Expression and Function of the Actin-severing Protein Adseverin in the Proliferation, Migration, and Differentiation of Dental Pulp Cells

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Abstract

Introduction: Adseverin is an actin-severing and actin-capping protein that is primarily expressed in secretory cells, where it regulates the filamentous actin cytoskeleton during cell differentiation and exocytosis. However, little is known regarding its regulatory role in dental pulp cells (DPCs). This study examined the expression and function of adseverin in the proliferation, migration, and odontoblastic differentiation of DPCs. **Methods:** DPCs were assayed for morphologic changes, proliferation, migration, alkaline phosphatase activity, and dentin sialoprotein and dentin matrix protein-1 protein levels *in vitro* after knockdown of adseverin by using small interfering RNA. Tooth germs isolated from Sprague-Dawley rats were processed for immunohistochemistry analysis of adseverin. **Results:** Adseverin expression was increased in a time-dependent fashion in the early stage of odontoblastic differentiation. When adseverin expression was suppressed in DPCs, their cellular morphology was altered, and their proliferation, migration, and odontoblastic differentiation were substantially decreased *in vitro*. Secretory odontoblasts in the tooth germ at day 5 post partum expressed a stronger adseverin signal compared with those at days 1 and 3 post partum. **Conclusions:** Adseverin may play a crucial role in the proliferation, migration, and odontoblastic differentiation of DPCs via filamentous actin cytoskeleton regulation. However, further investigations are required to clarify the underlying mechanisms. *(J Endod 2015;41:493–500)*

Key Words

Adseverin, dental pulp cells, differentiation, odontoblasts

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An odontoblast is a functional cellular unit responsible for the development of the physiological primary and secondary dentins (1). Dentin regeneration, mediated by the odontoblastic differentiation of potential precursor cells, is required in the case of intensive pathologic stimuli, which leads to the death of existing odontoblasts (2). Dental pulp cells (DPCs) are a group of potential precursor cells with the capacity to differentiate into new odontoblasts that are responsible for the secretion of dentin matrix, such as alkaline phosphatase (ALP), dentin sialoprotein (DSP), and dentin matrix protein-1 (DMP-1) (3, 4). This differentiation process is accompanied by drastic changes in a complex cytoskeleton of microtubules, intermediate filaments, and actin filaments (5, 6).

The cytoskeleton plays an important role in a variety of cell functions, including proliferation, migration, and differentiation (7–9). Adseverin, a member of the gelsolin superfamily, was first discovered in secretory cells such as platelets, megakaryocytes, and chromaffin cells (10–13). It governs the filamentous actin (F-actin) cytoskeleton remodeling via calcium-regulated and intracellular pH–regulated actin-severing and actin-capping activities. Adseverin also has an integral role in the differentiation program in a number of cell lineages, including chondrocyte differentiation and maturation (14). To date, the molecular links between cytoskeletal organization and cell differentiation in DPCs remain elusive despite a potential role of cytoskeleton proteins in the control of the early odontoblastic differentiation revealed by proteomic profiling (4).

In the present study, we hypothesized that adseverin-mediated actin reorganization controls cell morphology, which could directly affect the biofunctions of DPCs. The purpose of this study was to investigate the expression, distribution, and possible functions of adseverin associated with odontoblastic differentiation.

Materials and Methods

Cell Culture, Small Interfering RNA Transfection, and Induction of Odontoblastic Differentiation

Six sound impacted third molars were collected from 3 patients (14 to 25 years old) undergoing orthodontic treatment at the Department of Oral and Maxillofacial Surgery at the Affiliate Stomatology Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China. Informed consent was obtained from each patient. Separated DPCs were cultured as previously reported (15). Briefly, DPCs were cultured in Dulbecco modified Eagle medium (DMEM) (GIBCO-BRL Life Technologies, Guangzhou Genewindows Biotech Ltd, Guangzhou, China) supplemented with 10% fetal bovine serum (GIBCO-Invitrogen, Grand Island, NY), 10 U/mL penicillin, and 10 mg/mL streptomycin (Sigma-Aldrich, St Louis, MO).

DPCs (passages 3–5) were transfected with 50 nmol/L small interfering RNA (siRNA) targeting the human gene adseverin (si-Ads) by using Lipofectamine RNAi Max (Life Technologies) according to the manufacturer's instructions. Cells transfected with non-targeting siRNA (si-NT) or without siRNA (mock transfection) were used as negative controls. The human si-Ads (forward, 5′-GGAGAUUUGGCGUGUAGAAdTdT-3′, and reverse, 3′-dTdT CCUCUAAACCGCACAUCUU-5′) and si-NT were synthesized by Ribobio (Guangzhou, China).

Odontogenic medium (OM) containing 10 mmol β-glycerophosphate (Sigma-Aldrich), 0.2 mmol ascorbic acid (Sigma-Aldrich), and 100 nmol dexamethasone (Sigma-Aldrich) in growth medium was used to induce odontoblastic differentiation in the following experiments (16).

Immunofluorescence

DPCs transfected with si-Ads or si-NT were cultured in OM for 0–7 days. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% bovine serum albumin. Primary anti-human adseverin (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated overnight at 4°C. Fluorescein isothiocyanate–conjugated goat anti-mouse secondary antibody (Earth, San Francisco, USA) and rhodamine-phalloidin (Cytoskeleton Inc, Denver, CO), counterstained with DAPI, were used for detection under confocal microscopy. Cells were manually traced, and images for cell width, length, and area were acquired by using ImageJ software (National Institutes of Health, Bethesda, MD).

Cell Count Kit-8 Assay for Proliferation

DPCs were transfected with si-Ads or si-NT for 24, 48, or 72 hours. Mock-transfected cells were used as a negative control. Cell Count Kit-8 (Dojindo, Kumamoto, Japan) was added to each well in 10% volume, followed by incubation in the dark for 2 hours. The optical density was measured at 450 nm by using a microplate reader (Tecan, Reading, UK).

Cell Cycle and Apoptosis Analysis

DPCs transfected with si-Ads or si-NT for 72 hours were used for cell cycle and apoptosis analyses. For the cell cycle assay, treated cells were fixed in pre-chilled ethanol and incubated with RNase cocktail. Cell cycle staining kits (Multisciences, Hangzhou, China) were used for DNA staining in the dark. The annexin V/propidium iodide apoptosis assay was performed by using a cell apoptosis detection kit (KeyGEN BioTECH, Nanjing, China) according to the manufacturer's protocols. All of the samples were analyzed by using a FACSCalibur flow cytometer (BD Bioscience, Mississauga, ON, Canada).

Cell Migration Assay

The si-Ads and si-NT DPCs were trypsinized and resuspended in serum-free DMEM at a density of 3×10^5/mL. Aliquots of 200-μL suspension were seeded into the upper chamber of the Costar Transwell system (Corning Inc, Corning, NY). The lower chamber was filled with 600 μL DMEM containing 10% fetal bovine serum. The cells were allowed to migrate through the 8-μm pores for 16 hours. The cells present beneath the membrane were stained with DAPI. Ten fields per sample were randomly selected to perform the quantitative analysis of cell migration by counting the cell nucleus.

Western Blotting

DPCs with or without silenced adseverin were grown in OM for the scheduled time. Mock-transfected cells without OM incubation were used as a negative control, whereas cells grown in OM were used as a positive control. The lysates were harvested with radioimmunoprecipitation lysis buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 1% sodium dodecylsulfate, 1% Nonidet P-40, 1:1000 proteinase inhibitor cocktail, 50 mmol/L β-glycerophosphate, and 50 mmol/L sodium fluoride), separated by 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis, and transferred onto 0.22-μm nitrocellulose membranes (MILIPORE, Livingston, UK). Mouse anti-human adseverin, gelsolin (BD Transduction Laboratories, Le Pont de Claix, France), DMP-1 (Abcam, Cambridge, UK), DSP (Santa Cruz Biotechnology), or β-actin (Earth) were incubated overnight at 4°C. Final detection was completed by using the appropriate secondary antibody according to the enhanced chemiluminescence method. Densitometry analysis of the expressed bands was measured by using ImageJ software.

Alkaline Phosphatase Activity Assay

The si-NT or si-Ads DPCs seeded into 24-well culture plates were grown in OM for 7 days. Plates were washed twice, followed by the addition of 120 μL 1% Triton X-100. After incubation overnight at 4°C, 30-μL aliquots of cell lysate per well were subjected to an alkaline phosphatase (ALP) activity assay by using an ALP kit (Jiancheng, Nanjing, China). The protein concentration was measured by using a BCA protein assay kit (Boshide, Wuhan, China) to normalize the enzyme activity.

Immunohistochemistry

Nine Sprague-Dawley rats (Laboratory Animal Centre, Sun Yat-sen University) at days 1, 3, and 5 post partum were killed by using an animal study protocol approved by the University Ethics Committee. Mandibles containing the first molars were dissected, fixed in 4% paraformaldehyde, and decalcified in methanoic acid for 5 days, followed by paraffin embedding. Mesiodistal sections were used for immunohistochemical detection of adseverin, and the images were obtained by using an optical microscope (Zeiss, Oberkochen, Germany).

Statistical Analysis

The experiments were performed at least 3 times. The results were evaluated by one-way analysis of variance or the Bonferroni test and expressed as the mean and standard deviation. The Kruskal-Wallis test was used to assess the variance of heterogeneity. A P value less than .05 was considered statistically significant.

Results

Adseverin Acts as a Morphologic Regulator of DPCs

DPCs without OM incubation exhibited fusiform shapes with well-developed actin stress fibers running parallel to the long axis of the cell. Adseverin was primarily colocalized with the actin stress fibers within the cytoplasm (Fig. 1*A*). After odontogenic differentiation, the cells exhibited cuboidal and polygonal shapes with increased cell size and elongated cellular processes, and both adseverin and F-actin were strongly detected in the cellular processes. OM stimulation triggered F-actin network reorganization, which was rearranged to form a nondirectional pattern (Fig. 1*A*).

The si-Ads treatment altered the DPCs from a fusiform into a short rod–like morphology (Fig. 1*A*). After OM incubation, the si-Ads cells became round without any cellular processes (Fig. 1*A*). The length and spread area of adseverin-depleted cells decreased when compared with control cells ($P < .05$, Fig. 1*B*), whereas the cell width was not changed ($P > .05$, Fig. 1*B*). These results indicated that adseverin primarily acted as a morphologic regulator of DPCs during odontoblastic differentiation.

Involvement of Adseverin in the Proliferation and Migration of DPCs

Western blotting was used to investigate the efficiency of si-Ads transfection in DPCs. Our results showed that the protein level of adseverin was reduced by nearly 75% (3 days after si-Ads transfection) and