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# Elevated expression of hepatocyte and keratinocyte growth factor in cultured buccal-mucosa-derived fibroblasts compared with normal-skin-derived fibroblasts

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**Abstract**

Oral mucosa heals faster with less scar formation than skin and a hypertrophic scar is very rare in the oral cavity, but its mechanism has not been elucidated enough. To elucidate whether or not there are differences in growth factor expression between fibroblasts derived from buccal mucosal and normal skin, we investigated the expression of hepatocyte growth factor (HGF), keratinocyte growth factor (KGF) and stem cell factor (SCF) by cultured fibroblasts. The semiquantitative RT-PCR revealed that the expression of HGF and KGF transcripts by buccal mucosal fibroblasts was significantly elevated compared with that by dermal fibroblasts. In parallel, ELISA revealed the significant increase of HGF production by buccal mucosal fibroblasts. The level of production of SCF protein did not differ significantly. Our study suggests that increased expression of HGF and KGF by buccal mucosal fibroblasts may partly be responsible for the faster wound healing with less scar formation in the oral cavity compared with normal skin.


*Keywords:* Wound healing; Oral cavity; Buccal mucosa; Hepatocyte growth factor; Keratinocyte growth factor; Fibroblast; Epithelial–mesenchymal interaction

## 1. Introduction

It is generally approved that oral mucosa heals faster with less scar formation than skin and hypertrophic scar is very rare in the oral cavity, but its mechanism has not been elucidated enough. Deitch [1] reported that the most important indicator of whether a burn wound would develop hypertrophic scars was the time required for the burn to heal. Delayed wound healing results in the frequent development of hypertrophic scars in the burn site. The comparison between oral mucosal and epidermal keratinocytes showed that oral

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mucosal keratinocytes possessed greater proliferating ability than epidermal keratinocytes in culture [2]. Faster wound healing by faster regeneration of epithelium is one of the possible causes of less scar formation in the oral cavity, but there should be many other factors for the amount of scar formation, including an imbalance of extracellular matrices (ECM) synthesis and degradation [3] and the effect of cytokines [4–10]. Cytokine production by epidermal keratinocytes has been investigated [4,5] and the differences in the pro-inflammatory cytokine expression of oral versus epidermal keratinocytes (with or without stimulation) have been reported [6,7]. Compared with epidermal keratinocytes, interleukin (IL)-6 was produced by oral mucosal keratinocytes constitutively without prior stimulation, which may indicate that mucosal keratinocytes actively prepare for the regeneration of oral mucosa.

On the other hand, communication between epithelium and mesenchyme is crucial for skin regeneration and closely related to wound healing. Interaction between keratinocytes and fibroblasts involves both direct contact and soluble mediators that function in either an autocrine and/or paracrine manner. Thus, the subepithelial fibroblasts play an important role in wound healing and scar formation. Fibroblasts from different anatomical sites show heterogeneity [11] and the differences in the cellular behavior of fibroblasts between oral mucosa and skin and in their response to growth factors have been reported [8,9] and there is a report on the difference in pro-inflammatory cytokine production by mucosal and dermal fibroblasts [10]. However, differences in the expressions of fibroblast-derived growth factors have not been reported. Differences in growth factor expression by fibroblasts might, in part, be responsible for the different course of wound healing. The representative growth factors that act on the skin or mucosa include hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), and stem cell factor (SCF) [12–17]. The aim of this study is to elucidate whether or not there are differences in the growth factor expression between human fibroblasts cultured from buccal mucosa and normal skin.

## 2. Materials and methods

### 2.1. Cell isolation and cell culture

Buccal mucosa biopsies were obtained from five healthy Japanese donors with no clinical indications of periodontal diseases (M1–5; one female and four males, age $22.0 \pm 10.9$). Normal skin was obtained during plastic surgery from six age-matched healthy donors (S1–6; three females and three males, age $23.8 \pm 13.1$). Informed consent was obtained from all patients. The profiles of specimens are shown in Table 1. The specimens were washed three times in phosphate buffered saline (PBS) and finely shredded with scissors, incubated with 0.25% trypsin and 0.02% EDTA in PBS for 16–24 h at 4 °C and the epithelium was separated from the subepithelial tissue. From separated dermis and buccal mucosal proper lamina (BMPL), human fibroblasts were cultured for explant and maintained at 37 °C under a 5% $CO_2$ atmosphere in fibroblast growth medium (FGM), which consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 0.6 mg/ml glutamine. About 3 weeks later, primary cultures were subcultured. Cells at third passage were used for the experiments.

Table 1
Profiles of specimens

| Sample No. | Age/sex | Donor site |
|---|---|---|
| Mucosa 1 | 20/M | Buccal mucosa |
| Mucosa 2 | 33/M | Buccal mucosa |
| Mucosa 3 | 5/M | Buccal mucosa |
| Mucosa 4 | 22/F | Buccal mucosa |
| Mucosa 5 | 30/M | Buccal mucosa |
| Skin 1 | 12/F | Face |
| Skin 2 | 21/M | Abdomen |
| Skin 3 | 48/M | Back |
| Skin 4 | 22/F | Face |
| Skin 5 | 27/F | Abdomen |
| Skin 6 | 13/M | Abdomen |

### 2.2. Measurement of cytokines by ELISA

#### 2.2.1. Cytokine secretion into fibroblast-conditioned medium

HGF, KGF and SCF secretion by the cultured fibroblasts were measured by ELISA. Human fibroblasts were seeded in a 60 mm culture dish (three dishes per specimen) at a density of $5 \times 10^5$ cells/5 ml and cultured in FGM. After human fibroblasts had been cultured for 96 h at 37 °C under a 5% $CO_2$ atmosphere, the medium was collected and used for measurement of HGF, KGF, SCF and by ELISA. At the point of harvest, the fibroblasts were near confluent.

The ELISA kit was purchased from Techne Corporation (Minneapolis). These ELISA kit is a solid-phase enzyme immunoassay using the multiple-antibody sandwich principle. Levels of immunoreactive cytokine were measured as absorbency at 450 nm (wave length correction 540 nm) by ELISA plate reader (Microplate Reader Model 550; Bio-Rad Laboratories). Cytokine levels in culture samples were determined by comparing their absorbance with that produced by standards. The standard curve was linear from 62.5 to 4000 pg/ml for HGF derivatives, from 31.2 to 2000 pg/ml for KGF derivatives and from 31.2 to 2000 pg/ml for SCF derivatives. Comparison of the cytokine concentration was carried out using Student's *t*-test.

### 2.3. Semiquantitative RT-PCR analysis

Semiquantitative RT-PCR analysis was performed to examine whether the expression of HGF and KGF transcripts by cultured fibroblasts was increased in buccal mucosa compared with that in skin. It was performed on all five specimens in mucosal fibroblasts and the three representative specimens in dermal fibroblasts. After fibroblast-conditioned medium was collected for ELISA analysis, fibroblasts were used for the RT-PCR analyses to quantify the expression of HGF and SCF transcript. Total cellular RNA was extracted from cultured fibroblasts using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) and was quantified by measuring the optical density at 260 nm. About 1 μg of total RNA was obtained from each tissue sample. Reverse transcription was performed in 20 μl reaction mixtures containing 2 μl 10 × PCR buffer, 4 μl $MgCl_2$ (25 mM), 1 μl oligo-(dT) adaptor primer(2.5 μM), 0.5 μl RNase inhibitor (40 U/μl), 2 μl deoxynucleotide mixture (10 mM), 1 μl avian myeloblastosis virus reverse transcriptase (5 U/μl) and 9.5 μl RNA (1 μg in DEPC-treated water). Reaction mixtures were incubated at 42 °C for 30 min and heated to 99 °C for 5 min to inactivate the reverse transcriptase. For PCR amplification, 0.5 μl of each cDNA reaction mixture was added to 49.5 μl PCR mixture containing 5 μl $MgCl_2$ (25 mM), 5 μl 10 × PCR buffer, 1 μl deoxynucleotide mixture (10 mM), 0.5 μl each of the 3′ and 5′ primers (50 μM each) and 0.25 μl Taq polymerase and 37.25 μl DEPC-treated water. Reaction mixtures were amplified using Microplate Gradient Thermal Cycler PC-960G (Corbett Research). The PCR cycle conditions were: melting for 30 s at 94 °C, annealing for 30 s at 61 °C (HGF), 59 °C (KGF) and extension for 1.5 min at 72 °C. Reaction products (2 μl) were resolved on 2% agarose gels and were visualized by ethidium bromide staining. The sequences of the 3′ and 5′ primer pairs used in this study are shown in Tables 1 and 2. As negative controls, we conducted PCR without reverse transcriptase and confirmed that there was no amplification of the transcripts. To quantitate the expression of the transcripts, the intensities of PCR bands were measured by densitometry using Image-Pro Plus (version 3.0, Media Cybernetics, Silver Spring, MA) and are expressed as relative intensities to glyceraldehydes-3-phosphate dehy-

Table 2
Nucleotide sequences for 5′ and 3′ primers

| |
|---|
| *HGF 396 bp* |
| 5′primer 5′- GTT ATC GTG GGA ATG GCA A -3′ |
| 3′primer 3′- G TAT AGA CGC CTC CTA GTA -5′ |
| *KGF 247 bp* |
| 5′primer 5′- AAG TAA AAG GGA CCC AAG AGA TGA AG -3′ |
| 3′primer 3′- GT TGC CTC CCC TTT ACA AAC AAC -5′ |
| *Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) 226 bp* |
| 5′primer 5′- GAA GGT GAA GGT CGG AGT C -3′ |
| 3′primer 3′- CT TTA GGG TAG TGG TAG AAG -5′ |

drogenase (GAPDH). Comparison of the intensity was carried out using Student's *t*-test.

## 3. Result

### 3.1. Cytokine secretion into fibroblast-conditioned medium

ELISA of HGF released into the fibroblast-conditioned medium revealed that the concentration of HGF was significantly increased in buccal mucosa (mean ± S.D. = 1421 ± 966 pg/ml $P < 0.05$) compared with normal skin (mean ± S.D. = 108 ± 47 pg/ml) (Fig. 1A). Although the average value of KGF was 1.5-fold increased in buccal mucosa, the difference was not significant (Fig. 1B). The level of SCF did not differ significantly (Fig. 1C).

### 3.2. Semiquantitative RT-PCR analyses

Semiquantitative RT-PCR of HGF and KGF mRNA revealed that the expression of HGF and KGF transcripts in buccal mucosa was significantly accentuated (average HGF; 6.8-fold, $P < 0.001$, KGF; 2.6-fold, $P < 0.05$) compared with that in the normal skin (Fig. 2A,B).

## 4. Discussion

The communication between epithelium and mesenchyme is crucial for skin regeneration and the subepithelial fibroblasts are closely related to the wound healing and scar formation. Our present study suggests that increased expression of HGF and KGF by buccal mucosal fibroblasts might, in part, be responsible for the faster wound healing in the oral cavity compared with the skin.

In our preliminary study, the cell numbers were counted in the same culture conditions, which confirmed the cell numbers at harvest was within 10% difference on average between normal skin and mucosal fibroblasts and the difference was not significant. It was reported that oral mucosal fibroblasts proliferated more than dermal fibroblast [9] and the doubling times of oral mucosal fibroblasts were smaller than those of dermal fibroblasts [19]. However, our preliminary study also revealed that the cell numbers at confluent were smaller in mucosal fibroblasts than those in dermal fibroblasts (data not shown). It indicates that mucosal fibroblasts stop proliferating as they grow to confluent. On the other hand, dermal fibroblasts continue to proliferate after confluent. This difference may be related to the rare incidence of hypertrophic scar in the oral cavity.

Among the fibroblast-derived soluble mediators, HGF and KGF are the representative cytokines that have a variety of actions on keratinocytes [12–16]. SCF is also produced by fibroblasts and acts on the skin, although its main targets are melanocytes and mast cells [17,18]. While the roles of HGF and KGF are a little different in epidermal physiology [19], both similarly stimulate proliferation and migration of human keratinocytes [12–15]. In addition, HGF induces vascular permeability factor expression by keratinocytes [20], whereas KGF stimulates plasminogen activator activity of normal human keratinocytes [15]. Through a variety of action on keratinocytes stated above, HGF and KGF stimulate keratinocytes and are greatly related to the regeneration of epithelium. Thus, elevated expression of HGF and KGF by mucosal fibroblasts might be partly responsible for accelerated wound healing in the oral cavity.

HGF is also known to prevent fibrosis in pulmonary, liver, renal and myocardial injured models [21–24]. The fibrosis, including hypertrophic scars, is caused by the disordered accumulation of ECM [3]. HGF stimulates the fibroblasts to produce matrix metalloprotease-1 (MMP-1) and inhibit the expression of transforming growth factor (TGF)-β [24]. TGF-β stimulates the production of various extracellular matrix proteins and inhibits MMP-1 activity responsible for degradation of these matrix proteins [25,26]. In many diseases, excessive TGF-β contributes to a pathologic excess of tissue fibrosis that compromises normal organ function [27–29]. Thus, HGF inhibit the accumulation of ECM and prevent fibrosis and it is probable that less scar formation

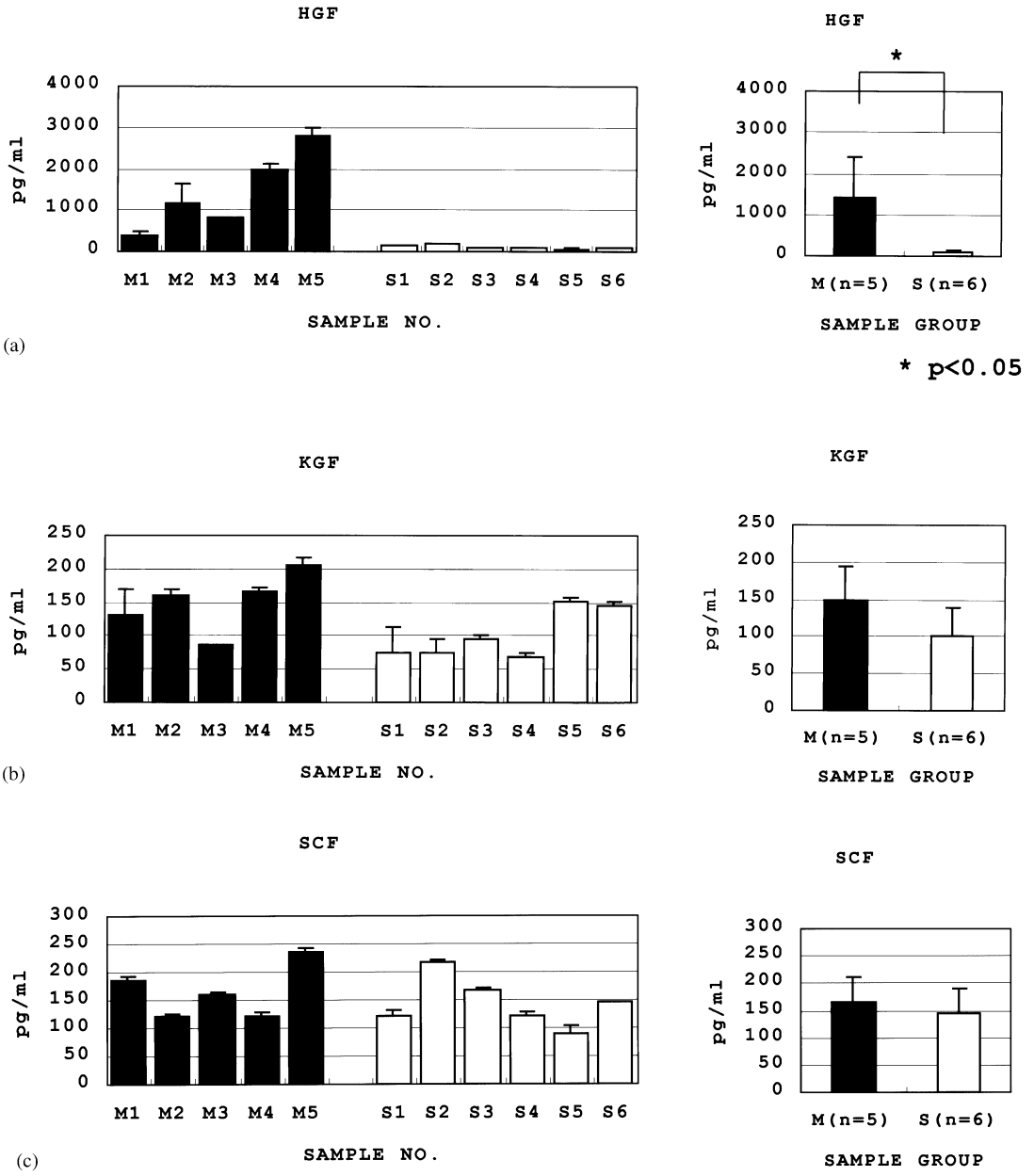


Fig. 1. Cytokine secretion by fibroblasts for 4 days in culture. After human fibroblasts were cultured for 4 days at 37 °C under a 5% $CO_2$ atmosphere, the fibroblast-conditioned medium was collected and used to quantify secreted cytokines by ELISA. Values are means ± S.D. derived from three wells of each specimens. (A) HGF; (B) KGF; (C) SCF. Right: M1–M5 indicate the specimen number of mucosa ($n = 5$). S1–S6 indicate the specimen number of normal skins ($n = 6$). Left: Comparison of the averaged value between two groups. *$P < 0.05$ to normal skin.

and very rare incident of hypertrophic scar in the oral cavity are related to the increased HGF expression by buccal mucosal fibroblasts.

The pro-inflammatory cytokines, interleukin-1α and β stimulate the expression of HGF mRNA and protein in cultured fibroblasts [30,31], while

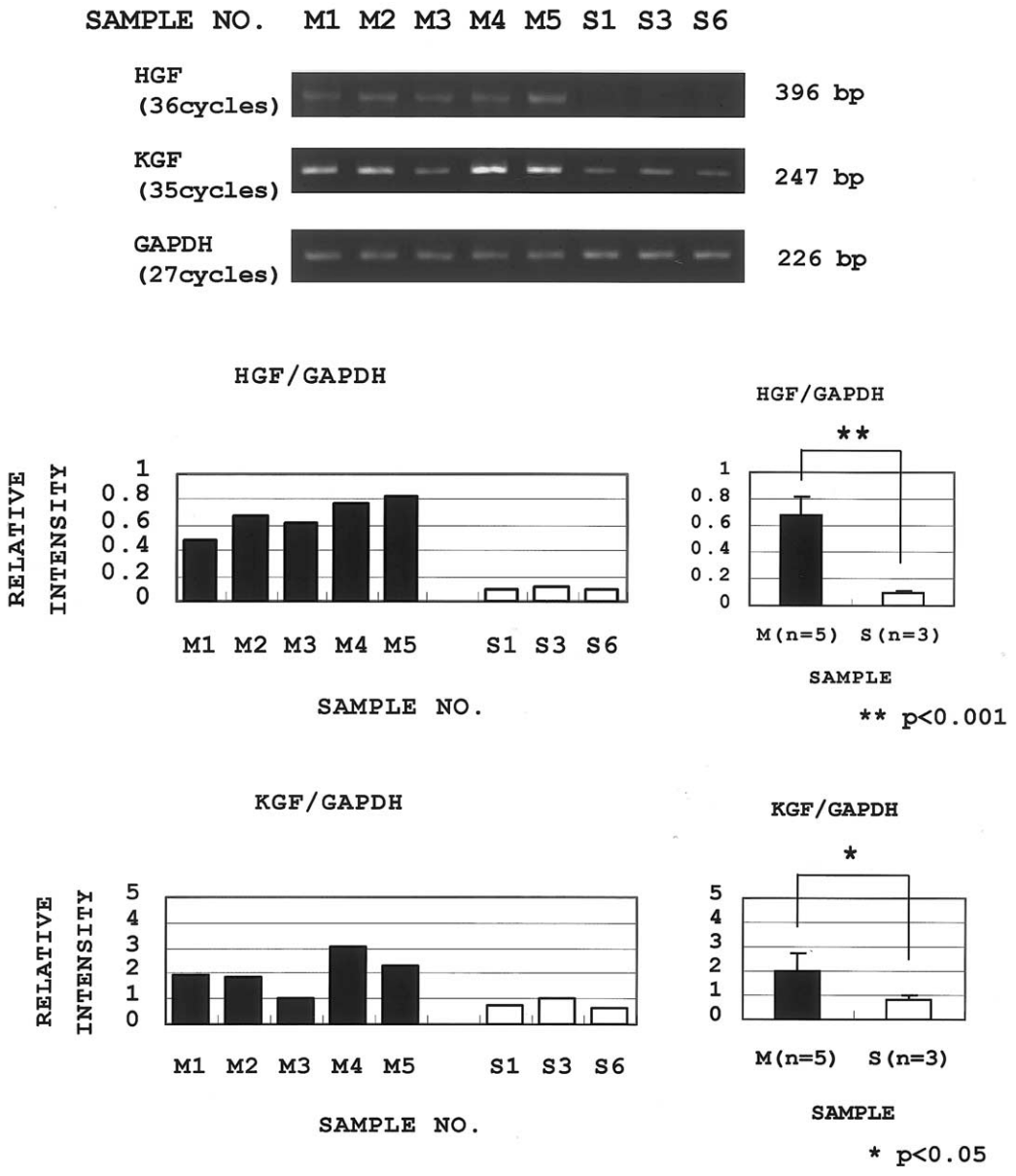


Fig. 2. Semiquantitative RT-PCR analysis of HGF and KGF mRNA expression. (A) Fluorogram semiquantitative RT-PCR analysis was performed in all five specimens in mucosal fibroblasts and representative three specimens in dermal fibroblasts. M1–M5 indicate the specimen number of mucosa ($n = 5$). S1, S3 and S6 indicate the specimen number of normal skins ($n = 3$). (B) Densitometric analysis. Left: The intensities of each PCR bands are expressed as relative intensities to GAPDH. Right: Comparison of the averaged intensity between two groups. $^{*}P < 0.05$, $^{**}P < 0.001$ to normal skin.

IL-6, IL-1α and β induce the expression of KGF mRNA and protein [32,33]. It was reported that the fibroblasts derived from oropharyngeal mucosa were capable of producing a number of cytokines without the need for additional stimuli; the proinflammatory cytokines IL-1, IL-6 and IL-8 were found at high levels in cultured fibroblasts obtained from the palatoglossal arch or from

uvulopalatopharyngoplasty specimens compared to the dermal fibroblasts [10]. Increased expression of HGF and KGF by buccal mucosal fibroblasts shown in our study might be the result of the autocrine stimulation by IL-1 or IL-6. Another possibility is that the mucosal fibroblasts might have a property of producing abundant HGF and KGF independently of autocrine stimulation by pro-inflammatory cytokines.

In addition to the greater proliferating ability and increased production of pro-inflammatory cytokines in oral keratinocytes [2,6,7], our present study suggest that the increased expression of HGF and KGF in oral mucosal fibroblasts is related to faster wound healing with less scar formation in the oral cavity.

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