RESEARCH ARTICLE

Nifedipine and phenytoin induce matrix synthesis, but not proliferation, in intact human gingival connective tissue *ex vivo*

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Abstract Drug-induced gingival enlargement (DIGE) is a fibrotic condition that can be caused by the antihypertensive drug nifedipine and the anti-seizure drug phenytoin, but the molecular etiology of this type of fibrosis is not well understood and the role of confounding factors such as inflammation remains to be fully investigated. The aim of this study was to develop an *ex vivo* gingival explant system to allow investigation of the effects of nifedipine and phenytoin alone on human gingival tissue. Comparisons were made to the histology of human DIGE tissue retrieved from individuals with DIGE. Increased collagen, fibronectin, and proliferating fibroblasts were evident, but myofibroblasts were not detected in DIGE samples caused by nifedipine and phenytoin. In healthy gingiva cultured in nifedipine or phenytoin-containing media, the number of cells positive for p-SMAD2/3 increased, concomitant with increased CCN2 and periostin immunoreactivity compared to untreated explants. Collagen content assessed through hydroxyproline assays was significantly higher in tissues cultured with either drug compared to control tissues,

which was confirmed histologically. Matrix fibronectin levels were also qualitatively greater in tissues treated with either drug. No significant differences in proliferating cells were observed between any of the conditions. Our study demonstrates that nifedipine and phenytoin activate canonical transforming growth factor-beta signaling, CCN2 and periostin expression, as well as increase collagen density, but do not influence cell proliferation or induce myofibroblast differentiation. We conclude that in the absence of confounding variables, nifedipine and phenytoin alter matrix homeostasis in gingival tissue explants *ex vivo*, and drug administration is a significant factor influencing ECM accumulation in gingival enlargement.

Keywords Gingival overgrowth · Gingiva · Fibrosis · Periostin · Nifedipine · Phenytoin

Introduction

Drug-induced gingival enlargement (DIGE), also termed gingival overgrowth, is a side effect that can arise from the systemic administration of the antihypertensive drug nifedipine and anti-seizure drug, phenytoin in a significant number of individuals. Statistics on the DIGE show the prevalence of the condition to be variable, but has been reported to be 5–85 % for nifedipine (Heasman and Hughes 2014) and 50 % for phenytoin (Dongari-Bagtzoglou 2004). DIGE is often evident within 1 month of the onset of drug therapy (Brew et al. 2000; Kaur et al. 2010). Current treatment options are limited to rigorous maintenance of oral hygiene, and in severe cases, surgical removal of the overgrown tissue (Brown et al. 1991). However, the recurrence rate of DIGE is very high, with patients requiring repeated surgical interventions (Ilgenli et al. 1999). Discontinuation of the drug therapy

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alleviates the condition, but this is rarely an option for patients (Nishikawa et al. 1996). Despite development of new drugs, both nifedipine and phenytoin are still widely prescribed (Heasman and Hughes 2014).

DIGE is classified as a fibrotic lesion (Brown et al. 1991; Dill and Iacopino 1997; Steinsvoll et al. 1999; Kataoka et al. 2000; Uzel et al. 2001); fibrosis is typically associated with inappropriate tissue remodeling that results in excessive extracellular matrix (ECM) deposition, and often the persistence of highly contractile myofibroblasts (Gabbiani 2003; Kim et al. 2015). An imbalance in the production and degradation of type I collagen, the major ECM component in gingiva (Trojanowska et al. 1998), has been shown to be prominent in DIGE (Kataoka et al. 2001; Kanno et al. 2008; McKleroy et al. 2013). Human gingival fibroblasts (HGFs) isolated from fibrotic gingiva produce significantly greater levels of collagen, but additionally exhibit reduced collagenase activity compared to non-fibrotic HGFs (Tipton et al. 1994). Although current evidence shows that phenytoin and nifedipine alter matrix production and degradation by gingival fibroblasts in vitro (Keith et al. 1977; Moy et al. 1985; Kato et al. 2006), how the drugs influence matrix production at the molecular level is still not well understood, particularly in intact tissue.

The transforming growth factor-beta (TGF- β) superfamily, a large family of growth and differentiation factors, is widely accepted as a central mediator in many fibrotic conditions. Previous reports have implicated TGF- β induction of the matricellular protein (MP) CCN2 in development of phenytoin and nifedipine-induced gingival enlargement where it alters ECM synthesis and accumulation (Uzel et al. 2001; Trackman and Kantarci 2015). Like CCN2, periostin, is a MP that is a critical regulator of events during connective tissue remodeling (Wen et al. 2010a; Zhou et al. 2010), but has also been shown to be associated with development and progression of various fibrotic disorders, by modulating collagen production and α -smooth muscle actin expression (α -SMA) (Oku et al. 2008; Vi et al. 2009; Zhou et al. 2010; Naik et al. 2012; Yamaguchi et al. 2012; Yang et al. 2012; Mael-Ainin et al. 2014). Interestingly, in excisional skin healing, CCN2 and periostin show a similar temporal expression pattern (Kapoor et al. 2008; Elliott et al. 2012). Interestingly, both periostin (Horiuchi et al. 1999; Arancibia et al. 2013) and CCN2 (Hong et al. 1999) are TGF- β inducible in HGFs. We recently described for the first time that periostin protein levels are significantly elevated in nifedipine-induced gingival enlargement and nifedipine increases periostin in a TGF- β -dependent mechanism in vitro (Kim et al. 2013). Despite the advances in the understanding of DIGE pathology, there is still a significant gap at the molecular level on how the drugs specifically contribute to gingival enlargement.

DIGE has been classified by the American Academy of Periodontology as an plaque-mediated condition (Armitage 1999); implicating bacterial infection and plaque as primary drivers of the condition. It is still unclear what the specific role of nifedipine and phenytoin are in the processes underlying gingival enlargement compared to plaque. However, a major roadblock to understanding DIGE are that current systems in which it can be studied have limitations; human tissue retrieved is of limited value for molecular studies and in vitro cell culture cannot mimic the complexities present in a tissue. The aim of this study was to develop an ex-vivo gingival explant system to assess the effects of nifedipine and phenytoin treatment on proliferation, matrix synthesis and accumulation, as well as whether myofibroblast differentiation is associated with DIGE.

Materials and methods

In situ study - tissue procurement and tissue preparation

Clinically healthy gingiva ($n=6$) was obtained with informed consent from six patients undergoing periodontal or implant therapies at the Oral Surgery Clinic at The University of Western Ontario. Gingival tissues from 11 patients with DIGE [nifedipine ($n=6$) and phenytoin ($n=5$)] were obtained from the oral pathology laboratory. The use of all tissue material was in accordance with the guidelines of the University's Research Ethics Board for Health Sciences Research involving Human Subjects requiring informed consent.

Gingival tissue explant cultures

Human gingival connective tissues were obtained from systematically healthy subjects. Tissues were cut into equal sizes, about 4×4 mm. Explants were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 1× antibiotics and antimycotics (AA; 100 μ g/ml penicillin G, 50 μ g/ml gentamicin, 25 μ g/ml amphotericin B), and 50 μ g/ml L-ascorbic acid (to facilitate collagen synthesis) (Sigma Aldrich, St. Louis, MO, USA) with no drug (DMSO alone), nifedipine (100 ng/ml), or phenytoin (30 μ g/ml) for 2 weeks. Media was changed every other day with freshly made up drugs and DMEM. Cultures were maintained at 37 °C in a humidified atmosphere of 95 % air 5 % CO₂. Three repeated independent experiments were done with explant tissues obtained from three different individuals.

Tissue preparation and immunohistochemistry

Tissues were 10 % neutral buffered formalin (Sigma Aldrich, St. Louis, MO, USA)-fixed, paraffin processed, embedded, and sectioned at 5 μ m for various staining.

Immunohistochemistry was performed as previously described (Wen et al. 2010b; Zhou et al. 2010; Elliott et al. 2012). In brief, tissues were deparaffinized and immunolabeled using primary antibodies against α -smooth muscle actin (α -SMA) (ab5694, Abcam, Cambridge, MA, USA; 1:400), phosphorylated-SMAD2/3 (p-SMAD2/3) (Ser 423/425) (sc-11769, Santa Cruz Biotechnology, Dallas, TX, USA; 1:100), proliferating cell nuclear antigen (PCNA) (ab2426-1, Abcam, Cambridge, MA, USA; 1:100), and CCN2 (ab6992; Abcam; 1:100). Primary antibodies were detected using the ImmPRESS Reagent Kit Peroxidase (Vector Laboratory, Burlingame, CA, USA) following the manufacturer's instructions. Antibody specificity was confirmed using primary delete controls. All sections were counterstained with haematoxylin (Sigma Aldrich, St. Louis, MO, USA). For collagen staining, tissues were stained with Masson's trichrome, Picrosirius red, and van Gieson stains. Images were taken with a DM1000 light microscope (Leica, Concord, Ontario, Canada) and Leica Application Suite Software (version 3.8).

Immunofluorescence

Deparaffinized sections were also fluorescently stained. Tissues were permeabilized with 0.1 % Triton X-100 (Caledon, Georgetown, ON, Canada) PBS, blocked with 10 % horse serum in 0.1 % Triton X-100 PBS, and incubated with fibronectin (sc-8422, Santa Cruz Biotechnology, Dallas, TX; 1:100), α -SMA (ab5694, Abcam, Cambridge, MA, USA; 1:400), periostin (sc49480; Santa Cruz Biotechnology, Dallas, TX; 1:100), fibroblast specific protein-1 (FSP-1) (07-2274, Millipore, Billerica, MA, USA; 1:100), or PCNA (ab2426-1, Abcam, Cambridge, MA, USA; 1:100) primary antibodies overnight. Primary antibodies were detected using Cy5-conjugated anti-mouse or anti-rabbit secondary antibodies (Molecular Probes, Carlsbad, CA, USA). All sections were counterstained with Hoechst 3342 dye (1:5000) for nuclei. Images were taken on Carl Zeiss Imager M2m microscope (Carl Zeiss, Jena, Germany) using Zen Pro 2012 software. To quantify percentages of proliferating fibroblasts, the number of PCNA-positive cells and DAPI-stained cells were counted in 10 sections per tissue.

Collagen labeling and visualization

To assess collagen density, deparaffinized histological sections were stained using Masson's trichrome (University Hospital, London, ON, Canada), Van Gieson's (Sigma-Aldrich, St. Louis, MO, USA), and Picrosirius red stains.

Images for Masson's trichrome and Van Gieson's stains were taken with DM1000 light microscope (Leica, Concord, Ontario, Canada) and Leica Application Suite Software (version 3.8). For Picrosirius red stain, sections were stained for 1 h in 0.1 % Picrosirius red (Sigma-Aldrich, St. Louis, MO, USA) and were imaged on a Carl Zeiss microscope. A1 Axio using Zen Pro 2012 under a polarized light to visualize birefringent collagen fibers.

Hydroxyproline assay

Hydroxyproline assays were performed on gingival tissue samples following manufacturer protocols (MAK008; Sigma Aldrich, St. Louis, MO, USA). Briefly, after 2 weeks of ex vivo culture, human gingival tissues were weighted and homogenized in water and hydrolyzed in 6 M at 95 °C overnight. Oxidized hydroxyproline reacted with 4-(Dimethylamino)benzaldehyde and resulting colorimetric (560 nm) product was read on the plate reader (Tecan Safire, Seestrasse, Männedorf, Switzerland). Experiments were done in triplicates and three independent experiments with explants from different individuals.

Tissue viability

In Situ Cell Death Detection Kit (Fluorescein) (Roche Diagnostics GmbH, Mannheim, Germany) was used to label apoptotic cells in histological sections of the explants using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end-labeling (TUNEL). Images were captured on Carl Zeiss Imager M2m microscope (Jena, Germany) using Zen Pro 2012 software. Subsequently, the numbers of cells positive for TUNEL and the total numbers of cells (DAPI-stained) per fields of view were analyzed with the ImageJ version 10.2 software (National Institutes of Health, Bethesda, MD, USA) using 10 images taken per explant from three independent experiments to get average percentages of apoptotic cells.

Statistical analysis

All statistical analysis was performed using Graphpad Software version 6 (Graphpad Software, San Diego, CA, USA) ($p < 0.05$ was considered significant). To compare hydroxyproline contents (Fig. 5c), cell proliferation (Fig. 6c), and apoptosis (Fig. 5b) between tissue explants treated with nifedipine or phenytoin and without drug, one-way ANOVA with Bonferroni's multiple comparisons test was used. For cell proliferation and apoptosis, 10 images per tissue explant were analyzed. Data are expressed as the mean $\pm$ standard deviation of three individual experiments with independent explant cultures from different individuals.

For quantification of percentage of proliferating fibroblasts in tissues from healthy individuals and patients with DIGE (Fig. 4c), data are expressed as the mean $\pm$ standard deviation of six healthy subjects, six patients with nifedipine-induced gingival enlargement, and five patients with phenytoin-induced gingival enlargement. To compare the percentage of cell proliferations between the tissues obtained from healthy individuals and patients with nifedipine or phenytoin induced gingival enlargement, 10 images of the connective tissue per individual were analyzed and significance was measured using one-way ANOVA with Bonferroni's multiple comparisons test.

Results

Periostin and p-SMAD2/3 immunoreactivity are increased in DIGE individuals

We qualitatively examined the patterns of phosphorylated-SMAD2/3 (p-SMAD2/3) levels and periostin protein in pathological samples isolated from patients. No immunoreactivity

for p-SMAD2/3 was evident in healthy gingival tissues, but in all DIGE samples, p-SMAD2/3 localized to the nuclei of many cells in the connective tissue (Fig. 1a). In healthy gingiva, periostin immunoreactivity was evident in the sub-epithelial connective tissue along the basal lamina ($n=6$) (Fig. 1a). In contrast, periostin immunoreactivity was significantly elevated throughout the gingival connective tissue in DIGE (nifedipine-induced gingival enlargement, $n=6$; phenytoin-induced gingival enlargement, $n=5$), with pockets of cells evident that did not label for periostin in all tissues (Fig. 1b).

Increased extracellular matrix deposition is evident tissue from DIGE individuals

In DIGE samples isolated from patients, fibronectin immunoreactivity was qualitatively greater in nifedipine and phenytoin DIGE compared to healthy tissues in the connective tissue area (Fig. 2a). Immunoreactivity for fibronectin was detected throughout the gingival connective tissues from all individuals and was always qualitatively higher in DIGE samples. While fibronectin was only

Fig. 1 Elevated periostin and p-SMAD2/3 in DIGE. **a.** Immunoreactivity for p-SMAD2/3 in the gingival tissues from healthy subjects and DIGE patients [nifedipine ($n=6$) and phenytoin ($n=5$)]. P-SMAD2/3 was detected using peroxidase-conjugate secondary antibody and DAB. P-SMAD2/3 positive cells (arrows) were detected in the connective tissues of DIGE. **b.** Fluorescent immunoreactivity for periostin in the gingival tissues from healthy subjects ($n=6$) and DIGE patients. Sections were incubated with a primary antibody against periostin, which was detected using Cy5-conjugated secondary antibody (red). Nuclei are stained with Hoechst 3342 dye (blue). Elevated periostin was observed in the connective tissues of DIGE, while periostin was only found in the basement membrane (arrows) in healthy tissue. Yellow dotted line outlines the border between the oral epithelium and the connective tissue. Scale bar, 50 μ m

Fig. 2 Excess matrix in the gingival tissues from patients experiencing DIGE. **a.** Immunofluorescence for fibronectin in the gingival tissues from healthy subjects ($n=6$) and DIGE patients [nifedipine ($n=6$) and phenytoin ($n=5$)] was detected with Cy5 (red) conjugated secondary antibody. Nuclei are stained with Hoechst 3342 dye (blue). Increased fibronectin levels are observed in the gingival tissues from patients experiencing DIGE. Arrows indicate fibroblasts positive for fibronectin. Scale bar, 50 μm . **b.** Collagen deposition in gingival tissues derived

healthy subjects and DIGE patients. Masson's trichrome (top panel) and picrosirius red (bottom panel) stains collagen fibers in blue and red, respectively. Polarized light microscope was used to image the sections stained with picrosirius red. Dense collagen accumulations are evident in the gingival tissues from patients experiencing DIGE. Yellow dotted line outlines the border between the oral epithelium and the connective tissue. Scale bar, 50 μm

associated with the extracellular compartment in healthy tissues, numerous cells in DIGE tissues were also positive for fibronectin, indicated by arrows. The structure and localization of collagen in healthy and DIGE samples was assessed using Masson's trichrome and Picrosirius red stain. Compared to healthy gingival tissues ($n=6$), the density of collagen was qualitatively higher indicated by dense collagen accumulation in gingival tissues from patients diagnosed with DIGE (nifedipine-induced gingival enlargement, $n=6$; phenytoin-induced gingival enlargement, $n=5$) (Fig. 2b).

Myofibroblasts are not present in DIGE

To evaluate whether myofibroblast differentiation is associated with DIGE, immunoreactivity for alpha-smooth muscle actin (α -SMA) was assessed histologically (Fig. 3a). In both groups of healthy and DIGE gingival tissues, α -SMA immunoreactivity was only evident in the vasculature, with no fibroblasts labeling positive for α -SMA.

Increased cell proliferation is evident in DIGE compared to healthy gingiva

To assess whether proliferation was evident in DIGE, sections were labeled with antibodies to proliferating cell nuclear antigen (PCNA) and fibroblast specific protein-1 (FSP-1). Proliferating cells were only sparsely observed in healthy gingiva (Fig. 3b), but in tissues from patients with DIGE, increased PCNA-positive cells were found throughout the connective tissue. Vimentin, a marker for mesenchymal cells showed a significant alteration in staining pattern in both nifedipine- and phenytoin-induced gingival enlargement indicating an increase in mesenchymal cells (Fig. 4a). FSP-1, a marker for remodeling fibroblasts was only sparsely observed in healthy tissue, but increased immunoreactivity was evident in both nifedipine and phenytoin DIGE tissue (Fig. 4b). The percentage of proliferating cells in the connective tissues of DIGE was significantly higher compared to healthy samples ($p<0.05$) (Fig. 4c). The numbers of both FSP-1-positive (FSP-1⁺) cells and total cell number in DIGE tissues were significantly greater compared to healthy tissue (Fig. 4d).

Fig. 3 Localization of α -SMA and PCNA in gingival tissues from patients experiencing DIGE. **a.** Immunoreactivity for α -smooth muscle actin (α -SMA), the marker for myofibroblasts, in gingival tissues from healthy subjects ($n=6$) and DIGE patients [nifedipine ($n=6$) and phenytoin ($n=5$)]. Sections were incubated with a primary antibody against α -SMA and detected using peroxidase-conjugate secondary antibody and DAB. All samples from both groups lacked α -SMA positive cells in the connective tissue, except in the smooth muscle of

the blood vessel walls, which is the internal positive control for α -SMA (arrows). The inset shows α -SMA-stained myofibrobloma tissue, as the experimental positive control. **b.** To detect levels of cell proliferation, histological sections were incubated with a primary antibody against PCNA and detected using peroxidase-conjugate secondary antibody and DAB, in gingival tissues from healthy subjects and DIGE patients. Black arrowheads indicate PCNA-positive fibroblasts, in the connective tissues

Gingival tissue is viable in explant culture for 2 weeks

To study the direct effects of nifedipine and phenytoin on tissue ECM composition, gingival tissue explants were cultured with nifedipine or phenytoin for 2 weeks and compared to untreated explants. To confirm viability, apoptotic cells were labeled in gingival explants using TUNEL (Fig. 5a). There were greater percentages of TUNEL-positive cells in gingival explants independently treated with nifedipine (2.4505 %) and phenytoin (3.3320 %) compared to control (1.3217 %) tissue ($p<0.05$) (Fig. 5b).

p-SMAD3, CCN2, and periostin are increased by nifedipine and phenytoin treatments in gingival connective tissues ex vivo

In gingival tissue explants independently cultured with nifedipine and phenytoin, immunoreactivity for p-SMAD2/3 was qualitatively increased to the nuclei of cells in the connective tissue, compared to explants cultured without either drug (Fig. 6a). Immunoreactivity for CCN2 (Fig. 6b) and periostin (Fig. 6c) was qualitatively higher in the connective tissues of

the explants cultured with either nifedipine or phenytoin compared to control explants. CCN2 localized to areas consistent with blood vessels, but periostin was associated primarily with the ECM of the connective tissue.

Nifedipine and phenytoin increase fibronectin and collagen deposition ex vivo

Levels of fibronectin and collagen were next assessed in gingival explants cultured with nifedipine, phenytoin or untreated for 2 weeks (Fig. 7). Greater immunoreactivity for fibronectin was observed in explants treated with either nifedipine or phenytoin compared to untreated explants (Fig. 7a). Increased accumulation of fibronectin was observed in the ECM, but fibroblasts in the explants treated with either nifedipine or phenytoin showed increased immunoreactivity.

Collagen deposition was assessed in all explants using Masson's trichrome, Picrosirius red, and Van Gieson's stains (Fig. 7b). Increased staining for collagen was evident in gingival connective tissues independently cultured with nifedipine and phenytoin, compared with control tissues at 2 weeks post-treatment (Fig. 7b). In Van Gieson's stain, bright pink

Fig. 4 Vimentin and FSP-1 labeling indicates increased cell density in DIGE tissues. **a.** Immunoreactivity for vimentin, which is a marker for mesenchymal cells, in gingival tissues from healthy subjects ($n=6$) and DIGE patients [nifedipine ($n=6$) and phenytoin ($n=5$)]. Sections were incubated with a primary antibody against vimentin and detected using peroxidase-conjugate secondary antibody and DAB. Increased vimentin immunoreactivity was seen in all DIGE samples. Scale bar; 50 μ m **b.** Fluorescent immunoreactivity for FSP-1 in the gingival tissues from healthy subjects and DIGE patients. Sections were incubated with a primary antibody against FSP-1, which was detected using Cy5-conjugated secondary antibody (red). Nuclei are stained with Hoechst 3342 dye (blue). White arrowheads indicate FSP-1⁺ cells. Yellow dotted line outlines the border between the oral epithelium and the connective tissue. Scale bar; 50 μ m **c.** Average percentages of PCNA-positive fibroblasts in the connective tissues from healthy subjects,

patients with nifedipine-induced gingival enlargement (NIGE) and phenytoin-induced gingival enlargement (PIGE) were quantified per field of view. Data represents mean percentage of PCNA-positive cells normalized to the total number of cells $\pm$ STD of 10 images per tissue. Percentages of proliferating cells were greater in the tissues from patients with nifedipine-induced gingival enlargement (NIGE) and phenytoin-induced gingival enlargement (PIGE) compared to healthy individuals. **d.** Average number of FSP-1⁺ cells and total number of cells in the connective tissues from healthy subjects, patients with nifedipine-induced gingival enlargement (NIGE) and phenytoin-induced gingival enlargement (PIGE) were quantified per field of view. Data represents mean number of FSP-1⁺ cells and total number of cells $\pm$ STD of 10 images per tissue. Data was analyzed using one-way ANOVA with Bonferroni's multiple comparisons test (* $p < 0.001$, ** $p < 0.0001$).

stained matrices indicating newly synthesized collagen were evident in tissues treated with either nifedipine and phenytoin in comparison with untreated explants (Fig. 7b). Quantitative assessment of collagen showed that hydroxyproline levels normalized to total tissue weights were significantly greater in gingival connective tissues each treated with nifedipine and phenytoin, compared to control tissue cultured without the drug ($p < 0.05$) (Fig. 7c).

No myofibroblasts are evident in gingival tissues treated with the nifedipine or phenytoin

To assess whether the drug influences myofibroblast differentiation, histological sections of gingival tissue explants were

stained for α -SMA. No myofibroblasts were observed in any of the explants cultured with nifedipine, phenytoin or without drug (Fig. 8a).

Proliferation in gingival tissue explants ex vivo

To assess whether nifedipine or phenytoin influenced cell proliferation, histological sections of the gingival explants were labeled for PCNA and FSP-1. PCNA-positive nuclei were evident in all samples (Fig. 8b), as were FSP-1⁺ cells (Fig. 8c). There were no significant differences in the percentages of proliferating cells in the sub-epithelial connective tissues among the explants cultured with nifedipine, phenytoin or without drug ($p > 0.05$) (Fig. 8d). Similarly, no significant

Fig. 5 TUNEL staining of gingival tissues treated with nifedipine or phenytoin ex vivo. Human gingival connective tissues cultured with nifedipine (100 ng/ml), phenytoin (30 μ g/ml), or without the drug for 2 weeks were stained using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end-labeling (TUNEL). **a.** Histological sections of gingival explants treated without drug or with nifedipine or phenytoin were stained with TUNEL assay. TUNEL labeling is detected with FITC. Nuclei are stained with Hoechst 3342 dye (blue). Yellow dotted line outlines the border between the oral epithelium and the connective tissue. Scale bar, 50 μ m. **b.** Average percentage of TUNEL-positive cells (excluding vessels) per field of view were quantified. Data represents mean percentage of cells $\pm$ STD of 10 images per explant condition from three independent experiments. Data was analyzed using one-way ANOVA with Bonferroni's multiple comparisons test (* p <0.05, ** p <0.01)

differences in FSP-1⁺ cells or total number of cells in the sub-epithelial connective tissues were observed between each condition (p >0.05) (Fig. 8e).

Discussion

Systemic administration of nifedipine and phenytoin are known to cause gingival enlargement in a significant number of individuals (Heasman and Hughes 2014). Gender, duration of administration, genetics, and plaque-induced inflammation have been suggested as contributing factors, but the exact role of the drugs on gingival fibroblasts and matrix accumulation has been difficult to investigate due to limitations in model systems to study the condition (Tavassoli et al. 1998; Miranda et al. 2001). In this study, we developed an explant system in which we show that ex vivo culture of gingival tissue explants is a suitable and valid model for investigating the effects of nifedipine and phenytoin on matrix homeostasis and fibrotic patterns of ECM deposition.

It is now apparent that DIGE is an extremely complex pathology, with the composition of the lesions differing depending on which drug is the causative agent (Trackman and

Kantarci 2015). It has been hypothesized that several variables including plaque accumulation contribute to gingival enlargement (Tavassoli et al. 1998), such that we developed a novel explant culture systems to understand the effects of nifedipine and phenytoin alone on gingival enlargement. Studying cells on 2-dimensional (2-D) tissue culture plastic substrates in vitro is a standard method for investigating complex biological phenomena (Jaiswal et al. 1997), but represent one of the most artificial surfaces cells can be cultured on. When cells are excised from their native 3-dimensional (3-D) tissues and confined to a monolayer system, many behaviours change significantly (Baker and Chen 2012). Culturing cells on 3-D systems such as collagen gels is closer to the in vivo environment (Petersen et al. 1992), but such gel systems do not possess the complex biochemical composition present in tissues, including gingiva (Bartold et al. 2000). As we have shown (Kim et al. 2013), analysis of tissue obtained from patients with DIGE provides important insights in the pathogenesis, but it has shortcomings; our access to primary DIGE tissues is extremely limited and studying the pathological influence of the drugs alone using formalin-fixed and paraffin-embedded tissues is not possible. As stated above, DIGE arising from different drugs is associated with varying levels of fibrosis and

inflammatory cell infiltration (Trackman and Kantarci 2015), but systems to investigate the molecular mechanisms underlying these differences have been lacking, which our ex vivo model overcomes.

We selected pSMAD2/3, periostin, fibronectin, and collagen as fibrotic markers in this study, and as a baseline, we first analyzed these markers in DIGE samples retrieved from patients. The involvement of CCN2 has been previously described (Uzel et al. 2001; Kantarci et al. 2006), and this was utilized in analysis of explant tissue only. Phenytoin and nifedipine are known to influence matrix synthesis and degradation (Yamada et al. 2000; Kataoka et al. 2001; Kanno et al. 2008), with phenytoin considered to result in a more fibrotic tissue than nifedipine (Trackman and Kantarci 2015). However, whether this excess matrix accumulation can be solely attributed to the effects of nifedipine and phenytoin alone has been unclear. In gingival tissues isolated from patients diagnosed with nifedipine and phenytoin DIGE, increased pSMAD2/3 nuclear translocation, periostin and fibronectin immunoreactivity, as well as increased collagen density were evident compared to gingiva from healthy individuals confirming previous studies (Takagi et al. 1991; Shikata et al. 1993; Pisoschi et al. 2014). Although only qualitative, staining levels for fibronectin, periostin as well as collagen density seemed higher in phenytoin DIGE compared to nifedipine, which is supported by previous research that phenytoin

results in a more fibrotic lesion (Uzel et al. 2001). Using our ex vivo system, we have now confirmed that gingival tissue explants cultured with either nifedipine or phenytoin show increased pSMAD2/3 nuclear localization, greater collagen deposition, as well as increased CCN2, periostin and fibronectin immunoreactivity compared to untreated controls 2 weeks after the onset of drug treatment (summarized in Fig. 9). While we have to quantify these protein levels, it shows that TGF- β signaling is activated in the presence of either drug. Nifedipine and phenytoin pharmacologically have different modes of action, but the histopathology of nifedipine and phenytoin-induced gingival enlargement are known to be similar (Heasman and Hughes 2014), which our initial results in explants appear to confirm in the presence of the drugs alone. Whether nifedipine and phenytoin specifically influence DIGE through the exact same molecular mechanism is yet to be determined and needs to be further studied, particularly in relation to inflammation where differences have been shown between nifedipine and phenytoin DIGE (Trackman and Kantarci 2015).

Previous studies have shown increased matrix accumulation and TGF- β signaling in DIGE, and we have shown that that nifedipine activates latent TGF- β in gingival fibroblasts, but does not increase TGF- β mRNA levels (Kim et al. 2013). Downstream of TGF- β signaling, it has long established that the matricellular protein CCN2 is upregulated in DIGE (Uzel

Fig. 6 Elevated p-SMAD2/3, CCN2, and periostin in the explants cultured with nifedipine or phenytoin. Immunoreactivity for p-SMAD2/3 (**a**) and CCN2 (**b**) in the gingival tissue explants cultured with nifedipine (100 ng/ml), phenytoin (30 μ g/ml), or without the drug for 2 weeks. P-SMAD2/3 and CCN2 were detected using peroxidase-conjugate secondary antibody and DAB. **c**. Immunofluorescence for periostin in the gingival tissue explants cultured with nifedipine (100 ng/ml), phenytoin (30 μ g/ml), or without the drug for 2 weeks. Sections were incubated with a primary antibody against FSP-1, which was detected using Cy5-conjugated secondary antibody (red). Nuclei are stained with Hoechst 3342 dye (blue). Yellow dotted line outlines the border between the oral epithelium and the connective tissue. Scale bar, 50 μ m

Fig. 7 Matrix contents in gingival explants are increased by nifedipine and phenytoin ex vivo. **a.** Fibronectin contents in gingival tissues treated with nifedipine or phenytoin or without drug ex vivo. Human gingival connective tissues cultured with nifedipine (100 ng/ml), phenytoin (30 μ g/ml), or without the drug for 2 weeks were stained for fibronectin. Fluorescent immunoreactivity for fibronectin was detected with Cy5-conjugated secondary antibody (red). Nuclei are stained with Hoechst 3342 dye (blue). Arrows indicate fibroblasts-positive for fibronectin. Yellow dotted line outlines the border between the oral epithelium and the connective tissue. **b.** Collagen contents in human gingival connective tissues cultured with nifedipine (100 ng/ml),

phenytoin (30 μ g/ml), or without the drug for 2 weeks had higher collagen contents indicated by Masson's trichrome, Van Gieson's, and Picrosirius red staining. Polarized light microscope was used to image the sections stained with picrosirius red. Scale bar, 50 μ m. **c.** Hydroxyproline contents normalized to weights of gingival tissue explants cultured with nifedipine (100 ng/ml), phenytoin (30 μ g/ml), or without the drug for 2 weeks. Data represents mean fold hydroxyproline levels $\pm$ STD relative to control (no drug) condition of three independent experiments in triplicates. Data was analyzed using one-way ANOVA with Bonferroni's multiple comparisons test (* p <0.05)

et al. 2001), and we now show that periostin is similarly increased in DIGE and in explants cultured with nifedipine and phenytoin. Both CCN2 and periostin are known pro-fibrotic molecules activated by TGF- β . Interestingly, a significant association between CCN2 and TGF- β in gingival overgrowth arising from nifedipine, phenytoin, or cyclosporine A administration was not found (Uzel et al. 2001). This supports our previous findings that it is activation of TGF- β , as opposed to

an increase in overall TGF- β expression that may underlie the condition. Periostin, like CCN2, is a critical modulator of matrix production during tissue remodeling, particularly in fibrosis (Norris et al. 2007; Wen et al. 2010a; Zhou et al. 2010; Naik et al. 2012). Future studies should focus on the interplay between CCN2 and periostin on the expression of collagen and fibronectin in DIGE. Studies examining the regulation of CCN2 in gingival fibroblasts have shown that

Fig. 8 Nifedipine and phenytoin do not induce myofibroblast differentiation or fibroblast proliferation *ex vivo*. **a** and **b**. Human gingival connective tissues cultured with nifedipine (100 ng/ml), phenytoin (30 μ g/ml), or without the drug for 2 weeks were stained for α -SMA (a marker for myofibroblast) and PCNA. To perform immunohistochemistry, sections were incubated with primary antibodies against α -SMA and PCNA and detected using peroxidase-conjugate secondary antibody and DAB. All samples from both groups lacked α -SMA positive cells in the connective tissue, except in the smooth muscle of the blood vessel walls, which is the internal positive control for α -SMA (arrows). The inset shows α -SMA-stained myofibroma tissue, used for the experimental positive control. **c**. Fluorescent immunoreactivity for FSP-1 in gingival tissues cultured with nifedipine (100 ng/ml), phenytoin (30 μ g/ml), or without the drug

for 2 weeks. Immunofluorescence of FSP-1 was detected with Cy5-conjugated secondary antibody (red). Nuclei are stained with Hoechst 3342 dye (blue). Yellow dotted line outlines the border between the oral epithelium and the connective tissue. Scale bar, 50 μ m. **d**. Average percentages of proliferating cells per field of view were quantified. Data represents mean percentage of PCNA-positive cells normalized to the total number of Hoechst 3342-stained cells $\pm$ STD of ten images per explant condition from three independent experiments. **e**. Average number of FSP-1⁺ cells and total number of cells in connective tissues of the explants were quantified per field of view. Data represents mean number of FSP-1⁺ cells and total number of cells $\pm$ STD of ten images per explant condition from three independent experiments. Data was analyzed using one-way ANOVA with Bonferroni's multiple comparisons test ($p > 0.05$; ns, not significant)

several pathways converge to upregulate the protein (for a comprehensive review, see (Trackman and Kantarci 2015), but whether periostin is increased through similar mechanisms is yet to be determined.

As gingival fibroblasts are one of the central cell types in the process of DIGE, we investigated whether nifedipine and phenytoin altered the fibroblastic content of the tissue. Using

vimentin and fibroblast specific protein-1 (FSP-1) as a marker, we demonstrated an qualitative increase in vimentin positive cells in DIGE samples, but only an increase in FSP-1⁺ cells in DIGE pathological samples, but not in healthy gingiva. FSP-1 expression in healthy gingival tissue is normally only faintly detectable at the mRNA level (Duarte et al. 1998), which our study confirms at the protein level. Of great interest, this

Fig. 9 Schematic representation of our hypothesis of the effects of nifedipine and phenytoin on DIGE. Nifedipine and phenytoin induce gingival enlargement by increasing matrix deposition

increase in FSP-1⁺ cells evident in pathological samples was not observed in our explant cultures in the presence of the drugs. While the exact specificity of this marker for fibroblasts has been questioned, particularly in relation to FSP-1 expression by inflammatory cells, it is expressed in fibroblasts in organs undergoing remodeling (Osterreicher et al. 2011), such that an increase in FSP-1⁺ cells in DIGE is expected. In remodeling, FSP-1 has been implicated in the control of migration, particularly in tumour metastasis (Boye and Maeldansmo 2010), but also in the regulation of metalloproteinases and tissue inhibitor of metalloproteinases (Elenjord et al. 2008). However, in DIGE pathological samples, the possibility of inflammatory cells expressing FSP-1 cannot be eliminated and should be further studied in the future.

We next investigated whether the increase in FSP-1⁺ cells in DIGE pathological samples could be attributed to the effects of nifedipine and phenytoin on cell proliferation. In our explant cultures, neither nifedipine nor phenytoin significantly increased proliferation, suggesting that the increase in cell number in DIGE is not directly due to drug administration. Previous studies showed that cell proliferation is elevated in both nifedipine and phenytoin induced gingival overgrowth (Kantarci et al. 2007). While elevated proliferation and decreased apoptosis have suggested to contribute to DIGE (Moy et al. 1985; Fujimori et al. 2001), others have reported that nifedipine and phenytoin do not influence proliferation (Kato et al. 2005; Pisoschi et al. 2014). Patients with gingival enlargement often have increased plaque accumulation, which causes a host inflammatory response (Pihlstrom et al. 2005) in the gingival tissues, resulting in the release of pro-inflammatory cytokines that are known to stimulate cell proliferation (Sugarman et al. 1985). However, Banthia et al. have suggested that although there is an association between poor oral health and the severity of DIGE, the cause and effect relationship has yet to be established (Banthia et al. 2014).

Indeed, a recent study has demonstrated that the presence of plaque and inflammation is not required for the development of gingival enlargement in patients (Sam and Sebastian 2014). Based on our data, we suggest that plaque may result in increased fibroblast proliferation, but the increase in matrix accumulation can be attributed to the presence of nifedipine and phenytoin. However, Kantarci et al., demonstrated that cell proliferation was independent of the level of inflammation (Kantarci et al. 2007). Future studies should investigate co-culturing of gingival explants with macrophages and/or inflammatory-mediating cytokines and assess how this influences proliferation.

In many types of fibrosis (Darby et al. 1990), the presence of myofibroblasts is a hallmark, and indeed it was suggested to be present in drug-induced gingival fibromatosis (Yamasaki et al. 1987; Dill and Iacopino 1997). However, we show here that myofibroblasts are absent in DIGE tissues, which we also confirmed in our ex vivo model; no myofibroblasts were evident in tissue explants cultured with nifedipine and phenytoin for 2 weeks (Figs. 3, 6). However, recent studies have shown an absence or very low levels of myofibroblasts in fibrotic gingiva, idiopathic gingival fibromatosis and in DIGE (Sakamoto et al. 2002; Martelli et al. 2010; Sobral et al. 2010; Pisoschi et al. 2014). TGF-β which mediates myofibroblast differentiation, requires adhesive signaling (Leask 2013) and it is known that the adhesion capacity of gingival fibroblasts to ECM is reduced in comparison with dermal fibroblasts (Guo et al. 2011), which would explain the absence of α-SMA expression. Interestingly, we have shown in skin that periostin modulates myofibroblast differentiation in a manner dependent on non-canonical TGF-β signaling through β1 integrin and focal adhesion kinase (Elliott et al. 2012). This suggests that requirement of periostin for myofibroblast differentiation is tissue-dependent, as its upregulation in DIGE and explant cultures is not associated

with α -SMA upregulation in gingival fibroblasts. As we have shown pSMAD2/3 activation in DIGE samples and explants cultured with nifedipine and phenytoin, the absence of α -SMA is likely due to a lack of non-canonical TGF- β signaling, although this does not inhibit matrix deposition by the cells.

In conclusion, this study demonstrates for the first time, an ex vivo gingival tissue model that allows investigation of the molecular pathogenesis of DIGE by nifedipine and phenytoin in a setting that closely simulates in vivo, without exposing the tissues to confounding variables such as plaque. Our ex vivo model is able to simulate ECM production that is characteristics of the fibrotic histopathology of DIGE associated with nifedipine and phenytoin, demonstrating that drug therapy is indeed a major causal factor resulting in gingival enlargement. We have shown that nifedipine and phenytoin treatments increase matrix deposition, but this is independent of cell proliferation. Frequent accumulation of plaque and associated inflammation that is either pre-existing or a consequence of gingival overgrowth could be augmenting gingival enlargement (Mishra et al. 2011), potentially by increasing cell proliferation and thus the number of matrix producing cells.

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Author contributions SSK performed the research, explant culture, immunohistochemistry, hydroxyproline assay, data analysis, prepared the figures, and wrote the manuscript. DWH designed the research study, as well as wrote and revised the manuscript.

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