論 文 要 旨

Effects of Dentin Phosphophoryn-derived RGD Peptides on Differentiation and Mineralization in Odontoblast-like Cells *in vitro*

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Introduction

Calcium hydroxide has been considered as the standard therapy for direct pulp capping. However, formation of reparative dentine is usually due to the irritant nature of calcium hydroxide (pH≈12) rather than any bioinductive characteristics. Dentin phosphophoryn is the most abundant non-collagenous protein in dentin, and is reported to initiate mineral deposition due to the post-translational phosphorylation which binds calcium ions with high affinity (*George A et al. Cells Tissues Organs 2005*). It has been demonstrated that dentin phosphophoryn promoted cell migration (*Yasuda Y et al. J Endod 2008*), differentiation and mineralization via its RGD motif near N-terminal. Moreover, the RGD moiety can bind with integrin and regulate gene expression via MAPK signaling pathway (*Sfeir C et al. J Biol Chem 2004*). The purpose of this study was to investigate the effects of dentin phosphophoryn-derived RGD peptides on a rat odontoblast-like cell line, MDPC-23, regarding differentiation and mineralization using carbodiimide chemistry assisted peptide immobilization method.

Materials and methods

1. Surface modification of tissue culture polystyrene (TCPS) with dentin phosphophoryn-derived RGD peptides

Vinyl methyl ether-maleic anhydride copolymer (VEMA) at the concentration of 1% (w/v) in DMSO was adsorbed to 35mm TCPS. After argon plasma irradiation, carboxyl groups were released by immersion in aqueous 0.1M NaOH. Released carboxyl groups in TCPS were activated by incubation of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) with *N*-Hydroxysuccinimide (NHS) for 3hrs, and then RGD-1 (SESDNNSSSRGDASYNSDES), RGD-2 (ANSESDNNSSSRGDA), RGD-3 (SRGDASYNSDESKD) with concentration of 0.1mg/ml and 0.5mg/ml (RGD-1-0.1, RGD-1-0.5, RGD-2-0.1, RGD-2-0.5, RGD-3-0.1, and RGD-3-0.5) were poured into the activated dishes for 60hrs. TCPS with released carboxyl groups was taken to be a negative control. Then surface carboxyl group quantification was analyzed by Toluidine Blue O method. Moreover, amino acid analysis was conducted to determine peptides coupled to TCPS.

2. Cell experiments *in vitro*

MDPC-23, a rat odontoblast-like cell line, was maintained in dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), under 5% CO_2, 37°C in a humidified atmosphere (5×10^4/35mm dish). For osteogenic induction, 10mM β-glycerophosphate and 50μg/ml ascorbic acid were incorporated into culture medium (osteogenic medium: OM) on the 6th day, on which cells reached confluence. Cell morphology on 1hr, 21hrs and the 3rd day was observed under phase contrast microscopy. Cell number was counted manually by hemocytometer on the 3rd day. To evaluate odontoblast differentiation, ALPase activity was quantified and conventional PCR and real time PCR were conducted for *ALP, BSP, OCN, DMP-1* and *Runx-2* gene expression analysis with β-actin as an internal control. For detection of calcific deposition in cells, alizarin red staining was performed.

Results and Discussion

1. Surface characterization

Carboxyl group density on VEMA-coated TCPS was 4.85±0.52 nmol/cm^2 which is significantly higher than the non-treated TCPS, 0.59±0.02 nmol/cm^2. Density of RGD peptides immobilized on TCPS was as follows; RGD-1-0.1: 70.83 pmol/cm^2, RGD-1-0.5: 141.67 pmol/cm^2, RGD-2-0.1: 208.33 pmol/cm^2, RGD-2-0.5: 258.33 pmol/cm^2, RGD-3-0.1: 133.33 pmol/cm^2, RGD-3-0.5: 130.21 pmol/cm^2.

Carboxyl group was successfully introduced to TCPS at the magnitude of nmol/cm^2. Amino acid analysis indicated that RGD peptides were successfully grafted onto TCPS at the scale of pmol/cm^2. It was reported that surface density of 10 fmol/cm^2 for GRGDY is enough to facilitate focal and stress fiber formation on glass substrates (*Hubbell JA et al. J Cell Biol 1991*). Thus, RGD peptides-immobilized specimens were used in the following studies.

2. *In vitro* experiment

In experimental groups, more cells adhered to the plates on the 3rd day as compared to negative control, and cell aggregation was observed in some portions. Cell number in RGD-1-0.5

RGD-2-0.1 and RGD-3-0.5 was all higher than negative control with significance. ALPase activity of RGD-3-0.5 was higher than negative control with significance. RGD-3-0.5 enhanced *DMP-1* mRNA expression compared to negative control significantly. RGD-1-0.1, RGD-1-0.5 and RGD-3-0.1 promoted *DMP-1* mRNA expression to different extent whereas are all lower than RGD-3-0.5. Experimental groups promoted *BSP*, *ALP, Runx-2* mRNA expression. Regarding *ALP* mRNA expression, the difference between negative control and RGD-2-0.1 was significant. Staining in experimental groups are obvious as compared to negative control, among them, RGD-3-0.5 had denser staining than the other experimental groups. Evident staining of RGD peptides groups indicate that cells mineralization occurred rapidly in contrast to negative control which only has scattered stained spots.

In experimental groups, more cells adhered on the plate at the beginning. It may be due to the cell migration effect of dentin phosphophoryn-derived RGD peptides as mentioned previously. Thus they reached confluence earlier comparatively and started to differentiate. It is noteworthy that cells formed aggregates on experimental groups in contrast to negative control. The formation of cell-cell aggregation indicates an increase in cell-cell interaction (*Schneider GB et al. J Dent Res 2005*). It is possible that evident and accelerated mineralization was observed due to increased cell-cell interaction which was caused by RGD peptides. Dentin phosphophoryn-derived RGD peptides, especially RGD-3-0.5 induced odontoblast differentiation through enhancing ALPase activity and promoting the odontogenic gene expression followed by mineralization.

Conclusion

The findings suggested that dentin phosphophoryn-derived RGD Peptides, especially RGD-3-0.5 promotes the differentiation and mineralization of odontoblasts. Therefore RGD-3 can be a promising candidate for formulating a new pulp capping material.