Hepatocyte growth factor exhibits anti-fibrotic effects in an in vitro model of nifedipine-induced gingival overgrowth

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Abstract

Purpose: The aim of this study was to establish an in vitro model of nifedipine-induced gingival overgrowth and characterize the anti-fibrotic effect of hepatocyte growth factor (HGF) using this model.

Methods: Human gingival fibroblasts were cultured-treated with 0.1, 1, or 10 µg/mL nifedipine or 10 ng/mL IL-1β + 0.1, 1, or 10 µg/mL nifedipine (0.1N, 1N, 10N, IL + 0.1N, IL + 1N, IL + 10N). Cell proliferation and levels of type I collagen, TGF-β1, CCN2/CTGF, and α-SMA were measured 48 h after the simultaneous addition of 10 and 50 ng/mL HGF (10 and 50HGF) along with IL-1β and nifedipine. Type I collagen was measured after administration of anti-HGF neutralizing antibody.

Results: Significant increases in type I collagen, TGF-β1, and CCN2/CTGF were observed after treatment in the 1N and IL + 0.1N groups. Levels of type I collagen and CCN2/CTGF differed significantly between the IL + 0.1N group and the IL + 0.1N + 50HGF group. Production of type I collagen increased significantly following addition of anti-HGF antibody.

Conclusion: This study demonstrated the establishment of an in vitro model of nifedipine-induced gingival overgrowth by showing increased collagen levels. Experiments using this model suggested that HGF exerts anti-fibrotic effects.

Keywords: drug-induced gingival overgrowth, hepatocyte growth factor, nifedipine

Introduction

Gingival overgrowth, also known as gingival enlargement or gingival hyperplasia, is a general term used to describe inflammatory and non-inflammatory gingival growth. Focal gingival overgrowth is often caused by mouth breathing and inflammatory periodontal disease. Pervasive gingival hyperplasia encompasses both drug-induced gingival overgrowth and hereditary gingival fibromatosis, each of which is classified as a periodontal disease [1]. Genetic background plays a role in hereditary gingival fibromatosis, a disease characterized by fibrous proliferation of the gingiva that occurs primarily during childhood. There is no difference between the sexes in the incidence of hereditary gingival fibromatosis, which has a frequency of 1 in 175,000 individuals [2]. Clinically, drug-induced gingival overgrowth is the most common type of gingival proliferation.

Drug-induced gingival overgrowth is defined as gingival enlargement occurring as a side effect of certain medications, including the anti-seizure medication phenytoin, the anti-hypertensive drug nifedipine, and the immunosuppressant drug cyclosporine [3]. Periodontal pockets formed due to thickening of the gingiva promote the development and worsening of periodontitis, as well as dental irregularities and esthetic and occlusal disorders. In general, patients with poor plaque control are more likely to develop the disease [4].

It has been estimated that approximately 43 million people in Japan have hypertension [5], with approximately 9.94 million receiving antihypertensive treatment, according to the Patient Survey 2017 by the Ministry of Health, Labor and Welfare. Calcium channel blockers, which show good antihypertensive effects and safety, are often selected as first-line drugs to treat hypertension. Among anti-seizure medications, anti-hypertensive drugs, and immunosuppressant drugs, anti-seizure medications are associated with the highest incidence of drug-induced gingival overgrowth [6]. In terms of absolute number of patients, however, gingival overgrowth associated with antihypertensive drugs is the most common. As such, the number of patients with drug-induced gingival overgrowth continues to increase.

Treatment of gingival overgrowth involves asking physicians to change a patient’s medication or reduce the dosage, concurrent with initial periodontal therapy. If no improvement is observed following initial treatment, gingivectomy is performed. However, in many cases, it is not possible to change or reduce the dose of anti-hypertensive medications to treat proliferative disease due to potential difficulties in maintaining and managing the patient’s general underlying condition. In addition, post-gingivectomy recurrence is common, which highlights the need to develop new treatment methods that do not require alternative drugs or gingivectomy.

Histopathologically, drug-induced gingival overgrowth is characterized by parakeratosis, rete pegs, and increased collagen deposition in the connective tissue [7]. These characteristics have led to speculation that drug-induced gingival overgrowth induces increased production of extracellular matrix (ECM) components, primarily collagen, and causes metabolic disorders in the connective tissue. Collagen is produced by fibroblasts, and pathologic overgrowth of collagen-based ECM can occur in a variety of organs, such as the liver and kidney, which is known as fibrosis, and type I collagen plays a major role in this condition [8,9]. Collagen production by fibroblasts is promoted by transforming growth factor (TGF)-β1 and platelet-derived growth factor [10,11]. TGF-β1 is considered to play a central role in the fibrotic process. Increased TGF-β1 levels enhance the expression of type I collagen [12], which, as stated above, is associated with the pathogenesis of various fibrotic diseases, such as pulmonary and renal fibrosis. A number of other proteins that contribute significantly to fibrosis and are activated by TGF-β1 have been identified, including various matrix cellular proteins that modulate cell-matrix interactions. One such protein is cellular communication network factor 2/connective tissue growth factor (CCN2/CTGF) [13]. CCN2/CTGF is a member of the CCN family and highly expressed in fibrotic tissues [14,15]. CCN2/CTGF functions in concert with various growth factors, growth factor receptors, the ECM, and extracellular receptors [16]. Previous research suggests that nifedipine-induced gingival overgrowth is associated with metabolic disorders in collagen metabolism in the gingival tissues, increased collagen production by gingival fibroblasts, suppression of collagenase activity by collagen-degrading enzymes such as matrix metalloproteinase (MMP)-1, increased production of inflammatory cytokines such as interleukin (IL)-1 and IL-6, and increased expression of TGF-β1, basic fibroblast growth factor, and CCN2/CTGF [3]. However, the precise cause remains to be identified. Elucidation of the mechanism of gingival overgrowth could facilitate the development of therapies that target specific molecules involved in the disease and methods to identify patients likely to develop gingival overgrowth.

Recent studies of fibrotic diseases, such as systemic sclerosis and pulmonary fibrosis, have shown increased expression of α-smooth muscle actin (SMA) [17,18]. α-SMA is abundantly expressed in smooth muscle cells and thus considered a marker of fibrosis. In organs affected by...
inflammation, fibroblasts are activated, express α-SMA, and differentiate into myofibroblasts. Myofibroblasts exhibit characteristics intermediate between those of fibroblasts and smooth muscle cells [19]. Myofibroblasts appear in the granulation tissue of wounds, where they are thought to produce ECM components and contract the wound during the subsequent repair process [20]. Because overproduction of ECM components such as collagen by myofibroblasts leads to fibrosis, the present study postulated that a similar mechanism underlies the development of nifedipine-induced gingival overgrowth and, therefore, focused attention on α-SMA.

Hepatocyte growth factor (HGF) was first discovered in 1984 by Nakamura et al. and partially purified from rat blood using DNA synthesis in primary cultured rat hepatocytes as an indicator [21]. HGF is primarily produced by mesenchymal cells such as fibroblasts and vascular smooth muscle cells and known to exert a variety of physiological effects, such as promotion of cell proliferation, dispersion, motility, and morphogenesis [22,23]. In addition, HGF reportedly exerts anti-fibrotic effects and inhibits the expression of TGF-β1 [24]. It has been reported that HGF not only prevents fibrosis by inhibiting the deposition of ECM components such as collagen through the suppression of TGF-β1, but it also promotes the degradation of already formed ECM by inducing the expression of MMP-1 [25].

From the above, the present study hypothesized that HGF exerts anti-fibrotic effects in drug-induced gingival overgrowth. To verify this hypothesis, an in vitro nifedipine-induced gingival overgrowth model was constructed using human gingival fibroblasts, and the anti-fibrotic effect of HGF was investigated.

Materials and Methods

Cell culture
Human gingival fibroblasts were obtained from normal human gingiva during extraction of impacted wisdom teeth (n = 5, patients 21-48 years of age). This study was approved by the Institutional Ethical Review Committee of The Nippon Dental University (NDU-T2018-32, 2018). Informed consent was obtained from each patient prior to the study. The gingival specimens were cut into small pieces, and the tissue fragments were transferred to wells of a 6-well plate containing a few drops of Dulbecco’s modified Eagle medium (DMEM)/F-12 (Life Technologies, Carlsbad, CA, USA) supplemented with penicillin (500 units/mL)-streptomycin (500 µg/mL)-amphotericin (1.25 µg/mL) (Life Technologies) and 20% fetal bovine serum (FBS) (Biowest, Nuaillé, France). Tissues were incubated in the wells until dissociated cells adhered to the bottom of the plate. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. The medium was replaced every 3 or 4 days until sub-confluence was achieved, at which time 0.25% trypsin-EDTA (Life Technologies) was added to remove the cells, which were then transferred to a 100-mm dish. The medium was replaced every 3 or 4 days until sub-confluence was achieved, at which time 0.25% trypsin-EDTA (Life Technologies) was added to remove the cells, which were then transferred to a 100-mm dish. The cells were maintained in DMEM/F-12 supplemented with 10% FBS and used in experiments between the fourth and eighth passages.

Development of an in vitro model of nifedipine-induced gingival overgrowth

Cell proliferation
The effects of IL-1β (Fujifilm Wako Pure Chemical Corp., Osaka, Japan) and nifedipine (Fujifilm Wako Pure Chemical Corp.) on proliferation of human gingival fibroblasts were assessed using WST-8 tetrazolium (Cell Systems, Minneapolis, MN, USA), and CCN2/CTGF (Arigo Biolaboratories Corp., Hsinchu, Taiwan). The cell lysates were used to measure the level of α-SMA (Abcam, Cambridge, UK). A similar mechanism underlies the development of nifedipine-induced gingival overgrowth.

Statistical analysis
All data are presented as means ± standard deviation. The normality of the distribution of the measured data was confirmed by the Shapiro-Wilk test. In addition, homoscedasticity of the data was tested using Levene’s test. Subsequently, for cell proliferation data that showed a normal distribution, Dunnett’s test was used to compare between groups. For those that did not
show a normal distribution, the Kruskal-Wallis test and post hoc Dunn’s test were used to compare between groups. In addition, for ELISA data that showed a normal distribution, one-way analysis of variance and post hoc Tukey or Games-Howell tests were used to compare between groups. For those that did not show a normal distribution, the Kruskal-Wallis test and post hoc Dunn’s test were used to compare between groups. A $P$-value of $<0.05$ was considered indicative of significance. All statistical processing was performed using SPSS version 25 (IBM, Chicago, IL, USA).

**Results**

**Development of an in vitro model of nifedipine-induced gingival overgrowth**

Effects of IL-1β & nifedipine on the proliferation of human gingival fibroblasts

The MTT assay was used to examine the effects of IL and nifedipine on the proliferation of gingival fibroblasts. Compared with the control group, fibroblast proliferation was inhibited in the 10N and IL + 10N groups ($P < 0.05$). In contrast, no significant differences compared with the control were observed in the 0.1N, 1N, IL + 0.1N, and IL + 1N groups, and the IL + 0.1N and IL + 1N groups showed an increasing trend after day 4 (Fig. 1).

**Effects of IL and nifedipine on the levels of type I collagen, TGF-β1, CCN2/CTGF, and α-SMA in human gingival fibroblasts**

To examine the effects of IL and nifedipine on the levels of type I collagen, TGF-β1, CCN2/CTGF, and α-SMA, we were measured in culture supernatants and cell lysates using ELISAs. Based on the abovementioned cell proliferation results, the 0.1N, 1N, IL + 0.1N, and IL + 1N groups were examined. A significant increase in type I collagen expression was observed in the 0.1N, 1N, and IL + 0.1N groups 48 h after treatment compared with 0 h ($P < 0.05$). Similarly, significant increases in TGF-β1 expression were observed in the 1N, IL + 0.1N, and IL + 1N groups 48 h after treatment compared with 0 h ($P < 0.05$), and significant increases in CCN2/CTGF expression were observed in the 0.1N, 1N, and IL + 0.1N groups 48 h after treatment compared with 0 h ($P < 0.05$). In contrast, there were no significant differences in α-SMA expression at 48 h in the 0.1N, 1N, IL + 0.1N, and IL + 1N groups compared with 0 h (Fig. 2).

**Anti-fibrotic effects of HGF**

**Effect of HGF on the proliferation of gingival fibroblasts**

The effect of HGF on the proliferation of gingival fibroblasts was assessed using the MTT assay. Compared with the control group, no significant differences in gingival fibroblast proliferation were observed in the 5HGF, 10HGF, 50HGF, and 100HGF groups (Fig. 3).

**Effects of HGF on the levels of type I collagen, TGF-β1, CCN2/CTGF, and α-SMA in the in vitro model of nifedipine-induced gingival overgrowth**

To examine the effects of HGF on the levels of type I collagen, TGF-β1, CCN2/CTGF, and α-SMA, we were measured in culture supernatants and cell lysates using ELISAs. Based on the abovementioned cell proliferation results, the 0.1N, 1N, IL + 0.1N, and IL + 1N groups were examined. A significant increase in type I collagen expression was observed in the 0.1N, 1N, and IL + 0.1N groups 48 h after treatment compared with 0 h ($P < 0.05$). Similarly, significant increases in TGF-β1 expression were observed in the 1N, IL + 0.1N, and IL + 1N groups 48 h after treatment compared with 0 h ($P < 0.05$), and significant increases in CCN2/CTGF expression were observed in the 0.1N, 1N, and IL + 0.1N groups 48 h after treatment compared with 0 h ($P < 0.05$). In contrast, there were no significant differences in α-SMA expression at 48 h in the 0.1N, 1N, IL + 0.1N, and IL + 1N groups compared with 0 h (Fig. 2).
and CCN2/CTGF in the in vitro model of nifedipine-induced gingival overgrowth, protein levels were measured in culture supernatants using ELISAs. Based on the above results, it was decided to simultaneously treat the 1N or IL + 0.1N groups with HGF for 48 h, and the expression of type I collagen was measured. Parametric data was represented using bar charts, and non-parametric data was represented using box plots. (a) Type I collagen: a decrease in expression is observed in the IL + 0.1N + 10HGF and IL + 0.1N + 50HGF groups compared with the IL + 0.1N group, but no significant differences are observed in the IN + 10HGF and IN + 50HGF groups compared with the IN group. Further, an increase in expression of type I collagen is observed with the addition of anti-HGF antibody. (b) Transforming growth factor (TGF)-β1: no significant differences are observed in the IN + 10HGF and IN + 50HGF groups compared with the IN group, and no significant differences are observed in the IL + 0.1N + 10HGF and IL + 0.1N + 50HGF groups compared with the IL + 0.1N group. (c) Cellular communication network factor 2/connective tissue growth factor (CCN2/CTGF): no significant differences are observed in the IN + 10HGF and IN + 50HGF groups compared with the IN group. However, a significant difference is observed in the IN + 0.1N + 50HGF group compared with the IL + 0.1N group. A significant difference is also observed in the IL + 0.1N + 50HGF group compared with the IL + 0.1N group (*P < 0.05). 1N, 1 µg/mL nifedipine; 1N + 10HGF, 1N + 10 ng/mL HGF; 1N + 50HGF, 1N + 50 ng/mL HGF; IL + 0.1N, 10 ng/mL IL-1β + 0.1 µg/mL nifedipine; IL + 0.1N + 10HGF, IL + 0.1N + 10 ng/mL HGF; IL + 0.1N + 50HGF, IL + 0.1N + 50 ng/mL HGF

To determine the effect of an anti-HGF antibody in neutralizing HGF-induced collagen production in the in vitro model of nifedipine-induced gingival overgrowth, type I collagen levels were analyzed in culture supernatants using an ELISA. The IL + 0.1N + 50HGF + anti-HGF group showed increased production of type I collagen compared with the IL + 0.1N + 50HGF group (*P < 0.05) (Fig. 4).

Discussion

In 1939, Kimball reported the first case of phenytoin-induced gingival overgrowth [26]. In 1984, Ramon et al. reported an association between nifedipine and gingival overgrowth [27], thereby focusing attention on nifedipine-induced gingival overgrowth. To date, it has not been possible to define the mechanism because the overgrowth occurs specifically in the gingiva and not in the connective tissues of other organs, and there has been no commonality in terms of the types of drugs investigated, their
pharmacologic action, or their chemical formula. Although several studies have attempted to elucidate the mechanism of drug-induced gingival overgrowth, the mechanism remains unclear, despite the large number of patients taking this medication. Therefore, in this study, an in vitro model of nifedipine-induced overgrowth of human gingival fibroblasts was established and investigated.

Drug-induced gingival overgrowth is generally thought to be caused by inflammation due to plaque accumulation, in addition to drug-induced side effects. However, Banthia et al. reported that, although an association between poor oral hygiene and the severity of drug-induced gingival overgrowth has been documented, no cause-and-effect relationship has been established [28]. Other studies have reported that plaque and inflammation are not required for the development of gingival overgrowth [29]. To date, no studies comparing the effects of nifedipine alone or in combination with inflammatory cytokines have been reported. Therefore, the present study first conducted an in vitro evaluation of nifedipine alone and in combination with IL-1β to elucidate the role of inflammation in the response to nifedipine.

The effect of nifedipine on cell proliferation was examined at various doses and exposure times. There were no significant differences in fibroblast proliferation between the 0.1N, 1N, IL + 0.1N, and IL + 1N groups compared with the control group. The present results were consistent with those of Pisoschi et al., who reported that nifedipine does not affect fibroblast proliferation [30]. The 0.1N, 1N, IL + 0.1N, and IL + 1N conditions were selected for further analyses because they did not affect fibroblast proliferation, and proliferation of fibroblasts was inhibited in the high-dose nifedipine groups. Levels of type I collagen, the primary factor in fibrosis, increased in a time-dependent manner in the 0.1N, 1N, IL + 0.1N, and IL + 1N groups. Nifedipine-induced gingival overgrowth is characterized by the over-accumulation of ECM components, particularly collagen. Lu et al. reported that fibroblast cultures established from gingival tissues obtained from patients with nifedipine-induced gingival overgrowth exhibit increased expression of type I collagen compared with fibroblasts obtained from healthy gingiva [31]. These data indicate that a suitable in vitro model of nifedipine-induced gingival overgrowth could be established by treating human gingival fibroblasts with nifedipine alone or with IL-1β and nifedipine.

TGF-β1 promotes collagen production and plays an important role in fibrosis. In addition, TGF-β1 strongly induces the expression of CCN2/CTGF, which plays a key role in fibrotic diseases such as scleroderma, renal fibrosis, bleomycin-induced pulmonary fibrosis, and liver fibrosis [32-35]. Since it has been reported that nifedipine-induced gingival overgrowth is associated with increased expression of TGF-β1 and CCN2/CTGF [3], it is further suggested that the model established in this study represents the pathogenesis of nifedipine-induced gingival overgrowth. The 1N and IL + 0.1N groups in particular exhibited significant increases in expression of type I collagen, TGF-β1, and CCN2/CTGF 48 h after treatment, and thus, the anti-fibrotic effects of HGF were examined in the 1N and IL + 0.1N groups. Considered collectively, the results of the cell proliferation and protein level assays suggest that the increases in the levels of type I collagen, TGF-β1, and CCN2/CTGF are not related to an increase in the number of cells, but rather to increased secretion by the cells.

Recent studies of fibrotic diseases in other organs have focused on the expression of α-SMA, a marker of fibrosis. The presence of myofibroblasts, which is characteristic of many fibrotic diseases, has also been reported in cyclosporine A-induced gingival overgrowth [36]. To date, however, no study of nifedipine-induced gingival overgrowth before the present study has examined the expression of α-SMA in vitro. Therefore, the involvement of α-SMA was examined, and no significant difference in α-SMA expression between the control and test groups was found in the established model. Immunohistochemical staining for α-SMA in an in vivo model of nifedipine-induced gingival overgrowth was negative [37]. In addition, immunohistochemical staining targeting α-SMA in gingival tissue from a patient with nifedipine-induced gingival overgrowth was positive in the lamina propria, but there were a small number of α-SMA-positive cells [30]. These results are consistent with the present results, and available data suggest that myofibroblasts may not play a role in nifedipine-induced gingival overgrowth.

In analyses of the difference in response to nifedipine in the presence and absence of inflammation, 48 h treatment with 1N or IL + 0.1N resulted in significant differences in the production of type I collagen and expressions of TGF-β1 and CCN2/CTGF. These results suggest that gingival overgrowth is affected more by inflammatory cytokines, although the increase in ECM is presumed to have been due to the presence of nifedipine. To date, various studies have described the role of inflammation in nifedipine-induced gingival overgrowth. However, the critical role of inflammation in nifedipine-induced gingival overgrowth remains to be fully elucidated.

In the present study of the anti-fibrotic effects of HGF, the levels of type I collagen were significantly suppressed, and the levels of CCN2/CTGF were also modestly, but significantly, suppressed, but TGF-β1 expression was not affected. Taken together, these results indicate that HGF attenuated the production of type I collagen in the in vitro model of nifedipine-induced gingival overgrowth, probably not via a reduction in TGF-β1 expression, but instead via direct blockade of CCN2/CTGF induction. These results are similar to those of a study by Sherriff-Tadano et al. examining the anti-fibrotic effect of HGF in scleroderma [38]. It has also been reported that, although TGF-β1 initiates the fibrotic response in fibroblasts obtained from patients with scleroderma, CCN2/CTGF sustains that response [39]. This suggests that the pathogenic mechanism of nifedipine-induced gingival overgrowth is similar to that of scleroderma. In addition, the inhibition of type I collagen production by HGF observed in the present study could be the result of HGF-mediated inhibition of the maintenance of CCN2/CTGF fibrosis.

In addition, HGF exhibited anti-fibrotic effects only in the model involving IL-1β and nifedipine, suggesting that the anti-inflammatory effect is related to an HGF-mediated decrease in type I collagen production. There has been no study in which HGF and IL-1β were administered simultaneously to examine the anti-fibrotic effect of HGF, and this paper is the first report of such a study. In a mouse model of type II diabetes-induced chronic renal failure, Cheng et al. reported that administration of HGF suppressed IL-1 expression via the NF-κB pathway [40], which suggests that HGF suppresses nifedipine-induced collagen production by inhibiting IL-1β expression. Therefore, further studies are needed to elucidate the reason why HGF shows anti-fibrotic effects only in the presence of IL.

Since HGF was shown to have an anti-fibrotic effect, this was further verified using a neutralizing anti-HGF antibody. When anti-HGF neutralizing antibody was applied, no suppression of type I collagen was observed. This anti-fibrotic effect was inhibited by the addition of anti-HGF antibody, clearly demonstrating that HGF mediates the anti-fibrotic effect in the in vitro model of nifedipine-induced gingival overgrowth.

As indicated above, the in vitro model of nifedipine-induced gingival overgrowth was established in the present study by treating human gingival fibroblasts with IL and nifedipine. The increases in the levels of type I collagen, TGF-β1, and CCN2/CTGF in this in vitro model did not depend on cell proliferation or α-SMA. It was also demonstrated that in vitro treatment with HGF suppressed the production of type I collagen and CCN2/CTGF expression. For clinical applications in the future, it is necessary to elucidate the mechanism by which HGF inhibits collagen production.

In conclusion, an in vitro model of nifedipine-induced gingival overgrowth was established, and the anti-fibrotic effects of HGF were investigated using this model. The established model involves treatment of gingival tissues with nifedipine alone or IL and nifedipine simultaneously. As a result, this is the first report suggesting that HGF exerts an anti-fibrotic effect in nifedipine-induced gingival overgrowth.

Conflict of interest
There is no conflict of interest to declare.

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