Morphologic Change of Parvalbumin-positive Myelinated Axons in the Human Dental Pulp

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Abstract

Introduction: Information on the nerve fibers innervating the dental pulp is crucial for understanding dental pain and hypersensitivity. This study investigated the morphologic differences of parvalbumin (PV)-positive (+) myelinated fibers in 3 different regions of the human dental pulp. **Methods:** Light and electron microscopic immunohistochemistry for parvalbumin, a marker for myelinated fibers, and quantitative analysis were performed in the apical root, core of coronal pulp, and peripheral pulp of human premolar teeth. **Results:** About 40% of the myelinated fibers in the apical root pulp became unmyelinated in the core of the coronal pulp, and virtually all the remaining fibers became unmyelinated at the peripheral pulp. The size of myelinated axons decreased from root to peripheral pulp. PV+ axons showed extensive axonal varicosities in the peripheral pulp. **Conclusions:** These findings suggest that the myelinated fibers innervating the human dental pulp undergo extensive morphologic change in the extrapulpal region and in the coronal and peripheral pulp, and that PV-mediated regulation of calcium concentration and its downstream events may occur primarily in axonal varicosities in the peripheral pulp. *(J Endod 2017;■:1–5)*

Key Words

Dental pulp, myelinated axon, parvalbumin, ultrastructure

Significance

This study provides information on the morphologic change of the myelinated axons in the human dental pulp, which is important for understanding dental pain and dentinal hypersensitivity and for developing its therapeutic strategy.

The dental pulp is densely innervated by both myelinated (Aδ) nerve fibers that carry the sensation of sharp, well-localized pain and unmyelinated (C) nerve fibers that carry the sensation of dull, poorly localized pain; the latter class is the majority (1–5). We showed previously that most myelinated axons in the rat root pulp lose their myelin and become thinner when they reach the peripheral pulp, that is, the same fiber may be thick and myelinated in the root pulp and thin and unmyelinated in the peripheral pulp (6). However, at present, little is known about the morphologic changes of the myelinated axons in the human dental pulp, especially in each pulpal segment, ie, root, coronal, and peripheral pulp. To know this is important because the 2 classes of fibers, myelinated and unmyelinated, have different functional properties, play different roles in dental pain and dentin hypersensitivity, and are targeted with different therapeutic strategies. In addition, the previous findings in the rat dental pulp (6) may be put into question by the uncertain selectivity of the most commonly used marker for myelinated fibers, neurofilament 200 (NF200); we have shown that it frequently labels unmyelinated fibers (7).

To address this, we used the calcium-binding protein parvalbumin (PV), which is a much more reliable marker for myelinated fibers including Aβ fibers because it is expressed exclusively in neurons that give rise to such fibers (8–11), to investigate the morphology and distribution of myelinated nerve fibers in 3 segments of the human dental pulp: apical root, core of coronal, and peripheral.

Materials and Methods

Tissue Preparation

All experimental procedures were performed under the approval of the Research and Ethics Committee of the Kyungpook National University. Informed consents were obtained from all human subjects who participated after explaining the nature of the procedure. First and second healthy maxillary premolars, extracted during the course of orthodontic treatment from eight 16- to 28-year-old patients at the Department of Oral Surgery, Kyungpook National University Hospital, South Korea, were cut longitudinally with a high-speed diamond bur, and the pulps were carefully isolated. Five dental pulps were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB) (pH 7.4) for light microscopy (LM), and 5 dental pulps were fixed in 4% paraformaldehyde

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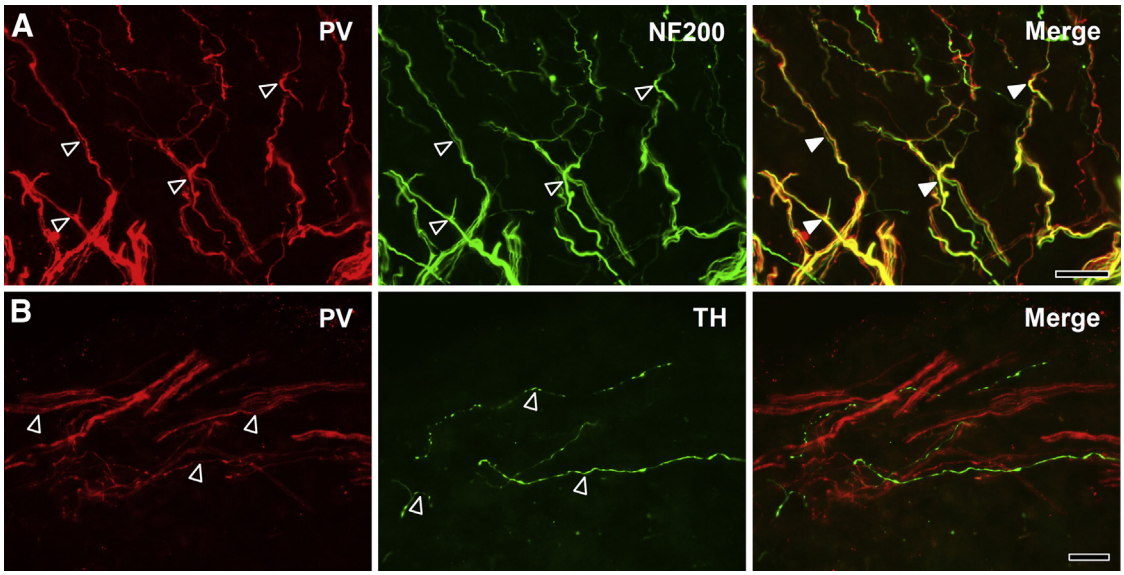

Figure 1. Double immunofluorescent staining for PV (red) and NF200 (marker for sensory afferents, green in *A*) or TH (marker for sympathetic efferents, green in *B*) in the coronal pulp of human premolar teeth; colocalization is represented in yellow in the merged images. Most PV+ axons are immunostained for NF200 but not for TH. *Open arrowheads*: NF200+ (*A*) or TH+ (*B*) axons. *Filled arrowheads*: axons immunopositive for PV and NF200 (*A*). Scale bar = 20 μm.

containing 0.01% glutaraldehyde for electron microscopy (EM) for 3 hours. Pulps were then cryoprotected in 30% sucrose in PB at 4°C overnight and cut longitudinally on a freezing microtome at 30 μm for LM immunohistochemistry and transversely on a Vibratome (Leica Biosystems, Wetzlar, Germany) at 60 μm for EM immunohistochemistry.

LM Immunohistochemistry

For immunoperoxidase staining, sections were treated with 50% ethanol, 3% H_2O_2, and 10% normal donkey serum (NDS) (Jackson ImmunoResearch, West Grove, PA), washed in PB, and incubated in mouse anti-PV antibody (1:3000; 235; Swant, Marly, Switzerland) overnight. After several washes in phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.4), sections were incubated in biotinylated donkey anti-mouse antibody (1:200; Jackson ImmunoResearch) for 2 hours and in ExtrAvidin peroxidase (1:5000; Sigma-Aldrich, St. Louis, MO) for 1 hour. Immunoperoxidase was visualized with nickel-intensified 3, 3′-diaminobenzidine tetrahydrochloride (Ni-DAB).

For double immunofluorescent staining for PV and NF200, a marker for sensory afferents, or tyrosine hydroxylase (TH), a marker for sympathetic efferents, sections were permeabilized with 50% ethanol, blocked with 10% NDS, and incubated in a mixture of primary antibodies in PBS overnight and in secondary antibodies for 3 hours. The primary antibodies were rabbit anti-PV (1:2000; PV 25, Swant) and a mouse anti-NF200 (1:50,000; N0142, Sigma-Aldrich) or a mouse anti-TH (1:200, MAB318; Millipore, Billerica, MA); the secondary antibodies were Cy3-conjugated donkey anti-rabbit and fluorescein isothiocyanate–conjugated donkey anti-mouse antibody (1:200; Jackson ImmunoResearch). Sections were mounted on slides and examined in a Zeiss (Carl Zeiss Microscopy, Jena, Germany) Axioplan 2 microscope.

EM Immunohistochemistry

Cryoprotected sections were frozen on dry ice, thawed in PBS, and treated with sodium borohydride in PBS to remove glutaraldehyde. Then, sections were incubated with 3% H_2O_2 for 10 minutes and blocked with 10% NDS for 30 minutes. After incubation with a mouse anti-PV antibody (1:3000; 235, Swant) overnight, sections were incubated with 2% NDS and in the biotinylated donkey anti-mouse antibody (1:200) for 2 hours, rinsed, and incubated with ExtrAvidin peroxidase (1:5000; Sigma-Aldrich) for 1 hour. Immunoperoxidase was visualized with DAB. Sections were postfixed in osmium tetroxide, dehydrated in graded alcohols, flat embedded in Durcupan ACM (Fluka, Buchs, Switzerland), and cured for 48 hours at 60°C.

Small resin chips containing dense cross-sectioned PV+ axons in sections of apical root, core of coronal pulp, and peripheral pulp were cut out and glued onto blank resin blocks. Ultrathin sections were cut, collected on formvar-coated single-slot nickel grids, and stained with uranyl acetate and lead citrate. Grids were observed on a Hitachi H-7500 (Hitachi, Tokyo, Japan) electron microscope at 80 kV accelerating voltage. Images were captured by using DigitalMicrograph software driving a SC1000 CCD camera (Gatan, Pleasanton, CA) attached to the microscope and saved as TIFF files.

To test for antibody specificity, we omitted the primary or secondary antisera, which abolished the staining. The specific immunostaining for PV was also completely abolished by preadsorption with 18 μg/mL blocking peptide.

Analysis of PV-immunopositive Axons

For quantitative analysis, we used 30–38 electron micrographs at $\times$10,000 magnification (corresponding to 7380–9348 μm^2 pulpal area) from each of apical root, core of coronal pulp, and peripheral pulp (a total of 150–190 micrographs from each pulp region in 5 pulps). The number, diameter, axon area, and myelin thickness were analyzed in 1353, 1017, and 1347 PV+ myelinated and unmyelinated axons in the root, coronal pulp, and peripheral pulp, respectively, by using Image J (NIH, Bethesda, MD). Statistical significance of differences between pulpal regions was examined by one-way analysis of variance (ANOVA) and Scheffé F test ($P < .05$).