Expressional Alterations of Fibrillin-1 during Wound Healing of Human Dental Pulp

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Abstract

Introduction: The degradation of fibrillins, the major constituents of microfibrils, is known to facilitate the release of active transforming growth factor-β (TGF-β), a signaling molecule contributing to mineralized tissue barrier formation in exposed dental pulps. To examine the involvement of fibrillins in the barrier formation, we examined the temporospatial expression of (1) genes and proteins of fibrillins and (2) factors possibly associated with fibrillin degradation and cytodifferentiation in exposed human pulps. Human pulp slice cultures were also examined for the role of fibrillins in mineralization. **Methods:** Clinically healthy pulps were mechanically exposed and capped with mineral trioxide aggregate. After 7 to 42 days, the teeth were processed for immunohistochemical and cytochemical staining of fibrillin-1, fibrillin-2, latent TGF-β-binding protein (LTBP)-1, matrix metalloproteinase-3 (MMP-3), alkaline phosphatase (ALP), and *in situ* hybridization of fibrillin-1. Pulp tissue slices cultured with β-glycerophosphate were analyzed for fibrillin-1, fibrillin-2, and ALP with the immunohistochemical/cytochemical staining and quantitative reverse-transcriptase polymerase chain reaction. **Results:** Fibrillin-1-immunoreactivity was seen until 7 days but turned into undetectable since 14 days in the pulpal area just beneath the exposure site. MMP-3-immunoreaction was transiently detected at 14 days. At 42 days when the mineralized barrier was evident, fibrillin-1-immunoreactivity and fibrillin-1 expression remained down-regulated. Fibrillin-2, LTBP-1, and ALP were constantly detected in the fibrillin-1–undetectable area. Pulp slices cultured with β-glycerophosphate showed mineralization with up-regulation of ALP and down-regulation of fibrillin-1. **Conclusions:** Degradation and down-regulation of fibrillin-1 expression took place during the mineralized tissue barrier formation in exposed pulps *in vivo* and β-glycerophosphate–induced pulpal mineralization *in vitro*. *(J Endod 2012;38:177–184)*

Key Words

Cytodifferentiation, fibrillin-1, fibrillin-2, human dental pulp, mineral trioxide aggregate, pulp capping, wound healing

Fibrillins-1 and -2 are the main structural components of extracellular microfibrils found in a wide variety of tissues, and they play a role in the maintenance of connective tissue architecture through their involvement in elastic fiber formation (1). Mutations in the fibrillin-1 gene give rise to Marfan syndrome, a systemic disorder of the connective tissue, and congenital contractural arachnodactyly is caused by mutations in the fibrillin-2 gene. Abnormal pulp chambers with obliteration and pulp stones have been reported in Marfan syndrome and congenital contractural arachnodactyly (2).

It has recently been recognized that fibrillins contribute to the extracellular regulation of transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) (3). Interestingly, fragments of fibrillin-1 possess signaling activities; fibrillin-1 fragments induce up-regulation of matrix metalloproteinase (MMP)-1 and MMP-3 (4), and a breakdown of fibrillin-1-containing microfibrils may be a common mechanism for the release of active TGF-β1 sequestered in microfibrils (5, 6). Moreover, fibrillins are associated with latent TGF-β-binding protein (LTBP)-1, which is involved in the proper sequestration and extracellular control of TGF-β (7, 8).

Wound healing is a complex process requiring cell proliferation, migration, differentiation, and matrix synthesis. With regard to the teeth, the exposure site of dental pulp is finally covered with a mineralized tissue barrier (dentin bridge) formed by newly differentiated odontoblast-like cells. TGF-β as well as BMP signaling is required for odontoblast differentiation and dentin formation during tooth morphogenesis and for reparative dentinogenesis (9, 10). Calcium hydroxide–containing materials are the most commonly used pulp-capping agents because of their inductive ability of mineralization. Recently, mineral trioxide aggregate (MTA), a calcium silicate–containing material, has also been used with a predictable clinical outcome, most probably because of its biocompatibility and high sealing ability (11). The hydrated MTA contains calcium hydroxide and releases Ca and OH ions whereby alkaline environment is maintained (11).

MMPs are a family of zinc-dependent enzymes that are capable of degrading different substrates of extracellular matrices (12). They are secreted by mesenchymal

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cells, macrophages, and polymorphonuclear leukocytes and are crucially important in a wide range of physiological and pathological processes (12). The temporal and spatial expression of MMPs and their natural inhibitors, the tissue inhibitor of metalloproteinases, likely contribute to tooth morphogenesis and pulp tissue development (13). Moreover, MMP-1, MMP-2, and MMP-3 play a role in tissue destruction in inflamed human dental pulp (14, 15), and MMP-2 (16) and MMP-3 (17) promote wound healing in injured rat pulp. Several MMPs are able to digest fibrillins; MMP-2, -3, -9, -12, -13, and -14 are known to degrade fibrillin-1, and MMP-2, -12, and -13 degrade fibrillin-2 (18, 19). MMP-3 would be one of the proteases that function in the alkaline environment of calcium hydroxyde or MTA-applied dental pulps because the pH activity profile of MMP-3 is unique in that it exhibits a broad shoulder in the pH range of up to 9.5 (20).

Taken together, we hypothesized that fibrillins, upon degradation, contribute to the mineralized tissue barrier formation of exposed dental pulps. Thus, we designed the present study to address this hypothesis by examining the temporospatial expression of (1) genes and proteins of fibrillins and (2) factors that may be associated with fibrillin degradation and cytodifferentiation in MTA-capped human pulps *in vivo*. We also performed human pulp slices cultures to examine the role of fibrillins in *in vitro* mineralization.

Materials and Methods

This research project was approved by the Niigata University Ethics Committee. Human healthy permanent teeth were obtained for orthodontic treatment from young patients (18–25 years old) who gave their informed consent.

Antibodies

We used the following antibodies: mouse antihuman fibrillin-1 (Chemicon, Temecula, CA), rabbit antihuman fibrillin-2 (Elastin Products Company, Owensville, MO), rabbit antihuman LTBP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit antihuman MMP-3 (Abcam, Cambridge, UK), goat antihuman TGF-β1 (R&D Systems, Minneapolis, MN), horseradish peroxidase–labeled swine antirabbit immunoglobulin (Ig) G secondary antibody (Dako, Glostrup, Denmark), horseradish peroxidase–labeled rabbit antimouse IgG secondary antibody (Dako), and horseradish peroxidase–labeled rabbit antigoat IgG secondary antibody (Dako).

Pulp-capping Procedures

A total of nine clinically intact teeth were used for pulp capping. Under local anesthesia and rubber dam isolation, a cavity was prepared with a diamond bur on the occlusal surface. The pulp was then exposed by means of a sterilized round steel bur (1.0 mm in diameter). After the surgical area was washed with sterile saline, the exposed pulp tissue was capped with MTA (Dentsply Tulsa Dental, Tulsa, OK). The cavity was then filled with composite resins. Three teeth were extracted under local anesthesia at 7, 14, and 42 days after the operation, respectively. They were then fixed with 4% paraformaldehyde and decalcified in 10% EDTA (pH = 7.4). Ten-micrometer-thick frozen sections were cut and mounted on poly-L-lysine–coated slides.

Tissue Culture of Dental Pulp

Human dental pulp tissue was obtained from 10 intact third molars. After extraction under local anesthesia, the teeth were immediately split open, and the coronal pulp was removed. The removed pulp did not contain odontoblasts because their processes remained in dentin. However, cells in the subodontoblast layer partially remained in the removed pulp, and, therefore, the circumferential tissue was removed with a knife. The pulp was subsequently sliced into approximately 1.5-mm-thick specimens with a knife and then placed on Millicell-CM filter inserts (Millipore, Billerica, MA) placed in 24-well plates. The explants were incubated at 37°C in a humidified incubator at 5% CO_2. The culture media were replaced every 3 days, and cultures were maintained for 7 days. The culture medium was composed of Dulbecco modified Eagle medium (Gibco, Paisley, Scotland) containing 10% fetal bovine serum, 10 IU/mL penicillin, 10 μg/mL streptomycin, 50 μg/mL ascorbic acid, and 10 mmol/L β -glycerophosphate (Calbiochem, Burlington, ON, Canada) was added to induce mineralization. A part of each cultured tissue was fixed in 4% paraformaldehyde, and 10-μm-thick frozen sections were cut for immunohistochemical and cytochemical staining. The rest of the tissue was processed for the quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis as described later.

Immunohistochemical and Cytochemical Staining

Indirect immunoperoxidase staining was performed on the cryosections according to standard protocols (21). Negative control staining was performed by replacing the primary antibodies with nonimmune mouse, rabbit, or goat sera. Alkaline phosphatase (ALP) was used as a cell differentiation marker for mineralized tissues. ALP detection was performed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt solution (Roche, Mannheim, Germany) on the cryosections. Matrix mineralization of cultured tissues was determined with a 2% alizarin red S solution (Sigma, St Louis, MO) on the cryosections.

RT-PCR

Total RNA was prepared from five normal human dental pulps using the RNeasy Plus Micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Single-strand complementary DNA was made from 1 μg total RNA by RT using an oligo (dT) primer. Polymerase chain reaction was performed using a gene amplification system (GeneAmp PCR system 9700; Applied Biosystems, Foster City, CA). The amplification was carried out through 40 cycles of 98°C for 5 seconds, 55°C for 5 seconds, and 72°C for 5 seconds. The primer sets used for RT-PCR were as follows: fibrillin-1 (sense: 5'-GGAACGTGAAGGAAACCAGA-3', antisense: 5'-GGCAAATGGGGACAATACAC-3', product size: 162 bp), fibrillin-2 (sense: 5'-GGCCAGTGTACCAACATTCC-3', antisense: 5'-GTCGCA GTTATGAGCACCAA-3', product size: 249 bp), LTBP-1 (sense: 5'-GTCTGTTCACCTCACCAAGCA -3', antisense: 5'-ATAACCCATTCCAC-CAGGACA -3', product size: 127 bp), ALP (sense: 5'-CTCCAAGACGCC-CAAGAAC-3', antisense: 5'-GAGTTCAGAGGGAAGGCA CA-3', product size: 198bp), MMP-3 (sense: 5'-GTGGAGTTCCTGACGTTGGT-3', antisense: 5'-AGCCTGGAGAATGTGAGTGG-3', product size: 181 bp), TGF-β1 (sense: 5'-CACGTGGAGCTGTACCAGAA-3', antisense: 5'-CTA AGGCGAAAGCCCTCAA-3', product size: 161 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a house keeping gene (sense: 5'-ACACCCACTCCTCCACCTTT-3', antisense: 5'-TTCCTCTTGT-GCTCTTGCTG-3', product size: 183 bp).

Quantitative RT-PCR Analysis

Total RNA was isolated from cultured human dental pulps using the RNeasy Plus Micro kit. Quantitative RT-PCR was performed using the One Step SYBR PrimeScript PLUS RT-PCR kit (Takara Bio Inc, Shiga, Japan) with the Opticon Real-Time PCR System (MJ Research, Arlington, MA). The same primers designed for RT-PCR were used for