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Flutamide inhibits nifedipine- and interleukin-1β-induced collagen overproduction in gingival fibroblasts

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Background and Objective: To understand the role of the androgen receptor in gingival overgrowth, the effects of flutamide on interleukin-1β- and nifedipine-induced gene expression of connective tissue growth factor (CTGF/CCN2) and collagen production in gingival fibroblasts were examined.

Material and Methods: Gingival fibroblasts from healthy subjects and patients with dihydropyridine-induced gingival overgrowth (DIGO) were used. Confluent cells were treated with nifedipine, interleukin-1β or both. The mRNA expression was examined using real-time polymerase chain reaction, and the concentration of total soluble collagen in conditioned media was analysed by Sircol Collagen Assay. In addition, the protein expressions of androgen receptor, CTGF/CCN2 and type I collagen in gingival tissue were determined by western blot.

Results: Interleukin-1β was more potent than nifedipine in stimulating CTGF/CCN2 and procollagen α1(I) mRNA expression, and there was an additive effect of the two drugs. Healthy cells exhibited an equal or stronger response of procollagen α1(I) than those with DIGO, but DIGO cells displayed a stronger response in the secretion of soluble collagen in the same conditions. Flutamide, an androgen receptor antagonist, inhibited stimulation by nifedipine or interleukin-1β. Additionally, the protein expressions of androgen receptor and type I collagen were higher in DIGO gingival tissue than those in healthy gingival tissue.

Conclusion: The data suggest that both nifedipine and interleukin-1β play an important role in DIGO via androgen receptor upregulation and that gingival overgrowth is mainly due to collagen accumulation. Flutamide decreases the gene expression and protein production of collagen from dihydropyridine-induced overgrowth cells.

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Many cases of gingival overgrowth in our clinics have been induced by type II dihydropyridines, such as amlodipine, nicardipine and felodipine. Type II dihydropyridines are second-generation calcium channel blockers that are pharmacokinetically similar to nifedipine but with a slower onset, longer duration of action and greater vascular/cardiac effect ratios (1–3). Since androgen receptor is upregulated in nifedipine-induced gingival overgrowth tissues (4), this may contribute to gingival fibrosis. In addition, interleukin-1β induces higher gene expression of the androgen receptor in gingival fibroblasts derived from patients with dihydropyridine-induced

gingival overgrowth (DIGO) than in those from healthy gingival tissue (5). These findings prompted us to suggest that proinflammatory cytokines, such as interleukin-1β, may be one of the major mediators responsible for the high gene expression of androgen receptor in responder cells of nifedipine-induced gingival overgrowth.

Cytokines and growth factors, including interleukin-1 and interleukin-6, are elevated in drug-induced gingival overgrowth (6–9), and interleukin-1 induces metabolic conversion of androgenic substrates in gingival fibroblasts (10). Therefore, upregulation of the androgen receptor in DIGO might be augmented by inflammation.

Connective tissue growth factor (CTGF/CCN2), a member of the CCN family of proteins, is highly expressed in fibrosing tissues (11–14). The CTGF/CCN2 works in concert with growth factors, growth factor receptors, the extracellular matrix and extracellular receptors (15,16). Levels of CTGF/CCN2 are elevated in tissues exhibiting phenytoin- and nifedipine-induced gingival overgrowth, but not in cyclosporine-A-stimulated gingival overgrowth (17,18). However, a possible link between androgen receptors, CTGF/CCN2 and collagen in gingival fibroblasts has not yet been investigated.

In this study, we have clarified the role of androgen receptors in nifedipine- and interleukin-1β-induced collagen overproduction in gingival overgrowth.

Material and methods

Patient selection

Patients with DIGO ($n = 4$) and matched periodontally healthy, non-smoking individuals ($n = 4$) were the participants in this study (Table 1). Informed consent was received from each participant under a protocol approved by the Ethics Committee of Taipei Medical University Hospital. Diagnostic criteria for the DIGO group included patients who had taken dihydropyridine (including amlodipine and felodipine) for 6–24 mo, and whose overgrowth of gingiva was classified as being a responder (with a score of 2–3; 19). Patients were selected for tissue sampling 6 wk after the control of soft-tissue inflammation by scaling and root planing. Areas chosen for tissue sampling from the DIGO group possessed probing depths of > 5 mm and were still enlarged after scaling and root planing, plaque control, recontouring of defective restorations, caries control and tissue re-evaluation. Gingival samples were gathered from the wedge tissues of healthy individuals who accepted crown lengthening procedures or implant surgery. None of the selected individuals had received any antibiotic therapy for any reason in the previous 3 mo, nor had any systemic condition that might contribute to periodontal conditions, or regularly consumed any non-steroidal anti-inflammatory drugs.

Table 1. Age, sex and gingival overgrowth index of the selected patients

	Age (years)	Mean age (years)	Sex	Gingival overgrowth index
Healthy group	—	52	—	—
H1	57	—	Male	—
H2	52	—	Female	—
H3	46	—	Male	—
H4	55	—	Male	—
DIGO group	—	55	—	—
N1	55	—	Male	2
N2	55	—	Male	2
N3	51	—	Male	2
N4	59	—	Female	2

DIGO, dihydropyridine-induced gingival overgrowth.

Gingival fibroblast cell culture

After the epithelial layers were removed, gingival tissues obtained from patients with DIGO and healthy subjects were cut into small pieces and cultured in 5 cm Petri dishes containing Dulbecco's modified Eagle's medium (Invitrogen-Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen-Gibco). After 10–12 d, the attached cells were subcultured in fresh media, and the cells from passages 3–8 were used for experimentation. For this investigation, 50 μg/mL of 3-aminopropionitrile fumarate (Sigma-Aldrich, St Louis, MO, USA) and 50 μg/mL of ascorbic acid were supplemented in cultured medium. Confluent fibroblasts were cultured in phenol red-free Dulbecco's modified Eagle's medium (Invitrogen-Gibco) containing 0.2% fetal calf serum for 24 h prior to the administration of nifedipine (10 μM), interleukin-1β (10 ng/mL) or both, according to doses described previously (5). Flutamide (10 μM) was added 30 min before stimulation with nifedipine and interleukin-1β. Forty-eight hours after drug administration, cells were harvested for gene expression analysis and the conditioned media were used for Sircol Collagen Assay (Biocolor, Belfast, UK).

Western blot analysis

The gingival explants selected from both DIGO patients (N2, N3 and N4) and healthy individuals (H2, H3 and H4) were ground to a powder in liquid nitrogen, then resuspended in 2 × sampling buffer (125 mM Tris pH 6.8, 4% sodium dodecyl sulphate, 10% glycerol, 0.006% bromophenol blue and 1.8% β-mercaptoethanol) and boiled for 5 min. Thirty microgram protein samples were separated by 10% sodium dodecyl sulphate–polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membranes. The primary antibodies used in this study were rabbit anti-androgen receptor, anti-CTGF/CCN2, anti-collagen type I (Abcam, Cambridge, MA, USA) and mouse anti-β-actin (Sigma-Aldrich). After incubation with the primary antibody, the membranes were probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit

secondary antibody (Pierce, Rockford, IL, USA). Immune complexes were visualized using a Supersignal West Pico ECL kit (Pierce). The fluorescent images were quantitatively compared with a densitometer.

Reverse transcription and real-time quantitative polymerase chain reaction

Total RNA was isolated with TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and treated with DNase-I (Sigma-Aldrich). Complementary DNA was synthesized from 2 μg of RNA with the First-Strand cDNA Synthesis kit (GE Healthcare, Piscataway, NJ, USA) using an oligo-dT primer in a 15 μL reaction mixture. The real-time quantitative polymerase chain reaction was based on the high-affinity double-stranded DNA-binding dye SYBR® Green I (Applied Biosystems, Foster City, CA, USA). For each sample, the amount of the gene of interest and of the independent endogenous reference 18S rRNA was determined from the appropriate standard curve in autonomous experiments. Results were normalized according to the average amount of the endogenous references. Primer sequences used in these studies were as follows: CTGF/CCN2 sense, 5′-TTAGAGCCAACTGCCTGGTC-3′ and antisense, 5′-CAGGAGGCGTTGTCATTGGTA-3′; procollagen α1(I) sense, 5′-GTGCTAAAGGTGCCAATGGT-3′ and antisense 5′-ACCAGGTTCACCGCTGTTAC-3′; and 18S rRNA sense, 5′-GGACACGGACAGGATTGACA-3′ and antisense, 5′-ACCCACGGAATCGAGAAAGA-3′.

Determination of collagen production

Total soluble collagen was measured in conditioned media using the Sircol Collagen Assay kit (Biocolor, Belfast, UK) according to a modified method (20). Briefly, 0.3 mL of Sirius Red reagent was added to an equal volume of test sample and mixed for 30 min at room temperature. The collagen–dye complex was precipitated by centrifugation and dissolved in 0.5 M sodium hydroxide; the absorbance was measured at 540 nm. The collagen level in each specimen was obtained as an average of three readings.

Statistical analysis

Owing to limited tissue samples, the Mann–Whitney *U*-test was applied to compare the protein expressions of the androgen receptor, CTGF/CCN2, and type I collagen in gingival tissue of DIGO patients and healthy individuals. Since the data for real-time polymerse chain reaction displayed a normal distribution as estimated by the Shapiro–Wilk test, Student's paired *t*-test was used to examine the effects of flutamide on the gene expression of CTGF/CCN2 or procollagen α1(I), and soluble total collagen in conditioned media. The results were considered significantly different when the *p*-value was < 0.05.

Results

Protein expression of the androgen receptor, CTGF/CCN2 and type I collagen in gingival tissue

Western blot showed that levels of androgen receptor and type I collagen in gingival tissue from DIGO patients were significantly higher than those in healthy individuals ($p < 0.05$; Fig. 1). However, CTGF/CCN2 levels were comparable in both groups ($p > 0.05$). This result implies that androgen receptor might upregulate collagen levels in gingival overgrowth.

The expression of androgen receptor is required for increasing expression of CTGF/CCN2 or procollagen α1(I) mRNA and total soluble collagen production in gingival fibroblasts

Gingival fibroblasts derived from four healthy subjects and four DIGO

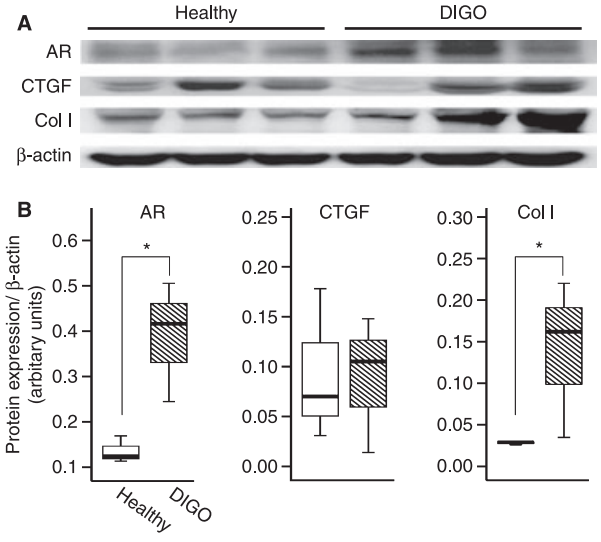

Fig. 1. The androgen receptor and type I collagen were upregulated in DIGO gingivae. (A) Gingival homogenates from healthy control subjects ($n = 3$) and DIGO patients ($n = 3$) were immunoblotted with anti-androgen receptor, anti-connective tissue growth factor (CTGF/CCN2), and anti-type I collagen antibodies. The western blot membrane was reprobed for β-actin to confirm the loading. Stronger expressions of the androgen receptor and type I collagen were observed in DIGO gingival tissue compared with those in healthy gingival tissue. (B) Densitometric analysis revealed a 3.4- and 5.6-fold increase in androgen receptor and type I collagen, respectively, in DIGO gingival tissue compared with tissue in the healthy group ($p < 0.01$). Protein expressions were quantified as a ratio to β-actin expression and are presented as median values (quartile range, 25–75%) and calculated with a Mann–Whitney *U*-test. Blots are representative of three independent experiments. Abbreviations: AR, androgen receptor; CTGF, connective tissue growth factor; Col I, type I collagen; and DIGO, dihydropyridine-induced gingival overgrowth.

patients were used for this study. When stimulated with nifedipine, CTGF/CCN2 mRNA expression slightly increased in both healthy and DIGO cells compared with unstimulated cells (Fig. 2A), but interleukin-1β significantly induced CTGF/CCN2 mRNA expression in both cell types. Furthermore, an additive effect of the two drugs on healthy and DIGO cells was observed. However, the stimulatory effect was entirely abolished when flutamide was added (*$p < 0.05$, **$p < 0.01$). Since flutamide is a non-steroidal androgen receptor antagonist, this result strongly suggests that androgen receptor activity is needed for *CTGF/CCN2* gene expression. Since CTGF/CCN2 promotes distinctive extracellular matrix accumulation (i.e. type I collagen) in fibroblasts, the effect of nifedipine and interleukin-1β on procollagen α1(I) RNA expression was also examined. The response of procollagen α1(I) mRNA expression in healthy cells (1.6–4.7 times the control value) was equal to, or stronger than, that in DIGO cells (1.6–3.6 times the control value; Fig. 2B). The additive induction of gene expression by nifedipine and interleukin-1β was also diminished by flutamide in both groups (*$p < 0.05$, **$p < 0.01$), but the DIGO cells still expressed higher collagen mRNA than did healthy cells when an androgen receptor competitor was used.

The response of soluble collagen secretion to stimulants was stronger in DIGO cells (1.8–3.2 times the control value) than in healthy cells (1.0–1.8 times the control value). Flutamide also blocked the accumulation of total collagen in culture media in both groups (Fig. 3, *$p < 0.05$). In this work, both healthy and DIGO fibroblasts secreted similar amounts of soluble collagen in untreated cells; however, the latter cells were more sensitive to drug stimulation than the former. In the presence of the stimulants nifedipine, interleukin-1β and nifedipine plus interleukin-1β, DIGO cells displayed more sensitivity to flutamide (40.1, 39.0 and 48.1% inhibition, respectively) than healthy cells (30.5, 29.5 and 28.0% inhibition, respectively).

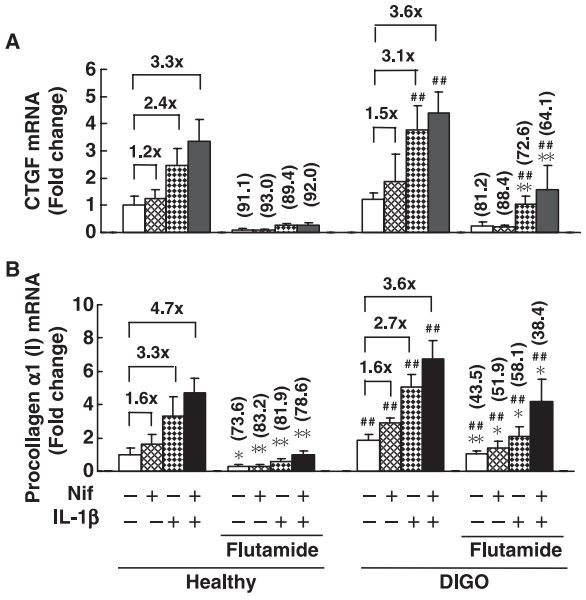

Fig. 2. Flutamide inhibited nifedipine-, interleukin-1β- or nifedipine plus interleukin-1β-stimulated mRNA expression of the connective tissue growth factor (CTGF/CCN2) and procollagen α1(I) in gingival fibroblasts derived from healthy subjects ($n = 4$) and DIGO patients ($n = 4$). Gingival fibroblasts were stimulated with nifedipine (10 μM), interleukin-1β (10 ng/mL) or both for 48 h, then mRNA expressions were measured by real-time polymerase chain reaction and compared with unstimulated fibroblasts. Interleukin-1β positively stimulated CTGF/CCN2 mRNA expression in gingival fibroblasts from healthy subjects and DIGO patients, and nifedipine enhanced the effect of interleukin-1β on the mRNA expressions (A). Furthermore, DIGO patients expressed higher levels of procollagen α1(I) mRNA than did the healthy control subjects regardless of the presence of stimulants. In addition, flutamide always had a stronger inhibitory activity on healthy cells whether stimulants were present or not (B). Results are presented as the means and standard deviation of multiples of change in the levels of mRNA relative to that in unstimulated healthy cells; values are normalized to the expression of 18S rRNA. Data in parentheses denotes percentage inhibition by flutamide. Statistically significant effects were analysed by Student's paired *t*-test (*$p < 0.05$ or **$p < 0.01$ compared with a control group submitted to the same drug treatment without flutamide; #$p < 0.05$ or ##$p < 0.01$ compared with a healthy group submitted to the same drug treatment). Abbreviations: CTGF, connective tissue growth factor; Nif, nifedipine; IL-1β, interleukin-1β; and DIGO, dihydropyridine-induced gingival overgrowth.

Discussion

The profound effects of the intracellular androgen receptor–CTGF/CCN2–collagen axis on downstream extracellular collagen matrix synthesis and tissue overgrowth have seldom been discussed. Androgen-deficient mice have significantly reduced levels of collagen in their tissues (21). The overexpression of androgen receptor in drug-induced gingival overgrowth tissues seemed to be important in the pathogenesis of nifedipine-induced gingival overgrowth (4). The present study shows that the protein expression of androgen receptor and type I collagen in DIGO fibrotic gingival tissue are concomitantly higher than in healthy cells. However, there is no difference in CTGF protein expression between these two tissues (Fig. 1). The cells derived from DIGO patients showed stronger gene expression of CTGF/CCN2 and procollagen α1(I) than those from healthy subjects, whether the stimulants were present or not (Fig 2). The response of procollagen α1(I) expression to nifedipine, interleukin-1β or the drug combination in healthy cells seemed stronger than that in DIGO cells (1.6, 3.3 and 4.7 times control value vs. 1.6, 2.7 and

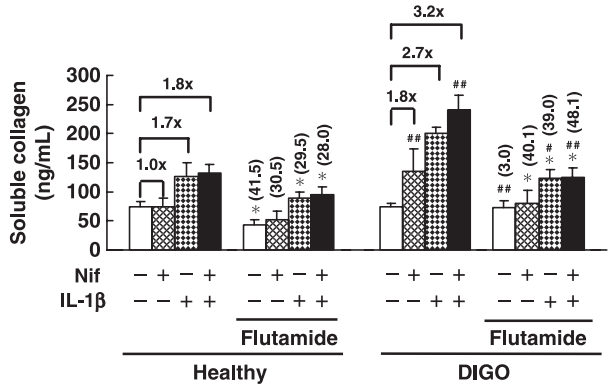

Fig. 3. Flutamide inhibited nifedipine-, interleukin-1β- and nifedipine plus interleukin-1β-stimulated soluble collagen secretion in gingival fibroblasts derived from healthy subjects and DIGO patients. The basal levels of soluble collagen released were similar in gingival fibroblasts derived from both groups, but the response to drug administration was stronger in DIGO patients than in healthy control subjects, while nifedipine alone did not increase the soluble collagen secretion in the healthy control subjects. Flutamide inhibited the basal and stimulant-enhanced collagen secretion in healthy control subjects and did not obviously inhibit the basal collagen secretion in DIGO patients. The inhibition of collagen secretion by flutamide tended to be stronger in DIGO patients than in healthy control subjects. See Fig. 2 for abbreviations.

3.6 times control value, respectively), but the response of collagen protein secretion to these drugs was quite different (1.0, 1.7 and 1.8 times control value vs. 1.8, 2.7 and 3.2 times control value, respectively). Furthermore, DIGO cells always produced more soluble collagen in the presence of stimulants (Fig 3, $p < 0.01$ or 0.05). Therefore, compared with healthy cells, we suggest that the high protein expression of collagen in DIGO tissue or fibroblasts derived from DIGO tissue is mainly due to a decrease in collagen degradation rather than an increase in collagen synthesis.

The androgen receptor is a member of the nuclear receptor superfamily. Binding to ligand, the androgen receptor is translocated from the cytosol into the nucleus, where it binds to specific promoter elements. Some co-regulators are bound to the androgen receptor, which then regulates the transcription of various target genes (22,23). The androgen–androgen receptor system plays a pivotal role in normal cardiac growth and in cardiac protection against angiotensin II-induced cardiac fibrosis (24). However, the role of the androgen receptor in gingival overgrowth has rarely been reported. In this study, by using flutamide as an androgen receptor inhibitor, we clarified the importance of the androgen receptor in gingival overgrowth. As shown in Fig. 2, whether the stimulants were administered or not, flutamide decreased the mRNA expressions of CTGF/CCN2 and procollagen α1(I) in both strains of gingival fibroblasts, but cells derived from DIGO patients were less sensitive to flutamide inhibition than those from healthy tissue. A possible reason is that DIGO cells express higher androgen receptor levels than healthy cells, and the concentration of flutamide used in this study may not be sufficient to inhibit the androgen receptor, or the androgen receptor is not the only pathway for signalling either gene expression in DIGO cells.

In addition to nifedipine, this investigation also revealed that interleukin-1β is one of the major mediators responsible for androgen receptor or *CTGF*/*CCN2* gene expression and collagen overproduction in dihydropyridine responders. Interleukin-1 is not only a potent pleiotropic cytokine involved in host immune and inflammatory responses, but also plays a crucial role in the pathogenesis of periodontal disease (25,26). The various biological activities attributed to interleukin-1 are due to its ability to induce a wide range of factors, which include matrix metalloproteinases, nitric oxide synthetase, prostanoids and other cytokines (27). Our study indicates that interleukin-1β not only mediates the well-established catabolic arm of the periodontal immunity and inflammatory responses, but also acts in conjunction with nifedipine to augment the anabolic activity of DIGO cells through the androgen receptor–CTGF/CCN2–collagen pathway. Furthermore, the androgen receptor can be positively regulated by interleukin-1β in gingival fibroblasts derived from DIGO patients (5).

Interleukin-1β reduces the intracellular calcium concentration ($[Ca^{2+}]_i$) through blockade of the voltage-operated P/O-type calcium channels (28). Nevertheless, an increase in $[Ca^{2+}]_i$ by the calcium ionophore effectively reduces androgen receptor expression in the human prostate cancer cell line and platelets (29). We infer that nifedipine reduces $[Ca^{2+}]_i$ as a result of a blockade of L-type Ca^{2+} channels in DIGO responder cells, and interleukin-1β may enhance the inhibition of Ca^{2+} influx from an independent non-genomic pathway through P/O-type calcium channels and additively increase the expression of androgen receptor in those cells (30,31). In this investigation, we suggest that the additive effects of nifedipine and interleukin-1β on gingival tissue may aggravate gingival overgrowth by augmenting androgen receptor activity through the regulation of intracellular $[Ca^{2+}]$ homeostasis (32).

Our investigations were initially based upon the postulated 'three models' that a combination of an increase in 5α-dihydrotestosterone receptors, deficient intracellular folic acid uptake and suppression of both interleukin-2 production and T-cell proliferation were the possible aetiological factors for drug-induced gingival hyperplasia (33). That model has been challenged in a recent review that focused on connective tissue metabolism (17,34). The CTGF/CCN2 plays an important role in drug-induced

gingival overgrowth and fibrosis (35). Nonetheless, the present study shows that the gene expressions of CTGF/CCN2 in both healthy and DIGO gingival fibroblasts significantly decreased after blockade of androgen receptor activity with flutamide. This implies that the androgen receptor plays an important role in up-regulating CTGF/CCN2 and procollagen α1(I) mRNA expressions, and in the accumulation of the extracellular collagen matrix in gingival fibroblasts.

Anti-androgens act by two primary mechanisms: inhibition of androgen binding to the androgen receptor and inhibition of androgen-independent activation of the receptor (36). The latter mechanism occurs via several pathways, including inhibition of nuclear co-activators, activation of co-suppressors and inhibition of a variety of androgen response elements in genes. Flutamide was the first non-steroidal anilide analogue to enter clinical use. It is more accurate to refer to flutamide as an androgen receptor antagonist, since it inhibits activation of the androgen receptor whether androgen-mediated or not (37). The long-term use of low-dose flutamide in treating hirsutism or polycystic ovary syndrome is a safe regime (38,39). In the present study, a low dose of flutamide non-specifically decreased the expression of CTGF/CCN2 and procollagen α1(I) mRNAs in all experimental conditions by inhibition of androgen receptor activity.

Gingival overgrowth induced by calcium channel blocker occurred 3.3 times more commonly in men than in women (40). One animal study showed that male rats were more prone to drug-induced gingival overgrowth than females (41). However, neither androgen metabolism (42) nor the androgen receptor-positive cell distribution (4) exhibited any sex differences in highly inflamed gingival tissue. Therefore, since samples were limited in this clinically oriented investigation, we consider that sex differences should not be a concern.

In conclusion, nifedipine in combination with interleukin-1β may possess potent additive interactions on androgen receptor, CTGF/CCN2 and procollagen α1(I) mRNA expressions by gingival fibroblasts. The high expression of androgen receptors may be one of the mechanisms for gingival overgrowth induced by dihydropyridines. Furthermore, the results encourage us to consider that DIGO may be either prevented or treated through a pharmaceutical strategy using non-steroidal anti-inflammatory agents to control interleukin-1-mediated inflammation, or by treatment with a reassuringly safe profile of low-dose androgen receptor antagonists to block the androgen receptor–CTGF/CCN2–collagen cascade and decrease collagen production.

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