Journal of Oral Biosciences ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Journal of Oral Biosciences

journal homepage: www.elsevier.com/locate/job

Original Article

Relationship between localization of proteoglycans and induction of neurotrophic factors in mouse dental pulp

Akira Yukita [a,*], Miroku Hara [b], Akihiro Hosoya [a], Hiroaki Nakamura [a]

[a] Department of Hard Tissue Research, Graduate School of Oral Medicine, Matsumoto Dental University, 1780 Hirooka-gobara, Shiojiri, Nagano 399-0781, Japan
[b] Department of Oral Histology, Matsumoto Dental University, 1780 Hirooka-gobara, Shiojiri, Nagano 399-0781, Japan

ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form
29 August 2016
Accepted 29 September 2016

Keywords:
Heparan sulfate
Perlecan
FGF-induced Erk1/2 signaling
GDNF

ABSTRACT

Objectives: Dental pulp is composed of an odontoblastic layer, a subodontoblastic layer, and a central part. Although extracellular matrices affect the cells residing in these regions, the precise role of proteoglycans in dental pulp is incompletely understood. Hence, we investigated the immunolocalization of glucosaminoglycans, such as heparan sulfate and chondroitin sulfate, as well as core proteins of proteoglycans, such as perlecan and versican, in adult mouse dental pulp.
Methods: Localization of glucosaminoglycans and proteoglycans in dental pulp was evaluated immunohistochemically. Dental pulp cells cultured with or without fibroblast growth factor 2 (FGF2) and heparin were stained with Alizarin red S to visualize calcified nodules. Real-time PCR was used to assess the levels of gene expression of neurotrophic factors in cultured cells.
Results: Heparan sulfate and perlecan were mainly localized in the subodontoblastic layer, whereas chondroitin sulfate and versican were restricted to the center of dental pulp. Similar to perlecan, Erk1/2, a signaling transducer of FGF, was localized in the subodontoblastic layer. Moreover, we show that simultaneous treatment of cultured dental pulp cells with recombinant human FGF2 and heparin induced the expression of glial cell-derived neurotrophic factor (*GDNF*).
Conclusions: These data suggest that heparan sulfate proteoglycans, such as perlecan, enhance the expression of *GDNF* by facilitating FGF-induced Erk1/2 signaling in the subodontoblastic layer.

1. Introduction

Dental pulp is composed of odontoblastic layer, subodontoblastic layer, and a central region. The distribution of the cells, such as odontoblasts, fibroblasts, and endothelial cells, which form these layers in dental pulp, is strictly regulated. Odontoblasts are localized in the odontoblast layer, situated at the edge of dental pulp near the predentin, whereas fibroblasts and mesenchymal stem cells mainly reside in the center of dental pulp [1,2]. Cells residing in the subodontoblastic layer, located between the odontoblastic layer and the center of dental pulp, can differentiate into odontoblast-like cells that form hard tissues in response to various stimuli [3–5]. Although extracellular matrices (ECMs) play important roles in the differentiation, survival, and distribution of cells residing in these regions, the precise function of ECMs in dental pulp remains obscure. Versican is a large chondroitin sulfate proteoglycan expressed in various tissues such as the brain, blood vessels, and cartilage; it is involved in water holding capacity, cell shape, proliferation, differentiation, and the inflammatory response [6,7]. Perlecan, one of the heparan sulfate proteoglycans, is localized in basement membranes as well as in the extracellular matrix [8,9]. Heparan sulfate, via its own negative charge, maintains heparin-binding growth factors such as FGFs, bone morphogenetic proteins (BMPs), and Wnts. Heparan sulfate proteoglycans regulate the signaling pathways involving these growth factors [10–15]. In the developing tooth, perlecan is localized in the intercellular space of the enamel organ, in dental mesenchymal tissues, and in Hertwig's epithelial root sheath [16,17]. In mice, overexpression of perlecan in the epithelial cells induces abnormal formation and poor crystallization of the molars [18]. Although these findings suggest that perlecan plays an important role in tooth development by regulating the activities of heparin-binding growth factors, the function of perlecan in adult dental pulp remains unclear.

Neurotrophic factors, such as neural growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-cell derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and neurotrophin-3 and -4/5 (NT-3, NT-4/5), play important roles in the survival and differentiation of neural cells [19]. Previous studies have shown that

* Corresponding author. Present address: Department of Education (Science), Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka-city, Shizuoka 422-8529, Japan.
E-mail address: yukita.akira@shizuoka.ac.jp (A. Yukita).

http://dx.doi.org/10.1016/j.job.2016.10.001
1349-0079/

these factors are expressed in the developing tooth; some are expressed more in dental pulp stem cells than in the bone marrow stromal cells [20–22]. Although these data suggest that neurotrophic factors are involved in tooth innervation, the mechanisms regulating the expression and function of these factors in the dental pulp of adult rodents remain obscure.

In this study, to clarify the differences in the functions of heparan sulfate, chondroitin sulfate, perlecan, and versican, we investigated the localization of these proteoglycans and core proteins in the dental pulp of adult mice. Furthermore, we examined the localization of the signaling transducers of heparin-binding growth factors such as Wnts, BMPs, and FGFs. Finally, we tested whether treatment with FGF2 affects the expression of neurotrophic factors in cultured dental pulp cells.

2. Materials and methods

2.1. Animals

Four-week-old male C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All procedures for animal care were approved by the Animal Management Committee of Matsumoto Dental University.

2.2. Immunohistochemistry

Mouse mandibles were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) and then demineralized in 10% EDTA for 3 weeks. After dehydration in a graded series of ethanol, the samples were embedded in paraffin and sectioned on a standard microtome (Type LS-113, Yamato Kohki Industrial Co., LTD., Saitama, Japan). For the detection of chondroitin sulfate, sections were reacted with chondroitinase ABC (SEIKAGAKU Co., Tokyo, Japan) for 1 h at 37 °C, pretreated with 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) for 30 min at 25 °C, and then incubated with the primary antibody for 12 h at 4 °C. The samples were subsequently washed with PBS and treated with Histofine Simple Stain mouse MAX-PO (Nichirei Co., Tokyo, Japan) for 1 h at room temperature. After one wash with PBS, immunoreactivity was visualized using a DAB kit (Dako, Glostrup, Denmark) and sections were counterstained with Carrazzi's hematoxylin solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

The following primary antibodies were used for immunohistochemistry: anti-heparan sulfate (clone F58-10E4, SEIKAGAKU), anti-chondroitin sulfate (clone 2-B-6 Di-4S and clone 1-B-5 Di-0S, SEIKAGAKU), anti-perlecan (clone HK-102, SEIKAGAKU), anti-versican (Chemicon International, Inc., Temecula, CA), anti-Erk1/2 (Cell Signaling Technology, Danvers, MA), anti-Smad1/5/8 (clone N-18, Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-catenin (Cell Signaling Technology), and anti-GDNF (Santa Cruz Biotechnology). Control sections were incubated with mouse and rabbit IgG preimmune serum (data not shown).

2.3. Cell culture

Incisors were removed from 4-week-old male mice. Dental pulp was isolated from the incisors and rinsed with PBS. Dental pulp samples were digested for 1 h at 37 °C in α-modified Eagle's medium (α-MEM; Sigma-Aldrich) containing 2 mg/ml collagenase (Wako Pure Chemical Industries, Ltd.), 0.25% trypsin (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), and 100 μg/ml kanamycin sulfate. The attached dental pulp cells were cultured in α-MEM containing 10% FBS, L-glutamine (Invitrogen), and an antibiotic–antimycotic (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 2–3 days.

2.4. Alizarin red staining

Subcultured dental pulp cells were plated in 48-well plates at a density of 2×10^4 cell/well and cultured in α-MEM containing 10% FBS, or in osteogenic medium (α-MEM containing 10% FBS, 5 mM β-glycerophosphate, 100 mg/ml ascorbic acid), with or without 5 nM recombinant human FGF2 (rhFGF2; Oriental Yeast Co., ltd., Tokyo, Japan) for 5 days. The cells were rinsed with PBS and stained with 0.1% Alizarin Red S solution (Wako Pure Chemical Industries, Ltd.) at room temperature for 5 min to visualize the calcified nodules.

2.5. Real-time PCR

Dental pulp cells were plated in 12-well plates (1×10^5 cell/well) containing α-MEM and 5 nM rhFGF2, or 0.25 nM rhFGF2 and 5 μg/ml heparin (Sigma-Aldrich), and cultured at 37 °C for 72 h. Total RNA was isolated from the cultured cells using an RNeasy Plus Micro kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Real-time PCR was performed with a One Step SYBR PrimeScript PLUS RT-PCR kit (TAKARA, Shiga, Japan). The following primer sets were used: BDNF, 5′- GTGACAGTATTAGCGAGTGGG-3′ (forward primer) and 5′-GGGTAGTTCGGCATTGC-3′ (reverse primer); GDNF, 5′-GCGTGCTCTTGCTCCCGACCT-3′ (forward primer) and 5′-GACAGCCACGACATCCCATAAC-3′; NT-3, 5′- CGACGTCCCTGGAAATAGTC-3′ (forward primer) and 5′-TGGACATCACCTTGTTCACC-3′ (reverse primer); NT-4/5, 5′- AGGAGACTACCTGTATCCTACAAAGG-3′ (forward primer) and 5′-AGCATGGCTTGCACACCT-3′ (reverse primer); NGF, 5′-AATTAGGCTCCCTGGAGGTG-3′ (forward primer) and 5′-TGGACTGCACGACCACAG-3′ (reverse primer); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′- TGTGTCCGTCGTGGATCTGA-3′ (forward primer) and 5′-TTGCTGTTGAAGTCGCAGGAG-3′ (reverse primer).

3. Results

3.1. Immunolocalization of heparan sulfate and chondroitin sulfate in dental pulp

We investigated the localization of glycosaminoglycans, such as heparan sulfate and chondroitin sulfate, in the dental pulp of mouse molars (Fig. 1). The immunoreactivity for heparan sulfate was strong in the subodontoblastic layer but weak in the center of the dental pulp and in the odontoblastic layer (Fig. 1a and b). Notably, chondroitin sulfate seemed to be localized complementary to heparan sulfate in the dental pulp; i.e., immunoreactivity for chondroitin sulfate was strong in the center of the dental pulp but weak in the odontoblastic and subodontoblastic layers (Fig. 1c and d).

3.2. Immunolocalization of perlecan and versican in dental pulp

The different patterns of localization between heparan sulfate and chondroitin sulfate raise the possibility that core proteoglycan proteins are involved in the distribution of glycosaminoglycans. We investigated the localization of perlecan and versican (Fig. 2), which are the core proteins of heparan sulfate and chondroitin sulfate proteoglycans, respectively. The immunolocalization for perlecan was similar to that for heparan sulfate; i.e., the signal intensity was strong in the subodontoblastic layer but rarely detected in the center of the dental pulp or in the odontoblastic layer (Fig. 2a and b). The immunolocalization for versican was similar to that for chondroitin sulfate; i.e., strong signal was

Please cite this article as: Yukita A, et al. Relationship between localization of proteoglycans and induction of neurotrophic factors in mouse dental pulp. J Oral Biosci (2016), http://dx.doi.org/10.1016/j.job.2016.10.001