CELL VIABILITY, FUNCTIONAL AND CELL SURFACE CHARACTERISTICS OF TOOTH DERIVED STEM CELLS

Ph.D. Thesis

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INTRODUCTION

Multipotent periodontal ligament stem cells (PDLSCs) are a potential promising candidate for the regeneration of lost/damaged periodontal tissue. Therefore, determining the functional and cell physiological properties of PDLSC is valuable for the further development of regenerative medicine.

Biocompatible material to provide proper cell adherence and promote cell growth is valuable in regenerative medicine and tissue engineering. Integrin binding synthetic RGD peptides are chemically defined, relatively inexpensive to produce, and minimize the risk of immune reactivity or pathogen transfer compared with native extracellular matrix proteins. In this study project, we evaluated three different synthetic RGD peptides immobilized with a poly L-lysine backbone: i) poly L-lysine backbone with oligo DL-alanine side chains carrying cyclo [RGDfC] on the N-terminal; refers to AK-c[RGDfC]; ii) poly L-lysine backbone with serine- oligo DL-alanine (SAK) side chains carrying cyclo [RGDfC] on the N-terminal; refers to SAK-c[RGDfC]; iii) poly L-lysine backbone with serine- oligo DL-alanine (SAK) side chains carrying linear osteopontin derivative (Ac-GRGDSVVYGLR-NH_2); refers to SAK-opn.

Periodontal ligament tissue is highly possible to contact antiseptic compounds during or post-dental procedures directly. Therefore, PDLSCs are suitable cell models for toxicity prediction studies. We studied the effect of antiseptics, including chlorhexidine (CHX), cetylpyridinium chloride (CPC), triclosan (TCS), and povidone-iodine (PVP-I), on the viability of PDLSCs.

OBJECTIVES

The primary purpose of this Ph.D. research project was to evaluate the human PDLSCs characteristics and responses to different materials, including novel biomimetic peptides and dental antiseptic compounds widely used in dental procedures.

1. We examined the functional and cell surface characteristics of PDLSCs. And our collaborators have synthesized integrin-binding RGD sequence contained adhesive peptides. We set our objectives to analyze:
   - adhesion and proliferation
   - migration
   - differentiation and
   - cell surface molecules of PDLSCs when cultivated on synthetic RGD peptide coatings
2. We aimed to study the cell viability of PDLSCs when exposed to dental antiseptic agents.
   Assayed antiseptic compounds are:
   - cetylpyridinium chloride
   - chlorhexidine
   - triclosan and
   - povidone-iodine

MATERIALS AND METHODS

Cell isolation and culture

Briefly, we isolated PDLSCs from extracted human third molars, human lung derived fibroblast cell line MRC-5 (Sigma-Aldrich), and human gingival epithelial cell line HGEP (CELLnTEC) were cultured and used as reference cells.

Preparation of adhesive coatings

The peptide constructs were synthesized at the MTA-ELTE Research Group of Peptide Chemistry (Budapest, Hungary). A simple adsorption method was used to coat tissue culture plastic or electric arrays with synthetic RGD peptides or human plasma fibronectin (Millipore), estimated with 1 $\mu g/cm^2$ density. The control remained uncoated.

Impedimetric analysis for cell adhesion and proliferation

xCELLigence SP system (Roche Applied Science) was operated to monitor PDLSCs adhesion and proliferation on RGD peptide coatings when cultured in media supplemented with 10% fetal bovine serum (FBS) or without FBS supplementation. The electric impedance on the electrode was recorded by a real-time cell analyzer

(RTCA), and it was expressed as a cell index (CI) value which is directly correlated to cell coverage.

Cell migration assay

Cell migration assay was performed using ibidi® Culture insert chamber (Ibidi®). The wound closure was monitored over the next 48 h and images were captured with Axio Observer A1 inverted microscope (Zeiss).

Flowcytometry

For detecting the cell surface antigens on PDLSCs before or after RGD synthetic peptide treatments, monoclonal antibodies against to MSCs representative markers: CD146 (MCAM), CD90 (Thy-1), CD73, CD271, CD105 (endoglin), and STRO-1; integrin subunits: CD49b (integrin α2), CD49c (integrin α3), CD49d (integrin α4), CD29 (integrin β1), integrin β7 and against integrin αVβ3; other cell adhesion molecules: CD106 (VCAM-1), CD166 (ALCAM) and CD54 (ICAM-1) were used.

Immunofluorescence

Detection of CD9 and STRO-1 markers on PDLSCs were performed with the immunofluorescence method, and nuclear staining was done with DAPI blue. Zeiss Axiophot fluorescence microscopy (Zeiss) was used for visualization.

Osteogenic differentiation

PDLSCs were maintained with osteogenic induction medium (OIM) on the peptide coatings for up to 3 weeks.

ALP assay

Detection of alkaline phosphatase was performed with Alkaline Phosphatase Yellow (pNPP) Lipid Substrate System for ELISA (Sigma-Aldrich) after 1 and 2 weeks post osteogenic induction.

Alizarin Red S staining

Detection of calcium deposits was done with Alizarin Red S (Sigma-Aldrich) staining after 1, 2, and 3 weeks of post-induction.

Real-time PCR

Quantitative real-time PCR was used to determine the expression of osteogenic genes: ALPL, RUNX2, IBSP, SP7, and BGLAP, and the RPLP0 gene was used as an internal control.

Adipogenic differentiation

PDLSCs were induced into adipogenesis, and after 2 and 3 weeks of post-induction, the cytoplasmic lipid droplets were detected with Oil Red O staining.

ECIS analysis

Osteogenic and adipogenic differentiation of PDLSCs were tracked with impedance-based ECIS® Zθ (Applied BioPhysics) instrument for a 2-weeks duration in real-time.

Preparation of antiseptic solutions

Stock solutions of cetylpyridinium chloride (CPC), chlorhexidine (CHX) and povidone-iodine (PVP-I) were solubilized in d.i water and triclosan (TCS) was solubilized in dimethyl sulfoxide (DMSO). Final concentrations were: CPC (Sigma-Aldrich) 0.0001, 0.001, 0.01 and 0.1 mg/ml; CHX (Sigma-Aldrich) 0.001, 0.01, 0.1 and 1 mg/ml; TCS (Sigma-Aldrich) 0.01, 0.1, 1 and 2 mg/ml; and PVP-I (Abcam) 0.1, 1, 2 and 4 mg/ml. Each concentration of 22 µl was added per well in 96-well plate cultures. Controls received d.i water or ×100 times dilution of DMSO regarding solvents.

Fluorescence-based alamarBlue® assay

The alamarBlue® assay was used for the detection of cell viability after exposure to different concentrations of antiseptic agents in ultra-short (10, 20, 30 seconds), short (10, 20, 30 minutes), and long term (24 and 48 hours). Fluorescence intensity was measured with a Spectrophotometer at wavelength 565; 590 nm.

xCELLigence analysis for cytotoxicity

Real-time xCELLigence SP (Roche Applied Science) system was used for monitoring cytotoxicity and cell proliferation of PDLSCs under stimuli of antiseptic agents for the ultra-short, short, and long term.

RESULTS

Synthetic RGD peptides improved cell adhesion

According to impedimetry results, the adhesion of PDLSCs was significantly increased on synthetic RGD peptide coatings.

Synthetic RGD peptides improved cell migration

Wound healing assay results indicated significantly improved PDLSCs migration on SAK-c[RGDfC] and AK-c[RGDfC] coated surfaces.

Cell surface molecules of PDLSCs

Among tested cell surface markers, CD90, CD73, CD166, and integrin α3 were expressed in 100% of the PDLSCs population, and integrin β1 (98.2%) and CD146 (83.3%) were expressed in a high fraction of cells. Compared with reference cells human lung derived fibroblast cell line MRC-5 and human gingival epithelial cell line HGEP, the expression percentage of CD90 and CD146 was higher in PDLSCs while CD105, CD106, CD54, and integrin β7 were expressed in a lower number of PDLSCs.

After being maintained on synthetic RGD peptide coatings for 1 week, CD105, CD146, and CD271 positive PDLSCs fractions were significantly increased while integrin β1, integrin α4 positive fractions were reduced.

Synthetic RGD peptides supported osteogenic differentiation of PDLSCs

SAK-c[RGDfC] and AK-c[RGDfC] coatings were able to significantly increase ALP activity (in 1-2 weeks) and Alizarin Red S staining (in 3 weeks) in PDLSCs without additional osteogenic induction.

According to qPCR analysis after 2 weeks of osteogenic induction, the level of osteogenic marker genes was increased in osteo-induced PDLSCs compared with absolute control (RUNX 1.3-1.5 fold; ALPL 1.-2.3 fold; osteocalcin over 2 fold and bone sialoprotein 5.0-7.0 fold). In a regular culture without additional osteogenic induction, SAK-opn elevated RUNX and ALPL genes expression compared with absolute control.

Impedance based ECIS monitoring of osteogenic and adipogenic differentiation of PDLSCs

Osteogenesis and adipogenesis presented different dielectric characteristics. Cellular impedance was significantly lowered due to adipogenic induction whereas it was increased in osteo-induced cells compared with control non-induced.

With synthetic RGD peptide coatings, the impedance was significantly increased than uncoated control in osteo-induced and adipo-induced groups respectively.

Dental antiseptic agents on the viability of PDLSCs

Real-time xCELLigence analysis end-point alamarBlue® assay results showed that ultra-short-term contact (10, 20, 30 sec) with cetylpyridinium chloride ≥0.01 mg/ml, chlorhexidine ≥1 mg/ml, triclosan ≥1 mg/ml and povidone-iodine ≥1mg/ml; and long-term exposure (10, 20, 30 min) to cetylpyridinium chloride ≥0.001 mg/ml, chlorhexidine ≥0.01 mg/ml, triclosan ≥1 mg/ml, povidone-iodine ≥1 mg/ml were able to reduce the viability of human PDLSCs significantly.

According to the half-maximal inhibitory concentration (IC50) the rank of cytotoxicity was cetylpyridinium chloride > chlorhexidine > triclosan > povidone-iodine

CONCLUSIONS

Major conclusions of our studies are as follows:

- Human PDLSCs presented MSCs cell surface molecules and successfully differentiated towards osteogenic and adipogenic lineages.
- Synthetic cyclic RGD peptides: SAK-c[RGDfC] and AK-c[RGDfC] promoted cell adhesion and migration, and supported osteogenic differentiation in PDLSCs.
- Our results suggest that cyclic RGD conjugates SAK-c[RGDfC] and AK-c[RGDfC] are the potentials for application for artificial cell substrates for cell attracting.
- Antiseptic agents exerted a cytotoxic effect on PDLSCs in a dose-dependent manner and reduced cell viability and proliferation at lower than practically recommended concentrations. Among tested compounds, CPC and CHX were highly cytotoxic; PVP-I and TCS were moderately toxic in PDLSCs in vitro culture.
- Our study results suggest a cautious use of antiseptic agents when there is possible exposure to an open periodontal wound or during stem cell-based regenerative therapies.

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