UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

HERCÍLIO MARTELLI JÚNIOR
CIRURGIÃO DENTISTA

Efeito de testosterona na proliferação celular e de TGF-β1, IL-6 e INF-γ na expressão e produção de colágeno tipo I, Hsp47 e metaloproteinases de matriz em fibroblastos de gengiva normal e de fibromatose gengival hereditária.

Orientador: **Prof. Dr. Ricardo Della Coletta**

Tese apresentada ao Programa de Pós-Graduação em Estomatopatologia da Faculdade de Odontologia de Piracicaba - UNICAMP, como parte dos requisitos para obtenção do Título de Doutor em Estomatopatologia.

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 17 de Abril de 2002, considerou o candidato HERCÍLIO MARTELLI JÚNIOR aprovado.

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DEDICATÓRIA

" Nada te perturbes

Nada te espantes

Deus não muda

A paciência tudo alcança

A quem a Deus possui

Nada há de faltar. "

Aos meus pais, pelos seus esforços e dignidade

transmítidos durante a nossa formação.

Aos meus irmãos, pelas lembranças agradáveis da infância

e pelos momentos felizes compartilhados.

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pelo amor, participação e compreensão

durante este período e seus familiares.

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"Nossos dias são preciosos, mas com alegria os vemos passando

se no seu lugar encontramos uma coisa mais preciosa crescendo:

uma planta rara e exótica, uma criança que estamos ensinando,

um livrinho que estamos escrevendo".

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SUMÁRIO

1 - LISTA DE ABREVIATURAS

Siglas	*Significado*
ATP	Trifosfato de Adenosina
C-terminal	Porção Carboxi-Terminal
DMEM	Meio de Cultura de Eagle Modificado por Dulbecco
DMSO	Di-Metil Sulfóxido
DTH	Diidrotestosterona
FGH	Fibromatose Gengival Hereditária
GN	Gengiva Normal
Grp75	Proteína Regulada por Glicose de 75 kDa
Grp78	Proteína Regulada por Glicose de 78 kDa
Grp94	Proteína Regulada por Glicose de 94 kDa
Hsps	Proteínas de Choque Térmico
Hsp47	Proteína de Choque Térmico de 47 kDa
Hsp70	Proteína de Choque Térmico de 70 kDa
Hsp90	Proteína de Choque Térmico de 90 kDa
IL	Interleucina
INF-γ	Interferon-γ
MEC	Matriz Extra-celular
MMP	Metaloproteinase de Matriz
N-terminal	Porção Amino-Terminal
PAGE	Eletroforese em Gel de Poliacrilamida
PBS	Tampão Fosfato Salino
RE	Retículo Endoplasmático
RT-PCR	Transcriptase Reversa-Reação da Polimerase em Cadeia
SDS	Sódio Dodecil Sulfato
Sv40	Vírus Simian 40
TGF-β	Fator de Crescimento Transformante β
TIMP	Inibidor Tecidual de Metaloproteinase de Matriz

2 - RESUMO

Fibromatose Ggengival Hereditária (FGH) representa uma condição oral incomum, caracterizada por aumento gengival fibrótico e generalizado. Para elucidar algumas características regulatórias que resultam nesta condição, linhagens celulares de fibroblastos de pacientes de uma mesma família com FGH foram isolados e analisados em relação ao efeito de diidrotestosterona (DHT) na proliferação celular e de TGF-β1, IL-6 e INF-γ na expressão e produção de colágeno tipo I, Hsp47, MMP-1 e MMP-2. Adicionalmente, analisamos o efeito de DHT na produção de IL-6 e determinamos os níveis de expressão de receptores de andrógenos em fibroblastos de GN e de FGH. Os resultados indicaram que DHT simultaneamente estimulou a proliferação celular e inibiu a produção de IL-6 em fibroblastos de GN e de FGH. A expressão de receptores para andrógenos foi detectada em fibroblastos de GN e de FGH; contudo, a expressão foi maior em fibroblastos de GN. Ensaios de RT-PCR, western blot, ELISA e enzimografia demonstraram que a expressão e produção de colágeno tipo I e Hsp47 foram significativamente maiores em fibroblastos de FGH comparados a fibroblastos de GN, enquanto a expressão de MMP-1 e MMP-2 foram menores em fibroblastos de FGH. A adição de TGF-β1 e IL-6 à fibroblastos de GN e de FGH promoveram um aumento na expressão de colágeno tipo I e Hsp47 e uma diminuição de MMP-1 e MMP-2. INF-γ reduziu a expressão de colágeno tipo I e Hsp47, apresentando menor efeito na expressão de MMP-1 e MMP-2. Estes resultados demostram que DHT coordena a proliferação celular e a produção de IL-6 em fibroblastos de GN e de FGH e que TGF-β1 e IL-6 estimulam a síntese de colágeno e reduzem a atividade proteolítica de fibroblastos de FGH, os quais favorecem o acúmulo de matriz extracelular.

Palavras Chaves: Fibromatose Gengival Hereditária; Diidrotestosterona; Colágeno Tipo I; Hsp47; Metaloproteinases de Matriz; Citocina

3 - ABSTRACT

Hereditary Gingival Fibromatosis (HGF) is an uncommon oral condition characterized by a fibrous gingival enlargement. To further elucidate some of the regulatory features resulting in this condition, the culture characteristics of cell lines of gingival fibroblasts derived from patients of the some family with HGF were isolated and analyzed on the effect of DHT on proliferation rate and on the effect of TGF-β1, IL-6 and INF-γ on expression and production of type I collagen, Hsp47, MMP-1 and MMP-2. Additionally, we analyzed the effect of DHT on IL-6 production and determined the expression levels of androgen receptors in NG and HGF fibroblasts. The results indicated that DHT simultaneously upregulated the cell stimulates proliferation and downregulated the production of IL-6 production by NG and HGF fibroblasts. Androgen receptor levels was identified in both NG and HGF fibroblasts; however, the levels in NG were higher than those observed in HGF. Our results obtained from RT-PCR, Western blot, ELISA and enzymography assays demonstrated that the expression and production of type I collagen and Hsp47 were significantly higher in fibroblasts from HGF than from NG, whereas MMP-1 and MMP-2 expression and production were lower in fibroblasts from HGF patients. Addition of TGF-β1 and IL-6 promoted an increase in type I collagen and Hsp47 and decrease in MMP-1 and MMP-2 expression. INF-γ reduced both type I collagen and Hsp47 expression, whereas had a slight effect on expression of MMP-1 and MMP-2. These results show that DHT coordinates the proliferation and production of IL-6 by NG and HGF fibroblasts and that enhanced TGF-β1 and IL-6 production simultaneously increase the

synthesis and reduce the proteolytic activies of HGF fibroblasts, which favor the accumulation of extracellular matrix.

Key Words: Hereditary Gingival Fibromatosis; Dihydrotestosterone; Type I Collagen; Hsp47; Matrix Metalloproteinases; Cytokines.

4 – INTRODUÇÃO

4.1 - Fibromatose Gengival Hereditária

Fibromatose gengival é um termo clinicamente utilizado para referir-se ao aumento de volume da gengiva, decorrente de excessivo acúmulo de colágeno e uma maior proliferação fibroblástica no tecido gengival (Page e Shroeder, 1982; Takagi et al., 1991; Coletta et ., 1998). As fibromatoses gengivais podem ser de natureza inflamatória, neoplásica, medicamentosa e hereditária. A fibromatose com padrão genético é denominada FGH, podendo ocorrer como fenótipo isolado ou componente de diversas síndromes (Gorlin et al., 1990). A FGH foi descrita inicialmente por Gross em 1856, sendo confecionado o primeiro heredograma por Nasse em 1895.

FGH é uma alteração bucal rara (1:750.000; Singer et al., 1993) sem predileção por sexo (Rushton, 1957), e que envolve a maxila e a mandíbula. Clinicamente, verifica-se um crescimento gengival lento e contínuo, sem regressão espontânea. A gengiva apresenta-se firme, indolor, não hemorrágica, com coloração rosa e pontilhado superficial característico, podendo resultar na cobertura das coroas dentais em variados graus (Sciubba e Niebloom, 1986; Bozzo et al., 1992; Bozzo et al., 1994; Martelli-Júnior et al., 2000).

Na maioria dos casos, a FGH é transmítida através de herança autossômica dominante, embora padrões recessivos tenham sido descritos. Os relatos de fenótipos autossômicos recessivos, que são minoria em relação ao fenótipo dominante, segundo alguns autores provém de casamentos cosangüíneos (Nevin, 1971). Porém, a consangüinidade não é a única e nem a explicação mais aceita. Descendentes de pessoas não aparentadas, cada qual portadora de um gene mutante, parece ser responsável pela maioria dos casos de doenças

autossômicas recessivas, principalmente se o caráter recessivo apresentar alta freqüência na população (Coletta et al., 1998). Bozzo et al. (1994) mostraram que a penetrância da FGH na mesma família deste estudo é completa, pois em nenhuma ocasião indivíduos não afetados tiveram filhos afetados. Além disso, há evidências de expressividade variável, uma vez que todos os indivíduos afetados apresentam diferentes manifestações clínicas da doença. É possível que a penetrância e a expressividade da FGH possam variar entre diferentes famílias.

A FGH foi descrita em microscopia óptica, pela primeira vez, por Tomes em 1879. Esta condição é caracterizada histologicamente por epitélio pavimentoso estratificado queratinizado, mostrando áreas de acantose (Johnson et al., 1986) e longas e delgadas projeções epiteliais que se estendem em direção ao conjuntivo subjascente (Danesh-Meyer e Holborow, 1993; Singer et al., 1993). O tecido conjuntivo apresenta densos e espessos feixes de fibras colágenas entremeadas por fibroblastos e um discreto infiltrado inflamatório nas áreas perivasculares (Redman et al., 1985). Pequenos e múltiplos focos de calcificação distrófica, ilhas de metaplasia óssea, áreas de ulceração e focos de células inflamatórias também foram descritos (Gunhan et al., 1995).

A FGH pode ocorrer como um achado clínico isolado ou mais raramente como componente de variadas síndromes, associado, entre outras condições, com hipertricose, retardo mental e epilepsia (Araiche e Brode, 1959; Ramon et al., 1967; Horning et al., 1985; Cuestas-Carneiro et al., 1988), surdez progressiva (Harstifield et al., 1985), múltiplos fibromas hialinos e defeitos das falanges terminais (Bakeen e Scully, 1991).

Embora transmítida geneticamente, os mecanismos bioquímicos e os genes responsáveis por esta alteração são pouco conhecidos. Contudo, os eventos envolvidos na patogênese do crescimento gengival resultam de distúrbios no equilíbrio homeostático envolvendo a síntese e degradação de colágeno e outras moléculas da matriz extracelular, bem como da proliferação fibroblástica. Hart et al. (1998) estudando uma família de 32 pessoas, sendo 12 membros afetados para FGH, mostrou uma relação da FGH com o cromossomo 2p21, entre os locus D2S1788 e D2S441. Estudos recentes de localização gênica mostram variações em relação a região mapeada por Hart et al. (1988), evidenciando assim diferentes padrões de herança mendeliana, sugerindo a heterogeneidade genética da FGH (Xiao et al., 2000; Hart et al., 2000; Xiao et al., 2001). A identificação das bases genéticas da FGH proverão uma melhor compreensão dos mecanismos envolvidos e possibilitarão perspectivas para terapias mais eficientes nestes pacientes.

Os mecanismos bioquímicos e moleculares que levam à formação excessiva de tecido gengival ainda são pobremente conhecidos e os estudos envolvendo cultura de células são controversos. Estudos prévios, envolvendo membros da família participante deste estudo, mostraram que fibroblastos da FGH são fenotipicamente distintos de fibroblastos de GN e apresentam uma taxa de proliferação maior comparado a fibroblastos de GN (Coletta et al., 1998). Por outro lado, Johnson et al. (1986) e Shirasuna et al. (1989) mostraram que fibroblastos da FGH apresentam proliferação menor que fibroblastos controle. Inconsistência nas descrições dos fibroblastos da FGH podem ser devido a heterogeneidade genética e diferenças funcionais em fibroblastos oriundos de diferentes fibroses gengivais (Hassell e Stanek, 1983). Coletta et al. (1999b) mostraram em culturas celulares que a produção e

expressão de MMPs, particularmente MMP-1 e MMP-2, foram reduzidas em fibroblastos de FGH comparado a fibroblastos de GN. Entretanto, os níveis de TIMP-1 e TIMP-2 não foram alterados nas mesmas culturas avaliadas.

4.2 -Colágeno

Colágeno compõe uma família de proteínas fibrosas encontradas em todos os organismos multicelulares. Esta proteína é secretada por células do tecido conjuntivo e constitui a proteína mais abundante de mamíferos. O colágeno representa aproximadamente 25% do total de proteínas em um organismo (Burgeson, 1988). A principal e mais freqüente característica das moléculas de colágeno é a estrutura firme de hélice tríplice. Três cadeias de polipeptídeos de colágeno chamadas de cadeias α (cada uma de 1000 aminoácidos) são entrelaçadas entre elas numa configuração de super-hélice formando uma molécula de colágeno com aproximadamente 300nm de comprimento e 1,5nm de diâmetro (Burgeson, 1988).

Vinte tipos distintos de moléculas de colágeno já foram descritas. As moléculas dos diferentes tipos de colágeno são formadas por combinações de vinte e cinco diferentes tipos de cadeias α, cada qual codificada por um gene distinto. Combinações diferentes destes genes são expressas em diferentes tecidos. Os colágenos melhor caracterizados e mais freqüentes são os tipos I, II, III e IV. Os tipos I, II e III são colágenos fibrilares e constituem os principais colágenos encontrados no tecido conjuntivo, sendo o tipo I o mais abundante (Darnell e Lodish, 1986). Após serem secretados no espaço extracelular, estes três tipos de colágeno se polimerizam em estruturas organizadas denominadas de fibrilas.

O colágeno tipo I é uma molécula heterotrimérica produzida por mRNAs individuais (Flug e Kopf-Maier, 1995). Este tipo é produzido principalmente por células produtoras de matriz orgânica, como fibroblastos, osteoblastos, odontoblastos e condroblastos. As cadeias individuais de polipeptídeos de colágeno são produzidas como precursores chamados de pró-α-cadeias. O colágeno heterotrimérico tipo I é composto por duas cadeias pró-α 1 idênticas e uma cadeia distinta pró-α 2, fornecendo a seguinte estequiometria [2 pró-α1 (I), 1 pró-α2 (I)]. Cada cadeia pró-α do colágeno tipo I possui composição química rica em glicina, representando aproximadamente 30% do total de aminoácidos da molécula e massa molecular de 140 kDa. Além da alta concentração de glicina, as cadeias α apresentam em sua composição hidroxiprolina e hidroxilisina, que raramente são observados em outras proteínas. Esta composição é caracterizada pela repetição da fórmula trimérica Pro-X-Gly, sendo a presença de prolina e glicina fundamentais para estabilização da tríplice hélice. As cadeias do colágeno tipo I são secretadas no meio extracelular na forma de pró-colágeno, contendo polipeptídeos N- e C- terminais que são removidos por enzimas específicas, seguindo-se a associação e processamento de pró-peptídeos de colágeno e formação de heterotrímeros (Flug e Kopf-Maier, 1995).

4.3 - Hsp47

A conformação estrutural das proteínas depende, entre vários fatores, da participação de um grupo de proteínas com atividade catalisadora denominadas *chaperones* ou proteínas que se ligam a cadeias peptídicas nascentes. Estas proteínas ligam-se a peptídeos em formação, tão logo inicia a síntese protéica propriamente dita com a deposição de

aminoácidos nos ribossomos, sem detectar qualquer sequência específica. Estas *chaperones* agem facilitando o empacotamento (*folding*), polimerização das proteínas (*assembly*), prevenindo a secreção de proteínas mal formadas e assegurando que estes eventos ocorram com alta fidelidade (Hass e Wabl, 1983; Dorner et al., 1987; Kassenbrock et al., 1988; Flynn et al., 1989; Da Silva et al., 1990, 1993; Gething e Sambrook, 1992; Dafforn et al., 2001). Proteínas *chaperones* são atualmente descritas como uma classe de proteínas capazes de associar e dissociar de forma transitória a porções hidrófobas das cadeias nascentes de polipeptídeos em processos de aquisição de estrutura terciária e polimerização, a proteínas que falham em se empacotar corretamente ou em se agregar em complexos oligoméricos. Alguns membros apenas são expressos em condições de estresse celular, enquanto outros são sintetizados constitutivamente (Hurtley e Helenius, 1989).

Hsp47, uma proteína residente no RE, tem sido colocada na função principal de *chaperone*, garantindo assim a produção de colágeno I heterotrimérico (Ferreira et al., 1996; Dafforn et al., 2001). Esta *chaperone* é expressa constitutivamente e também é afetada por estresse ambientais, como elevação de temperatura (Nagata et al., 1986, Nagata et al., 1988). Hsp47 foi inicialmente descrita como uma glicoproteína em células endodérmicas de camundongo, sendo também capaz de ligar-se a gelatina (Kurkinen et al., 1984). Esta proteína também é denominada coligina, por se ligar a vários tipos de colágeno (Taylor et al., 1985; Saga et al., 1987). Esta glicoproteína de 47 kDa liga-se à cadeias nascentes de colágeno tipos I, II, III, IV e V (Sauk et al., 1994; Nagata et al., 1986; Satoh et al., 1996), sendo detectada apenas em células produtoras de colágeno (Nakai et al., 1992). Adicionalmente, foi demonstrado que a produção de Hsp47 é diminuída em fibroblatos de

embriões de galinha transformados pelo vírus do Sarcoma Rous (Nagata e Yamada, 1986) e em células BALB/3T3 transformadas pelo SV40 (Nakai et al., 1990). Porém, os níveis de Hsp47 são aumentados durante a diferenciação de células F9 (Takechi et al., 1992; Ferreira., 1996). Células primitivas F9 são induzidas a diferenciar em células viscerais na presença do ácido retinóico ou pela combinação de ácido retinóico e dibutiridil AMPcíclico. Quando diferenciadas, estas células são capazes de produzir grandes quantidades de colágeno tipo IV (Strickland e Mahdavil, 1978; Ferreira et al., 1996). É interessante observar que células indiferenciadas mostraram pouca expressão de Hsp47, mas quando diferenciadas, a produção de Hsp47 foi significantemente aumentada (Ferreira et al., 1996).

A associação de Hsp47 e pró-colágeno *in vitro* foi verificada por imunoprecipitação usando anticorpos anti-Hsp47, demonstrando que esta associação é transitória e ocorre antes da secreção das moléculas de colágeno (Nakai et al., 1992). Shroff et al. (1993) demonstraram a presença de Hsp47 nos compartimentos intermediários entre o RE e o aparelho de Golgi. É proposto que após a dissociação da molécula de tropocolágeno via provavelmente acidificação do pH, Hsp47 recicle para o RE por um processo envolvendo a seqüência KDEL e receptores erd2P (Nagata, 1996). Recentemente foi demonstrado que Hsp47 é expressa em associação com o receptor para a sequência KDEL (Lis-Asp-Glu-Leu) na superfície celular (Sauk et al., 1998) e na membrana externa de células tumorais (Sauk et al., 2000). Uma possível função para esta expressão ainda é desconhecida.

Hsp47 tem sido relacionada a uma série de alterações fibróticas, as quais tem demonstrado um aumento na expressão de Hsp47 paralelamente a progressão destas doenças, tanto em modelos humanos como em modelos animais (Razzaque e Taguchi, 1999). Kuroda

et al. (1998) estudando culturas de fibroblastos da pele de nove pacientes portadores de esclerose sistêmica, doença do tecido conjuntivo caracterizada por fibrose da pele, tecidos subcutâneos e vários órgãos internos, observaram um aumento nos níveis de Hsp47 mRNA e proteínas nos fibroblastos da esclerose sistêmica quando comparado a fibroblastos de pele normal. Estas culturas de fibroblastos da esclerose sistêmica mostraram também expressão acentuada de pró-colágeno tipo I. Os autores investigaram também o efeito de citocinas na expressão de Hsp47 nas culturas de fibroblastos normais. TGF-β1 e IL-4 aumentaram os níveis de Hsp47 mRNA, enquanto INF-γ reduziu a expressão de Hsp47. O mesmo padrão das citocinas na expressão dos níveis de pró-colágeno I foi observado. Estes resultados indicam que a expressão de Hsp47 esta aumentada podendo estar envolvida na produção abundante de pró-colágeno I em fibroblastos de esclerose sistêmica.

4.4- Metaloproteinases de Matriz

MMPs compreendem uma família de enzimas dependentes de zinco que são conhecidas pela capacidade de degradar diferentes moléculas da MEC, desempenhando papéis fundamentais em processos fisiológicos e patológicos (Coletta et al., 1999b). As proteínas pertecentes a família das MMPs possuem grande homologia entre si, embora codificadas por diferentes genes. Em humanos, a família das MMPs são representadas por 17 membros, sendo estes divididos em 4 grupos em função da especificidade ao substrato e de sua estrutura (Bernot et al., 1997). Estes grupos são: colagenases (MMP-1, -8 e –13), gelatinases ou colagenases tipo IV (MMP-2 e -9), estromelisinas (MMP-3, -10 e –12) e as enzimas representantes do quarto grupo são denominadas de MMPs de membrana (MT-

MMPs) pelo fato de representarem moléculas transmembrânicas (Coletta et al., 1999b; Cotrim et al., in press).

A expressão de MMPs pode ocorrer de forma constitutiva nas células ou ser induzida, sendo regulada por variados agentes, incluindo a citocina TGF-β1 (Overall et al., 1991). MMP-1 e MMP-2 são expressas de forma constitutiva em várias células. Quando em gel de poliacrilamida, a colagenase de fibroblastos (MMP-1) aparece como uma banda com massa molecular variando entre 57 e 52 kDa. Esta variação provavelmente se deve a proteólise parcial da molécula. MMP-1 é sintetizada por fibroblastos, queratinócitos, células endoteliais, macrófagos, osteoblastos, condrócitos e osteoclastos (Bikedal-Hansen, 1993). A MMP-2 é a MMP mais abundante, sendo sintetizada por fibroblastos, queratinócitos, células endoteliais, macrófagos, osteoblastos e condrócitos, estando também presente no plasma sangüíneo (Bikedal-Hansen, 1993). MMP-2 é capaz de clivar a região helicoidal do colágeno tipo IV (Murphy et al., 1989), além de degradar colágeno tipo V e VII, elastina e gelatina (colágeno desnaturado). A MMP-2 possui massa molecular de 72 kDa e sua especificidade sobre o colágeno tipo IV parece indicar sua participação na remodelação e degradação da membrana basal (Aimes e Quigley, 1995).

4.5- Citocinas

Citocinas representam proteínas efetoras que modulam as interações celulares, envolvendo respostas inflamatórias, imunológicas e proliferativas. As citocinas podem também alterar o comportamento ou as propriedades celulares em nível local e sistêmico (Abbas et al., 1997). Entre as citocinas estão incluídas: as interleucinas, os interferons, os

fatores de crescimento, os fatores citotóxicos, os fatores inibidores ou ativadores e os fatores estimulantes de colônia (Alberts et al., 1994).

4.5.1- Fator de Crescimento Transformante-β

Constituem um grupo de citocinas multifuncionais, conhecidas por sua importância em eventos de cicatrização e processos fibróticos (Border e Ruoslahti et al., 1992). Representam uma família de proteínas formada por, pelo menos, cinco membros (TGF-β1 a TGF-β5), os quais são codificadas por diferentes genes. Estas citocinas são produzidas por diferentes células, incluindo células inflamatórias, células endoteliais e fibroblastos (Wright et al., 2001). Em humanos, três isoformas (TGF-β1, TGF-β2 e TGF-β3) podem ser distinguidas por seus efeitos no crescimento celular e respectivos receptores celulares (Fahey et al., 1996). Acredita-se que alterações na expressão de isoformas de TGF-β possam ser responsáveis por processos fibróticos (Wright et al., 2001). TGF-β é um potente fator mitogênico para fibroblastos, não apenas por induzir as células a entrarem na fase S, mas também por encurtar a fase G1 do ciclo de divisão celular. TGF-β também inibe a degradação da MEC, por inibir a síntese de MMPs e estimular a síntese de inibidores teciduais de MMPs (Kim et al., 1998).

Aumento na expressão de TGF-β1 tem sido demonstrado em vários efeitos colaterais induzido pelo tratamento com ciclosporina, incluindo os aumentos gengivais e as fibroses renais (Coupes et al., 1994). Níveis elevados de TGF-β1 também tem sido verificados em culturas de fibroblastos gengivais tratadas com ciclosporina (James et al., 1998), e aumento nos níveis gengivais de TGF-β1 e TGF-β2 foram também observados em aumentos

gengivais induzidos por fenitoína e nifedipina (Saito et al., 1996). Coletta et al. (1999b) mostraram que a diminuição na expressão de MMP-1 e MMP-2 em fibroblastos de FGH comparado a fibroblastos de GN é mediada pela ação de TGF-β1. Recentemente, Andrade et al. (2001) observaram estimulação na capacidade proliferativa de fibroblastos gengivais de FGH comparado a fibroblastos de GN, através dos efeitos autócrinos de TGF-β1.

4.5.2- Interleucina – 6

IL-6 é uma potente citocina envolvida com a regulação da resposta imune e inflamatória, principalmente na fase aguda, e nos eventos hematopoiéticos. IL-6 é responsável por mediar a comunicação entre um grande número de células e controlar a proliferação e diferenciação de linfócitos B, hepatócitos e linfócitos T (Ebersole e Cappelli, 2000).

Níveis aumentados de IL-6 tem sido verificado no córtex cerebral de pacientes com doença de Alzheimer e em esclerose sistêmica (Ebersole e Cappelli, 2000). Estudos têm demonstrado que fibroblastos derivados de doenças fibróticas como esclerose sistêmica da pele e fibrose pulmonar induzida por drogas expressam elevados níveis de IL-6 mRNA e proteínas em paralelo com aumento na produção de colágeno (Takemura et al., 1998; Coker e Laurent, 1998). Além disso, esta citocina tem sido associada com processos fibróticos do tecido gengival (Williamson et al., 1994; Fries et al., 1994). Achados microscópicos de crescimento gengival induzido por ciclosporina mostraram elevação na expressão de IL-6 em células do tecido conjuntivo gengival (Morton e Dongari-Bagtzoglou, 1999), sendo estes mesmos dados encontrados no soro e urina de pacientes submetidos a transplantes renais, sob

medicação com ciclosporina. IL-6 também tem sido associada à regulação da produção de colágeno e glicosaminoglicanas (Duncan e Berman, 1991; Fries et al., 1994).

4.5.3- Interferon-γ

Interferons constituem um grupo de proteínas que exibem diferentes funções biológicas. Foram primeiramente caracterizadas como substâncias inibidoras da replicação viral, sendo suas isoformas (INF-α e INF-β) produzidas por células infectadas por vírus, enquanto a isoforma INF-γ é produzida durante a resposta imune por linfócitos T e células natural Killer recrutadas por IL-2 (Page e Kornman, 1997). INF-γ participa da maturação de linfócitos B e secreção de imunoglobulinas. Além disso, esta citocina inibe diversas atividades induzidas por IL-4, sugerindo que estas duas citocinas reciprocamente regulam diferentes eventos biológicos (O'Garra, 1989).

Cornelissen et al. (1999) mostraram utilizando culturas de fibroblastos de ratos condicionados com INF-γ, em concentrações variando de 100 a 10000 U/ml, uma diminuição na síntese de proteínas colagênicas e não colagênicas, determinadas pela incorporação de [3H]-prolina. Culturas de fibroblastos da pele e de pulmão condicionadas por INF-γ demonstraram níveis diminuídos na síntese de colágeno tipo I e III (Czaja et al., 1987; Narayanan et al., 1992). Gillery et al. (1992) verificaram um aumento na produção de MMPs em culturas celulares de pacientes com escleroderma. Em função da participação de INF-γ na redução da síntese de colágeno, sugere-se a possibilidade desta citocina poder ser usada terapeuticamente no tratamento de diversos quadros de fibrose (Jimenez et al., 1984; Granstein et al., 1987).

4.6- Testosterona

Os testículos secretam vários hormônios sexuais masculinos que são coletivamente chamados de andrógenos, compreendendo a testosterona, androstenodiona e DTH. Contudo, a testosterona é mais abundante que os demais hormônios, apesar de, grande parte dela, se não sua maioria, ser convertida no hormônio mais ativo, a DTH nos tecidos-alvo (Byskov, 1986).

Todos os andrógenos são compostos esteróides. Tanto nos testículos quanto nas adrenais, os andrógenos podem ser sintetizados a partir do colesterol ou diretamente a partir da acetilcoenzima A. Parte da testosterona secretada é fixada nos tecidos ou degradada a produtos inativos que subsequentemente são excretados. A parte que se fixou aos tecidos é convertida dentro das células, sob influência da enzima 5-α-redutase, em DTH, a qual fixa em uma proteína receptora citoplasmática. O complexo DTH-receptor migra para o núcleo, onde se liga a uma proteína nuclear e induz a transcrição, seguindo-se de aumento progressivo de proteínas nucleares (Guyton e Hall, 1996). Entre outras funções, a testosterona, quando em grandes quantidades, pode aumentar em até 15% a taxa de metabolismo basal. Esta taxa aumentada do metabolismo é possivelmente o resultado indireto do efeito da testosterona sobre o anabolismo induzindo um aumento nas atividades celulares (Guyton e Hall, 1996).

Diferentes estudos sugerem que hormônios sexuais, incluindo testosterona e seus metabólitos têm importante participação nos aumentos gengivais medicamentosos (Sooriyamoorthy et al., 1988; Nyska et al., 1994) e em inflamações gengivais, muitas vezes

presentes em gestantes (Gornstein et al., 1999). Aumento na incidência de gengivite tem sido observado durante a puberdade e associado ao uso de contraceptivos orais, contendo altas doses de hormônios. A etiologia e os mecanismos envolvidos nestas condições permanecem desconhecidos (Gornstein et al., 1999). Fibroblastos derivados de crescimentos gengivais induzidos por drogas metabolizam testosterona em níveis significantemente maiores comparado a fibroblastos derivados de GN (Sooriyamoorthy et al., 1990). Lapp et al. (1995) mostraram em fibroblastos gengivais de humanos que a progesterona modula a produção de IL-6, tendo esta citocina um nível de diminuição entre 40 a 50% em relação a células controle quando do efeito da progesterona. A proliferação de fibroblastos é regulada por diferentes moléculas, incluindo a testosterona (Sooriyamoorthy et al., 1990; Andrade et al., 2001). Gornstein et al. (1999) avaliaram a ação de andrógenos, testosterona e DTH em fibroblastos gengivais, verificando que níveis elevados de testosterona e DTH modulam a produção de IL-6.

5 – PROPOSIÇÃO

Baseado nos conhecimentos atuais sobre a patogênese da FGH e nos diversos mecanismos envolvidos nesta condição fibrótica, este estudo teve como objetivos:

1. Determinar os níveis basais de expressão e produção de colágeno tipo I, Hsp47, MMP-1 e MMP-2 de fibroblastos de GN e de FGH.
2. Avaliar o efeito de TGF-β1, IL-6 e INF-γ na expressão e produção de colágeno tipo I, Hsp47, MMP-1 e MMP-2 em fibroblastos de GN e FGH.
3. Avaliar o efeito da diidrotestosterona na proliferação celular e na produção de IL-6 por fibroblastos de GN e de FGH.
4. Determinar os níveis de receptores de andrógenos em fibroblastos de GN e de FGH.

Effect of TGF-β1, IL-6 and IFN-γ on the expression of type I collagen, Hsp47, MMP-1 and MMP-2 by fibroblasts from normal gingiva and hereditary gingival fibromatosis.

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ABSTRACT

Background: Increased collagen and extracellular matrix deposition within the gingiva is the main characteristic feature of hereditary gingival fibromatosis (HGF). To date, it is not well established if these events are a consequence of alterations in the collagen and other extracellular matrix molecules synthesis or disturbances in the homeostatic equilibrium between synthesis and degradation of extracellular matrix molecules. Cytokines are important regulators of expression of the pro-fibrogenic genes, including type I collagen and its molecular chaperone Hsp47 and proteolytic enzymes degrading extracellular matrix such as matrix metalloproteinases-1 and -2 (MMP-1 and MMP-2).

Methods: In this study, we analyzed the expression and production of type I collagen, Hsp47, MMP-1 and MMP-2 in normal gingival (NG) and HGF fibroblasts, and investigated the effects of transforming growth factor-beta1 (TGF-β1), interleukin-6 (IL-6) and interferon-gamma (IFN-γ) on the main processes controlling type I collagen synthesis and degradation using NG and HGF fibroblasts.

Results: Our results obtained from semi-quantitative reverse transcriptase-polymerase chain reactions (RT-PCR), Western blots, enzyme-linked immunosorbent assays (ELISA) and enzymographies clearly demonstrated that the expression and production of type I collagen

and Hsp47 were significantly higher in fibroblasts from HGF than from NG, whereas MMP-1 and MMP-2 expression and production were lower in fibroblasts from HGF patients. Addition of TGF-β1 and IL-6, which are produced in greater amounts by HGF fibroblasts, promoted an increase in type I collagen and Hsp47 and a decrease in MMP-1 and MMP-2 expression. IFN-γ reduced both type I collagen and Hsp47 expression, whereas had a slight effect on the expression of MMP-1 and MMP-2.

Conclusion: These patterns of expression and production suggest that enhanced TGF-β1 and IL-6 production simultaneously increase the synthesis and reduce the proteolytic activities of HGF fibroblasts, which may favor the accumulation of extracellular matrix.

KEY WORDS

Hereditary gingival fibromatosis; type I collagen; Hsp47; matrix metalloproteinases; cytokines.

RUNNING TITLE

Effect of cytokines in NG and HGF fibroblasts.

INTRODUCTION

Hereditary gingival fibromatosis (HGF) is a rare oral disease characterized by a slowly and progressive enlargement of both the maxilla and mandible gingiva.[1] The enlarged gingiva has normal color, fibrous consistency and is non-hemorrhagic and asymptomatic. The gingival hyperplasia may be generalized or partial, involving only localized portions of maxilla and mandible, and the degree of enlargement may vary from mild to severe.[2] HGF has an autosomal dominant mode of inheritance with variable penetrance and expressivity, however, autosomal recessive cases have been reported.[3] The most prominent pathologic manifestation of this disease is an excessive accumulation of extracellular matrix, predominantly type I collagen.[4,5] Many studies have shown increased transcriptional and translational levels of type I collagen in both tissue and fibroblasts cultures derived from gingiva of HGF patients.[6-8] During the collagen biosynthesis, nascent single procollagen polipeptides, immediately after translation, undergo modification in the endoplasmatic reticulum, form triple helical chains, and are secreted as procollagen into the extracellular space via the Golgi apparatus. Therefore, post-translational processing mechanisms may be important for the understanding of the precise mechanism of gingival fibrosis in HGF patients, where abundant nascent procollagen polypetides result in overproduction of collagen.

Heat shock protein 47 (Hsp47) is a 47 kDa collagen-binding glycoprotein localized in endoplasmatic reticulum of collagen producing cells.[9,10] Hsp47 primarily binds to collagens particularly to procollagen, but may also associate with fetulin.[11] Immunoprecipitation studies have revealed that Hsp47 is closely associated with the translation-translocation

machinery during the production of procollagen I, suggesting that Hsp47 is a pivotal element during the biosynthesis and secretion of procollagen.[12,13] This association of Hsp47 and type I collagen is also observed in a number of physiological and pathological conditions, including the up-regulation of Hsp47 and type I collagen mRNA during the progression of CCl_4-induced liver fibrosis.[14] Moreover, up-regulation of Hsp47 has been shown to occur during the progression of interstitial fibrosis following unilateral ureteral obstruction[15] and in bleomycin-induced pulmonary fibrosis.[16]

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases capable of degrading various components of the extracellular matrix.[17] These enzymes have been implicated in a variety of physiological and pathological conditions, including embryogenesis, wound healing, inflammation, arthritis and cancer.[18-21] Alterations in the MMPs expression have been also implicated in the etiology of other diseases that include fibrosis as a component. For example, cultured skin fibroblasts from patients with systemic scleroderma, a disease marked by extracelular matrix accumulation in the skin and various internal organs, have been shown to produce less MMP-3 and overexpress the tissue inhibitor of MMPs, favoring the accumulation of matrix.[22] Similarly, cultured fibroblasts derived from radiation-induced skin fibrosis show low MMP-1 activity, reduced steady-state levels of MMP-1 mRNA and increased inhibitor expression.[23] Furthermore, recent data have shown reduced steady-state levels of MMP-1 and MMP-2 mRNA in HGF fibroblasts leading to the net accumulation of extracelular matrix.[24]

Besides the fact that Hsp47 acts as a specific molecular chaperone for nascent chains of type I collagen and that MMPs are key enzymes for the maintenance of composition of

extracellular matrix, it is not clear yet whether these molecules are associated with the excessive accumulation of type I collagen in the gingiva of HGF patients. In this study we investigated the expression and production of type I collagen, Hsp47, MMP-1 and MMP-2 in normal gingival and HGF fibroblasts and examined the regulation of these genes by cytokines believed to be involved in the pathogenesis of gingival fibrosis: transforming growth factor-beta 1 (TGF-β1), interleukin-6 (IL-6) and interferon-gamma (IFN-γ).

MATERIALS AND METHODS

Cell Culture

Human gingival fibroblast primary cultures from 6 patients affected by HGF and from 6 patients with normal gingiva (NG) were obtained using standard explant culture as described previously.[25] Cells were cultured in Dulbecco's modified Eagle's medium[1] (DMEM) containing 10% fetal bovine serum (FBS), 100 μg/ml penicillin, 100 μg/ml streptomycin, 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. All patients were informed about the study's purpose before they consented to participate.

RT-PCR

Total cellular RNAs were isolated from NG and HGF cells after the methods of Chomezynski and Sacchi[26] using the Trizol™ kit*. The concentration of RNA in each

[1] Gibco, BRL, Gaithersburg, MD.

sample was determined by the absorption at 260/280 nm using a spectrophotometer[2]. Two micrograms of total RNA from NG and HGF cells per sample were then used to generate cDNA using the Superscript II RNase H-reverse transcriptase[#]. The resulting cDNAs were subsequently amplified in a 50 µl reaction mixture containing 1 µM of each primer, 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 for type I collagen, Hsp47, MMP-1, MMP-2 and β-actin are showed in Table 1. For Hsp47, type I collagen and β-actin, 30 cycles of amplification were performed in a thermocycler model 9700[3], followed by a final extension of 10 min at 72°C. The cycling parameters were: denaturation for 45 s at 94°C, annealing for 45 s at 56°C, extension for 1.5 min at 72°C. For MMPs, 40 cycles of amplification were made with the following parameters: denaturation for 45 s at 93°C, annealing for 45 s at 58°C, extension for 1.5 min 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.[27] The PCR amplification yield of target sequences were expressed in arbitrary units as the ratio the optical density of type I collagen, or MMPs/β-actin electrophoretic bands.

Western blot analysis

NG and HGF cells were incubated in lysis buffer containing 50 mM Tris-HCl, 1% Tween 20, 5 mM EDTA, 150 mM $NaCl_2$, 2 mM phenylmethylsulfony fluoride[4] (PMSF), 2 mM N-

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[#] Genesys 2, Spectronic Inst., Rochester, NY.
[□] Perkin Elmer, Foster City, CA.
[4] Sigma Chemical Co., St. Louis, MO.

ethylmaleimide[§] (NEM) pH 7.2 at 4°C for 30 min. After centrifugation, the protein concentration was determined as described by Bradford[28] using bovine serum albumin[§] (BSA) as standard. Equivalent amounts of protein per sample were resolved in a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions,[29] and transferred to nitrocellulose membranes (0.45 um pore size type BA85)[5]. The membranes were blocked for 1 h with 10% non-fat dry milk in phosphate buffered saline (PBS), rinsed in the same buffer, and incubated for 1 h with anti-Hsp47 antibodies[6] diluited 1:2,000 in PBS. After washing, the membranes were developed using the Enhanced Chemiluminescent Western blot[R] kit[7].

Type I collagen production

The production of type I collagen was determined by enzyme-linked immunosorbent assays (ELISA). To obtain conditioned cell culture medium and cells for this assay, NG and HGF fibroblasts were plated in 24-well culture plates at a density of 100,000 cells/well, in DMEM containing 10% FBS and antibiotics. After 16 h, the cells were rinsed with PBS and the medium replaced with serum-free DMEM containing 50 μg/ml of ascorbic acid[§]. After 4 days, the culture supernatants and the cells were harvested. The wells of replicate plates were treated in a like manner and used for cell counts. The cells were collected by scraping in 0.5 M acetic acid for 30 min. The supernatants and the cellular contents of each well were

[5] Shleicher&Schell Inc. Krebe, NH.
[6] StressGen, Victoria, BC, CA.
[7] Amershan Life Sci., Arlington, IL.

pooled, lyophilized and reconstituted in 300 µl of 0.5 M acetic acid. For the ELISA assays, microtiter plate wells were coated with the samples diluted in PBS (1:100) for 2 h at room temperature. The wells were then washed 3 times with 400 µl of 1% Tween 20 in PBS and nonspecific binding sites were blocked with 3% BSA in PBS for 2 h. After washing, biotinylated anti-human type I collagen antibodies[8], diluted 1:2,000 in PBS, were added to the wells and incubated for 2 h. After another washing steps, peroxidase-conjugated streptavidin[§§] diluted 1:500 in PBS was added and maintained for 1 h. The reaction was developed with 0.5 mg/ml of o-phenyllenediamine[§] in citric buffer containing 0.01% H_2O_2 for 20 min. After terminating the reaction with 50 µl of 2 N H_2SO_4, absorbance was read at 450 nm with λ correction at 570 nm. A standard curve was constructed using human placenta type I collagen purified protein[§§] diluted in PBS, ranging of 0 to 800 ng/ml. The cells plated in a second replicate were harvested using 0.2% trypsin and counted with a Coulter Counter[9]. The values were expressed as ng type I collagen/cell.

Enzymographic analysis

Enzymographic analysis was performed as described previously.[30] Equivalent amounts of protein per sample (0.8 µg) from culture supernatants of NG and HGF cells were mixed with non-reducing sample buffer, loaded, and run on a 10% SDS-PAGE containing 1.6 mg/ml of gelatin[10] as substrate. After electrophoresis the gel was soaked for 1 h in 2% Triton X-100 and for 16 h in activation buffer (10 mM Tris-HCl pH 8.0, 5 mM $CaCl_2$) at 37°C. Gelatinolytic activity was visualized by staining with Coomassie blue R-250[§]. To confirm

[8] Chemicon Int., Temecula, CA.
[9] Coulter Electronics, Luton, England.

the identity of these enzymes 0.5 mM of 1,10-phenanthroline[§], a specific inhibitor of MMP activity by chelating Zn^{+2} ions of their catalytic domain, was added.

Production of TGF-β1, IL-6 and IFN-γ

TGF-β1, IL-6 and IFN-γ in culture supernatants of fibroblasts were quantified using ELISA. Briefly, fibroblasts from NG and HGF were plated in 24-well culture plates at a density of $5x10^4$ cells/well in DMEM containing 10% FBS and antibiotics. After 16 h, the cells were rinsed with PBS, and the medium replaced with serum-free DMEM for an additional of 24 h. Following serum starvation, the cells were incubated for 4 days in serum-free DMEM. The culture supernatants were assayed using ELISA kits[§§§] according to the manufacturer's instructions. For the TGF-β1 assays, the supernatants containing the latent form of TGF-β1 were converted to an active form, before measurement, by treatment with 0.167 M HCl for 10 min, followed by neutralization with 1.2 M NaOH in 0.5 M HEPES. The cells of each well were harvested using 0.2% trypsin and counted with a Coulter Counter***. Data were expressed as ρg of protein/cell.

Cell treatments

NG and HGF fibroblasts were compared after the following treatments: (a) exposure to 0.1, 1 and 10 ng/ml of TGF-β1[§§§] for 24 h at 37°C; (b) exposure to 0.1, 1 and 10 ng/ml of IL-6[§§§] for 24 h at 37°C; (c) exposure to 250, 500 and 100 IU/ml of IFN-γ[§§§] for 24 h at 37°C. The

[10] Bio Rad Lab, Hercules, CA.
[§§§] R&D Systems, Minneapolis, MN.

cells were then harvested and RT-PCR assays performed as described above. The data were expressed as percentage of stimulation in comparison with the controls (without treatment) and represented the mean ± SEM of 6 cell lines for each group.

Statistical analysis

Non-parametric one-way analysis of variance (ANOVA) and multiple comparison were used to test group effects and treatments based on Kruskal-Wallis test at 5% significance ($P<0.05$). All assays were performed at least three times in triplicates or quadruplicates.

RESULTS

To determine whether cells derived from patients with HGF expressed and produced altered levels of type I collagen and its molecular chaperone Hsp47 as well as the proteolytic enzymes MMP-1 and MMP-2, we have employed RT-PCR, ELISA, Western blot and enzymography. RT-PCR analysis was performed using total RNA isolated from fibroblast cultures established from 6 patients with HGF and from 6 NG used as controls. Scanning densitometry of the PCR products, after normalization by the value from β-actin housekeeping gene, demonstrated statistically higher levels of type I collagen mRNA in fibroblast cultures from HGF patients than in those from healthy controls ($p<0.05$; Fig. 1A). The levels of Hsp47 mRNA were also significantly higher in HGF fibroblasts than in normal fibroblasts ($p<0.05$; Fig. 1B). Interestingly, HGF fibroblasts that contained increased levels of type I collagen mRNA also exhibited high levels of Hsp47 expression. On the other hand,

the levels of MMP-1 and MMP-2 mRNAs were significantly lower in HGF fibroblasts compared with those from control cells ($p<0.01$; Fig. 1C and D).

Type I collagen production as measured by ELISA was significantly higher in HGF fibroblasts that in normal control fibroblasts ($0.76\pm0.11 \times 10^{-3}$ ng/cell vs $0.42\pm0.08 \times 10^{-3}$ng/cell; $p<0.05$; Fig. 2). Western blot analysis with anti-Hsp47 monoclonal antibody performed on cell lysates revealed that both NG and HGF fibroblasts produced Hsp47 and an incompletely post-translationally processed form of Hsp47 that migrated with a Mr=46 kDa (Fig. 3A). The relative magnitudes of the 46 and 47 kDa bands were assessed by densitometry, demonstrating significantly higher levels of Hsp47 in fibroblast cultures from HGF patients in comparison with the cells from healthy controls ($p<0.05$; Fig. 3B). HGF fibroblasts with high levels of Hsp47 protein also showed levels of type I collagen. Gelatin enzymography performed with culture supernatants of NG and HGF fibroblasts revealed gelatinolytic activities at Mrs= ~70 kDa and ~46 kDa (Fig. 4). These bands probably correspond to the active forms of MMP-2 and MMP-1, respectively.[24] To further characterize the nature of these proteases, their response to a specific inhibitor was investigated as earlier described.[31] All gelatinolytic bands were completely inhibited by 1,10-phenanthroline, which confirms that these enzymes belong to the MMP family (Fig 4, lane 13). Densitometric analysis demonstrated that MMP-2 activity of HGF cells was dramatically reduced to 40-80% of that observed in corresponding NG cell lines ($p<0.005$), whereas MMP-1 activity was slight higher in NG cells.

Since TGF-β1, IL-6 and IFN-γ are known to alter fibrotic gene expression, [32-37] we analyzed the effects of these cytokines on type I collagen, Hsp47, MMP-1 and MMP-2

expression in NG and HGF cultured fibroblasts. Supernatants collected from NG and HGF fibroblasts were used to perform the TGF-β1, IL-6 and IFN-γ immunoassays. TGF-β1 production from NG and HGF cells are depicted in Fig. 5A and reveal that TGF-β1 levels were 1.6 to 20 times higher in supernatants from HGF than from NG cells ($22.89\pm8.84 \times 10^{-4}$ ρg/cell vs $4.21\pm3.88 \times 10^{-4}$ρg/cell). These differences in TGF-β1 production for all HGF cell lines were statistically different from the control cells ($p<0.001$). The production of IL-6 was also significantly higher in HGF fibroblasts than in NG fibroblasts ($231.42\pm46.53 \times 10^{-3}$ ρg/cell vs $79.69\pm51.98 \times 10^{-3}$ ρg/cell; $p<0.0005$, Fig. 5B). IFN-γ was not detected in both HGF and NG supernatant cultures using these experimental conditions. The effect of TGF-β1, IL-6 and IFN-γ on the expression of type I collagen, Hsp47, MMP-1 and MMP-2 by fibroblasts from NG and HGF are depicted in figure 6. TGF-β1 and IL-6 enhanced significantly type I collagen and Hsp47 mRNA levels in both cell lines (Fig. 6A and B). The TGF-β1 concentration of 0.1 ng/ml produced the highest stimulation on type I collagen and Hsp47 expression. TGF-β1 and IL-6 regulated type I collagen mRNA levels synchronously to that of Hsp47 mRNA. At the same concentrations, TGF-β1 and IL-6 significantly inhibited MMP-1 and MMP-2 expression by NG and HGF fibroblasts (Fig. 6C and D). No significant differences in the relative expression rate for all studied concentrations between groups were observed. Addition of IFN-γ to fibroblast cultures suppressed mRNA levels of type I collagen and Hsp47 of a dose-independent manner (Fig. 6A and B). Conversely, similar assays for MMPs expression revealed that MMP-1 and MMP-2 expression by NG

and HGF cells treated with IFN-γ were quite equivalent to those of control cells (without IFN-γ) when adjusted to actin levels (Fig. 6C and D).

DISCUSSION

HGF is characterized by an overgrowth of the gingiva with deposition of dense collagen fibers. Although some reports of HGF have documented increased synthesis of extracellular matrix including collagen, the biochemical mechanisms involved in the development of this disease are unknown. There are few controversial studies examining the *in vitro* production of collagen in HGF. Both increase and decrease in collagen synthesis have been reported.[6,7,38-40] Our previous investigations showed that HGF fibroblasts are metabolically more active than normal fibroblasts.[5,8,24,25,41] In the present study, we have shown that both type I collagen and Hsp47 mRNA and protein levels were significantly increased in cultured HGF fibroblasts compared to NG fibroblasts. Furthermore, HGF fibroblasts with increased levels of type I collagen mRNA and protein also exhibited high levels of Hsp47.

The binding of Hsp47 to the nascent type I procollagen peptides prevents premature folding and aggregation of procollagen chains.[42,43] Previous study demonstrated that transfection of antisense oligonucleotides for Hsp47 into mouse collagen-secreting embryonic cells causes a decrease in fully elongated nascent procollagen α1 (I).[44] Hsp47 has also been reported to have an inhibitory effect on the degradation of procollagens in the endoplasmatic reticulum.[45] Therefore, in HGF fibroblasts with enhanced type I collagen synthesis, an increase in the Hsp47 levels may be necessary to maintain the stoichiometry of the interaction between the two molecules. Hsp47 may play an important role in the post-

translational processing of the overproduced type I procollagen chains, leading to its accumulation in HGF. The results presented here also reinforce reported evidences that embrace Hsp47 as a pivotal element during the biosynthesis and secretion of type I procollagen.[15,16,43,44] Although all fibroblast strains from HGF patients had higher expression of type I collagen and Hsp47 than fibroblasts from NG patients, the results were not uniform. Many studies have shown that the expression of type I collagen and other proteins varies between different gingival fibroblast strains. This finding is in accord with the "cells population theory" proposed by Hassel & Cooper[46] and substantiated by Phipps et al.[47] Despite the variability, the expression and production of type I collagen and Hsp47 were significantly higher in HGF than in the NG group.

In addition to the strong association between type I collagen and Hsp47 expression in HGF fibroblasts, our cytokine studies demonstrated a coordinated regulation. TGF-β1 and IL-6 increased whereas IFN-γ decreased the expression of both type I collagen and Hsp47 in NG and HGF fibroblasts. Previous studies have also demonstrated co-regulation of collagen and Hsp47.[8] Furthermore, type I collagen and Hsp47 synthesis decreases after fibroblast transformation[42] and increases during differentiation of F9 teratocarcinoma cells after treatment with retinoic acid.[12] TGF-β and epidermal growth factor (EGF) were reported to coordinately regulate the expression of type I collagen and Hsp47 in L6 myoblasts: TGF-β stimulates and EGF suppresses the expression of both type I collagen and Hsp47.[48] Exceptionally, Hsp47 is not co-regulated with collagen by heat induction.[8] The regulation of heat shock protein gene expression with respect to procollagen synthesis is not well understood. Co-regulation might be due to the sharing of common regulatory sequences in

the promoter region of the type I collagen and Hsp47 genes such as AP-1 binding site,[49] whereas heat induction may require the presence of heat shock elements found only in the promoter regions of Hsp47.[50]

The studies presented here also revealed that the expression and activity of MMP-1 and MMP-2 in NG cells were higher than in HGF cells. The role of MMP-1 in effecting interstitial collagen degradation is well documented, and although MMP-2 acts predominantly on type IV collagen, MMP-2 has also been shown to degrade type I collagen in its native form.[51] Moreover, MMP-2 inhibition may contribute to an abnormal accumulation of glycosaminoglycans and proteoglycans, substrates for MMP-2, in the HGF gingival tissues. Consequently, the reduction of the degradative capacity of HGF cells may contribute to the increased collagen content even in the absence of type I collagen overexpression. Interestingly, similar inhibitions of MMP-1, MMP-2 and MMP-3 have been described in the cyclosporin-induced gingival overgrowth.[30] Similarly, inhibitions in the MMPs expression have been associated with a variety of other fibrotic diseases.[22,23,52-54]

TGF-β1 and IL-6 are recognized as key mediators responsible for the accumulation of extracellular matrix in both drug-induced gingival overgrowth and HGF.[7,25,55-59] It is well established that *in vitro* TGF-β1 and IL-6 upregulate collagens, fibronectins and laminins in a variety of cell types.[60] However, increased synthesis does not necessarily lead to extracellular matrix accumulation. Rather, fibrosis seems to be a disturb in the balance between degradation and deposition of extracellular matrix. We have recently demonstrated that cyclosporin simultaneously increases the TGF-β1 levels and inhibits the levels of MMP-1 and MMP-2.[61] In addition, neutralizing antibodies against TGF-β1 increase MMP-1 and

MMP-2 expression by HGF fibroblasts, affecting a state conducive to the net accumulation of extracellular matrix in HGF.[24] In primary human lung fibroblasts, TGF-β1 and IL-6 control a net increase in the deposition of extracellular matrix, especially collagen, a decrease in MMP-1 secretion, and an increase in the expression of MMPs inhibitors.[62,63] On the other hand, IFN-γ is a potent inhibitor of collagen production.[64,65] Our observations thus describe the effects of TGF-β1 and IL-6 in NG and HGF fibroblasts and emphasize the biological role of these cytokines in the pathogenesis of the gingival fibrosis of HGF patients.

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FIGURE LEGENDS

Figure 1.

Expression of type I collagen, Hsp47, MMP-1 and MMP-2 in gingival cells from NG and HGF. Total RNA isolated from gingival fibroblasts from 6 normal individuals and from 6 patients with HGF were subjected to RT-PCR assays using specific primers for type I collagen (A), Hsp47 (B), MMP-1 (C) and MMP-2 (D). The inserts show representative samples of the PCR products. Comparison of expression of type I collagen, Hsp47, MMP-1 and MMP-2 in NG and HGF cells indicated that in HGF fibroblasts, type I collagen mRNA was increased approximately in 53% and Hsp47 mRNA in 72% of the value observed in corresponding normal control fibroblasts, while MMP-1 and MMP-2 mRNA were reduced to 41% and 61% of the value observed in corresponding normal control, respectively. Values are expressed as the optical density units relative with normalizing to equivalent amounts of the β-actin mRNA.

Figure 2.

The rate of type I collagen production in monolayers of NG and HGF cells as determined by ELISA. The values represent the mean of ng x 10^{-3}/cell of type I collagen production for triplicate wells ± SEM. The amount of type I collagen production by HGF cells was significantly greater than NG cells ($p<0.05$).

Figure 3.

(A) Western blot analysis of total cellular proteins from NG and HGF cells utilizing anti-Hsp47 antibodies. (B) Densitometric analysis of the Hsp47 bands demonstrated that Hsp47 production by HGF fibroblasts was approximately 1.4 greater than HG fibroblasts ($p<0.05$).

Figure 4.

Enzymographic analysis of the culture supernatants of NG and HGF fibroblasts. Gelatinolytic activities were detected at ~70 kDa and ~46 kDa in NG and HGF cells culture supernatants, consistent with the presence of the MMP-2 and MMP-1, respectively. Lanes I show the complete inhibition of gelatinolytic activities of MMP-1 and MMP-2 in the conditioned medium obtained from HGF cells incubated with 0.5 mM of 1,10-phenanthroline. Densitometric analysis of the MMP-1 and MMP-2 bands demonstrated a reduction in the activities of MMP-1 and MMP-2 in the HGF group.

Figure 5.

Production of TGF-β1 (A) and IL-6 (B) by NG and HGF cells. Data are expressed as ρg of protein/cell. The amount of TGF-β1 and IL-6 produced by HGF cells was significantly higher than NG cells. ($p<0.05$)

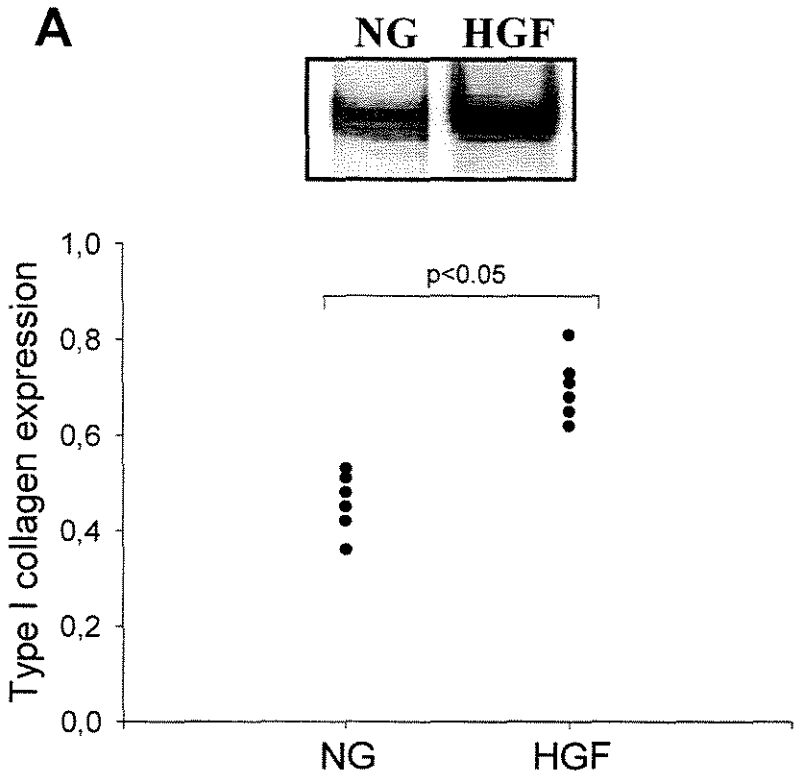
Figure 6.

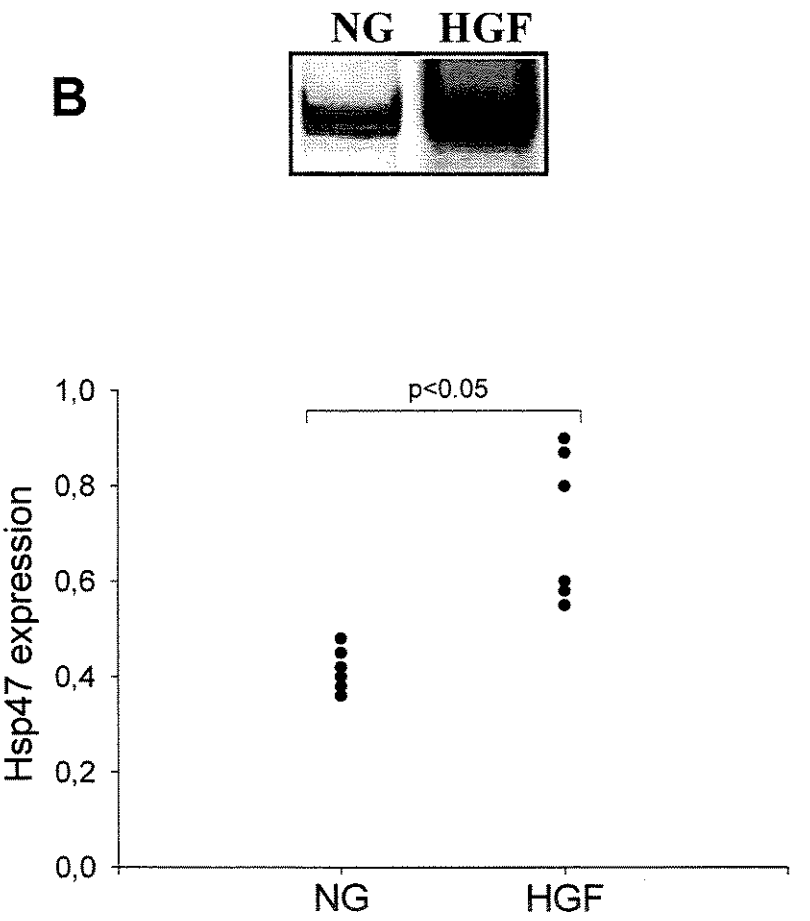
Effect of TGF-β1, IL-6 and IFN-γ on expression of type I collagen, Hsp47, MMP-1 and MMP-2 by NG and HGF fibroblasts. Gingival fibroblasts were incubated with increasing concentrations of TGF-β1 (0.1, 1 and 10 ng/ml), IL-6 (0.1, 1 and 10 ng/ml), or IFN-γ (250, 500 and 1000 IU/ml), cultured for 24 h, and the levels of type I collagen (A), Hsp47 (B), MMP-1 (C) and MMP-2 (D) expression determined by RT-PCR. Values represent the means ± SEM from independent experiments with 6 cell lines for each group and are expressed as percentage of stimulation compared to the control (without treatment).

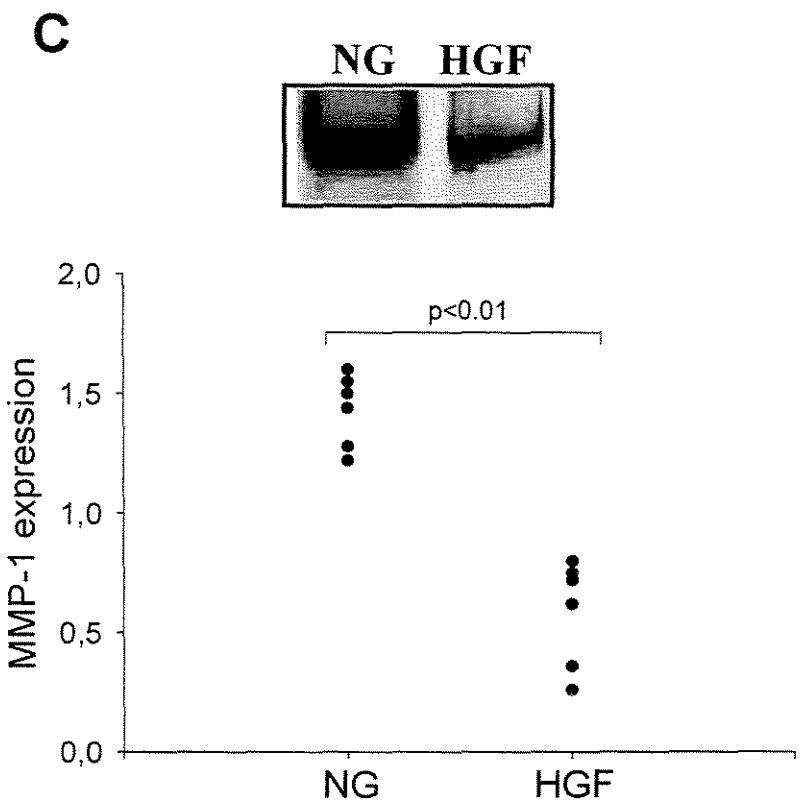
Table 1. Description of primers used in RT-PCR.

	Primer	Sequence 5' → 3'	Predicted size (base pairs)
Type I collagen	Sense	CTG GCA AAG AAG GCG GCA AA	98
	Antisense	CTC ACC ACG ATC ACC ACT CT	
Hsp47	Sense	CAC ACT GGG ATG AGA AGT TTC	503
	Antisense	TGT CCG GTG CAT CAT GCT AAC	
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	
β-actin	Sense	TCA GAA GGA CTC CTA TGT GG	506
	Anti-sense	TCT CTT TGA TGT CAC GCA CG	

Figure 1


A
NG
HGF
Type I collagen expression
1,0
0,8
0,6
0,4
0,2
0,0
p<0.05
NG
HGF


B
NG
HGF
p<0.05
Hsp47 expression
1,0
0,8
0,6
0,4
0,2
0,0
NG
HGF


C
NG
HGF
2,0
1,5
1,0
0,5
0,0
MMP-1 expression
p<0.01
NG
HGF

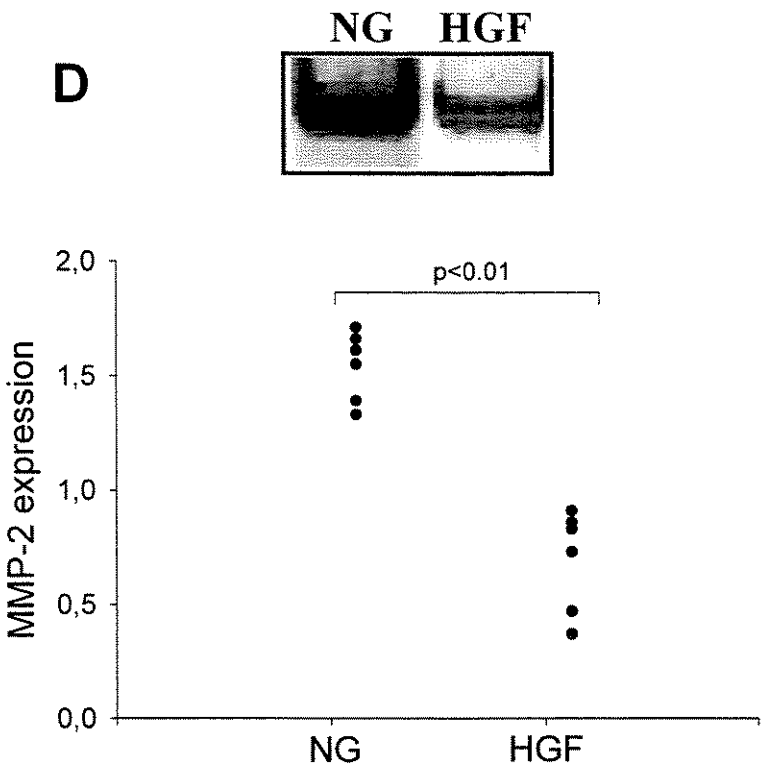

D
NG
HGF
p<0.01
MMP-2 expression
2,0
1,5
1,0
0,5
0,0
NG
HGF

Figure 2

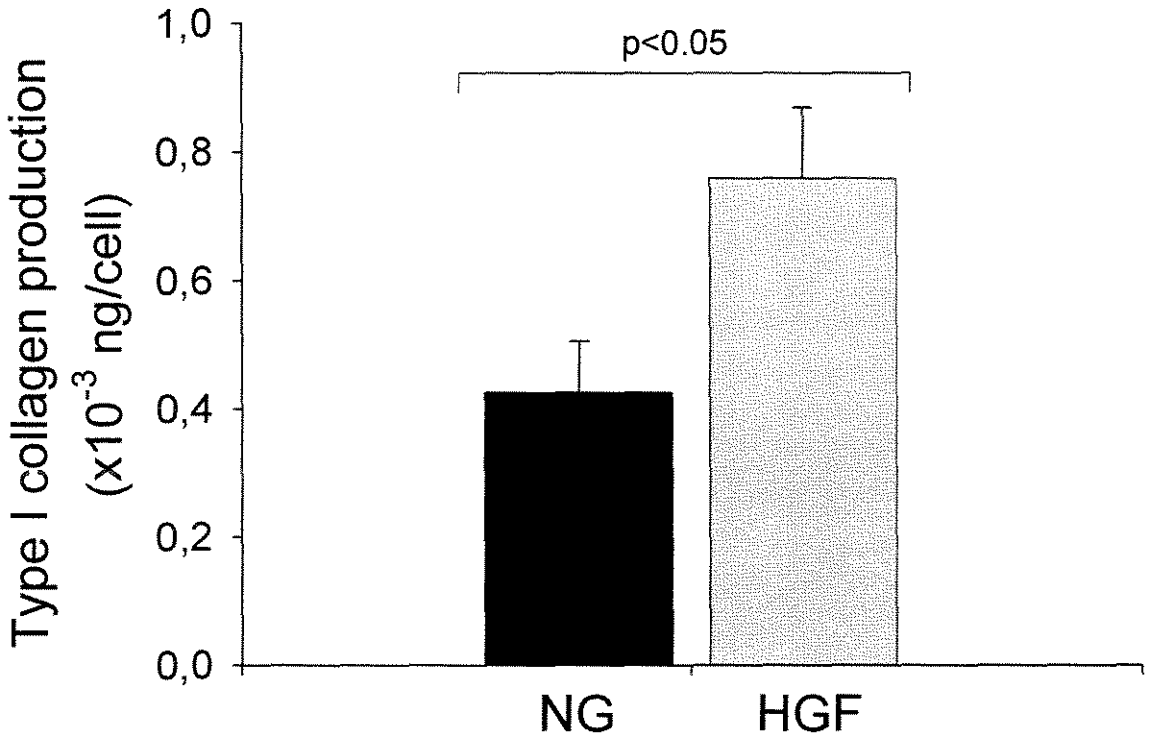

Type I collagen production
(x10-3 ng/cell)
1,0
0,8
0,6
0,4
0,2
0,0
p<0.05
NG
HGF

Figure 3A

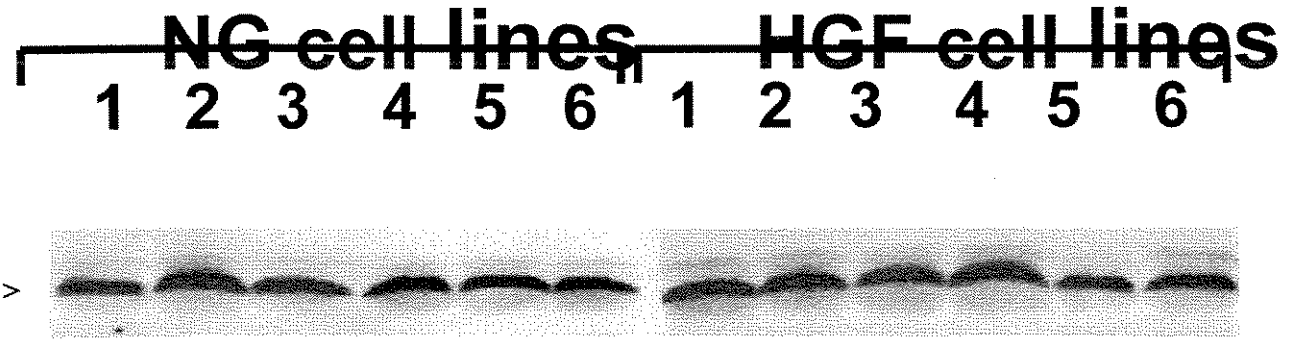


Figure 3B

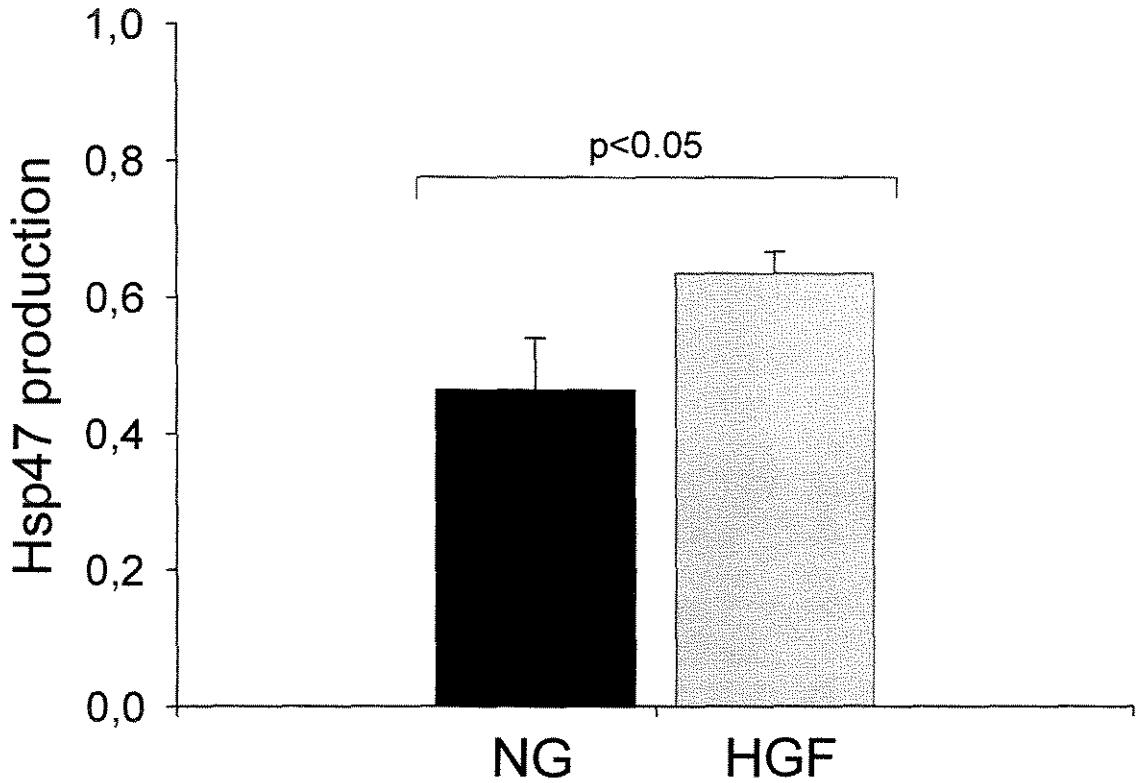


Figure 4

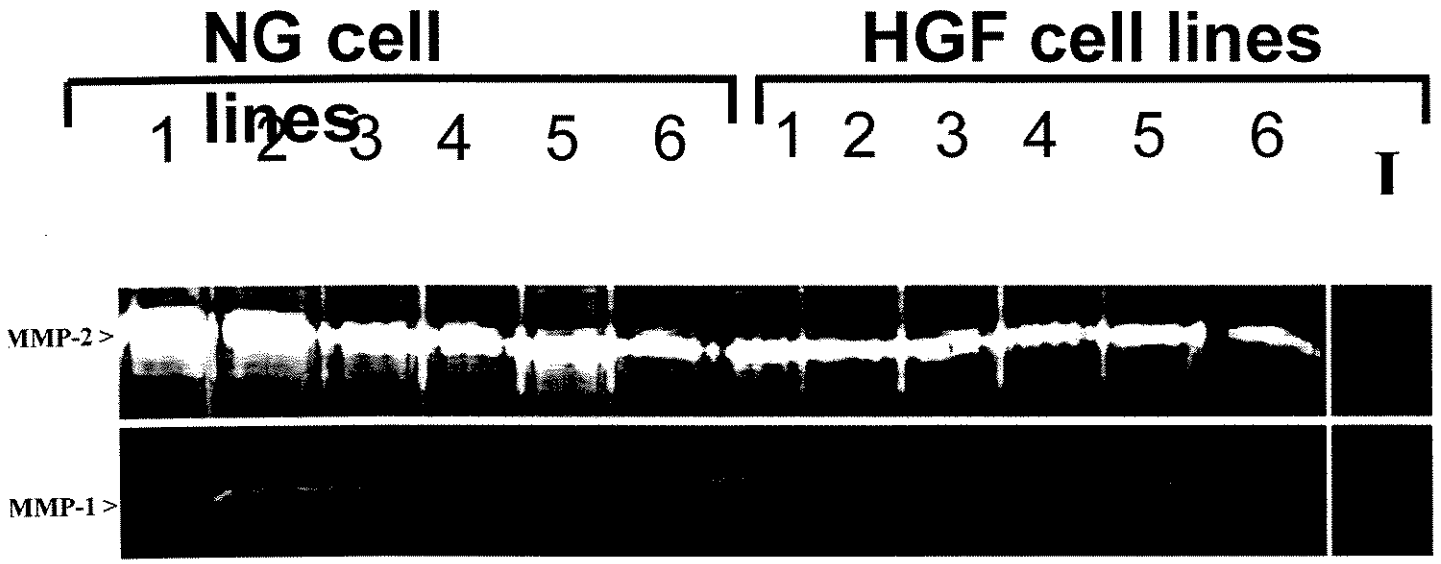


Figure 5

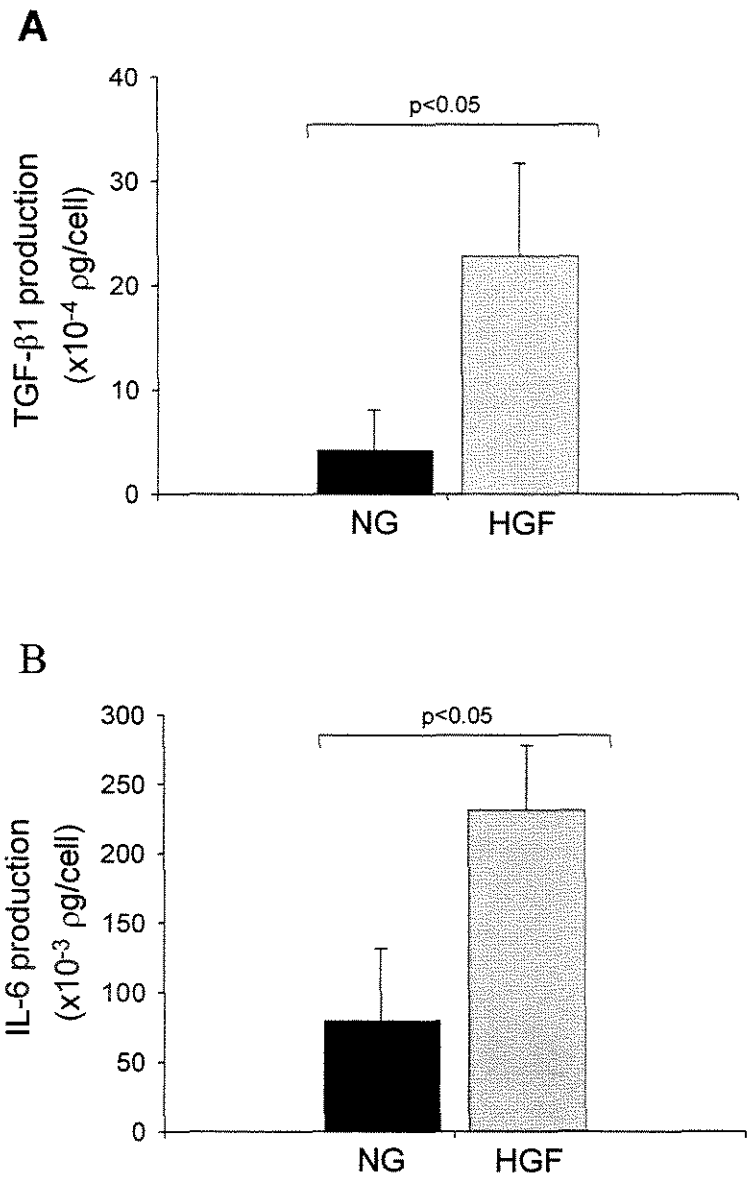
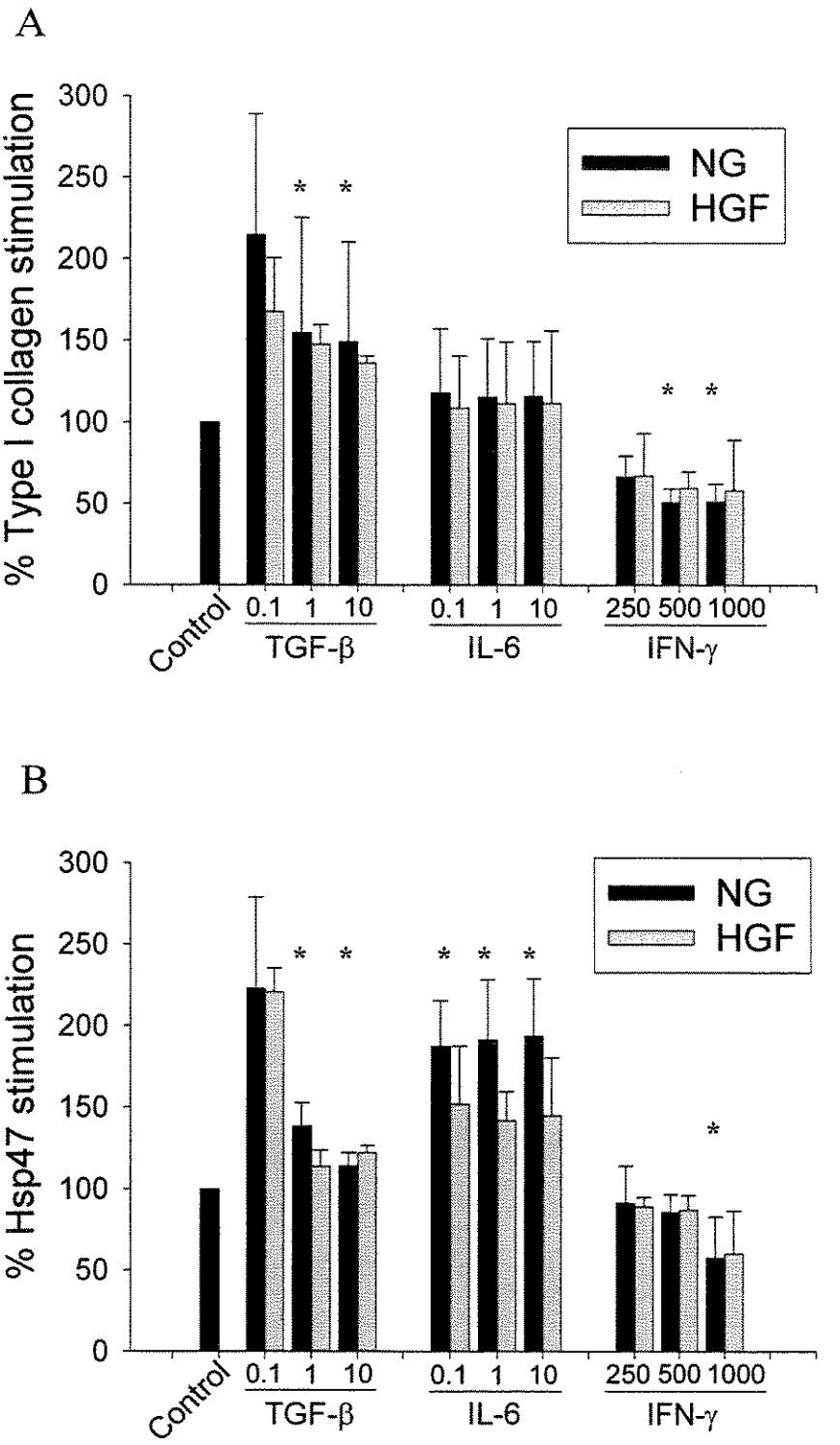
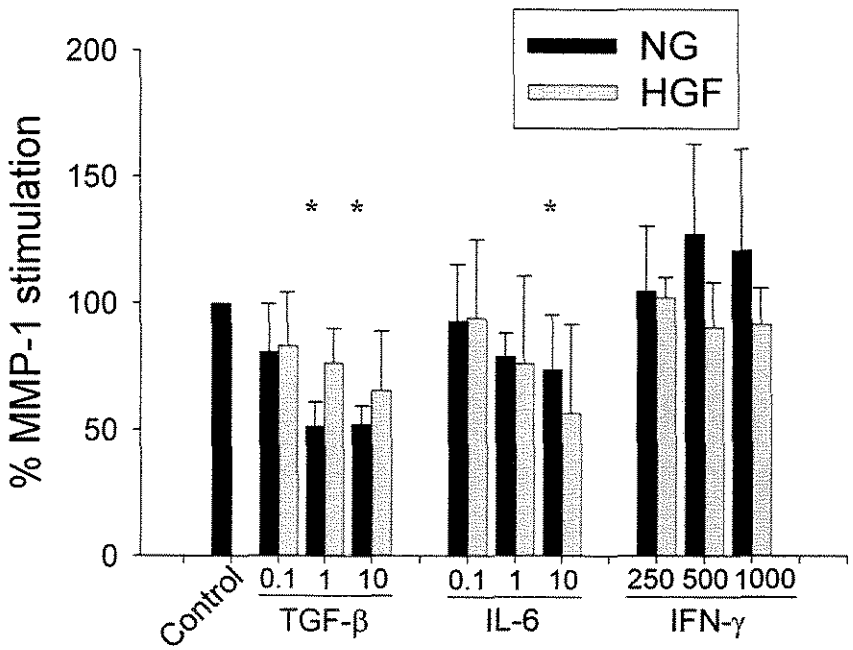

A
40
30
20
10
0
TGF-β1 production
(x10-4 ρg/cell)
p<0.05
NG
HGF
B
300
250
200
150
100
50
0
IL-6 production
(x10-3 ρg/cell)
p<0.05
NG
HGF

Figure 6


A
% Type I collagen stimulation
300
250
200
150
100
50
0
NG
HGF
*
Control
0.1
1
10
TGF-β
0.1
1
10
IL-6
250
500
1000
IFN-γ
B
% Hsp47 stimulation
300
250
200
150
100
50
0
NG
HGF
Control
0.1
1
10
TGF-β
0.1
1
10
IL-6
250
500
1000
IFN-γ

C


NG
HGF
% MMP-1 stimulation
200
150
100
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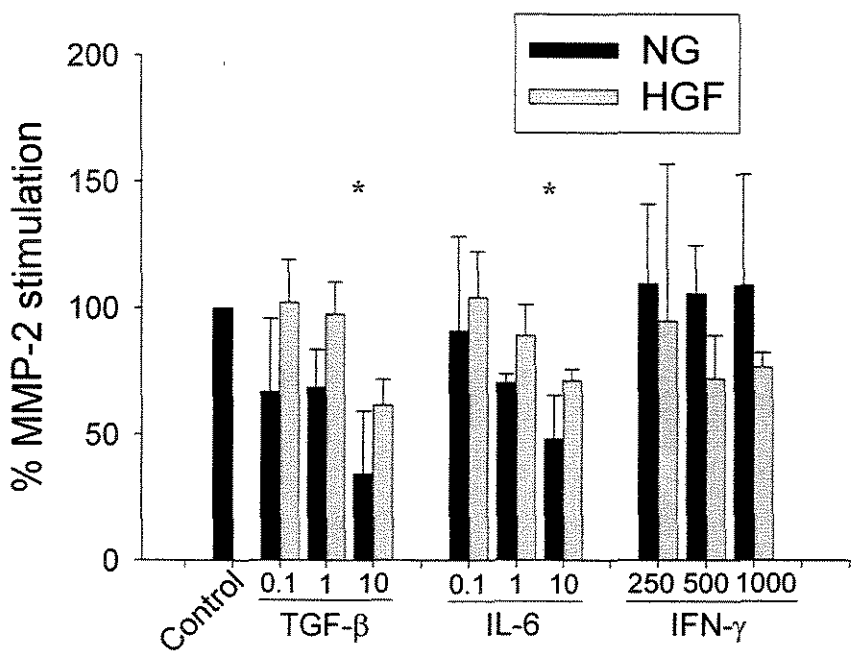
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Testosterone stimulates proliferation and inhibits interleukin-6 production by normal and hereditary gingival fibromatosis fibroblasts.

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ABSTRACT

Hereditary gingival fibromatosis (HGF) is a rare oral condition characterized by a slow and progressive enlargement of the gingiva, involving both the maxilla and mandible. In vitro, HGF fibroblasts demonstrate a proliferative index significantly higher than fibroblasts from normal gingiva (NG). The objective of this study was to determine the effect of dihydrotestosterone on the proliferation of gingival fibroblasts derived from patients with HGF (n=4) and from 4 healthy individuals. Additionally, we analyzed the effect of dihydrotestosterone on interleukin-6 (IL-6) production and determined the expression levels of androgen receptors in NG and HGF fibroblasts. Gingival fibroblasts from NG and HGF were incubated with increasing concentrations of dihydrotestosterone with or without androgen blockers, cultured for 24 h, and the proliferation index determined by automated cell counter. IL-6 production, in this system, was quantified using a "capture" enzyme-linked immunosorbent assay (ELISA). Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to measure androgen receptors mRNA expression. The results indicated that dihydrotestosterone simultaneously downregulates the production of IL-6 and upregulates the cell proliferation. Finasteride and cyprosterone acetate, two anti-androgens, reversed partially these effects. Androgen receptor mRNA expression was identified in both NG and HGF fibroblasts; however, the levels in NG were higher than those

observed in HGF. These results show that testosterone coordinates the proliferation and production of IL-6 by normal and HGF fibroblasts.

Key words: hereditary gingival fibromatosis; dihydrotestosterone; interleukin-6; proliferation; fibroblasts.

Running title: Effect of testosterone in NG and HGF fibroblasts

Introduction

Sex hormones have a major role in the growth, development and regulation of target tissues including the mammary and prostate glands as well as sex organs, where they interact with other hormones, growth factors and cytokines, precisely regulating cell proliferation and differentiation (1, 2). The sex hormones induce their functions via specific intracellular receptors, which in turn regulate the transcription of target genes. These include adhesion molecules, proteolytic enzymes, extracellular matrix macromolecules, cytokines and cell cycle regulatory proteins (3-6). Sex hormones are closely related to the onset and progression of periodontal diseases and to the gingival hyperplasia induced by nifedipine and cyclosporin (7, 8). In addition, castration prevents calcium channel blocker-induced gingival overgrowth in beagle dogs (9). Although the molecular basis of these interactions remains poorly understood, Parkar et al. (10) have shown, using semi-quantitative reverse transcriptase-polymerase chain reactions (RT-PCR), that the androgen receptors but not the estrogen receptors are expressed by gingival fibroblasts, and that sex hormones influence the metabolism of fibroblasts derived from periodontal tissues (11, 12). Moreover, recent studies have documented that the expression and production of interleukin-6 (IL-6) is downregulated by dihydrotestosterone, a potent metabolite resulted of metabolism of testosterone (13, 14). IL-6 possesses powerful profibrogenic activity and is believed to be a pivotal cytokine in fibrotic diseases including keloids, systemic sclerosis, and gingival overgrowth induced by cyclosporin-A (15-17).

Hereditary gingival fibromatosis (HGF) is a rare oral disease characterized by a slowly and progressive enlargement of both the maxilla and mandible gingiva (18). The

enlarged gingiva is of normal color, firm consistency, non-hemorragic and assymptomatic (19). HGF occurs as an isolated finding or associated with other features such as hypertrichosis (20), mental retardation (21), epilepsy (22), progressive sensoneural hearing loss (23), and abnormalities of the extremities, particularly of fingers and toes (24). HGF has an autosomal dominant mode of inheritance with variable penetrance and expressivity, however, autosomal recessive cases have been reported (25). Histologically, HGF tissues are composed mainly of dense connective tissue rich in collagen fibers; the epithelium is hyperplastic with long rete pegs (26, 27).

The cellular and molecular mechanisms underlying the characteristic accumulation of the dense fibrous gingival connective tissue in HGF are unknown, and the results of cell culture studies are controversial (28-30). Our previous experiments using four strains of HGF fibroblasts from patients of the same family demonstrated altered proliferative behavior and production of excessively large amounts of protein, particularly type I collagen (31, 32). These results are consistent with the reports of Tipton et al. (33) and Tipton and Dabbous (34), demonstrating that HGF fibroblasts synthesize greater amounts of collagen and proliferate more rapidly than NG fibroblasts. The purpose of the present study was to determine the relative levels of androgen receptor mRNA and to analyze the effect of dihydrotestosterone on the proliferation rates and production of IL-6 by gingival fibroblasts isolated from NG and HGF.

Materials and Methods

Cell culture

Human gingival fibroblasts from volunteers with NG and patients with autosomal dominant HGF were obtained using standard explant culture as described previously (31). All patients with HGF were members of the same family, and no systemic alterations commonly seen in association with HGF were observed. The mean age of these patients with HGF was 22.1 years and included 3 males and 1 female. Normal gingiva was obtained from 3 males and 1 female, with a mean age of 26 years. Informed consents were obtained from all patients and the study protocol was approved by the Ethical Committee in Research at the University of Campinas Dental School. Cells were cultured in Dulbeccos modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA), 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine at 37° C in a 5% CO_2 atmosphere. All experiments were performed using cells between the third and tenth passages. Normal human prostate epithelial cells were purchased from Clonetics Co. (San Diego, CA, USA) and cultured as manufacturer's recommendations.

Androgen Receptor Expression

The expression of androgen receptor was assessed by RT-PCR. Total cellular RNAs were isolated from NG, HGF, and normal human prostate epithelial cells using the methods of Chomezynski and Sacchi (35) and the Trizol kit (Gibco BRL, Gaithersburg, MD, USA). The concentration and purity of RNA in each sample was determined by 260/280 nm readings using a Genesys 2 spectrophotometer (Spectronic Inst., Rochester, NY, USA). Two

micrograms of total RNA from each cell line were reverse transcribed to cDNA using a Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA). For androgen receptor expression analysis, the resulting cDNAs were amplified in a 25 µl reaction mixture containing 1µM of each primer, 2 mM $MgCl_2$, 0.8 mM dNTPs, 0.025 U/µl Taq DNA polymerase and 0.5 µCi ^{32}P dCTP (New England Nuclear, Boston, MA, USA). Primers for androgen receptor were sense 5' GCT GCA AGG TCT TCT TCA A 3' and anti-sense 5' TCG TCC ACG TGT AAG TTG CG 3' (10). After denaturation for 5 min at 94° C, 35 cycles of amplification were performed followed by final extension of 5 min at 72° C. The cycling parameters were denaturation for 45 s at 94° C, annealing for 1 min at 60° C and extension for 1.5 min at 72° C. After amplification, 5 µl of PCR product were electrophoresed on a 5% polyacrylamide gel, dried, and autoradiographed. To assess the quality of total RNA and as an internal control, β-actin housekeeping gene was used. Primers for β-actin were previously described (36). The PCR amplification yield of target sequences was expressed in arbitrary units as the ratio the optical density of androgen receptor/β-actin electrophoretic bands.

Effect of dihydrotestosterone and IL-6 treatment on cell proliferation

Fibroblasts from NG and HGF were plated in 24-well culture plates at a density of $1x10^4$ cells/well, in DMEM containing 10% FBS and antibiotics. After 16 h, the cells were rinsed with PBS, and the medium replaced with serum-free DMEM for an additional of 24 h. Following serum starvation cells were stimulated with 2.5% charcoal-treated (CT)-FBS/DMEM supplemented by either 0, 10 and 20 ng/ml of dihydrotestosterone (Sigma

Chemical Co., St. Louis, MO, USA) or by 0, 5 and 10 ng/ml of IL-6 (Calbiochem Novabiochem, San Diego, CA, USA). Cells were harvested after 24 h using 0.2% trypsin in PBS and counted with a Coulter Counted (Coulter Electronics, Luton, England).

To investigate the effect of anti-androgens on proliferation of NG and HGF fibroblasts, these cells cultured as above were treated by either cyprosterone acetate, a steroidal binding of androgen receptor, at a concentration of 10^{-6} and 10^{-5} M or by finasteride, an inhibitor of androgen metabolism by specifically inhibit the enzyme 5α-reductase, at a concentration of 1 and 10 μg/ml, in the absence and presence of 20 ng/ml dihydrotestosterone. Following treatment, the number of cells was determined. All experiments were done three times with triplicate wells.

Production of IL-6

IL-6 in culture supernatants of fibroblasts treated with dihydrotestosterone or dihydrotestosterone plus anti-androgens was quantified using a "capture" enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The results were expressed as pg IL-6/cell and represent the average of six replicate wells.

Statistical analysis

Overall treatment and group effects were assessed using one-way analysis of variance (ANOVA), with post-hoc comparisons base on the Newman-Keuls test. In our comparisons, $P \leq 0.05$ was considered to indicate statistical significance.

Results

In accordance with previous results (10), we detected the expression of androgen receptors in both NG and HGF fibroblasts cells lines (Fig. 1A). Normal human prostate epithelial cells were also positive (data not shown). Figure 1B shows that NG fibroblasts have a higher androgen receptor expression levels than HGF strains. All NG cell lines showed more androgen receptor transcripts than HGF cells, with exception of NG5 that showed androgen receptor mRNA levels lower than the mean of HGF group. NG5 cell line is the sample derived from only female of NG group. Conversely, similar assays for the cell line isolated from a seventeen-years-old female affected with HGF revealed that androgen receptor expression was quite similar to those of HGF cells isolated from males.

The effect of dihydrotestosterone on the proliferation of fibroblasts from NG and HGF are depicted in figure 2. Both cell lines were positively stimulated by dihydrotestosterone ($P<0.0001$). No significant differences in the relative growth rate for both studied concentrations between groups were observed. However, proliferation of fibroblasts from HGF without dihydrotestosterone treatment was significantly higher than those of NG ($P<0.05$). These data indicate that testosterone has direct effect on the proliferation of gingival fibroblasts. To confirm this hypothesis, anti-androgens, which inhibit the androgen metabolism or compete by their receptor, were added to the culture medium of NG3 and HGF2 fibroblasts stimulated by 20 ng/ml dihydrotestosterone. These studies demonstrated that both anti-androgens reduced the mitogenic effects of dihydrotestosterone, while their combination practically abrogated this effect (Fig. 3). The effect of cyprosterone acetate was independent of concentration and reduced the number of

NG3 and HGF2 cells in 11% and 15%, respectively, in comparison with the respective control (20 ng/ml dihydrotestosterone alone) (P=0.12). In the presence of finasteride, NG3 proliferation was inhibited by approximately 27-33% (P<0.001), whereas HGF2 proliferation was inhibited by 23-25% (P<0.01). Cyprosterone acetate and finasteride simultaneously added to the cultures reduced the dihydrotestosterone effect in the proliferation of NG3 in 42% and of HGF2 in 38% (P<0.0005).

Testosterone has been shown to alter IL-6 expression by normal gingival and periodontal ligament fibroblasts (13, 14). Since dihydrotestosterone affects gingival fibroblast proliferation, we sought to determine if dihydrotestosterone stimulation could be supported by alterations in IL-6 levels. The results showed in figure 4 indicate that, in the presence of dihydrotestosterone, NG and HGF cells produced decreased levels of IL-6. Both concentrations of dihydrotestosterone (10 and 20 ng/ml) inhibited IL-6 production of NG3 fibroblasts by approximately 29% and 55% respectively (P<0.0001), whereas HGF2 production was inhibited by approximately 22% and 50% respectively (P<0.001). The addition of finasteride to dihydrotestosterone treated cells increased in 25% the IL-6 production comparing with IL-6 production by cells stimulated by dihydrotestosterone alone. In contrast, cyprosterone acetate had slight effect on IL-6 production when added alone or in association with dihydrotestosterone. The combination of both anti-androgens increased in approximately 33% and 40% the production of IL-6 by NG3 and HGF2 fibroblasts respectively in presence of 20 ng/ml dihydrotestosterone (Fig. 4). Although the effect of dihydrotestosterone on IL-6 production was not reverted by anti-androgens to statistically significant levels, the reversion caused by finasteride was stronger than that of cyprosterone

acetate ($P<0.05$). In order to establish whether the stimulatory effect of dihydrotestosterone on cell proliferation occurred because of down-regulation of IL-6 production, NG and HGF fibroblasts were incubated directly with increasing concentrations of IL-6 for 24 h. These studies showed that the treatment with IL-6 at various doses caused a progressive decrease in growth rate of NG and HGF fibroblasts ($P<0.0001$) (Fig. 5).

Discussion

Our previous studies showed that HGF fibroblasts proliferate more rapidly than NG fibroblasts (31). Because gingival fibroblasts from drug-induced gingival overgrowth metabolize testosterone in levels significantly higher than gingival fibroblasts derived from normal tissues (7), we were interested in determining whether HGF fibroblasts express androgen receptors and if testosterone modulates their proliferation. To the best of our knowledge, no previous investigation has analyzed the effect of testosterone metabolites on cell proliferation of gingival fibroblasts derived from normal or diseased tissues. In this study we have determined that androgen receptor expression by HGF fibroblasts is lower than by NG fibroblasts, and that dihydrotestosterone causes a significant stimulation on the proliferation of both type of cells. Since the response to testosterone depends on androgen receptor expression, the similar proliferative rates found in NG and HGF cells after dihydrotestosterone treatment suggests that the NG fibroblasts are more sensitive to dihydrotestosterone than HGF fibroblasts, probably due to its higher androgen receptor levels. Additionally, although gingival fibroblasts isolated from female patients (NG5 and HGF5) showed lower androgen receptor levels, we did not find any difference on proliferation between males and females cells treated with dihydrotestosterone. Similarly,

Southren et al. (37) showed no correlation between dihydrotestosterone metabolism and age or sex of the patient. Recently, Xia et al. (38) have shown that the expression of androgen receptor isoforms varies in different cell lines, and that different androgen receptor isoforms may modulate distinct activation or repression of gene transcriptional activity through activation of cytokine responsive promoters. Furthermore, androgens are known to regulate expression of the androgen receptor (39).

A number of studies have suggested that sex hormones, including testosterone and its metabolites, may also have an important role in drug-induced gingival overgrowth (7, 40, 41). While it is well established that sex steroids are associated with periodontal function and disease, the mechanisms underlying its effects are not known. Our findings demonstrate that increasing concentrations of dihydrotestosterone progressively upregulate the proliferation of NG and HGF fibroblasts. The dose dependent dihydrotestosterone-induced proliferation of NG and HGF cells is consistent with the results of other studies showing that fibroblasts from the prostate stroma can be stimulated by this androgen metabolite (42, 43). A number of studies have shown that the androgen receptor has affinity for other steroids in addition to dihydrotestosterone, including synthetic anti-androgenic drugs (44). To determine whether the stimulatory effect of dihydrotestosterone on cell proliferation was mediated by androgen receptors, we analyzed the effect of 2 anti-androgen blockers: cyprosterone acetate, which compete with dihydrotestosterone for the androgen receptor binding site, and finasteride, an potent inhibitor of dihydrotestosterone by specifically inhibit the enzyme 5α-reductase. Anti-androgen alone or in combination showed partial inhibition of the proliferation induced by dihydrotestosterone.

IL-6 is a multifunctional cytokine with impact on a wide variety of body systems (45). Dysregulation of its expression may result in a variety of disorders including osteoporosis, lymphomas, and autoimmune disease. Although its role in periodontal disease is not completely clear, several lines of evidence suggest it may be important in either bone resorption or in mediating inflammatory response (46). Recently, Parkar et al. (13) and Gornstein et al. (14) have demonstrated that dihydrotestosterone causes a marked reduction in the relative levels of IL-6 mRNA in the gingival fibroblasts. Dihydrotestosterone inhibits IL-6 gene expression by maintaining IkBα protein levels, resulting in continued sequestration of NFkB, the promoter factor of IL-6 expression, in the cytoplasm (47). Furthermore, other steroids such as dexamethasone and 17-β-estradiol have been demonstrated to inhibit IL-6 expression (48, 49). Accordingly, we demonstrate here that IL-6 production decreases in both NG and HGF fibroblasts treated with dihydrotestosterone. In the present study, we were unable to establish an optimal concentration of cyprosterone acetate to block the dihydrotestosterone-induced downregulation of IL-6. However, finasteride, a specific inhibitor of dihydrotestosterone metabolism, was more effective than cyprosterone acetate. In contrast, gingival fibroblasts stimulated by cyclosporin-A showed significant upregulation of IL-6 gene expression (15).

In this study we have demonstrated that dihydrotestosterone simultaneously upregulates the cell proliferation and downregulates IL-6 production by fibroblasts from NG and HGF. Addition of IL-6 directly to the cell culture medium of NG and HGF fibroblasts resulted in an anti-proliferative effect. Taken together, our results suggest that the dihydrotestosterone-induced proliferation may be mediated by IL-6. Dihydrotestosterone treatment has been

associated with transforming growth factor-beta 1 (TGF-β1) production. Kim et al. (50) have demonstrated that androgens increase the secretion of TGF-β1, a potent cell stimulator of proliferation. Moreover, TGF-β1 is a potent inducer of IL-6 mRNA and protein expression in primary human lung fibroblasts by active the transcription factor AP-1 (51). However, the addition of neutralizing antibodies against TGF-β1 did not modify the cell proliferation caused by dihydrotestosterone (Coletta RD; unpublished results). Additional work is required to better understand the events trigged by dihydrotestosterone stimulation in NG and HGF fibroblasts, as well as the role of this hormone in the HGF pathogenesis.

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Figure Legends

Fig. 1. (A) RT-PCR analysis of androgen receptor expression in NG and HGF fibroblasts. Total RNA was isolated and cDNA synthesized by RT. After amplification using specific primers to androgen receptor (panel I) and β-actin (panel II), the products were resolved in a 5% polyacrylamide gel and autoradiographed. Lanes A, B, C, and D represent NG1, NG3, NG4, and NG5 cells, and lanes E, F, G, and H represent HGF2, HGF3, HGF5, and HGF7 cells, respectively. (B) Comparison of androgen receptor mRNA expression in HGF and NG cells. Androgen receptor expression was approximately 30% greater in NG than in HGF fibroblasts. Values are expressed as optical density units normalized by β-actin.

Fig. 2. Influence of dihydrotestosterone (DHT) on the proliferation rates of NG and HGF fibroblasts. Gingival fibroblasts were cultured for 24 h in serum-free medium to ensure serum starvation, and then exposed to 10 or 20 ng/ml dihydrotestosterone in medium supplemented with 2.5% CT-FBS. After 24 h, the cells were harvested and counted with a counter cells. Both cell lines were stimulated in a dose dependent manner by dihydrotestosterone ($P<0.0001$).

Fig. 3. Effect of androgen blockers on cell proliferation of NG3 and HGF2 fibroblasts stimulated with dihydrotestosterone (DHT). Fibroblasts were incubated for 24 h with 20 ng/ml dihydrotestosterone in presence of 10^{-6} and 10^{-5} M of cyprosterone acetate (CYPA)

or 1 and 10 ug/ml of finasteride (FIN), as described in Methods. Following the treatment, the number of cells was determined. Untreated and treated with 20 ng/ml dihydrotestosterone cells were used as controls.

Fig. 4. Effect of dihydrotestosterone (DHT) and androgen blockers on IL-6 production by NG3 and HGF2 fibroblasts. Gingival fibroblasts were incubated with increasing concentrations of dihydrotestosterone alone or in association with finasteride (FIN; 1 and 10 ug/ml), cyprosterone acetate (CYPA; 10^{-6} and 10^{-5} M), or both, cultured for 24 h, and the levels of IL-6 in culture supernatant determined by "capture" ELISA. The addition of increasing concentrations of dihydrotestosterone significantly inhibited IL-6 synthesis ($P<0.001$). Finasteride, the inhibitor of androgen metabolism, reverted the effect of 20 ng/ml dihydrotestosterone at significantly levels ($P<0.05$), whereas cyprosterone acetate, the androgen receptor blocker, not.

Fig. 5. The effect of IL-6 on the cell proliferation rate of NG and HGF fibroblasts. Data are the mean ± SEM of three triplicate determinations. The treatment with IL-6 reduced significantly the growth rate of both NG and HGF fibroblasts ($P<0.0001$).

Figure 1A

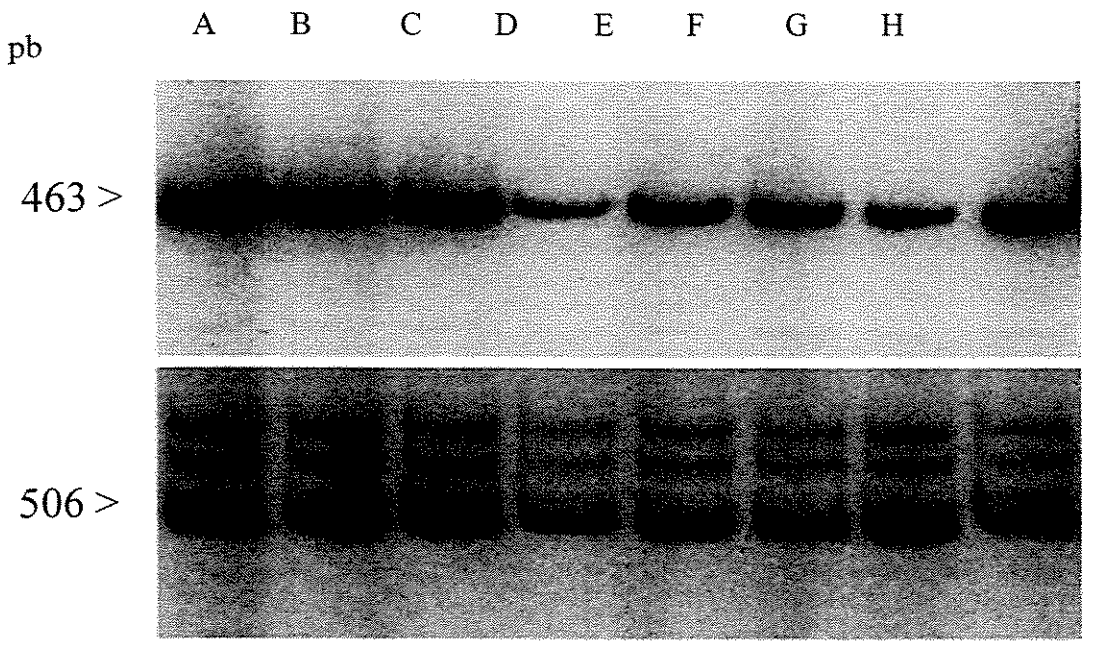


Figure 1B

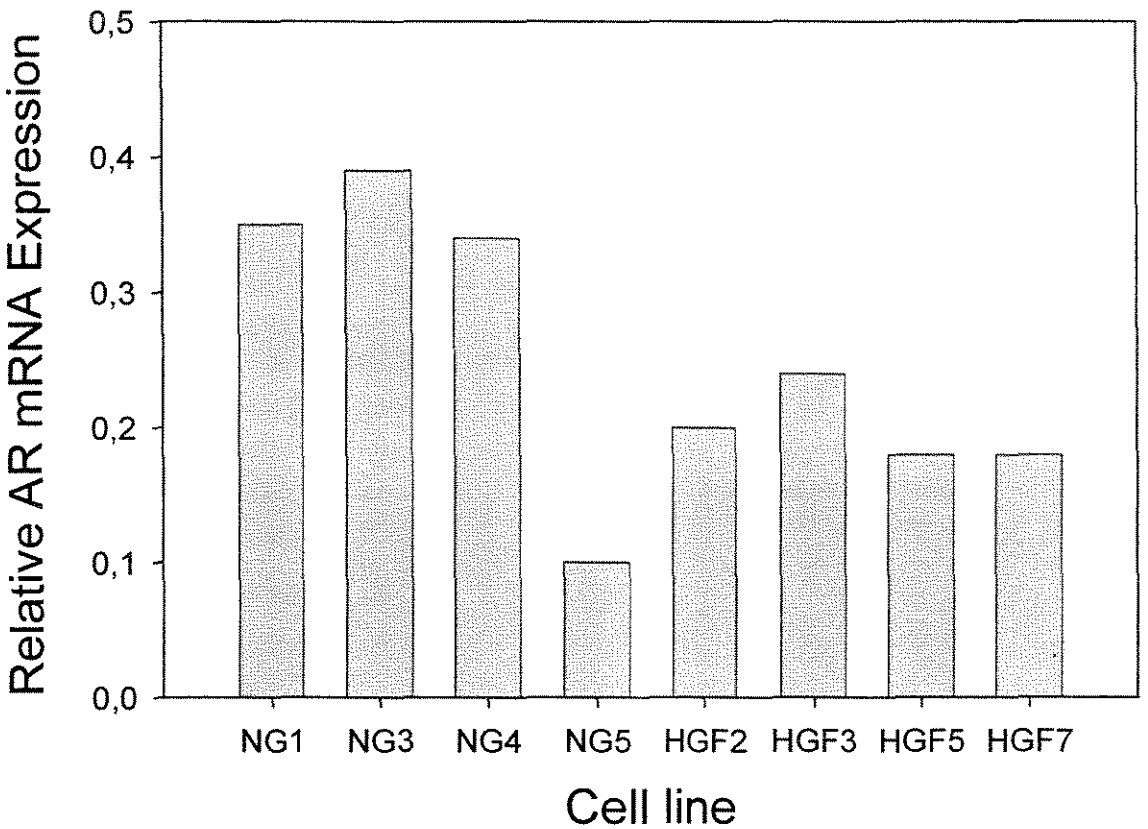

Relative AR mRNA Expression
0,5
0,4
0,3
0,2
0,1
0,0
NG1
NG3
NG4
NG5
HGF2
HGF3
HGF5
HGF7
Cell line

Figure 2

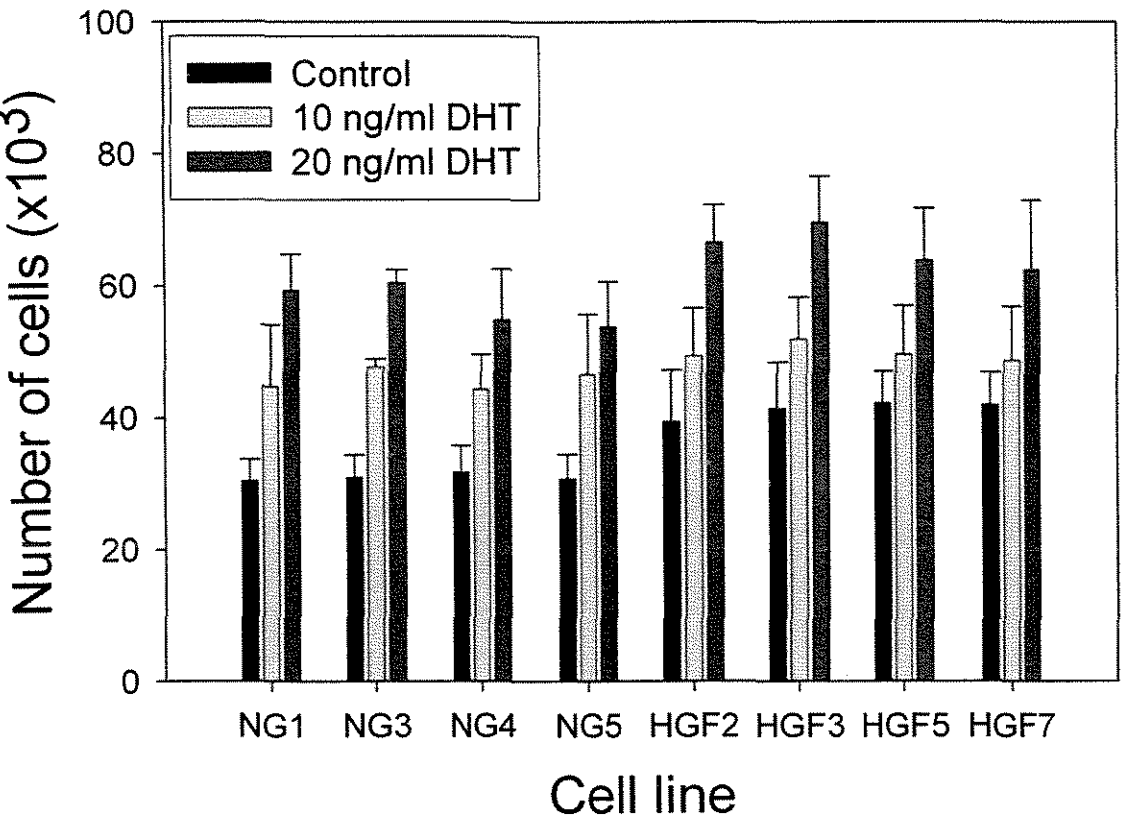

Control
10 ng/ml DHT
20 ng/ml DHT
Number of cells (x10^3)
100
80
60
40
20
0
NG1
NG3
NG4
NG5
HGF2
HGF3
HGF5
HGF7
Cell line

Figure 3

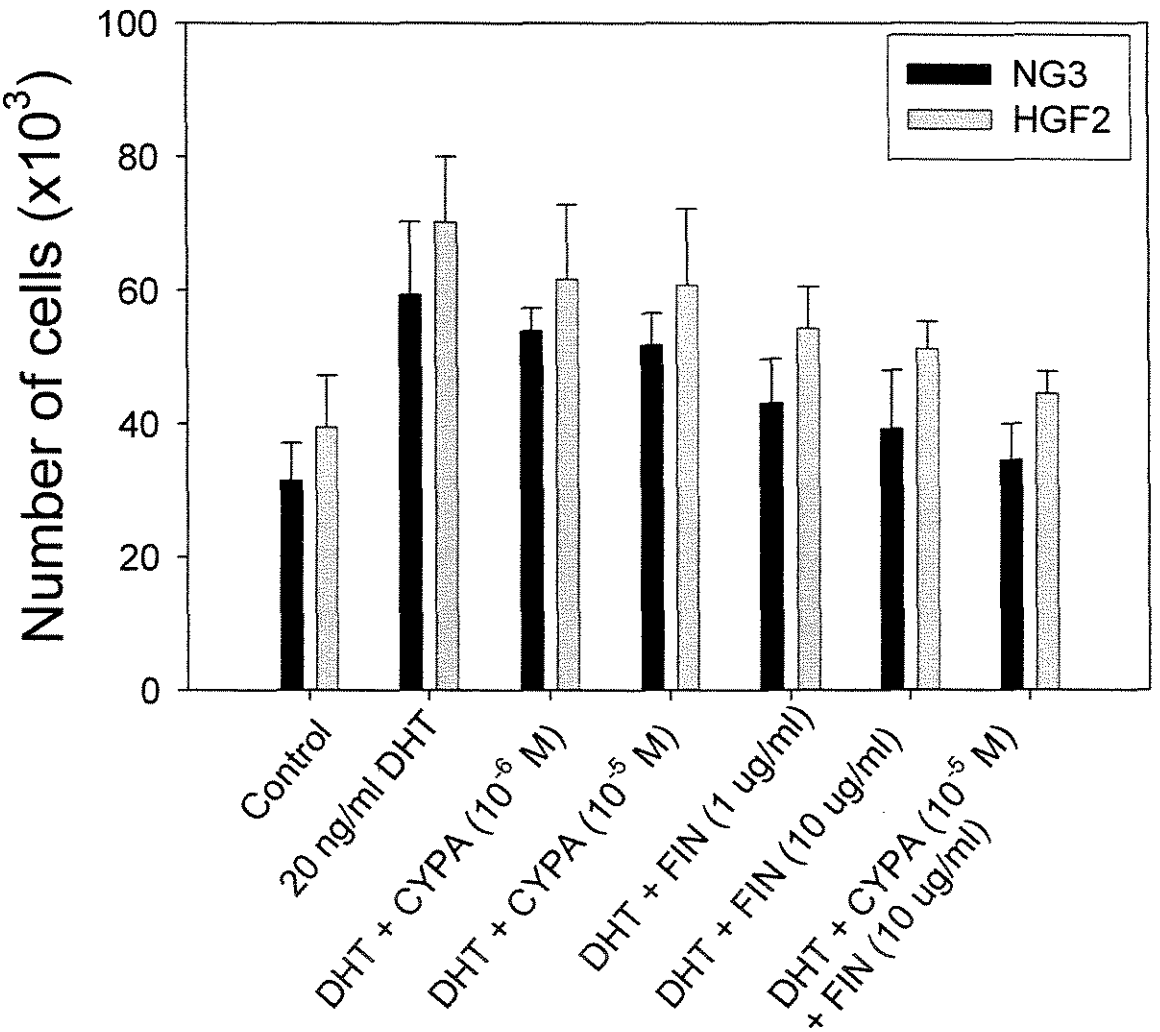

Number of cells (x10^3)
100
80
60
40
20
0
NG3
HGF2
Control
20 ng/ml DHT
DHT + CYPA (10^{-6} M)
DHT + CYPA (10^{-5} M)
DHT + FIN (1 ug/ml)
DHT + FIN (10 ug/ml)
DHT + CYPA (10^{-5} M) + FIN (10 ug/ml)

Figure 4

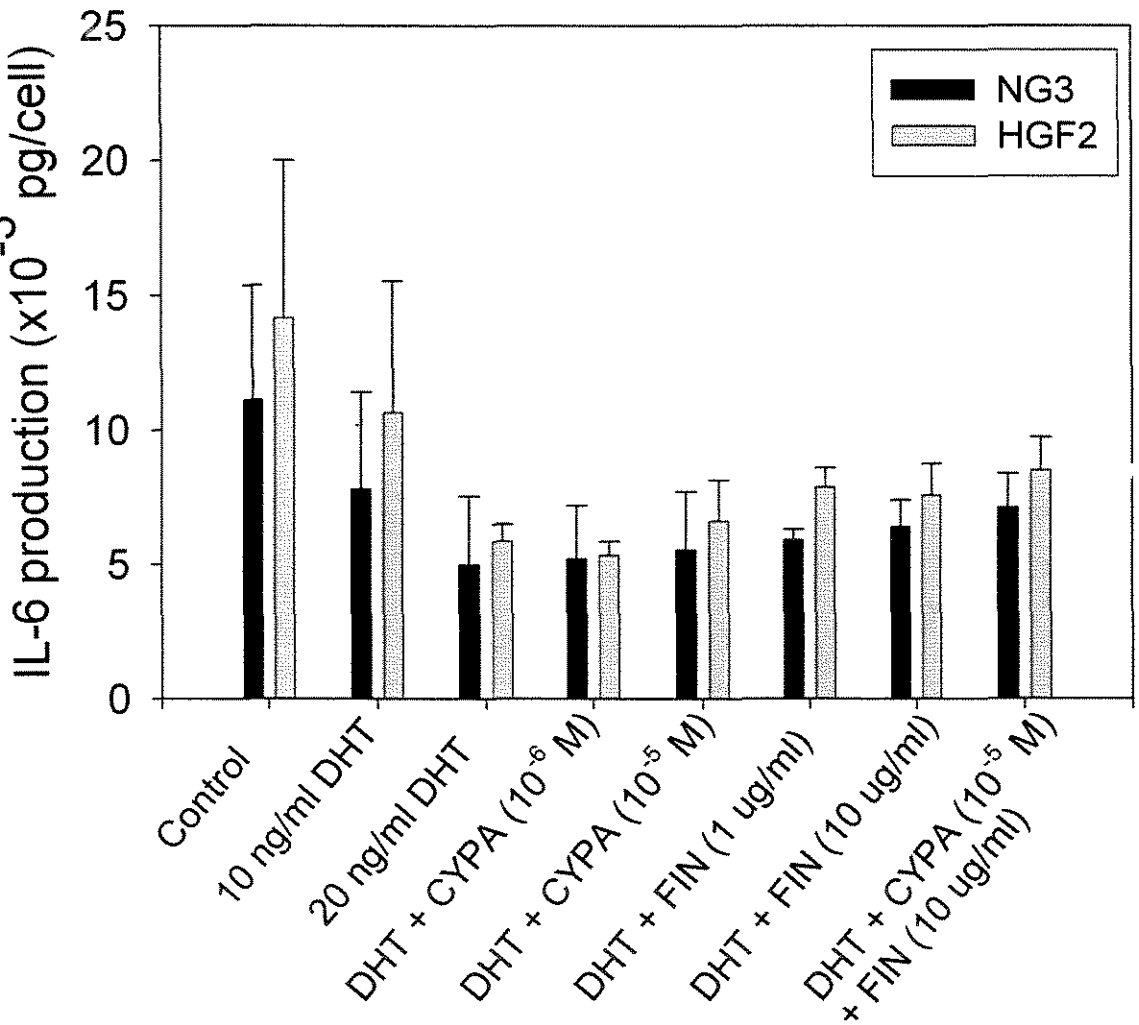
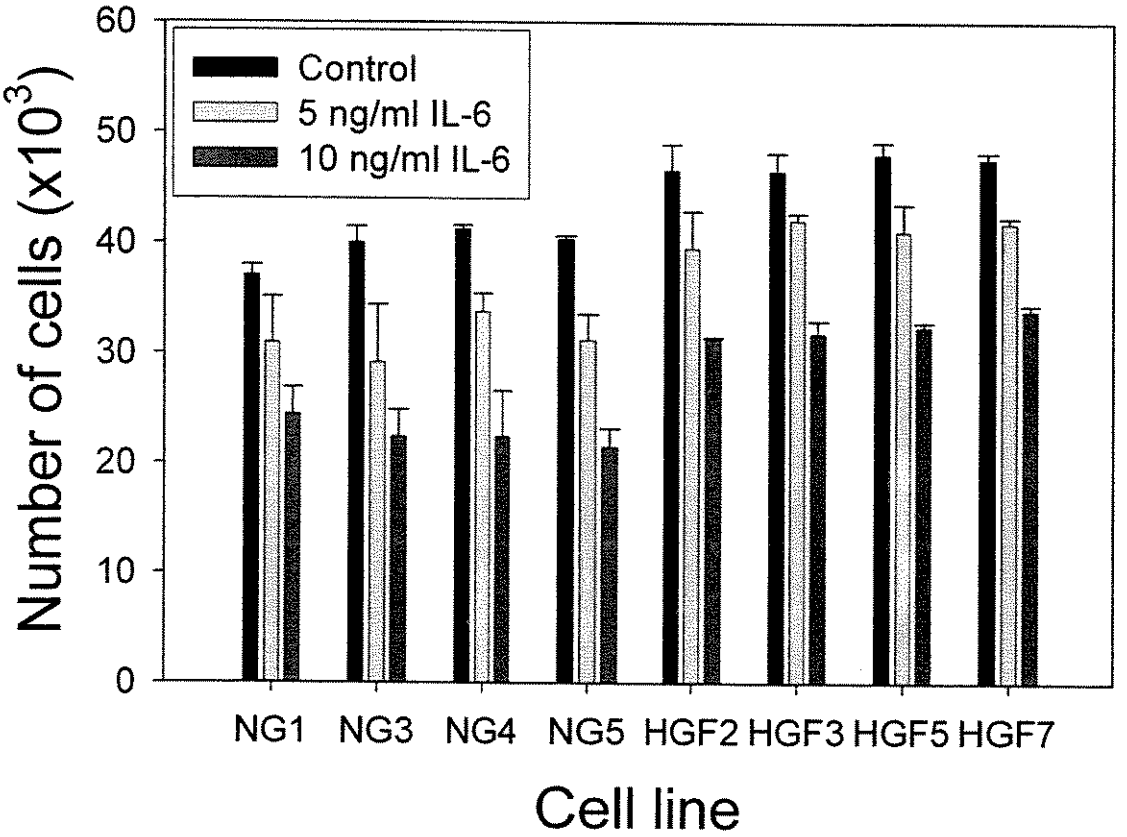

IL-6 production (x10-3 pg/cell)
25
20
15
10
5
0
NG3
HGF2
Control
10 ng/ml DHT
20 ng/ml DHT
DHT + CYPA (10-6 M)
DHT + CYPA (10-5 M)
DHT + FIN (1 ug/ml)
DHT + FIN (10 ug/ml)
DHT + CYPA (10-5 M) + FIN (10 ug/ml)

Figure 5


Control
5 ng/ml IL-6
10 ng/ml IL-6
Number of cells (X10^3)
60
50
40
30
20
10
0
NG1
NG3
NG4
NG5
HGF2
HGF3
HGF5
HGF7
Cell line

8 – CONCLUSÕES

1. Diidrotestosterona simultaneamente estimula a proliferação celular e inibe a produção de IL-6 em fibroblastos de GN e de FGH.
2. A adição de IL-6 à cultura de fibroblastos de GN e de FGH resulta em uma inibição da proliferação celular.
3. Os níveis de mRNA de receptores de andrógenos são maiores em fibroblastos de GN que em FGH.
4. A expressão e produção de colágeno tipo I e Hsp47 são maiores em fibroblastos de FGH comparado a fibroblastos de GN, enquanto que a expressão e produção de MMP-1 e MMP-2 são menores em fibroblastos de FGH.
5. TGF-β1 e IL-6 promovem um aumento na expressão de colágeno tipo I e Hsp47 em fibroblastos de GN e de FGH e uma diminuição na expressão de MMP-1 e MMP-2.
6. INF-γ reduz a expressão de colágeno tipo I e Hsp47 em fibroblastos de GN e de FGH e não apresenta um efeito significante na produção e expressão de MMP-1 e MMP-2 em fibroblastos de GN e FGH.

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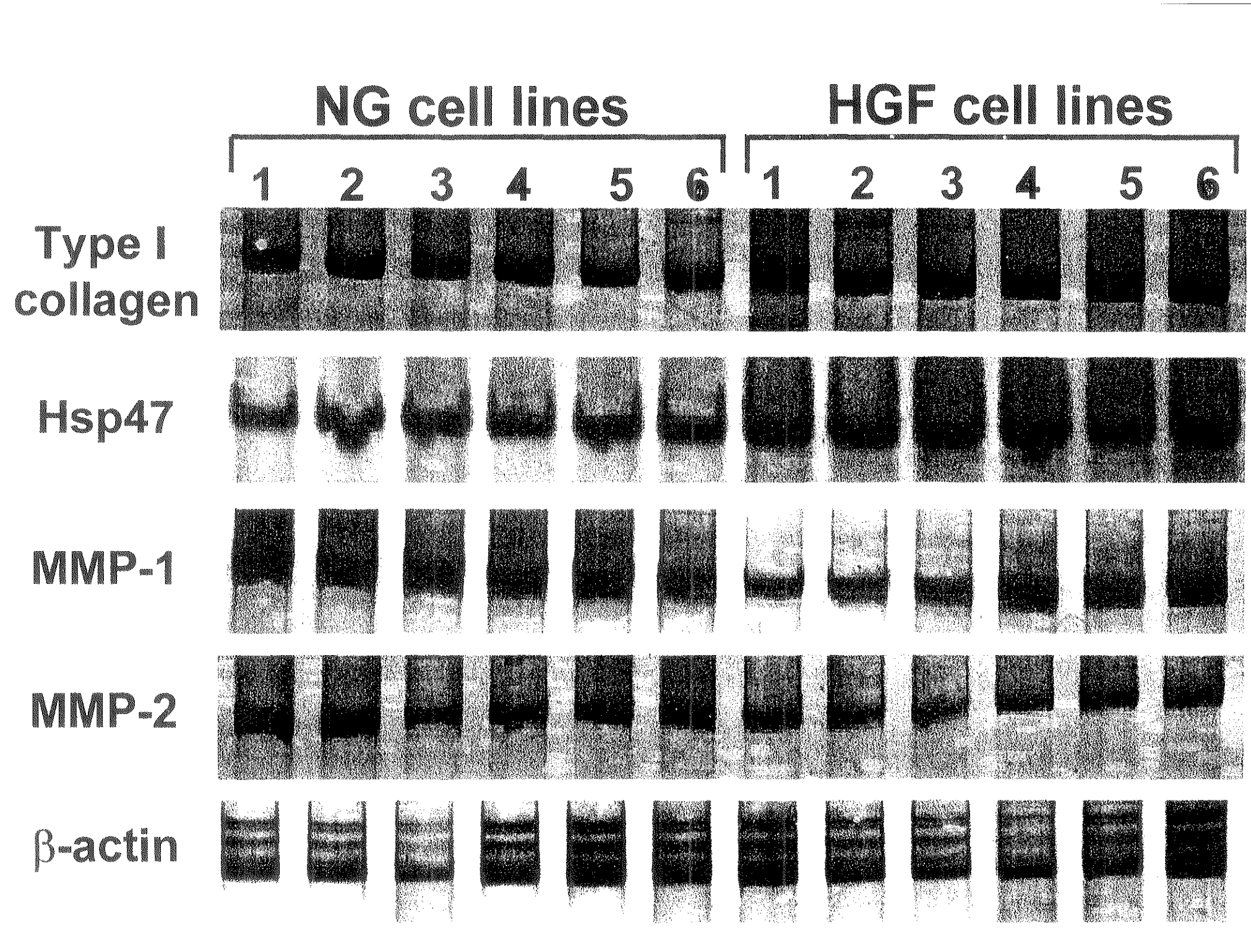
ANEXO 1

Análise por RT-PCR de expressão de colágeno tipo I, Hsp47, MMP-1 e MMP-2 em fibroblastos de GN e de FGH. Fibroblastos de GN e de FGH foram cultivados, submetidos a análise por RT-PCR e avaliados por eletroforese em gel de poliacrilamida revelado por coloração de prata. A análise densitométrica da expressão dos genes específicos é mostrada na figura 1 (A-D), nas páginas 70 a 73.

ANEXO 2

Cópia da carta de aceitação do trabalho contido no capítulo 2 intitulado: "Testosterone stimulates proliferation and inhibits interleukin-6 production by normal and hereditary gingival fibromatosis fibroblasts", para publicação no *Journal of Periodontal Research.*


NG cell lines
HGF cell lines
1
2
3
4
5
6
1
2
3
4
5
6
Type I collagen
Hsp47
MMP-1
MMP-2
β-actin

PERIODONTAL
RESEARCH

11/8/2001

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Caixa Postal 52
13414-018 Piracicaba-SP
BRAZIL

Dear Dr. Coletta;

I am pleased to inform you that your paper entitled "Testoterone stimulates proliferation and inhibits interleukin-6 production by normal and hereditary gingival fibromatosis fibroblasts" has been accepted for publication in Journal of Periodontal Research. Please complete the enclosed copyright release form and return it to me.

Thank you for your contribution

Sincerely yours,

Jorgen Slots

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