Investigation of Human Dental Pulp Cells on a Potential Injectable Poly(lactic-co-glycolic acid) Microsphere Scaffold

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Abstract

Introduction: Poly(lactic-co-glycolic acid) (PLGA) has been extensively explored in the tissue engineering field with good biocompatibility and biodegradability. PLGA microspheres' injectable potency makes it highly desirable in dentin-pulp complex regeneration. Therefore, we investigated the cell adhesion, proliferation, odontogenic differentiation, and matrix mineralization of human dental pulp cells (HDPCs) on a PLGA microsphere scaffold. We hypothesized that this scaffold might be suitable for dentin-pulp complex regeneration. **Methods:** PLGA microsphere scaffolds were fabricated using the double-emulsion solvent extraction technique with or without type I collagen surface modification. HDPCs were isolated from freshly extracted premolar or third molar teeth with patients' informed consent and ethical approval. Fourth-passage HDPCs (1×10^5 cells/ml) were seeded onto surface-modified or -unmodified PLGA microspheres and cultured *in vitro*. Cell adhesion, proliferation, and alkaline phosphatase activity were evaluated at different time points. Odontogenic-related gene expression (*DMP1*, *DSPP*, *COL1*, *OPN*, and *OCN*) were analyzed using quantitative real-time polymerase chain reaction. After 8 weeks of culture, samples were observed under scanning electron microscopy. **Results:** Surface modification using type I collagen significantly enhanced HDPC attachment to the PLGA microspheres and promoted cell spreading. Alkaline phosphatase activity and odontogenic-related gene expression of HDPCs cultured with PLGA microsphere scaffolds were enhanced significantly compared with HDPCs cultured without PLGA microsphere scaffolds. After 8 weeks of culture, HDPCs combined with PLGA microspheres formed 3-dimensional structures. Partial degradation of the scaffolds and matrix mineralization were also observed. **Conclusions:** HDPCs can adhere to the PLGA microspheres, proliferate and differentiate into odontoblastlike cells, and form a 3-dimensional complex with matrix mineralization. This study may provide insight into the clinical dentin-pulp complex restoration with HDPCs and PLGA microsphere constructs. *(J Endod 2017;■:1–6)*

Key Words

Human dental pulp cells, microsphere, odontogenic differentiation, poly(lactic-co-glycolic acid)

Significance

A PLGA microsphere scaffold might be suitable for dentin-pulp complex regeneration, which can be an ideal option to maintain or rebuild a vital pulp or vital dentin-pulp complex.

Healing of infected dental pulp tissue is not only the desire of people suffering from dental pulp diseases but also the ambition of dentists taking care of these patients. Current root canal therapy showed acceptable success and tooth survival rates in the long-term (1). However, it can only stop dental pulp infection without restoring fresh living pulp tissue. Other treatment options such as pulp capping or pulp revascularization also encounter substantial challenges (2, 3).

Recently, pulp or dentin-pulp complex regeneration using dental pulp cells and scaffolds to maintain or rebuild a vital pulp or vital dentin-pulp complex have gained great attention. As a natural scaffold, a treated or cryopreserved dentin matrix has shown superior potential in dentin-pulp complex regeneration (4, 5). However, using a dentin matrix from other donors may generate pathogen transmission, immunorejection, or other risks. Synthetic scaffolds such as silk fibroin scaffolds, polycaprolactone/submicron bioactive glass hybrid composites, hyaluronan-based nonwoven mesh scaffolds, and magnetic nanofiber scaffolds have also shown odontogenic differentiation stimulation effects (6–9). Injectable scaffolds such as hydrogel, nanofibrous spongy microspheres have also been reported to enhance the regeneration of pulp tissues. However, these highly promising systems still have a number of limitations such as a lack of control over tissue formation and development (10, 11).

As a well-characterized biomaterial, poly(lactic-co-glycolic acid) (PLGA) has been extensively explored in the tissue engineering field with good biocompatibility and biodegradability. The combined qualities such as controllable biodegradation and surface functionalization of PLGA make it ideal for tissue engineering scaffolding, drug or vaccine delivery, and so on (12, 13). Subcutaneous implantation of rabbit dental pulp cells with 50/50 PLGA scaffolds using 2 different porogen particle sizes generates osteodentin and tubularlike dentin structures (14). Root canals containing PLGA

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scaffolds with swine dental pulp cells show dentin-pulplike tissue regeneration after reimplantation into the fresh postextraction socket of the minipig jaw (15). PLGA microspheres can be injected into the pulp chamber using a syringe, which makes them a potential candidate in dentin-pulp complex regeneration. However, this scaffold can cause human dental pulp cell (HDPC) degeneration and apoptosis, which may be caused by the adverse effects of the stabilizer N-methy-2-pyrrolidone (16). Therefore, we fabricated a PLGA microsphere scaffold with 75:25 polymer composition (molecular weight 11,0000 g/mol) and investigated HDPC attachment, proliferation, and odontogenic differentiation on this scaffold. We hypothesized that this injectable PLGA microsphere scaffold might be suitable for dentin-pulp complex regeneration.

Materials and Methods

PLGA Microsphere Scaffold Fabrication and Surface Modification

A 75:25 polymer composition of PLGA (molecular weight 11,0000 g/mol) was used to generate PLGA microspheres using the double-emulsion solvent extraction technique. NH_4HCO_3 solution (W_1) and PLGA solution (O phase) were mixed using a homogenizer in an ice bath to obtain the initial emulsion liquid (W_1/O) and then using polyvinyl alcohol solution (W_2) with a mechanical agitator at room temperature to get the second emulsion liquid ($W_1/O/W_2$). The fabrication conditions were as follows: W_1 phase, 1.25 mL 5% NH_4HCO_3 solution; oil phase, 4 mL 6.25% PLA/CH_2Cl_2 solution; and W_2 phase, 150 mL 0.1% poly vinyl alcohol solution. The primary emulsion (W_1/O) was emulsified using a homogenizer at level 5 for 3 minutes in an ice bath. The secondary emulsion ($[W_1/O]/W_2$) was emulsified by an impeller at 400 rpm for 4 hours. During the organic solvent emulsification process of the second emulsion liquid, PLGA porous microspheres were finally generated. The PLGA porous microspheres were dispersed in excess amounts of ethylenediamine solution, stirring at room temperature for decentralized 30 minutes. After hydrolysis, microspheres were washed with distilled water repeatedly to remove the residual ethylenediamine solution. For surface modification, PLGA microspheres were soaked in 0.5% type I collagen solution at 4°C overnight and then repeatedly washed with distilled water for further use.

Isolation and Culture of HDPCs

HDPCs were isolated from freshly extracted premolar or third molar teeth for orthodontic or impaction reasons from patients (3 patients in total, including 2 women and 1 man, aged 18–24 years) attending the Department of Oral Surgery at Tianjin Stomatological Hospital, Tianjin, China. Teeth were obtained with patients' informed consent and with ethical approval from the Ethics Committee of Tianjin Stomatological Hospital. The external tooth surfaces were cleaned and washed with phosphate-buffered saline (PBS) twice. Then, the teeth were cracked open, and the pulp tissues were collected, cut into pieces, and placed in T25 flasks (Corning, Amsterdam, Netherlands) containing alpha minimum essential medium supplemented with 100 U/mL penicillin and streptomycin and 10% fetal bovine serum in an incubator at 37°C under standard conditions with 5% CO_2. The culture medium was changed every 5 days. Cells of the third passage were used in the following experiments.

Cells Cultured with PLGA Microsphere Scaffolds

Fourth-passage HDPCs were seeded in 6-well plates and cultured with surface-modified PLGA microspheres (1 mL microspheres added to 1 mL cell culture medium and 1 mL cell suspension, final seeding concentrations at 1×10^5 cells/mL according to our preliminary experiment results) for up to 8 weeks. Cells cultured with unmodified scaffolds served as the control for cell adhesion and proliferation experiments. Cells cultured with a monolayer in 6-well plates without PLGA microspheres served as the control for alkaline phosphatase (ALP) activity and gene expression analysis. An inverted phase-contrast microscope was used to observe cellular morphological changes. Cells were harvested after 4 and 24 hours of seeding for cell adhesion and after 3 and 7 days of culture for cell proliferation by measuring double-stranded DNA (dsDNA) content with a fluorometric dsDNA quantitation kit (PicoGreen dsDNA quantitation kit; Invitrogen, Carlsbad, CA). Odontogenic differentiation was assessed using ALP activity and quantitative reverse-transcription polymerase chain reaction analysis at 7 and 14 days after culture. After 8 weeks of culture, samples were collected for scanning electron microscopic analysis. The protocols are briefly described later.

Cell Adhesion and Proliferation Assays. Before measuring the DNA content of real samples of HDPCs seeded on PLGA scaffolds, the DNA content per single HDPC was validated. Briefly, a series of HDPC suspensions ranging between 2 and 2000 cells/μL were prepared in 0.1% (v/v) Triton X-100 (Sigma-Aldrich, Munich, Germany) in 1× PBS (pH = 7.4). Cells were lysed by freeze thawing twice to release DNA; 20 μL of each lysed sample was mixed with 80 μL 1× tris-HCl, ethylene diamine tetraacetic acid buffer and 100 μL PicoGreen fluorescence reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Fluorescence was measured using a microplate fluorometer (Fluoroskan Ascent; Thermo Scientific, Waltham, MA) (excitation = −480 nm, emission = −520 nm), and the DNA content of each sample was calculated from a standard calibration curve (λDNA 20–20,000 ng/mL was used for calibration). Standard curves of the DNA released from known numbers of cells were drawn to check the linearity of the assay and used to calculate the DNA content per single HDPC. Thereafter, the calculated total cell number of each sample was determined via the total DNA content divided by the single-cell dsDNA content.

After the relationship between fluorescence DNA content and cell number had been shown to be linear, the Quant-iT PicoGreen dsDNA assay (Life Technologies) was used to evaluate the adhesion and proliferation of HDPCs on PLGA scaffolds. At different time points, HDPCs were washed with 1× PBS 3 times and lysed in 0.1% Triton X-100. The DNA content of each sample was measured and normalized to the cell number.

Measurement of ALP Activity. For ALP activity detection, each sample was mixed with 500 μL 0.1% (v/v) Triton X-100 and freeze thawed twice. Cell lysis was confirmed by light microscopy. ALP activity of the lysate was measured using the p-nitrophenylphosphate method. After 30 minutes of incubation at 37°C, the absorbance of p-nitrophenylphosphate at 405 nm was determined using a microplate reader. DNA content was accessed with the PicoGreen dsDNA quantitation kit. ALP-specific activity was expressed as nmol substrate hydrolyzed/h/μg DNA.

Quantitative Reverse-transcription Polymerase Chain Reaction Analysis. For odontogenic-related gene expression analysis, HDPCs cultured in 6-well plates with or without PLGA microsphere scaffolds were digested and used for RNA extraction, respectively. The total RNA was isolated from the cells with an RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA quality was tested by denaturing agarose gel electrophoresis, and the content was measured using the NanoDrop spectrophotometer (ND-1000; Labtech, East Sussex, UK) at 260 nm. DNA was eliminated with DNase 1, Amplification Grade (Invitrogen), and 2 μg RNA was reverse transcribed with a high-capacity RNA-to- cDNA kit (Applied Biosystems, Foster City, CA). The messenger RNA (mRNA) expressions were determined by quantitative real-time reverse-transcription polymerase chain reaction in TaqMan gene expression assays (Applied Biosystems) for the