Role of Mechanical Stress-induced Glutamate Signaling-associated Molecules in Cytodifferentiation of Periodontal Ligament Cells※§

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In this study, we analyzed the effects of tensile mechanical stress on the gene expression profile of in vitro-maintained human periodontal ligament (PDL) cells. A DNA chip analysis identified 17 up-regulated genes in human PDL cells under the mechanical stress, including HOMER1 (homer homolog 1) and GRIN3A (glutamate receptor ionotropic N-methyl-D-aspartate 3A), which are related to glutamate signaling. RT-PCR and real-time PCR analyses revealed that human PDL cells constitutively expressed glutamate signaling-associated genes and that mechanical stress increased the expression of these mRNAs, leading to release of glutamate from human PDL cells and intracellular glutamate signal transduction. Interestingly, exogenous glutamate increased the mRNAs of cytodifferentiation and mineralization-related genes as well as the ALP (alkaline phosphatase) activities during the cytodifferentiation of the PDL cells. On the other hand, the glutamate signaling inhibitors riluzole and (+)-MK801 maleate suppressed the alkaline phosphatase activities and mineralized nodule formation during the cytodifferentiation and mineralization. Riluzole inhibited the mechanical stress-induced glutamate signaling-associated gene expressions in human PDL cells. Moreover, in situ hybridization analyses showed up-regulation of glutamate signaling-associated gene expressions at tension sites in the PDL under orthodontic tooth movement in a mouse model. The present data demonstrate that the glutamate signaling induced by mechanical stress positively regulates the cytodifferentiation and mineralization of PDL cells.

The ability of cells to sense and respond to physical stress is required for tissue homeostasis and normal development. In muscle, bone, tendon, periodontium, and the cardiovascular system, applied forces of physiological magnitude regulate cellular processes that are critical for normal tissue and organ functions, such as differentiation, proliferation, and migration

(1). The periodontal ligament (PDL)3 is a connective tissue interposed between the roots of teeth and the inner wall of the tooth-supporting bone (alveolar bone) socket. The PDL constitutively and iatrogenically receives mechanical stress, such as occlusal pressure and orthodontic forces, which have effects on the homeostasis of the PDL (2). Proper mechanical stress on teeth induces not only the proliferation and differentiation of PDL cells into osteoblasts and cementoblasts but also the synthesis and degradation of extracellular matrix (ECM) molecules (3). For example, during orthodontic tooth movement, two types of sites (tension sites and pressure sites) arise around the tooth through the orthodontic force. At the tension sites, the PDL is stretched, and the expressions of bone-related genes, such as osteocalcin (4) and bone sialoprotein (5), are up-regulated, such that bone formation is finally induced on the alveolar bone facing the tooth root (6). On the other hand, at the pressure sites, the PDL is compressed, and osteoclasts are activated. Consequently, resorption of the alveolar bone is induced. An orchestrated balance between bone formation and resorption controls tooth movement (7). In contrast, elimination of mechanical stress on teeth is known to cause atrophy of the PDL in vivo (8). Kaneko et al. (9) reported that loss of occlusal function by extraction of the antagonistic upper molars of rats caused atrophic changes in the PDL of the lower molars, such as narrowing of the space, disorientation of collagen fibers, and decreases in proteoglycans. These findings indicate that mechanical stress on teeth affects the remodeling of the PDL, cementum, and alveolar bone. Thus, it is important to clarify the physiological functions of mechanical stress on the PDL.

To clarify the molecular basis of the mechanical stress-regulated PDL functions, we analyzed the gene expression profile of human PDL cells receiving tensile mechanical stress in vitro. Interestingly, an oligo-DNA chip analysis identified two glutamate signaling-associated genes, HOMER1 (homer homolog 1) and GRIN3A (glutamate receptor ionotropic N-methyl-D-aspartate 3A), among the up-regulated genes. Glutamate is the most abundant amino acid in the central nervous system and plays important roles in neurotransmission (10). Stimulation of

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3 The abbreviations used are: PDL, periodontal ligament; MK801, (+)-MK801 maleate; CREB, cAMP-response element-binding protein; ECM, extracellular matrix; Ca2+; calcium ion; NMDAR, N-methyl-D-aspartate receptor; IP3, inositol 1,4,5-trisphosphate; aRNA, amino allyl RNA; DIG, digoxigenin.
presynaptic cells promotes the release of glutamate, and the released glutamate induces glutamate signaling by binding to glutamate receptors on the postsynaptic cells. In addition to the central nervous system, glutamate signaling has been observed in non-neural tissues, such as the taste buds (11), spleen (12), and bone (13), and modulates various functions of each tissue (14). However, involvement of glutamate signaling in the PDL has not been reported. In the present study, we further analyzed the gene expression and functions of glutamate signaling-associated molecules in PDL cells to elucidate the roles of glutamate signaling in the PDL.

EXPERIMENTAL PROCEDURES

Reagents—α-Modified Eagle’s medium was obtained from Nikken Biomedical Laboratory (Kyoto, Japan). Fetal calf serum (FCS) was purchased from JRH Biosciences (Lenexa, KS). Riluzole, bisbenzimidazole (Hoechst 33258), and DNA sodium salt (from calf thymus) were products from Sigma. Kanamycin, l-glutamate, β-glycerophosphate, ascorbic acid, and p-nitrophenyl phosphate were obtained from Wako Pure Chemical Industries (Osaka, Japan). (−)-MK801 maleate (MK801) was purchased from Tocris Cookson (Bristol, UK).

Cell Culture—Human PDL cells were isolated as described previously (15). The cells were cultured in α-modified Eagle’s medium supplemented with 10% FCS, 50 units/ml penicillin G, and 50 μg/ml streptomycin at 37 °C under 5% CO2. For the induction of cytodifferentiation, human PDL cells were cultured in α-modified Eagle’s medium with 10% FCS in the presence of 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid (mineralization-inducing medium). The mineralization-inducing medium was replaced every 3 days.

Application of Mechanical Stress—To allow cell attachment, 10-cm2 silicon membrane chambers (Scholertec, Osaka, Japan) were coated with 0.3 mg/ml pepsin-digested collagen type I derived from swine (Nitta Gelatin, Osaka, Japan) according to the Scholertec manufacturer’s instructions. Human PDL cells were transferred to the chambers at a density of 1.5 × 104 cell/cm2 and cultured in α-modified Eagle’s medium with 10% FCS for 3 days. The chambers were applied to a stretch apparatus, Scholertec NS-350 (Scholertec), and the cells were repeatedly stretched and relaxed at 37 °C under 5% CO2 in vitro. The force conditions were 0.5 Hz (30 cycles/min) with 110% elongation for 0, 24, and 48 h. This force represented the physiological conditions of occlusal force (16). Cells seeded on the chambers without stretching served as controls.

Oligo-DNA Chip Analysis—Total RNA (1 μg) extracted from the stretched human PDL cells using RNA Bee (Tel-Test Inc., Friendswood, TX) was targeted to synthesize experimental amino allyl RNA (aRNA) using an Amino Allyn MessageAmp aRNA kit (Ambion Inc., Austin, TX) according to the manufacturer’s protocol. Cy3 or Cy5 fluorescent dyes (Amersham Biosciences) were incorporated into the aRNAs by an indirect labeling method. The labeled aRNAs were fragmented and hybridized to an AceGene Human Oligo Chip 30K (Hitachi Software Engineering Co., Ibaraki, Japan) consisting of 30,000 human oligonucleotide features. After a 12-h hybridization, the DNA chip was washed according to the manufacturer’s instructions, and the fluorescence intensities were scanned with a ScanArray Lite (PerkinElmer Life Sciences). The resulting image was analyzed using Scan Array Express version 2.0. Normalization using LOWELL was performed for data obtained from the raw data.

Real-time RT-PCR Analysis—cDNA was synthesized from the purified total RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer’s instructions. The obtained cDNA was mixed with Power PCR SYBR Master Mix (Applied Biosystems) and gene-specific primers (Takara Bio, Shiga, Japan). The sequences of the primers are shown in supplemental Table 1. Real-time PCR was performed using a 7300 fast real-time RT-PCR system (Applied Biosystems) according to the manufacturer’s instructions. The amplification conditions consisted of an initial 15-min denaturation step at 95 °C, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. The dissociation curves were analyzed to ensure the amplification of a single PCR product. Three independent assays were performed for each primer. The amount of cDNA was calculated for each sample from the standard curve. The relative expression is shown after normalization by the gene expression of HPRT (hypoxanthine phosphoribosyltransferase).

Conventional RT-PCR Analysis—The purified total RNA was reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen) and an oligo(dT) primer (Invitrogen). The synthesized cDNA was mixed with AmpliTaq Gold DNA polymerase (Applied Biosystems) and gene-specific primers synthesized by GeneDesign Inc. (Osaka, Japan). The sequences of the primers are shown in supplemental Table 2. PCR was performed using a PTC-200 Peltier thermal cycler (Bio-Rad). The amplification conditions consisted of an initial incubation at 94 °C for 9 min, followed by cycles of denaturation at 94 °C for 60 s, annealing at the temperatures indicated in supplemental Table 2, and elongation at 72 °C for 60 s. The PCR products were evaluated by agarose gel electrophoresis.

Determination of Released Glutamate—The levels of glutamate were determined by a modification of an enzyme-linked fluorometric assay according to a previously published protocol (17). In the presence of glutamate dehydrogenase (Oriental Yeast Co., Osaka, Japan) and β-nicotinamide adenine dinucleotide phosphate (β-NADP+) (Oriental Yeast Co.), released glutamate is oxidized to α-ketoglutarate with the production of NADPH, which can be determined fluorometrically to quantify the glutamate concentration. Briefly, human PDL cells were grown in 6-well plates or stretched on chambers. The supernatants were collected from each well, filtered with a Minisart 0.2-μm filter (Sartorius Stedim Biotech, Goettingen, Germany), and prewarmed to 25 °C with 7 mM β-NADP+ (pH 7.4). The reaction was initiated by the addition of glutamate dehydrogenase (9 IU/ml). After incubation for 15 min, the absorbance was measured at 320 nm using a GeneQuant pro “S” spectrophotometer (Amersham Biosciences) and compared with standard curves constructed using known concentrations of l-glutamate. The DNA concentrations in the cell layers were determined by subtracting the amount of glutamate in the medium as the absorbance of the background in advance.
Western Blotting Analysis of Phosphorylation of cAMP-response Element-binding Protein (CREB) in Human PDL Cells—Human PDL cells stimulated by glutamate or mechanical stress were lysed with cell lysis buffer (50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mg/ml aprotinin, leupeptin, and pepstatin; 1 mM Na3VO4; 1 mM NaF; 1 μM microcystin). The protein concentrations of the cell lysates were measured using a BCA protein assay kit (Pierce) according to the manufacturer’s instructions. The cell lysates were subjected to 7.5% SDS-PAGE and then electroblotted onto polyvinylidene difluoride membranes. A rabbit anti-phospho-CREB antibody (1:1000; Milli-pore, Temecula, CA) and a rabbit anti-CREB antibody (1:1000, Milli-pore) were used as the primary antibodies. A horseradish peroxidase-linked goat anti-rabbit IgG antibody (Cappel, Aurora, OH) was used as the secondary antibody. Immunoreactive proteins were detected using an ECL Plus kit (GE Healthcare).

Measurement of Intracellular Ca2+ Content—Fluorescence measurements of the intracellular Ca2+ contents were performed using a Fluoskan Ascent FC spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) and a Calcium Kit-Fluo 3 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturers’ protocols. Briefly, human PDL cells were seeded at a density of 1.5 × 10⁴ cells/well on a black 96-well plate. After incubation for 24 h, the cells were washed with PBS and incubated in loading buffer containing Fluo 3-AM for 1 h. The cells were washed with PBS, and then 100 μl of recording medium containing 0.04% Pluronic F-127 and 1.25 mM probenecid was added to each well. The PDL cells were then stimulated with 20 μM L-glutamate (600 μM). The fluorescence intensities were immediately measured at 1-s intervals using wavelengths of 485 nm for excitation and 538 nm for emission.

Cell Survival Analysis—Human PDL cells were seeded on 12-well dishes at a density of 1.0 × 10⁵ cells/well. After 24 h of serum starvation, the cells were cultured in the presence or absence of inhibitors at specified concentrations for 0, 24, and 48 h. The cells were then treated with trypsin for 5 min at 37 °C and centrifuged at 1,200 x g for 5 min. The cell pellets were resuspended with PBS. The live and dead cells were manually counted with a hemocytometer after 0.5% trypan blue staining.

Determination of ALP (Alkaline Phosphatase) Activity—ALP activities were measured according to the procedure described by Bessey et al. (18). Briefly, human PDL cells were placed in 12-well plates at a density of 1.0 × 10⁵ cells/well. After 24 h of serum starvation, the cells were cultured in the presence or absence of inhibitors at specified concentrations for 0, 24, and 48 h. The cells were then treated with trypsin for 5 min at 37 °C and then washed with PBS. The cells were then resuspended in 1 ml of incubation buffer (50 mM Tris-HCl, pH 9.0) and sonicated for 1 min. The cell lysates were then centrifuged at 10,000 x g for 5 min. The supernatants were then mixed with 150 μl of the supernatants. The addition of 100 μl of p-nitrophenyl phosphate (5 mM) as a substrate was used to deter-
Mechanical Stress-induced Glutamate Signaling in PDL Cells

A.

| Metabotropic glutamate receptors | Ionotropic glutamate receptors |
|----------------------------------|--------------------------------|
| mGluR1 Brain                     | GRIA1 HPDL Brain              |
| mGluR2 Brain                     | GRIA2 HPDL Brain              |
| mGluR3 Brain                     | GRIA3 HPDL Brain              |
| mGluR4 Brain                     | GRIA4 HPDL Brain              |
| mGluR5 Brain                     | GRIA5 HPDL Brain              |
| mGluR6 Brain                     | GRIA6 HPDL Brain              |
| mGluR7 Brain                     | GRIA7 HPDL Brain              |
| mGluR8 Brain                     | GRIA8 HPDL Brain              |
| Glutamate vesicle transporters   | GRIA9 HPDL Brain              |

B.

- None
- Glutamate (100 μM)

Fluorescence intensity

C.

P-CREB CREB

Glutamate

Preparation of a Mouse Mechanical Stimulation Model by Experimental Tooth Movement and Tissue Preparation—The first molars of 6-week-old male ICR mice were moved according to the method described Sakai et al. (21). Briefly, a nickel-titanium wire, 0.012 inches in diameter, was fixed to the maxillary incisor with resin, and the right maxillary first molar was moved toward the palatal side for 3 days. The force loaded was directly measured on a plaster model before and after the placement of the wire in each mouse using a dial tension gauge (DTG-10NP, Mitani, Fukui, Japan).

Alizarin Red Staining—Histochmical staining of Ca²⁺ was performed by a modification of the method described by Dahl (20). Briefly, human PDL cell layers were washed twice with PBS and then fixed with dehydration ethanol for 10 min. After fixation, the cell layers were stained with 1% alizarin red S (Wako Pure Chemical Industries) in 0.1% NH₄OH (pH 6.5) for 5 min. The wells were washed with distilled water and scanned with a GT-9700F system (Epson, Nagano, Japan). The density of calcified nodules in each well was calculated using the software Win ROOF version 5.6 (Mitani, Fukui, Japan).

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mine the ALP activities. The samples were incubated at 37 °C for 30 min, and 0.25 ml of 1 N NaOH was added to stop the reaction. The absorbance of each sample was measured at 405 nm using a microplate reader (model 680; Bio-Rad). One unit of activity was defined as the enzyme activity hydrolyzing 1 nmol of p-nitrophenyl phosphate in 30 min.

Determination of Cellular DNA Contents—The DNA content was measured by a modification of the method of Labarca and Paigen (19). Briefly, human PDL cells were washed twice with PBS and sonicated on ice in 2 ml of distilled water. Hoechst 33258 was prepared by dissolution in NaCl (2 M) plus Tris-HCl (25 mM, pH 7.5), and 25 μl of the resulting Hoechst 33258 solution (5 μg/ml) was added to 100 μl of the supernatants. The fluorescence was monitored at an emission wavelength of 450 nm after excitation at 356 nm using a spectrophotometer (Fluoskan Ascent FC, Thermo Fisher Scientific Inc.). The concentration of DNA in the samples was determined by a standard curve based on various concentrations of calf thymus DNA.

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Mechanical Stress-induced Glutamate Signaling in PDL Cells

Probe Preparation—Commercial mouse Homer1, Vglut1, Grin1 (glutamate receptor ionotropic N-methyl-D-aspartate 1), mGluR3 (metabotropic G-protein-coupled receptor 3), mGluR5, and mGluR6 full-length cDNA clones were purchased from Source BioScience Plc Geneservice (Cambridge, England). By using these clones as templates for RT-PCR, we obtained the corresponding cDNA fragments. The DNA fragment for Homer1 was 501 bp (site location from 388 to 888 bp of accession number NM_147176), that for Vglut1 (vesicular glutamate transporter 1) was 364 bp (site location from 515 to 878 bp of accession number NM_182993), that for Grin1 was 478 bp (site location from 1 to 477 bp of accession number NM_001081414), that for mGluR3 was 509 bp (site location from 202 to 710 bp of accession number NM_00181850), that for mGluR5 was 505 bp (site location from 98 to 602 bp of accession number NM_001081414), and that for mGluR6 was 485 bp (site location from 58 to 542 bp of accession number NM_173372). The DNA fragment for mouse Runx2 was 701 bp (site location from 3324 to 4024 bp of accession number NM_001146038), that for mouse osteocalcin was 403 bp (site location from 39 to 441 bp of accession number NM_007541), and that for mouse type I collagen was 241 bp (site location from 119 to 359 bp of accession number NM_007742). The fragments were subcloned into pGEM-T Easy vectors (Promega, Madison, WI) and used to generate sense and antisense probes. Digoxigenin (DIG)-11-UTP-labeled single-stranded RNA probes were prepared using a DIG RNA labeling kit (Roche Applied Science) according to the manufacturer's instructions.

In Situ Hybridization—In situ hybridization was performed as described previously (4), with minor modifications. Briefly, sections were deparaffinized and fixed with 4% paraformaldehyde for 20 min at room temperature. The sections were then washed and incubated with 5 μg/mL proteinase K (Roche Applied Science) in 10 mM Tris-HCl (pH 8) and 1 mM EDTA for 15 min at room temperature. The sections were then washed and incubated with 5 μg/mL proteinase K (Roche Applied Science) in 10 mM Tris-HCl (pH 8) and 1 mM EDTA for 15 min at room temperature. The sections were washed and incubated with 5 μg/mL proteinase K (Roche Applied Science) in 10 mM Tris-HCl (pH 8) and 1 mM EDTA for 15 min at room temperature. The sections were then washed and incubated with 5 μg/mL proteinase K (Roche Applied Science) in 10 mM Tris-HCl (pH 8) and 1 mM EDTA for 15 min at room temperature. The sections were then washed and incubated with 5 μg/mL proteinase K (Roche Applied Science) in 10 mM Tris-HCl (pH 8) and 1 mM EDTA for 15 min at room temperature. The sections were then washed and incubated with 5 μg/mL proteinase K (Roche Applied Science) in 10 mM Tris-HCl (pH 8) and 1 mM EDTA for 15 min at room temperature. HCl for 10 min at room temperature, washed again, and equilibrated with 0.1 μM triethanolamine-HCl buffer (pH 8) for 2 min. Acetylation of the sections was performed by incubation with freshly prepared 25% acetic anhydride for 10 min at room temperature. The sections were dehydrated and used

FIGURE 3. Effects of human PDL cells under mechanical stress on glutamate signaling. A, RT-PCR analyses were performed for the expressions of glutamate signaling-associated genes in human PDL cells after the application of mechanical stress for 24 h. The numbers of PCR cycles were 35 for mGluR2-R6 and VGLUT1, 30 for GRIA3 and HPRT, 40 for GRIN1 and GRIN3B, and 38 for GRIN2C and GRIN2D. A quantitative analysis of the PCR products using the software Win ROOF is shown as the ratio of the intensities of each gene expression and HPRT. Control, non-stretched human PDL cells;Stretch, stretched cells. Representative results of three independent experiments are shown.

B, The release of glutamate was determined in supernatants from human PDL cells after the application of mechanical stress for the indicated times. Values are shown as the ratio of each PCR product to the internal control HPRT. Values represent the means ± S.D. (error bars) of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown.

C, Western blotting analysis was carried out for phospho-CREB and CREB proteins in human PDL cells after the application of mechanical stress for the indicated times. A quantitative analysis is shown as the ratio of the intensities of phospho-CREB and CREB. Similar results were obtained in three separate experiments, and representative data are shown.
Mechanical Stress-induced Glutamate Signaling in PDL Cells

RESULTS

Gene Expression Profile of Human PDL Cells under Mechanical Stress—First, we confirmed the application system of tensile mechanical stress to human PDL cells. After the application of tensile mechanical stress for 48 h, the axes of human PDL cells were aligned perpendicularly to the direction of the stretch force, whereas the control cells were aligned randomly (data not shown). To biologically evaluate whether the mechanical stress worked on human PDL cells in this system, we examined the gene expression of C-FOS (FBJ murine osteosarcoma viral oncogene homolog), which is known to be a mechanical stress-responsive gene (22). Real-time PCR analysis showed that the application of mechanical stress to human PDL cells for 48 h significantly up-regulated the gene expression of C-FOS (data not shown). Next, utilizing oligo-DNA chips, we analyzed the gene expression profile of human PDL cells after the application of mechanical stress for 48 h. The DNA chip analysis identified 17 up-regulated genes stress to human PDL cells for 48 h significantly up-regulated the gene expression of C-FOS (data not shown). Next, utilizing oligo-DNA chips, we analyzed the gene expression profile of human PDL cells after the application of mechanical stress for 48 h. The DNA chip analysis identified 17 up-regulated genes, including the following genes with specific functions: for signal transduction, the gene expression of C-FOS was examined (data not shown). Next, utilizing oligo-DNA chips, we analyzed the gene expression profile of human PDL cells after the application of mechanical stress for 48 h. The DNA chip analysis identified 17 up-regulated genes, including the following genes with specific functions: for signal transduction, the gene expression of C-FOS, for cytodifferentiation and mineralization, the gene expression of ALP, and for cell proliferation, the gene expression of CREB and IP3. The expressions of CREB and IP3 were normalized by the HPRT expression. Values are shown as the relative ratios to day 0. Values represent the means ± S.D. (error bars) of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with PBS alone. B, human PDL cells were cultured in the mineralization-inducing medium. Every 3 days, the supernatants of the cultured cells were harvested, and the released glutamate was determined. Values represent the means ± S.D. (error bars) of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with PBS alone. C, real-time RT-PCR was performed for the expressions of CREB and IP3 transcripts during the cytodifferentiation and mineralization of human PDL cells. The expressions of CREB and IP3 were normalized by the HPRT expression. Values are shown as the relative ratios to day 0. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. D, real-time RT-PCR was performed for the expressions of CREB and IP3 transcripts during the cytodifferentiation and mineralization of human PDL cells. The expressions of CREB and IP3 were normalized by the HPRT expression. Values are shown as the relative ratios to day 0. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. E, real-time RT-PCR was performed for the expression of RUNX2 during the cytodifferentiation of human PDL cells. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. F, real-time RT-PCR was performed for the expression of ALP during the cytodifferentiation of human PDL cells. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. G, real-time RT-PCR was performed for the expression of C-FOS during the cytodifferentiation of human PDL cells. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. H, real-time RT-PCR was performed for the expression of IP3 during the cytodifferentiation of human PDL cells. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0.

Functional analysis of glutamate signaling in human PDL cells. A, real-time RT-PCR was performed for the expressions of cytodifferentiation- and mineralization-related genes, such as C-FOS, RUNX2, and ALP, after a 12-h stimulation with exogenous glutamate (100 μM). The expressions of C-FOS, RUNX2, and ALP were normalized by the HPRT expression. Values are shown as the relative ratios to the non-stimulated controls. Values represent the means ± S.D. (error bars) of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. C-FOS expression was examined (data not shown). Next, utilizing oligo-DNA chips, we analyzed the gene expression profile of human PDL cells after the application of mechanical stress for 48 h. The DNA chip analysis identified 17 up-regulated genes, including the following genes with specific functions: for signal transduction, the gene expression of C-FOS was examined (data not shown). Next, utilizing oligo-DNA chips, we analyzed the gene expression profile of human PDL cells after the application of mechanical stress for 48 h. The DNA chip analysis identified 17 up-regulated genes, including the following genes with specific functions: for signal transduction, the gene expression of C-FOS, for cytodifferentiation and mineralization, the gene expression of ALP, and for cell proliferation, the gene expression of CREB and IP3. The expressions of CREB and IP3 were normalized by the HPRT expression. Values are shown as the relative ratios to day 0. Values represent the means ± S.D. (error bars) of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with PBS alone. C, real-time RT-PCR was performed for the expressions of CREB and IP3 transcripts during the cytodifferentiation and mineralization of human PDL cells. The expressions of CREB and IP3 were normalized by the HPRT expression. Values are shown as the relative ratios to day 0. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. D, real-time RT-PCR was performed for the expressions of CREB and IP3 transcripts during the cytodifferentiation and mineralization of human PDL cells. The expressions of CREB and IP3 were normalized by the HPRT expression. Values are shown as the relative ratios to day 0. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. E, real-time RT-PCR was performed for the expression of RUNX2 during the cytodifferentiation of human PDL cells. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. F, real-time RT-PCR was performed for the expression of ALP during the cytodifferentiation of human PDL cells. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. G, real-time RT-PCR was performed for the expression of C-FOS during the cytodifferentiation of human PDL cells. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0. H, real-time RT-PCR was performed for the expression of IP3 during the cytodifferentiation of human PDL cells. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05, compared with day 0.
Mechanical Stress-induced Glutamate Signaling in PDL Cells

transduction, CNTFR (ciliary neurotropic factor receptor) and DSCR1 (Down syndrome critical region gene 1); for ECM remodeling, MMP15 (matrix metallopeptidase 15); for ECM components, LRRFIP1 (leucine-rich repeat interacting protein 1) and MUC4 (mucin 4 cell surface-associated). Interestingly, we also identified two glutamate signaling-associated genes among the up-regulated genes: HOMER1 (homer homolog 1) and GRIN3A (glutamate receptor ionotropic N-methyl-d-aspartate 3A). HOMER1 is a glutamate receptor-binding protein, and GRIN3A is one of the glutamate receptor subunits. The HOMER1 and GRIN3A mRNA expressions in the mechanically stressed human PDL cells were significantly up-regulated in the real-time PCR analysis (Fig. 1B).

Functional Expression of Glutamate Signaling-associated Molecules in Human PDL Cells—Glutamate receptors are divided into two groups: metabotropic G-protein-coupled receptors (mGluR1 to -8) and ionotropic ligand-gated channels. The ionotropic ligand-gated channels are subdivided into α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptors (GRIA1 to -4), kainate receptors (GRIK1 to -5), and N-methyl-d-aspartate receptors (NMDARs; GRIN1, GRIN2A to -2D, GRIN3A, and GRIN3B) (23). Vesicular glutamate transporters (VGLUT1 to -3) (24) and adaptor molecules, such as HOMER1, also participate in glutamate signaling (25).

Through our RT-PCR analysis, we found for the first time that human PDL cells constitutively expressed the mRNAs for mGluR2 to -6, GRIA3, GRIN1, GRIN2C, GRIN2D, GRIN3B, and VGLUT1 (Fig. 2A). Similar results were observed in human PDL cells derived from three different donors (data not shown). Next, we assessed whether the glutamate receptors were functional in human PDL cells. Determination of the intracellular Ca\(^{2+}\) influx showed that 100 μM exogenous glutamate apparently increased the fluorescence intensity compared with the control cells (Fig. 2B). These findings showed that exogenous glutamate induced an intracellular Ca\(^{2+}\) influx, followed by glutamate signaling in human PDL cells. We also analyzed the phosphorylation of CREB, which is mediated via mGluRs (26–28) and promotes the cytodifferentiation of osteoblasts in vitro and in vivo (29). Western blotting analysis showed that glutamate stimulation induced the phosphorylation of CREB in human PDL cells (Fig. 2C). These results indicated that the glutamate receptors expressed on human PDL cells were functional and that intracellular glutamate signal transduction was activated.

Effects of Mechanical Stress on Glutamate Signaling in Human PDL Cells—After the application of mechanical stress to human PDL cells for 24 h, we extracted total RNA and performed RT-PCR analyses. The analyses revealed that the mechanical stress up-regulated the gene expressions of mGluR2–6, VGLUT1, GRIN1, GRIN2C, and GRIN3B (Fig. 3A). Interestingly, we also found that human PDL cells spontaneously released glutamate and that the mechanical stress significantly increased the glutamate release after 48 h (Fig. 3B). We then assessed whether the mechanical stress induced the phosphorylation of CREB in human PDL cells. Western blotting analyses revealed that the mechanical stress induced the phosphorylation of CREB after 48 h (Fig. 3C).

Functional Analyses of Glutamate Signaling in Human PDL Cells—As shown in Fig. 4A, glutamate stimulation of human PDL cells up-regulated the gene expressions of C-FOS, ALP, and RUNX2 (Runt-related transcription factor 2), which are known to be cytodifferentiation- and mineralization-related genes. These findings suggest the involvement of glutamate signaling in the cytodifferentiation and mineralization of human PDL cells. Thus, we measured the release of glutamate during the cytodifferentiation of human PDL cells into mineralized tissue-forming cells. We confirmed that the ALP activities
Mechanical Stress-induced Glutamate Signaling in PDL Cells

FIGURE 6. Effects of inhibition of glutamate signaling via NMDARs on the cytodifferentiation and mineralization of human PDL cells.

A. The viabilities of human PDL cells were assessed after incubation for 0, 24, and 48 h in the presence or absence of MK801 for 0, 24, and 48 h. Values are shown as percentages relative to the numbers of the surviving cells at 0 h. Values represent the means ± S.D. (error bars) of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown.

B. The Ca²⁺ influxes in human PDL cells in the presence or absence of MK801 were determined after stimulation of human PDL cells with 100 μM exogenous glutamate. The fluorescence intensity was measured to assess the Ca²⁺ influx into human PDL cells. Zero seconds indicates the time of the stimulation with glutamate. A representative experiment of four independent experiments is shown.

C. Human PDL cells were cultured in the mineralization-inducing medium in the presence or absence of MK801, and the ALP activities were measured. The mineralized nodule formation was analyzed on day 24. A picture of the alizarin red staining is shown on the left, and the densities of the alizarin red staining analyzed by the software Win ROOF are shown on the right. Values represent the means ± S.D. of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown.

D. To examine the effects of riluzole on the cytodifferentiation and mineralization of human PDL cells, cultured human PDL cells in the mineralization-inducing medium in 21 days were treated with riluzole for 15 days after the induction of the cytodifferentiation in a dose-dependent manner.

Effects of Inhibition of Glutamate Release on the Cytodifferentiation and Mineralization of Human PDL Cells—To investigate the effects of endogenous glutamate signaling on the cytodifferentiation and mineralization of human PDL cells, we utilized a glutamate release inhibitor, riluzole. First, we examined the effect of riluzole on the survival of human PDL cells. The cell survival assay showed that riluzole at 12.5 μM or less did not affect the viability of human PDL cells after a 48-h incubation (Fig. 5A). We then confirmed the effect of riluzole on the inhibition of glutamate release from human PDL cells. As shown in Fig. 5B, we found that riluzole significantly decreased the glutamate release from human PDL cells during the cytodifferentiation in a dose-dependent manner.

To examine the effects of riluzole on the cytodifferentiation and mineralization of human PDL cells, we utilized a glutamate release inhibitor, riluzole. First, we examined the effect of riluzole on the survival of human PDL cells. The cell survival assay showed that riluzole at 12.5 μM or less did not affect the viability of human PDL cells after a 48-h incubation (Fig. 5A). We then confirmed the effect of riluzole on the inhibition of glutamate release from human PDL cells. As shown in Fig. 5B, we found that riluzole significantly decreased the glutamate release from human PDL cells during the cytodifferentiation in a dose-dependent manner.

Effects of Inhibition of Glutamate Signaling via NMDARs on the Cytodifferentiation and Mineralization of Human PDL Cells—NMDARs are specific glutamate ionotropic receptors and are composed of heteromeric assemblies between the glutamate receptors and are composed of heteromeric assemblies between the glutamate PDL cells.

gradually increased during culture of human PDL cells in the mineralization-inducing medium for 21 days (data not shown). Under these experimental conditions, the release of glutamate from human PDL cells was increased during the process of the cytodifferentiation and reached its peak on day 9 of long-term culture (Fig. 4B). Next, we assessed the expressions of CREB and inositol 1,4,5-trisphosphate (IP₃), which is one of the second messengers of glutamate signaling via mGluRs (26). Real-time PCR analyses revealed up-regulation of CREB and IP₃ during the cytodifferentiation of PDL cells with the exact same pattern of glutamate secretion (Fig. 4C). These results suggested that glutamate secreted by PDL cells induces CREB and IP₃ expressions during the course of the cytodifferentiation. Subsequently, we examined the effect of the exogenous addition of glutamate on the cytodifferentiation of human PDL cells. As shown in Fig. 4D, the ALP activities of human PDL cells were significantly enhanced by the addition of glutamate for 15 days after the induction of the cytodifferentiation of human PDL cells. RINX2 expression was also significantly enhanced by the addition of glutamate during the early stage of the cytodifferentiation of human PDL cells (Fig. 4E). These results suggest that glutamate signaling is involved in the cytodifferentiation of human PDL cells into mineralized tissue-forming cells.
Mechanical Stress-induced Glutamate Signaling in PDL Cells

![Graphs and Images]

Effects of glutamate signaling on mechanical stress-induced gene expressions. Real-time RT-PCR was performed for the gene expressions of HOMER1, GRIN3A, C-FOS, RUNX2, and ALP in human PDL cells after the application of mechanical stress for 48 h in the presence or absence of riluzole (12.5 μM). Each gene expression was normalized by the HPRT expression. Values represent the means ± S.D. (error bars) of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. *, p < 0.05.

essential GRIN1 subunit and the other subunits (30, 31). These receptors play an important role in the initiation of neuroplasty and modulate intracellular Ca²⁺ influx, activate Ca²⁺-dependent enzymes such as Ca²⁺/calmodulin-dependent protein kinases (CaMKII) and calcineurin, and regulate the phosphorylation of proteins and gene transcription (32).

To analyze the effects of glutamate signaling via NMDARs on the cytodifferentiation and mineralization of human PDL cells, we utilized an antagonist of NMDARs, MK801. First, we examined the effect of MK801 on the survival of human PDL cells. The cell survival assay revealed that MK801 at 100 μM or less had no effect on the viability of human PDL cells after a 48-h incubation (Fig. 6A). Human PDL cells were stimulated with 100 μM exogenous glutamate in the presence of MK801 to confirm the antagonistic effect of MK801 on the activation of NMDARs in human PDL cells. Determination of the Ca²⁺ influx showed that MK801 inhibited the fluorescence intensity in a dose-dependent manner (Fig. 6B).

To examine the effects of MK801 on the cytodifferentiation and mineralization of human PDL cells, we cultured human PDL cells in the mineralization-inducing medium in the presence of MK801. As shown in Fig. 6C, MK801 significantly suppressed the ALP activities in a dose-dependent manner during the cytodifferentiation of human PDL cells. Alizarin red staining revealed that MK801 significantly reduced the mineralized nodule formation on day 24 during the course of the cytodifferentiation and mineralization of human PDL cells (Fig. 6D).

Effects of Glutamate Signaling on Mechanical Stress-induced Gene Expression—To analyze the relationships among glutamate signaling, mechanical stress, and the cytodifferentiation and mineralization of human PDL cells, we examined the effects of riluzole on the cytodifferentiation and mineralization-related gene expressions in human PDL cells under mechanical stress. Real-time RT-PCR analyses demonstrated that the gene expressions of HOMER1 and GRIN3A were up-regulated after a 48-h mechanical stress in the absence of riluzole. On the other hand, riluzole significantly inhibited the up-regulation of the HOMER1 and GRIN3A expressions (Fig. 7, upper panels). Likewise, the mechanical stress-induced gene expressions of C-FOS, RUNX2, and ALP as cytodifferentiation and mineralization-related genes were also down-regulated in the presence of riluzole (Fig. 7, bottom).

In Vivo Induction of the Gene Expressions of Glutamate Signaling-associated Molecules in PDL Tissue by Orthodontic Tooth Movement—To establish the biological relevance of the in vitro studies to the in vivo situation, we investigated the gene expressions of the glutamate signaling-associated molecules in PDL tissue by utilizing a mouse model of orthodontic tooth movement (21). We selected probes for Homer1, Vglut1, Grin1, mGluR3, mGluR5, and mGluR6. The upper molar teeth were moved by an orthodontic force mediated by a closed-coil spring for 0, 12, and 24 h. We observed horizontal cross-sections of the tooth roots at the indicated time points by hematoxylin-eosin (H&E) staining (Fig. 8A, a–c). At 12 and 24 h after the application, the root was moving to the right side in each image. In Fig. 8, tension sites were created on the left side of the root, and pressure sites were present on the right side. At the indicated time points, the teeth were moving in the direction of the force within the dental sockets, and resorption and remodeling of the alveolar bone were initially activated (Fig. 8A, a–c). In situ hybridization analyses revealed that the PDL faintly expressed the glutamate signaling-associated molecule genes Homer1, Vglut1, Grin1, mGluR3, mGluR5, and mGluR6 at the base line (Fig. 8A, d, g, j, m, p, and s). Of note was the observation that these gene expressions were up-regulated at the tension sites at 12 and 24 h after the application (Fig. 8A, e, f, h, i, k, l, n, o, q, r, t, and u). On the contrary, the mRNA expression levels of these molecules were not changed at the pressure sites at 12 and 24 h after the application (Fig. 8A, e, f, h, i, k, l, n, o, q, r, t, and u). We also analyzed the expressions of the cytodifferentiation and mineralization-related genes Runx2, type I collagen, and osteocalcin. In situ hybridization analyses revealed that the mRNA expression of Runx2 was observed even in normal PDL cells (at 0 h) and thereafter was only increased at the tension sites after the application of orthodontic force (Fig. 8B, a–c). This finding indicated that cytodifferentiation of PDL cells at the tension
Mechanical Stress-induced Glutamate Signaling in PDL Cells

Mechanical stress is one of the most important factors for maintaining the homeostasis of a variety of tissues, such as bone, muscle, skin, and blood vessels. PDL tissues are also influenced and regulated by mechanical stresses, such as occlusal pressure and orthodontic forces. The physiological levels of the forces regulate cellular functions and remodeling of the PDL adequately, whereas pathophysiological forces can induce connective tissue destruction and bone and teeth resorption in periodontal diseases or inadequate orthodontic tooth treatments clinically.

The signaling pathways linking mechanical stress to cell functions are still not well described. In the present study, we carried out a comprehensive microarray analysis to assess the influences of physiological tensile mechanical stress on human PDL cells in vitro and identified the up-regulation of glutamate signaling-associated genes. We further revealed that a mechanical stretch applied to human PDL cells induced their cytodifferentiation and mineralization through activation of glutamate signaling pathways.

In this study, we identified 17 up-regulated genes, including CNTFR, DSCR1, MMP15, LRRFIP1, PLXND1, and ANGPTL1, in human PDL cells after a 48-h mechanical stress using a DNA chip analysis. Interestingly, de Araujo et al. (33) reported a microarray analysis of human PDL cells under compressive forces using an in vitro three-dimensional culture system. They identified several up-regulated genes, including inflammation-related molecules, such as COX-2 (cyclooxygenase-2), PGE2 (prostaglandin E2), IL-6 (interleukin-6), and IL-1β (interleukin-1β), which were not listed in our present study. These findings suggest that mechanical compressive stress tends to induce inflammation and destructive responses, whereas tensile stress induces cytodifferentiation and remodeling responses in PDL cells in vivo, similar to those in orthodontic treatments. CNTFR encodes a receptor for ciliary neurotropic factor, which is known to be a growth factor. Ciliary neurotropic factor has been implicated in the regulation of cell survival and cytodifferentiation in the brain (34). DSCR1, the product of a chromosome 21 gene highly expressed in the brain, is located in the minimal candidate region for the Down syndrome phenotype. DSCR1 protein encoded by the gene interacts with calcineurin A to inhibit calcineurin-dependent signaling pathways and induce anti-inflammatory reactions (35). MMP15 is a member of the matrix metalloproteinase (MMP) family and promotes ECM remodeling (36). LRRFIP1 (leucine-rich repeat (in FLII)-interacting protein 1), which is known to be a growth factor, occupies a tumor necrosis factor-α (TNF-α) promoter site and appears to act as a repressor of TNF-α production (37). PLXND1 (Plexin D1) (38) and ANGPTL1 (angiotropin-like 1) (39) are both associated with vasculogenesis and angiogenesis. In summary, the differentiation, proliferation, ECM remodeling, and angiogenesis induced by mechanical stress may contribute to arranging the optimal microcircumstances for the homeostasis and remodeling of PDL tissue. Among these up-regulated genes, we found two glutamate signaling-associated genes, HOMER1 and GRIN3A. HOMER1 encodes a member of the homer family of dendritic proteins and regulates intracellular Ca²⁺. HOMER connects group 1 mGluRs, such as mGluR1 and mGluR5, to other scaffolding proteins (40). GRIN3A encodes a subunit of NMDAR, which...
Mechanical Stress-induced Glutamate Signaling in PDL Cells

belongs to the superfamily of glutamate-regulated ion channels. Further analyses revealed that in addition to HOMER1 and GRIN3A, human PDL cells constitutively and functionally expressed glutamate signaling-associated molecules, including glutamate and its receptors. We also demonstrated that the application of mechanical stress to human PDL cells up-regulated the expressions of glutamate signaling-associated genes, increased the secretion of glutamate from the cells, and induced intracellular signal transduction. These results reveal that mechanical stress activates glutamate signaling pathways in human PDL cells.

Recently, glutamate signaling has been reported to be involved in bone metabolism (41). There are some similarities between PDL cells and osteoblasts, in that PDL cells can differentiate into mineralized tissue-forming cells when they are cultured in mineralization-inducing medium in vitro (42). The induction of the cytodifferentiation of human PDL cells increased the release of glutamate from human PDL cells as well as CREB and IP3 transcription. Exogenous glutamate enhanced the ALP activities and RUNX2 expression during the cytodifferentiation of human PDL cells. These results suggest that glutamate signaling promotes the cytodifferentiation and mineralization of human PDL cells. The suppression of ALP activities and calcified nodule formation of human PDL cells by riluzole, an inhibitor of glutamate release, and MK801, an antagonist of NMDARs, strengthens the notion that glutamate signaling is crucial for the cytodifferentiation and mineralization of human PDL cells. The analyses utilizing MK801 suggested that glutamate signaling via NMDARs contributes to the enhancement of the cytodifferentiation and mineralization of human PDL cells. The effects of antagonists for other glutamate receptors on the cytodifferentiation and mineralization of human PDL cells warrant further investigation.

Mechanical stress has been reported to induce the expressions of cytodifferentiation and mineralization-related genes in osteoblasts (43). However, the molecular mechanism of the activation of osteoblasts that leads to bone remodeling has not been fully elucidated. In this study, we confirmed that human PDL cells up-regulated the gene expressions of C-FOS, RUNX2, and ALP under mechanical stress. On the other hand, riluzole inhibited the up-regulation of the mechanical stress-induced gene expressions of C-FOS, RUNX2, and ALP. These results suggest that glutamate signaling controls the upstream of the mechanical stress-induced gene expressions and clearly demonstrate the involvement of glutamate signaling in the mechanical stress-induced cytodifferentiation and mineralization of human PDL cells.

Orthodontic tooth movement has been defined as the result of biological responses to mechanical stress applied to a tooth and is achieved by PDL and alveolar bone remodeling. Bone formation and resorption are induced at the tension sites and pressure sites, respectively. The in situ hybridization analyses revealed that the expressions of the glutamate signaling-associated genes Homer1, Vglut1, Grin1, mGluR3, mGluR5, and mGlur6 were clearly up-regulated in the tension sites of the PDL under orthodontic tooth movement in vivo. Up-regulation of the cytodifferentiation and mineralization-related genes Runx2, type I collagen, and osteocalcin was observed in the tension sites. These findings strongly support our in vitro data indicating that glutamate signaling was induced by the tensile mechanical stress and promoted the cytodifferentiation and mineralization of PDL cells. According to a previous report that tensile force activates PDL cells and modulates PDL remodeling by the induction of ECM synthesis and degradation (32), we consider that the glutamate signaling-associated molecules are also modulated together with the activation of PDL cells and PDL remodeling in response to mechanical stress.

In conclusion, we have demonstrated that mechanical stress induces glutamate signaling in the PDL, resulting in enhancement of the cytodifferentiation and mineralization of PDL cells. These findings suggest that mechanical stress-induced glutamate signaling is involved in the homeostasis, remodeling, and regeneration of periodontal tissue.

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Mechanical Stress-induced Glutamate Signaling in PDL Cells

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