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Cytodifferentiation activity of synthetic human enamel sheath protein peptides

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Background and Objective: Enamel sheath protein (ESP) is involved in the construction of the enamel sheath during tooth development. The 17 kDa ESP is a one-step cleavage product processed by proteolysis from the N-terminal side of sheathlin (ameloblastin/amelin), one of the porcine enamel matrix proteins. Enamel sheath protein exhibits periodontal ligament and cementum regeneration activity in a buccal dehiscence model in dogs, and promotes the cytodifferentiation of cultured human periodontal ligament (HPDL) cells. The aim of this study was to determine the peptide segment on the C-terminal side sequence of the human ESP that possesses a cytodifferentiation activity on cultured HPDL cells.

Material and Methods: The peptides synthesized on the basis of human ESP C-terminal side sequence were tested for their ability to increase the alkaline phosphatase (ALP) and mineralization activity of cultured HPDL cells. The expressions of osteocalcin, osteopontin and bone sialoprotein were measured by semi-quantitative PCR and therefore were determined to be specific indicators of mineralized tissue differentiation.

Results: Multiple synthetic peptides from the human ESP increased the ALP activity and stimulated matrix mineralization in long-term cultures of HPDL cells. Semi-quantitative PCR demonstrated the osteocalcin, osteopontin and bone sialoprotein expressions to increase relative to the control values. The peptide SDKPPKPELPGVDF had the strongest cytodifferentiation activity among all the synthetic peptides tested.

Conclusion: A specific peptide sequence derived from the C-terminal side of the human ESP promotes the cytodifferentiation and mineralization activity of HPDL cells in a cell culture system.

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Enamel protein extracts induce osteogenesis and/or cementogenesis (1–4). Commercially available enamel protein preparations such as Emdogain® are used clinically to stimulate periodontal regeneration activity. However, the molecules and the mechanisms behind the bioactivities of these complex, multicomponent enamel protein preparations are unclear. The cementum regeneration activity, which is one aspect of the periodontal ligament regeneration activity, is carried out by proteins in newly formed porcine secretory stage enamel (5,6), including the 25 kDa amelogenin, 89 kDa enamelin and 17 kDa enamel sheath protein (ESP; 7, 8).

Amelogenins are the major structural components in the secretory stage enamel matrix, in which the growth of enamel crystals occurs. Amelogenins assemble to form micelle structures (10), which support the elongating enamel crystal ribbons. Amelogenins are progressively degraded by the enamel proteases, Mmp-20 (11) and Klk4 (EMSP-1; 12).The slow degradation of amelogenin assemblies that separate the crystals provides space for the

progressive thickening of the enamel crystals, which is necessary for hardening the enamel. The 89 kDa enamelin resides mainly in the enamel rod of newly formed secretory stage enamel and has high affinity for binding to the enamel crystallites (7,8). The function of enamelin is critical for proper dental enamel formation and it thus play a role in crystal formation (13). The 17 kDa ESP is the N-terminal cleavage product of sheathlin (14), which has also been called ameloblastin (15) or amelin (16), and it becomes concentrated within the sheath space between enamel rods (8,9,17).

When these proteins are individually tested using the buccal dehiscence model in dogs, cementum regenerating activity is exhibited by 17 kDa ESP purified from newly formed secretory stage enamel, but not by the amelogenin and enamelin fractions. Significantly, 13 and 15 kDa ESPs, which are cleavage products of 17 kDa ESP lacking the C-terminal peptide, do not exhibit any cementum regeneration activity (5).

In cell culture systems, the alkaline phosphatase (ALP) activity is the standard marker for the cytodifferentiation of osteoblast-like cells. Various purified ESPs have been used to stimulate human periodontal ligament (HPDL) cells, which were subsequently assayed to determine their ALP activity. Only 17 kDa ESP shows cytodifferentiation activity in a cell culture system, while other lower molecular weight ESPs do not, thus suggesting that the periodontal ligament inducing activity and cytodifferentiation activity are carried by the C-terminal segment of the 17 kDa ESP that is missing from the smaller molecular weight ESPs. Synthetic peptides were synthesized that corresponded to segments of the C-terminal sequence of porcine 17 kDa ESP, and thereby showed a cytodifferentiation activity similar to the 17 kDa ESP itself when their ALP-inducing activities of HPDL cells were evaluated in a cell culture system (6). Peptides corresponding to this same region of human ESP were therefore synthesized to identify any peptides demonstrating cytodifferentiation activity using an HPDL cell culture system.

Material and methods

The study protocol was approved by the Ethics Committee of the Institute of Tsurumi University, Yokohama, Japan.

The peptides synthesized based on the human sequence were purchased from Shimadzu Biotech (Kyoto, Japan).

Determination of the human ESP sequence

The extracted premolar tooth for orthodontic reasons was cleaned with tissue paper and cut longitudinally by cracking with a bone chisel and dental bar into two pieces to reveal the pulp cavity. After the pulp was removed, the odontoblast cell layer on the predentin surface was directly suspended in RNA sol (StrataPrep Total RNA Miniprep Kit; Invitrogen, Carlsbad, CA, USA) in order to obtain total RNA. Cloned DNA was synthesized with Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). PCR primer sets for human enamel sheath protein were designed using the nucleotide sequence in the GenBank database (18).

The two primer-pairs used were 5′-TGAAGGACCTGATACTGATCC and 5′-TGATTTGCTCCAAAAGGCACG, which generates a 718 bp amplification product.

Synthetic peptides

Several peptides were synthesized based on the sequence of the C-terminal side peptide of human and porcine ESPs. Their sequences are shown in Table 1. The P-1 peptide was used as a control because it was shown in a previous study to possess the highest ALP inducing activity among all the porcine ESP synthetic peptides tested (6).

Cell cultures of HPDL cells

Normal human periodontal ligament fibroblasts (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) were maintained in an α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS; Asahi Technoglass, Chiba, Japan), either with or without 10 nM 1α-25dihydroxy-vitamin D_3 (Calbiochem, La Jolla, CA, USA) and 1% antibiotics (100 U/mL of penicillin G and 100 μg/mL of streptomycin sulfate; Gibco BRL, Grand Island, NY, USA) at 37°C in a humidified atmosphere of air containing 5% CO_2.

Table 1. The peptides synthesized on the basis of the human C-terminal extra peptide sequence

H-1: EGELPLVQQQVAPS
H-2: SDKPPKPELPGVDF
H-3: FADPQGPSLPGMDF
H-4: FPDPQGPSLPGLDF
H-5: FADPQGSTIFQIAR
H-2′: SDKPPKPELPVDF
H-2a: SDKPPKPELP
H-2b: SDKPPKPEL
H-2c: KPPKPELPVDF
H-2d: VAPSDKPPKP
H-2e: PELPGVDF
P-1: QVEGPMVQQQVAPSEK

Another cell line, ST2 cells (Riken Cell Bank, Tsukuba, Japan), were obtained from an osteoblast-like mouse bone marrow stromal cell line. These cells were cultured in the same conditions as those described above.

Alkaline phosphatase activity assay

The ALP inducing activities of synthetic peptides using the cell culture of HPDL cells and ST2 cells were examined. The HPDL cells were distributed in 96-well plates at a density of approximately 5×10^5 cells per well and incubated for 24 h. The medium was then changed to α-MEM containing 10 nM of 1α-25dihydroxy-vitamin D_3 and either synthetic peptides or growth factors dissolved in ultrapure water. After 96 h of incubation, the cells were washed once with phosphate-buffered saline, and 10 mM *p*-nitrophenylphosphate in 100 mM 2-amino-2-methel-1,3-propanediol–HCl buffer (pH 10.0) containing 5 mM $MgCl_2$ was added. The ALP activity was determined after 10 min incubation at 37°C and the absorbance at 405 nm was read using a plate reader, after adding 0.2 M NaOH to stop the reaction. Positive controls included the use of recombinant growth factors, bone morphogenetic protein-2 (BMP-2) (1 μg/mL; Techne Co., Lexington

Kentucky, Minneapolis, MN, USA) and transforming growth factor-β1 (TGF-β1, 0.5 or 1 ng/mL; R&D Systems, Inc., MN, USA). When the synthetic peptides were applied in the cell culture system, their final concentrations were 25 or 50 μg/mL. A TGF-β1 receptor inhibitor (SB431542, 10 μM) was applied to the HPDL cell culture system to examine their influence relative to the ALP inducing activity of the synthetic peptides.

Semi-quantitative PCR with the LightCycler instrument

Total RNA was extracted using RNAzol™ B (Tel-Test Inc., Friendswood, TX, USA) from cultured HPDL cells. The cDNA was synthesized from 3 μg of the total RNA obtained from HPDL cells cultured for 4 or 21 d using an oligo-dT primer and the You-primed First-Strand Beads kit (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's protocol. The PCR primers were designed, based upon the *Homo sapiens* mRNA sequences. The primer pairs were as follows: osteopontin, 5′-TGACCTCTGTGAAAACAGCGT-3′ and 5′-TGTACATTGTGAAGCTG TGAA-3′ (301 bp); osteocalcin, 5′-TTGTGTCCAAGCAGGAGGGCA-3′ and 5′-ACATCCATAGGGCTGG GAGGT-3′ (304 bp); and bone sialo protein, 5′-GCAGAAGTGGATGAA-AACGA-3′ and 5′-TGGTGGTAG TATTCTGACCA-3′ (448 bp). A primer set amplifying glyceraldehyde-3-phosphate dehydrogenase (Clontech, Palo Alto, CA, USA) mRNA was used as a control.

The cDNAs were generated from HPDL cells by means of the DNA Master SYBR Green I kit and protocol and a LightCycler instrument (Roche Molecular Biochemicals, Mannheim, Germany). The relative amount of each mRNA was determined at 50% levels of PCR product, and normalized with use of the relative amount of GAPDH mRNA.

Mineralization activity

The HPDL cells were plated in six-well plates at an initial density of 1×10^5 cells per well. After 24 h of incubation, the medium was replaced with growth medium containing 50 μM ascorbic acid, 10 mM β-glycerophosphate and 10 nM 1α-25dihydroxy-vitamin D_3 (differentiation medium) and 25 μg/mL of samples. The medium was changed every 72 h. The cells were maintained for 28 d and the medium was discarded to examine the biomineralization activity by Alizarin Red S staining and also to measure the calcium content.

Analytical methods

For Alizarin Red S staining, the compartments of 28 d cultured cells were fixed in 100% methanol, stained with Alizarin Red S for 10 min, then washed with ultrapure water and photographed. The Alizarin Red S staining solution was 1% Alizarin Red S (sodium alizarin sulfonate; Sigma) dissolved in ultrapure water and adjusted to pH 6.4 with 0.1 N ammonium hydroxide. To measure the calcium content, the cell compartments were dissolved with 0.5 N hydrochloric acid. The resulting solution was measured using a Calcium C-test kit and protocol (Wako Pure Chemical Industries Ltd, Osaka, Japan). The absorbance at 570 nm was read with a plate reader. The DNA sequencing of cDNA corresponding to the human enamel sheath protein was carried out using an ALF DNA zSequencer (Pharmacia LKB ALF, Stockholm, Sweden).

Statistical analysis

All values are represented as the means ± SEM. Statistical significance was determined using Student's unpaired *t*-test, and $p < 0.05$ was considered to be statistically significant.

Results

Figure 1 shows the amino acid sequence of human ESP deduced from its cDNA sequence obtained from odontoblasts. The human ESP has an extra peptide near its C-terminus, comprised of 25 amino acid residues, that is encoded by exons 8 and 9. These exons are derived from a tandem duplication of exon 7 in the line to humans and are not found in most mammalian species, including pig. The GAG codon in *AMBN* exon 7 that encodes Gly^{154} is only present in some people (18); thus, the human ESP peptides tested in this study included variants that lacked (H-2′) or included Gly^{154} (H-2). Based on the 40 amino acid C-terminal segment of the pig 17 kDa ESP that effectively induced cytodifferentiation of HPDL cells (6), the corresponding 65 amino acid residues of the human homologue were targeted for analysis.

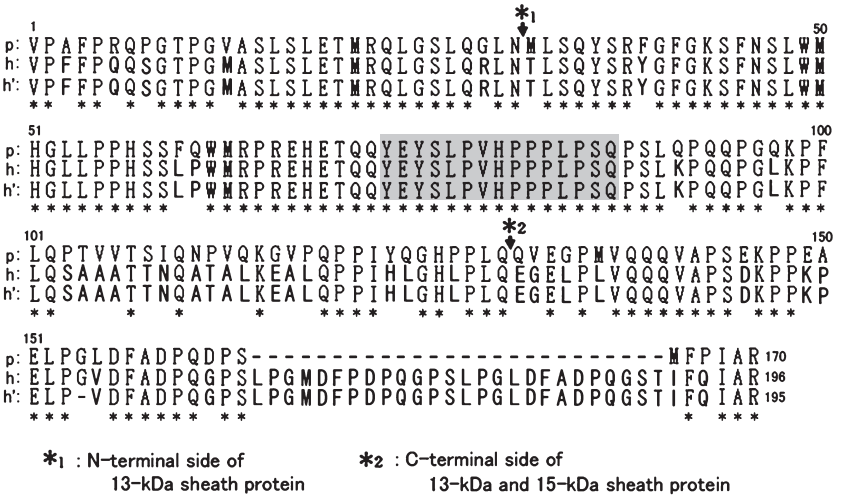

Fig. 1. Porcine and human enamel sheath protein (ESP) sequences. The shaded portion indicates the peptide results obtained from alternative splicing. Abbreviations: p, porcine ESP sequence; h. human ESP sequence obtained by database; and h′, human ESP sequence obtained from the odontoblast sample.

One porcine and eleven human peptides were synthesized (Table 1) and evaluated for their bioactivity using a cell culture system consisting of HPDL cells and ST2 cells.

The levels of ALP activity of HPDL cells were enhanced and stabilized by the addition of 1α-25dihydroxy-vitamin D_3 into the cell culture system with the application of both ESP synthetic peptides and TGF-β1 (Fig. 2). Therefore, the addition of 1α-25dihydroxy-vitamin D_3 to the cell culture system was employed for examination of the ALP inducing and mineralization activities of the HPDL cells. The ALP activity of HPDL cells in these conditions characteristically increased after the application of recombinant TGF-β1, but it was not influenced by BMP-2 (Fig. 3). The multiple synthetic peptides also induced ALP activity in HPDL cells, although higher concentrations were required than with TGF-β1 and none achieved ALP activity levels equal to those of TGF-β1 (Figs 2 and 3). The H-2 peptide had the highest ALP inducing activity of HPDL cells among the human synthetic peptides and P-1 peptide. The synthetic peptides shorter than H-2 peptide showed only a weak activity, except for the H-2′ peptide (Fig. 3). These activities were not affected by the addition of SB431542, the TGF-β1 receptor inhibitor, which significantly inhibited the activity of TGF-β1 (Fig. 4). No synthetic peptide enhanced the ALP activity of ST2 cells, which was enhanced by BMP-2, but not by TGF-β1 (data not shown).

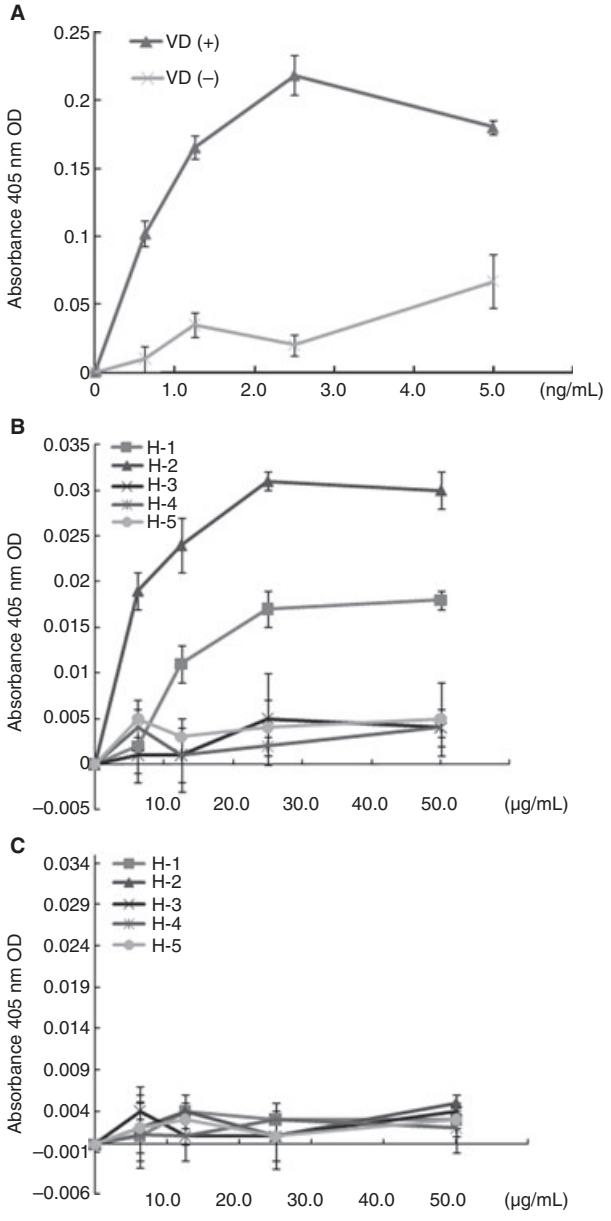

Fig. 2. The alkaline phosphatase (ALP) inducing activity of human synthetic peptides on human periodontal ligament fibroblast (HPDL) cells either with or without 1α-25dihydroxy-vitamin D_3. (A) The ALP inducing activity of HPDL cells by transforming growth factor-β1 (TGF-β1) with or without 1α-25dihydroxy-vitamin D_3 (VD). The ALP activity of HPDL cells was enhanced in a dose-dependent manner by TGF-β1 with 1α-25dihydroxy-vitamin D_3. (B) The ALP inducing activity of HPDL cells by ESP peptides (H-1 to H-5) with 1α-25dihydroxy-vitamin D_3. (C) The ALP inducing activity of HPDL cells by ESP peptides (H-1 to H-5) without 1α-25dihydroxy-vitamin D_3.

The mineralization tests of the H-2 peptide were examined using the HPDL cells in long-term cell culture because H-2 peptide was demonstrated higher ALP inducing activity than the other peptides. The application of H-2 peptide induced mineralization by HPDL cells in 28 d cell culture in comparison to the control cells, in which mineralization was also detected, although its level was much lower than that of cultures containing TGF-β1 as a positive control (Fig. 5).

The expressions of osteopontin, osteocalcin and bone sialoprotein, which are mineralized tissue markers related to the state of differentiation of HPDL cells, were examined in the total mRNA obtained from 4, 14 and 28 d cultured HPDL cells in the condition medium inducing the mineralization. They were expressed in all cases and increased during the culture. These expressions by the application of H-2 peptide were also closely similar to those of TGF-β1 (Fig. 6).

Discussion

Enamel sheath protein is produced by proteolytic cleavage, possibly by Mmp-20 (19), from the amino-terminal side of sheathlin (14) (ameloblastin and amelin; 15,16) and is involved in the

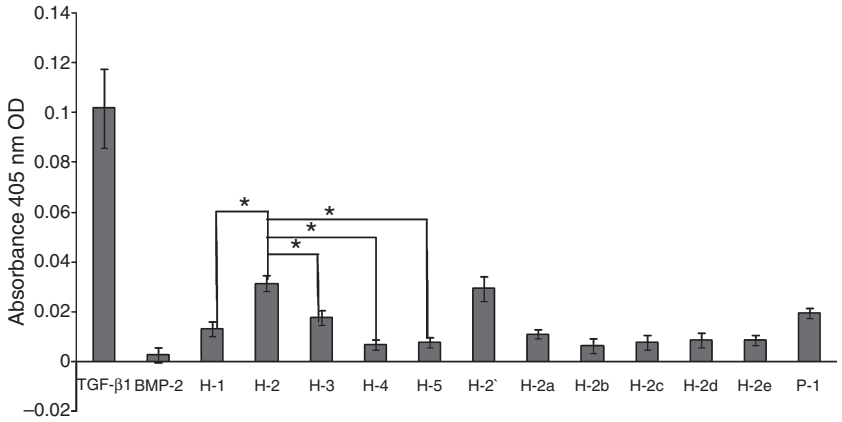

Fig. 3. The ALP inducing activity of HPDL cells by all synthetic ESP peptides and TGF-β1. The synthetic peptide H-2 induced higher ALP activity than the other peptides. The data represent the means ± SEM of five culture wells. * Significantly different from H-2 at a value of $p < 0.01$. All peptides were evaluated at a final concentration of 50 μg/mL in the culture system with the addition of 10 nM 1α-25dihydroxy-vitamin D_3. Transforming growth factor-β1 was evaluated at a final concentration of 1 ng/mL.

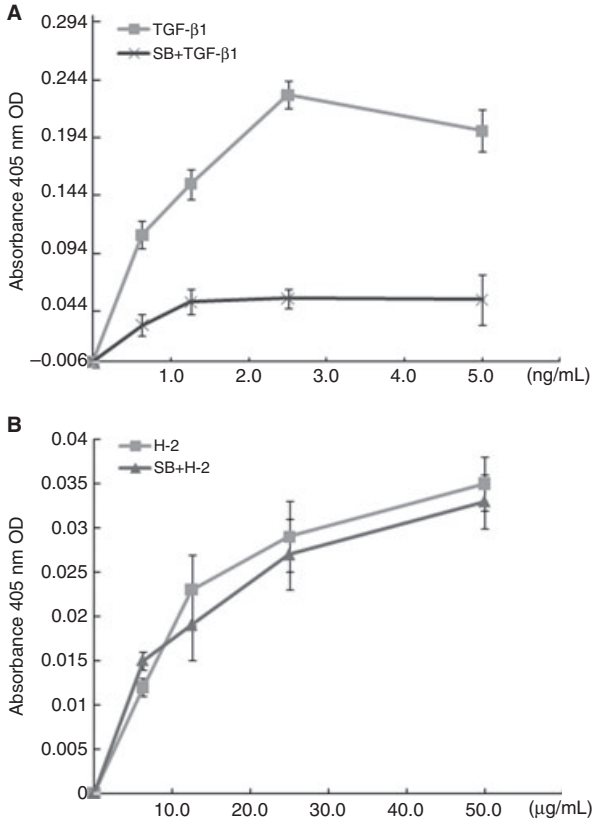

Fig. 4. The ALP inducing activity of human synthetic peptides and the effect of SB431542 (SB). The HPDL cells were cultured with the addition of 10 nM 1α-25dihydroxy-vitamin D_3. To investigate SB341542, 10 μM of SB431542 was added in every well. Transforming growth factor-β1 was evaluated at a final concentration range of 0–5 ng/mL.

construction of the enamel sheath (8,9,17). Porcine ESP, with an apparent molecular mass of 17 kDa, has a stronger cementum regeneration promoting activity, which plays an important part in periodontal regeneration, than that of TGF-β1, based on the findings of an *in vivo* system of experimental cavities prepared in the buccal dehiscence dog model. The C-terminal region of the 17 kDa ESP appears to be the true biologically active component in this fraction, because the 15 kDa ESP, which is a cleavage product of the 17 kDa ESP lacking the C-terminal peptide, has scarcely any cementum regeneration activity at all. The 17 kDa ESP also shows a cytodifferentiation activity in a cell culture system of HPDL cells, although this activity is weaker than that of TGF-β1; however, other lower molecular weight ESPs do not demonstrate such an activity. These activities are carried by the C-terminal segment of the 17 kDa ESP; synthetic peptides of this sequence also show cytodifferentiation activity similar to the 17 kDa ESP itself in the cultured HPDL cells (6). Therefore, the cell culture system of HPDL cells is employed for determination of the biologically active sequence, which shows both periodontal ligament inducing activity and cytodifferentiation activity, in the C-terminal segment of the 17 kDa ESP that is missing from the smaller molecular weight ESPs.

For the purposes of periodontal ligament regeneration, peptides were synthesized corresponding to this same region of human ESP and were then evaluated to show their ALP inducing activity in the HPDL cells.

The odontoblast cell layer on the predentin of healthy human premolars was used, since enamel protein mRNAs are expressed in the porcine odontoblast cell layer (20). After determining the deduced amino acid sequence of the human ESP, several peptides were synthesized and evaluated for their ALP inducing activity in HPDL cells in a cell culture system. These peptides induced various amounts of HPDL cell cytodifferentiation.

The peptide SDKPPKPELPGVDF had the most efficient cytodifferentiation activity of the human synthetic peptides and also promoted mineralization in long-term cell culture. As suspected, the peptide SDKPPKPEL-PVDF, which lacks Gly^{154}, showed the

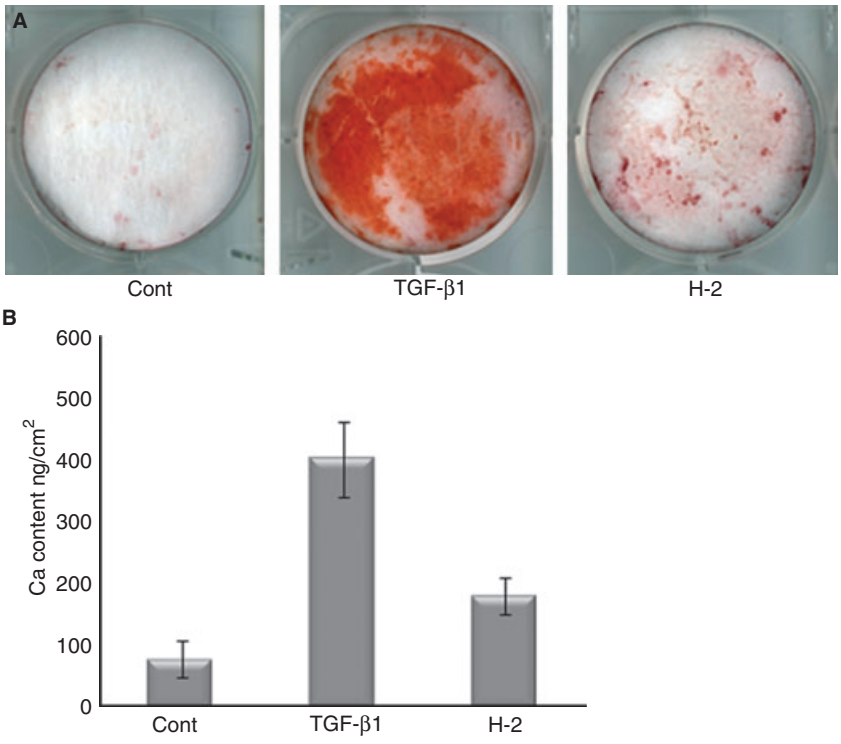

Fig. 5. Biomineralization activity of H-2 peptide and TGF-β1. The HPDL cells were cultured for 28 d with H-2 peptide and recombinant TGF-β1 to examine the biomineralization activity and stained with Alizarin Red S (A) and measured by the calcium test (B). Transforming growth factor-β1 was evaluted at a final concentration of 1 ng/mL and H-2 peptide was at 25 μg/mL. Cont, control.

same inductive properties. However, these sequences were different from the P-1 peptide, which carries the highest ALP inducing activity among the synthetic porcine ESP peptides tested in a previous study (6). There is no clear relationship between the amino acid sequences of these active synthetic peptides of human and pig ESPs.

When a TGF-β1 receptor inhibitor (SB431542) was added to the HPDL cell culture system, the ALP inducing activity of TGF-β1 was distinctly inhibited. However, the increased ALP inducing activity of HPDL cells in response to the synthetic peptide was not inhibited by adding SB431542. This suggested that the cytodifferentiation activity of the synthetic peptides is not induced via the TGF-β1 receptor. It also demonstrated that the bioactivity of the synthetic peptides was not due to TGF-β1 induced by the peptides in the HPDL cells.

The synthetic peptides had less ALP inducing activity, and higher concentrations were needed for the induction of the activity in comparison to TGF-β1. This may suggest that they are incorporated into the cells by

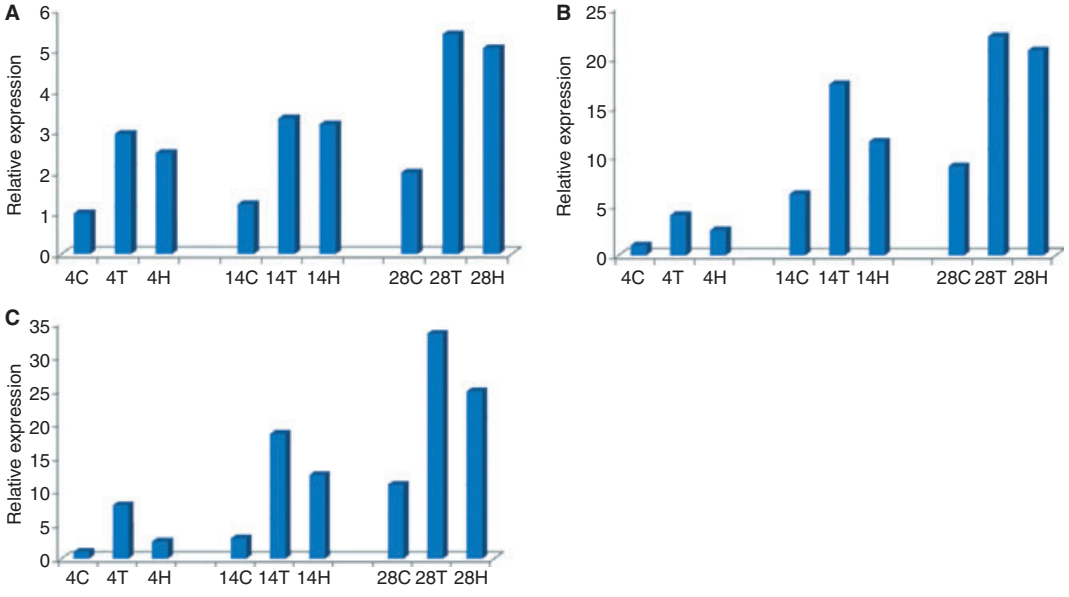

Fig. 6. Semi-quantitative PCR with the LightCycler instrument. The expression of bone sialoprotein gene (A), osteocalcin gene (B) and osteopontin gene (C) in HPDL cells is shown in comparison with the values of 4C. Abbreviations: 4C, 4 d cultured control; 4T, cultured 4 d with TGF-β1; 4H, cultured 4 d with H-2; 14C, 14 d cultured control; 14T, cultured 14 d with TGF-β1; 14H, cultured 14 d with H-2 peptide; 28C, cultured 28 d control; 28T, cultured 28 d with TGF-β1; and 28H, cultured 28 d with H-2. Transforming growth factor-β1 was tested at a final concentration of 1 ng/mL and H-2 peptide was at 25 μg/mL.

another mechanism without the activation of a receptor, although this is currently unclear. After they are incorporated into the cells, they may be involved in the induction of ALP activity via the same cell signaling pathway as the TGF-β1, since both increase the ALP inducing activity and stimulate the mRNA of mineralized tissue markers.

In conclusion, the synthesis and evaluation of peptides corresponding to the human ESP C-terminal sequence identified the peptide sequence of the human ESP that is able to induce the cytodifferentitaon of HPDL cells in a cell culture system. Since the cytodifferentiation activity of HPDL cells may correlate with the cementum regeneration promoting activity, these results may be clinically useful for periodontal ligament regeneration.

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