Increased expression of integrin α2 and abnormal response to TGF-β1 in hereditary gingival fibromatosis

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OBJECTIVE: To investigate the possible correlation between integrin α1, α2, and β1 expression and excessive collagen synthesis in fibroblasts from 3 unrelated Chinese families with hereditary gingival fibromatosis (HGF).

DESIGN: Gingival fibroblasts from three Chinese HGF patients and three healthy subjects were included. The expression of α1, α2, and β1 integrin subunits was examined by immunohistochemistry, quantitative PCR, and flow cytometry. We also investigated the effects of transforming growth factor-β1 (TGF-β1) on the expression of these integrin subunits.

RESULTS: Our results demonstrate that the expression of α2 was significantly higher in HGF fibroblasts compared with control fibroblasts (P < 0.01). No significant differences in the expression of α1 and β1 were detected. Furthermore, TGF-β1 promoted the expression of α1 and α2 in fibroblasts from both HGF patients and controls. However, it had a larger effect on the expression of α2 in HGF fibroblasts than in control cells. In contrast, α1 expression was stimulated more in control fibroblasts.

CONCLUSION: The increased expression of integrin α2 and the increased response to TGF-β1 of HGF fibroblasts may be related to the excessive collagen deposition in HGF patients.


Keywords: hereditary gingival fibromatosis; integrin expression; TGF-β1

Introduction

Hereditary gingival fibromatosis (HGF) is a rare oral condition manifested by a slowly progressive, benign, localized or generalized enlargement of the gingiva (Häkkinen and Csiszar, 2007). Clinically, HGF results in both esthetic and functional problems such as delayed tooth eruption and malocclusion. The incidence and severity of this disease appear to depend on the penetrance of the mutated genes (Hart et al, 1998; Xiao et al, 2001; Ye et al, 2005). The excessive accumulation of extracellular matrix (ECM) is the most prominent pathologic feature of this disease. The increase in collagen is reported for both tissues and fibroblast cultures from the gingiva of HGF patients (Tipton et al, 1997; Sakamoto et al, 2002; Meng et al, 2007, 2008; Kather et al, 2008). However, it is not well established whether the increased collagen content results from an increase in production by fibroblasts or a decreased degradation.

Integrins, major cell surface receptors, mediate cell adhesion, control cell proliferation, and regulate gene expression (Hynes, 1992; Miyamoto et al, 1998). In higher vertebrates, the integrin family is composed of 18α subunits and 8β subunits, which combine to 24 different heterodimeric integrins (Hynes, 1986). Fibroblasts interact with their surrounding matrix mainly through the β1 family of integrins. Of these integrins, α1β1, α2β1, α3β1, α10β1, and α11β1 act as receptors for native collagens. In many cell lines, α3β1 integrin is not a receptor for collagen, whereas α10β1 and α11β1 integrins are mainly present in embryonic tissues (Popova et al, 2007). It has been established that α1β1 and α2β1 regulate collagen and MMP-1 gene expression. Integrin α1β1 reduces collagen expression upon ligand binding, whereas integrin α2β1 stimulates collagen and collagenase gene expression in both two-dimensional and three-dimensional cell cultures. However, the response was much stronger in three-dimensional collagen gels than in monolayer cultures (Ivarsson et al, 1993; Langholz et al, 1995; Riikonen et al, 1995).
Changes in the expression of these two integrins are associated with fibrotic diseases such as scleroderma, keloids, and hypertrophic scars, but the results were partly contradictory (Kozlowska et al., 1996; Herzhoff et al., 1999; Szulgit et al., 2002). Excess ECM accumulation is a common feature of HGF and the above conditions. However, it has never been studied whether integrins are related to this process.

TGF-$\beta$'s are multifunctional growth factors implicated in both healing and fibrosis (Sportun and Roberts, 1990; Border and Ruoslahti, 1992). It has been proposed that increased expression of TGF-$\beta$1 plays a critical role in the pathogenesis of HGF by upregulating type I collagen expression and downregulating MMP expression (Tippon and Dabbous, 1998; Coletta et al., 1999; Martelli-Junior et al., 2003). $\alpha_1\beta_1$ and $\alpha_2\beta_1$, which are known to be upregulated in response to TGF-$\beta_1$, mediate the cell attachment to collagen and collagen gel remodeling (Kagami et al., 1999; Kondo et al., 2004). Therefore, we hypothesized that TGF-$\beta_1$ leads to excessive collagen deposition in HGF probably by upregulating integrins expression.

The purpose of our study was to investigate the expression of $\alpha_1$, $\alpha_2$, and $\beta_1$ integrin subunits in normal gingiva (NG) and HGF tissue samples and cell cultures. We also examined the additional effect of exogenous TGF-$\beta_1$ on the expression of these integrins by NG and HGF fibroblasts.

**Materials and methods**

**Tissue collection**
A total of six subjects were included in this study. Gingival tissue samples were obtained from three unrelated HGF patients without clinical signs of periodontal inflammation by gingivectomy, and from three healthy individuals by routine surgical crown lengthening (Table 1). The samples included the marginal and attached gingiva. The diagnosis of HGF was based on our previous criteria (Ye et al., 2005; Meng et al., 2007). According to clinical and family histories, all three HGF patients have the non-syndromic form with autosomal dominant inheritance. The study was approved by the Institutional Review Board of Hospital and School of Stomatology, Wuhan University, China. All patients were informed about the study before they consented to participate.

**Table 1** Clinical details of patients and controls

<table>
<thead>
<tr>
<th>Number/age/gender</th>
<th>Diagnosis</th>
<th>Degree of overgrowth</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/16/M</td>
<td>HGF</td>
<td>Severe</td>
<td>No</td>
</tr>
<tr>
<td>2/21/F</td>
<td>HGF</td>
<td>Severe</td>
<td>No</td>
</tr>
<tr>
<td>3/28/M</td>
<td>HGF</td>
<td>Severe</td>
<td>No</td>
</tr>
<tr>
<td>4/16/M</td>
<td>Gingiva health</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>5/20/F</td>
<td>Gingiva health</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6/25/M</td>
<td>Gingiva health</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

HGF, hereditary gingival fibromatosis.

**Immunohistochemistry**

Immunohistochemical studies were performed using the following antibodies: mouse monoclonal anti-human integrin $\alpha_1$ (1 mg ml$^{-1}$, dilution 1:300), $\alpha_2$ (0.2 mg ml$^{-1}$, dilution: 1:60), and $\beta_1$ (1 mg ml$^{-1}$, dilution 1:200) (CHEMICON international, Inc., Temecula, CA, USA). All immunostainings were performed on frozen sections (10 $\mu$m). Sections were fixed with cold acetone. To block endogenous peroxidase activity, sections were treated with 3% hydrogen peroxide in methanol for 30 min. Following treatment with normal goat serum for 15 min, sections were incubated with primary antibodies overnight at 4°C. Negative controls were without the primary antibodies. Subsequently, the standard streptavidin-biotin-peroxidase complex method was performed using a SP kit (MaiXin Ltd, Fu Zhou, China). The immunostaining was visualized by developing in diaminobenzidine, and counterstaining with Mayer’s hematoxylin. The sections were viewed by light microscopy (Olympus, Tokyo, Japan). In the end, the section was analyzed with the Qwin system (Leica Imaging System, Wetzlar, Germany). In the macro program for the Qwin system, the image was edited by marking the basal layer to analyze the connective tissues (CT) and gingival epithelium (GE) separately. Protein expression was determined as the percentage of positive area in the total relevant cell area.

**Cell culture**

Six strains of fibroblasts were cultured from tissue samples of three HGF patients and three controls according to standard methods (Meng et al., 2007). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 mg ml$^{-1}$ penicillin, and 100 mg ml$^{-1}$ kanamycin sulfate at 37°C in 5% CO$_2$ in air. Cells were trypsinized and passaged when they were about 80–90% confluent. Each experiment was performed with cultures at a passage number of 4–8, and cells from the similar passage number were used in individual experiments.

**TGF-$\beta_1$ treatment**

Cells from NG and HGF were plated in 6-well plates, cultured to 80–90% confluence and washed twice with PBS. The serum-free medium was then added. After 24 h, three concentrations (0.1, 1 and 10 mg ml$^{-1}$) of recombinant human TGF-$\beta_1$ (PeproTech EC Ltd, London, UK) were added. Control cells were incubated under identical conditions but in the absence of TGF-$\beta_1$. After 24 h, the cells were collected for analysis of mRNA expression.

**RNA isolation and real-time quantitative PCR**

Total cellular RNA was extracted from fibroblasts according to a standard protocol (Total RNA isolation kit; Takara, Tokyo, Japan). The concentration and purity of extracted total RNA was quantified by a standard spectrophotometric method. Without previous DNase treatment, two micrograms of total RNA was...
then reverse-transcribed by incubating the sample for 30 min at 42°C. cDNAs were amplified and quantified using the relative standard curve method on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). PCR amplification used Taq polymerase (HotStarTaq; Takara), 0.64 μM integrin primers and 0.4 μM Taqman probe (GeneCore, Shanghai, China). Gene specific Taqman probes were labeled at the 5'-ends with fluorescent FAM for each gene, and TAMRA (quencher) at the 3'-ends (Table 2). The standard samples were constructed and serially diluted as a standard curve. Human housekeeping gene β-actin was used as an internal control. Test samples, positive control samples (a series of dilutions of standard samples and a series of dilutions of β-actin standard), and negative control samples (without cDNA template) were included in each run. The PCR conditions were 95°C for 10 s (1 cycle), 95°C for 10 s, and 60°C for 25 s (40 cycles). Each sample was run in triplicate. A standard reaction curve was run. The PCR conditions were 95°C for 10 s, and 60°C for 25 s (40 cycles). Each sample was run in triplicate. A standard reaction curve was run. The PCR conditions were 95°C for 10 s, and 60°C for 25 s (40 cycles). Each sample was run in triplicate. A standard reaction curve was run. The PCR conditions were 95°C for 10 s, and 60°C for 25 s (40 cycles). Each sample was run in triplicate. A standard reaction curve was run.

Flow cytometry

After trypsinization and washing, the cell suspension was adjusted to a concentration of 1–5 × 10⁶ cells ml⁻¹. Cells were blocked in PBS containing 10% bovine serum albumin (BSA) for 1 h on ice. Antibodies used were as follows: primary antibodies used in immunohistochemistry with a dilution of 1:100, 1:20, 1:100 separately, FITC-labeled affinity purified primary antibody to mouse immunoglobulin (IgG) (KPL, Gaithersburg, MD, USA). Staining for integrins was carried out in 10% BSA in PBS on ice for 1 h. Negative controls were incubated without primary antibodies. The cells were then washed three times in PBS and incubated with FITC-labeled affinity purified antibody to mouse IgG. Following 1 h on ice in the dark, the cells were washed again with PBS and finally resuspended in 400 μl BSA/PBS for analysis on a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA, USA). Ten thousand events were measured. Quantitative flow cytometric analysis was performed with the aid of CellQuest software (Becton Dickinson), measuring the percentage of positive cells and the mean fluorescence intensity.

Statistical analysis

All data were presented as mean ± s.d. Student’s t-tests were used for statistical analysis, and P < 0.05 was considered to indicate statistical significance. When multiple testing was performed, the Bonferroni correction was applied.

Results

Integrin expression in tissue samples

Immunohistochemistry was performed to determine the presence and expression of integrin α1, α2, and β1 in the gingival tissues from NG and HGF. These three subunits were all expressed in fibroblasts, and no significant differences were found in the percentage of positive staining area for each integrin between NG and HGF (Figure 1). In both NG and HGF tissue samples, α1 subunit was only expressed in CT, and mainly expressed in the blood vessel walls, with faint staining in some fibroblasts (Figure 1a,b). The percentage of α1-positive staining area for HGF fibroblast-like cells was 2.97 ± 0.21%, compared to 3.50 ± 0.45% in NG cells. In contrast, α2 was expressed abundantly in the GE, with the strongest expression in the basal cell layer (Figure 1c,d). In GE, the percentage of the staining area in HGF (31.93 ± 6.23%) was higher than NG (26.77 ± 5.61%), but there was no significant difference. In the CT, integrin α2 antibody reacted with fibroblasts and vascular endothelial cells (Figure 1e,f). The percentage of the labeling area for fibroblast-like cells was 7.23 ± 1.43% for HGF and 6.11 ± 0.48% for NG. As shown in Figure 1g,j, integrin β1 had the similar expression pattern as α2 in both GE and CT. The percentage of staining area in either GE (g, i) or CT (h, j) was similar between NG and HGF gingiva.

Integrin mRNA expression in cells

We used quantitative PCR to analyze the mRNA expression of α1, α2, and β1 integrin subunits in

### Table 2. Primers and probes used in quantitative PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5'–3'</th>
<th>Amplimer size, bp</th>
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</thead>
<tbody>
<tr>
<td>Integrinα1 (NM_181501)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>AAATATTGTTTTCAGTTTAACACATCTCTATCTTC</td>
<td>115</td>
</tr>
<tr>
<td>Reverse</td>
<td>ATGTCCACTACATATAGAAGGTTCTCA</td>
<td></td>
</tr>
<tr>
<td>Taqman probe</td>
<td>AGTGCACGACTGACAGCGAAAGAACCCTCC</td>
<td></td>
</tr>
<tr>
<td>Integrinα2 (NM_002203)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GGTGACCAGATTGGCTCCTATCT</td>
<td>79</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGACGACTCCTGTAATGTTGTCCT</td>
<td></td>
</tr>
<tr>
<td>Taqman probe</td>
<td>ACTCCATCAACTGAAACAGCACACTACCA</td>
<td></td>
</tr>
<tr>
<td>Integrinβ1 (NM_002211)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CCTGAAATGTCCCAAGTGTATGTA</td>
<td>129</td>
</tr>
<tr>
<td>Reverse</td>
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<tr>
<td>Taqman probe</td>
<td>ATGACACTGACGACAGCGC</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
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<td>Forward</td>
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</tr>
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<td>Reverse</td>
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<td>Taqman probe</td>
<td>ATCAAGATCAGACTGTCCTGAGA</td>
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fibroblasts from HGF patients and controls. The mRNA expression of α2 subunit in HGF fibroblasts was significantly higher than in controls after normalization for the β-actin housekeeping gene (P < 0.01) (Figure 2). No apparent differences in the expression of α1 and β1 integrin were found.

Integrin protein expression in cells
To confirm the PCR findings, flow cytometry was performed to investigate the protein expression of integrin α1, α2, and β1 subunits in fibroblasts from controls and HGF patients. All strains revealed the same fluorescence pattern with one uniform peak of integrin expression (Figure 3a). HGF fibroblasts demonstrated a similar number of positive cells, but a stronger fluorescence intensity for integrin α2 compared with control (P < 0.01) (Figure 3a,b). The percentage of positive cells and the relative fluorescence intensity of integrin α1 and β1 did not differ (Figure 3a,b). The integrin β1 levels were the highest (Figure 3a,b). This observation was expected, as β1 dimerizes with several α chains.

To determine whether FBS modulates integrin expression, fibroblasts were serum starved for 24 h and compared with fibroblasts maintained in DMEM with 10% FBS. The result showed that integrin subunits expression was not affected (data not shown).

Response to TGF-β1
We analyzed the effects of TGF-β1 on the expression of α1, α2, and β1 subunits in control and HGF fibroblasts by quantitative PCR. TGF-β1 significantly enhanced integrin α1 and α2 levels in both controls and HGF

Figure 1 Immunohistochemical analysis of integrin α1, α2, and β1 in normal and HGF gingival tissues. Values represent the mean ± s.d. Expression of α1 in a variety of cell types within connective tissues (CT) of NG (a) and HGF (b) gingiva. Panels (c) and (d) represent α2, which was expressed in gingival epithelium (GE) from NG (e) and HGF (d). α2 was also expressed in CT of NG (e) and HGF (f) gingival. Panels (g), (h), (i), and (j) represent the expression of β1 in GE and CT from NG (g, i) and HGF (h, j) separately. Bar, 200 μm

Figure 2 Quantitative PCR analysis of integrin α1, α2, and β1 mRNA in HGF and NG fibroblasts. Values represent the mean ± s.d. from three independent experiments with all cell lines. **Significant difference between NG (n = 3) and HGF (n = 3) (P < 0.01, Student’s t-test)
patients (Figure 4). Integrin α1 expression increased less in fibroblasts from HGF patients than in those from controls at each concentration (P < 0.01) (Figure 4a). In contrast, integrin α2 expression was stimulated more in HGF patients at each concentration (P < 0.01) (Figure 4b). A concentration of 10 ng ml⁻¹ gave the highest stimulation of α1 and α2 subunits expression. TGF-β1 had no effect on the expression of integrin β1 (data not shown).

Discussion

In our study, we examined the expression of the α1, α2, and β1 integrin subunits in gingival samples of three unrelated Chinese HGF patients and three controls. We also investigated the expression levels in fibroblasts cultured from these tissues at both mRNA and protein level. Our previous study has demonstrated increased mRNA and protein levels of type I collagen in these fibroblasts (Meng et al., 2007, 2008). The present results show that gingival fibroblasts express integrin α1, α2, and β1 in both tissue samples and cell cultures, although the expression of the α1 subunit was relatively low. An increased α2 expression was found in these HGF fibroblasts compared with NG cells. Furthermore, HGF fibroblasts show an increased response to exogenous TGF-β1 in the expression of α2.

Integrin α1/β1 has been shown to be a feedback inhibitor of collagen synthesis (Langholz et al., 1995). The α1 null mouse had an increase in steady state collagen content in the dermis (Gardner et al., 1999). This might be explained by the lack of α1 feedback
study shows that integrin α1 was expressed at relatively low levels, and no differences were observed in both tissues and fibroblasts between HGF and NG. Either α1 or α2 subunit contains functional domain: I domain, which plays a central role in ligand binding and intercellular adhesion (Takada et al., 2007). It has been demonstrated that cells expressing the α1 I domain preferentially adhere to collagen IV (Dickeson et al., 1999). As gingiva only contains minor amounts of collagen IV compare to collagen I (Buduneli et al., 2001), α1 expression might be low. In contrast, cells expressing the α2 I domain preferentially adhere to collagen I (Dickeson et al., 1999). Therefore, integrin α1 may only play a minor role in collagen I synthesis in HGF.

Immunohistochemical and flow cytometric data did not show a difference in the extent of α2-positive staining between NG and HGF. In flow cytometry, however, there was a significant difference staining intensity. Also, we found a significant difference in the mRNA expression of α2 by real time quantitative PCR. These findings show that the expression of α2 integrin subunit is significantly higher in HGF fibroblasts than in control cells both at mRNA and protein levels. It has been reported that integrin α2 stimulates collagen synthesis by competing with a “negative regulator”, possibly α1 integrin (Riikonen et al., 1995). Our data show that α2 subunit expression is increased in HGF fibroblasts. Moreover, the expression of integrin α2 is higher than that of α1. These findings suggest that the increased collagen I synthesis in HGF is due to an altered expression of α2 rather than α1. Interestingly, integrin α2 also plays a critical role in collagen phagocytosis (Lee et al., 1996). Some observations indicate that cyclosporine-A-induced gingival overgrowth is caused by an inhibition of collagen phagocytosis through reducing α2 expression in fibroblasts (Bolcato-Bellemin et al., 2003; Kataoka et al., 2003). Cyclosporine-A-induced gingival overgrowth samples showed lower expression of type I collagen than control gingiva (Kataoka et al., 2000). These findings indicate that collagen accumulation in this type of gingival overgrowth is related to impaired collagen degradation rather than collagen synthesis. In contrast, excessive collagen synthesis has been well-established in both tissues and fibroblast cultures from HGF patients (Tipton et al., 1997, 2008; Kather et al., 2008). Our present data suggest a different mechanism of collagen accumulation in HGF patients, which might involve α2. However, the effects of increased α2 expression on collagen phagocytosis in HGF are not known.

Interestingly, α2 and β1 subunits were mainly expressed in the basal layer of GE. Increased expression of certain integrins in the epithelium was shown to participate in controlling the formation of elongated rete ridges and tissue fibrosis in drug-induced gingival overgrowth (Walsh et al., 2007). Our present results show no significant difference in the percentage of α2- and β1-positive area between NG and HGF in GE. However, it is hard to deny the role of these genes in HGF without other accurate methods.

### Figure 4
Effect of TGF-β1 on the expression of integrin α1 and α2 by NG and HGF fibroblasts. Gingival fibroblasts were cultured with increasing concentrations of TGF-β1 (0.1, 1, 10 ng ml⁻¹), cultured for 24 h and the levels of integrin α1 (a) and α2 (b) expression determined by real-time PCR. Values represent the mean ± s.d. from three independent experiments with all cell lines and are expressed as the fold level of stimulation compared with the control (without treatment), which was set at onefold. **Significant difference between NG (n = 3) and HGF (n = 3) (P < 0.01, Student’s t-test with Bonferroni’s correction).**

inhibitor on collagen synthesis. The expression of α1 in fibrotic diseases, such as scleroderma and keloids has been investigated, but the results were in part contradictory (Ivarsson et al., 1993; Herzhoff et al., 1999; Szulgí et al., 2002), suggesting that integrin α1 might play a different role in different fibrotic diseases. Our
TGF-β1 is recognized as a key mediator for the accumulation of extracellular matrix in HGF. Exogenous or autocrine TGF-β1 can upregulate type I collagen and downregulate MMP expression in NG and HGF fibroblasts (Tipton and Dabbous, 1998; Coletta et al, 1999; Martelli-Junior et al, 2003). Interestingly, some studies also show that TGF-β1 enhances cell adhesion to collagen and collagen gel contraction via increased expression of α1 and α2 integrin subunits in rat mesangial cells and human renal fibroblasts (Kagami et al, 1999; Kondo et al, 2004). This suggests that TGF-β1 might affect collagen production through the stimulation of integrin expression. Our results show that TGF-β1 upregulates the expression of α1 and α2 subunits in both controls and HGF fibroblasts, but with a much stronger response by HGF fibroblasts with regard to the expression of α2 subunit. Based on previous findings that HGF tissues and fibroblasts constitutively express an elevated level of TGF-β1 (Wright et al, 2001; Martelli-Junior et al, 2003; Bitu et al, 2006), our findings support the notion that the increased expression of integrin α2 may be due to an elevated expression of TGF-β1 in HGF tissues and fibroblasts. In contrast, compared with NG cells, HGF fibroblasts showed a weaker response to TGF-β1 in the stimulation of α1 expression. No change expression of α1 in HGF may be caused by the relatively weak responsiveness of HGF fibroblasts to endogenous TGF-β1. Together with our findings, the available data indicate a correlation between excessive collagen synthesis and elevated expression of TGF-β1 and α2 in our HGF cells. To elucidate the exact mechanism, further studies on TGF-β1 expression, its effects on collagen metabolism and the role of integrins are required. As fibroblast behavior in three-dimensional cultures is closer to the in vivo situation than that in monolayer cultures, the former might be more suitable (Grinnell, 1994).

In conclusion, our results suggest that the increased integrin α2 expression in response to TGF-β1 is related to the excessive collagen accumulation in HGF patients. In addition, the main mechanism of collagen accumulation in HGF might be increased collagen synthesis, which is different from decreased collagen degradation in drug-induced gingival overgrowth.

Author contributions

Xiaoqian Ye, Liuyan Meng and Jie Zhou collected samples and cultured cells included in this manuscript. Jie Zhou designed this study with Zhaow Bian, Liuyan Meng and Xiaoqian Ye’s help. Jie Zhou performed the experiments, analyzed the data and wrote the text of this manuscript. Zhaow Bian, Johannes W. Von den Hoff and Liuyan Meng reviewed and revised this manuscript.

References


