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ORIGINAL ARTICLE

Effects of HB-EGF and epiregulin on wound healing of gingival cells *in vitro*

JM Kim[1,2], EJ Bak[3], JY Chang[1], S-T Kim[4], W-S Park[5], Y-J Yoo[1,2,3], J-H Cha[1,2,3]

[1]*Department of Oral Biology, BK21 Project, Oral Science Research Center, Yonsei University, Seoul;* [2]*Department of Applied Life Science, The Graduate School, Yonsei University, Seoul;* [3]*Oral Cancer Research Institute, Yonsei University, Seoul;* [4]*Department of Prosthodontics, Yonsei University, Seoul;* [5]*Department of Advanced General Dentistry, Yonsei University, Seoul, Korea*

OBJECTIVE: Gingival wound healing is important to periodontal disease and surgery. This *in vitro* study was conducted to assess the manner in which heparin-binding epidermal growth factor-like growth factor (HB-EGF) and epiregulin cooperatively participate in the wound-healing process in the gingival epithelial and fibroblast cells of the oral mucosa.

MATERIAL AND METHODS: Gingival epithelium and fibroblast were separated from gingival tissue biopsies and prepared to primary cultures. The changes in the mRNA expression were evaluated via real-time PCR. The effects on cell proliferation, migration, and repopulation were evaluated *in vitro*.

RESULTS: The different regulation of expressions of HB-EGF, epiregulin, and epidermal growth factor receptors was observed over time and with different gingival cell types. HB-EGF exerted a cell migration-inducing effect on both epithelial and fibroblast cells, whereas epiregulin did not. Both growth factors functioned as mitogens for epithelial cell proliferation, but not for fibroblast proliferation. HB-EGF strongly promoted epithelial cell repopulation and mildly promoted fibroblast repopulation, whereas epiregulin promoted only fibroblast repopulation.

CONCLUSION: These results indicated that both growth factors might function importantly in the wound-healing process of human gingival tissue via the different regulation of the expression, cell migration, proliferation, and repopulation.

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Keywords: heparin-binding epidermal growth factor-like growth factor; epiregulin; gingival tissue; wound healing

Correspondence: Jeong-Heon Cha, PhD, Department of Oral Biology, Yonsei University College of Dentistry, 134 Sinchon-dong, Seodaemoon-gu, Seoul 120-752, Korea. Tel: +82 2 2228 3061, Fax: +82 2 2227 7903, E-mail: jcha@yuhs.ac
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Introduction

The gingiva is a part of the oral mucosa that covers the alveolar bone processes of the mandibula and the maxilla. The gingiva is composed of the epithelium and the underlying connective tissue. The principal functions of the gingival epithelium are to protect the underlying structures against physical stress during mastication and to function as a primary barrier against bacterial invasion. Gingival fibroblast cells are the most prominent cellular element in gingival connective tissue. The fibroblast is considered to be a mass of multipotent cells with the capacity to differentiate into other cell types. The gingival fibroblasts synthesize collagen and elastic fibers and participate in the formation of granulation tissue at the wound site (Hakkinen *et al*, 2000; Newman *et al*, 2002). Therefore, gingival epithelial and fibroblast cells inevitably perform a key role in the formation, maintenance, and regeneration of gingival tissue.

Wound healing has been studied extensively in skin and corneal tissue and is known to be an extremely complex process (Martin, 1997; Lu *et al*, 2001). Successful wound healing involves a number of processes, which include cell proliferation, migration, and repopulation (Martin, 1997). Cell migration and proliferation, which are driven by growth factors and cytokines released in a coordinated manner into the injured sites, are critical in this regard (Howell *et al*, 1996; Cochran and Wozney, 1999; King and Cochran, 2002). Most prominent among these injured cell-derived factors are ligands for the epidermal growth factor receptor (EGFR), the epidermal growth factor (EGF) family (Fitsialos *et al*, 2007). A number of studies have previously demonstrated that an autocrine manner by EGFR–ligand interactions functions an important role in epithelial wound healing (Green and Kehinde, 1974, 1979; Sidhu, 1979). Heparin-binding epidermal growth factor-like growth factor (HB-EGF) and epiregulin are members of the EGF family, which includes the EGF, transforming growth factor-α, amphiregulin, and betacellulin (Higashiyama *et al*, 1991; Toyoda *et al*, 1995). Analyses of the amino acid sequence of the HB-EGF and epiregulin precursors revealed a signal sequence, an

extracellular domain, a transmembrane domain, and a carboxylterminal cytoplasmic domain (Naglich *et al*, 1992; Toyoda *et al*, 1997; Cha *et al*, 1999). On the cell surface, the precursor is cleaved and released as a mature/soluble growth factor (Goishi *et al*, 1995). The released mature growth factor can function as a mitogen via binding to EGFRs (Higashiyama *et al*, 1991). The mature growth factors of HB-EGF and epiregulin have been demonstrated to bind specifically to two EGFRs, EGFR1/erbB1/HER1 and EGFR4/erbB4/HER4, among EGFRs 1–4 (Higashiyama *et al*, 1991; Elenius *et al*, 1997; Nishi *et al*, 2001). The ligand–receptor complexes form homo-dimers with themselves or hetero-dimers with other EGFRs, and activate intracellular signals to function as a mitogenic and chemotactic factor (Ullrich and Schlessinger, 1990; van der Geer *et al*, 1994). Interestingly, no ligand for EGFR 2 with high affinity has yet been identified, and the analysis of the structure of EGFR 2 showed that EGFR 2 unbound to ligand could hetero-dimerize with other activated EGFRs (Xu *et al*, 2004).

Heparin-binding epidermal growth factor-like growth factor was originally detected in conditioned medium from the U937 macrophage-like cell line (Besner *et al*, 1990; Higashiyama *et al*, 1991, 1992), and epiregulin was purified from the conditioned medium of the T7 NIH3T3 cell line (Toyoda *et al*, 1995). The mitogenic activity of mature HB-EGF was initially demonstrated using smooth-muscle cells and fibroblasts (Dluz *et al*, 1993; Blotnick *et al*, 1994; Fukuda *et al*, 1995), but other cell types, such as keratinocytes, hepatocytes, kidney tubule cells, and gastrointestinal epithelial cell lines, also respond to this factor (Raab and Klagsbrun, 1997). Epiregulin has been shown to perform a crucial role in the proliferation and migration of human keratinocyte, human corneal epithelial, and renal proximal tubular cells (Shirakata *et al*, 2000; Morita *et al*, 2007; Zhuang *et al*, 2007). Although normal tissues express relatively low levels of HB-EGF and epiregulin mRNA, expression increases in response to tissue damage (Marikovsky *et al*, 1993; Powell *et al*, 1993; Homma *et al*, 1995; Kiso *et al*, 1995, 1996; McCarthy *et al*, 1996). In addition, we previously demonstrated using periodontal ligament (PDL) cells that HB-EGF might perform an important function in the oral wound-healing process (Lee *et al*, 2009). Considering the mounting body of evidence suggesting that HB-EGF and epiregulin may be important growth factors in the oral wound healing, the principal objective of our study was to understand how HB-EGF and epiregulin cooperatively participated in the wound-healing process, including cell migration, proliferation, and repopulation in gingival epithelial and fibroblast cells of the oral mucosa.

Materials and methods

Materials

The materials in the cell culture were purchased from Gibco BRL (Grand Island, NY, USA). Keratinocyte growth medium (KGM) containing keratinocyte basal medium-2 (KBM-2) and supplements (KGM-2 SingleQuots) was obtained from Lonza (Walkersville, MD, USA). Collagenase A and dispase II were acquired from Roche (Mannheim, Germany). Human recombinant HB-EGF and epiregulin were obtained from R&D Systems (Minneapolis, MN, USA). FNC Coating Mix® that includes fibronectin, collagen, and albumin was obtained from AthenaES (Baltimore, MD, USA). Rat tail tendon collagen type I was acquired from Upstate (Billerica, MA, USA), and the gelatin was provided by Sigma Chemical (Louis, Mo, USA).

Cell culture

Human gingival epithelial and fibroblast cells were obtained and cultured from the explant tissues of healthy human gingiva. The healthy explant tissues were obtained from patients who had undergone crown lengthening procedures (Yonsei University College of Dentistry, Seoul, Korea). Written informed consent was received from the patients for the use of their explant tissues in this study. Gingival epithelium and connective tissues were separated by collagenase A and dispase II, after which the epithelial cells were separated as single cells by treatment with trypsin/EDTA. The epithelial cells were cultured in KGM, and the fibroblast cells were cultured in DMEM containing 10% fetal bovine serum, streptomycin (100 μg ml^{-1}), amphotericin B (0.5 mg ml^{-1}), and penicillin (100 units ml^{-1}) in a 5% CO_2 incubator at 37°C. All experiments were conducted within three to four cellular passages.

Real-time PCR assay

Human gingival epithelial and fibroblast cells were cultured on 60-mm dishes. After achieving confluence, approximately 50% of the cells were scratch wounded with a sterile plastic hair comb, after which the cells were incubated for the indicated times. Total RNA was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) in accordance with the manufacturer's instructions. One microgram of total RNA was converted to cDNA using the RT premix kit (Bioneer, Seoul, Korea) according to the manufacturer's instructions. Quantitative real-time PCR was conducted using a SYBR®GREEN PCR MASTER Mix (Applied Biosystems, Warrington, UK) and MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The specific primers and annealing temperature utilized for real-time PCR of HB-EGF, epiregulin, EGFR 1–4, and GAPDH were listed in Table 1. A 627-bp insert of human HB-EGF gene and a 510-bp insert of human epiregulin gene were cloned in pGEM®-T Easy to establish standard curves of HB-EGF and epiregulin mRNA. The standard curve equation was used to calculate the absolute copy number of HB-EGF and epiregulin mRNA (Chini *et al*, 2007). For EGFRs, relative quantification was performed in relation to GAPDH mRNA expression by the application of the $2^{-\Delta\Delta C_t}$ analysis method (Livak and Schmittgen, 2001). All real-time PCR procedures were completed with a melting curve analysis to verify the specificity of amplification and the lack of primer dimers. All

Table 1 Condition of real-time PCR

Gene	*5′ – 3′*	*Annealing temperature (°C)*	*Product size (bp)*
hu HB-EGF	F: GTTCTCTCGGCACTGGTGA R: TGGTCCGTGGATACAGTGG	55	101
hu epiregulin	F: ATCACAGTCGTCGGTTCCA R: ACTTGCGGCAACTCTGGAT	60	116
hu EGFR	F: GGTGGCATTTAGGGGTGAC R: CCAAGCCTGAATCAGCAAA	52	112
hu EGFR2	F: CTACTCGCTGACCCTGCAA R: GTGTGCACGAAGCAGAGGT	62	117
hu EGFR3	F: AGCCAGCTGTCCCCATAAC R: CCCACAAGGCTCACACATC	53	112
hu EGFR4	F: GGCTTTCAACATCCCACCT R: GGAGGGCTGTGTCCAATTT	52	81
hu GAPDH	F: GAGTCCACTGGCGTCTTCA R: CAGAGGGGGCAGAGATGAT	56	88

HB-EGF, heparin-binding epidermal growth factor-like growth factor; EGFR, epidermal growth factor receptor.

experiments were conducted in triplicate for each data point on two separate experiments.

To compare the mRNA expression patterns among HB-EGF, epiregulin, and EGFRs, their relative mRNA expressions were recalculated relative to the amount of GAPDH mRNA by the application of the $2^{-\Delta\Delta C_t}$ analysis method with the standardization of GAPDH mRNA cycle threshold (C_t).

Cell migration assay

For analyzing the chemotactic effects of HB-EGF and epiregulin, 5×10^4 cells in the media without 10% fetal bovine serum (FBS) or supplements were placed in the upper chambers of 48 trans-well plates with polycarbonate filters with 8-μm pores (Corning Incorp., Lowell, MA, USA). The upper side of membrane was precoated with 0.35 mg ml^{-1} of rat tail tendon type I collagen, and the lower side was precoated with 1 mg ml^{-1} of gelatin. Serum-free medium or KBM-2 medium including either HB-EGF (0, 1, and 10 ng ml^{-1}) or epiregulin (0, 1, and 20 ng ml^{-1}) was added to the lower chamber. After 16 h of incubation, cells that remained on the upper side of the membrane were removed with a cotton swab. Cells that migrated to the lower side of the membrane were fixed with methanol, stained with hematoxylin (Sigma), and then counted. Cell migration was measured as the number of migrated cells per membrane. The assay was conducted in duplicate in three separate experiments.

Cell proliferation assay

Heparin-binding epidermal growth factor-like growth factor and epiregulin at the indicated concentrations were utilized to determine their effects on the proliferation of gingival epithelial and fibroblast cells. The epithelial and fibroblast cells were seeded into six-well plates at a density of 5×10^3 cells per well and cultured in KGM or DMEM media including 10% FBS, respectively. After 24 h, the epithelial and fibroblast cells were incubated for 5 days in the KBM-2 or serum-free DMEM media in the presence of either HB-EGF (0, 1, and 10 ng ml^{-1}) or epiregulin (0, 1, and 20 ng ml^{-1}) in a 5% CO_2 incubator at 37°C. The medium was exchanged every 48 h. The cells were photographed after 5 days using the Olympus CKX41 inverted microscope system (Olympus, Tokyo, Japan) at 40× magnification. Afterward, the cells were stained with 0.4% trypan blue stain and counted with a hemocytometer. The assays were conducted in duplicate in three separate experiments.

Cell repopulation assay

Gingival epithelial and fibroblast cells were plated on 12-well plates precoated with FNC Coating Mix® and then grown to confluence. The epithelial and fibroblast cells were then cultured overnight in KBM-2 or serum-free DMEM media, respectively. The cells were then scratched with a sterile yellow tip, producing a single wound line. After one washing with phosphate-buffered saline, HB-EGF (0, 1, and 10 ng ml^{-1}) or epiregulin (0, 1, and 20 ng ml^{-1}) was added to the media. After 24 h of incubation in a 5% CO_2 incubator at 37°C, wound closure was photographed using an Olympus CKX41 inverted microscope system (Olympus) at 40× magnification. The assay was conducted in duplicate in three separate experiments.

Statistical analysis

The SPSS 12.0 statistical package program (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The unpaired *t*-test was conducted to evaluate the statistical differences between groups. A P value of < 0.05 was considered to indicate a statistically significant difference.

Results

mRNA expression patterns of HB-EGF, epiregulin, and EGFRs regulated by wounding

Thus far, the expression patterns and roles of HB-EGF and epiregulin in wounding have yet to be identified in the gingiva of the oral mucosa. First, the manner in which wounding regulates the endogenous expressions of HB-EGF, epiregulin, and EGFRs 1–4 in human gingival epithelial and fibroblast cells was determined.

Quantitative real-time PCR was conducted for 24 h after approximately 50% of the cells were scratch wounded *in vitro* (Figure 1). The results demonstrated that gingival epithelial and fibroblast cells expressed relatively low levels of HB-EGF mRNA prior to the scratch wounding procedure (at 0 h). The epithelial cells expressed 6139 copies ng^{-1} of total RNA at 0 h, whereas the fibroblast cells expressed 271 copies ng^{-1} of total RNA. The low level expression was maintained for a brief time after the wounding up to 0.2 h in the case of the epithelial cells and 0.5 h in the fibroblast cells (Figure 1a,b). Then, the wounded gingival epithelial cells evidenced significant, gradual increases in HB-EGF mRNA expression to 2.6-, 3.2-, and 5.0-fold at 0.5, 1, and 2 h and then another increase to 2.8-, 4.2- and 6.3-fold at 3, 4, and 6 h, respectively, as compared to the expression measured at 0 h (Figure 1a). The trends of decrease from 2 to 3 h and from 6 to 8 h and increase from 8 to 24 h were shown, although these trends were not statistically significant. In gingival fibroblast cells, the level of HB-EGF mRNA expression increased significantly and gradually to 5.9- and 25.4-fold at 1 and 2 h as compared to that measured at 0 h (Figure 1b), and especially the maximum level of HB-EGF expression occurred at 2 h. After the decrease at 3 and 4 h, the HB-EGF expression was increased to 5.5-fold at 6 h (Figure 1b). Epiregulin mRNA expression in gingival epithelial and fibroblast cells was also relatively low prior to the wounding. The epithelial cells expressed 4904 copies ng^{-1} of total RNA at 0 h, whereas the fibroblast cells expressed 372 copies ng^{-1} of total RNA. The expression did not increase significantly after wounding for up to 1 h in the gingival epithelial cells and for up to 6 h in the fibroblast cells. Epiregulin mRNA expression in the epithelial cells significantly increased to 5.3-, 5.4-, 10.5-, 3.2-, and 4.6-fold at 2, 3, 4, 6, and 24 h, respectively, and increased gradually at 3–4 h, after the wounding (Figure 1a). The maximum level of epiregulin expression occurred at 4 h. The expression of epiregulin mRNA in the fibroblast cells was increased significantly, by 5.7-fold at 8 h (Figure 1b). In the epithelial cells, an initial induction occurred at 0.5 h in HB-EGF and 2 h in epiregulin, and the induction of the fibroblast cells began at 1 h in HB-EGF and at 8 h in epiregulin. Therefore, in both human gingival epithelial and fibroblast cells, HB-EGF upregulation occurred earlier than in epiregulin.

In the gingival epithelial cells, EGFR 1, 2, and 3 were expressed, but EGFR 4 was not (Figure 1c). EGFR 1 evidenced no significant changes in response to wounding. EGFR 2 mRNA expression increased significantly from 3 to 24 h. The induction of EGFR 2 was increased to 2.2-, 6.4-, 5.1-, 4.0-, and 5.1-fold at 3, 4, 6, 8, and 24 h, respectively. EGFR 3 mRNA increased by 3.8-fold at 24 h. In the gingival fibroblast cells, the wounding did not significantly alter the mRNA expression levels of all four EGFRs (data not shown). Therefore, to compare the mRNA expression patterns among HB-EGF, epiregulin, and EGFRs in the gingival epithelial cells, their relative mRNA expression was calculated and plotted on a graph (Figure 1d). Three peaks of HB-EGF expression were observed at 2, 6, and 24 h, while two peaks of epiregulin expression occurred at 2 and 4 h. EGFR2 showed three peaks at 2, 4, and 6 h, and

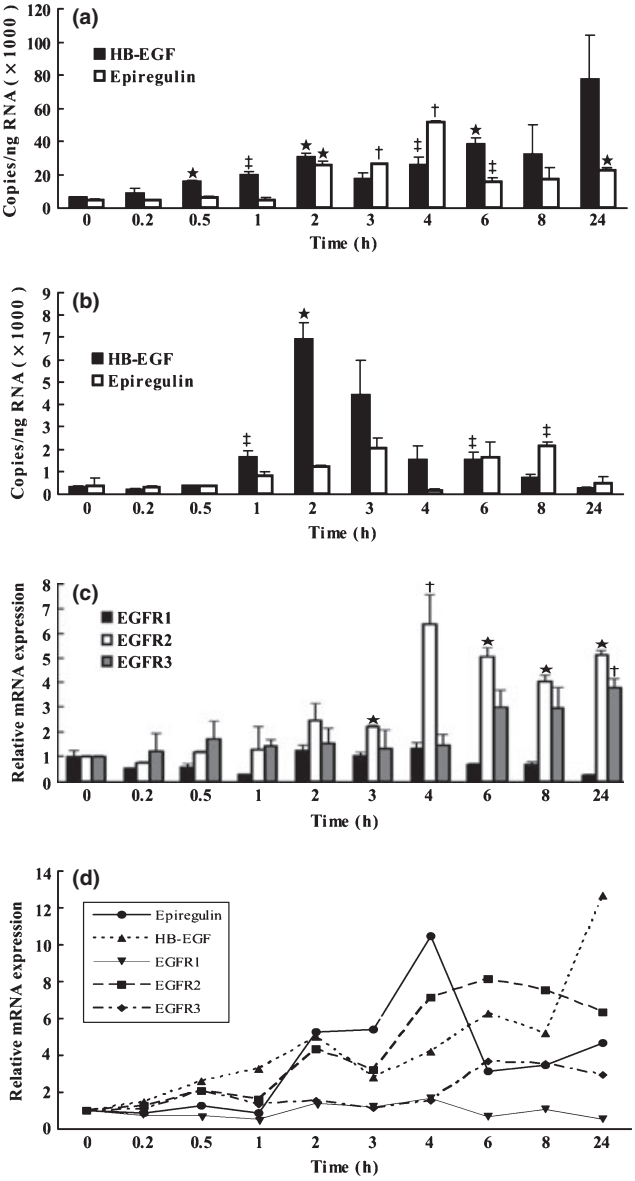

Figure 1 Quantitative mRNA expression levels of heparin-binding epidermal growth factor-like growth factor (HB-EGF) and epiregulin in human gingival epithelial and fibroblast cells after scratch wounding. Gingival epithelial and fibroblast cells were cultured to confluence, and a monolayer of cells was scratch wounded. RNA was isolated from the cells remaining on the dish from 0 to 24 h after scratch wounding. Quantitative real-time PCR was performed with RNA isolated at the indicated times after scratch wounding. The absolute quantification for HB-EGF and epiregulin mRNA in gingival epithelial (**a**) and gingival fibroblast cells (**b**) was calculated using standard curves. To identify the expression patterns in gingival epithelial cells among epidermal growth factor receptors (EGFRs) (**c**), and among HB-EGF, epiregulin, and the EGFRs (**d**), the relative quantification was calculated by the application of the $2^{-\Delta\Delta C_t}$ analysis method with the standardization of GAPDH mRNA cycle threshold (C_t). The error bar represents the standard error of the mean. The symbols (‡, *, †) indicate significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively

EGFR3 had a peak at 6 h while the expression of EGFR1 showed no peak.

The results suggested that gingival wounding might regulate the expression of HB-EGF, epiregulin, and EGFRs differently over time and with different cell types, and probably in a coordinated manner.

Effects of HB-EGF and epiregulin on cell migration

To determine the effects of HB-EGF and epiregulin on the migration of gingival cells, human gingival epithelial and fibroblast cells were treated with HB-EGF or epiregulin for 12 h at the indicated concentrations, after which cell migration was evaluated (Figure 2). The treatment of HB-EGF at 10 ng ml^{-1} induced the migration of epithelial cells by up to 12-fold ($P < 0.05$) as compared with the controls in the absence of HB-EGF (Figure 2a), whereas HB-EGF significantly induced the migration of fibroblast cells in a dose-dependent manner, with increases to 3.6- and 6.3-fold at 1 and 10 ng ml^{-1}, respectively (Figure 2b). By way of contrast, epiregulin evidenced no significant effects on the migration of both gingival epithelial and fibroblast cells. These results demonstrated that HB-EGF exerts a cell migration-inducing effect on both cell types, whereas epiregulin does not.

Effects of HB-EGF and epiregulin on cell proliferation

To determine the effects of HB-EGF and epiregulin on the proliferation of gingival cells, human gingival epithelial and fibroblast cells were treated with HB-EGF or epiregulin for 5 days at the indicated concentrations, and the viable cells were counted with a hemocytometer (Figure 3). HB-EGF and epiregulin stimulated the proliferation of epithelial cells in a dose-dependent manner. The numbers of cells increased to 2.4- and 4.5-fold at 1 and 10 ng ml^{-1} of HB-EGF, and 2.0- and 2.7-fold at 1 and 20 ng ml^{-1} of epiregulin, respectively (Figure 3a). However, HB-EGF and epiregulin did not significantly affect the proliferation of fibroblast cells (Figure 3b). The results indicated that HB-EGF and epiregulin were important growth factors for the induction of epithelial cell proliferation, but did not induce fibroblast cell proliferation.

Effects of HB-EGF and epiregulin on cell repopulation

In an effort to evaluate the effects of HB-EGF and epiregulin on gingival cell repopulation, a monolayer of human gingival epithelial or fibroblast cells was scratch wounded with a sterile yellow tip and then incubated with HB-EGF or epiregulin at the indicated concentrations for 24 h, after which the wound closure was photographed (Figure 4). When the epithelial cells were treated with HB-EGF, the wounded gap closed in a dose-dependent manner, whereas after epiregulin treatment, very slight improvement, if any, in wound closure was noted (Figure 4a). However, when the fibroblast cells were treated with epiregulin, the wound closure improved significantly in a dose-dependent manner, whereas a slight improvement in wound closure was noted after treatment with HB-EGF (Figure 4b). This result showed that HB-EGF strongly promoted the repopulation of epithelial cells and mildly promoted the repopulation of fibroblast cells, whereas epiregulin promoted only the repopulation of fibroblast cells.

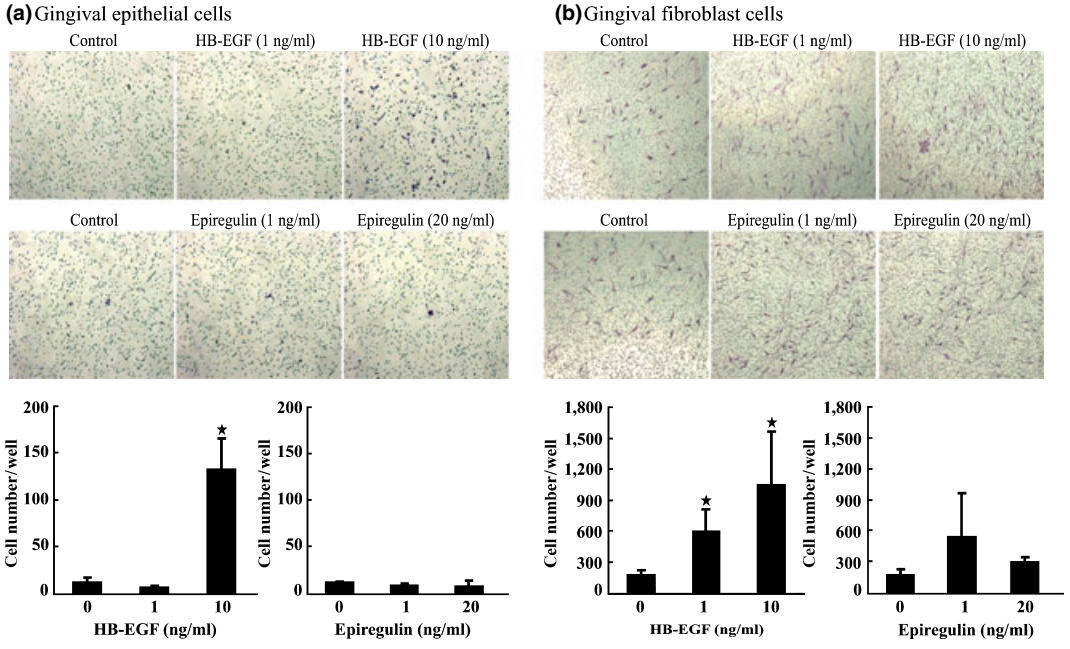

Figure 2 Effect of heparin-binding epidermal growth factor-like growth factor (HB-EGF) and epiregulin on the migration of human gingival epithelial and fibroblast cells. Gingival epithelial (**a**) and fibroblast cells (**b**) were plated in the upper chamber, and HB-EGF and epiregulin at the indicated concentrations were added to the bottom chamber. After 16 h, cells that migrated through membrane pores were stained and counted. The error bars represent the standard error of the mean. The asterisk indicates a significant difference at $P < 0.05$. Bar = 50 μm

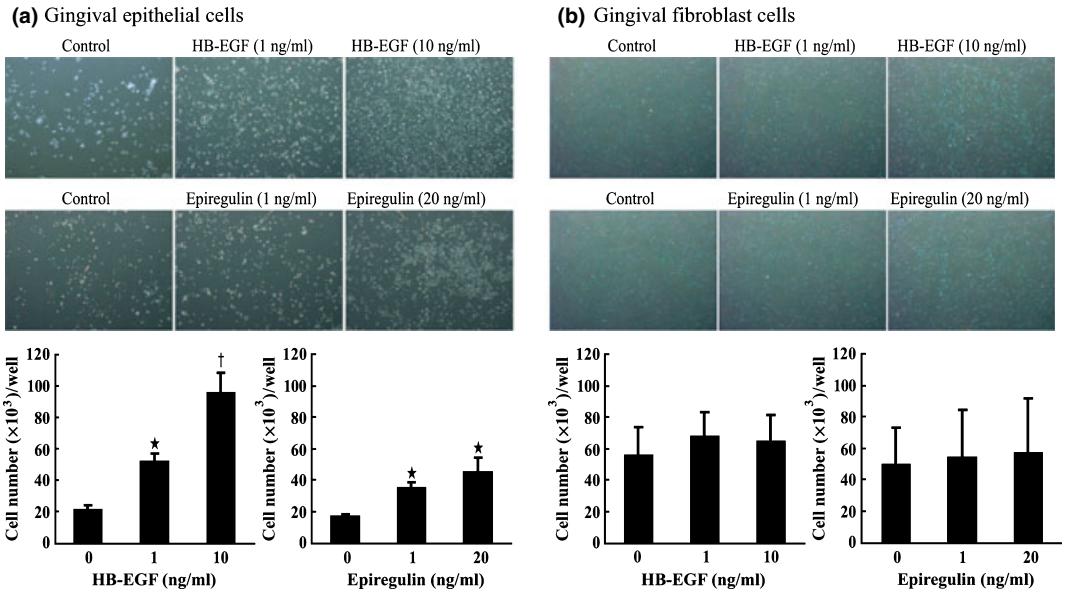

Figure 3 Effect of heparin-binding epidermal growth factor-like growth factor (HB-EGF) and epiregulin on the proliferation of human gingival epithelial and fibroblast cells. Gingival epithelial (**a**) and fibroblast cells (**b**) were plated and grown for 1 day. The cells were treated with HB-EGF and epiregulin at the indicated concentrations for 5 days. The cells were counted using the trypan blue exclusion method. The error bars represent the standard error of the mean. The symbols (*, †) indicate significant differences at $P < 0.01$ and $P < 0.001$, respectively. Bar = 50 μm

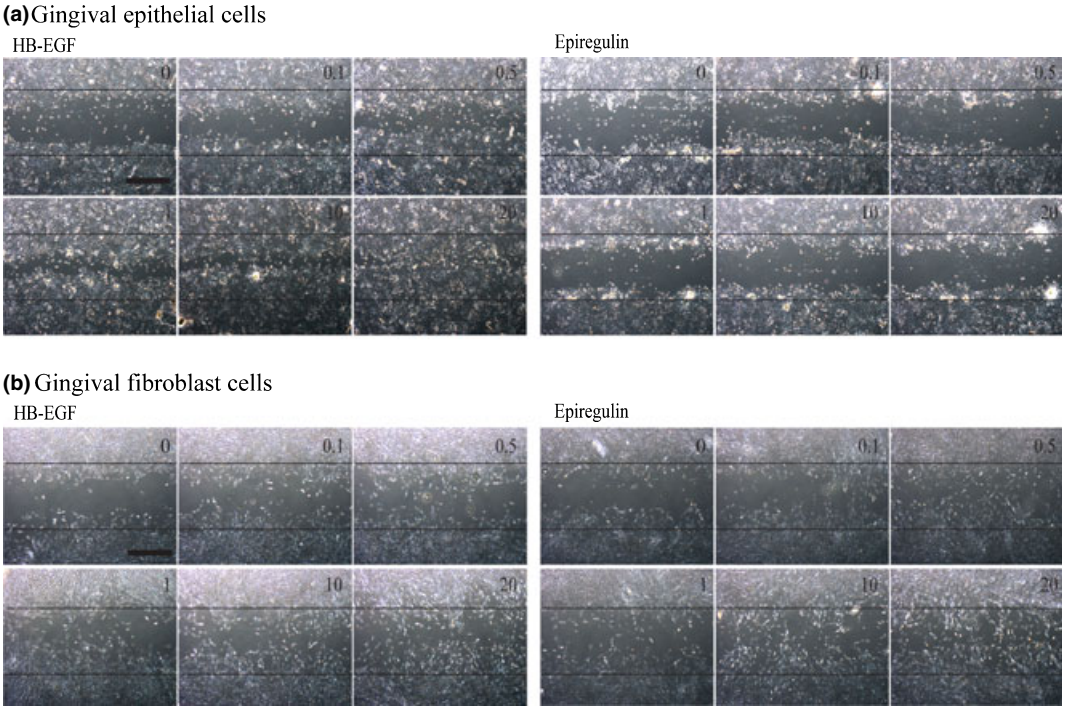

Figure 4 Effect of heparin-binding epidermal growth factor-like growth factor (HB-EGF) and epiregulin on the repopulation of human gingival epithelial and fibroblast cells. A monolayer of gingival epithelial (**a**) and gingival fibroblast cells (**b**) was scratched with a sterile yellow tip, producing a single straight wound line, and cultured with HB-EGF and epiregulin at the indicated concentrations. Cell repopulation was photographed at 24 h after the wounding. The lines represent the position of the scratch wound. Bar = 50 μm

Discussion

When gingival tissue is wounded as the result of an accident, and a variety of dental procedures such as the treatment of periodontitis, avulsion of teeth, and surgical processes, gingival epithelial and fibroblast cells at the wounded site contribute to the wound healing. In an effort to gain greater insight into the process of oral wound healing, we investigated and attempted to identify the effects of HB-EGF and epiregulin on the

wound healing of human gingival epithelial and fibroblast cells, which are the primary cells contained in the gingiva of oral mucosa.

We detected different regulation of wounding for endogenous HB-EGF and epiregulin expression in time and in the different cell types in the gingiva. The *in vitro* scratch wounding upregulates HB-EGF mRNA expression earlier than induction of epiregulin expression in both human gingival epithelial and fibroblast cells. This finding is similar to the microarray data reported previously by Fitsialog's group (Fitsialos *et al*, 2007). In human keratinocytes isolated from neonatal foreskin, the expression of HB-EGF was induced at 1 h after *in vitro* wounding, after which epiregulin was upregulated at 3 h. Since the upregulation of HB-EGF at 30 min and at 2 h in the gingival epithelial cells, gingival epithelial cells respond more promptly to the wounding by earlier expression of both growth factors than keratinocytes. Collectively, our results show that HB-EGF may function earlier in the wound-healing process than epiregulin. Additionally, the epithelial cells increase HB-EGF expression earlier than in fibroblast cells after wounding, and the epithelial cells also evidence increased epiregulin expression earlier than is observed in fibroblast cells, thereby indicating that the epithelial cells may need more agile response to wounding in terms of the expression of the growth factors. It is interesting to note that gingival epithelial cells express much higher mRNA levels of HB-EGF and epiregulin than are measured in the gingival fibroblast cells. Before the wounding, the epithelial cells expressed levels that were 22 times higher than those of the fibroblasts; after wounding, the epithelial cells expressed HB-EGF mRNA at maximum levels 44 times higher. The epithelial cells also expressed 13-fold higher levels of epiregulin mRNA prior to wounding, and then maximum 51-fold higher levels after wounding, than were measured in the fibroblasts. This indicated that gingival epithelial cells are a primary cell type that provides growth factors in response to wounding. In the case of EGFRs, gingival epithelial cells express EGFR 1, 2, and 3, whereas the fibroblast cells express EGFR 1, 2, 3, and 4. The upregulation of mRNA expression after wounding was observed in EGFR 2 and 3 of the epithelial cells, whereas the expression of those EGFRs was not altered significantly in the fibroblast. In the case of HB-EGF, it was reported that heterodimers containing EGFR 2 induce the healing process more profoundly than does interaction with other EGFRs (Bindels *et al*, 2002; Ueda *et al*, 2004; Xu *et al*, 2004); this may explain why the strongest gingival epithelial cell repopulation was observed in response to HB-EGF. It is worth noting that when the mRNA expression of HB-EGF is high, EGFR2 also shows relatively high expression in gingival epithelial cells (Figure 1d), and therefore, it would be speculated that the EGFR2 expression may have the correlation with the HB-EGF expression. Interestingly, no EGFR 4 mRNA was detected in the gingival epithelial cells, either prior to and after wounding. It was previously reported in a related study that EGFR 4 is not expressed *in vivo* in the epidermis, primary-cultured keratinocytes, PDL cells, or the HaCat cell line originated from human keratinocytes (Marques *et al*, 1999; Stoll *et al*, 2001; Piepkorn *et al*, 2003; Lee *et al*, 2009). EGFR1 should be considered a major receptor for HB-EGF and epiregulin in the wound responses of gingival epithelial cells, whereas EGFR 1 and 4 may function as the receptor in gingival fibroblast cells, as HB-EGF and epiregulin are known to be capable of binding only to EGFR 1 and 4.

Our results demonstrated that HB-EGF induces cell migration in both human gingival epithelial and fibroblast cells, but epiregulin does not, thereby indicating that HB-EGF can function as a cell migration-inducing factor for both cell types during wound healing, whereas epiregulin does not. The cell migration-inducing effect of HB-EGF is stronger in fibroblast cells, as the response of the fibroblast cells to HB-EGF at 10 ng ml^{-1} was 7.9-fold higher than that of the epithelial cells. HB-EGF and epiregulin promote epithelial cell proliferation but not fibroblast cell proliferation, thereby showing that HB-EGF and epiregulin can function as mitogenic factors only for epithelial cells, and not for fibroblast cells. Additionally, epithelial cell repopulation is strongly improved in the presence of exogenous HB-EGF after wounding but not in the presence of epiregulin, whereas fibroblast cell repopulation is mildly induced by both HB-EGF and epiregulin. The process of repopulation of the gingival epithelial cells may be critical for wound healing because the wound closure needs to form a primary barrier against microorganism invasion and thus epithelial cell repopulation should happen fast. Our findings made us to speculate that wounding increases HB-EGF expression at an early stage (30 min) in gingival epithelial cells and increases gradually the expression up to 2 h; this may induce epithelial cell migration as chemokinetic factor, proliferation as a mitogenic factor, and repopulation at the wound edge. Interestingly, when HB-EGF expression maintains the similar levels at 2–4 h, epithelial cell proliferation may be continuously and additively induced by epiregulin upregulated at 2 h and further epiregulin expression increases up to 4 h. Thus, the expression at different times between HB-EGF and epiregulin may be regulated tightly to form the critical primary barrier. In addition, the stimulatory effect of HB-EGF and epiregulin on repopulation for the fibroblast cells may be critically important to the healing process, because the fibroblast cells reconstruct damaged extracellular matrix and form granulation tissue to fill damaged connective tissue. According to the results of our study, the significant increase in HB-EGF expression at 1 and especially 2 h may induce cell migration as a chemotactic factor, and cells repopulate in the fibroblast at the damage site of the gingival connective tissue, and after the decrease at 3–4 h, HB-EGF expression increases again at 6 h. Then, the upregulation of epiregulin expression at a later stage (8 h) induces continuous fibroblast cell repopulation for the subsequent oral wound-healing process. We previously demonstrated in vitro using PDL cells that the wounding stimulates the phosphorylation of Erk1/2 and p38, but not of Akt (Lee *et al*, 2009). Erk inhibition

blocked the cell repopulation induced by HB-EGF, and p38 inhibition delayed the process in PDL cells. The further investigation may elucidate whether the similar mechanisms of signaling transduction regulate the wound healing of gingival tissue.

The results of our study indicated that HB-EGF and epiregulin may constitute critical factors in the wound healing of human gingival tissue by regulating complex processes, including cell migration, proliferation, and repopulation. Thus, with caution of the possible cancerous proliferation effect on epithelial cells, HB-EGF and epiregulin may potentially be applied as therapeutic agents for the healing of gingival wounds inflicted in an accident and also for a variety of dental procedures.

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Author contributions

J.-H. Cha designed the project and involved in writing the manuscript. J.M. Kim, E.J. Bak and J. Chang involved in research and writing the manuscript. S. Kim and W. Pak undertook the research. Y.-J. Yoo involved in discussion and writing the manuscript.

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