Original Article

Effect of Phenytoin and Age on Gingival Fibroblast Enzymes

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Abstract

Objective: The alteration of cytokine balance is stated to exert greater influence on gingival overgrowth compared to the direct effect of the drug on the regulation of extracellular matrix metabolism. The current study evaluated the effect of phenytoin on the regulation of collagen, lysyl oxidase and elastin in gingival fibroblasts.

Materials and Methods: Normal human gingival fibroblasts (HGFs) were obtained from 4 healthy children and 4 adults. Samples were cultured with phenytoin. MTT test was used to evaluate the proliferation and ELISA was performed to determine the level of IL1β and PGE2 production by HGFs. Total RNA of gingival fibroblasts was extracted and RT-PCR was performed on samples. Mann-Whitney U test was used to analyze the data with an alpha error level less than 0.05.

Results: There was a significant difference in the expression of elastin between the controls and treated samples in both adult and pediatric groups and also in the lysyl oxidase expression of adult controls and treated adults. No significant difference was found between collagen expression in adults.

Conclusion: The significant difference in elastin and lysyl oxidase expression between adult and pediatric samples indicates the significant effect of age on their production.

Keywords: Phenytoin; Fibroblasts; Lysyl oxidase; Elastin; Collagen1; RT-PCR

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INTRODUCTION

Phenytoin is a common antiepileptic drug; which acts on voltage-gated sodium channels in neural cells [1]. It has been in the limelight of research for its effect on clinically detectable gingival overgrowth with a quoted prevalence figure of 50% [2]. Despite similar clinical manifestation of gingival overgrowth induced by antiepileptic (phenytoin) and calcium channel blockers (nifedipine), various degrees of inflammation exist between them [3].

The severity of overgrowth has been correlated with the drug concentration in the gingival crevicular fluid (GCF), degree of protein binding and bioavailability of the drug [4, 5]. However, not all patients on phenytoin develop gingival overgrowth. Existence of various types of fibroblasts in gingival and other tissues explains their heterogeneity due to factors such as protein synthesis, collagen production, glycosaminoglycan accumulation, replicative life span in culture, proliferation rate, cell size distribution and response to exogenously added substances [6, 7]. Resultantly, phenytoin may either stimulate or inhibit synthetic or proliferative activity in the aforementioned subpopulations [8-12]. As clearly stated in previous studies, extracellular collagen degradation is controlled by collagenase, a known matrix metalloproteinase (MMP), responsible for pathologic turnover of connective tissue [4, 13-15]. Its intracellular degradation, however, is controlled by fibroblast phagocytes known as normal turnover. Phenytoin's inhibitory effect on collagen phagocytosis by fibroblasts is shown to lead to the decrease of collagen degradation [16]. This indicates that overgrowth is not solely attributed to the increase of collagen synthesis but also to the decrease in its degradation. This collagen is resistant to the tissue MMP of inflammatory bacterial collagenase [17, 18]. Another study has demonstrated up regulation of collagen 1, 2 and glycosaminoglycans [19].

The alteration of cytokine balance is suggested to exert greater influence on gingival overgrowth compared to the direct effect of the drug on the regulation of extracellular matrix metabolism or proliferation of gingival overgrowth [20]. Decreased Laminin 5 and discontinued expression of collagen IV were also reported in the disrupted basal membrane of overgrown gingival tissue [21]. On the other hand, TGFB1 was shown to decrease E-Cadherin and increase the expression of MMP2, MMP9 and MMP 13 [22].

As for the role of lysyl oxidase in early passage of fibroblasts, a TGF-B stimulated CTGF was shown to contribute to biosynthesis of lysyl oxidase and collagen [23, 24]. Lysyl oxidase is a copper dependant enzyme; which plays an important role in connective tissues [25]. It finalizes the ECM production by cross-linking elastin-collagen [26].

Recently revealed functions of lysyl oxidase include regulation of cellular and gene transcription due to its ability of oxidizing substrates and adjustment of cell function, hypothetically by modulation of growth factors [27].

Formation and maturation of collagen and elastin are vastly dependent on post-translational modification/alteration. Alteration of lysyl oxidase leads to the changes in the ratio of synthesis of elastin and collagen [28]. Epidemiologic studies have revealed drug induced gingival overgrowth to be more dominant in male adolescents and children [29]. One study has introduced age as an important risk factor for drug induced gingival overgrowth [30].

Alteration of fibroblasts, decrease of collagen and non-collagenous protein, change in the fibroblast size, and their mitotic activity with age could be contributory [31-33].

There is continuous debate in the literature regarding the difference of mechanism of action and cellular effect of phenytoin between fibroblasts of children and adults. Also despite today's broad insight into the molecular basis of gingival overgrowth, the exact relationship between cytokine levels and extracellular matrix, elastin biosynthesis and mRNA levels remains yet unexplained.

There has only been an assumption of age's effect; thus, further studies are indeed needed. In this study we aimed to assess the effect of phenytoin on the regulation of connective tissue proteins such as lysyl oxidase, elastin and collagen1 genes in fibroblasts of adults and children using RT-PCR.

MATERIALS AND METHODS

Cell Culture:

Pediatric samples were obtained from four 4-11 year-old healthy patients while performing impacted tooth extraction. Adult fibroblast samples were derived from four 34-42 year-old healthy adults who underwent crown lengthening surgery. Following informed consent of donors, samples were removed from excess tissue during the surgery under local anesthesia. The experimental protocol was approved by the ethics committee of our university. Cells were plated in Dulbecco's Modified Eagle's Medium (DMEM; Biochrom AG, Berlin, Germany) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), streptomycin (50 U/ml), and penicillin (50 U/ml). Samples were then cultured in 25cm2 plates, and incubated at 37°C containing 5% CO2. When necessary, samples were fed with fresh medium at their regular control intervals for contamination and cell growth. Fibroblasts were trypsinized when out grown from the explants. Then, they were transferred to succeeding flasks (Nunc, Copenhagen, Denmark) for secondary culture. The fourth passages of the cells were used for the experiments.

Enzyme-Linked Immunosorbent Assay (ELISA):

24 well plates (Nunc, Copenhagen, Denmark) were divided equally into control and experimental groups. Gingival fibroblasts were seeded into wells at a density of 60x – 103cells/well. After 48 hours, phenytoin (Sigma-Aldrich, St. Louis, MO, USA) (20 µg/ml) was added to the experimental wells. Samples were then incubated at 37°C in 95% humidified atmosphere containing CO2. Due to natural constraints in initial cell culture, several pediatric samples were ruined leading to final total number of 22 adult and 19 pediatric samples. Supernatant fluid of each control and experimental well was selected to be used for assessment of inflammatory mediators secreted by fibroblasts. The concentration of PGE2 and IL-1β was evaluated by ELISA (R&D systems, Minneapolis, MN, USA) following the manufacturer's instructions.

MTT Assay: The MTT assay was performed according to the manufacturer's instructions. Gingival fibroblasts were seeded into 96-well plates (Nunc, Copenhagen, Denmark) at a density of 5×1000 cells/well and were cultured in a 200cc medium. Samples were divided into control and experimental groups following 48 hours of incubation. Phenytoin was then added to the experimental wells. After 48 hours of incubation, the medium was substituted with 100 cc of a fresh medium containing a 0.5 mg/ml solution of tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA). Incubation was carried out at 37°C in 95% humidified atmosphere containing CO2 for 4 hours. Dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) was used to dissolve the crystals of formazan produced by the living cells. The quantity of formazan was assessed at a wavelength of 570 nm with ELISA plate reader (Stat Fax, Florida, USA) according to the manufacturer's instructions.

RT-PCR: Total RNA of gingival fibroblasts was extracted with R Neasy Minikit (Qiagen, USA). RT reaction was performed in a mixture containing 1mM MgCl2(Fermentas, Lithuania), 0.2mM dNTP, 1X RT buffer , 0.5µg oligo dT primer , 200U MMuLV reverse transcriptase and DEPC water up to 20 µl final volume reaction. The primers' sequences and RT-PCR program are respectively listed in Tables 1 and 2. PCR reactions were carried out in a final volume of 30 µl consisting of 5µl cDNA, 0.2mM of dNTPs mix, 1pmol of each primer (lysyl oxidase, elastin and collagen) and 1.25U Taq DNA polymerase (Fermentas, Lithuania).

Table 1. The Oligonucleotide primers for RT-PCR

Primer	Sequence	Product Base Pairs
COLLAGENLAGEN1	F 5'actttcggac ggccgagctg cag3' R 5'aacctctagttcctggttgaccag3'	300bp
Lysyl oxidase	F 5'atccaatgggagaacaacgggcagg 3' R 5'cgcccaccatgccgtcctcgcggctg 3'	
Elastin	5' TGGAGCCCTGGGATATCAAG GAAGCACCAACATGTAGCAC 3'	170bp

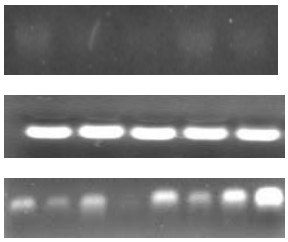
ELN F 5'TTCCTGGTGGAGTTCCTGGAGG3'
ELN R 5'TTCCCAGGCTTCACTCCGGCTC3'

Table 2. PCR thermal program

Genes	Program			Repeats
COLLAGENLAGEN1	Denaturation	94°C	30s	35
	Annealing			
	Elongation	72°C	1min	
Lysyl oxidase	Denaturation	94°C	30s	35
	Annealing			
	Elongation	72°C	1min	
Elastin	Annealing temp 60			
	PCR program is like the others			
	PCR product 170bp			

Gene expression

PCR products quantification which showing mRNA levels



Collagen 1

Elastin

Lysyl oxidase

Results were analyzed via 2% agarose gel electrophoresis and SYBR® Green staining. The quantification of PCR bands was performed with Work-Lab software.

Protein regulation in treatment groups was performed using Mann Whitney U test. The mean average of the elastin, lysyl oxidase and collagen expression in control groups of both pediatric and adult samples is shown in Table 3. Kruskal-Wallis test was used to determine the difference between expression of elastin, lysyl oxidase and collagen in the control and treated samples in both pediatric and adult groups. For the study of protein regulation in treatment groups statistical analysis was performed using Mann Whitney U test which was applied to determine the difference between expression of elastin, lysyl oxidase and collagen in the control and treated samples in both pediatric and adult groups. P values were set less than 0.05 according to the Table 4. Data were entered and statistically analyzed using SPSS 16 for Windows.

RESULTS

The mean average of the elastin, lysyl oxidase and collagen protein expression in control groups of both pediatric and adult samples are shown in Table1.Significant differences were found in the expression of elastin between controls and treated samples in both adult and pediatric groups and also in the lysyl oxidase expression of controls and treated adults ($P<0.05$).

No significant difference was found between collagen expression in adult controls and treated adults. Similarly, there was no difference in lysyl oxidase and collagen gene expression in the control and treated groups of pediatric samples. Assessment of the effect of age on each of the treatment groups was performed by Mann Whitney U test.

Comparison of protein expression between adult/ pediatric samples of control group and adult/pediatric samples of treated group, for the effect of age, was assessed via the Mann Whitney U test (Table 2).

Table 3. Means of the Elastin, Lysyl oxidase and Collagen Protein expression in control groups of both pediatric and adult samples

Protein / Control Group	Elastin	Lysyl Oxidase	Collagen
Pediatric	219.3 ±28.6	43.6±22.6	84.90±61.9
Adult	161.0±74.8	60.0±7.1	90.63±34.8

Table 4. Difference between expression of Elastin, Lysyl oxidase and Collagen Proteins in the control and treated samples in both Pediatric and adult groups.

Protein / Sample Group	Elastin	Lysyl Oxidase	Collagen
Pediatric control and PHENYTOIN treated	P=0.042	P=0.253	P=0.253
Adult control and PHENYTOIN treated	P=0.042	P=0.002	P=0.470

The only significant difference was in the elastin and lysyl oxidase expression between adult and pediatric samples of control group and also between the adult and pediatric samples of phenytoin treated groups. This indicates that age had a significant effect on the expression of elastin and lysyl oxidase in both control and phenytoin treated samples.

DISCUSSION

The present study analyzed the effect of phenytoin on the regulation of connective tissue proteins such as lysyl oxidase, collagen and elastin. It also investigated the effect of age on the regulation of aforementioned proteins. In a previous study on the cytokine regulation of collagen, lysyl oxidase and elastin in gingival fibroblasts, no investigation was done on the effect of age [34].

In the control group of our study, collagen and lysyl oxidase were expressed more in adult fibroblasts compared to pediatric samples; whereas, elastin was expressed more in pediatric fibroblasts. The pattern was similar in the phenytoin treated group.

Phenytoin significantly down-regulated elastin expression of adult and pediatric samples. It had an up-regulatory effect on the lysyl oxidase and collagen expression of adult and pediatric fibroblasts, the latter being insignificant. It has already been shown that the drug induced gingival overgrowth is partially due to cytokines such as bFGF, TGF-B CTGF, IL-1B and IL-6 [35-40]. Receptors of bFGF and TGF-B1 were detected in the lamina propria of gingival overgrowth assuming their collagen elaborative effect on gingival overgrowth [3]. CTGF and bFGF were reported to have a regulatory effect on lysyl oxidase and collagen [39, 40]. In contrast to collagen, elastin and elastic fibers are estimated to have a small proportion of 5% in the gingival tissue [41, 42]. Study of gingival overgrowth showed an increased amount of ECM while the increase of collagen and elastic fibers was witnessed in some lesions [43-45]. The increase of lysyl oxidase has been reported in diseases such as oral submucous fibrosis and lung and liver fibrosis [46, 47]; this highlights the role of lysyl oxidase in the balance of ECM metabolism.

Table 5. The mean of Protein expression in pediatric and adult samples of control groups

PHENYTOIN Groups / Protein	Pediatric	Adult
Elastin	243.84±24.3	122.47±58.5
Lysyl oxidase	49.83±22.4	83.55±22.4
Collagen	104.14±77.0	107.60±21.8

Table 6. Comparison of protein expression between adult/ pediatric samples of control group and adult/ pediatric samples of treated group, indicating the effect of age, was assessed via Mann-Whitney test

Studied Groups / Protein	Control Groups of Pediatric and Adult Samples	Phenytoin Groups of Pediatric and Adult Samples
Elastin	P=0.114	P=0.000
Lysyl oxidase	P= 0.253	p=0.012
Collagen	P=0.253	P=0.114

Elastin and collagen are mainly formed and matured by lysyl oxidase in the post-translational modification. Their cross-linking and fixation into ECM are done by lysyl oxidase [48]. Therefore, increase of lysyl oxidase may alter the ratio of elastin to collagen synthesis [49]. TGF-B has an up-regulatory effect on the lysyl oxidase and collagen mRNAs. It also has been shown to induce CTGF; which in turn increases lysyl oxidase activity via increase of insoluble collagen [39]. It has been shown that regulation of elastin mRNA levels and elastin synthesis in connective tissue cells is implicated by mRNA stability. TGF-B can affect this mRNA stability as well as mRNA stability of some other connective tissue genes such as collagen1 [49]. The effect of TGF-B on elastin mRNA of skin fibroblasts leads to an increase of elastin mRNA concentration and elastin synthesis [49-53]. In our study, lysyl oxidase was increased in both treated groups; however, the increase of lysyl oxidase coincided with the increase of elastin in pediatric samples but decrease of elastin in adult samples. This outcome may be explained by fibroblast heterogeneity or the difference of connective tissues between adults and pediatrics. TGF-βs and BMPs have an up-regulatory effect on collagen [54-58]. A decrease of mRNA for type 1collagen after 1-2 day incubation with phenytoin was reported while no change was observed in type IV collagen mRNA [59].

Kato et al, who studied the effect of TNFa and phenytoin on collagen metabolism demonstrated that these factors lead to collagen accumulation by impairing collagen metabolism in gingival fibroblasts. Both these factors inhibit collagen endocytosis. On the other hand, phenytoin increases the effect of TNF-a. As previously stated, collagen synthesis is regulated by MMPs and its antagonist TIMPS. In their study, phenytoin decreased mRNA expression of MMp1 and 2 and increased TIMP-1 mRNA expression leading to the suppression of collagen degradation. They concluded that phenytoin enhances collagen accumulation in gingival fibroblasts exposed to low levels of TNF-a. Since this factor exists in inflammation, it explains why overgrowth is worsened in cases of chronic gingival inflammation [55-57]; this is in agreement with our study where collagen was insignificantly increased in the phenytoin treated samples of both adults and pediatrics. .Increase of TIMP and TGF-B and decrease of MMP1 were observed in both adult and pediatric samples. It should be pointed out that the changes in pediatric samples were significant; whereas, those in adults where insignificant [54]. In our study, there was a significant difference in the regulatory effect of phenytoin on elastin and lysyl oxidase between adult and pediatric treated fibroblasts. This indicates the effect of age on the regulation of these proteins. No significant difference was reported in the expression of collagen between phenytoin treated fibroblasts of adults and pediatrics indicating that age did not affect on the regulation of collagen. To explain this, one should look deeper into the effect of age on the homeostasis of connective tissue; all body tissues undergo alterations with age and gingival tissue is no exception. Previous studies documented decrease of collagen and non-collagenous proteins' metabolism with age as well as modification in the size of fibroblasts and mitotic activity of both fibroblasts and epithelial cells [59-63].

Gagliano et al. reported a decrease in the collagen gene expression and TIMP-1 with no change in MMP-1 and TGF ℵ1 mRNA levels in adult fibroblasts compared to young ones [64]. In drug treated samples, there were higher levels of collagen-1 in (CsA) treated young fibroblasts. Studies have reported down-regulation of MMP-1 protein levels in young fibroblasts treated with CsA [64, 65]. In their study, similar collagen content was reported in young and aging fibroblasts.

An unchanged level of MMP-1and TIMP-1 mRNA was reported.

They documented similar MMP-1 and MMP-2 protein levels assuming that gingival collagen content is not regulated at its degradation level. There was also no alteration in TGF-1 gene expression in aging fibroblasts. This assumes similar tone of this factor influencing collagen turnover. Likewise, in our study no significant change was observed in the MMP-1, 2, TIMP and TGF-B levels in adult and pediatric fibroblasts. As previously stated, TGF B does have an up-regulatory effect on collagen. However, the up-regulatory effect of the drug on collagen content in our study might be due to different drugs used in our study compared to Gagliano's report [64]. It is suggested that collagen regulation in young and aged gingiva could partly depend on maturation pathways and post-translational modifications such as collagen cross-linking. This study is in agreement with the result of our study which may implicate the pathways of collagen turnover might be similarly affected by drug treatment in young and aging fibroblasts. Thus, young and aged fibroblasts may respond similarly to drugs in terms of collagen regulation. This suggests that phenytoin treatment affects collagen turnover pathways to a similar extent in young and aging gingival fibroblasts.

As for lysyl oxidase, its enzyme activity is reported to be stimulated by addition of 1 to 50 ng/ml CTGF to gingival fibroblasts. TGF-beta 1 is also reported to increase lysyl oxidase activity and its mRNA levels [67]. This effect was shown to be dose- and time-dependent. But, no such effect was observed on elastin. This is in agreement with our study where increase of TGF B and consequently lysyl oxidase led to the increase of elastin in pediatric samples but decreased in adult samples. The highest stimulatory effect of TGF-beta1 on lysyl oxidase mRNA activity occurred after 48 hours of fibroblast treatment with 500 pM of TGF-beta1. A study on lysyl oxidase and its mRNA level, quantified by real-time reverse transcriptase-polymerase chain reaction, showed their decrease in adult skin fibroblasts when compared with fibroblasts from children [68]. The elastin mRNA level in contrast remained stable at all ages. The inconsistency of this finding with our result might be due to the difference between skin and gingival fibroblasts and also the heterogeneity within gingival fibroblasts. Observation of rat aorta showed high lysyl oxidase expression in early development with significant reduction in adulthood [69]. In our study lysyl oxidase and collagen content was more in adults. The outcome disagrees with the aforementioned data; the reason might be sample differences. While aging is defined by decreased elastin/collagen ratio and decreased expression of lysyl oxidase tropocollagen and collagen 1, this could vary in different cell types and also in different samples especially when exposed to drugs [69].

These are incompatible with our study where collagen and lysyl oxidase levels were shown to be less in pediatric fibroblasts compared to adult fibroblasts both in control and treated samples. This might be due to the type of studied fibroblasts. Apart from the differences between skin and gingival fibroblasts, the heterogeneity within gingival fibroblasts should also be taken into account. Phenytoin stimulated pediatric fibroblasts to produce more levels of IL1β while there was no change in the adult group. Also, the mild decrease of T cells and the decrease of TGF-B with age should be regarded when the difference of adult and pediatric connective tissue components is considered. It is obvious that the increased mass of gingival tissue consists of increased amount of substance; it is said that glycosaminoglycans also influence the rate and quality of fibril formation by its interaction with collagen.

This emphasizes the different reaction to drugs of the responder cell type compared to its morphologically identical gingival counterpart [70].

CONCLUSION

Pediatric fibroblasts of all 3 proteins increased by phenytoin. However, in adult samples, the

increase of collagen and lysyl oxidase was parallel with the decrease of elastin.

The protein expression between adult and pediatric samples indicated that age had a significant effect on the expression of elastin and lysyl oxidase in both control and phenytoin treated samples.

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