Short communication

Increased expression of collagen prolyl 4-hydroxylases in Chinese patients with hereditary gingival fibromatosis

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ABSTRACT

Objectives: Hereditary gingival fibromatosis (HGF) is characterized by excess accumulation of interstitial collagen. However, until now, there has been controversy about the mechanism of collagen accumulation in HGF gingivae. The present study aimed to clarify the pathogenic mechanisms potentially involved.

Design: Gingival fibroblasts from three Chinese HGF patients and three healthy subjects were cultured. Cell proliferation was assessed by MTT assay. The mRNA levels of type I collagen, MMP-1, MMP-3, TIMP-1, prolyl 4-hydroxylase (P4H)α(I), α(II), α(III) and P4Hβ were analyzed in gingival fibroblasts by RT-PCR. The protein production of type I collagen and P4H was examined respectively by ELISA and Western blot.

Results: In culture, HGF gingival fibroblasts showed similar growth characteristics to fibroblasts isolated from control gingivae. The mRNA and protein levels of type I collagen and P4Hα in HGF fibroblasts were higher than those in controls. There were no detected differences in mRNA expression levels of MMP-1, MMP-3, TIMP-1, P4Hα(II), α(III) and P4Hβ between HGF and control fibroblasts.

Conclusions: These data suggest that increased collagen post-translational modification by P4H may be one mechanism by which increased collagen accumulation occurs in some forms of HGF.

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1. Introduction

Hereditary gingival fibromatosis (HGF) is a rare benign disorder manifested by progressive fibrous gingival overgrowth. HGF is characterized by excess accumulation of extracellular matrix (ECM) molecules and it has been reported that HGF fibroblasts synthesized predominantly type I collagen.[1] However, until now, there has been controversy about the mechanism of collagen accumulation in HGF gingivae: gingival fibrosis is a result of increased collagen synthesis and/or decreased degradation, or alteration in fibroblast proliferation.[2–5] Currently, there is no report that has systematically investigated these possible mechanisms.

Collagen prolyl 4-hydroxylases (P4Hs) have an important role as rate-limiting enzymes in the synthesis of collagens as they catalyze the formation of 4-hydroxyproline that is essential for the folding of the newly synthesized collagen polypeptide chains into triple-helical molecules.[6] P4Hs are

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$\alpha_2\beta_2$ tetramers containing two catalytic sites localized in the α-subunits. α-Subunits occur as three isoforms $\alpha(I)$, $\alpha(II)$ and $\alpha(III)$, which possess very similar but not identical catalytic properties.[7] Increased levels of P4H activity have been reported in many diseases associated with fibrosis such as hepatic fibrosis and keloid.[8,9] Excess ECM accumulation in HGF is similar to those of the above diseases and might involve abnormal expression of P4Hs which have not been studied in this condition.

Matrix metalloproteinases (MMPs) are a family of Zn-containing proteases important for the degradation of ECM proteins. The balance between ECM synthesis and degradation by MMPs, as well as the level and activity of their regulators (tissue inhibitors of metalloproteinases, TIMPs), is crucial for ECM remodeling process and the development of fibrosis.[5,10] MMP-1 is one of the main collagenases present in gingival tissues. Several studies have shown decreased mRNA level of MMP-1 and normal mRNA level of TIMP-1 in cultured HGF gingival fibroblasts compared with those from controls.[5] MMP-3 is able to degrade some ECM such as proteoglycans and is also recognized as a potent activator of procollagenases,[11] and may play an important role in the pathologenesis of HGF.

The purpose of the present study was, therefore to analyze gingival fibroblasts from Chinese HGF patients by molecular methods to investigate the roles of P4Hs, MMP-1, MMP-3, and TIMP-1 in the pathogenesis of HGF.

2. Materials and methods

Gingival tissue samples with no clinical signs of periodontal inflammation were obtained from three unrelated HGF patients (Fig. 1) (two males and one female, aged 16–28 years) by gingivectomy and from three healthy individuals (two males and one female, aged 16–22 years) by routine surgical crown lengthening. The diagnosis of HGF was based on our previous criteria.[12] In brief, enlarged gingiva covering at least one third of clinical dental crowns of five or more teeth; affected members have not been exposed to any inducible drugs, including nifedipine, phenytoin, cyclosporine, and other calcium channel blockers without other systemic diseases. The whole study was approved by the Institutional Review Board of Hospital and School of Stomatology, Wuhan University, China. According to clinical and family histories, all three HGF patients belong to the non-syndromic form with autosomal dominant inheritance. The affected gene localized to chromosome 2p but the SOS1 gene was excluded as the causative gene.[12]

2.1. Cell culture

Gingival tissues were washed with PBS, minced, plated in culture flasks and incubated in Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2 in air. When fibroblasts grew out from the explants, they were trypsinized for secondary cultures. Fibroblasts, identified by morphology and being vimentin positive and keratin
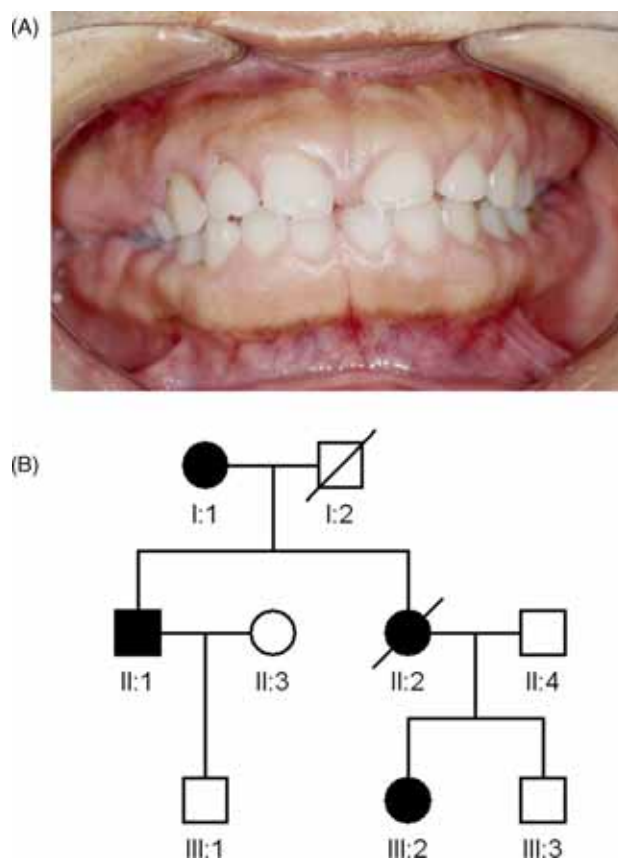
Fig. 1 – Clinical picture and a scheme of family lineage tree of a HGF patients. (A) Clinical picture of gingivae from a 24-year-old female HGF patients. (B) A scheme of family lineage tree of this patient. Blackened circles and squares represent affected members. The patient is III:2.

negative by immunohistochemistry, were used between passages 3–6. Cells from the same passage number were used in individual experiments comparing control and HGF derived fibroblasts in order to remove the differences due to cell passage.

2.2. Cell proliferation

Fibroblasts from HGF and CG were seeded in triplicate in 96-well plates (10^3/well), and after 24 h, cells were washed with PBS, and DMEM containing 10%FBS was added. The MTT assay was done after incubation for 1–5 days using a cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, 10 μl of cell counting kit solution was added to each well. The plates were then incubated at 37 °C for 3 h, and the absorbance at 450 nm was measured.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNAs were extracted according to the manufacturer's protocol (Total RNA isolation kit, Takara, Tokyo, Japan). RNA concentration and purity were determined by standard spectrophotometric method. Two micrograms of
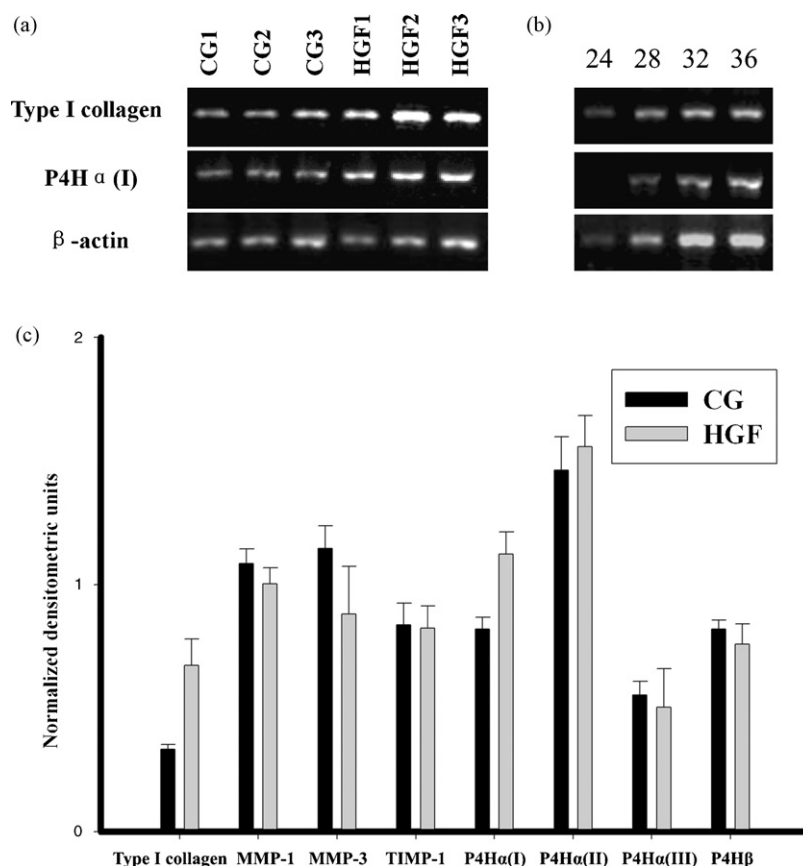
Fig. 2 – RT-PCR analysis of type I collagen, MMP-1, MMP-3, TIMP-1, P4Hα(I), α(II) and α(III) and P4Hβ mRNA in HGF and CG gingival fibroblasts. (a) Agarose gel showing the PCR products of the type I collagen, P4Hα(I) and β-actin genes of HGF and CG fibroblasts. (b) Agarose gel showing the PCR products in the 24th, 28th, 32nd, and 36th cycles of the type I collagen, P4Hα(I) and β-actin genes. (c) Representative experiment showing RT-PCR analysis of HGF and normal gingival fibroblasts. Values represent the means ± S.D. from three independent experiments with all cell lines.

total RNA extracted was reverse-transcribed by incubating the samples for 30 min at 42 °C. The cDNA was then used for the amplification of type I collagen, P4Hα(I), α(II) and α(III), P4Hβ, MMP-1, MMP-3 and TIMP-1 as described previously.[10,13,14] PCR amplifications were performed for 28 cycles for type I collagen, MMP-1, MMP-3, TIMP-1, P4Hβ and β-actin and for 30 cycles for P4Hα(I), α(II) and α(III), which were in the exponential range of amplification (Fig. 2b). After amplification, the PCR products were for electrophoresis on 1% agarose gel and the PCR amplification yields of target sequences were expressed in arbitrary units as the ratio the optical density of target genes/β-actin electrophoretic bands.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Fibroblasts were plated in 6-well culture plates at a density of 1.2×10^4/well in DMEM containing 10% FBS in triplicate. After 24 h, the cells were rinsed with PBS and the medium replaced with fresh DMEM containing 10% FBS and 1 mM ascorbic acid 2-phosphate. After 5 days, the cells were collected in 0.5 ml 0.05 M acetic acid (pH 3.0). The production of type I collagen was measured using human type I collagen detection kit (Chondrex, Redmond, Washington, USA), according to the manufacturer's instructions.
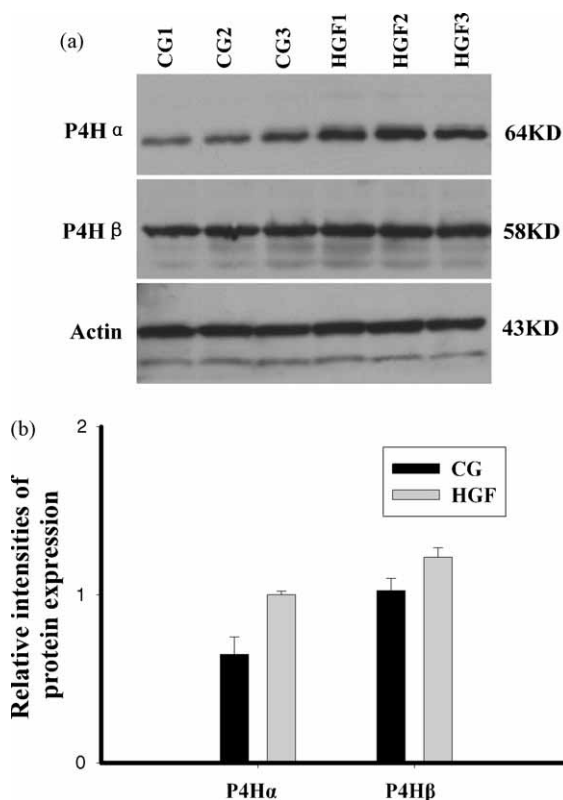
2.5. Western blot analysis

Fibroblasts were lysed by cell lysis buffer (Beyotime, Shanghai, China) supplemented with 1 mM PMSF. Protein extracts (30 μg/sample) were separated by 10% SDS poly-acrylamide gel electrophoresis and then electrophoretically transferred to polyvinylidene fluoride membranes. After blocking with 5% BSA, the membranes were incubated with mouse monoclonal antibodies against human P4Hα (Clone 9-47H10) and P4Hβ (Clone 3-2B12) (1:1000, MP Biomedicals, London, UK) and rabbit polyclonal antibodies against human actin (1:400, Santa Cruz Biotechnology, CA, USA) at 4 °C overnight. Secondary anti-mouse or anti-rabbit IgGs con-jugated to horseradish peroxidase (Pierce Biotechnology, Rockford, IL, USA) were incubated with the membranes for 1 h at room temperature at a 1:5000 dilution in PBS containing 0.1% Tween 20. The blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

2.6. Statistical analysis

All data were presented as mean ± S.D. One-way analysis of variance (ANOVA) was used for statistical analysis of
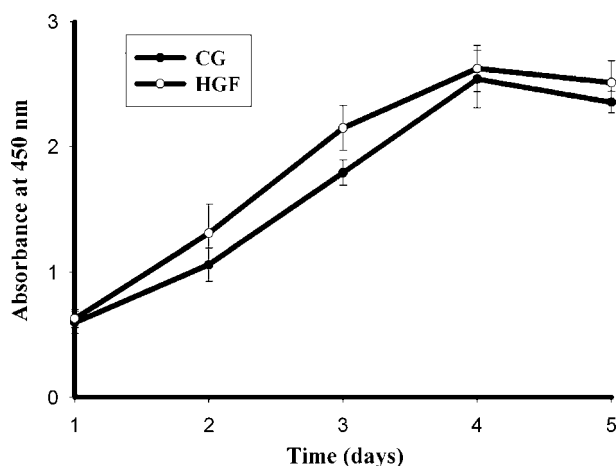
Fig. 3 – Cell proliferation curves of CG (n = 3) and HGF (n = 3) gingival fibroblasts at the indicated intervals of time.

Fig. 4 – Western blot analysis of P4Hα and P4Hβ protein in CG and HGF fibroblasts. (a) Western blot bands of P4Hα, P4Hβ and actin in CG and HGF fibroblasts. (b) Representative experiment showing Western blot analysis of HGF and normal gingival fibroblasts. Values represent the means ± S.D. from three independent experiments with all cell lines.

differences between groups of CG (n = 3) and HGF (n = 3) in the experiments of cell proliferation and ELISA. In our comparisons, $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Proliferation of cultured fibroblasts

Proliferation, as determined by the MTT assay, of fibroblasts from CG (n = 3) and HGF patients (n = 3) reached a peak on the 4th day (Fig. 3). There was no significantly difference in growth between fibroblasts from CG and HGF at any of the time points studied.

3.2. Type I collagen expression by HGF and CG fibroblasts

RT-PCR analysis demonstrated that the level of type I collagen mRNA, as a ratio to β-actin gene expression, was two-fold higher in HGF fibroblasts than in CG (Fig. 2). This difference was supported by the ELISA results which showed greater production of type I collagen by HGF fibroblasts (170.00 ± 26.91 ng/ml) compared with CG fibroblasts (74.67 ± 13.01 ng/ml) ($p < 0.01$).

3.3. P4Hα and P4Hβ expressions by HGF and CG fibroblasts

RT-PCR analysis indicated that P4Hα(I) mRNA levels in HGF fibroblasts were higher than those of CG fibroblasts (Fig. 2). However, there were no apparent differences between HGF and CG fibroblasts in P4Hα(II), P4Hα(III) and P4Hβ gene expression. The protein levels of P4Hα and P4Hβ from CG and HGF fibroblasts, as measured by Western blot and densitometry (Fig. 4), demonstrated that fibroblasts from HGF had higher levels (54.96%) of P4Hα than those from CG, with little difference in the protein level of P4Hβ.

3.4. mRNA expression of MMP-1, MMP-3 and TIMP-1 by HGF and CG fibroblasts

There were no detectable differences in the mRNA levels of MMP-1, MMP-3 and TIMP-1 between HGF and CG fibroblasts (Fig. 2).

4. Discussion

The present study suggests that excess ECM accumulation in HGF results from a local increase in collagen synthesis that is not associated in increased growth rate in vitro. It has been report that HGF fibroblasts have a higher growth rate than normal fibroblasts.[4] By contrast, others have suggested decreased proliferation rates[2,3] or that HGF fibroblasts grow normally[2]. Our data, based on the MTT assay, support the latter finding. These conflicting results might be partly due to genetic heterogeneity and differences in the particular type of gingival fibrosis, or variations among individuals.[4] Several researchers observed a decrease of proliferative activity with increased cell passages.[15] In order to limit the variation due to differences in culture conditions, we used fibroblasts in early passages 3–6 and in the same passages for each experiment between HGF and CG in the study. This might also be the reason that the individual variation is small within each group in the present study. It was reported that gingival fibroblasts

from passages 4–10 had no obvious alteration in proliferative activity.[16] HGF fibroblasts in the previous studies, including ours, were used below passage 10,[2–4] the differences in cell passages were therefore possibly excluded from the causes for the conflicting results.

Interstitial collagen accumulation is the main feature of HGF. Our quantitative analysis of fibroblasts showed higher collagen production in HGF than in CG, which is in agreement with the results of previous studies on gingival fibromatosis.[1,17] Collagen homeostasis is not only determined by the synthesis of procollagen chains themselves, but also by the expression and activity of modifying and degrading enzymes like P4Hs and MMPs.

P4Hs play a central role in the biosynthesis of collagens, as 4-hydroxyproline residues are essential for the formation of the collagen triple helix. The expression of α subunit of P4Hs limits the rate of active P4H formation and reduction of P4Hα mRNA results in the decreased P4H activity and collagen production.[18] It has been reported that the activity of prolyl hydroxylase was increased in one case of idiopathic gingival overgrowth and two cases of drug-induced gingival overgrowth.[19] But the enzyme was not identified to be from P3H, P4H or their isoforms. In our study, P4H isoforms: P4Hα(I), α(II) and α(III) mRNA expressions were all detected in HGF and CG fibroblasts. P4Hα(I) mRNA and protein expression levels were higher in HGF fibroblasts, compared with those from controls, while those of type II and III P4Hα were not. These findings suggest that P4Hα(I) might be involved in the pathogenesis of HGF and confirms that HGF fibroblasts showed increased regulation at the level of post-translational protein modification.

In this study, the mRNA levels of MMP-1 and MMP-3 in HGF fibroblasts were similar to those of control fibroblasts. Degradation of interstitial collagen might not therefore be the main mechanism responsible for fibrosis in these three Chinese patients. However, our results on MMPs are different from previous work, in which MMP-1 and MMP-2 levels were reported to be lower in HGF fibroblasts compared with normal subjects, while TIMP-1 and TIMP-2 were not different.[5] This may reflect differences in cell origin.

Physiological effects of increased collagen accumulation in HGF are likely to be the result of regulation roles of growth factors. Transforming growth factor-β (TGF-β) expression by gingival fibroblasts have been detected in HGF.[20] TGF-β1 up-regulates the expression of P4Hα(I), and increases type I collagen production and decrease MMP synthesis.[5,21] Thus, TGF-β1 might promote excessive collagen deposition by both decreasing proteolysis via reduced MMP activity and increasing synthesis via upregulation of P4H.

Our results suggest that the pathologic process of HGF is characterized by an increased collagen and P4Hα expression indicating that increased collagen synthesis rather than decreased degradation or increased cell proliferation, might be the main mechanism in collagen accumulation in the gingiva of the Chinese HGF families studied.

Acknowledgements

REFERENCES

1. Sakamoto R, Nitta T, Kamikawa Y, Kono S, Kamikawa Y, Sugihara K, et al. Histochemical, immunohistochemical, and ultrastructural studies of gingival fibromatosis: a case report. *Med Electron Microsc* 2002;**35**:248–54.
2. Johnson BD, el-Guindy M, Ammons WF, Narayanan AS, Page RC. A defect in fibroblasts from an unidentified syndrome with gingival hyperplasia as the predominant feature. *J Periodontal Res* 1986;**21**:403–13.
3. Shirasuna K, Okura M, Watatami I, Hayashido Y, Saka M, Matsuya T. Abnormal cellular property of fibroblasts from congenital gingival fibromatosis. *J Oral Pathol* 1988;**7**:381–5.
4. Coletta RD, Almeida OP, Graner E, Page RC, Bozzo L. Differential proliferation of fibroblasts cultured from hereditary gingival fibromatosis and normal gingiva. *J Periodontal Res* 1998;**33**:469–75.
5. Coletta RD, Almeida OP, Reynolds MA, Sauk JJ. Alteration in expression of MMP-1 and MMP-2 but not TIMP-1 and TIMP-2 in hereditary gingival fibromatosis is mediated by TGF-beta 1 autocrine stimulation. *J Periodontal Res* 1999;**34**:457–63.
6. Kivirikko KI, Myllyharju J. Prolyl 4-hydroxylases and their protein disulfide isomerase subunit. *Matrix Biol* 1998;**16**:357–68.
7. Kukkola L, Hieta R, Kivirikko KI, Myllyharju J. Identification and characterization of a third human, rat, and mouse collagen prolyl 4-hydroxylase isoenzyme. *J Biol Chem* 2003;**278**:47685–93.
8. Abergel RP, Pizzurro D, Meeker CA, Lask G, Matsuoka LY, Minor RR, et al. Biochemical composition of the connective tissue in keloids and analysis of collagen metabolism in keloid fibroblast cultures. *J Invest Dermatol* 1985;**84**:384–90.
9. Pereira TN, Lewindon PJ, Smith JL, Murphy TL, Lincoln DJ, Shepherd RW, et al. Serum markers of hepatic fibrogenesis in cystic fibrosis liver disease. *J Hepatol* 2004;**41**:576–83.
10. Kubota T, Nomura T, Takahashi T, Hara K. Expression of mRNA for matrix metalloproteinases and tissue inhibitors of metalloproteinases in periodontitis-affected human gingival tissue. *Arch Oral Biol* 1996;**41**:253–62.
11. Suzuki K, Enghild JJ, Morodomi T, Salvesen G, Nagase H. Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). *Biochemistry* 1990;**29**:10261–70.
12. Ye X, Shi L, Cheng Y, Peng Q, Huang S, Liu J, et al. A novel locus for autosomal dominant hereditary gingival fibromatosis, GINGF3, maps to chromosome 2p22. 3-p23. 3. *Clin Genet* 2005;**68**:239–44.
13. Qian H, Xiao Y, Bartold PM. Immunohistochemical localization and expression of fibromodulin in adult rat periodontium and inflamed human gingiva. *Oral Dis* 2004;**10**:233–9.
14. Fahling M, Perlewitz A, Doller A, Thiele BJ. Regulation of collagen prolyl 4-hydroxylase and matrix metalloproteinases in fibrosarcoma cells by hypoxia. *Comp Biochem Physiol C Toxicol Pharmacol* 2004;**139**:119–26.
15. Pendergrass W, Angello J, Norwood TH. The relationship between cell size, the activity of DNA polymerase alpha and proliferative activity in human diploid fibroblast-like cell cultures. *Exp Gerontol* 1989;**24**:383–93.
16. Kent LW, Dyken RA, Rahemtulla F, Allison AC, Michalek SM. Effect of in vitro passage of healthy human gingival fibroblasts on cellular morphology and cytokine expression. *Arch Oral Biol* 1996;**41**:263–70.
17. Bonnaure-Mallet M, Tricot-Doleux S, Godeau GJ. Changes in extracellular matrix macromolecules in human gingiva after treatment with drugs inducing gingival overgrowth. *Arch Oral Biol* 1995;**40**:393–400.

18. Han XY, Wang W, Myllyla R, Virtanen P, Karpakka J. Takala TE. mRNA levels for alpha-subunit of prolyl 4-hydroxylase and fibrillar collagens in immobilized rat skeletal muscle. *J Appl Physiol* 1999;**87**:90–6.

19. Huang JS, Ho KY, Chen CC, Wu YM, Wang CC, Ho YP, Liu CS. Collagen synthesis in idiopathic and dilantin-induced gingival fibromatosis. *Kaohsiung J Med Sci* 1997;**13**:141–8.

20. Wright HJ, Chapple IL, Matthews JB. TGF-beta isoforms and TGF-beta receptors in drug-induced and hereditary gingival overgrowth. *J Oral Pathol Med* 2001;**30**:281–9.

21. Koslowski R, Seidel D, Kuhlisch E, Knoch KP. Evidence for the involvement of TGF-beta and PDGF in the regulation of prolyl 4-hydroxylase and lysyloxidase in cultured rat lung fibroblasts. *Exp Toxicol Pathol* 2003;**55**:257–64.