Beneficial Effects of Periodontal Ligament Stem Cells in Alzheimer’s Disease Cell Model

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Abstract

Objective: Alzheimer’s disease (AD) is a chronic neurodegenerative disease with multiple etiological factors, characterized by neurofibrillary tangles of hyperphosphorylated tau (pTau) protein. Periodontal ligament stem cells (PDLSCs) have been utilized to reconstruct tissues destroyed by chronic pathology, but the use of PDLSCs for treating AD remains unstudied.

Methods: We investigated the in vitro effects of PDLSCs on the function of SH-SY5Y cells that had been established as models of AD via use of okadaic acid (OA). To this end, we utilized immunofluorescence, CCK-8 assay, flow cytometry, western blot, and confocal and transmission electron microscopy.

Results: Co-culturing OA-treated SH-SY5Y cells with PDLSCs for 24 h was associated with improved cell shape, dendrites, cytoskeleton structure, and cell viability relative to the model without PDLSCs, while cell apoptosis and pTau protein levels were lower.

Conclusion: These findings suggest that treatment of AD-model cells with PDLSCs promoted the recovery of cell shape, structure, and function. PDLSCs may be a novel research target for clinical treatment of patients with AD. Further investigations of the effect of PDLSCs in AD from the behavioral or molecular levels are warranted.

Keywords: Periodontal ligament stem cell (PDLSC); Alzheimer’s disease (AD); Okadaic acid (OA); pTau; Cell model; SH-SY5Y cells

Abbreviations: Aβ: Amyloid Beta; AD: Alzheimer’s Disease; BMSCs: Bone Marrow Stromal Cells; ECL: Enhanced Chemiluminescence; OA: Okadaic Acid; PDLSCs: Periodontal Ligament Stem Cells; PP: Protein Phosphatases; SD: Standard Deviation

Introduction

Alzheimer’s disease (AD) is a chronic neurodegenerative central nervous system disease of dementia with multiple risk factors, including genetic. Many AD patients have histories of head injuries, depression or hypertension, especially in those older than 65 years [1,2]. The major brain features of AD molecularly are intracellular neurofibrillary tangles of aggregated hyper phosphorylated tau (pTau) protein and GSK3β (glycogen synthase kinase 3 beta), extracellular senile plaques containing amyloid-beta (Aβ) peptide, neurotransmitter alterations and inflammatory cascade reactions [3]. Tau protein is a microtubule-associated protein responsible for maintaining the stability of axonal microtubules, axonal transport and axon growth [4]. The hyper phosphorylation of tau protein has been associated with accumulated Aβ, nerve inflammation, and neurological toxicity, which has a crucial role in AD development [5,6]. Thus, treatment of AD that focuses on pTau regulation has been a major research direction in recent years.

Phosphoserine/phosphothreonyl protein phosphatases (PP) found in the neurons of the human brain is putatively responsible for the regulation of tau phosphorylation. In particular, PP2A and PP1 have been shown to dephosphorylate tau in vitro [7]. Okadaic acid (OA) is a marine toxin that specifically inhibits the dephosphorylation of tau protein by PP2A and PP1. Treatment with OA leads to the hyper phosphorylation and accumulation of tau that is able to mimic pathological changes of AD, including the assembly of neurofibrillary tangles [3].

Intra hippocampal injection of OA in rats has been shown to result in memory impairment accompanied by neuropathic changes, which include hippocampal neurodegeneration, dysfunction of hippocampal glucose uptake, hyper phosphorylated tau, paired helical filament-like structures, and increased Aβ deposition in plaque-like structures [8,9]. Wu et al. [10] found that injection of OA into the rat lateral ventricle established an ideal model for studying AD. Maidana et al. [11] reported that OA altered short- and long-term memory in rats, and also promoted oxidative stress in the hippocampus. Taken together, OA contributes to the hyper phosphorylation of tau protein in neurons, the production of neurofibrillary tangles, the accumulation of Aβ and inflammatory reactions.

The use of stem cells in murine models to treat AD has shown promise in recent years. Examples include neural stem cells [12,13], bone marrow mesenchymal stem cells [14], adipose stem cells [15] and mesenchymal stem cells derived from umbilical cord blood [16,17]. Although stem cells in these models reversed the behavioral and pathological changes in AD, yet problems remain that prevent...
clinical application. Such problems include the source of stem cells, immune rejection, medical ethics and distribution and survival after transplantation [18]. In addition, the therapeutic effects of stem cells may be primarily paracrine [19-21], with secreted nutrients and immune regulatory factors specific to the stem cell source. Therefore, the selection of the specific stem cells for therapy must be appropriate to the disease.

Periodontal ligament stem cells (PDLSCs) are multipotent cells that were first isolated by Seo et al. [22] in 2004. PDLSCs have promising rates of growth, self-renewal, and repair. These cells originate at the neural crest and can be obtained after the natural loss or surgical removal of adult teeth, this avoiding secondary damage and ethical controversies [23]. PDLSCs can develop into cementum, adipo genesis or cartilage and may differentiate into neurons and glia cells under certain conditions [22], also demonstrated by Widera et al. [24]. Thus, PDLSCs, similar to original stem cells, may be used to treat diseases of the nervous system. Yet, the use of PDLSC in AD therapy has never been studied.

In this investigation, we studied the in vitro effects of PDLSCs on the structure and function of SH-SY5Y cells that were established as models of AD with the use of OA.

Methods

All patients signed a written informed consent to agree to participate in this study. The research protocol of this study was approved by the Ethics Committee of the General Hospital of People’s Liberation Army.

PDLSC culture

Ten healthy third molars were removed from four patients (female, 16-25 years old). The periodontal ligament tissue in the 1/3 root zone was scraped and transferred to a centrifuge tube under sterile conditions. The tube was gently shaken with 3 mg/ml type I collagenase (Gibco, NY, USA) and 4 mg/ml Dispase (Roche, Basel, Switzerland) and incubated at 37°C for 1 h. A single discrete cell was collected by passage through a 70 μm filter (Biolinx, Lenexa, KS, USA). The cells of all the patients were suspended at 1 × 10⁴/mL in 20% fetal bovine serum (FBS; Gibco) and alpha-minimum essential medium (α-MEM; Gibco), and incubated at 37°C for 1 h. The P1 generation of cells at 10-15 cells/mL density were used for PDLSC culture.

The P4 generation of human PDLSCs were digested and recovered cells were incubated with H-DMEM containing 20 nmol/L 490 nm. The P4 generation of PDLSCs was digested and transferred to 6-well plates with trypsin after spreading 1/3-1/2 area of the bottom surface of the well.

PDLSC verification

The P4 generation of PDLSs was digested and distributed among 12 tubes with 1 × 10⁵ cells in each tube. These were incubated serially with the following conjugated antibodies for 30 min at 4°C without light: cluster of differentiation (CD)166-phycocerythrin (PE); CD31-allophycocyanin (APC); CD44-APC-H7; CD34-phycocerythrin (PE); CD29-PE; CD73-PE; CD90-PE; CD105-fluorescein isothiocyanate (FITC); CD45-PE; CD14-FITC (all from BD Biosciences, San Jose, CA, USA); STRO-1-PE (Santa Cruz, CA, USA); CD3-FITC; human leukocyte antigen-antigen D related (HLA-DR)-PerCP/Cy5.5 (both from ebioscience, USA); and CD38-FITC (BioLegend, USA). The cells were washed in 1x phosphate-buffered saline (PBS) and after filtering the markers on the cell surface were analyzed by flow cytometry (BD Biosciences).

AD cell model

To establish the AD cell model in vitro, OA (Sigma) was used to damage the SH-SY5Y cells. The SH-SY5Y cells (Shanghai Cell Bank of Chinese Academy of Sciences) were recovered in high-glucose Dulbecco’s modified Eagle’s medium (H-DMEM; Sigma) with 10% FBS and seeded in 6-well plates, 6 × 10⁴ per well. After adherent the recovered cells were incubated with H-DMEM containing 20 nmol/L OA, and after 24 h the cells were ready for the following experiments.

Co-culture of PDLSCs and AD cell model

The cells treated with OA were transferred to 6-well plates with transwell (Millipore, USA). The P4 generation of human PDLSCs were seeded in the transwell at 8 × 10⁴ per well. 3 ml of α-MEM medium were added in each well to incubate for 24 h.

Cells in this study were divided into 3 groups: SH-SY5Y cells without any treatments (control group); SH-SY5Y cells treated with OA (OA group); and SH-SY5Y cells treated with OA and co-cultured with human PDLScs (OA+PDLSC).

Analysis of cell morphology

An inverted microscope (Nikon, Tokyo, Japan) was used to observe and analyze 10 random images from each experimental group (OA, OA+PDLSC and control). The 8 longest axons were selected and analyzed by Image-Pro plus 6.0 software.

Cells were fixed in 4% parafomaldehyde for 30 min and then stained with DiI (Beyotime Biotechnology, Shanghai, China) for 25 min without light at room temperature. Cell morphology was noted using a confocal microscope (Nikon). DiI is a lipophilic membrane stain that diffuses laterally to stain the entire cell [25].

Cell viability test

The cells were seeded in 96-well plates at 4 × 10³/well with 8 replications of each group, after washing in PBS for 3 times and incubated with cell counting kit-8 (CCK-8; Beyotime Biotechnology) for 2 h. The absorbance value was measured by microplate reader at 490 nm.

Cell apoptosis test

The cells were seeded in a confocal dish (Corning) at 1 × 10⁴/well with 8 replications of each group. After washing in PBS for 3 times, the cells were fixed with 4% paraformaldehyde for 30 min and then stained with Hoechst 33342 (Beyotime Biotechnology) for 5 min without light. The nuclei of apoptotic cells were observed by confocal microscopy. The percentage of dark staining and broken cells among the total cell number were calculated in 10 images from each group.

Immunofluorescence

The cells were seeded in a confocal dish (Corning) at 1 × 10⁴/well, with 8 replications of each group. After washing in PBS 3 times, cells were fixed with 4% paraformaldehyde for 30 min, incubated with 0.1% triton for 15 min and 10% goat serum (Gibco) for 30 min.

Cells were treated with the primary mouse antibody α-tubulin (1:4000 dilution; Cell Signaling Technology, Beverly, MA, USA) for 2 h in 37°C and then FITC-conjugated secondary antibody (1:400 dilution;
ZSGB Biotechnology, Beijing, China) and tetramethylrhodamine (TRITC)-labeled phalloidin (1:100 dilution; Thermo Fisher) for 30 min without light. After washing with PBS for 3 times, samples were stained with DAPI (4',6-diamidino-2-phenylindole) and observed by confocal microscopy.

Transmission electron microscopy

The cells were seeded in 96-well plates for electron microscopy at 1 × 10^5/well with 8 replications of each group, after fixed with 2.5% glutaraldehyde for 2 h in 4°C, 0.1 mol/L phoshoric acid buffer was used to wash 15 min for 3 times. Cells were fixed with 1% osmium tetroxide for 2 h, washed in PBS for 3 times, dehydrated in graded ethanol, infiltrated with epoxy resin, embedded with fresh resin. Ultrathin sectioning and staining followed. The samples were observed and analyzed by transmission electron microscopy (JEM-1400, JEOL, Japan).

Western blot

The cells were seeded in 6-well plates with 8 replications of each group. RIPA lysis buffer (500 μL) with protease inhibitor (100:1) and phosphatase inhibitor (1001, Roche) was used to suspend cells in a shaker for 1 h at 4°C. After centrifugation at 16000 g for 20 min the supernatants were collected as protein samples.

A 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel was used to resolve the proteins during electrophoresis and then transferred to nitrocellulose membranes. Membranes were blocked with 5% of skimmed milk in TBST for 1 h in room temperature. These were incubated serially with the following primary antibodies at 4°C overnight: GAPDH (1:2000, Invitrogen, USA), pTau in Ser 396 (1:1000, Invitrogen), and total tau (1:800, Proteintech, Chicago, IL, USA).

Membranes were incubated with secondary antibodies (1:400 dilution; ZSGB Biotechnology, Beijing, China) for 1 h at room temperature. After washing in TBST for three times, the membranes were incubated with enhanced chemiluminescence (ECL) buffer (Merck Millipore) for 1 min and then exposed to x-ray film in a darkroom and scanned by Gel image analysis.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). The statistical analysis was performed using SPSS 17.0 software. The lengths of axons and cell viability were compared by one-way analysis of variance. The percentage of apoptotic cells was compared by chi-squared test. P<0.05 was considered a significant difference.

Results

Cell morphology and phenotypic characteristics of human PDLSCs

Using an inverted microscope, we observed that the human PDLSCs had a fibroblast-like morphology with a vortex-like arrangement (Figure 1A). We utilized flow cytometry to detect cell surface proteins on the human PDLSCs. The following were present: CD166, CD44, CD29, CD73, CD90, CD105 and STRO-1. The following were not found: CD31, CD34, CD45, HLA-DR, CD14 and CD38 (Figure 1B).

PDLSCs treatment improved the cell shape and dendritic length

Using inverted phase microscopy (Figure 2A) and confocal microscopy with DiI staining (Figure 2B), we observed that the cells of the OA group (SH-SY5Y cells treated with OA) had less cytoplasm than did the cells of the control group (SH-SY5Y cells without any treatment), with collapsed soma and broken or shortened dendrites. However, compared with the OA group, the OA+PDLSC group (SH-SY5Y cells treated with OA and co-cultured with PDLSCs) had a better cell shape, cytoplasm and dendrites, similar to the control group (Figures 2A and 2B).

The dendritic length of the OA group (26.64 ± 9.12 μm) was shorter than that of the control group (52.73 ± 9.94 μm). The dendritic length of the OA+PDLSC group (42.55 ± 10.65 μm) was longer than that of the OA group (26.64 ± 9.12 μm). This suggests that exposure to PDLSC reversed the effect of OA on the SH-SY5Y cells (Figure 2C).

Effect of PDLSC exposure on cell viability and apoptosis

A Cell Counting Kit-8 (CCK-8) was used for cell proliferation and...
cytotoxicity assays to determine the cell viability of SH-SY5Y cells [25]. We found that the absorbance value at 490 nm of the OA group (0.85 ± 0.01) was less than that of the control group (0.89 ± 0.04). This suggests that OA treatment resulted in reduced cell viability. In addition, the absorbance of the OA+PDLSC group (0.93 ± 0.04) was higher than that of the OA group (0.85 ± 0.01). This indicates that exposure to PDLSCs improved the cell viability relative to the OA group (Figure 3A).

The Hoechst 33258 dye for DNA revealed that the cell nuclei of the control group were similarly round with uniform blue staining. In the OA group, the cell nuclei were various in shape and shrink fragments and staining was uneven. The cell nuclei of the...
OA+PDLSC group appeared less injured than that of the OA group (Figure 3B).

The percentage of apoptotic SH-SY5Y cells was higher in the OA group (22.21 ± 3.42%) than the control group (8.05 ± 0.83%), while that of the OA+PDLSC group (13.77 ± 1.51%) was lower than that of the OA group (Figure 3C).

**Effect of PDLSC exposure on microtubule-associated proteins and structure**

In this study, α-tubulin labeled by FITC was used to reveal microtubule-associated protein [27,28] and F-actin labeled by TRITC was used to show the cytoskeleton [29,30]. The microtubules and microfilaments of the OA group were in disarray, shorter, and fewer compared with that of the control group, while those of the OA+PDLSC group were more similar to the control group (Figure 4A).

Quantitative analyses were conducted of the mean dendritic length, fluorescence intensity, and fluorescence area of the SH-SY5Y cells, using Volocity Demo 6.1.1 software (Figure 4B). In the OA group, the mean dendritic length (20.25 μm ± 4.18 μm), fluorescence intensity (2157.35 ± 459.58) and fluorescence area (309.75 ± 27.92 μm²) were each less than that of the control group (55.75 μm ± 6.93 μm, 3525.88 ± 627.31 and 514.33 ± 46.27 μm², respectively). In the OA+PDLSC group, the mean dendritic length, fluorescence intensity and fluorescence area data fell between that of the OA and control groups (50.03 μm ± 5.24 μm, 2796.21 ± 520.98 and 421.92 ± 33.81 μm²) (Figures 4C and 4D).

**Effect of PDLSC exposure on microtubule structure induced by OA**

The ultrastructure of the SH-SY5Y cells was observed via transmission electron microscopy (Figure 5). The cells of the OA group showed obvious damage relative to that of the control group, in which microtubules were blurred or lost and vacuolar-like structures in cytoplasm. In the OA+PDLSC group, the ultrastructure of the cells was less damaged, with clear microtubules arranged in fiber bundles that were more similar to the control group.

**Effect of PDLSC exposure on pTau levels**

The results of the western blot showed that the ratio of pTau at Ser-396 to the total Tau of the OA group (1.86 ± 0.20) was significantly higher than that of the control group (0.64 ± 0.20), while that of the OA+PDLSC group (0.99 ± 0.24) was closer to that of the control group (Figures 6A and 6B).

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**Figure 4:** Effects of PDLSC treatment on cytoskeleton-associated protein expression. Alpha-tubulin was labeled by FITC (green). F-actin was labeled by TRITC (red). (A) The structures of microtubules and microfilaments were observed by confocal microscopy. Scale bar: 50 μm. (B) Quantitative analysis of dendritic length; (C) Mean fluorescence intensity (MFI); (D) Mean area of fluorescence. N=3 in each group. Data are expressed in mean ± SD

*P<0.05, **P<0.01, ***P<0.001
Discussion

In this study, we found for the first time that exposure to PDLSCs is associated with beneficial effects in an in vitro AD model established using SH-SY5Y cells by OA induction. Co-culture with PDLSCs was associated with improved cell morphology, biological activity, and cytoskeletal structure, and a lower rate of cell apoptosis, perhaps by way of inhibiting hyper phosphorylation of Tau protein.

PDLSCs originate from the cranial neural crest during tooth development, and are located in periodontal ligament tissue. The periodontal ligament connects the bone and cementum in the roots of the teeth, adapting the position of the tooth in the socket. PDLSCs are characterized by plasticity and the ability to proliferate [22], with a crucial role in the dynamic renovation of teeth. Compared to bone marrow stromal cells (BMSCs), PDLSCs have stronger clonal ability [31]. For example, under the same conditions, within 96 h of incubation the number of PDLSCs was twice that of the BMSCs [31]. Phenotypic analysis has shown that PDLSCs express the associated proteins on surface of mesenchymal stem cells, and also express specific markers of the neural crest such as Slug, Snail, Sox10, Nestin, NeuN and β3-tubulin [32,33]. Although the amount of periodontal ligament tissue and PDLSCs is limited compared with other types of stem cells, the strong proliferative ability of PDLSCs has a potential advantage in neural regeneration.

Studies concerning PDLSCs have mainly focused on the periodontal area, with far fewer investigations of the use of PDLSCs to treat nervous system diseases. The periodontal ligament is rich in nerves and sensitive to various stimuli, with a proprioceptive function. The PDLSCs can secrete multiple neurotrophic factors. Co-culture with trigeminal neurons promotes the growth of axons [34]. PDLSCs are able to differentiate into neurofilament protein-positive neurons, glial fibrillary acidic protein (GFAP)-positive astrocytes, and CNP (2',3'-cyclic nucleotide phosphodiesterase)-positive oligodendrocytes [35]. Byers et al. [36] found that periodontal ligaments express nestin and GFAP, which are the precursors of neurons and glial cells respectively, indicating that cultured PDLSCs, could differentiate into neurons. Li et al. [37] reported that, in a rat model of peripheral nerve injury, human PDLSCs injected into a crushed-injury left mental nerve improved recovery of sensory function and axonal regeneration, especially after 4 weeks. This supported the efficient therapeutic effect of PDLSCs, which was comparable to autologous Schwann cells in axonal regeneration. These investigations provide evidence for applying PDLSCs in the treatment of AD.

Indeed, the stem cell therapy in AD has gained support in recent years. For example, intravenous or intracranial injection of mesenchymal stem cells into mouse models of AD was shown to have a beneficial effect [38,39], although the underlying mechanism remains unclear because of the various in vivo factors. The cell paracrine cytokines and partial differentiation after cell transplantation may be possible mechanisms of PDLSC efficiency. In this study, we co-cultured PDLSCs and SH-SY5Y cells treated with OA in trans-well way and found that the impaired functions of cells and dendrites induced by...
OA were rescued by PDLSCs. This rescued effect was believed to be paracrine and not via differentiation, since cells were not in direct contact. These results may suggest that PDLSCs could promote neural regeneration by secreting multiple cytokines or regulating the local microenvironment.

To clarify the effects of PDLSCs on cytoskeleton, we utilized confocal and transmission electron microscopy to reveal the cytoskeleton proteins and structure with α-tubulin and F-actin. In AD-model cells treated by PDLSCs, the structure of the cytoskeleton was significantly less damaged and in less disarray than that of AD-model cells not exposed to PDLSCs. Furthermore, there was less hyper phosphorylated Tau protein in the AD-model cells treated by PDLSCs, which may have contributed to the better cell morphology observed in these cells.

**Conclusion**

In summary, herein we report that OA treatment of SH-SY5Y cells established a reliable model of AD. Furthermore, the PDLSCs co-cultured in trans-well way showed definite differences in cell morphology and function compared with AD-model cells that were not exposed to PDLSC treatment. The effect of the PDLSC treatment may be due to inhibiting phosphorylation of tau protein. This study suggests that PDLSCs may be a potential therapy in AD, although the underlying mechanisms warrant further study.

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