論　文　題　目

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., 2005). It has been demonstrated that dentin phosphophoryn promoted cell migration (Yasuda Y et al., 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 (Jadlowiec J et al., 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, in terms of 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 dimethyl sulfoxide (DMSO) was coated to 35mm tissue culture polystyrene dish (TCPS) (Sasai Y et al., 2009). The coated dish was dried up *in vacuo* at room temperature overnight and irradiated by argon plasma. After argon plasma irradiation, carboxyl groups were released by immersion in aqueous 0.1 M NaOH. Released carboxyl groups in TCPS were activated by incubation of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC, 0.2 M) with *N*-Hydroxysuccinimide (NHS, 0.1 M) for 3 hours, and then RGD-1 (SESDNNSSSRGDASYNSDES), RGD-2 (ANSESDNNSSSRGDA), RGD-3 (SRGDASYNSDESKD) with concentration of 0.1 mg/ml and 0.5 mg/ml respectively (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 derivatized dishes for another 60 hours. TCPS with released carboxyl

groups was taken to be a negative control. Then surface carboxyl group quantification was carried out by Toluidine Blue O method (Sano S et al., 1993). Moreover, amino acid analysis was conducted to determine the density of 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 (seeding number: 5×10^4/35mm dish). For odontogenic induction, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid were incorporated into culture medium (odontogenic medium: OM) from day 6, on which cells reached confluence. Cell morphology on 1 hour, 21 hours and day 3 was observed using phase contrast microscopy. Cell number was counted manually by hemocytometer on day 3. To evaluate odontoblast differentiation, ALP activity assay, conventional RT-PCR and real time RT-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. Tukey's multiple comparison test was used for statistical analysis.

Results

1. Surface characterization

Carboxyl group density on VEMA-coated TCPS was 4.85±0.52 nmol/cm^2 which is significantly higher than that of the non-treated TCPS, 0.59±0.02 nmol/cm^2 ($p<0.05$). 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.

The data showed that carboxyl group was successfully introduced to TCPS. Meanwhile, amino acid analysis indicated that RGD peptides were successfully grafted onto TCPS on the order of pmol/cm^2. It was reported that surface density of 10 fmol/cm^2 for G<u>RGD</u>Y was enough to facilitate focal and stress fiber formation on glass substrates (Massia SP et al., 1991). In this regard, the method of peptides immobilization employed in the current study was enough to trigger a cell response.

2. *In vitro* experiment

2.1: Cell morphology and number

The cell morphology remained unchanged in experimental and control groups. However, the cell number in RGD-1-0.1 ($7.58 \pm 1.04 \times 10^4$), RGD-1-0.5 ($10.00 \pm 2.46 \times 10^4$), RGD-2-0.1 ($8.83 \pm 0.52 \times 10^4$), RGD-2-0.5 ($8.08 \pm 0.95 \times 10^4$), RGD-3-0.1 ($8.58 \pm 0.88 \times 10^4$), RGD-3-0.5 ($9.54 \pm 0.89 \times 10^4$) was higher than that of control ($5.42 \pm 0.14 \times 10^4$), with the difference between RGD-1-0.5, RGD-2-0.1, RGD-3-0.5 and control to be statistical significant ($p<0.05$).

2.2: ALP activity

All the experimental groups presented higher ALP activity as compared to control (0.82 ± 0.11 units/μg protein) on day 7, and RGD-3-0.5 (1.12 ± 0.09 units/μg protein) exhibited the highest ALP activity ($p<0.05$).

2.3: Conventional and real time RT-PCR

No statistically significant difference was observed by real time RT-PCR in the expression of these five genes on day 7, although conventional RT-PCR on day 7 suggested an enhanced expression of DMP-1 mRNA in the RGD-1-0.5, RGD-2-0.1, RGD-2-0.5, and RGD-3-0.1 treated cultures. On day 10, the RGD-3-0.5 culture showed 2.15 times the expression of DMP-1 mRNA compared to the control ($p<0.05$), and RGD-2-0.1 enhanced ALP mRNA expression 1.30 times as compared to the control ($p<0.05$). There was no statistically significant difference in the expression of BSP, OCN and Runx-2 mRNA in any of the RGD groups.

2.4: Alizarin red staining

Consistent with the cell number and ALP activity data, the control showed negligible staining for mineralized matrix. In contrast, the immobilized RGD groups presented evident staining, with the strongest staining on the surfaces having the highest RGD-3 concentration (RGD-3-0.5).

Discussion

DPP is the major and most acidic component in dentin matrix and is an essential

regulator of mineralization (George A et al., 2005). As a member of SIBLING family, RGD is the defining character of it. The RGD motif in DPP is conserved over species, to name a few, human, elephant, cow, pig, horse, cat, dog and mouse (McKnight DA et al., 2009), suggesting its critical importance in regulating the biological activity of DPP.

In this study, we successfully immobilized artificially synthesized RGD peptides onto the TCPS dishes as demonstrated by amino acid analysis, further found that the potentiality of the three peptides in enhancing the biocompatibility of TCPS dish in terms of increased cell number on day 3, promoting the early cell differentiation and accelerating mineralization of cells at late stage. Among the peptides, RGD-3, which contains a lysine residue in its carboxyl terminus, turned out to be the most potential one in the in vitro performance. This is in agreement with a previous research carried out by Lee (Lee MH et al., 2007), they compared the detachment strength of cell on various RGD-immobilized substrates, and observed that RGDSPK was the strongest anchor in facilitating attachment of cells. Lee further showed that the lysine-containing RGDSPK resulted in the highest levels of ALP activity and mineralization after long term incubation in comparison with other RGD peptides and the RGES control. It is believed that the physiological pH turns extra amine in side chain of lysine to be positively charged, which interacts with negatively charged glycocalyx (i.e. glycoprotein-polysaccharide covering that surrounds the cell membranes of cells) on surface of cells in an non-specifically manner. Therefore, the binding of RGD-3 with cell integrin was further enhanced by this non-specific force.

The present data suggested RGD peptides unique to human DPP especially RGD-3 induced the MDPC-23 differentiation and mineralization. Nonetheless, it should be pointed out that the effect of isolated RGD peptides was lower as compared to their parental DPP (Jadlowiec J et al., 2004). Plausible explanation for the phenomena may be attributable to the absence of regulatory domain near or removed from the motif, consequently made it difficult to recapitulate the whole effect of intact protein (Aota S et al., 1994; Mardon HJ et al., 1994). Moreover, the RGD containing fibronectin and other proteins in serum and secreted by cells implicated that the immobilized RGD domain did not act in isolation,

indeed the effect originated from RGD peptides could have been masked by the other proteins. A solution involves using protein adsorption resistant substrates (for example: polyethylene glycol (PEG)) so that the effect of RGD alone could possibly be evaluated.

Conclusion

This study described the procedure of immobilizing peptides onto TCPS, and characterized the surfaces by carboxyl group quantification and amino acid analysis. Cell response on the RGD-immobilized dishes was evaluated with respect to differentiation and mineralization. The RGD peptides-grafted TCPS promoted differentiation and mineralization of MDPC-23 as evidenced by up-regulated ALP activity, elevated odontogenic gene expression, and accelerated matrix mineralization. Despite the limitation of the work, it is suggested that RGD-3 might be a potential candidate in formulating new pulp capping materials.

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