Dynamics of Bromodeoxyuridine Label–retaining Dental Pulp Cells during Pulpal Healing after Cavity Preparation in Mice

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Abstract

Introduction: This study aimed at clarifying the dynamics of bromodeoxyuridine (BrdU) label-retaining cells (LRCs) and their relationship to cell proliferation and apoptosis during pulpal healing after cavity preparation in mice. **Methods:** A groove-shaped cavity was prepared on the mesial cervical surface of the upper first molars, and the samples were collected at intervals of 12 hours–14 days. The demineralized paraffin sections were processed for immunohistochemistry for BrdU, nestin, and Ki-67 and apoptosis assay using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) and *in situ* hybridization for dentin sialophosphoprotein (*Dspp*). **Results:** During hour 12–day 1, odontoblasts and subodontoblastic cells beneath the affected dentin showed degenerative features and TUNEL-positive reactions, and the expressions of nestin and *Dspp* were lost in the damaged odontoblasts. TUNEL-positive reactions were observed even in the center of the pulp tissue, whereas dense and granular LRCs remained there. On days 2–3, Ki-67–positive cells were significantly increased in number in the center of mesial dental pulp. During days 3–5, granular and few dense LRCs were committed into some (not all) nestin-positive newly differentiated odontoblast-like cells, and these differentiated cells began to express nestin and *Dspp*. Until day 14, tertiary dentin formation occurred next to the preexisting dentin at the mesial pulp floor in addition to the mesial coronal pulp. **Conclusions:** These results suggest that odontoblasts and subodontoblastic cells degenerate after tooth drilling, and, subsequently, dental pulp stem/progenitor cells actively proliferate and differentiate into new odontoblast-like cells. *(J Endod 2013;39:1250–1255)*

Key Words

Adult Stem Cells, apoptosis, bromodeoxyuridine, cell differentiation, cell proliferation, dental cavity preparation, ICR mice, regeneration, tooth injuries

Numerous organs contain committed multipotent stem cells, or adult stem cells, even in adults. Adult stem cells are crucial for physiological tissue renewal and regeneration after injury, and it is assumed that a single quiescent population of stem cells resides in a specialized niche of a given tissue. Emerging evidence indicates that both quiescent (out of cell cycle and in a lower metabolic state) and active (in cell cycle and not able to retain DNA labels) stem cell subpopulations may coexist in several tissues (1). It is now accepted that stem/progenitor cells reside within the postnatal dental pulp (2). Because tissue self-renewal rarely occurs in dental pulp in a steady state, dental pulp/stem cells are supposed to be a type of quiescent stem cells and can shift to the active state in response to damage. Indeed, there are numerous reports on the contribution of pericytes or side population cells to the process of pulpal healing after tooth injury (3–5). Our recent study showed that 3 daily intraperitoneal injections of bromodeoxyuridine (BrdU) into pregnant Crlj:CD1 (ICR) mice enable the successful identification of dense BrdU-label retaining cells (LRCs) (ie, adult stem/progenitor cells) in the mature tissue of postnatal animals and that dense LRCs mainly reside in the center of the dental pulp and coexpress mesenchymal stem cell markers such as STRO-1 and CD146 (6). We applied the prenatal labeling method to several damage models such as tooth replantation and tooth or tooth crown transplantation and revealed that these LRCs possessed a high proliferative capacity and maintained the differentiation capacity into the odontoblast-like cells (7, 8). However, these experimental models using mice are severe damage models in which the tissue damage affects the whole dental pulp, resulting in almost total degeneration of odontoblasts. So far, there are no available data on the dynamics of dental pulp stem/progenitor cells after a mild damage model such as tooth drilling in which the tissue damage is localized in the pulp tissue to induce the partial degeneration of odontoblasts although a recent study suggests that Lymphoid enhancer-binding factor 1 (*Lef1*) might play a key role in odontoblast differentiation through dentin sialophosphoprotein (*Dspp*) expression (9). Thus, it is important to recognize differences in the contribution of LRCs to pulpal healing between severe and mild damage in order to clarify the mechanisms regulating the pulpal regenerative process after tooth injury.

Recent studies have shown that active cell proliferation occurs in dental pulp after tooth injury such as tooth replantation/transplantation in alveolar sockets, tooth crown transplantation in the sublingual region, and cavity preparation (7,10–12). In the heavy injury models such as tooth replantation and tooth or tooth crown transplantation, cell proliferation precedes odontoblast differentiation. In contrast, newly differentiated odontoblasts are arranged along the pulp dentin border, followed by active cell proliferation in a wide range of dental pulp in a mild injury model such as cavity preparation using rats (12). These findings suggest that progenitors equipped in the subodontoblastic layer first migrate and differentiate into new odontoblasts without

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proliferation to compensate for the loss of the odontoblast layer. In addition, our previous studies have shown that transplanted LRCs suffer from extensive apoptosis following cell proliferation after allogenic tooth crown transplantation (8). A previous study using an experimental model for cavity preparation showed that 2 waves of apoptosis were induced in dental pulp after the operation in rats (13). However, there are no available data on cell proliferation or apoptosis in the process of pulpal healing after cavity preparation using mice. Thus, cell proliferation and apoptosis assays are necessary for understanding the precise process of pulpal regeneration after tooth injuries, in addition to the analysis of the dynamics of LRCs. This study aimed to clarify the dynamics of LRCs and their relationship to cell proliferation and apoptosis during pulpal healing after cavity preparation in mice.

Materials and Methods

BrdU Labeling

All experiments were reviewed by the Committee on the Guidelines for Animal Experimentation, Niigata University, Niigata, Japan, and performed according to the recommendations or under the conditions proposed by the committee. Three peritoneal injections (once a day on embryonic days 15, 16, and 17) of BrdU (150 mg/kg) were administered to 5 pregnant Crlj:CD1 (ICR) mice, 8–10 weeks old, to map LRCs in the mature tissues of birthed animals. The labeled birthed animals at 5–6 weeks of age were used for cavity preparation.

Cavity Preparation

A groove-shaped cavity was prepared on the mesial cervical surface of the upper first molars using an air turbine with a tungsten carbide bur (diameter = 0.6 mm) under water cooling (Supplemental Video S1 is available online at www.jendodn.com). The nontreated upper first molars were used as controls.

Histologic Procedure

Materials were collected in groups of 3–6 animals at intervals of 12 hours and 1, 2, 3, 5, 7, and 14 days after the operation ($N = 37$). At each stage, the animals were perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH = 7.4) under deep anesthesia using an intraperitoneal injection of chloral hydrate (350 mg/kg). After decalcification in a 10% EDTA-2Na solution for immunohistochemical analysis or Morse solution for *in situ* hybridization, the specimens were dehydrated, embedded in paraffin, and cut sagittally at 4 μm. Sections were processed for hematoxylin-eosin staining, immunohistochemistry, and *in situ* hybridization.

Immunohistochemical Analysis

Immunohistochemistry was conducted essentially according to our previous report (8), with the Calbiochem BrdU Immunohistochemistry System (EMD Millipore Biosciences, Darmstadt, Germany; catalog number: HCS30) for BrdU immunostaining, a mouse anti-rat anti-inestin monoclonal antibody diluted to 1:500 (Chemicon International Inc, Temecula, CA; catalog number: MAB353), and a rat anti-mouse anti–Ki-67 monoclonal antibody diluted to 1:100 (Dako Japan, Tokyo, Japan; catalog number: M7249). The Envision + Horseradish Peroxidase System (Dako Japan; catalog number: K5027) and the avidin-biotin peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) method using biotinylated antirat immunoglobulin G (Vector Laboratories; catalog number: BA-4000) were used for nestin and Ki-67 immunohistochemistry, respectively. For the final visualization of the sections, 0.05 mol/L Tris-HCl buffer (pH = 7.6) containing 0.04% 3-3′-diaminobenzidine tetrahydrochloride and 0.0002% H_2O_2 was used. The immunostained sections were counterstained with hematoxylin. In double-staining experiments, nestin immunohistochemistry was performed first followed by BrdU immunohistochemistry. BrdU immunohistochemistry was performed as described previously, except that the sections were stained with 4-nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics Corp, Indianapolis, IN) for color development. Apoptosis was quantified by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Massachusetts, MA; catalog number: S7100). Negative controls were performed by replacing the primary antibodies or terminal deoxynucleotidyl transferase (TdT) enzyme with phosphate-buffered saline. These controls contained no specific reactions (Supplemental Figure S1 is available online at www.jendodn.com).

In Situ Hybridization

Section *in situ* hybridization was performed as described elsewhere (14). Digoxigenin-labeled probes for *Dspp* (15) were prepared according to the manufacturer's protocol (Roche, Manheim, Germany).

Statistical Analysis

The numbers of BrdU-, Ki-67-, and TUNEL-positive cells in the odontoblasts and subodontoblastic layer and the center of the pulp tissue of each specimen (50×200 or 100×100 μm^2 grids were selected) were calculated separately. Data were obtained from the samples of 37 teeth (after 12 hours [$n = 4$] and 1 [$n = 4$], 2 [$n = 5$], 3 [$n = 3$], 5 [$n = 3$], 7 [$n = 4$], and 14 days [$n = 4$] for BrdU labeling analysis; 12 hours [$n = 4$] and 1 [$n = 6$], 2 [$n = 4$], 3 [$n = 4$], 5 [$n = 4$], 7 [$n = 5$], and 14 days [$n = 5$] for cell proliferation assay using Ki-67 immunoreactivity; and 12 hours [$n = 5$], 1 [$n = 4$], 2 [$n = 4$], 3 [$n = 3$], 5 [$n = 3$], 7 [$n = 4$], and 14 days [$n = 4$] for TUNEL assay). All data were presented as the mean and standard deviation of each group. Furthermore, the number of cells in the pulp chamber at different times after the operation (12 hours–14 days) was compared using the Bonferroni test (1-way analysis of variance) using statistical software (SPSS 16.0J for Windows; SPSS Japan Inc, Tokyo, Japan).

Results

Changes of the Nestin and *Dspp* Expressions in Prepared Teeth

In the control teeth, odontoblasts showed an intense nestin immunoreactivity (Fig. 1*A* and *B*), which is an odontoblast differentiation marker (16, 17). Odontoblasts at the mesial cervical and root areas expressed intense *Dspp* messenger RNA although odontoblasts in the other areas showed faint expression (Fig. 1*C*). During 12 hours, 2 days after cavity preparation, odontoblast and subodontoblastic layers beneath the affected dentin were disturbed (Fig. 1*D*). The expressions of nestin and *Dspp* were lost in the damaged odontoblast layer, whereas nestin-positive reactions persisted in odontoblasts on the pulp floor, and the filamentous structures in the center of the pulp tissue showed positive reactions (Fig. 1*E* and *F*). On days 3–5, nestin-positive newly differentiated odontoblast-like cells were arranged along the pulp-dentin border and began to express nestin and *Dspp* (Fig. 1*G–I* and *K*). Subsequently, odontoblast-like cells both beneath the affected dentin and on the mesial pulp floor and root expressed *Dspp* on day 7 (Fig. 1*L*). Until day 14, tertiary dentin formation occurred next to the preexisting dentin at the mesial pulp floor in addition to the mesial coronal pulp (Fig. 1*J*).