos que são comuns tanto para o reparo tecidual quanto para o desenvolvimento de algumas doenças, incluindo angiogênese, quimiotoxia de fibroblastos, linfócitos, macrófagos e neutrófilos; proliferação de fibroblastos, controle de diferenciação celular, apoptose e o controle da síntese e degradação das proteínas que constituem a matriz extracelular, como colágenos, fibronectinas e proteoglicanos. (Govinden and Bhoola 2003; Ignotz and Messangue, 1986: Raghow et al., 1987; Laiho and Keski-Oja, 1989; Overall et al., 1989). Desde a definição inicial de TGF-β em 1983, mais de 40 membros diferentes da família TGF-β foram descritos em espécies que vão da alga marinha aos humanos. Todas essas proteínas têm em comum a característica básica de serem formadas por um centro composto por sete

moléculas de cisteina. Em humanos encontramos 3 isoformas, o original descrito TGF-β que foi posteriormente chamado de TGF-β1 além de TGF-β2 e TGF-β3, todos sendo secretados como homodímeros na forma latente (Flanders and Roberts, 2000). As alterações que TGF-β1 pode causar na dinâmica da matriz extracelular, modificando a síntese de MMPs e TIMPs e influênciando a proliferação celular, quando ocorrem de forma controlada (ou desejável), são importantes para os processos fisiológicos como por exemplo para o reparo tecidual. Já quando estas alterações ocorrem de forma descontrolada (ou indesejável) estão intimamente relacionadas a patogênese de doenças fibroproliferativas, carcinogênese, invasões e metástases em diferentes tipos de cânceres, doenças autoimunes e doenças parasitárias (Flanders and Roberts, 2000; Alberts et al. 2002).

A influência de TGF-β1 na proliferação de vários tipos celulares em processos fisiológicos e patológicos esta bem estabelecida na literatura. Artigos recentes relacionam a resposta proliferativa de fibroblastos dermais a TGF-β1 em úlceras crônicas (Lal BK et al., 2003). TGF-β1 esta relacionado com a patogênese da doença de Dupuytren, uma doenca fibroproliferativa afetando palmas e dedos das mãos, onde o efeito proliferativo e fibrogênico de TGF-β1 se apresenta exacerbado (Bayat A et al., 2003). Na regulação do processo proliferativo de fibroblastos cardíacos, TGF-β1 induz a produção de CNP (C-type natriuretic peptide) um regulador autócrino que evita o excesso de fibrose cardiaca.(Horio T et al.2003) Em pulmões de pacientes com fibrose pulmonar idiopática, abestosis crônica ou pneumonite, a expressão de TGF-β1 se mostra bastante elevada, inicialmente este aumento na expressão é notado

em macrófagos alveolares e posteriormente também nas células epiteliais (Flanders and Roberts, 2000). Simmons et al. mostraram que TGF-β1 estimula a proliferação de fibroblastos intestinais, além de estimular a síntese de colágeno tipo 1 nestas mesmas células (Simmons et al. 2002). Em fibromatose gengival hereditária, uma doença rara que afeta a cavidade oral e é caracterizada por um lento e progressivo aumento gengival, nós demostramos uma íntima relação de TGF-β1 com o maior potencial proliferativo de fibroblastos gengivais (Andrade et al). Nossos resultados demonstraram que a neutralização de TGF-β1 significantemente reduz a proliferação celular de fibroblastos gengivais isolados de pacientes com esta doença, trazendo os índices de proliferação próximos aos níveis de fibroblastos gengivais normais. Recentemente, nós demonstramos que a adição de TGF-β1 a fibroblastos gengivais humanos simultaneamente induz a expressão de colágeno tipo I e reduz os níveis de MMP-1 e MMP-2 (Martelli-Junior et al., 2002).

Tradicionalmente é inferida a produção local de matriz extracelular como causa do aumento gengival em pacientes com fibromatose gengival hereditária e hiperplasias gengivais induzidas por drogas. Contudo, recentes estudos demostram a importância dos mecanismos que regulam a proliferação celular e à síntese e degradação da matriz extracelular pelo acúmulo excessivo de tecido fibroso. A matriz extracelular é composta por várias famílias de proteínas, entre elas encontramos os colágenos, as lamininas, as tenascinas e os proteoglicanos. Além dessas quatro familias serem compostas por proteínas grandes e de alta massa molecular, elas também apresentam dominios

estruturais similares, favorecendo uma grande variedade de interações entre e[illegible] (Bosman and Stamenkovik, 2003). Apresentando uma estrutura [illegible]amica, sua composição (em especial os colágenos), sendo regulada por [illegible]ários fatores como a cinética celular, produção de citocinas e fatores de crescimento, atividade fagocitária, indução de apoptose, expressão de metaloproteinases de matriz (MMPs) e seus inibidores teciduais (TIMPs). De forma interessante, todos estes processos ja foram avaliados nos aumentos gengivais induzidos por drogas. Embora estes fatores sejam importantes na regulação da matriz extracelular, as MMPs parecem ser as principais enzimas relacionadas a dinâmica da matriz extracelular (Bosman and Stamenkovik, 2003; Stamenkovik, 2003).

As MMPs são endopeptidases dependentes de zinco, conhecidas pela sua capacidade de degradar várias macromoléculas da matriz extracelular. (Birkedal-Hansen 1993) Estas enzimas desempenham um papel fundamental em muitos processos fisiológicos como remodelamento tecidual durante o desenvolvimento, involução pós-parto, angiogênese, cicatrização e migração celular (Woessner, 1991). Na estrutura das MMPs é possível identificar um domínio de ativação e outro domínio do núcleo enzimático, o qual contém o sítio de ligação ao zinco (Birkedal-Hansen 1993). Todos os 23 membros da família MMP compartilham um razoável grau de homologia entre si. Apesar disto, existe uma especificidade de substratos. Por exemplo, apesar dos 50% de homologia entre colagenase de fibroblasto (MMP-1) e estromelisina (MMP-3), ambas as enzimas apresentam substratos diferentes. Enquanto a estromelisina é capaz de degradar vários substratos incluindo proteoglicanos e

gelatina, a colagenase tem sua atividade dirigida primariamente contra moléculas de colágeno nativo (Harris et al., 1984; Gross & Nagai, 1965). A especificidade das colagenases pelo colágeno nativo é surpreendente. Elas clivam ambas as cadeias da tripla hélice em um único ponto da molécula entre um resíduo de glicina e um de leucina ou isoleucina dependendo da cadeia alfa em questão (Harris et al., 1984). A atividade catalítica das MMPs é regulada em múltiplos níveis, os quais incluem transcrição, secreção, ativação proteolitica da forma zimógeno e inibição da enzima ativa por inidores endógenos, entre estes os principais são os TIMPs. A expressão gênica das MMPs é regulada por uma variedade de agentes, incluindo a citocina TGF-β1. Em resposta a TGF-β1, fibroblastos reduzem a produção de MMP-1 e 2, enquanto elevam os níveis de expressão e produção de e TIMPs e colágeno (Overall et. al. 1989; Wahl et. al. 1993).

Visto que TGF-β1 regula muitos, se não todos, os eventos biológicos sugeridos estarem associados aos aumentos gengivais hereditários e induzidos por drogas (proliferação celular e desequilíbrio síntese/degradação da matriz extracelular), o objetivo deste estudo é determinar o efeito da neutralização do estímulo autócrino de TGF-β1 na proliferação e expressão de MMPs e seus inibidores teciduais em fibroblastos gengivais normais tratados com CyA.

2. OBJETIVO

1. *Determinar o efeito de ciclosporina A na expressão e produção de TGF-β1 em fibroblastos gengivais humanos.*
2. *Analisar o efeito de Ciclosporina A na expressão e atividade enzimática de MMP-1 e 2 e TIMP-1 e 2 em fibroblastos gengivais.*
3. *Verificar o efeito de Ciclosporina A e TGF-β1 na proliferação de fibroblastos gengivais.*
4. *Analisar a influência da neutralização do estímulo autócrino de TGF-β1 na proliferação celular e na expressão de MMPs e TIMPs em fibroblastos gengivais humanos tratados com ciclosporina A.*

3. CAPÍTULO 1

Expression of Matrix Metalloproteinases in Cyclosporin – Treated Gingival Fibroblasts is Regulated by Transforming Growth Factor (TGF) – β1 Autocrine Stimulation

Expression of Matrix Metalloproteinases in Cyclosporin-Treated Gingival Fibroblasts Is Regulated by Transforming Growth Factor (TGF)-β1 Autocrine Stimulation

P. Cotrim,* C.R. de Andrade,* H. Martelli-Junior,* E. Graner,* J.J. Sauk,† and R.D. Coletta*

Background: Gingival overgrowth is a common side effect following the administration of cyclosporin A (CsA). The pathogenesis of this condition is not fully understood; however, recent studies show that CsA regulates the transcription of several cytokines including transforming growth factor-β1 (TGF-β1). The aim of this study was to investigate the potential role of TGF-β1 in the pathogenesis of CsA-induced gingival overgrowth, exploring a possible autocrine stimulation of TGF-β1 as a cellular regulator of synthesis of matrix metalloproteinases (MMPs) and its tissue inhibitors (TIMPs).

Methods: Gingival fibroblasts from human normal gingiva were incubated with increasing concentrations of CsA, cultured for 24 hours, and the expression and production of TGF-β1 determined by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. MMPs and TIMPs mRNA expression levels were also analyzed by RT-PCR. To determine the effect of TGF-β1 on the expression of MMPs and TIMPs by human gingival fibroblasts under CsA treatment, human gingival fibroblasts cultures were treated with sense oligonucleotides (SON) or antisense oligonucleotides (AON).

Results: CsA simultaneously stimulated TGF-β1 expression and production and inhibited expression of MMP-1 and MMP-2 by human gingival fibroblasts, whereas has a slight effect on TIMP-1 and TIMP-2 expression. AON reduced TGF-β1 production as demonstrated by ELISA, whereas TGF-β1 mRNA expression levels were not significantly modified. The inhibition of TGF-β1 production by AON modulated MMPs expression, demonstrating the autocrine inhibitory effect of TGF-β1 in CsA-treated human gingival fibroblasts.

Conclusions: The data presented here suggest that TGF-β1 in an autocrine fashion may contribute to a reduction of proteolytic activity of human gingival fibroblasts in CsA-induced gingival overgrowth, which favors the accumulation of extracellular matrix. *J Periodontol 2002;73:1313-1322.*

KEY WORDS

Cyclosporin A; transforming growth factor-β1; matrix metalloproteinases; gingival overgrowth.

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Cyclosporin A (CsA) is a hydrophobic cyclic endecapeptide with unsurpassed immunosuppressive activity.[1] CsA is used for the treatment of several diseases such as rheumatoid arthritis, psoriasis, nephrotic syndrome, and inflammatory bowel disease and to prevent organ rejection in transplantation.[2] Studies on the biological mechanisms of CsA have shown that the drug-cyclophilin complex binds and inhibits calcineurin, a calcium and calmodulin-dependent serine threonine phosphatase. This inactivation prevents dephosphorylation of nuclear factor of activated T cells (NF-AT), the nuclear import of NF-AT, and the formation of a transcriptionally active NF-AT complex. The net consequence, inhibition of interleukin-2 (IL-2) gene expression at the transcriptional level, is considered to be the primary mechanism for the immunosuppressive activity of CsA.[3] The use of CsA is associated with significant side effects, including nephropathy, hypertension, hepatotoxicity, thromboembolic complications, neurotoxicity, hypertricosis, and gingival overgrowth.[4-9] Gingival overgrowth is observed in 25% to 81% of the patients undertaking CsA.[10] Such individual susceptibility to CsA-induced gingival overgrowth may be due to variations in drug responsiveness and tolerance. Clinically, gingival overgrowth is observed initially as a papillary enlargement, which is prominent on the labial aspects and coalesces, resulting in a lobulated appearance.[11]

Despite extensive studies, the exact mechanism of induction of gingival overgrowth is unknown. Evidence obtained from both human and animal models shows that CsA, in contrast to its inhibitory activity on IL-2 gene transcription, stimulates the production of the cytokine TGF-β1. This potent CsA effect on TGF-β1 transcription and secretion has been observed in vitro in human T lymphocytes, mouse proximal tubular cell lines and tubulointerstitial fibroblasts, and in vivo in mouse and rat kidneys.[12-14] Recently, we demonstrated that CsA at similar concentrations found in the serum of patients undergoing CsA treatment inhibits significantly the production and activity of MMPs.[15] MMPs are zinc-dependent endopeptidases known for their ability to cleave several extracellular matrix molecules.[16] Extensive evidence exists on the role of MMPs in mediating normal and pathological processes, including embryogenesis, wound healing, inflammation, arthritis, cancer, and fibrotic diseases.[17-23] The catalytic activity of the MMPs is regulated at multiple levels including transcription, secretion, activation, and inhibition. The latter is accomplished by members of the TIMP family (TIMP-1 to TIMP-4). MMPs and TIMPs gene expression is regulated by a variety of agents including the TGF-β1 stimulation.[24-26] Our previous results demonstrated that increased levels of TGF-β1, in autocrine fashion, reduce the levels of MMP-1 and MMP-2 affecting a state conducive to the net accumulation of extracellular matrix in hereditary gingival fibromatosis.[27] The aim of the present study was to analyze the effect of CsA on expression and production of TGF-β1, MMPs, and TIMPs by human gingival fibroblasts. Additionally, we assessed the role of autocrine stimulation by TGF-β1 on the MMPs and TIMPs expression of these cells using antisense oligonucleotides directed against and overlapping the translation-start site of the TGF-β1 mRNA.

MATERIALS AND METHODS

Cell Culture

Human normal gingival fibroblast primary cultures were established using standard explant culture method as described previously.[28] Gingival samples from the attached gingiva were obtained by biopsies using 0.5 mm punchs from 2 volunteers with clinically normal gingiva without evidence of inflammation, hyperplasia, or history of taking drugs associated with gingival overgrowth. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)[‡] containing 10% fetal bovine serum (FBS), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C in a 5% CO_2 air atmosphere. All experiments were performed using cells between the third and tenth passages. The study protocol was approved by the Ethical Committee in Research at the University of Campinas Dental School.

Treatment of Human Gingival Fibroblasts With CsA

Human gingival fibroblasts grown to confluence in 25 cm^2 culture flasks[§] in DMEM containing 10% FBS were washed with phosphate buffered saline (PBS), and cultured in serum-free DMEM containing 0, 100, or 200 ng/ml of CsA[‖] for 48 hours. Following serum starvation, the medium was replaced to fresh serum-free DMEM containing the same concentrations of CsA and the cells incubated by an additional period of 24 hours. After treatment, the effects of CsA on TGF-β1 expression and production by human gingival fibroblasts were analyzed using semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The effect of CsA on MMPs and TIMPs expression was also analyzed.

RNA Extraction and RT-PCR Analysis

The expression of TGF-β1, MMP-1, MMP-2, TIMP-1, and TIMP-2 in human gingival fibroblasts after CsA treatment was assessed by RT-PCR. Total cellular RNA was isolated after the methods of Chomezynski and Sacchi[29] using a reagent kit.[‡] The concentration and purity of RNA in each sample was determined by spectrophotometry.[¶] Two micrograms of total RNA per sample were used to generate cDNAs using H-reverse transcriptase.[‡] The resulting cDNAs were subsequently amplified in a 50 μl reaction mixture containing 1 μM of each primer,[30,31] 2 mM $MgCl_2$, 0.8 mM dNTPs, and 0.25 U/μl Taq DNA polymerase. β-actin was used as a housekeeping control. Primers for TGF-β1, MMPs, TIMPs, and β-actin are showed in Table 1. For TGF-β1 and β-actin amplifications, 34 cycles of amplification were performed in a thermocycler,[#] followed by a final extension of 10 minutes at 72°C. The cycling parameters were denaturation for 30 seconds at 94°C, annealing for 1 minute at 55°C, and extension for 1 minute at 72°C. For MMPs and TIMPs, 40 cycles of amplification were made with the following parameters denaturation for 45 seconds at 93°C, annealing for 45 seconds at 58°C, and extension for 90 seconds at 72°C. After amplification, 2 μl of each PCR product were electrophoresed on a 5% non-denaturing polyacrylamide gel and the PCR products silver stained as described by Sanguinetti et al.[32] The intensities of the PCR product bands were determined.** Comparisons between different RT-PCR assays were made after normalization of the TGF-β1, MMPs, or TIMPs densitometric scans dividing each optical density value by β-actin value. The resulting relative optical values were transformed in percentage of CsA stimulation in ratio to control and used for statistical analysis.

‡ Gibco, BRL, Gaithersburg, MD.
§ Nunc A/S, Roskilde, Denmark.
‖ Sandimmun-Sandoz, Brazil.
¶ Genesys 2; Spectronic Inst., Rochester, NY.
# Perkin Elmer, Foster City, CA.
** Bio Rad Lab., Hercules, CA.

Table 1.
Description of Primers Used in RT-PCR

	Primer	Sequence 5′ → 3′	Predicted size (base pairs)
TGF-β1	Sense	AAG TGG ATC CAC GAG CCC AA	247
	Antisense	GCT GCA CTT GCA GGA GCG CAC	
MMP-1	Sense	GGT GAT GAA GCA GCC CAG	438
	Anti-sense	CAG TAG AAT GGG AGA GTC	
MMP-2	Sense	CCA CGT GAC AAG CCC ATG GGG CCC C	480
	Anti-sense	GCA GCC TAG CCA GTC GGA TTT GAT G	
TIMP-1	Sense	TGC ACC TGT GTC CCA CCC CAC CCA CAG ACG	551
	Anti-sense	GGC TAT CTG GGA CCG CAG GGA CTG CCA GGT	
TIMP-2	Sense	CCG AAT TCT GCA GCT GCT CCC CGG TGC ACC CG	590
	Anti-sense	GGA AGC TTT TAT GGG TCC TCG ATG TCG AG	
ß-actin	Sense	TCA GAA GGA CTC CTA TGT GG	506
	Anti-sense	TCT CTT TGA TGT CAC GCA CG	

ELISA

TGF-β1 in culture supernates of human gingival fibroblasts treated with different concentrations of CsA was quantified by ELISA. Before measurement, the supernates containing the latent form of TGF-β1 were converted to an active form by treatment with 0.167 M HCl for 10 minutes, followed by neutralization with 1.2 M NaOH in 0.5 M HEPES. The remaining cells were harvested using 0.2% trypsin[††] and counted.[‡‡] The samples were assayed using an ELISA kit[§§] according to the manufacturer's instructions.

Neutralization of TGF-β1 Translation Using Antisense Oligonucleotides

Neutralization of TGF-β1 autocrine stimulation on CsA-treated human gingival fibroblasts was performed as described by Le Roy et al.[33] with some modifications. Fibroblasts grown to 70% confluence in 25 cm^2 flask were incubated with serum-free DMEM containing 200 ng/ml of CsA for 48 hours. The cells were transfected in serum free-DMEM with sense oligonucleotides (SON) or antisense oligonucleotides (AON) by a cationic liposome-mediated transfection method. Antisense and sense 15-base deoxyribonucleotides corresponding to the translation-initiation region of TGF-β1 mRNA were: antisense 5′ GGA GGG CGG CAT GGG 3′ and sense 5′ CCC ATG CCG CCC TCC 3′. Briefly, oligonucleotides dissolved in one volume of DMEM without FBS were mixed with lipofectAMINE reagent[‡] dissolved in the same volume of medium and incubated for 45 minutes at room temperature. Thereafter, the oligonucleotide-liposome complexes were diluted with 8 volumes of medium resulting in DMEM containing 200 ng/ml of CsA, 1 μg/ml of each oligonucleotides, and 1.25% of lipofectAMINE, which was used to overlay to the cells for 24 hours. After incubation, the efficiency of the inhibition of TGF-β1 was determined using RT-PCR and ELISA. The role of autocrine stimulation of TGF-β1 on expression of MMPs and TIMPs by CsA-treated human gingival fibroblasts was determined as described above.

Statistical Analysis

The data are expressed as percentage of CsA stimulation in ratio to control (without CsA) and represent the mean ± SEM of 2 cell lines. Non-parametric one-way analysis of variance (ANOVA) and multiple comparisons were used to test the treatments based on Kruskal-Wallis test at 5% significance ($P < 0.05$). All assays were performed at least three times in independent experiments for each cell line.

RESULTS

Effect of CsA on the Expression and Production of TGF-β1 by Human Gingival Fibroblasts

Both RT-PCR and ELISA were used to verify whether CsA could affect TGF-β1 expression and production by human gingival fibroblasts. The semiquantitative RT-PCR assay was used in this study for the measurement of steady state levels of TGF-β1 mRNA. Total RNA was isolated from human gingival fibroblasts treated with 0, 100, or 200 ng/ml of CsA for 24 hours, and TGF-β1 mRNA and the constitutively expressed β-actin mRNA (control for RT-PCR) were amplified using sequence-specific primers pairs (Fig. 1A). The mRNA levels were semiquantitated by densitometric scanning and TGF-β1 mRNA expression was normalized to that of β-actin mRNA display. For densitometric scans, all bands of the β-actin PCR products were used. RT-PCR studies revealed that CsA treatment results in an increased expression of TGF-β1 mRNA (Fig. 1B; $P < 0.01$). The TGF-β1 expression in human gingival fibroblasts treated with 100 and 200 ng/ml of CsA was elevated in 29.8 ± 10.1% and 81.4 ± 40.5% (mean ± SEM of stimulation) of the value observed in corresponding normal control fibroblasts, respectively. To confirm these findings ELISA was performed revealing that normal cells treated with CsA dramatically elevated the TGF-β1 production in 60-120% of that observed in corre-

†† Sigma Chemical Co., St. Louis, MO.
‡‡ Coulter Electronics, Luton, UK.
§§ R & D Systems, Minneapolis, MN.

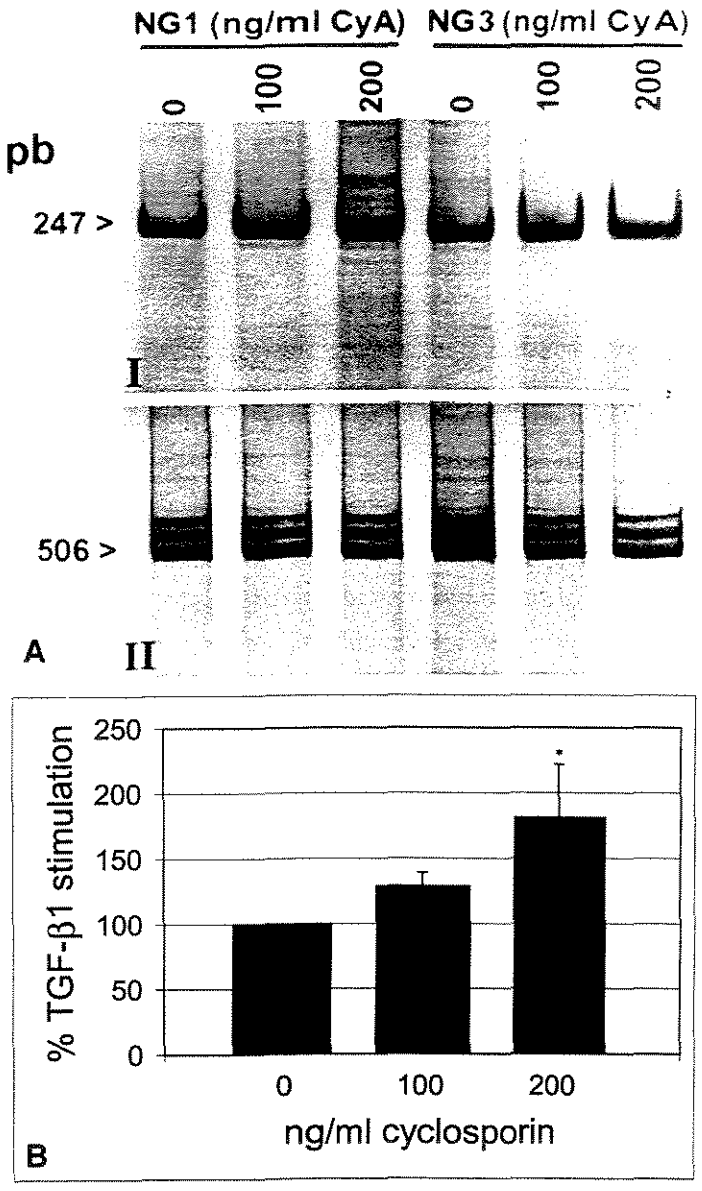


Figure 1.
Effect of CsA on TGF-β1 expression by human gingival fibroblasts. ***A.*** *Total RNA was isolated and cDNA synthesized by reverse transcriptase. After amplification using specific primers to TGF-β1 (panel I) and β-actin (panel II), the products were resolved in a 5% polyacrylamide gel and silver stained. Lanes are indicated as CsA concentration utilized.* ***B.*** *Comparison of TGF-β1 mRNA expression in human gingival fibroblasts (NG1 and NG3 lines) treated with CsA. CsA at 200 ng/ml significantly stimulated the expression of TGF-β1. Values represent the means ± SEM from 3 independent experiments for each cell line and are expressed as the percentage of TGF-β1 stimulation normalized by β-actin. *P <0.01.*

sponding human gingival cell lines without CsA treatment (Fig. 2; $P<0.001$). TGF-β1 production was 62.1% higher in human gingival fibroblasts treated with 100 ng/ml of CsA in comparison with control cells, whereas for human gingival fibroblasts treated with 200 ng/ml of CsA was 118.8% higher.

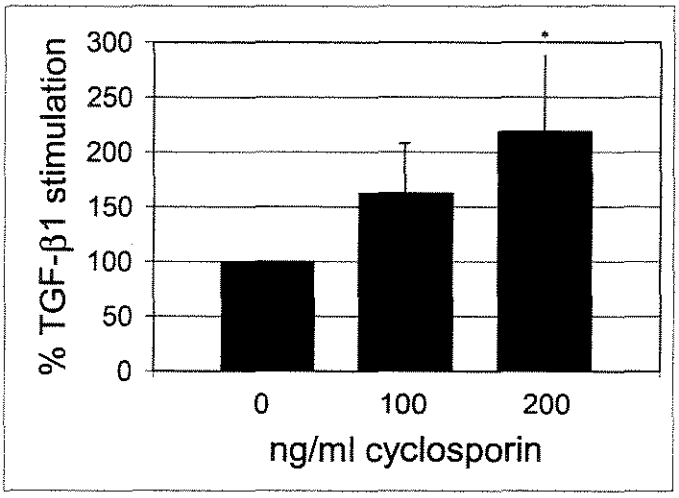


Figure 2.
*Effect of CsA on production of TGF-β1 by human gingival fibroblasts. TGF-β1 in culture supernates of CsA-treated human gingival fibroblasts was activated by brief acidification followed by capture ELISA. Data are the mean ± SEM and represent 3 experiments in triplicate for each cell line. The amount of TGF-β1 produced by human gingival fibroblasts was significantly stimulated by CsA *P <0.001.*

Effect of CsA on the Expression of MMPs and TIMPs by Human Gingival Fibroblasts

We also determined whether CsA could modulate MMPs expression in the human gingival fibroblasts. Figure 3A (panels I and II) depicts the expected amplification products of 438 and 480 base pairs that were detected in CsA-treated human gingival cells with the MMP-1 and MMP-2 primers, respectively. Normalization for differences in mRNA loading revealed that the treatment of human gingival fibroblasts with CsA produced a significant reduction of MMP-1 and MMP-2 expression in a dose dependent manner (Fig. 3B; $P<0.01$). The MMP-1 expression in human gingival fibroblasts treated with 200 ng/ml of CsA was reduced to 65.3% of the value observed in corresponding normal control fibroblasts, whereas MMP-2 expression was reduced to approximately 49.3% of the value observed in control fibroblasts. Conversely, similar assays for TIMP expression revealed that TIMP-1 and TIMP-2 mRNA levels were increased after CsA treatment (Fig. 3B, panels III and IV). However, TIMP expression differences were not statistically significant.

Effect of Antisense Oligonucleotides on Expression and Production of TGF-β1 by Human Gingival Fibroblasts

Since CsA stimulates TGF-β1 expression and production by human gingival fibroblasts, we sought to determine if the excessive production of this cytokine by normal fibroblast, in an autocrine fashion, could mediate the proteolytic activities of those cells. In an attempt to block endogenous TGF-β1, human gingival fibro-

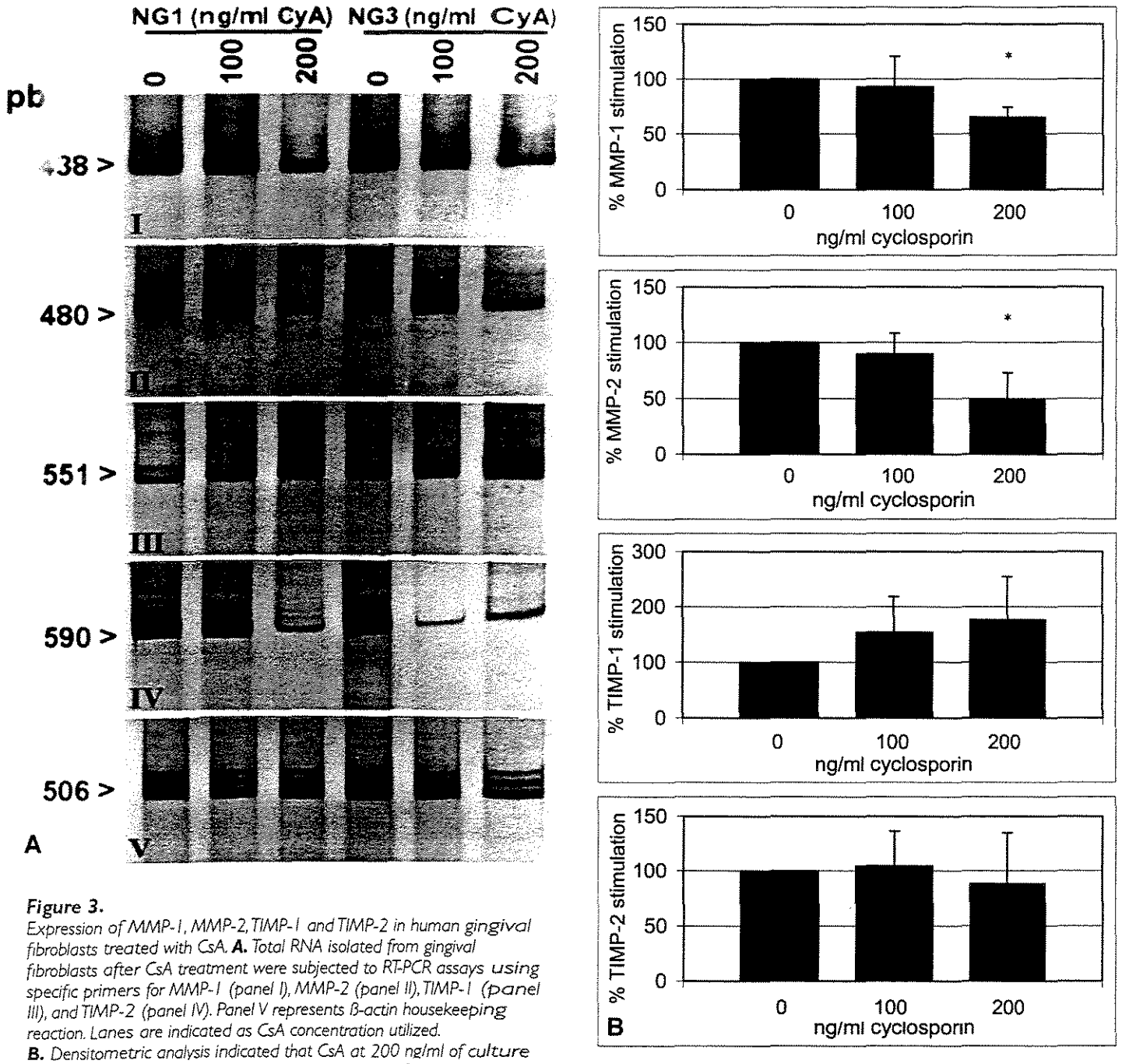


Figure 3.
Expression of MMP-1, MMP-2, TIMP-1 and TIMP-2 in human gingival fibroblasts treated with CsA. ***A.*** *Total RNA isolated from gingival fibroblasts after CsA treatment were subjected to RT-PCR assays using specific primers for MMP-1 (panel I), MMP-2 (panel II), TIMP-1 (panel III), and TIMP-2 (panel IV). Panel V represents ß-actin housekeeping reaction. Lanes are indicated as CsA concentration utilized.*
B. *Densitometric analysis indicated that CsA at 200 ng/ml of culture medium reduced MMP-1 mRNA approximately to 65% and MMP-2 mRNA to 49% of the value observed in corresponding normal control fibroblasts. TIMP-1 and TIMP-2 mRNA levels after CsA treatment were slightly greater or equivalent than those in control cells. Values are expressed as percentage of stimulation mean to specific mRNA levels ± SEM of 3 independent experiments for each cell line. Densitometric reading were normalized to equivalent amounts of ß-actin housekeeping mRNA. *P <0.01.*

blasts were transfected with 1 µg/ml of AON complementary to the translation initiation region of human TGF-β1 mRNA. As a control, a corresponding SON was used. The results showed (Fig. 4) that neither AON nor SON modified the levels of TGF-β1 mRNA. In contrast, the investigation of amount of TGF-β1 secreted in culture supernates by CsA-treated normal cells by ELISA revealed that AON significantly reduced TGF-β1 production in approximately 65% (65.8 ± 25.38%) in comparison with SON. Similar results were obtained by Le Roy et al.[33] using fibroblast-like cells isolated from interstitial tissue from testis and by Andrade et al.[34] using gingival fibroblasts. In so doing, these studies revealed the efficacy of AON on the neutralization of TGF-β1 production without modify TGF-β1 mRNA levels.

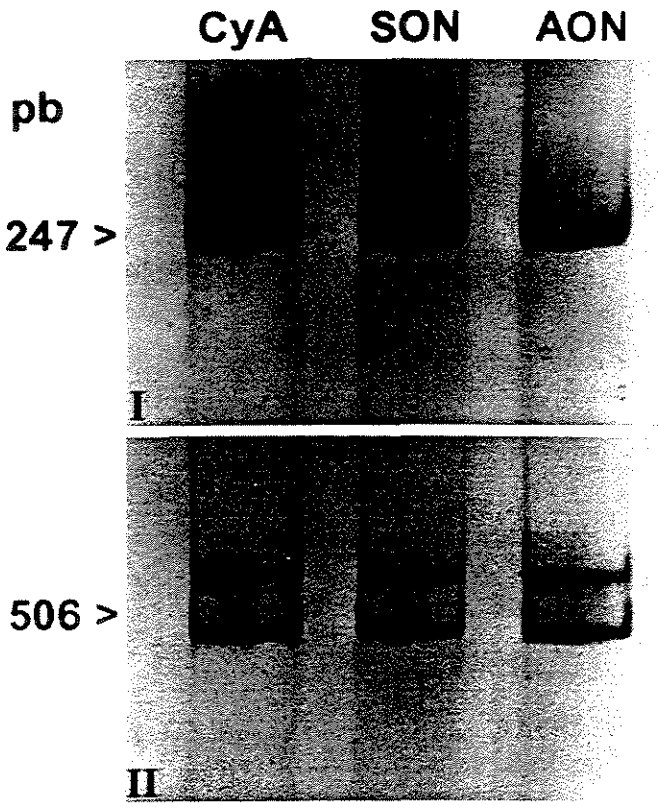


Figure 4.
Representative RT-PCR assay of effect of sense oligonucleotides (SON) and antisense oligonucleotides (AON) on TGF-ß1 mRNA expression by human gingival fibroblasts. Panel I represents specific amplification reaction for TGF-ß1 and panel II for ß-actin. Neither AON nor SON modified significantly the levels of TGF-ß1 transcripts.

Effect of Antisense Oligonucleotides on MMPs and TIMPs Expression by Human Gingival Fibroblasts

Since CsA treatment elevated the expression and production of TGF-β1 and simultaneously inhibited MMPs expression, we examined whether elevate levels of TGF-β1 protein induced by CsA correlates with MMPs and its tissue inhibitors mRNA levels. The results clearly show that the treatment with AON partially abrogated the effects of 200 ng/ml of CsA on expression of MMP-1 and MMP-2 by human gingival fibroblasts in comparison with the treatment with SON. Results show (Fig. 5) that AON increased significantly the MMP-2 expression to CsA treatment about 50% compared to SON ($P < 0.05$). The effect of CsA on MMP-1 expression was partially reverted by AON, but not at significant levels ($P = 0.15$). The expression of TIMP-1 and TIMP-2 was unaffected with AON or SON treatment.

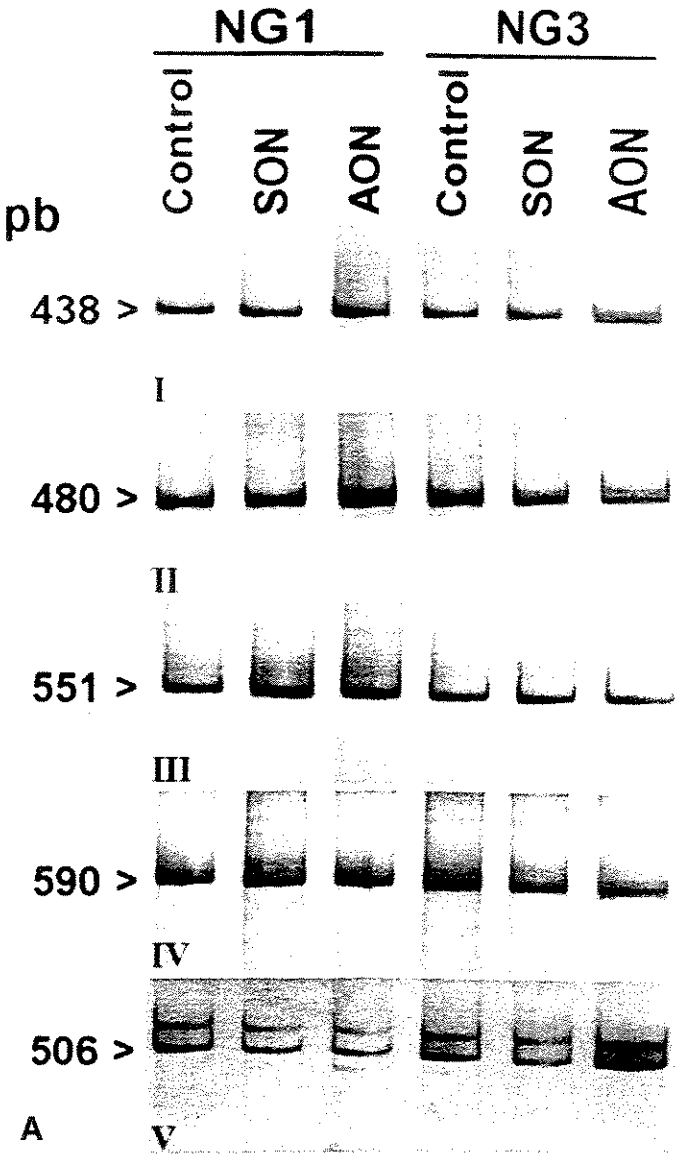
To further test the specificity of the AON, we examined whether exogenous TGF-β1 could reverse the effects of antisense treatment. We demonstrated that 10 ng/ml of TGF-β1 completely reversed the effects of the AON on MMP-1 and MMP-2 expression by CsA-treated human fibroblast cells (Fig. 6).

DISCUSSION

A number of histological and biochemical studies have investigated the changes in tissue composition and cellular function that accompanies CsA-induced gingival overgrowth. It has been demonstrated that CsA affects gingival fibroblast proliferation,[35] promotes abnormal synthesis of collagen and other extracellular matrix molecules,[36] decreases collagen turnover by inhibiting collagen phagocytosis and collagen degrading enzymes,[37,38] and also appears to act on abnormal cytokines production.[39,40] Although the exact mechanism by which CsA induces gingival overgrowth remains unclear, our understanding of the biochemical mechanisms involved in producing these tissue alterations is evolving. For example, increased TGF-β1 production has recently been demonstrated in CsA-induced gingival overgrowth[41] as well as in other fibrotic side effects of CsA-treatment.[42,43]

TGF-β1 is a multifunctional peptide that regulates diverse biologic activities including cell growth, cell death or apoptosis, cell differentiation, and extracellular matrix synthesis.[44] TGF-β1 is believed to be a key mediator of tissue fibrosis as a consequence of extracellular matrix accumulation in pathologic states such as hereditary gingival fibromatosis[27] and progressive renal diseases including CsA-induced nephropathy.[45] TGF-β1 actions are mediated by the heteromeric interactions of types I and II serine/threonine kinase receptors. Initiation of signaling requires binding of TGF-β1 to TGF-β type II receptor, a constitutively active serine/threonine kinase, which subsequently transphosphorylates TGF-β type I receptor. Following the initial receptor interaction with ligand, evidence suggests the transcriptional activation of mitogen-activated protein kinase (MAPK) and CTF-1, the prototypic member of the CTF/NF-I family of transcription factors. This induction correlates with the proposed role of CTF/NF-I binding sites in collagen and MMP gene inductions by TGF-β. However, the exact mechanism of TGF-β signal transduction remains poorly understood.[46]

Our previous study showed that CsA significantly inhibits the production and activity of MMP-1, MMP-2 and MMP-3 at the same concentrations found in the serum of patients undertaken CsA-treatment.[15] Since TGF-β1 expression is stimulated by CsA and its effect can modulate MMPs and TIMPs production as well as stimulate fibroblasts proliferation and collagen synthesis, we were interested in determining whether human gingival fibroblasts after CsA treatment alter TGF-β1 expression and production. To the best of our knowledge, no previous investigation has analyzed the direct effect of CsA on TGF-β1 expression and production by normal gingival fibroblasts. In this study we have determined that CsA simultaneously upregulates the expression and production of TGF-β1 and downregulates MMP expression from human gingival



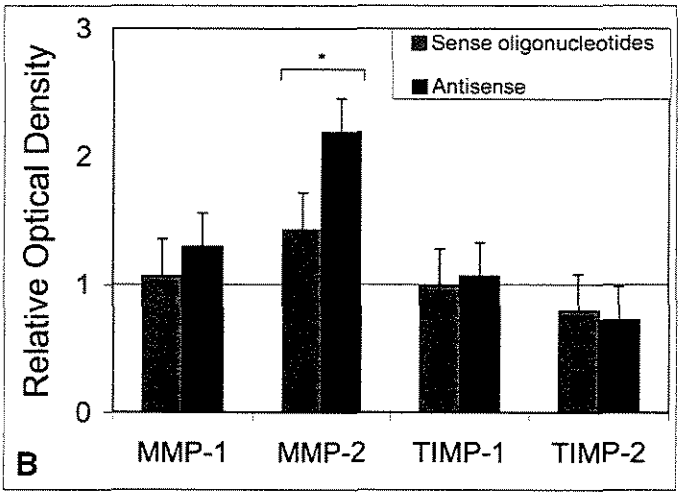
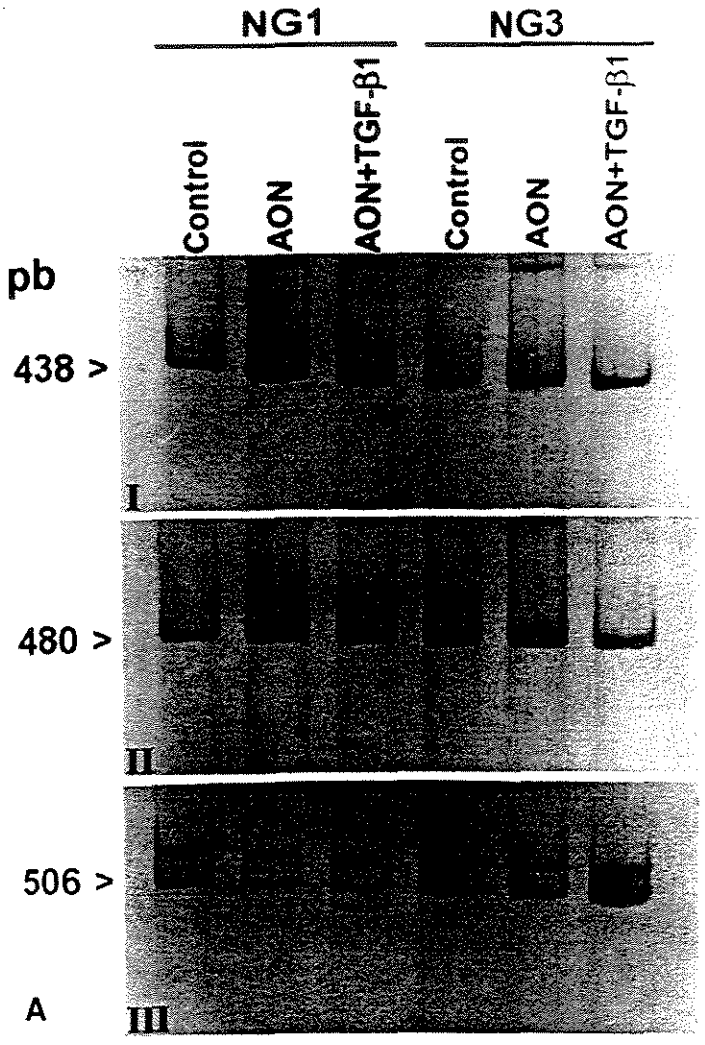


Figure 5.
Effect of sense oligonucleotides (SON) and antisense oligonucleotides (AON) on MMP-1, MMP-2, TIMP-1, and TIMP-2 expression by CsA-treated human gingival fibroblasts. ***A.*** *Total RNA was isolated from human gingival fibroblasts treated with 200 ng/ml of CsA and with 200 ng/ml of CsA plus SON or AON. After reverse transcriptase, cDNA was amplified for MMP-1 (panel I), MMP-2 (panel II), TIMP-1 (panel III), TIMP-2 (panel IV), and ß-actin (panel V).* ***B.*** *Densitometric analysis showing that AON reversed significantly CsA effect on MMP-2 mRNA expression levels. Values are expressed as optical density units normalized by ß-actin and represent the mean ± SEM of 3 independent assays for 2 cell lines.* *$P < 0.05$.

fibroblasts. Furthermore, blocking TGF-β1 synthesis with AON resulted in an increased effect of CsA on the MMP-1 and MMP-2 expression by gingival fibroblasts, whereas TIMP-1 and TIMP-2 were unaffected. Additionally, our data also revealed that the TGF-β1 signaling pathways are not compromised by the treatment with oligonucleotides, since exogenous TGF-β1 reversed the effects of the AON.

The collagen turnover in gingival tissues promoted by fibroblasts occurs by 2 systems: the phagocytic pathway and the MMP-dependent pathway.[18,47] CsA may alter both pathways, reducing phagocytosis or inhibiting the secretion of MMPs by gingival fibroblasts. The role of MMP-1 in interstitial collagen degradation is well documented. On the other hand, MMP-2 inhibition may contribute to an abnormal accumulation of glycosaminoglycans and proteoglycans, substrates for MMP-2, in the CsA-treated gingival tissue. Consequently, the scenario in which a net reduction in degradative activity exists in CsA-treated normal gingival cells may contribute to the increased collagen content even in the absence of increased collagen gene expression. Recently, Arora et al.[38] have demonstrated that CsA inhibits fibroblast collagen phagocytosis by blocking calcium release from endoplasmatic reticulum. Since TGF-β1 induces calcium release from endoplasmatic reticulum and mitochondria, stimulating several cellular effects as cell proliferation,[48,49] the decrease on fibroblasts phagocytosis under CsA treatment may be due to TGF-β1 overexpression.

The studies presented here also revealed that TIMP levels after CsA treatment were slightly greater than those in control cells and remained unaltered following neutralization with TGF-β1 antisense oligonucleotides. Although the effect of CsA on TIMP expression levels was not statistically significant, the stimulation of TIMP-1 was stronger than of TIMP-2. The effects of CsA on TIMP-1 expression by human fibroblasts strains display both inter-individual and intra-individual heterogeneity,[50] and in human dermal fibroblast cultures CsA does not affect TIMP-1 expression.[51] On the other hand, CsA-induced focal interstitial fibrosis is associated with locally increased expression of TMP-1 rather than a decrease of MMP expression.[52] Interestingly, TIMPs expression pattern is the same in hereditary gingival fibromatosis fibro-



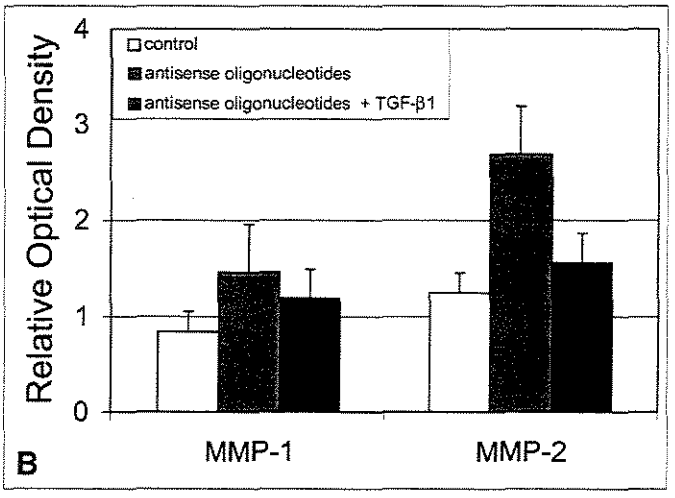


Figure 6.
Reversion of effect of antisense oligonucleotides (AON) on MMP-1 and MMP-2 expression by addition of exogenous TGF-ß1. Human gingival fibroblasts were cultured for 24 hours in presence of 200 ng/ml of CsA, 1 µg/ml of AON, and 10 ng/ml of TGF-ß1. RT-PCR ***(A)*** *and densitometric analysis* ***(B)****. Panel I represents MMP-1, panel II represents MMP-2, and panel III represent ß-actin. The reversion showed that TGF-ß1 pathway is independent of treatment with AON.*

blasts and in gingival fibroblasts after CsA treatment.[27] The reciprocal findings of MMPs and TIMPs in hereditary gingival fibromatosis compared to CsA-induced gingival overgrowth would suggest that different populations of gingival cells are affected, or that the TGF-β1 is not the only pathway involved. Analogously, alterations in the MMPs and TIMPs expression have been implicated in a variety of other diseases that include fibrosis as a component.[53-55] Collectively, these studies indicate that dysregulation of the proteolytic equilibrium is a common basis for pathological fibrosis in various organ systems including the gingiva of patients with hereditary gingival fibromatosis as well as in CsA-induced gingival overgrowth.

In summary, our investigation of the molecular events that lead to CsA-induced gingival overgrowth shows a stimulation of TGF-β1 expression and production and dowregulation of MMP-1 and MMP-2 mRNA levels. Additionaly, these effects seem to be mediated by TGF-β1 in an autocrine fashion. Thus a CsA-induced alteration in the TGF-β1 profile within gingival tissues may underlie the clinical changes which present as gingival overgrowth.

ACKNOWLEDGMENTS

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4. CAPÍTULO 2

Cyclosporin A Induces Proliferation in Human Gingival Fibroblasts Via Induction of Transforming Growth Factor- beta1

Cyclosporin A induces proliferation in human gingival fibroblasts via induction of transforming growth factor-beta1

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Background: Cyclosporin A (CyA) is a widely used immunosuppressant which causes significant side effects including gingival overgrowth. The pathogenesis of this condition is not fully understood; however, recent studies show that CyA regulates the transcription of several cytokines including transforming growth factor-beta1 (TGF-β1). In this work we evaluated the effects of CyA and TGF-β1 on human normal gingival (NG) fibroblast proliferation, and explored a possible autocrine stimulation of TGF-β1 as a cellular regulator of proliferation induced by CyA in NG fibroblasts.

Methods: NG fibroblast cell lines were incubated with increasing concentrations of CyA or TGF-β1 and the proliferation index determined by automatic cell counting, BrdU incorporation, PCNA expression and mitotic potential. To determine the effect of TGF-β1 on the proliferation rate of NG fibroblasts under CyA treatment, NG fibroblast cultures were simultaneously treated with CyA and antisense oligonucleotides against the translation-start site of the TGF-β1 mRNA.

Results: Treatment of NG fibroblasts with CyA or TGF-β1 stimulated significantly the cell proliferation in a dose-dependent manner. Furthermore, neutralization of TGF-β1 production in CyA-treated NG fibroblast inhibited CyA effect on the NG fibroblasts proliferation, demonstrating an autocrine stimulatory effect of TGF-β1 in CyA-treated NG fibroblast proliferation.

Conclusion: The results presented here suggest that CyA stimulatory induction of NG fibroblast proliferation is mediated through of TGF-β1 in an autocrine fashion.

KEY WORDS

Cyclosporin A; transforming growth factor-β1; gingival overgrowth; proliferation.

RUNNING TITLE

CyA and TGF-β1 in NG fibroblast proliferation.

Cyclosporin A (CyA) is a cyclic undecapeptide with highly specific immunosuppressive effects that is commonly used in the treatment of allograft rejection.[1] Previous immunological studies have revealed that CyA blocked calcium and antigen-dependent proliferation of T lymphocytes by inhibiting specific nuclear transcription factors that regulate expression of cytokine genes such as interleukin-2.[2] Formation of complexes between CyA and cyclophilin A blocks the calcium-activated phosphatase activity of calcineurin resulting in the inhibition of the translocation of nuclear factor of activated T-cell dephosphorylation into the nucleus, thus leading to the lack of interleukin-2 expression.[3] The use of CyA is associated with significant side effects, including nephropathy, hypertension, hepatotoxicity, thromboembolic complications, neurotoxicity, hypertricosis and gingival overgrowth.[4-9]

In spite of extensive studies, the exact mechanism by which CyA induces gingival overgrowth is unknown. Evidence obtained from both human and animal models shows that CyA, in contrast to its inhibitory activity on interleukin-2 gene transcription, stimulates the production of the cytokine transforming growth factor-beta1 (TGF-β1). This potent CyA effect on TGF-β1 expression and production has been observed in vitro in T lymphocytes, mouse proximal tubular cell lines and tubulointerstitial fibroblasts, and in vivo in mouse and rat kidneys.[10-12] Recently, we demonstrated that concentrations of CyA that are similar to those found in the serum of immunossupressed patients stimulate significantly the expression and production of TGF-β1 by normal gingival (NG) fibroblasts.[13] TGF-β1 is a cytokine produced by different cell types which acts on various cells including lymphocytes,

macrophages, hepatocytes as well as keratinocytes and fibroblasts.[14] Among its pleiotropic effects, TGF-β1 stimulates cell growth, cell death or apoptosis, cell differentiation and extracellular matrix synthesis.[15] TGF-β1 is believed to mediate tissue fibrosis as a consequence of stimulation of cell proliferation and of extracellular matrix accumulation in pathologic states such as hereditary gingival fibromatosis[16,17] and progressive renal diseases including CyA-induced nephropathy.[18] Fibroblast proliferation can be upregulated by a variety of agents including the cytokine TGF-β1.[19-21] The aim of the present study was to analyze the effects of CyA and TGF-β1 on the proliferation of NG fibroblasts. Additionally, we assessed the role of autocrine stimulation of TGF-β1, which is produced in higher amounts by CyA-treated NG fibroblasts,[13] on the proliferation rates of these cells using antisense oligonucleotides directed against and overlapping with the translation-start site of the TGF-β1 mRNA.

Material and Methods

Cell culture

Human NG fibroblast primary cultures were established using standard explant culture method as described previously.[22] Gingival samples were obtained from the attached gingiva of one Caucasian male and one Caucasian female, with age of 26 and 29 years old respectively. The gingivas were clinically normal without evidences of inflammation or hyperplasia. Cells were cultured in Dulbecco modified Eagle's medium* (DMEM) containing 10% fetal bovine serum (FBS), 100 μg/ml

* Gibco, BRL, Gaithersburg, MD.

penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C in a 5% CO_2 air atmosphere. All experiments were performed using cells between the third and tenth passages. The study protocol was approved by the Ethical Committee in Research at the University of Campinas Dental School.

Effect of CyA and TGF-β1 treatment on cell proliferation

Fibroblasts from NG were plated in triplicate in 24-well culture plates at a density of 30,000 cells/well in DMEM containing 10% FBS and antibiotics. After 16 h, the cells were rinsed with phosphate buffered saline (PBS), and the medium replaced with serum-free DMEM for an additional 24 h. Following serum starvation cells were stimulated with 2% charcoal-treated (CT)-FBS/DMEM supplemented by either 0, 50, 100, 200, 400 and 800 ng/ml of CyA[†] or by 0, 0.001, 0.01, 0.1, 1 and 10 ng/ml of TGF-β1.[‡] Cells were harvested after 24 h using 0.2% trypsin in PBS and counted with a Coulter Counter.[§]

Bromodeoxyuridine-labeling (BrdU) index

NG fibroblasts were plated in 8-well chamber slides[**] at a density of 30,000 cells per well, in 500 μl of DMEM containing 10% FBS. After 16 h, the cells were washed with PBS and cultured in serum-free DMEM for an additional 24 h. Following serum starvation, the medium was replaced by 2% CT-FBS/DMEM containing either 100 and 200 ng/ml of CyA or 0.1 and 1 ng/ml of TGF-β1.

[†] Sandimmun-Sandoz, Sao Paulo, Brazil.
[‡] R&D Systems, Minneapolis, MN.
[§] Coulter Electronics, Luton, England.
[**] Nunc A/S, Roskilde, Denmark.

Proliferation rates were determined 24 h after treatment by measuring BrdU incorporation into DNA. Briefly, BrdU antigen at a dilution of 1:1,000 was added to the cultures and kept for 1 h at 37°C in 5% CO_2. After incubation, cells were washed in PBS and fixed in 70% ethanol for 1 h. BrdU incorporation in proliferating cells was measured using an immunohistochemical analysis kit.[††] The BrdU-labeling index, expressed as the percentage of cells labeled with BrdU, was determined by counting 1,000 cells in three independent reactions for each concentration using an image analysis system.[‡‡]

Proliferating cell nuclear antigen (PCNA) index

Cell suspensions (30,000 cells) were cultured in culture chamber slides at 37°C in humidified air containing 5% CO_2 for 24 h. After incubation with CyA or TGF-β1 for 24 h as described above, the cells were fixed in 70% ethanol for 1 h and washed with PBS. Cells were then treated with 1% bovine serum albumin[§§] (BSA) diluted in PBS for 1 h, incubated with monoclonal antibodies against PCNA[***] (clone PC-10 diluted 1:3,000), and followed by the ABC method (StrepABC Complex/HRP). Reactions were developed with 0.6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) containing 0.01% H_2O_2. The PCNA index was calculated using an image analysis system by counting labeled nuclei of 1,000 cells in three independent reactions, and was expressed as percentage of PCNA positive cells.

[††] Amershan Pharmacia Biotech Inc., Piscataway, NJ.
[‡‡] KONTRON 400, Zeiss, Eching bei Munich, Germany.
[§§] Sigma Chemical Co., St. Louis, MO.
[***] Dako Corp., Carpenteria, CA.

Mitotic index

NG fibroblasts were plated in glass chamber slides and treated with 100 and 200 ng/ml of CyA or 0.1 and 1 ng/ml of TGF-β1. After 24 h, the cells were fixed in 70% ethanol and then stained with hematoxylin and eosin (H&E). Thirty randomly selected microscopic fields of 0.0256 mm^2 (approximately 1,000 cells) were examined for each fibroblast cell line and the number of cells in mitosis determined. Mitotic index was expressed as the percentage of cells in mitosis per field ± SD.

Neutralization of TGF-β1 translation using antisense oligonucleotides

Neutralization of TGF-β1 autocrine stimulation on CyA-treated NG fibroblasts was performed as described by Le Roy et al.[23] with some modifications. Fibroblasts grown to 70%-confluence in 25-cm^2 flasks were incubated with serum-free DMEM containing 200 ng/ml of CyA for 48 h. The cells were transfected in serum free-DMEM with sense or antisense oligonucleotides by a cationic liposome-mediated transfection method. Antisense and sense 15-base deoxyribonucleotides corresponding to the translation-initiation region of TGF-β1 mRNA were: antisense 5' GGA GGG CGG CAT GGG 3' and sense 5' CCC ATG CCG CCC TCC 3'. Briefly, oligonucleotides dissolved in one volume of DMEM without FBS were mixed with lipofectAMINE reagent* dissolved in the same volume of medium and incubated for 45 min at room temperature. Thereafter, the oligonucleotide-liposome complexes were diluted with 8 volumes of medium resulting in DMEM containing 200 ng/ml of CyA, 1 μg/ml of each oligonucleotides and 1.25% of lipofectAMINE,

which was used to treat the cells for 24 h. After incubation, the efficiency of the inhibition of TGF-β1 was determined using enzyme-linked immunosorbent assay (ELISA) and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The role of autocrine stimulation of TGF-β1 on proliferation rate of CyA-treated NG fibroblasts was assessed by automatic counting as described above.

ELISA

After oligonucleotide treatments, the conditioned cell culture medium was used to quantify TGF-β1 production by CyA-treated NG cells, and the cells were incubated in guanidine thiocyanate for total cellular RNA extraction (see below). Before measurement, the latent form of TGF-β1 in the conditioned cell culture medium was converted into the active form, by treatment with 0.167 M HCl for 10 min, followed by neutralization with 1.2 M NaOH in 0.5 M HEPES. The samples were assayed using an ELISA kit[‡] according to the manufacturer's instructions. The values were expressed as percentage of inhibition of TGF-β1.

RNA extraction and RT-PCR analysis

The expression of TGF-β1 in CyA-treated NG fibroblasts after each oligonucleotide treatment was assessed by RT-PCR. Total cellular RNAs were isolated after the methods of Chomezynski and Sacchi[24] using the Trizol™ reagent kit.* The concentration and purity of RNA in each sample was determined by the absorption at 260/280 nm using a spectrophotometer.[†††] Two micrograms of total RNA per

[†††] Genesys 2, Spectronic Inst., Rochester, NY.

sample were used to generate cDNAs using Superscript II RNase H-reverse transcriptase.* The resulting cDNAs were subsequently amplified in a 50 µl reaction mixture containing 1 µM of each primer,[25] 2 mM $MgCl_2$, 0.8 mM dNTPs and 0.025 U/µl Taq DNA polymerase.* β-actin was used as a housekeeping control. Primers used to amplify TGF-β1 were 5' AAG TGG ATC CAC GAG CCC AA 3' (sense) and 5' GCT GCA CTT GCA GGA GCG CAC 3' (antisense). For β-actin, primers used were 5' TCA GAA GGA CTC CTA TGT GG 3' (sense) and 5' TCT CTT TGA TGT CAC GCA CG 3' (antisense). After denaturation for 3 min at 93°C, 34 cycles of amplification were performed using a model 9700 thermocycler,‡‡‡ followed by final extension of 10 min at 72°C. The cycling parameters were: denaturation for 30 s at 94°C, annealing for 1 min at 55°C, extension for 1 min at 72°C. After amplification, 2 µl of PCR product were electrophoresed on a 5% non-denaturing polyacrylamide gel and the PCR products stained with silver as described by Sanguinetti et al.[26]

Statistical analysis

The data represent the mean ± SD of 2 cell lines. One-way analysis of variance (ANOVA) and multiple comparison were used to test group effects and treatments based on Newman-Keuls test at 5% significance ($P<0.05$). All assays were performed at least three times in triplicate or in quadruplicate.

‡‡‡ Perkin Elmer, Foster City, CA.

Results

Effect of CyA on NG cell proliferation

The effect of CyA on the proliferation of NG fibroblasts was initially assessed by automatic counting of cell number. The effect of increasing concentrations of CyA on proliferation of NG fibroblasts is depicted in Fig. 1. CyA at 50-200 ng/ml increased NG proliferation with the maximal stimulation being observed at concentration of 200 ng/ml ($p<0.05$). With increasing concentrations of CyA (400 and 800 ng/ml), NG fibroblast proliferation was inhibited. Our data in conjunction with previous studies[27] that demonstrated a serum level of CyA range between 100-250 ng/ml in patients undergoing CyA-treatment, let us perform all subsequent studies with CyA concentrations of 100 and 200 ng/ml.

Figure 1.

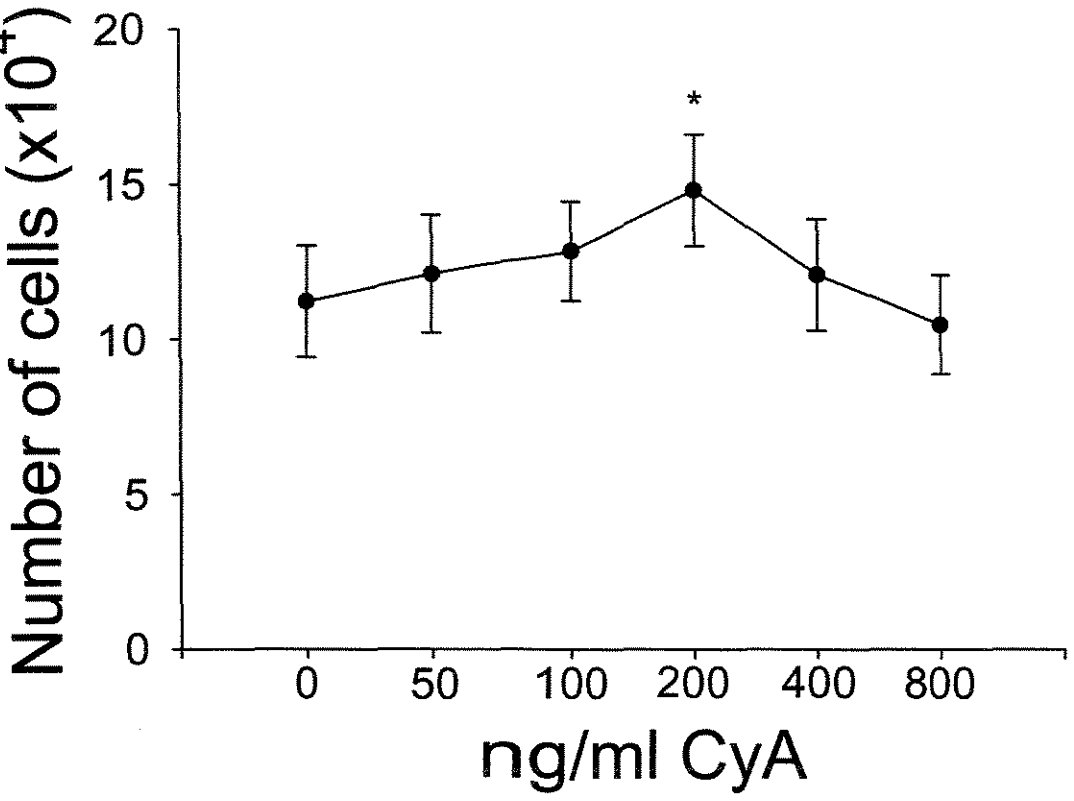

Fig. 1. Effect of CyA on NG fibroblast proliferation. NG fibroblasts were exposed for 24 h to increasing concentrations of CyA, harvested with 0.2% trypsin, and counted with an automated cell counter. Each concentration-point represents the mean of 16 counts for each cell line and is expressed as the mean ± SD of 2 fibroblast cell lines. *$p<0.05$

Our cell growth assays indicated that CyA has a direct effect on the proliferation of NG fibroblasts. To confirm these results, proliferation indexes of NG fibroblasts cultured for 24 h with 100 or 200 ng/ml of CyA were assessed by measuring BrdU incorporation into DNA, PCNA expression, and determination of mitotic potential of the cells. Nuclear immunoreactivity for BrdU and PCNA were clearly and easily identified. Nuclei with a clear brown color, regardless of the intensity of staining, were interpreted as positive. Fig. 2A shows that CyA at 200 ng/ml significantly increased the BrdU-labeling index of NG fibroblasts ($p<0.001$). NG fibroblasts treated with 100 and 200 ng/ml of CyA enhanced BrdU-labeling index by approximately 52.3% and 96.5% respectively, in comparison with the controls without CyA. PCNA levels in NG fibroblasts that were incubated with 200 ng/ml of CyA were also significantly higher than those of control (Fig. 2B; $p<0.01$). In the presence of 100 and 200 ng/ml of CyA, NG fibroblast PCNA indexes were stimulated by approximately 15.4% and 61.4%, respectively, compared to the controls. Mitotic activity measured by direct counting showed significantly higher percentage of cells in mitosis in NG cells treated with 200 ng/ml compared to the controls (Fig. 2C; $p<0.05$).

Figure 2

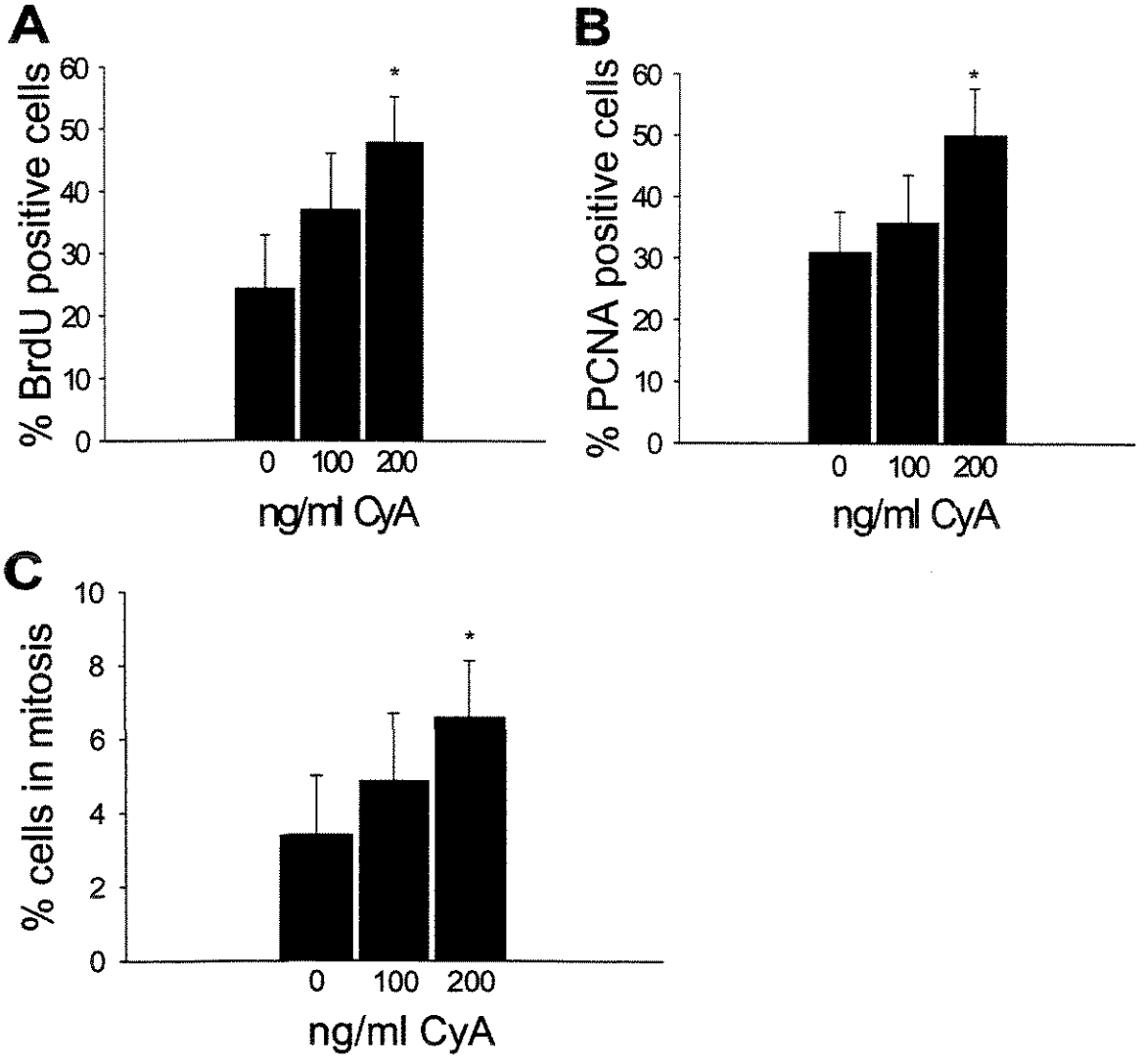

Fig. 2. BrdU-labeling (A), PCNA (B) and mitotic (C) indexes of NG fibroblasts incubated with 100 and 200 ng/ml of CyA for 24 h. The data of BrdU-labeling, PCNA and mitotic indexes correspond to the mean percentage of positive cells of 2 fibroblast cell lines. Addition of 200 ng/ml of CyA significantly stimulates the proliferation rate of NG fibroblasts. *$p<0.05$

Effect of TGF-β1 on NG cell proliferation

A 24 h incubation with TGF-β1 produced a significant increase in the proliferative rate of NG fibroblasts relative to controls (without TGF-β1). A significant increase in cell proliferation was observed at a TGF-β1 concentration of 0.1 and 1 ng/ml ($p<0.05$). However, at 10 ng/ml, TGF-β1 decreased NG cell proliferation (Fig. 3). No significant difference in the relative proliferative rate between 0.1 and 1 ng/ml of TGF-β1 was observed. The effect of TGF-β1 on NG fibroblast proliferation was also determined using BrdU-labeling, PCNA and mitotic indexes. The data of these three different cellular proliferation assays are depicted in Fig. 4 (A, B and C). The BrdU-labeling, PCNA and mitotic indexes of NG fibroblasts treated with 1 ng/ml of TGF-β1 were significantly higher than those of controls ($p<0.01$ for BrdU-labeling and mitotic indexes and $p<0.05$ for PCNA index). BrdU-labeling and mitotic indexes of NG fibroblasts incubated with 0.1 ng/ml of TGF-β1 were also significantly higher than those of controls ($p<0.05$), but not different from those treated with 1 ng/ml of TGF-β1.

Figure 3.

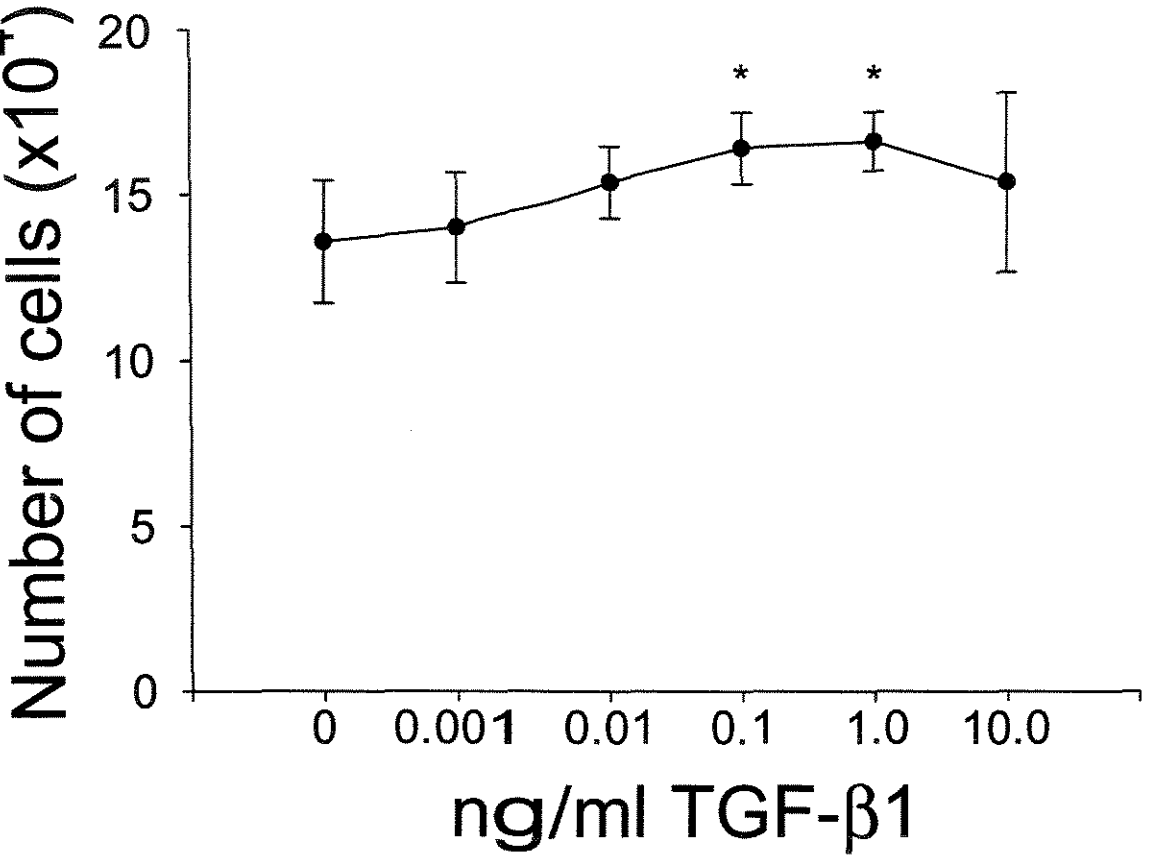

Fig. 3. Effect of TGF-β1 on NG fibroblast proliferation. Serum-starved cells were cultured in presence of various concentrations of TGF-β1 for 24 h. The combined results from 4 experiments for each cell line are presented as the number of cells ± SD. *$p<0.05$

Figure 4

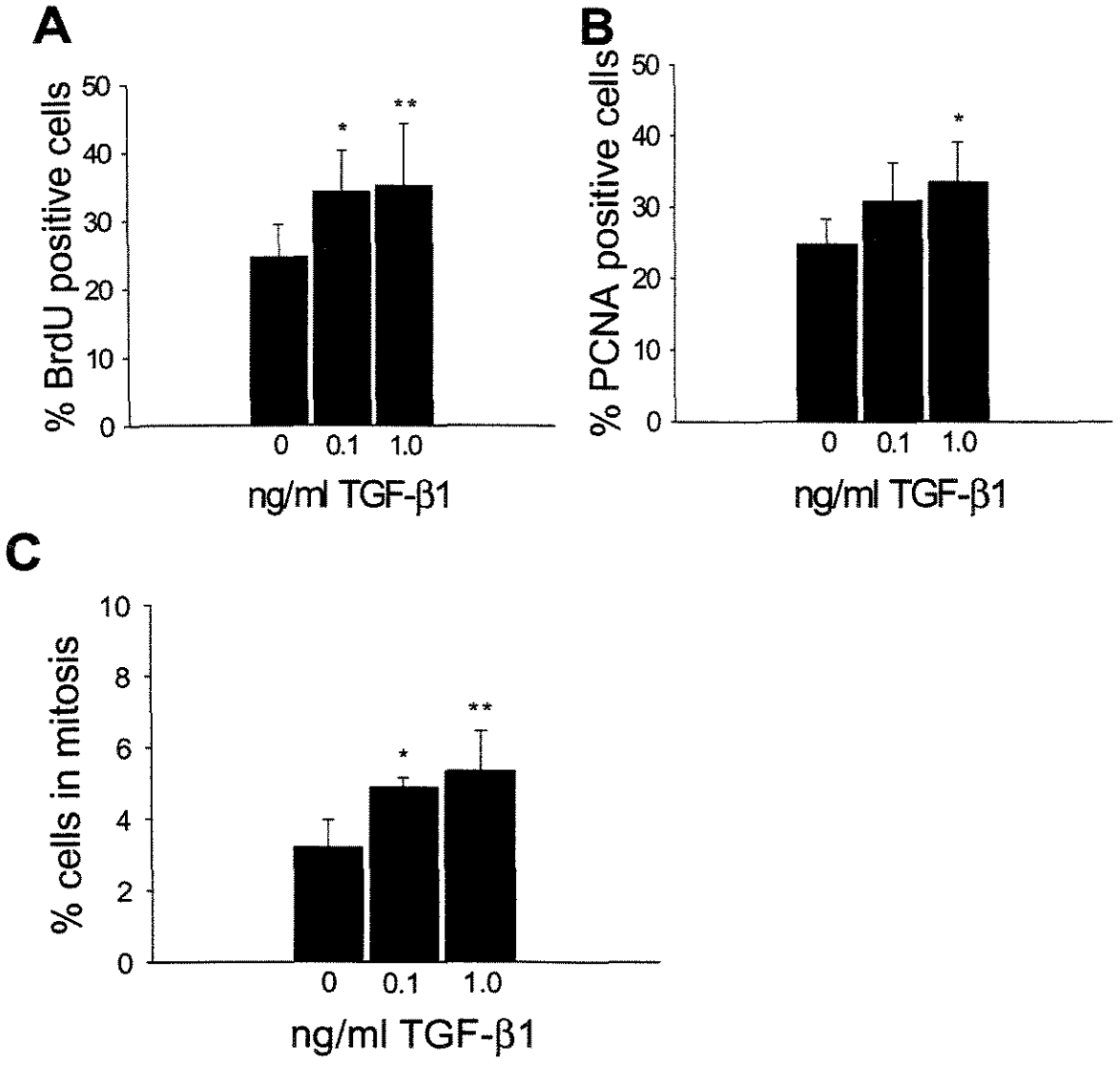

Fig. 4. Proliferation analysis of NG fibroblasts after addition of TGF-β1. NG fibroblasts were grown for 24 h in presence of 0.1 or 1 ng/ml TGF-β1. Following treatment, BrdU-labeling (A), PCNA (B) and mitotic (C) indexes were determined. The BrdU-labeling, PCNA and mitotic indexes of NG fibroblasts treated with 1 ng/ml of TGF-β1 were significantly higher than those of control. BrdU-labeling and mitotic indexes of NG fibroblasts incubated with 0.1 ng/ml of TGF-β1 were also significantly higher than those of control. *$p<0.05$; **$p<0.01$

Effect of antisense oligonucleotides on expression and production of TGF-β1 by NG fibroblasts

Since TGF-β1 stimulates NG fibroblast proliferation, we sought to determine if the excessive production of this cytokine by CyA-treated NG cells, in an autocrine fashion, could mediate the proliferative behavior of those cells. In an attempt to block endogenous TGF-β1, NG fibroblasts were transfected with 1 μg/ml of antisense oligonucleotides complementary to the translation initiation region of human TGF-β1 mRNA. As a control, corresponding sense oligonucleotide was used. To determine the efficacy of antisense oligonucleotides on the neutralization of TGF-β1 production, we investigated the amount of TGF-β1 secreted in culture supernatants by NG cells lines by ELISA. These studies revealed that antisense oligonucleotides significantly reduced TGF-β1 production by approximately 65% (65.8 ± 25.38%) in comparison with sense oligonucleotides. Similar results were obtained by Le Roy et al.[23] using fibroblast-like cells isolated from interstitial tissue from testis and by Andrade et al.[17] using gingival fibroblasts. To confirm that TGF-β1 antisense oligonucleotides promoted a translation arrest of TGF-β1 mRNA, we studied the TGF-β1 mRNA levels by RT-PCR. Neither antisense nor sense oligonucleotides modified significantly the levels of TGF-β1 transcripts (data not shown).

Effect of antisense oligonucleotides NG cell proliferation

To determine whether CyA regulates NG cell proliferation by TGF-β autocrine stimulation, antisense oligonucleotides were used to block TGF-β1 production by CyA-treated NG fibroblasts. Cell proliferation was then assessed by cell number counting as shown in Fig. 5. These results clearly show that the treatment with antisense oligonucleotides abrogated the effects of 200 ng/ml of CyA on proliferation of NG fibroblasts in comparison with the treatment with sense oligonucleotides ($p<0.05$). To further test the specificity of the antisense oligonucleotides, we examined whether exogenous TGF-β1 could reverse the effects of antisense treatment. We demonstrated that TGF-β1 at concentrations of 0.1 ng/ml or greater reversed significantly the effects of the antisense oligonucleotides on NG cell proliferation (Fig. 5 and data not shown; $p<0.01$). These data suggest that CyA upregulates cell proliferation in human NG fibroblasts via a TGF-β1-dependent mechanism. Furthermore, these results indicate that the TGF-β1 signaling pathways are not compromised by the treatment with antisense oligonucleotides.

Figure 5.

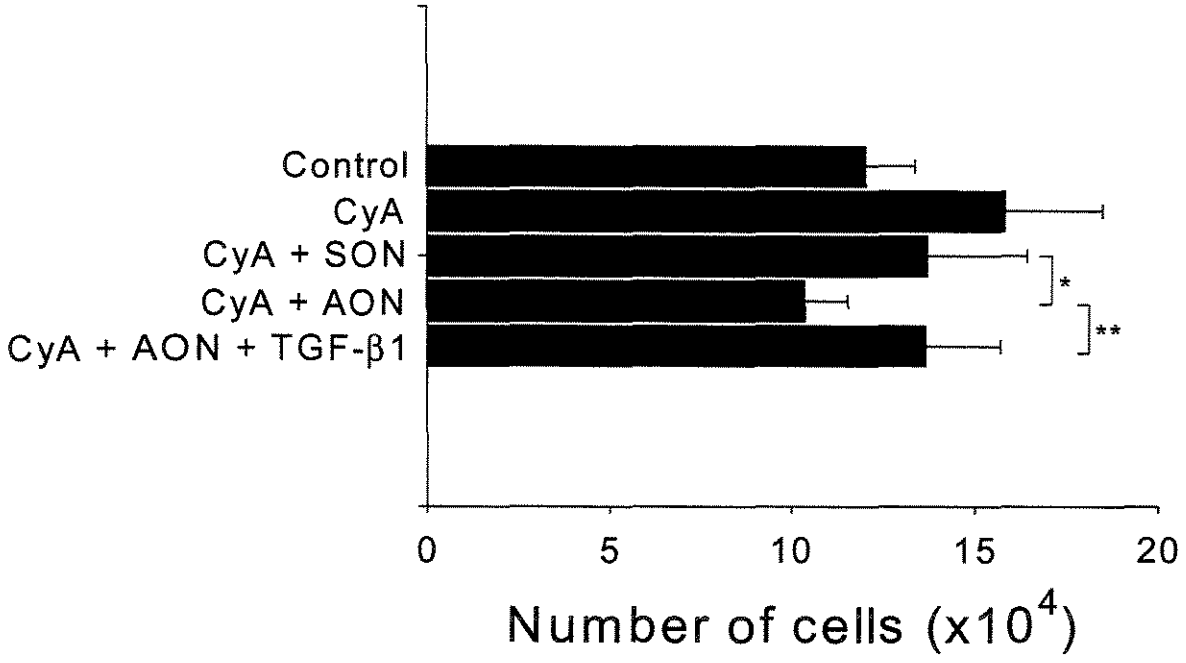

Fig. 5. Effect of antisense oligonucleotides on CyA-treated NG cell proliferation. NG fibroblasts were cultured in 24-well plates in DMEM with 10% FBS for 16 h and replaced with serum-free medium. After 24 h, 200 ng/ml of CyA in association with 1 μg/ml of antisense oligonucleotides (AON) or sense oligonucleotides (SON) were administered. To further test the specificity of the antisense oligonucleotide effect, NG fibroblasts were cultured in presence the both 1 μg/ml of antisense oligonucleotides and 0.1 ng/ml of TGF-β1. Following treatment, proliferation rates were determined by automated cell counting. The proliferation rate of NG fibroblasts treated with antisense oligonucleotides was significantly lower than that cells those treated with sense oligonucleotides. The reversal of inhibition after addition of exogenous TGF-β1 showed that this pathway is independent of treatment with antisense oligonucleotides. *$p<0.05$; **$p<0.01$

Discussion

Generalized gingival overgrowth can be caused by a number of factors including inflammation, leukemia, inheritance and drugs.[28] The most common form is induced by drugs such as phenytoin, nifedipine and CyA.[29] Despite extensive studies, the mechanism that leads to the accumulation of abnormal amounts of gingival tissue in CyA-induced gingival overgrowth is still unknown. The excess accumulation of connective tissue has traditionally been regarded to result from local increases in collagen synthesis, which is consistent with the histological characteristics of gingiva of patients with CyA-induced gingival overgrowth.[30] However, recent studies have recognized the equally important role of connective tissue degradation and resident cell proliferation in the pathogenesis of this side effect.[31] Additionally, we have recently shown that elevated expression and production of TGF-β1 in CyA-treated NG fibroblasts is associated with reduced expression and activity levels of matrix metalloproteinases, in particular MMP-1 and MMP-2, the most important enzymes associated with the extracellular matrix degradation and remodeling.[13]

We demonstrated in this study that CyA stimulates NG fibroblast proliferation at the same concentration found in the serum of patients undergoing CyA treatment (human serum levels range of 100-250 ng/ml).[27] Interestingly, CyA at similar doses utilized in this study significantly induces expression and synthesis of TGF-β1 by NG fibroblasts.[13] Addition of TGF-β1 significantly affected NG fibroblast proliferation in a dose-dependent manner. Moreover, the studies presented here revealed that CyA stimulates NG fibroblast proliferation through

autocrine stimulation of TGF-β1, and that neutralization of CyA-treated NG fibroblast proliferation by specific TGF-β1 antisense oligonucleotides is reversed by the addition of exogenous TGF-β1.

There are only a few and controversial previous studies demonstrating the proliferative response of NG fibroblasts to CyA. Increase, decrease or absence of effect in the proliferation rates after different drug concentration treatments have been reported.[32-36] Such variations among these studies may be attributed to subsets of fibroblasts that exist in gingiva and periodontum.[37,38] These studies suggest that alterations of the gingival connective tissue may result from clonal imbalance of resident fibroblast subtypes rather than from the presence of altered cells. Furthermore, the relative proportions of various subtypes of fibroblasts and their functional activities in the tissue could be regulated by specific cellular interactions with molecules such as cytokines. Moreover, inter-individual susceptibility to CyA-induced gingival overgrowth may also be related to a genetic predisposition, including variations in drug responsiveness and tolerance.[39,40] Our data showing the effect of CyA on the proliferation of NG fibroblasts are in agreement with various reports, including those of Bartold.[33] This author has demonstrated that CyA stimulates DNA synthesis and proliferation of NG fibroblasts and that this ability is retained even in the presence of lipopolysaccharide (LPS) that would normally inhibit these cells. Furthermore, the same author used similar concentrations of CyA, demonstrating that the stimulatory effect on cell proliferation occurs within the concentration ranges found in plasma and tissue of patients taking CyA.[27,41]

TGF-β1 promotes wound healing in vivo not only by stimulating the production of other growth factor such as platelet-derived growth factors (PDGF), but also by upregulating the proliferation of fibroblasts.[20,42] In the TGF-β1 dose-response analysis, low concentrations (0.1 and 1 ng/ml) were more efficient than higher concentrations (10 ng/ml). Although the levels of response of these low concentrations were not significantly different from each other in this experiment, the relative levels of response were consistent with those of the dose-response experiments suggesting a concentration-dependent effect. Previous studies have demonstrated that TGF-β1 is a mitogen for the human periodontal ligament fibroblasts with a bimodal effect.[43] This is in contrast to the results here presented using fibroblasts derived from normal gingiva. Although human periodontal ligament and gingival fibroblasts share common morphological features, it is not known how periodontal ligament fibroblasts compare to NG fibroblasts, and potential differences in these cell types may account for the distinct responses to growth factors such as TGF-β1. Additionally, Dennison et al.[21] demonstrated that the effect of 10 ng/ml of TGF-β1 on cell proliferation is different between periodontal ligament and NG fibroblasts.

The studies presented here also revealed that CyA induces proliferation in NG fibroblasts via induction of TGF-β1. The effect of CyA on NG proliferation was significantly reduced by blocking endogenous production of TGF-β1. Furthermore, our data suggested that the TGF-β1 signaling pathways are not compromised by the treatment with oligonucleotides, since exogenous TGF-β1 reversed the effects of the antisense oligonucleotides. TGF-β1 is the prototype of the TGF-β

superfamily of multifunctional peptides that control cell proliferation, differentiation and other functions in many cell types.[44] TGF-β1 has a profound growth inhibitory effect in cultured keratinocytes. On the other hand, TGF-β1 enhances fibroblast proliferation, not only by increasing the G_1/S transition and DNA synthesis, but also by shortening the G_1 phase of the cell cycle.[45] Although recent studies have delineated the binding of TGF-β1 to different serine-threonine receptor subtypes, little is known about its exact signaling pathways.[46] Various elements responsible for TGF-β1 gene induction have been identified, including phorbol myristate acetate (PMA), cAMP and AP-1.[47,48] Although calcineurin substrates, also known as phosphatase 2B (PP2B), are not yet clearly identified, it has been suggested that dephosphorylation of the RII regulatory subunit of protein kinase A by PP2B could be a mechanism by which Ca^{+2} increases the rate of inactivation of protein kinase A.[49] Van der Pouw Kraan[50] demonstrated that CyA enhanced the production of IL-13 from T-lymphocytes stimulated with anti-CD3 antibodies. This observation was comparable to the one obtained with PMA in the same conditions leading to the hypothesis that protein kinase C activation is responsible for the positive regulation of IL-13 production whereas Ca^{+2} mobilization has a negative effect. Thus, it can be hypothesized that inhibition of calcineurin with CyA could avoid the negative regulatory effect of this calcium-dependent phosphatase on cAMP/protein kinase A or protein kinase C-mediated TGF-β1 promoter activity leading to an enhancement of TGF-β1 gene transcription and stimulation of cell proliferation. However, additional work is required to better understand the

downstream events triggered by CyA on proliferative stimulation of NG fibroblasts via TGF-β1.

In summary, we have demonstrated that enhanced proliferation of CyA-treated gingival fibroblasts is mediated by TGF-β1 autocrine stimulation. Recently, our investigations of the molecular events that lead to CyA-induced gingival overgrowth showed a stimulation of TGF-β1 expression and production, which is associated, in an autocrine fashion, with a dowregulation of MMP-1 and MMP-2.[13] Furthermore, TGF-β1 upregulates type I collagen expression in fibroblasts from normal and hereditary gingival fibromatosis gingiva.[51] Taken together, our data suggest that TGF-β1 is a key regulator of the biochemical mechanisms associated in the pathogenesis of gingival overgrowth induced by CyA. The mechanisms involved in CyA inducing gingival overgrowth will be better understood when clinical, microscopic and microbiological data are considered in conjunction with new molecular techniques that are becoming available.

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5. CONCLUSÕES

1. *Ciclosporina A estimula a expressão e produção de TGF-β1 em fibroblastos gengivais humanos.*

2. *Ciclosporina A e TGF-β1 estimulam a proliferação e alteram a expressão de MMPs em fibroblastos gengivais*

3. *A neutralizacao do estímulo autócrino de TGF-β1 em fibroblastos gengivais tratados com ciclosporina A reverte o efeito da droga na proliferação e expressão de MMPs.*

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