Abstract

The effects of human dentin phosphophoryn-derived RGD peptides

on human dental pulp stem cells *in vitro*

2021

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Abstract

Vital pulp therapy is a treatment in which a protective agent is applied to injured dental pulp tissue due to deep dental caries or traumatic injury to promote pulp healing and preserve tooth vitality. When such damage occurs, new odontoblasts differentiated from dental pulp stem cells migrate toward the site of injury and form reparative dentin to protect the pulp tissue from harmful stimuli. Calcium hydroxide ($Ca(OH)_2$) has conventionally been used as the standard material for direct pulp capping to induce reparative dentin formation in areas of exposed pulp. However, according to some studies, the long-term effects of $Ca(OH)_2$ remain unpredictable. Disadvantages include incompatibility with biological tissues due to high alkalinity, lack of close adaptation to the tissues which leads to tunnel defect formation and controversial cytotoxic effects due to the formation of permanent necrotic tissue. Later, mineral trioxide aggregate (MTA) showed significantly improved sealing properties compared with $Ca(OH)_2$. However, this material still lacks the biocompatibility and biodegradability. Therefore, the clinical performance of current pulp-capping agents remains unsatisfactory in terms of biocompatibility and reparative dentin formation rate.

Extracellular matrix (ECM) proteins have complex roles in various biological processes such as osteogenesis, embryogenesis, wound healing, and tissue regeneration. Dentin phosphophoryn (DPP) is the most abundant noncollagenous protein in the dentin matrix. It has been reported that bovine DPP has the potential for dentin mineralization and formation. Thus, DPP has been suggested for application in vital pulp therapy. Previously the potential effect of the N-terminal RGD domains of DPP on the differentiation of odontoblast-like cells was reported. However, its effect on the differentiation of dental pulp stem cells into odontoblasts remains less understood. Thus, it is necessary to examine whether it possesses the ability to determine the direction of differentiation of dental pulp stem cells into odontoblasts. This study aimed to investigate the *in vitro* effects of peptides including the N-terminal RGD domains of DPP, that is, human DPP-derived RGD peptides, on human dental pulp stem cell (hDPSC) proliferation, differentiation, and mineralization, and to explore the mechanism of their function.

In the present study, RGD-1 (SESDNNSSSRGDASYNSDES), RGD-2 (ANSESDNNSSSRGDA) and RGD-3 (SRGDASYNSDESKD) were used. For comparison results, RAD-1 (SESDNNSSSRADASYNSDES), RAD-2 (ANSESDNNSSSRADA) and RAD-3 (SRADASYNSDESKD) were used. The present study was divided into three independent experiments. In experiment one, 1 M soluble

DPP-derived RGD peptides (0.5 mg/mL RGD-1, 0.36 mg/mL RGD-2 and 0.36 mg/mL RGD-3) were coated onto non tissue-culture polystyrene (TCPS) plates. In addition, 1 M RAD peptides (0.52 mg/mL RAD-1, 0.36 mg/mL RAD-2 and 0.37 mg/mL RAD-3) were coated onto non TCPS plates and dH_2O-coated plates served as the control. Cell proliferation was assessed using CCK-8 assay. Cell differentiation was analyzed via an alkaline phosphatase (ALP) activity assay and real-time RT–PCR was used to quantify the mRNA expression of odontogenic genes. Mineralization was evaluated via Alizarin Red S (ARS) staining. To explain the mechanism behind the *in-vitro* effects of RGD-3 on hDPSCs, several cell signaling pathways are hypothesized among which the MAP kinase (MAPK) pathway is one potential mechanism. The MAPK pathway includes extracellular signal-related kinases (ERK) 1/2, c-Jun N-terminal kinases (JNK) 1/2/3, and p38 isoforms (α, β, ɣ, δ) among others. In experiment two, specific MAPK pathway inhibitors were introduced to RGD-3-coated plates and hDPSCs were cultured, followed by ALP activity assays on Days 8 and 22, and ARS staining on Day 31. The selected MAPK inhibitors were SP600125 for JNK, SB202190 for p38 and PD98059 for ERK. In experiment three, hDPSCs were cultured on RGD-3-coated plates, and real-time RT–PCR was performed to observe the mRNA expression levels of the integrin subunit genes hITGA1 to hITGA8, hITGAV, hITGB1 and hITGB3. One-way ANOVA and post hoc Tukey's HSD test were used for statistical analysis with a significance level of $p<0.05$.

Cell proliferation of hDPSCs was promoted by all three RGD peptides in a concentration-dependent manner, with the greatest effect observed at 1 M concentration. All three RGD peptides had significantly higher ALP activity than the control on Days 8, 15 and 22. RGD-3 induced the highest ALP activity compared to the control on all three days (0.49 ± 0.03 vs. 0.14 ± 0.02 units/μg protein, 0.92 ± 0.03 vs. 0.2 ± 0.04 units/μg protein and 1.74 ± 0.04 vs. 0.27 ± 0.04 units/μg protein, respectively). hDMP-1, hDSPP, hALP and hBSP mRNA expression were significantly upregulated by RGD-3 as follows: hDMP-1 1.69-fold, hDSPP 1.99-fold, hALP 1.51-fold, and hBSP 2.31-fold compared to the control. The mineralization of hDPSCs was accelerated by the addition of all three RGD peptides. RGD-3 exhibited the greatest results in the proliferation, differentiation, and mineralization experiments. However, RAD peptides showed no significant difference in these experiments compared to the control, which suggests the crucial role of the RGD domain in the DPP sequence, in extracellular matrix-mediated signaling and the differentiation of hDPSCs into odontoblasts. The MAPK p38 inhibitor SB202190 inhibited the effect of RGD-3 to a level comparable to the control in both the ALP activity assay and ARS staining. The other two inhibitors did not have obvious inhibitory effects.

This result suggests that the p38 pathway is responsible for eliciting the differentiation and mineralization effects of DPP-derived RGD peptides in the hDPSCs. The mRNA expression levels of the integrins ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA7, ITGB1 and ITGB3 were significantly upregulated. Among them, expression of ITGA5 was promoted 1.9-fold, ITGA7 1.58-fold, ITGB1 1.75-fold and ITGB3 1.9-fold compared to the control. This finding suggests the involvement of different subunit combinations of these integrin channels in facilitating signal transduction for the differentiation of hDPSCs into odontoblasts.

The findings of the present study indicate that human DPP-derived RGD peptides RGD-1, RGD-2 and RGD-3 promote the proliferation, differentiation, and mineralization of hDPSCs *in vitro*. RGD-3 (SRGDASYNSDESKD) had the most significant results. Thus, it is suggested that RGD-3 may be a promising adjunctive formula for dentin regeneration. It is also suggested that RGD-3 binds to the integrin receptors on the surface of hDPSCs and regulates odontogenic gene expression and differentiation via the activation of p38 of the MAPK pathway. The integrins ITGA5, ITGA7, ITGB1 and ITGB3, either alone or in combination, might be involved in intracellular signaling in hDPSCs. Inclusion of DPP-derived RGD-3 in the formulation of a novel pulp-capping agent may induce undifferentiated pulp cells into odontoblasts and form reparative dentin in areas of the exposed pulp.