Combination of ions promotes cell migration via extracellular signal-regulated kinase 1/2 signaling pathway in human gingival fibroblasts

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Abstract. Wound healing is a dynamic process that involves highly coordinated cellular events, including proliferation and migration. Oral gingival fibroblasts serve a central role in maintaining oral mucosa homeostasis, and their functions include the coordination of physiological tissue repair. Recently, surface pre-reacted glass-ionomer (S-PRG) fillers have been widely applied in the field of dental materials for the prevention of dental caries, due to an excellent ability to release fluoride (F). In addition to F, S-PRG fillers are known to release several types of ions, including aluminum (Al), boron (B), sodium (Na), silicon (Si) and strontium (Sr). However, the influence of these ions on gingival fibroblasts remains unknown. The aim of the present study was to examine the effect of various concentrations of an S-PRG filler eluate on the growth and migration of gingival fibroblasts. The human gingival fibroblast cell line HGF-1 was treated with various dilutions of an eluent solution of S-PRG, which contained 32.0 ppm Al, 1,488.6 ppm B, 505.0 ppm Na, 12.9 ppm Si, 156.5 ppm Sr and 136.5 ppm F. Treatment with eluate at a dilution of 1:10,000 was observed to significantly promote the migration of HGF-1 cells. In addition, the current study evaluated the mechanism underlying the mediated cell migration by the S-PRG solution and revealed that it activated the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), but not of p38. Furthermore, treatment with a MEK inhibitor blocked the cell migration induced by the solution. Taken together, these results suggest that S-PRG fillers can stimulate HGF-1 cell migration via the ERK1/2 signaling pathway, indicating that a dental material containing this type of filler is useful for oral mucosa homeostasis and wound healing.

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Introduction

Human gingival fibroblasts (HGFs) are the most abundant resident cells in the periodontal tissue (1), and serve pivotal roles in the maintenance of tissues and oral wound healing. When tissue damage occurs, the wound healing process begins immediately to prevent further damage or infection. This is a well-coordinated complex process that involves four sequential overlapping phases, including hemostasis, inflammation, proliferation and remodeling (2), which are orchestrated by cross-talking of various cytokines, chemokines, growth factors and cells that participate in the process (3). Among these, the proliferation phase is characterized by migration and subsequent proliferation of fibroblasts, and is important for proper healing and rebuilding of damaged areas in the wound. Subsequently, fibroblasts secrete a new collagen matrix and participate in wound closure by formation of granulation tissue in preparation for the last remodeling phase. Thus, fibroblasts have crucial roles in wound healing, and elucidation of their associated characteristics is anticipated to lead to the development of appropriate therapeutic agents.

Surface pre-reacted glass-ionomer (S-PRG) fillers contain a stable glass ionomer that is generated by the reaction of fluoro-aluminosilicate glass with polyacrylic acid (4). These fillers have been developed as GIOMER products for use as dental materials, such as fissure sealants (5), direct filing composite resins (6), and tooth bonding and coating materials (7). S-PRG fillers are characterized by the ability to release and recharge fluoride (F), which makes them attractive materials for the prevention of secondary dental caries (4). Furthermore, S-PRG fillers release multiple other ions, including aluminum (Al), boron (B), sodium (Na), silicon (Si), and strontium (Sr), which have been reported to be effective in the prevention of oral bacteria adhesion (8),

suppression of biofilm formation (9), resistance to acid demineralization (10) and protection against dental caries (5,11,12). Recently, an eluate solution from S-PRG fillers was demonstrated to have a suppressive effect on periodontal disease in model mice (13). Thus, the use of S-PRG fillers containing dental materials may benefit oral health. However, to the best of our knowledge, no known studies have examined the influence of the multiple ions released from such fillers on HGFs.

In the current study, the effects of multiple ions released from S-PRG fillers on the proliferation, migration and signaling of the HGF-1 cell line were investigated. This is the first report to demonstrate the promotion of HGF-1 migration via the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway, induced by multiple ions present in the solution eluted from S-PRG fillers. These results suggested that the combination of multiple ions promotes cell migration, which assists in oral wound healing.

Materials and methods

Reagents. The mitogen-activated protein kinase kinase (MEK) inhibitor U0126 (cat. no. 9903), anti-p44/42 antibody (cat. no. 9102), anti-phosphorylated (p)–p44/42 antibody (cat. no. 9101S), anti-p38 MAPK antibody (cat. no. 86905), anti-p-p38 MAPK antibody (cat. no. 9215) and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G secondary antibody (cat. no. 7074) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (cat. no. 152121-47-6) and β-actin antibody (cat. no. GTX629630) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and GeneTex, Inc. (Irvine, CA, USA), respectively. The multiple-ion solution (cat. no. 041402) eluted from S-PRG fillers was provided by Shofu Dental Corporation (Kyoto, Japan). For the preparation of the multiple-ion solution, S-PRG filler was mixed with an equal volume of distilled water by a tumbler mixer at 23˚C for 24 h, followed by centrifugation at 3,000 x g and 23˚C for 6 h to separate the filler and the liquid. Next, the supernatant was filtered to remove any residual insoluble material and used as the S-PRG elute. The multiple-ion solution contained 32.0 ppm Al, 1,488.6 ppm B, 505.0 ppm Na, 12.9 ppm Si, 156.5 ppm Sr and 136.5 ppm F. The S-PRG elute was diluted with a 1:1 mixture of Dulbecco's Modified Eagle's medium (DMEM) and F12 nutrient mixture (DMEM/F-12, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at various dilution ratios (0, 1:100, 1:1,000 and 1:10,000).

Cell culture. HGF-1 cells were purchased from ScienCell Research Laboratories, Inc. (cat. no. 2620; San Diego, CA, USA). The cells were cultured in DMEM/F-12, supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France). Cultured fibroblasts were maintained at 37˚C in a humidified atmosphere of 5% CO_2. For the experiments, HGF-1 cells were cultured in DMEM/F-12 containing the multiple ion solution at various dilution ratios.

Cell proliferation. The influence of the multiple-ion solution on HGF-1 cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8) purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Briefly, cells were seeded into a 96-well plate at a density of 1,000 cells/well, and then incubated with various dilutions of the multiple-ion solution (0, 1:100, 1:1,000 and 1:10,000) for 24, 48 or 72 h. At each time point, 10 μl of solution from the CCK-8 kit was added to each well and then incubation was continued in an atmosphere that included 5% CO_2 at 37˚C for 1 h. The absorbance at 450 nm was measured using a plate reader (MultiSkan FC Basic; Thermo Fisher Scientific, Inc.).

Cell proliferation was also assessed using a cell count method. For this, HGF-1 cells were seeded at a density of $1.0x10^3$ cells/well in a 24-well plate and maintained with various concentrations of the multiple-ion solution (0, 1:100, 1:1,000 and 1:10,000) for 24, 48 or 72 h. Subsequently, the total cell number was counted in ten randomly selected fields of view in a 24-well plate under an inverted microscope (magnification, x20).

Cell migration. For *in vitro* cell migration assays, ibidi Culture-Inserts (ibidi GmbH, Martinsried, Germany) were used in a 35 mm dish. The ibidi Culture-Insert has two cell culture wells, which are separated by a 500-μm wall. HGF-1 cell suspension (70 μl; $5x10^5$ cells/ml) was added to each well on the two sides of the culture insert and cultured with DMEM/F-12 supplemented with 10% FBS for 24 h at 37˚C. Then, the Culture Insert was gently removed with sterile tweezers, and cells were cultured with various concentrations of the multiple-ion solution (0, 1:100, 1:1,000 and 1:10,000) in medium containing 5 or 10% FBS for 12, 16 and 22 h at 37˚C. Cell migration was observed and recorded using a Nikon inverted microscope system (Nikon ECLIPSE TE2000-U; Nikon Corporation, Tokyo, Japan). To quantify cell migration, the uncovered area in which no cells were present was measured using ImageJ 1.48v (National Institutes of Health, Bethesda, MD, USA).

Furthermore, in order to assess the effect of inhibitors on cell migration, HGF-1 cells were cultured in the presence of the multiple-ion solution (diluted to 1:10,000) with or without 10 μM U0126 (MEK inhibitor) or SB203580 (p38 MAPK inhibitor). Subsequently, cell migration was examined as mentioned earlier. An equivalent volume of dimethyl sulfoxide was used as control treatment.

Western blotting. Following serum deprivation for 1 h, HGF-1 cells were cultured in the presence of the multiple-ion solution (diluted to 1:10,000) for various time periods and then washed three times with ice-cold phosphate-buffer saline containing 1 mM sodium vanadate (Na_3VO_4). Next, the cells were solubilized with lysis buffer (containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl F and 20 units/ml aprotinin). The lysed cells were centrifuged at 11,177 x g for 5 min at 4˚C, and the protein concentration in each sample was measured using a micro-BCA protein assay reagent (Pierce; Thermo Fisher Scientific, Inc.). The samples were then denatured in SDS sample buffer, and 20 μg/lane of lysate protein was separated via 12% SDS-PAGE. Following separation, the proteins were transferred onto a PVDF membrane and blocked with 5% blocking solution (Cell Biolabs, Inc., San Diego, CA, USA) in PBS-0.05% Tween 20 for 1 h at room temperature (RT). Then, the blotted membrane was incubated for 1 h at RT using

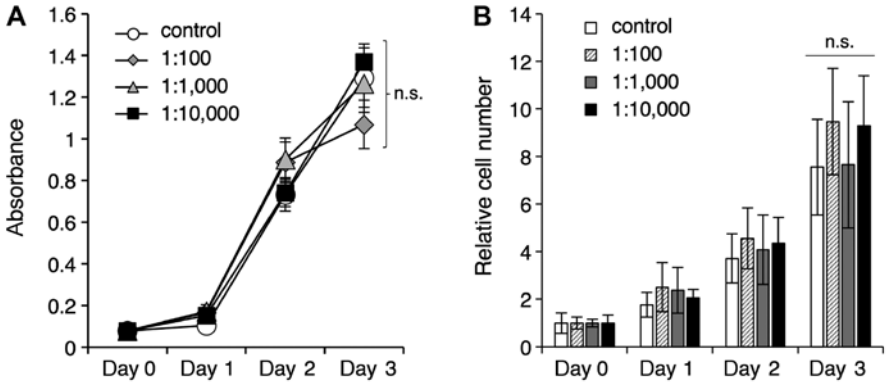

Figure 1. Eluted solution from S-PRG filler containing multiple ions has no effects on HGF-1 proliferation. HGF-1 cells were cultured with various dilutions of the multiple-ion solution (0, 1:100, 1:1,000 and 1:10,000) for 3 days. (A) Cell Counting Kit-8 assay and (B) a cell count method were used to assess cell proliferation. Values are presented as the mean ± standard error of the mean (n=8 in assay A and n=10 in assay B). Repeatability of the results obtained experimentally was confirmed by performing three independent trials. S-PRG, surface pre-reacted glass-ionomer; HGF, human gingival fibroblast; n.s., not significant.

primary antibodies (anti-p44/42, anti-p–p44/42, anti-p38 MAPK, anti-p-p38 MAPK and anti-β-actin; all 1:1,000 in the blocking solution). Membranes were subsequently incubated with the HRP-conjugated secondary antibody (1:2,000) for 1 h at RT. Subsequently, samples were visualized using an enhanced chemiluminescence kit (GE Healthcare Life Sciences, Little Chalfont, UK). The blot images were acquired using an Amersham Imager 600 (GE Healthcare Life Sciences).

Statistical analysis. All experiments were repeated at least three times. All values are presented as the mean ± standard error of the mean. Comparisons between more than two groups were assessed with analysis of variance, followed by Bonferroni multiple comparisons test. Comparisons between two groups were conducted with Student's t-test. Differences with $P<0.05$ were considered as statistically significant.

Results

S-PRG filler solution has no effect on cell proliferation. The solution eluted from the S-PRG filler contained multiple ions, including F, Al, B, Na, Si, and Sr. Initially, the study examined whether this multiple-ion solution at various dilution ratios (1:100, 1:1,000 and 1:10,000) had an effect on the proliferation of HGF-1 cells after 24, 48, and 72 h using a CCK-8 assay (Fig. 1A) and a cell count method (Fig. 1B). At the final time point of 72 h that was examined in the current study, no significant differences were observed in regard to cell proliferation between the control and experimental groups (Fig. 1). These results suggested that the multiple-ion solution eluted from S-PRG fillers did not have an effect on the proliferation of gingival fibroblasts.

S-PRG filler solution promotes cell migration. Proper wound healing requires fibroblast migration; thus, a cell migration assay was conducted in the present study using culture inserts to examine whether the multiple-ion solution eluted from the S-PRG filler had an effect on cell migration. HGF-1 cells were cultured with various dilutions of the multiple-ion solution (1:100, 1:1,000 and 1:10,000) in medium containing 5% or 10% FBS for 12, 16 or 22 h. The results revealed that medium containing multiple-ion solution at a dilution of 1:10,000 and 5% FBS exhibited significant promotion of cell migration at 16 h of incubation, as compared with the control cells (Fig. 2); however, other conditions did not induce an increase in cell migration. These results suggested that the multiple-ion solution eluted from the S-PRG filler promoted the migration of gingival fibroblasts in a concentration and time-dependent manner.

ERK signaling pathway is involved in fibroblast migration induced by S-PRG filler solution. The effect of the multiple-ion solution eluted from the S-PRG filler on intracellular signaling was also examined. MAPKs, including ERK, p38 MAPK and Jun N-terminus kinase (JNK), have a principal role in cell migration (14). In addition, a previous study has suggested that sodium fluoride strongly induced the activation of ERK1/2 and ERK5 signal transduction, rather than that of JNC and p38 in MC3T3-E1 cells (15). Therefore, the current study examined whether the multiple ion-containing solution eluted from the S-PRG filler induced the phosphorylation of ERK1/2 proteins. Marked ERK 1/2 phosphorylation was observed at 5 min after stimulation with the diluted multiple-ion solution, whereas p38 MAPK phosphorylation was not induced at any of the investigated time points up to 60 min after treatment (Fig. 3). These results indicated that activation of ERK1/2, but not p38 MAPK, may be involved in the promotion of HGF-1 cell migration by the multiple-ion solution. Furthermore, the study examined whether the MEK inhibitor U0126 had an effect on cell migration induced by the S-PRG filler solution. Clear inhibition of cell migration was observed following the addition of U0126, as compared with the control (Fig. 4A and B). To confirm the inhibition of ERK activity by U0126, western blotting was performed in cells treated with U0126. It was observed that U0126 evidently inhibited the phosphorylation of ERK1/2 in HGF-1 cells (Fig. 4C). By contrast, the p38 MAPK inhibitor SB203580 had no effect on cell migration (Fig. 4D and E) and the phosphorylation of p38 MAPK (Fig. 4F) in HGF-1 cells. Since phosphorylation of p38 MAPK was not observed in HGF-1 cells treated with a multiple ion-containing solution (Fig. 3), p38 MAPK signaling may not be involved in HGF-1 cell migration. Thus, these results suggested that the multiple-ion solution may promote the migration of gingival fibroblasts via an intracellular ERK1/2 signaling pathway.

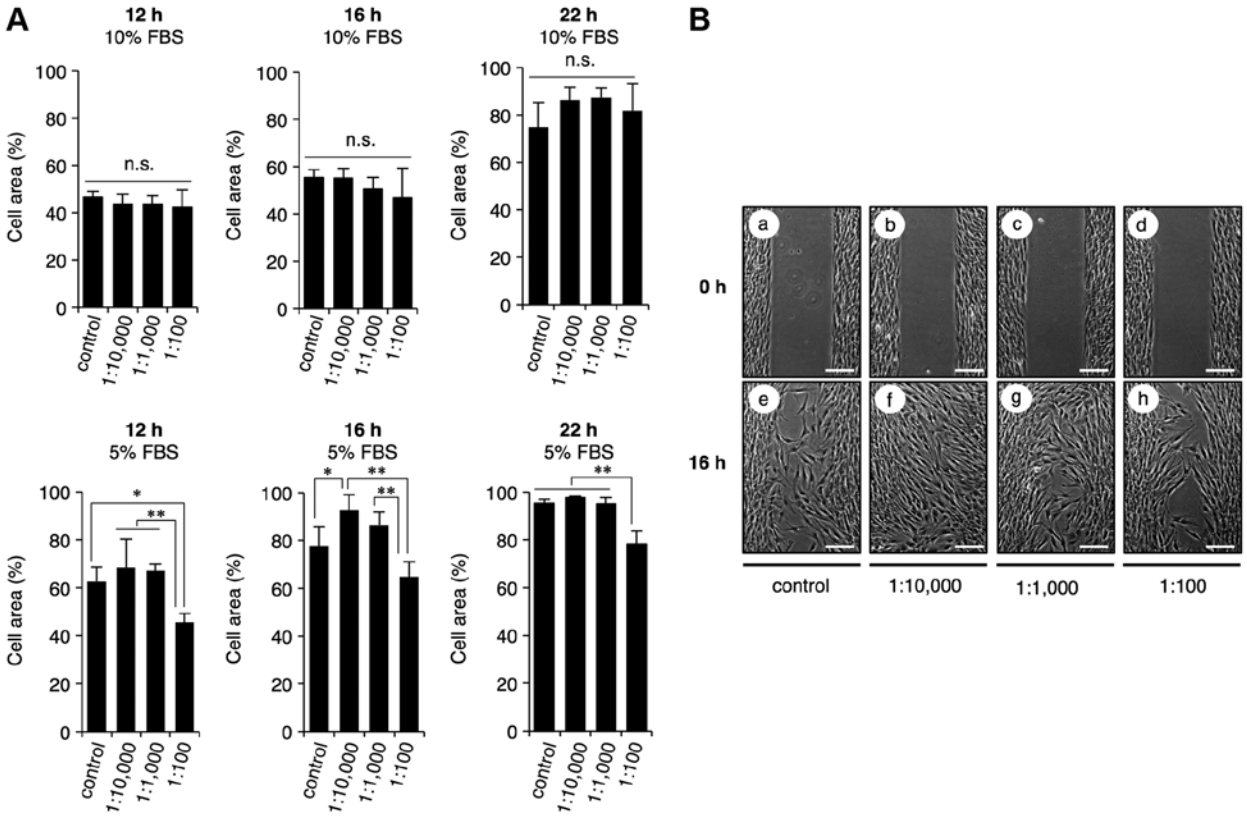

Figure 2. Eluted solution from S-PRG filler containing multiple ions promotes HGF-1 migration. HGF-1 cells were seeded into wells on both sides of a cell culture insert, cultured for 24 h and then exposed to the multiple-ion solution at various dilutions (0, 1:100, 1:1,000 and 1:10,000) containing 5 or 10% FBS at 12, 16 and 22 h for cell migration analysis. (A) Cell area graphs and (B) images of cell culture (magnification, x20; scale bar, 200 μm) at different conditions are shown. Medium containing the multiple-ion solution diluted at a ratio of 1:10,000 and supplemented with 5% FBS was observed to significantly promote cell migration as compared with the control cells at 16 h. To quantify cell migration, the uncovered area was measured using ImageJ software. Values are expressed as the mean ± standard error of the mean (n=4). Repeatability of the results obtained experimentally was confirmed by performing three independent trials *P<0.05 and **P<0.01. S-PRG, surface pre-reacted glass-ionomer; HGF, human gingival fibroblast; n.s., not significant.

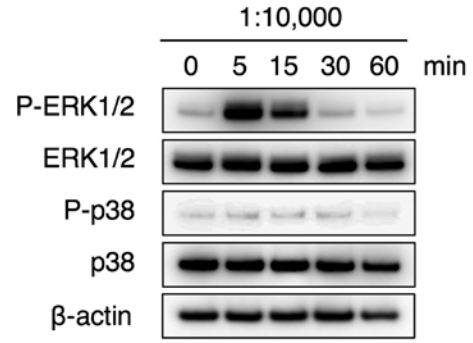

Figure 3. Phosphorylation or ERK and p38 following treatment with multiple ion solution. Following serum deprivation for 1 h, phosphorylation of ERK1/2 in cells was induced by incubation with the solution containing multiple ions that was eluted from the S-PRG filler (dilution, 1:10,000). The time course of ERK1/2 and p38 phosphorylation was analyzed by western blotting. S-PRG, surface pre-reacted glass-ionomer; ERK1/2, extracellular signal-regulated kinase 1/2; p-, phosphorylated.

Discussion

The oral cavity functions as an entry way for the digestive and respiratory systems, in addition to its important roles in daily activities, such as eating, speaking and breathing; thus, it is often exposed to various stimuli or injuries from contact with external factors. Gingival fibroblasts serve crucial roles in maintaining tissue homeostasis and recovery to a normal condition following acute inflammation, migrating to the wound site and proliferating in order to reconstitute connective tissue, events that are regulated by various growth factors and cytokines, including epidermal growth factor (16), basic fibroblast growth factor (bFGF) (17), platelet-derived growth factor (PDGF) (18) and transforming growth factor-beta (TGFβ) (19). The molecular mechanisms of these events are complex and have not been fully elucidated.

In the present study, it was observed that the eluted S-PRG solution containing multiple ions promoted the migration, but not the proliferation, of HGF-1 cells. S-PRG fillers, composed of a powdery reaction product containing polyalkenoic acid and fluorine-containing glass, are characterized by sustained F release and have cariostatic properties (4). In fact, a previous study reported that an S-PRG filler containing flowable resin had a high level of F release as compared with other flowable resins tested (20). Furthermore, S-PRG filler is produced by an acid-base reaction between acid-reactive glass and polyacids in the presence of water (4), and has properties similar to glass ionomer cement (21). Given these properties, S-PRG filler can release Al, B, Na, Si and Sr ions, in addition to F ion, and therefore the solution eluted from this filler contains multiple ions. According to the results of the present study, it is thus suggested that the combination of these multiple ions promotes fibroblast migration.

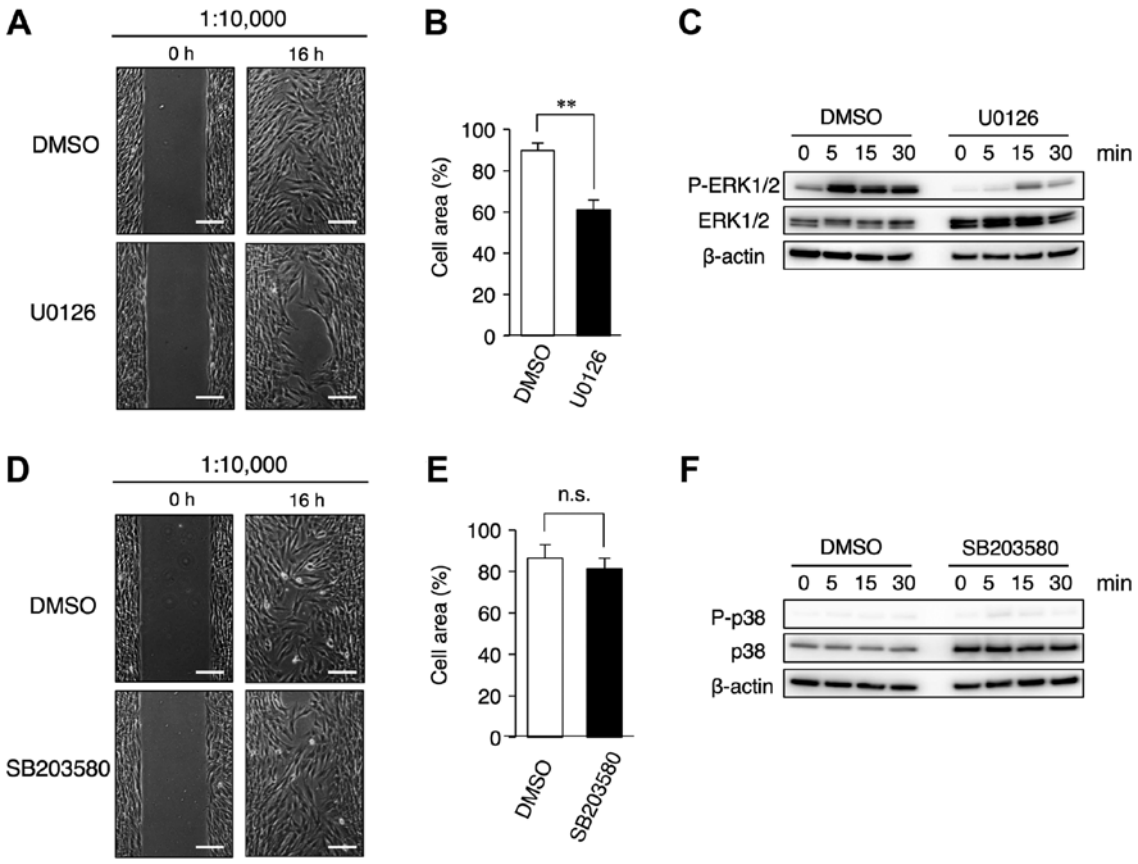

Figure 4. Inhibition of cell migration is induced by co-incubation with a MEK inhibitor and multiple-ion solution eluted from the S-PRG filler. HGF-1 cells were cultured in the presence of solution diluted to 1:10,000, and with or without 10 μM U0126 (a MEK inhibitor) or SB203580 (a p38 MAPK inhibitor) for 16 h. An equal volume of DMSO was added to the control cells. (A) Cell area percentage, (B) cell culture images (magnification, x20; scale bar, 200 μm), and (C) western blot results following incubation with U0126 are shown. (D) Cell area percentage, (E) cell culture images (magnification, x20; scale bar, 200 μm), and (F) western blot results following incubation with SB203580 are displayed. To quantify cell migration, the uncovered area was measured using the ImageJ software. The time course of ERK1/2 and p38 phosphorylation in the presence of U0126 and SB203580, respectively, was analyzed by western blotting. Values are presented as the mean ± standard error of the mean (n=4). Repeatability of the results obtained experimentally was confirmed by performing three independent trials. **P<0.01. S-PRG, surface pre-reacted glass-ionomer; HGF, human gingival fibroblast; ERK1/2, extracellular signal-regulated kinase 1/2; p-, phosphorylated; DMSO, dimethyl sulfoxide; n.s., not significant.

Extracellular ions are extracellular environment factors that have great effects on the physiological activity of cells (22-30). The respective influence of individual ions on cellular activity results in different effects depending on the conditions present, including concentration and combination with other ions. For instance, although Al is known to be a toxic agent (22), a previous study reported that micromolar concentrations of Al ions had a direct effect on osteoblasts, stimulating their proliferation and differentiation (23). Conversely, another study observed that low concentrations of Al ions induced no effects on osteoblast behaviors; however, in combination with titanium ions (Ti), Al enhanced the deleterious effects of Ti on osteoblast differentiation (24). Furthermore, long-term exposure to F ions (>1 mM) inhibited the proliferation of L-929 fibroblasts, whereas short-term exposure stimulated the cell proliferation, with the stimulatory effect further enhanced by 1 μM Al ions (29). A low-dose Sr was also found to stimulate osteogenic differentiation, while higher doses induced apoptosis of human adipose-derived stem cells (30). Thus, extracellular ions have important functions in supporting fundamental cell activities and cell death, and a change in extracellular ion composition can have a great influence, either positive or negative, on affected cells.

ERK1/2, a member of the MAPK family, is a dynamic cell signaling pathway that functions with various cell responses, including proliferation, migration, differentiation and death (31). In the current study, the examined solution eluted from S-PRG filler promoted the phosphorylation of ERK1/2, but not of p38 MAPK. Furthermore, U0126 (a MEK inhibitor), but not SB203580 (a p38 MAPK inhibitor), inhibited cell migration induced by the solution. Several studies have indicated that the p38 inhibitors SB203580 and SB202190 also inhibited cell migration that was induced by pigment epithelium-derived factor PDGF, TGFβ and IL-1β (32-34). These results suggest that p38 is involved in cell migration, while it also has a principal role in growth factor or cytokine-induced cell migration. Although further studies are required to identify the molecular mechanism regulating cell migration in HGF-1, these results indicated that the activation of ERK signaling may be responsible for gingival fibroblast migration induced by the multiple ions in the solution examined in the present study.

Dental restorative materials are designed to have specific characteristics based on the biological, chemical and mechanical properties of their components, and must not trigger inflammation, toxic reactions or allergenic symptoms. However, those materials in fact often cause allergic reactions,

with research in recent decades focusing on dental metals in particular. The intraoral environment is susceptible to sudden changes in temperature and pH caused by ingested food or drink, and is subjected to mechanical or electronic forces caused by occlusion, which have significant influence on the corrosion properties of dental metals (35). Furthermore, oxygen and chloride ions in saliva are involved in corrosion caused by chemical processes (36). The first reported dental metal allergy was in relation to mercury as part of amalgam dental materials, which was demonstrated to cause stomatitis and dermatitis (37). Thereafter, nickel, chromium, palladium and cobalt, commonly used as dental materials, were also reported to have associations with dental metal allergies (38). Resins are frequently used as an alternative dental material instead of metals, including methyl methacrylate, 2-hydroxyethyl methacrylate, ethylene glycol dimethacrylate and triethylene glycol dimethacrylate; however, these can also be a cause of allergies (39,40). Allergic reactions caused by resins are mainly considered to be associated with an unreacted residual monomer (41).

For effective use, biomaterials should be harmless to the body; thus, the concept of the biocompatibility of biomaterials used in regenerative medicine (42), associated with proper response by surrounding tissues, has recently become an important issue. Second generation biomaterials are characterized by their resorbable or biological activity, while third generation materials possess both of these characteristics (43). For instance, hydroxyapatite, a widely-used bone graft biomaterial with excellent biocompatibility and osteoconductive properties, promotes self-healing processes (44). Thus, biomaterials are required to have bioactive properties to induce and regulate specific cellular responses at the molecular level, indicating that biocompatibility is an essential concept for the design of dental materials.

A limitation of the current study is that only *in vitro* experiments were conducted to evaluate the effect of the multiple ion-containing solution eluted from the S-PRG filler. Therefore, it is possible that a multiple-ion solution at a higher dilution than 1:10,000 or a different combination of ions may have a greater effect on cell migration. Furthermore, the current study did not focus on which ion eluted from filler affected HGF-1 cell migration. Mechanistic analysis to address whether the eluted multiple ions had a direct or indirect effect on cell migration is also lacking. The individual ion function and different combination of ions should be assessed in further studies.

In conclusion, the results of the present study demonstrated that the multiple ion-containing solution eluted from S-PRG filler promoted the migration of HGF-1 cells via the ERK1/2 signaling pathway, potentially promoting oral mucosa wound healing. These findings suggested that the application of such an S-PRG filler may contribute not only to the prevention of dental caries, but also to homeostasis of the oral mucosa. In addition, the present study provides useful information for the development of novel therapeutic drugs for oral diseases with materials composed of multiple ions.

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Availability of data and materials

The datasets generated and analyzed in this study are available from the corresponding author upon reasonable request.

Authors' contributions

TI conceived and designed the experiments, analyzed the data, and drafted the manuscript. KYU, YA, KK, AS, HN, AM, RK, KI, TK and TH performed the experiments, analyzed the data, prepared the figures and reviewed cited literature. AY and SF analyzed the data and reviewed drafts of the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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