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Effects of heparin-binding epidermal growth factor-like growth factor on cell repopulation and signal transduction in periodontal ligament cells after scratch wounding *in vitro*

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Background and Objective: A growing amount of attention has been placed on periodontal regeneration and wound healing for periodontal therapy. This study was conducted in an effort to determine the effects of heparin-binding epidermal growth factor-like growth factor on cell repopulation and signal transduction in periodontal ligament cells after scratch wounding *in vitro*.

Material and Methods: Human periodontal ligament cells were acquired from explant tissue of human healthy periodontal ligament. After the wounding of periodontal ligament cells, the change in expression of heparin-binding epidermal growth factor-like growth factor and epidermal growth factor receptors 1–4 mRNA was assessed. The effects of heparin-binding epidermal growth factor-like growth factor on periodontal ligament cell proliferation and repopulation were assessed *in vitro* via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and by photographing the injuries, respectively. Extracellular signal-regulated kinase (Erk)1/2, p38 and Akt phosphorylation was characterized via western blotting.

Results: Scratch wounding resulted in a significant up-regulation of heparin-binding epidermal growth factor-like growth factor mRNA expression, whereas wounding had no effect on the expression levels of epidermal growth factor receptors 1–4. Interestingly, no expression of epidermal growth factor receptors 2 and 4 was detectable prior to or after wounding. Heparin-binding epidermal growth factor-like growth factor treatment promoted the proliferation and repopulation of periodontal ligament cells. The scratch wounding also stimulated the phosphorylation of Erk1/2 and p38, but not of Akt, in periodontal ligament cells, and heparin-binding epidermal growth factor-like growth factor treatment applied after wounding amplified and extended the activations of Erk1/2 and p38, but not of Akt. Furthermore, Erk1/2 inhibition blocked the process of cell

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repopulation induced by heparin-binding epidermal growth factor-like growth factor, whereas the inhibition of p38 delayed the process.

Conclusion: These results indicate that heparin-binding epidermal growth factor-like growth factor may constitute a critical factor in the wound healing of human periodontal ligament cells by a mechanism that requires the activation of Erk1/2 via specific interaction with epidermal growth factor receptor 1.

The periodontal ligament is a dense fibrous connective tissue that is located between the root cementum and alveolar bone and it performs a crucial role in the anchoring of tooth and alveolar bone, as well as in the maintenance of their structural integrity. The ligament consists of a number of different cell types, including fibroblasts, osteoblasts, osteoclasts, cementoblasts and their precursors (1,2). In particular, fibroblasts, of which periodontal ligament cells principally consist, are considered to be multipotent cells, or heterogeneous cell populations, with the capacity to differentiate into other cell types. In this regard, the periodontal ligament cells apparently perform a key role in the formation, maintenance and regeneration of this connective tissue.

Cells such as corneal epithelial cells respond rapidly to injury, resulting in a healing process that involves the migration of cells, which form a sheet to cover up the affected area and restore its functions (3). Immediately after injury, the cells at the margin of the wound begin to lose their hemidesmosome attachment sites and to change shape from a columnar to a more elongated morphology, as they begin to send out lamellipodia and move across the injured bed (4). Successful wound healing and regeneration involves a number of processes, including cell proliferation, cell migration, matrix deposition and tissue remodeling, in addition to inflammation, vascular permeability and angiogenesis (5). Cell migration and proliferation, which are driven by growth factors and cytokines released in a coordinated manner into the injured sites, are crucial in this regard (6–8). Most prominent among these injured cell-derived factors are ligands for the epidermal growth factor (EGF) receptor, the EGF family. A number of studies have previously shown that epithelial wound healing is, at least in part, mediated in an autocrine manner by EGF receptor–ligand interactions (9–11).

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the EGF family, which includes the EGF, transforming growth factor-α, amphiregulin, betacellulin and epiregulin (12). HB-EGF was originally detected in conditioned medium from the U937 macrophage-like cell line (12–14). Analyses of the amino acid sequence of the HB-EGF precursor revealed a signal sequence, an extracellular domain, a transmembrane domain and a carboxylterminal cytoplasmic domain (15,16). On the cell surface, the HB-EGF precursor is cleaved and released as a mature/soluble HB-EGF (17). The released HB-EGF is capable of functioning as a mitogen via binding to the EGF receptor (12), whereas the remaining cell surface-bound HB-EGF precursor is capable of functioning as a juxtacrine growth factor via the EGF receptor (18). Two EGF receptors – EGF receptor 1/erbB1/HER1 and EGF receptor 4/erbB4/HER4 – among EGF receptors 1 to 4 have been shown to bind to mature HB-EGF (12,19,20) and to activate intracellular signals. The mitogenic activity of soluble HB-EGF was initially demonstrated using smooth-muscle cells and fibroblasts (12–14), but other cell types, including hepatocytes, keratinocytes, kidney tubule cells and gastrointestinal epithelial cell lines, also respond to this factor (21). Although normal tissues express relatively low levels of HB-EGF mRNA, expression increases in response to tissue damage (22–27). This indicates that HB-EGF may be an important growth factor for wound healing.

In this study, we assessed the biological role of HB-EGF in the process of periodontal ligament cell repopulation and discussed the intracellular signals that may be required for the HB-EGF process.

Material and methods

Cell culture

The materials indicated in the cell culture were purchased from Gibco BRL (Grand Island, NY, USA). Human periodontal ligament cells were obtained and cultured from the explant tissue of human healthy periodontal ligament. The explant tissue was taken from several first premolars extracted from patients (Yonsei University College of Dentistry, Seoul, Korea) for orthodontic reasons. Written informed consent was received from the patients for use of their explant tissue in this study. Briefly, after removing calculus and plaque in the mouth, the premolar was extracted and rinsed three times with Hanks' balanced salt solution to remove the blood clot from the root surface. The periodontal ligament tissues from the middle third of the roots were minced, put into culture dishes and incubated in α-modified Eagle's minimal essential medium containing 10% fetal bovine serum and the antibiotics streptomycin (100 mg/mL), amphotericin B (0.5 mg/mL) and penicillin (100 units/mL) at 37°C in a humidified atmosphere of 5% CO_2. All the experiments were carried out within five to 10 cellular passages.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

About 2×10^4 periodontal ligament cells were seeded and grown on 96-well

plates for 1 d in α-modified Eagle's minimal essential medium containing 2% fetal bovine serum. Then, the culture medium was replaced with α-modified Eagle's minimal essential medium containing HB-EGF (R&D systems, Minneapolis, MN, USA) at the concentrations of 0, 0.01, 0.1, 1, 10 and 50 nM. Cell proliferation was measured using the MTT assay (Promega, Madison, WI, USA) after 3 and 5 d of culture, according to the manufacturer's instructions. The absorbance was measured on an MRX II microplate reader (Dynatech Laboratories, Chantilly, VA, USA) at 570 nm. All assays were performed in triplicate on two separate experiments.

Table 1. Polymerase chain reaction primer sequence and condition

Gene	5′–3′	Annealing temperature (°C)	Cycles
HB-EGF	F: CTAGGAGGCGGCCGGGACCGGAAAGTC R: GGTTGTGTGGTCATAGGTATATAAGC	55	35
EGFR1	F: ATGCGACCCTCCGGGACGGCCG R: CCTTCAGTCCGGTTTTATTTGC	52	30
EGFR2	F: ATGGAGCTGGCGGCCTTGTGCC R: GTGACAGGGGTGGTATTGTTCAGC	52	40
EGFR3	F: ATGAGGGCGAACGACGCTCTGC R: TCTCGGTGAGCTGAGTCAAGCG	53	33
EGFR4	F: GCAGACACCATTCATTGGCAAG R: TGCTCCATATGTGTACTTTGC	52	40
GAPDH	F: CGGAGTCAACGGATTTGGTCGTAT R: AGCCTTCTCCATGGTGGTGAAGAC	56	27

EGFR, epidermal growth factor receptor; F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HB-EGF, heparin-binding epidermal growth factor-like growth factor; R, reverse.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis

Human periodontal ligament cells were cultured on 60-mm dishes. After reaching confluency, the cells were scratch wounded using a sterile plastic hair comb, washed in phosphate-buffered saline and then the cells were incubated for the indicated times. Total RNA was extracted using the Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA), according to 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. Gene expression was analyzed using semiquantitative RT-PCR. The primers and conditions are listed in Table 1. The plasmids (pHuEGFR2 and pHuEGFR4), pBabe-puro containing cDNAs of human EGF receptor 2 and 4 were used as a PCR template for the positive control of EGF receptor 2 and 4, respectively. RT-PCR products were separated by agarose-gel electrophoresis on a 1% gel, stained with ethidium bromide and digitally photographed using CHEMIIMAGER (Alpha Innotech, San Leandro, CA, USA). The data were normalized to the corresponding value of glyceraldehyde-3-phosphate dehydrogenase gene expression using Spot Denso Analysis of FROG 2000 software (Alpha Innotech) and expressed as relative intensity. All assays were performed in duplicate on two separate experiments.

Cell repopulation assay

Human periodontal ligament cells were cultured to confluence on 12-well tissue culture plates. The cells were then starved in serum-free α-modified Eagle's minimal essential medium overnight. The monolayer was scratch-wounded with a yellow tip. After being washed once with phosphate-buffered saline, the cells were refed with serum-free α-modified Eagle's minimal essential medium in the presence of 10 nM HB-EGF, 10 μM AG1478 (Calbiochem, La Jolla, CA, USA), 10 μg/mL of CRM197 (Calbiochem), 30 μM PD98059 (Sigma Chemical Co., St Louis, MO, USA) and 10 μM SB203580 (Sigma Chemical Co.). Cell repopulation was photographed at the time-points indicated after the wounding using an Olympus CKX41 inverted microscope system (Olympus, Tokyo, Japan) with a ×40 magnification. All assays were performed in duplicate on three separate experiments.

Western blot analysis

Phosphorylation of extracellular signal-regulated kinase (Erk)1/2, p38 mitogen-activated protein kinase (MAPK) and Akt in wounded human periodontal ligament cells was measured by using immunoblot analysis. A serum-starved cell monolayer on 100-mm dishes was wounded using a sterile plastic hair comb. After wounding, the cells were washed with phosphate-buffered saline and incubated for the periods of time indicated, with or without 10 nM HB-EGF. For treatment with AG1478, periodontal ligament cells were pretreated with 1 μM AG1478 for 20 min, and 10 nM HB-EGF was added in the continuous presence of the inhibitor. After incubation for the periods of time indicated, the cells were placed on ice, rinsed twice with ice-cold phosphate-buffered saline and then lysed with lysis buffer containing a protease inhibitor cocktail. Cell lysates were prepared, normalized using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA) to load equal amounts of protein and then subjected to western blot analysis, as described previously (28). Blots were blocked with 5% nonfat dry powdered milk and then incubated with the primary antibodies (Cell Signaling Technology, Beverly, MA, USA) against phospho-Erk1/2, phospho-p38, phospho-Akt, Erk1/2, p38 and Akt at a 1:1000 dilution. This was followed by incubation with a secondary peroxidase-conjugated anti-rabbit immunoglobulin (Jackson ImmunoResearch, West Grove, PA, USA) at a 1:2500 dilution. Immunopositive bands were visualized using the ECL kit (Amersham Bioscience, Uppsala, Sweden). Band intensities were measured using TINA2.0 software (TINA, Straubenhardt, Germany). The data were nor-

malized to the corresponding value of total protein and expressed as relative intensity. All assays were performed in duplicate on two separate experiments.

Statistical analysis

Statistical analysis was performed using the SPSS 12.0 statistical package program (SPSS Inc., Chicago, IL, USA). The results, between groups, of the RT-PCR quantitative densitometric analyses and of absorbance in the MTT assay were compared using one-way analysis of variance and Tukey's *post hoc* test. A p value of < 0.05 was considered to indicate a statistically significant difference.

Results

Expression of HB-EGF and EGF receptors in human periodontal ligament cells

It was first determined, via RT-PCR analysis, whether wounding affects the endogenous expression of HB-EGF and EGF receptors 1–4 in human periodontal ligament cells. A monolayer of periodontal ligament cells was scratch wounded, and RT-PCR was conducted 0, 10, 30 and 60 min after wounding (Fig. 1A,B). The results indicated that periodontal ligament cells expressed extremely low levels of HB-EGF mRNA prior to wounding and 10 min after wounding. However, the wounded periodontal ligament cells showed a gradual increase of HB-EGF mRNA expression, to 2.4-fold ($p < 0.05$) and 3.3-fold ($p < 0.001$) at 30 and 60 min, respectively, compared with the expression observed at 0 min (Fig. 1A,C). In the case of EGF receptors, periodontal ligament cells expressed EGF receptor 1 and 3 mRNA prior to wounding, and the wounded periodontal ligament cells showed no changes in EGF receptor 1 and 3 mRNA expression for at least 60 min (Fig. 1B,C). Interestingly, the expression of EGF receptor 2 and 4 mRNA in periodontal ligament cells was undetectable prior to and after scratch wounding, even after 40 cycles of RT-PCR (Fig. 1B). A positive control was utilized to test the primers of EGF receptors 2 and 4, as no bands appeared (Fig. 1B). This finding indicated that scratch wounding significantly up-regulates the expression of HB-EGF mRNA, but does not alter the expression of EGF receptors 1 to 4.

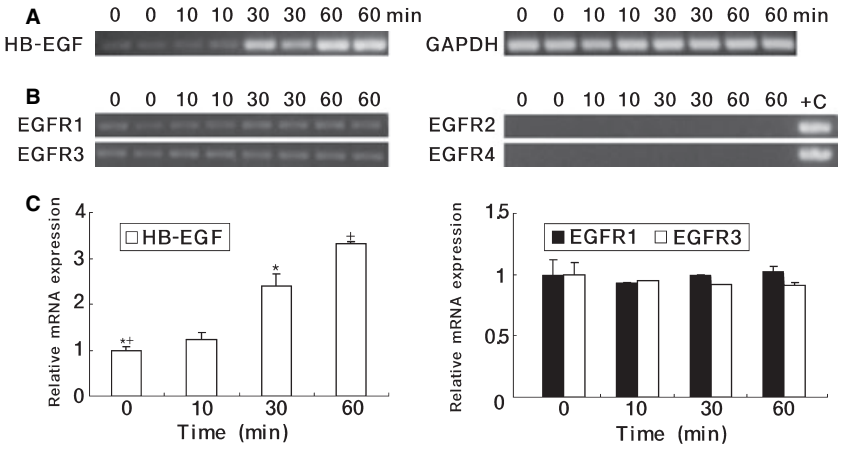

Fig. 1. Time course of the mRNA expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF) and epidermal growth factor (EGF) receptors 1–4 in human periodontal ligament cells after wounding. Human periodontal ligament cells were cultured to confluence, and a monolayer of periodontal ligament cells was scratch wounded. RNA was isolated in duplicate from the cells remaining on the dish at 0, 10, 30 and 60 min after scratch wounding, and semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was run in duplicate for HB-EGF (A), EGF receptors 1–4 (B) and glyceraldehyde-3 phosphate dehydrogenase as a loading control (A) using the primers and conditions listed in Table 1. PCR amplification of positive controls (+C) for EGF receptors 2 and 4 was performed using pHuEGFR2 or pHuEGFR4. (C) The expression value at 0 min was set as 1 to permit comparison of the relative mRNA expression levels with those at subsequent time-points The error bars represent the standard error of the mean. The symbols (★, +) indicate significant differences at $p < 0.05$ and $p < 0.001$, respectively. EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; HB-EGF, heparin-binding epidermal growth factor-like growth factor.

Effects of HB-EGF on human periodontal ligament cell proliferation

In order to determine the effects of HB-EGF on the proliferation of human periodontal ligament cells, periodontal ligament cells were treated with HB-EGF at the indicated concentrations, and the proliferation was evaluated at days 3 and 5 via the MTT assay (Fig. 2). HB-EGF induced proliferation in a dose-dependent manner up to a concentration of 10 nM, but proliferation was reduced significantly at a concentration of 50 nM ($p < 0.05$) (Fig. 2). On day 3, the proliferation of periodontal ligament cells in the presence of 10 nM HB-EGF had increased significantly, to 33.5% ($p < 0.05$), and proliferation on day 5 had increased significantly, to 83.6% ($p < 0.05$), compared with the control (Fig. 2). Therefore, HB-EGF has a stimulatory effect on the proliferation of human periodontal ligament cells, and the optimal concentration of HB-EGF is 10 nM.

Effect of HB-EGF on periodontal ligament cell repopulation

The effect of HB-EGF on the periodontal ligament cell repopulation was determined via a cell repopulation assay in order to assess the ability of periodontal ligament cell migration to close the wounded gap for 24 h after the human periodontal ligament cells were mechanically scratch wounded. The wounded gap of periodontal ligament cells closed more effectively at higher doses of HB-EGF (Fig. 3A), thereby suggesting that HB-EGF promotes periodontal ligament cell

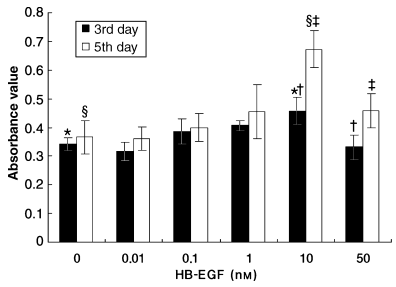

Fig. 2. Effect of heparin-binding epidermal growth factor-like growth factor (HB-EGF) on human periodontal ligament cell proliferation. Periodontal ligament cells were grown for 1 d in α-modified Eagle's minimal essential medium containing a low concentration of serum (2% fetal bovine serum). The culture medium was then replaced with α-modified Eagle's minimal essential medium containing HB-EGF at concentrations of 0, 0.01, 0.1, 1, 10 or 50 nM. Cell proliferation was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 3 d (black bar) and 5 d (white bar). The error bars represent the standard error of the mean. The symbols (★, §, † and ‡) indicate significant differences at $p < 0.05$. HB-EGF, heparin-binding epidermal growth factor-like growth factor.

repopulation in a dose-dependent manner. Furthermore, the induction of cell repopulation by 10 nM HB-EGF in the periodontal ligament cells was blocked by AG1478 (EGF receptor 1 inhibitor) or CRM197 (HB-EGF inhibitor) (Fig. 3B). The human HB-EGF precursor has been determined to be the endogenous receptor for diphtheria toxin (15,29). CRM197, a mutated form of the diphtheria toxin, conveys no toxicity, but it neutralizes the binding of HB-EGF to EGF receptor and can be used as a specific HB-EGF inhibitor. These results suggest that the specific interaction of HB-EGF with EGF receptor 1 is crucial for human periodontal ligament cell repopulation.

Phosphorylation of Erk1/2, p38 MAPK and Akt in human periodontal ligament cells

In order to characterize the mechanism underlying the repopulation of periodontal ligament cells, we assessed the activation of Erk1/2, p38 MAPK and Akt. These cellular signals are known to act downstream of EGF receptor signaling, or are implicated in the mediation of wound healing in other cell types. Periodontal ligament cells were cultured, scratch wounded and incubated in the absence or presence of 10 nM HB-EGF for the time periods indicated. The phosphorylation levels and total protein amounts of Erk1/2, p38 MAPK and Akt were determined via immunoblot analysis.

As shown in Fig. 4A,C, scratch wounding alone, without HB-EGF treatment, increased the phosphorylation of Erk1/2 and p38 MAPK, whereas Akt phosphorylation was not altered. The initial increase (*c.* 1.6-fold) observed at 5 min of Erk1/2 phosphorylation declined slowly to control levels (*c.* 1.1-fold) at a later time-point (120 min). In the case of p38 MAPK, the increased phosphorylation (*c.* 1.4-fold) was observed only at 5 min and diminished to approximately 1.1-fold at 10 min, thereby indicating a short period of activation. HB-EGF administered after scratch wounding enhanced the phosphorylation of Erk1/2 by more than twofold in comparison with the phosphorylation (*c.* 1.6-fold) observed after scratch wounding only (Fig. 4B,C). Furthermore, the induced phosphorylation of Erk1/2 and p38 MAPK persisted for longer, up to 240 and 30 min, respectively, than the 120 and 5 min observed after scratch wounding alone. In the case of Akt, HB-EGF applied after scratch wounding did not significantly increase Akt phosphorylation, nor did wounding alone. In an effort to determine whether the phosphorylation of Erk1/2 and p38 MAPK induced by HB-EGF requires EGF receptor 1 activation, periodontal ligament cells were pretreated with AG1478 (EGF receptor 1 inhibitor) prior to treatment with HB-EGF. The HB-EGF-induced phosphorylation of Erk1/2 and p38 MAPK was almost completely blocked by AG1478 at an early time-point and later was slightly induced (Fig. 4B,C), thereby showing that the interaction between HB-EGF and EGF receptor 1 activate the Erk1/2 and p38 MAPK pathways.

Involvement of Erk1/2 and p38 MAPK pathways in the cell repopulation induced by HB-EGF

In order to determine whether the stimulatory effects of HB-EGF on the process of periodontal ligament cell repopulation require the Erk1/2 and p38 MAPK pathways, a cell repopulation assay was conducted to measure the inhibition of HB-EGF-induced periodontal ligament cell repopulation by PD98059 (Erk1/2 inhibitor) or SB203580 (p38 inhibitor). At 24 h, HB-EGF-induced cell repopulation was blocked by treatment with PD98059 or SB203580, almost to control levels (Fig. 5A). At 48 h, the migration was still inhibited by PD98059 to control levels, but the SB203580-treated samples showed significantly better migration characteristics than the controls. We noted that none of them exhibited toxicity (data not shown). This finding indicates that HB-EGF promotes periodontal ligament cell repopulation, principally via the Erk1/2 pathway, and the inhibition of p38 activation probably delays the process induced by HB-EGF.

Discussion

Periodontitis is characterized by the loss of periodontal supporting tissues. The ultimate goal of periodontal therapy is regeneration of the lost supporting dental apparatus, including the alveolar bone, periodontal ligament and cementum surrounding a previously diseased tooth, in addition to the control and elimination of the infection. Therefore, a broad range of therapeutic procedures has been developed to achieve this primary goal. Periodontal treatment includes conventional methods, such as scaling and root planing, and periodontal surgery with or without osseous surgery, as well as some more novel approaches, including root conditioning agents, guided tissue regeneration, the use of different grafting materials and combinations of these approaches (30). Advances in molecular and cellular biology have resulted in the study of the potential roles of growth factors such as HB-EGF in the promotion of

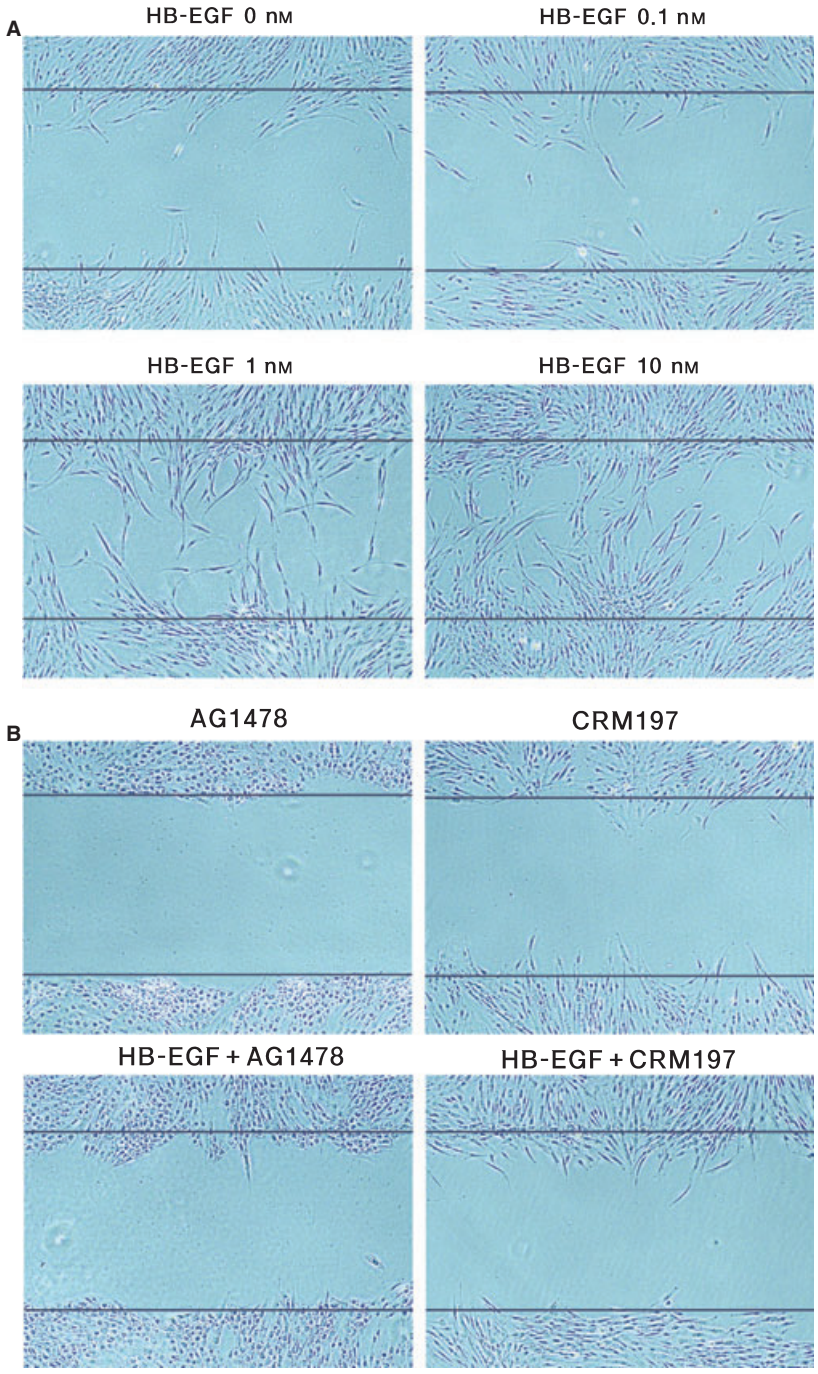

Fig. 3. Effects of heparin-binding epidermal growth factor-like growth factor (HB-EGF) on human periodontal ligament cell repopulation. A monolayer of serum-starved human periodontal ligament cells was scratch-wounded and cultured (A) in the presence of HB-EGF at concentrations of 0, 0.1, 1 and 10 nM, or (B) in the presence of 10 μM AG1478 alone, 10 μg/mL of CRM197 alone, 10 nM HB-EGF + 10 μM AG1478, and 10 nM HB-EGF + 10 μg/mL of CRM197. Cell repopulation was photographed 24 h after wounding. The lines represent the position of the scratch wound. HB-EGF, heparin-binding epidermal growth factor-like growth factor.

periodontal regeneration and their use in alternative therapeutic approaches. Also, an avulsion may cause damage to the attachment apparatus in periodontal ligament. Because the viable cells of periodontal ligament attached to the avulsed teeth are important for successful replantation, the optimal storage medium containing growth factors for the avulsed tooth should be capable of preserving the viability, mitogenicity and clonogenic capacity of injured periodontal ligament cells and their progenitors.

Growth factors have been reported to influence the regeneration of periodontal ligament cells. EGF, a member of the EGF family, induces a mitogenic response in human periodontal ligament cells, which appears to be associated with the rapid and selective activation of the Erk1/2 pathway (31). Platelet-derived and fibroblast growth factors induce mitogenic and chemotactic responses, which are extremely important during the healing and repair of periodontal tissues (6,30,32). It was also determined that transforming growth factor-β1 enhances the proliferation of human periodontal ligament and gingival fibroblasts at a concentration of 10 ng/mL after 48 h of cell culture (33). Another set of biological mediators – insulin-like growth factors I and II – regulate many cellular events, and may enhance the healing and regeneration of periodontal wounds by altering periodontal ligament cell turnover (34,35), and insulin-like growth factor I can synergistically interact with other growth factors, including platelet-derived growth factor, transforming growth factor-β1 and basic fibroblast growth factors, thereby enhancing the regeneration of periodontal soft and hard tissue (6,30,36).

Cell repopulation is critical in the wound-healing process. The purpose of the *in vitro* cell repopulation assay was to examine the ability of cells to migrate towards the center of a scratch wound in cultured cells. The scratch wound on the cell monolayer creates a situation of injury. Immediately after wounding, the cells move across the scratch-wound margin. The complex interactions of cells with extracellular matrix play crucial roles in mediating and regulating the wound-healing process. Integrins are the principal cell-surface adhesion receptors mediating cell–extracellular matrix adhesion. Integrins are heterodimeric receptors

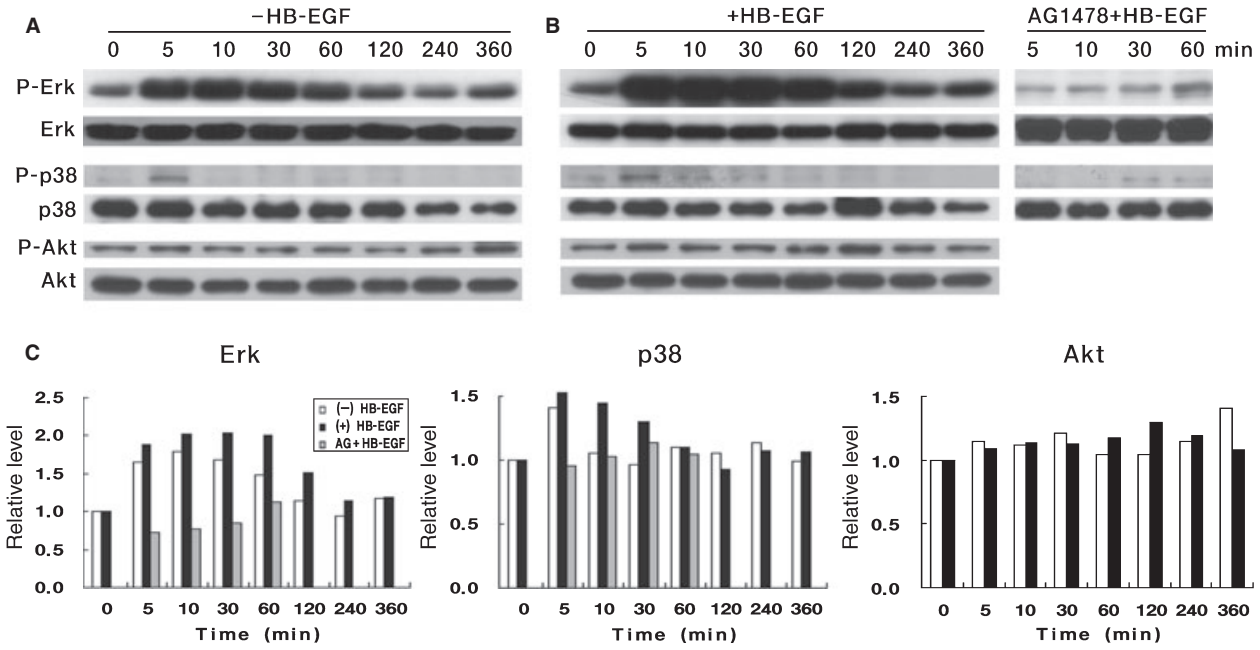

Fig. 4. Effects of heparin-binding epidermal growth factor-like growth factor (HB-EGF) on wound-induced phosphorylations of extracellular signal-regulated kinase (Erk)1/2, p38 mitogen-activated protein kinase (MAPK) and Akt. Serum-starved human periodontal ligament cells were scratch wounded and cultured at the time-points indicated without (A) and with (B) 10 nM HB-EGF. For treatment with AG1478 (an epidermal growth factor receptor 1 inhibitor), periodontal ligament cells were pretreated for 20 min with 1 μM AG1478. Then, 10 nM HB-EGF was added in the continuous presence of the inhibitor and cultured for the periods of time shown. Cell lysates were subjected to immunoblot analysis, with each antibody recognizing the phosphorylation of Erk1/2, p38 MAPK or Akt (upper panel) and with each antibody recognizing total Erk1/2, p38 MAPK or Akt as loading controls (lower panel). (C) The phosphorylation value at 0 min was set as 1 to permit comparison with the relative phosphorylation levels at subsequent time-points. Erk, extracellular signal-regulated kinase; HB-EGF, heparin-binding epidermal growth factor-like growth factor; P, phosphorylation.

generated by selective pairing between 18 α and 8 β subunits. There are 24 distinct integrin receptors that bind various extracellular matrix ligands with different affinities (37). Cells *in vivo* encounter multiple extracellular matrix proteins, such as several collagen isoforms, laminin isoforms and fibronectin. Even though cells grown in two-dimensional vs. three-dimensional matrices have dramatic differences in organization (38,39), the cells adapted to grow *in vitro* are routinely cultured on a two-dimensional substratum. For studies of the cell–extracellular matrix interaction in wound healing, cellular responses to growth have been characterized on a two-dimensional substratum coated with extracellular matrix proteins.

In the present study, we utilized human periodontal ligament cells to investigate the effects of HB-EGF on cell repopulation and to establish the underlying mechanisms by which HB-EGF regulates periodontal ligament cell repopulation. In the current study, it was shown that the periodontal ligament cells expressed extremely low levels of HB-EGF mRNA, but that scratch wounding significantly induced the expression of HB-EGF mRNA. In the case of EGF receptor, the periodontal ligament cells, prior to scratch wounding, expressed two EGF receptors – EGF receptor 1 and EGF receptor 3 – whereas the expression of mRNA for EGF receptors 2 and 4 was undetectable. Scratch wounding did not alter the mRNA expression of EGF receptors 1–4. Therefore, EGF receptor 1 should be considered as a major receptor for HB-EGF in the wound response of periodontal ligament cells, because HB-EGF is capable of binding only to EGF receptors 1 and 4. Treatment with exogenous HB-EGF promoted, in a dose-dependent manner, the processes of periodontal ligament cell proliferation and repopulation. Additionally, the EGF receptor inhibitor, AG1478, and the HB-EGF inhibitor, CRM197, blocked the cell repopulation promoted by HB-EGF. This finding shows that the specific interaction occurring between HB-EGF and EGF receptor 1 is critical for the repopulation of periodontal ligament cells. Taken together, wounding may enhance the interaction between HB-EGF and EGF receptor 1 by increasing the expression of HB-EGF, and may subsequently enhance the process of cell repopulation. The phosphorylation of EGF receptor in the context of ligand binding results in the activation of effectors, including Erk and phosphatidylinositol 3-kinase. Erk is the best-characterized cascade, and has been shown to mediate proliferation and chemotaxis in a variety of cell types (40–43). Phosphatidylinositol 3-kinase activates several downstream kinases, and Akt is a principal substrate of phosphatidylinositol

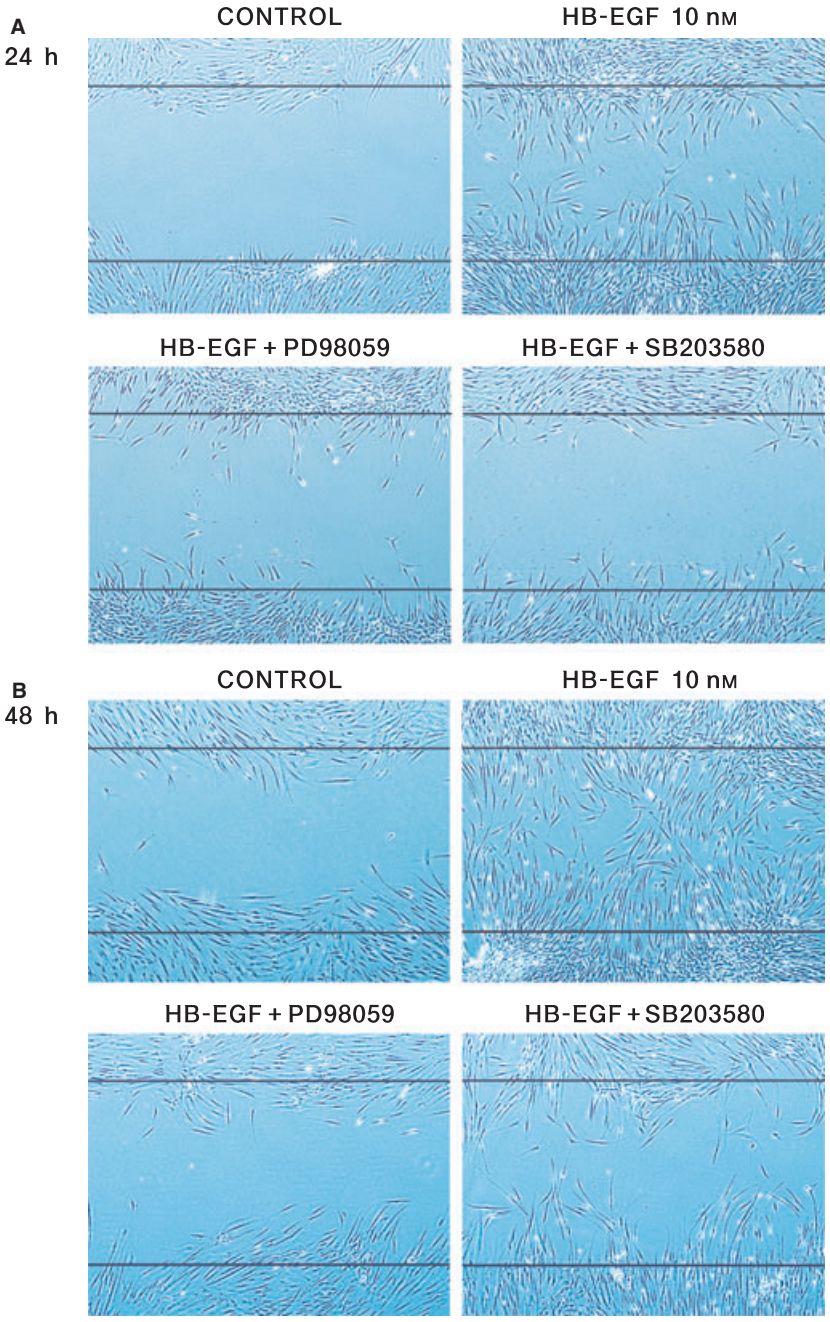

Fig. 5. Human periodontal ligament cell repopulation assay with the combination of heparin-binding epidermal growth factor-like growth factor (HB-EGF) and extracellular signal-regulated kinase (Erk)1/2 or p38 inhibitor. A monolayer of serum-starved human periodontal ligament cells was scratch wounded and cultured for 24 h (A) or 48 h (B) in the absence of HB-EGF as a control, and in the presence of 10 nM HB-EGF alone, 10 nM HB-EGF + 30 μM PD98059 (Erk1/2 inhibitor), or 10 nM HB-EGF + 10 μM SB203580 (p38 inhibitor). Cell repopulation was photographed 24 and 48 h after wounding. The lines represent the position of the scratch wound. HB-EGF, heparin-binding epidermal growth factor-like growth factor.

3-kinase. Phosphatidylinositol 3-kinase /Akt has been suggested to be critical for the generation of cell polarity and the regulation of cell migration, particularly in the control of the direction of chemotaxis (44,45). Another p38 MAPK is usually associated with stress stimuli, but has also been shown to stimulate proliferation and migration (43,46). In the present study, scratch wounding activated Erk1/2 and p38 MAPK phosphorylation, but did not activate Akt phosphorylation, and exogenous HB-EGF treatment amplified and extended the Erk1/2 and p38 MAPK signals in response to scratch wounding, although it did not amplify the Akt signal. Moreover, the inhibition of Erk1/2 by PD98059 blocked the cell repopulation promoted by HB-EGF, whereas the inhibition of p38 MAPK by SB203580 probably delayed it. It is worth noting that HB-EGF treatment induced no increases in the expression of Erk1/2, p38 MAPK and Akt, because their total protein expression was not altered by HB-EGF treatment. The results indicate that Erk1/2 is a principal pathway in the cell repopulation induced by HB-EGF. Consistent with our results, Fitsialos *et al*. recently demonstrated that the inhibition of the Erk pathway completely blocks wound closure in normal human keratinocytes, and that the inhibition of p38 MAPK only delays wound healing (43). They suggest that, unlike Erk1/2, p38 may not directly engage in the immediate scratch-generated signaling, but is probably involved in the propagation of events induced immediately after scratch wounding. Interestingly, they also reported that phosphatidylinositol 3-kinase/Akt inhibition accelerates scratch closure. This may explain, in part, our observation that HB-EGF did not activate Akt signaling.

Ellis *et al*. (47) reported that the scratch wounding of epithelial cell monolayers induces HB-EGF mRNA expression by a mechanism that probably requires the activation of Erk1/2. Consistent with the findings of Ellis' study, our study detected similar timing of HB-EGF induction and Erk1/2 phosphorylation after wounding. HB-EGF mRNA expression was induced 30 min after scratch wounding, whereas the phosphorylation of Erk1/2 was induced more rapidly, at 5 min, and the level of induction was maintained for up to 120–240 min. Therefore, it remains possible that HB-EGF mRNA expression may be induced by Erk1/2 activation in human

periodontal ligament cells, as previously reported by Ellis *et al*., and that the induced HB-EGF expression, in turn, may activate Erk1/2 phosphorylation at a later point and may maintain Erk1/2 activation for a prolonged duration. It has been reported that HB-EGF mRNA levels increase in response to tissue damage, such as skin wounding (26,27), lungs with hyperoxia (22), kidneys subjected to ischaemia/reperfusion injury (23) and liver after partial hepatectomy or chemical damage (24,25), thereby suggesting a physiological role for induced HB-EGF expression in promoting repair after injury.

Our results suggest that HB-EGF may constitute a critical factor in wound healing in human periodontal ligament cells, and may operate through a mechanism requiring Erk activation via specific interaction with EGF receptor 1. Therefore, HB-EGF may potentially be applied as a therapeutic agent for periodontal remedies and as a prereplantation conditioner for avulsed teeth in storage media.

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