Transforming growth factor-β-induced gene product-h3 inhibits odontoblastic differentiation of dental pulp cells

Suguru Serita[a], Atsushi Tomokiyo[b], Daigaku Hasegawa[b], Sayuri Hamano[a], Hideki Sugii[a], Shinichiro Yoshida[b], Hiroyuki Mizumachi[a], Hiromi Mitarai[a], Satoshi Monnouchi[a], Naohisa Wada[c], Hidefumi Maeda[a,b,*]

[a] Department of Endodontology and Operative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Division of Endodontology, Kyushu University Hospital, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[c] Division of General Dentistry, Kyushu University Hospital, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

ARTICLE INFO

ABSTRACT

Objective: The aim of this study was to investigate transforming growth factor-β-induced gene product-h3 (βig-h3) expression in dental pulp tissue and its effects on odontoblastic differentiation of dental pulp cells (DPCs).
Design: A rat direct pulp capping model was prepared using perforated rat upper first molars capped with mineral trioxide aggregate cement. Human DPCs (HDPCs) were isolated from extracted teeth. βig-h3 expression in rat dental pulp tissue and HDPCs was assessed by immunostaining. Mineralization of HDPCs was assessed by Alizarin red-S staining. Odontoblast-related gene expression in HDPCs was analyzed by quantitative RT-PCR.
Results: Expression of βig-h3 was detected in rat dental pulp tissue, and attenuated by direct pulp capping, while expression of interleukin-1β and tumor necrosis factor-α was increased in exposed pulp tissue. βig-h3 expression was also detected in HDPCs, with reduced expression during odontoblastic differentiation. The above cytokines reduced βig-h3 expression in HDPCs, and promoted their mineralization. Recombinant βig-h3 inhibited the expression of odontoblast-related genes and mineralization of HDPCs, while knockdown of βig-h3 gene expression promoted the expression of odontoblast-related genes in HDPCs.
Conclusions: The present findings suggest that βig-h3 in DPCs may be involved in reparative dentin formation and that its expression is likely to negatively regulate this process.

1. Introduction

Dental pulp tissue does not become mineralized under physiological conditions. However, reparative dentin is formed in response to nociceptive stimulation by caries, tooth cutting, or trauma. Dental pulp cells (DPCs) are considered to differentiate into odontoblasts prior to the production of reparative dentin. Several extracellular matrix (ECM) proteins in dentin, such as dentin sialophosphoprotein (DSPP), osteocalcin (OCN), and osteopontin (OPN), are considered to be related to odontoblastic differentiation. Various growth factors or cytokines, such as bone morphogenetic proteins, Wnt, and tumor necrosis factor (TNF)-α, were reported to be involved in odontoblastic differentiation of human DPCs (HDPCs), resulting in reparative dentinogenesis (Peng et al., 2010; Qin et al., 2012; Ueda et al., 2014). Moreover, activation of immunocompetent cells and secretion of inflammatory cytokines were observed in pulp tissue treated with pulp capping agents prior to reparative dentin formation (Goldberg, Njeh, & Uzunoglu, 2015), suggesting that these processes may be involved in dentinogenesis. However, the underlying mechanism for odontoblastic differentiation of DPCs has not been revealed.

Transforming growth factor (TGF)-β is a multifunctional growth factor expressed not only in many types of adult tissues, but also in developing cartridge and pituitary gland (Bazina, Vukojevic, Roje, & Saraga-Babic, 2009; Moroco, Hinton, Buschang, Milam, & Iacopino, 1997). TGF-β is known to regulate cell proliferation, cell migration, ECM synthesis, and inflammatory

processes (Cubela et al., 2016; Fujii et al., 2010; ten Dijke & Arthur, 2007). TGF-β-induced gene product-h3 (βig-h3) is a one of the ECM proteins induced by TGF-β (Skonier et al., 1992). βig-h3 has a signal sequence at the N-terminus, an RGD sequence in the C-terminus, and a FAS1 domain composed of four repeating sequences. The protein has been detected in various tissues, such as skin, blood vessels, and smooth muscle (Billings et al., 2000; LeBaron et al., 1995; Schorderet et al., 2000), and is involved in cell attachment, cell spreading, angiogenesis, and tumorigenesis (Aitkenhead et al., 2002; Ma et al., 2008; Ohno et al., 1999).

βig-h3 contributes to cell-matrix attachment through interactions of its RGD sequence with integrin subtypes, such as αvβ3 and αvβ5 (Choi et al., 2015; Ma et al., 2008). βig-h3 was also shown to interact with other ECM components, such as collagen I (Hashimoto et al., 1997). βig-h3 expression was observed in osteoblasts and periodontal ligament cells, while recombinant βig-h3 inhibited osteoblastic maturation (Thapa, Kang, & Kim, 2005) and osteoblastic differentiation of periodontal ligament cells (Ohno et al., 2002). However, the expression and functions of βig-h3 in dental pulp tissue have not been examined.

We hypothesized that βig-h3 is involved in reparative dentinogenesis and odontoblastic differentiation of DPCs. To explore this hypothesis, we investigated βig-h3 expression in dental pulp tissue after direct pulp capping, and its effects on odontoblastic differentiation of DPCs.

2. Materials and methods

2.1. Cell culture

Three samples of HDPCs were isolated from healthy teeth of a 24-year-old male (HDPC-3R), 27-year-old female (HDPC-3F), and 24-year-old female (HDPC-5A) with informed consent as described previously (Yoshida et al., 2016). Briefly, the extracted teeth were split and the dental pulp tissue was removed. The obtained pulp tissue was digested with 0.2% collagenase and 0.25% trypsin (Wako Pure Chemical Industries, Osaka, Japan) for 20 min at 37 °C to isolate single-cell populations. HDPCs at passages 3–8 were maintained in control medium (CM), defined as α-minimal essential medium (Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Biowest, Nuaille, France), at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.2. Reagents

The goat anti-rat βig-h3 polyclonal antibody, rabbit anti-rat interleukin (IL)-1β polyclonal antibody, normal goat IgG, and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Alexa Fluor 568-conjugated rabbit anti-goat IgG antibody was purchased from Invitrogen (Carlsbad, CA). The rabbit anti-rat TNF-α polyclonal antibody, recombinant human IL-1β, and recombinant human TNF-α were purchased from PeproTech EC (London, UK). Recombinant human βig-h3 was purchased from R&D Systems (Minneapolis, MN). βig-h3 small-interfering RNA (siRNA) and control siRNA were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Direct pulp capping model

A direct pulp capping model was prepared in rats as described previously (Yoshida et al., 2016). Briefly, the upper first molars of 8-week-old male Wistar rats (Kyudo, Saga, Japan) were perforated through the occlusal surface with a no. 1/2 round steel bur. Direct pulp capping was performed with ProRoot mineral trioxide aggregate (MTA; Dentsply-Sankin, Tokyo, Japan) and sealed with glass ionomer cement (Fuji IX; GC Corporation, Tokyo, Japan).

2.4. Immunofluorescence analysis

HDPCs cultured in CM for 24 h were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) containing 0.5% dimethyl sulfoxide (Wako Pure Chemical Industries) for 20 min, and washed with phosphate-buffered saline (PBS). After blocking with 2% bovine serum albumin (BSA; Nacalai Tesque, Kyoto, Japan) in PBS for 1 h, the cells were incubated with anti-βig-h3 goat antibody or normal goat IgG for 1 h, washed with PBS, and incubated with Alexa Fluor 568-conjugated rabbit anti-goat IgG antibody for 30 min. After washing with PBS, nuclear staining was performed with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Fluorescence images were observed using a Biozero digital microscope (Keyence, Osaka, Japan).

2.5. Immunohistochemical analysis

Immunohistochemical analysis was performed as described previously (Hasegawa et al., 2015). Briefly, the upper first molars of direct pulp capping model rats or normal rats were embedded in paraffin and sectioned at 5-μm thickness. After blocking with 2% BSA in PBS for 1 h, the sections were incubated with anti-βig-h3 goat antibody, anti-IL-1β rabbit antibody, anti-TNF-α rabbit antibody, or normal IgG overnight. The sections were then treated with biotinylated anti-goat IgG antibody or anti-rabbit IgG antibody, followed by avidin-peroxidase conjugate (Nichirei Bioscience, Tokyo, Japan). Positive reactions were visualized using DAB solution (Nichirei Bioscience). Nuclear staining was performed with Mayer's hematoxylin solution (Wako Pure Chemical Industries).

2.6. Odontoblastic differentiation of HDPCs

HDPCs were cultured in CM or CM containing 2 mM $CaCl_2$ as differentiation medium (DM), based on a recent report that this DM promoted osteoblastic differentiation of human periodontal ligament cells (Koori et al., 2014). The expression of odontoblast-related genes (DSPP, OCN, and OPN) in HDPCs was assessed by quantitative RT-PCR. Mineralization of HDPCs was evaluated by Alizarin red-S staining.

2.7. Semi-quantitative RT-PCR

Total cellular RNA was harvested using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcription (RT) with an ExScript RT Reagent Kit (Takara Bio, Shiga, Japan). Polymerase chain reaction (PCR) assays were performed with a cycle consisting of heat denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 30 s. The primer sequences, product sizes, annealing temperatures, and cycle numbers for integrin αv, β3, and β5, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown in Table 1. The PCR products were electrophoresed in a 2% agarose gel (Seakem ME; BioWhittaker Molecular Applications, Rockland, ME).

2.8. Quantitative RT-PCR

Total cellular RNA was harvested using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized by RT with an ExScript RT Reagent Kit (Takara Bio). Quantitative PCR assays were performed with a SYBR Green RT-PCR Kit (Takara Bio) using a Thermal Cycler Dice Real Time System (Takara Bio) with the following program: 95 °C for 10 s; 40 cycles at 95 °C for 5 s and 60 °C for 30 s; dissociation cycle at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. A human β-actin