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Epithelial and Connective Tissue Cell CTGF/CCN2 expression in Gingival Fibrosis

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Abstract

Gingival overgrowth and fibrosis is a side effect of certain medications and occurs in non-drug induced forms either as inherited (human gingival fibromatosis) or idiopathic gingival overgrowth. The most fibrotic drug-induced lesions develop in response to therapy with phenytoin, the least fibrotic lesions are caused by cyclosporin A, and intermediate fibrosis occurs in nifedipine-induced gingival overgrowth. Connective tissue growth factor (CTGF/CCN2) expression is positively related to the degree of fibrosis in these tissues. In the present study, the hypothesis was investigated that CTGF/CCN2 is expressed in human gingival fibromatosis tissues and contributes to this form of non-drug-induced gingival overgrowth. Histopathology/immunohistochemistry studies show that human gingival fibromatosis lesions are highly fibrotic, similar to phenytoin-induced lesions. Connective tissue CTGF/CCN2 levels were equivalent to the expression in phenytoin-induced gingival overgrowth. The additional novel observation was made that CTGF/CCN2 is highly expressed in the epithelium of fibrotic gingival tissues. This finding was confirmed by in situ hybridization. Real time PCR analyses of RNA extracted from control and drug-induced gingival overgrowth tissues for CTGF/CCN2 were fully consistent with these findings. Finally, normal primary gingival epithelial cell cultures were analyzed for the basal and TGF-β1 or lysophosphatidic acid stimulated CTGF/CCN2 expression at the protein and RNA levels. Data indicate that fibrotic human gingival tissues express CTGF/CCN2 in both the epithelium and connective tissues and cultured gingival epithelial cells express CTGF/CCN2, and lysophosphatidic acid further stimulates CTGF/CCN2 expression. These findings suggest that interactions between epithelial and connective tissues could contribute to gingival fibrosis.

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Keywords

gingival overgrowth; fibrosis; connective tissue growth factor; epithelium; fibroblast; epithelial-mesenchymal transition

INTRODUCTION

Gingival overgrowth occurs as a side effect of medications including the anti-seizure drug phenytoin, the immunosuppressant cyclosporin A, and calcium channel blockers including nifedipine used to control hypertension (reviewed in [1]). The clinical appearance of lesions resulting from different medications is similar [2,3]. Non-drug induced inherited forms of gingival overgrowth are known as human gingival fibromatosis, and have been linked to more than one genetic locus in different families and populations [4–6].

Although clinical features of drug-induced gingival overgrowth are similar, studies from this laboratory have shown that the histopathology of drug-induced gingival overgrowth lesions varies as a function of inducing drug [3]. Phenytoin-induced lesions are more fibrotic and less inflamed than nifedipine- and cyclosporin A-induced gingival overgrowth [3]. Levels of connective tissue growth factor (CTGF/CCN2) are highest in gingival tissues from phenytoin-induced lesions, intermediate in nifedipine-induced lesions, and nearly absent from cyclosporin A-induced overgrowth. CTGF/CCN2 levels correlate positively with fibrosis, consistent with the role of CTGF/CCN2 in promoting and maintaining fibrosis [3]. CTGF/CCN2 is a member of the CCN family of factors whose members contain conserved cysteine-rich domains, and have a variety of biological activities [7,8]. CTGF/CCN2 itself stimulates proliferation of diverse cell types [9–11], and promotes fibrosis [12,13]. CTGF/CCN2 is highly expressed in a wide variety of fibrotic lesions including skin [14] and kidney fibrosis [15], atheroscleorosis [16], and phenytoin-induced gingival overgrowth [3,17].

Preliminary histologic analyses of tissues from human gingival fibromatosis, a non-drug-induced gingival overgrowth, suggested that they are highly fibrotic [18]. This raised the possibility that CTGF/CCN2 could contribute to the observed fibrosis and might be present at elevated levels [3]. We, therefore, determined whether human gingival fibromatosis tissues were in fact generally more fibrotic and whether they expressed high levels of CTGF/CCN2 as predicted. In the course of these studies we noticed that epithelial layers of fibrotic gingiva were positive for CTGF/CCN2. We now report results from studies that investigate gingival epithelial and connective tissue CTGF/CCN2 expression performed at both the protein and RNA levels in fibrotic and non-fibrotic lesions, and in cultured human primary gingival epithelial and fibroblastic cells.

MATERIALS AND METHODS

Gingival Tissues

Gingival tissue samples were obtained from patients undergoing periodontal surgery in the Department of Periodontology and Oral Biology and the Clinical Research Center of Boston University at the Goldman School of Dental Medicine, the Franciscan Children's Hospital and Rehabilitation Center, the Department of Periodontology, Istanbul University, and the Department of Periodontology, Ege University. Samples from 42 donors were included: phenytoin-induced gingival overgrowth (n=12), cyclosporin-A-induced gingival overgrowth (n=9), hereditary gingival fibromatosis (n=9) and control tissues (n=12) from systemically healthy donors without gingival overgrowth. Consent from the patients was obtained prior to the study as approved by the Institutional Review Boards of Boston University Medical Center, Franciscan Children's Hospital and Rehabilitation Center, the Department of Periodontology

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of the Istanbul University, and the Department of Periodontology, Ege University. All individuals were 20 years of age or older. Prior to periodontal surgeries, patients received initial periodontal treatment including professional cleaning and oral hygiene instructions. Gingival biopsies were obtained from the attached gingiva of the anterior buccal, mesial, or distal aspects of the teeth during gingivectomy and flap surgery for pocket elimination or preprosthetic crown lengthening procedure.

Immunohistochemistry and histomorphometric analyses

Tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4° C for 4 hours, incubated in 30% sucrose overnight, and stored in 2-methylbutane at −80°C. Serial 5 μm frozen sections were made for each tissue sample on a cryostat. Peroxidase-immunohistochemistry was carried out using a temperature controlled staining system in order to standardize the staining conditions [3,17]. Primary antibodies for immunostaining were affinity purified rabbit polyclonal antibodies against CTGF/CCN2 (AbCam) and non-immune goat IgG, each used at 8 μg/ml. Secondary antibodies and peroxidase-based detection was performed with Elite Vecta Stain kits (Vector Laboratories). Quantitative histomorphometric analyses for fibrosis, inflammation, and for intracellular and extracellular CTGF/CCN2 immunostaining was performed as previously described [3]. Five sites with corresponding areas of 0.09 mm^2 were defined and utilized for quantitative analyses by computer-assisted image-analysis [3] (Image-Pro Plus 4.0, Media Cybernetics, Silver Spring, MD). Data were collected from 3–5 serial sections per tissue specimen per assay. CTGF was evaluated by counting the number of stained cells per unit area. Pre-immune stained slides from serial sections exhibited negligible staining. A semi-quantitative grading system was used to analyze the degree of fibrosis in hematoxylin-stained sections [3,17]. The number of inflammatory cells per 0.09 mm^2 was determined from hematoxylin-stained sections to assess the relative degree of inflammation. Results are expressed as mean ± standard deviation for each evaluated site. For statistical analysis, the Mann-Whitney test was used to compare differences. Statistical significance was declared at $p<0.05$.

In Situ Hybridization

A 445-bp cDNA fragment of human CTGF/CCN2 was generated from RNA from TGF-β1-treated gingival fibroblasts. RNA was first reverse transcribed and CTGF/CCN2 cDNA was amplified by the polymerase chain reaction using custom-made sense and anti-sense primers: 5′-TTCCAGAGCAGCTGCAAGTA-3′ and 5′-TGGAGATTTTGGGAGTACGG-3′ (Invitrogen) and cloned into pCR2.1 TOPO vector (Invitrogen). Clones were sequenced and plasmids were linearized with Hind III (Invitrogen) and incubated with T7-RNA polymerase to generate digoxigenin-labeled sense and anti-sense RNA transcripts, respectively (DIG RNA Labeling Kit, Roche Applied Science®). Slides were incubated with PBS containing 100mM glycine and then with PBS containing 0.3% Triton X-100, rinsed, incubated for 30 min at 37° C with TE-Buffer containing 1μg/ml RNAse-free Proteinase K (Qiagen®), post-fixed with PBS containing 4% paraformaldehyde, and immersed in freshly prepared 0.25% acetic anhydride, 0.1M triethanolamine. The sections were then hybridized for 18h in 40% deionized formamide, 10% dextran sulfate, 1xDenhardt’s solution, 4xSSC, 10 mM DTT, 1mg/ml yeast t-RNA, 1mg/ml sperm DNA containing 2μg/ml of the appropriate probe, incubated in 20μg/ml RNaseA (Sigma), and then blocked with 100mM Tris-HCl, 150mM NaCl, 0.1% Triton X-100 and 2% sheep serum. Detection of bound digoxigenin-labeled RNA probes was accomplished using in situ hybridization kit (Roche) with color development reagents of 0.18 mg/ml BCIP, 0.34 mg/ml NBT and 3 mM Levamisole (Sigma) for 16 hrs at room temperature. The color reaction was stopped by incubating slides in 10mM Tris-HCl, 1 mM EDTA before covering sections with Supermount.

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RNA isolation from gingival tissues

Gingival tissues were placed in RNAlater (Qiagen) upon surgical removal, and stored at −80° C until processed with the midi-RNeasy kit (Qiagen) according to the manufacturer's instructions that include treatment of tissue extracts with proteinase K. RNA quality was determined by agarose gel electrophoresis and detection of intact 28S and 18S rRNA bands at the expected clarity and intensity after ethidium bromide staining.

Primary gingival epithelial cell cultures

Biopsy samples were placed in PBS supplemented with 10% penicillin-streptomycin (Cellgro), briefly rinsed in 70% ethanol, and were cut into 2–3 mm cubes, placed in trypsin-PBS (1:1, v/v) overnight at 4°C, and the epithelial layer was detached from the connective tissue to isolate the epithelial cells. Cells and tissue debris were collected, washed with MCDB medium supplemented with pyruvic acid (0.11g powder/l), HEPES (7.15g/l) and sodium bicarbonate (1.2g/l) (Sigma-Aldrich). The sample was centrifuged for 10 minutes at 800 rpm (120 × g) at room temperature. The pellet was suspended in KSFM medium (Life Technologies) containing penicillin-streptomycin (10000 IU/ml), Amphotericin B (50μg/ml), insulin (10 μg/ml), transferrin (5 μg/ml), β-mercaptoethanol (10 μM), 2-ethanolamine (10 μM), and sodium selenite (10 nM) (Sigma Aldrich). Aliquots were added to type I collagen coated dishes (BD Biosciences). Cultures were maintained at 37C, 5% CO_2 and fed twice weekly. Cells in the logarithmic growth phase passaged at a ratio of 1:3 and were used either at the third or fourth passage. At confluence, cells were washed and treated for 2 or 6 hours with or without 5 ng/ml TGFβ1 (Peprotech), or 10 μM LPA (Avanti). Cells exhibited a characteristic cuboidal epithelial morphology at confluence, and Western blotting showed clear expression of E-cadherin, a specific marker of epithelial cells.

Gingival fibroblast cultures

Human gingival fibroblast cultures were grown from frozen stocks made from tissue explants in DMEM with high glucose, L-glutamine, pyridoxine hydrochloride, supplemented with 10% FBS heat-inactivated, 1% non-essential amino acids and 100 μg/ml of penicillin-streptomycin [19]. Experiments were performed after no more than two passages from one set of cell stocks. Cells exhibited a typical fusiform elongated morphology characteristic of fibroblasts, and contained no E-cadherin determined by Western blotting, as expected. Confluent cells were treated with serum-free medium containing 0.1% bovine serum albumin overnight prior to treatment with or without 5ng/ml TGF-β1 or 10μM LPA. RNA was isolated using the RNeasy Mini Kit (Qiagen) was used according to the manufacturer's instructions.

Western Blotting

Samples containing 60 μg of protein were subjected to SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% milk-TBST for 1 hour at room temperature, incubated with rabbit-anti-CTGF/CCN2 IgG antibodies (1 μg/ml) (FibroGen) in 5% milk-TBST, overnight at 4°C, washed with TBST, incubated with secondary goat anti-rabbit antibody conjugated to horseradish peroxidase [3], washed and visualized with ECL Western Blotting Detection Reagents (Amersham Biosciences). After stripping with Restore Western Blot Stripping Buffer (Pierce) for 20 minutes at room temperature, membranes were processed similarly with β-actin antibody as a loading control.

Real-time Quantitative PCR

1 μg intact RNA was subjected to reverse transcription in 30μl reactions (Applied Biosystems Reagents). Conditions for reverse transcription were 25°C for 10-minutes, 37°C for 60-minutes, and 95°C for 5-minutes. cDNA was stored at −20°C. Four microliters of each reverse transcription reaction was used for 50 μl real-time PCR reactions using a standard 96-well

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format. Taqman probes for CTGF/CCN2 and GAPDH were utilized in the same reactions. Real-time PCR reactions were run in an Applied Biosystems GeneAmp Prism 7700 System. Data was analyzed using the $2^{-\Delta\Delta ct}$ method and CTGF/CCN2 mRNA levels were calculated compared to corresponding control samples run at the same time each normalized to GAPDH mRNA internal controls.

RESULTS

CTGF/CCN2 expression in gingival fibrosis

Histologic analyses of gingival samples from patients diagnosed with phenytoin-induced gingival fibrosis or non-drug induced human gingival fibromatosis revealed hyperplasia with dense, elongated, and thin rete pegs inserted into deep connective tissue (Figure 1A). These elongations are more obvious and extended deeper into the connective tissue compared to gingival specimens from individuals with no overgrowth, consistent with previous reports [20]. In order to further compare phenytoin-induced gingival overgrowth with human gingival fibromatosis; semi-quantitative histomorphometric measurements of fibrosis and inflammation were studied. Fibrosis, as determined by the relative abundance of fibroblasts and fibers, is significantly higher in phenytoin overgrowth and gingival fibromatosis compared to healthy tissues ($p<0.05$; Figure 1B), and there was no significant difference between gingival fibromatosis and phenytoin overgrowth ($p>0.05$). The degree of inflammation, as assessed by the number of inflammatory cells per unit area, was significantly lower in human gingival fibromatosis and phenytoin overgrowth compared to control specimens, which exhibited no gingival fibrosis ($p<0.05$; Figure 1B).

CTGF/CCN2 protein expression was then assessed in gingival samples by immunohistochemistry. Representative images shown in Figure 1A demonstrate that CTGF/CCN2 was higher in phenytoin overgrowth and gingival fibromatosis compared to controls. Extracellular CTGF/CCN2 localization was predominant in connective tissue stroma, perivascular areas, and the endothelial layer of the blood vessels. Pre-immune CTGF/CCN2 staining for all groups was negligible (Figure 1A). This observation was further supported by the quantitative analysis of intracellular and semi-quantitative grading of extracellular CTGF/CCN2 expression (Figure 1B). Intracellular CTGF/CCN2 staining was higher in phenytoin overgrowth and gingival fibromatosis compared to healthy tissues (Figure 1B). There was no significant difference between phenytoin overgrowth and gingival fibromatosis in CTGF/CCN2 expression while control tissues expressed low extracellular CTGF/CCN2. Thus, CTGF/CCN2 staining is similarly elevated in phenytoin overgrowth and human gingival fibromatosis tissues.

As seen in Figure 1A, phenytoin-induced and human gingival fibromatosis gingival overgrowth samples contained elevated CTGF/CCN2 levels in both the stroma and the epithelial layer. The finding of elevated CTGF/CCN2 levels in gingival epithelium in fibrotic gingival tissues is novel and has not been previously reported. CTGF/CCN2 levels were elevated in phenytoin overgrowth and human gingival fibromatosis compared to control tissues. The basal layer of epithelial cells at the border of connective tissue appeared to express the highest levels of CTGF/CCN2 (Figure 1A).

We next wished to investigate CTGF/CCN2 expression by gingival epithelial cells more closely. CTGF/CCN2 has been found in cells that do not produce it [21]. Thus, we undertook in situ hybridization to identify cells that express CTGF/CCN2 mRNA as an indicator of CTGF/CCN2 biosynthesis. Figure 2 demonstrates that phenytoin-induced gingival overgrowth sections hybridized with the antisense CTGF/CCN2 probe show abundant levels of CTGF/CCN2 RNA expression that appears to be the highest in basal epithelial cells. Connective tissue fibroblasts were also positive in these sections, though the density of staining was weaker than

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the epithelium in parallel with immunohistochemistry results in Figure 1. Sections hybridized with control sense probe show low background staining. Our previous report indicates that cyclosporin A-induced gingival overgrowth does not contain elevated CTGF/CCN2 protein determined by immunohistochemistry [3]. *In situ* hybridization data in Figure 2A indicate that gingival epithelium and stroma contain few CTGF/CCN2-expressing cells in cyclosporin A-induced overgrowth.

To further support these findings, total RNA was isolated from phenytoin and cyclosporin A-induced gingival overgrowth tissues and subjected to real time PCR for CTGF/CCN2. Figure 2B indicates that phenytoin-induced gingival overgrowth tissues contain high CTGF/CCN2 mRNA levels compared to cyclosporin A-induced gingival overgrowth, fully consistent with new *in situ* hybridization data reported here and immunohistochemistry results reported previously [3].

CTGF/CCN2 expression in cultured gingival epithelial cells

We next wished to establish whether CTGF/CCN2 production could be demonstrated in cultured human gingival epithelial cells. Primary epithelial cells were cultured from human gingival specimens as outlined in the methods section from subjects without gingival overgrowth and were treated with or without 5 ng/ml TGF-β1 or 10 μM LPA for 2 and 6 hours. In similar experiments, primary gingival fibroblast cultures were cultured and treated with the same factors for comparison. TGF-β1 and LPA are known inducers of CTGF in epithelial cells and fibroblasts, though TGF-β1 does not induce CTGF/CCN2 in dermal epithelial cells [22, 23]. Real-time PCR quantification of total RNA samples normalized to GAPDH levels shows that CTGF/CCN2 was transiently induced in epithelial cells after 2 hours of LPA treatment, whereas TGF-β1 did not alter CTGF/CCN2 expression (Figure 3A). In contrast to epithelial cells, primary fibroblasts incubated with TGF-β1 expressed significantly higher levels of CTGF/CCN2 (Figure 3B). LPA transiently induced CTGF/CCN2, similar to what was seen in epithelial cells. Finally, we compared the basal levels of CTGF/CCN2 mRNA expression in epithelial cells to cultured human gingival fibroblasts normalized, respectively, to GAPDH levels. Real-time PCR data from three independent cultures indicate that basal CTGF/CCN2 levels were 25-fold higher than basal levels seen in gingival fibroblasts. These findings showed that cultured epithelial cells constitutively express CTGF/CCN2 and do not respond to TGF-β1, while LPA stimulates CTGF/CCN2 expression in both epithelial and fibroblastic cells. The lack of stimulation of CTGF/CCN2 expression by TGF-β1 in epithelial cells but potent stimulation in fibroblasts is consistent with studies in skin [22], but the constitutively high expression of CTGF/CCN2 in epithelial cultures was not expected.

In order to further characterize the expression of CTGF/CCN2 in epithelial cells, we performed Western blotting analysis of cell layer extracts of epithelial cells and fibroblasts treated with LPA or TGF-β1 for 2 or 6 hours. Figure 4 shows that CTGF/CCN2 is constitutively expressed in epithelial cells whereas untreated fibroblasts express little or no CTGF/CCN2. In response to TGF-β1, fibroblast CTGF/CCN2 expression is highly increased in fibroblasts after both 2 and 6 hours whereas in epithelial cells CTGF/CCN2 levels were not increased after 2 hours of TGF-β1 treatment, and only modestly at 6 hours (Figure 4). LPA resulted in a transiently increased CTGF/CCN2 expression at 2 hours in epithelial cells, and a slower increase in fibroblasts seen after 2 hours and before 6 hours. Multiple CTGF bands seen in Figure 4 are glycosylated and non-glycosylated forms of full length CTGF, and proteolytically processed forms of CTGF as previously reported [17]. These results are fully consistent with RNA data presented in Figure 3.

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DISCUSSION

CTGF/CCN2 promotes and sustains fibrosis initiated by TGF-β [12,13]. CTGF/CCN2 expression, activity and function in fibrosis are well-documented in skin, lung, and kidney, but few studies have examined its expression and role in gingival overgrowth and fibrosis. Previous studies from this laboratory have identified CTGF/CCN2 to be strongly induced by TGF-β1 in gingival fibroblast cultures [17]. Immunohistochemistry studies showed that CTGF/CCN2 is highly expressed in connective tissue stroma of phenytoin-induced gingival overgrowth [3, 17]. CTGF expression is related to the degree of fibrosis, as cyclosporin A-tissues show little CTGF expression and were demonstrated to be highly inflamed and not fibrotic [3]. In more recent studies, we have obtained tissues from patients suffering from human gingival fibromatosis, a non-drug-induced form of gingival overgrowth. Preliminary histopathology suggested that these tissues were highly fibrotic, perhaps more fibrotic than phenytoin-induced overgrowth lesions [18]. We predicted that gingival fibromatosis tissues would express higher levels of CTGF/CCN2 than phenytoin-induced gingival overgrowth.

Systematic analyses of human gingival fibromatosis tissues were, therefore, performed and reported here. Data indicate that hereditary gingival fibromatosis tissues are indeed highly fibrotic, but do not appear to be more fibrotic that phenytoin-induced gingival overgrowth. Interestingly, CTGF/CCN2 expression levels were essentially equivalent between phenytoin-induced fibrotic lesions and hereditary gingival fibromatosis. Thus, CTGF/CCN2 levels are related to the degree of fibrosis suggesting that common pathways exist between drug-induced and non-drug induced gingival fibrosis.

A novel finding is that CTGF/CCN2 is expressed both in the connective tissue stroma and in gingival epithelial cells in vivo in fibrotic tissues, but not in normal tissues. In addition to promoting fibrosis in connective tissue stroma, CTGF/CCN2 is expressed and has biological functions in epithelial and endothelial cells. For example, CTGF/CCN2 and proteolytic fragments isolated from uterine flushing enriched with endothelial cell products stimulate proliferation of a variety of cell types [9]. As gingival overgrowth is characterized by increases in both epithelial and connective tissue layers, it is possible that CTGF/CCN2 plays a role in the proliferation of basal gingival epithelial cells and promotes fibrosis in the connective tissue stroma. The observed high constitutive expression of CTGF/CCN2 in cultured gingival epithelial cells could suggest that these cells contain an autocrine loop of production and response to CTGF/CCN2 stimulation of gingival epithelial cell proliferation that might be required for gingival epithelial cells to survive in culture. Such a pathway could normally occur at an early proliferative stage of basal epithelial cell differentiation. At a later stage of differentiation, when proliferation of epithelial cells ceases, this pathway could be down-regulated, thus limiting proliferation and favoring terminal differentiation. Thus, in gingival overgrowth induced either by phenytoin or hereditary factors, we suggest that epithelial cell proliferation could be stimulated, and differentiation could be delayed, mediated by a persistent CTGF/CCN2 autocrine pathway leading to hyperplasia. Both the high constitutive expression of CTGF/CCN2 in proliferating gingival epithelial cells in culture, and the presence of strong in vivo CTGF/CCN2 expression in basal gingival cells in the present study supports this model.

Development of fibrotic pathology can involve a process known as epithelial/mesenchymal transition [24]. In this process, partial destruction of the basement membrane can lead to inappropriate diffusion of factors between the connective tissue and epithelial layers of gingival tissues. These factors, in turn, can stimulate epithelial cells to lose cell-cell contacts, decrease E-cadherin expression, increase cell motility, and promote invasion into the underlying connective tissue stroma, where they differentiate further into cells that are indistinguishable from fibroblasts and myofibroblasts. These fibroblastic cells, in turn, produce connective tissue proteins that contribute to fibrosis. The histopathology of gingival overgrowth is characterized

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by extensions of epithelial cells (known as rete pegs) deep into the connective tissue stroma. This, combined with our finding of elevated CTGF/CCN2 levels in epithelial cells in fibrotic gingiva, suggests that the process of epithelial-mesenchymal transition could be a process that contributes to gingival fibrosis. Increased CTGF/CCN2 expression has recently been found to occur in epithelial-mesenchymal transition in renal proximal tubular epithelial cells [25,26] and alveolar epithelial cells in vitro and in fibrotic lungs [27,28] and in normal tooth development [29]. Thus, the observation of production of CTGF/CCN2 in fibrotic gingival epithelium is highly significant providing a major molecular insight into biology and etiology of gingival fibrosis. Epithelial-mesenchymal transition can be inhibited by BMP-7 and HGF [30–32]. If epithelial-mesenchymal transition contributes significantly to gingival fibrosis, this new understanding could provide major new therapeutic strategies to address this clinical problem.

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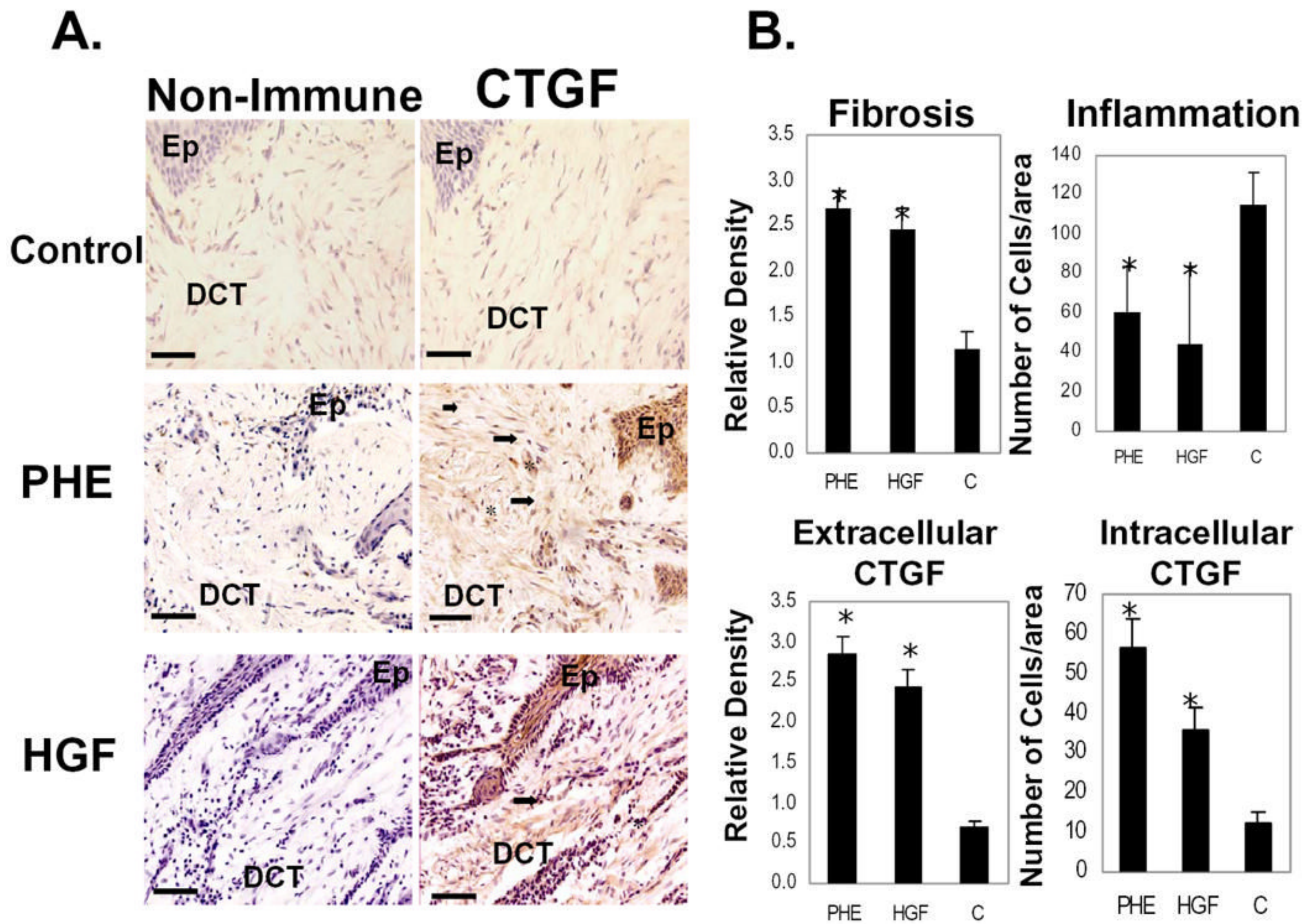

Figure 1. CTGF/CCN2 protein expression in gingival biopsies from fibrotic and normal control tissues

Representative immunohistochemical analysis for CTGF/CCN2 protein expression (A) shows the extracellular (asterisk) and intracellular (arrows) CTGF in deep connective tissue (DCT) in phenytoin (PHE) and human gingival fibromatosis (HGF) samples and not in controls. Epithelium (Ep) of fibrotic lesions stained for CTGF/CCN2 in PHE and HGF samples. Both gingival fibrosis lesions exhibited elongated rete pegs. Non-immune IgG stained serial sections showed no CTGF/CCN2 staining. Measure bars represent 200 μm at a magnification of 200×. (B); quantitative analyses of fibrosis, inflammation, and CTGF expression in connective tissue stroma are means +/− SE, (*, $p<0.05$), n = 12, PHE; n = 9, HGF; n = 12, no overgrowth controls.

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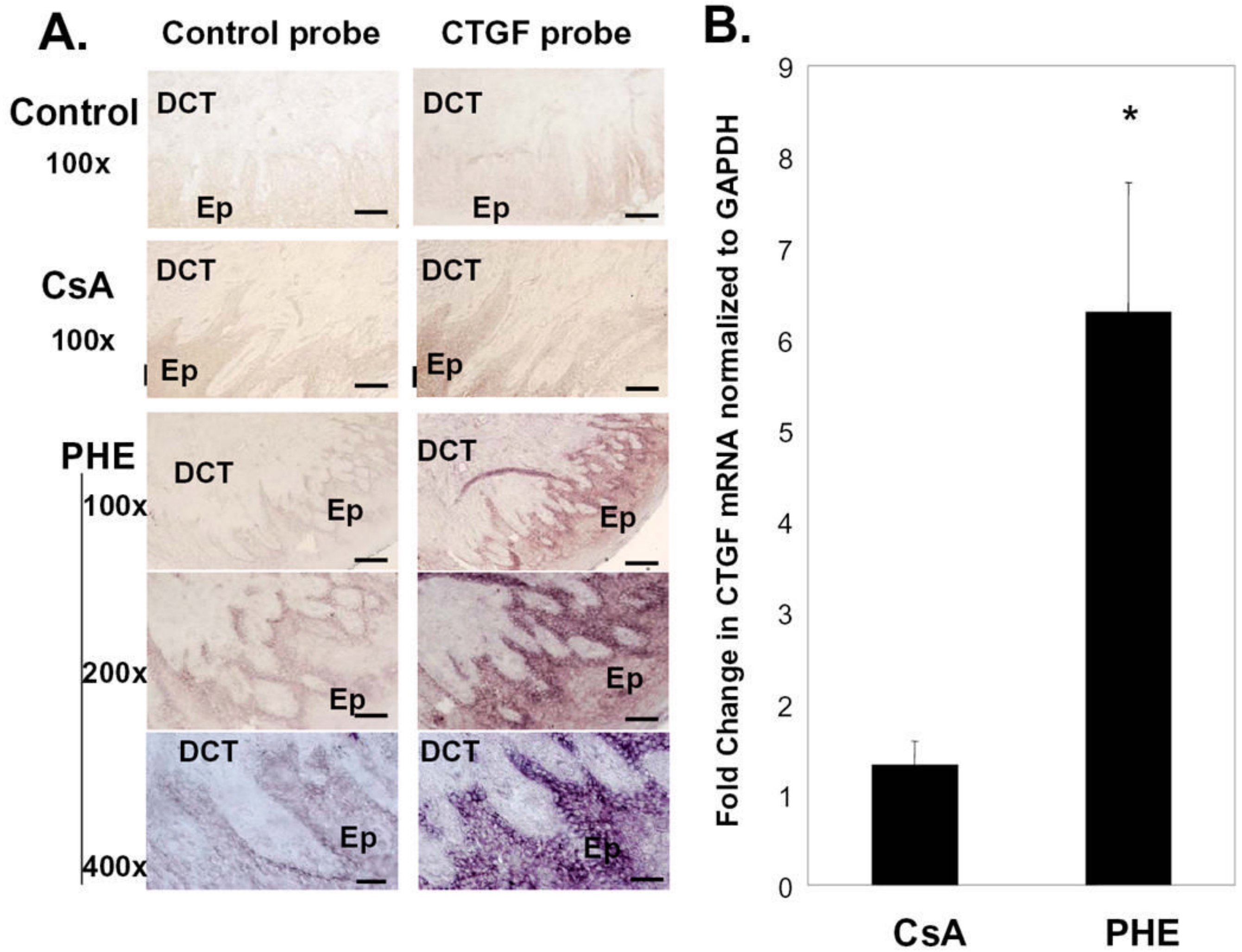

Figure 2. Expression of CTGF/CCN2 mRNA in gingival fibrosis

(A) Representative in situ hybridization sections of cyclosporin induced gingival overgrowth samples (CsA) and phenytoin (PHE) shows connective tissue and epithelial cell expression in PHE and not in CsA or no overgrowth controls. Measure bars are 400 μm, 200 μm, and 100 μm at 100×, 200×, and 400×-magnifications, respectively. (B) Real-time PCR quantification of CTGF/CCN2 mRNA expression isolated from PHE and CsA tissues normalized to GAPDH compared to RNA isolated from a non-inflamed no gingival overgrowth control tissue, expressed as fold induction compared to the same control sample +/− SD. PHE tissues (n=3) expressed significantly higher levels of CTGF/CCN2 mRNA (~7-fold) compared to CsA tissues (n=3) where CTGF/CCN2 expression was close to the no overgrowth controls (1.2-fold); *, $p<0.05$.

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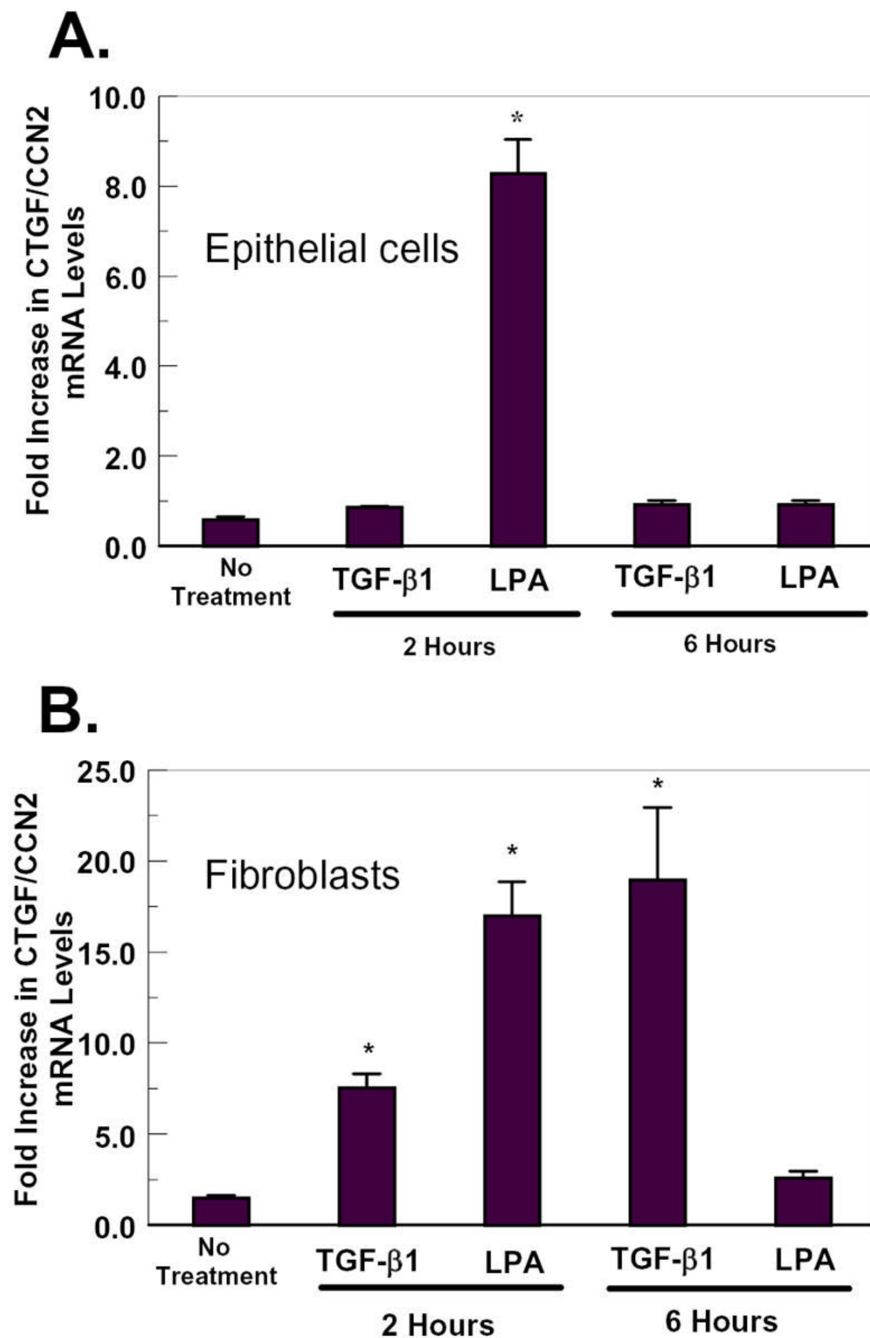

Figure 3. CTGF/CCN2 mRNA expression and regulation in primary gingival epithelial cell and fibroblast cultures

Real time PCR analyses of CTGF/CCN2 RNA expression of primary human gingival epithelial cells (A) and human gingival fibroblasts (B). Cells were cultured and treated with or without TGF-β1 or LPA as described in “Materials and Methods”. No treatment control RNA’s were isolated from cultures not treated with vehicle; fold changes were relative to vehicle treated controls at each time point +/− SD; all values are normalized to GAPDH internal controls; *, $p<0.001$ compared to no treatment control cultures. Data are from triplicate real time PCR assays of all samples.

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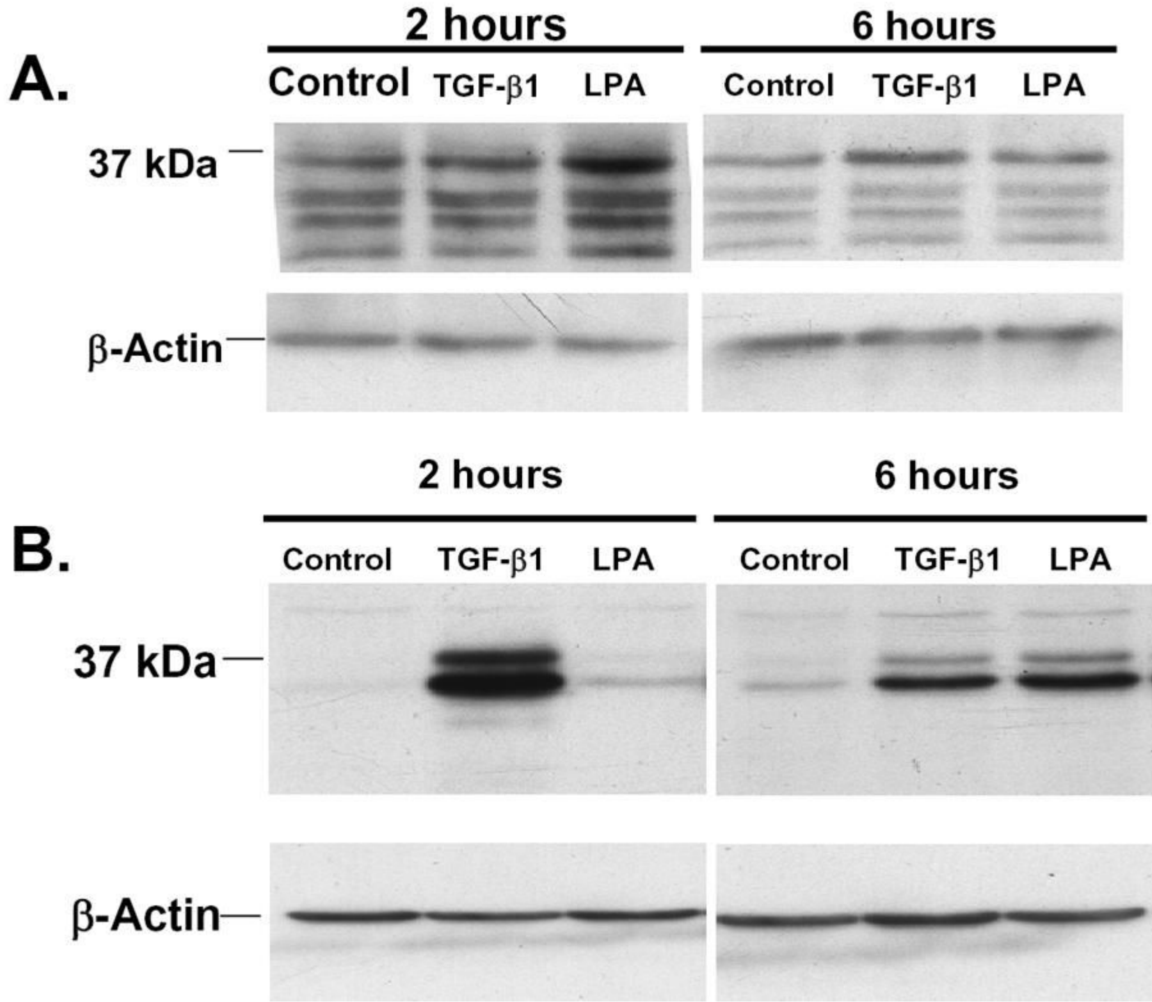

Figure 4. CTGF/CCN2 protein expression in primary gingival epithelial and fibroblast cultures determined by Western blotting

Western blots of cell layer extracts of primary human gingival epithelial cells (A) and human gingival fibroblasts (B) cultured and treated as described in "Methods and Materials" with 5 ng/ml TGF-β1, 10 μM LPA, or no treatment control. Blots analyzed with non-immune IgG gave no background signal. β-actin antibody was used to verify equal loading of gels. Blots are representative of four experiments with consistent results.