Short communication

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

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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

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)a(I), a(II), a(III) and P4Hb 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 P4Ha(I) 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, P4Ha(II), a(III) and P4Hb 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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αβ₂ tetramers containing two catalytic sites localized in the α-subunits. α-Subunits occur as three isoforms α(I), α(II) and α(III), which possess very similar but not identical catalytic properties. Increased levels of P4H activity have been reported in many diseases associated with fibrosis such as hepatic fibrosis and keloid. 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. 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. MMP-3 is able to degrade some ECM such as proteoglycans and is also recognized as a potent activator of procollagenases, and may play an important role in the pathogenesis 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. 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.

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% CO₂ 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 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 (10⁴/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 (CKK-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
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) and P4Hβ mRNA in HGF and CG gingival fibroblasts. PCR amplifications were performed for 28 cycles for type I collagen, MMP-1, MMP-3, TIMP-1, P4Hα(I), α(II) 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 x 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% polyacrylamide 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 conjugated 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
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\(^5\). 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.\textsuperscript{16} HGF fibroblasts in the previous studies, including ours, were used below passage 10,\textsuperscript{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.\textsuperscript{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 P4H α mRNA results in the decreased P4H activity and collagen production.\textsuperscript{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.\textsuperscript{19} But the enzyme was not identified to be from P3H, P4H or their isoforms. In our study, P4H isoforms: P4Hα(1), α(II) and α(III) mRNA expressions were all detected in HGF and CG fibroblasts. P4Hα(1) 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(1) 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.\textsuperscript{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.\textsuperscript{20} TGF-β1 up-regulates the expression of P4Hα(1), and increases type I collagen production and decrease MMP synthesis.\textsuperscript{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**

This study was supported by grants (No. 30670115 and 30500562) from the National Natural Science Foundation of China, China.

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