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Analysis of the Contribution of Nonresident Progenitor Cells and Hematopoietic Cells to Reparative Dentinogenesis Using Parabiosis Model in Mice

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Abstract

Introduction— The aim of this study was to analyze the contribution of nonresident progenitor/stem cells and hematopoietic cells to reparative dentinogenesis.

Methods— Parabiosis was established between C57BL/6-TgN(ACTbEGFP)10sb/J transgenic mice (GFP+) and C57BL/6 wild-type mice (GFP–) to ensure blood cross-circulation between animals. Reparative dentinogenesis was stimulated by pulp exposures and capping on the first maxillary molar in the GFP– mice. Histologic sections of injured molars from GFP– mice were analyzed by epifluorescence microscopy to examine the contributions of GFP+ cells (nonresident progenitor cells and hematopoietic cells originating from GFP+ mice) to reparative dentinogenesis.

Results— GFP+ cells were detected in close association with reparative dentin formed at the site of pulp exposure in the maxillary first molars of the GFP– mice.

Conclusions— The present study suggests the participation of the nonresident progenitor cells and hematopoietic cells in reparative dentinogenesis.

Keywords

Dental pulp; green fluorescence protein; odontoblast-like cells; parabiosis; reparative dentinogenesis

The ability of the dentin-pulp complex responding to injuries by hard-tissue formation has long been recognized (1, 2). Unlike primary or secondary dentin that is formed along the entire pulp-dentin border, tertiary dentin is formed focally in response to an injury (3–5). Tertiary dentin is divided into reactionary and reparative dentin (6, 7). Reactionary dentin is secreted by preexisting odontoblasts after a mild injury (6, 8). Reparative dentin is secreted by a new generation of odontoblast-like cells after intense injuries that lead to odontoblast

death (7, 9) and involves a cascade of inflammatory and healing events that occur rapidly after the degeneration of odontoblasts (9–11).

The molecular mechanisms involved in reparative dentinogenesis (most notably the origin of the cells giving rise to odontoblast-like cells) are not fully understood (6, 12–14). Available evidence suggests that the pulp contains several niches of potential progenitors involved in reparative dentinogenesis. These include the cell-rich layer of Höhl (subodontoblast layer), progenitors/stem cells (12, 13), and perivascular cells (15). However, the origin/identity of the progenitor cells and signaling pathways involved in reparative dentinogenesis remains elusive. Furthermore, in addition to the roles of resident progenitor populations within dental pulp in reparative dentinogenesis, the possible roles and involvement of the nonresident populations including progenitor/stem cells from other sites and the hematopoietic cell population in reparative dentinogenesis have not been examined. The parabiosis model in which 2 mice are surgically joined to allow the establishment of common blood circulation has provided an excellent model for studying various biological processes including the roles and involvement of nonresident progenitors/stem and hematopoietic cells migrating to the site of injury from circulating blood in tissue remodeling and repair (16–20).

The present study was designed to examine the contributions of the nonresident progenitor/stem cells and hematopoietic cells to reparative dentinogenesis using a parabiosis model. Parabiotic pairs were established between mice expressing enhanced green fluorescent protein (EGFP) (referred to as GFP+) (21) and nonfluorescent wild-type mice (referred to as GFP–) to establish common blood circulation. After 2 weeks of parabiosis, pulp exposures were created in the maxillary first right molar of the GFP– mice to stimulate reparative dentinogenesis. The contribution of the nonresident progenitor/stem cells and hematopoietic cells to reparative dentinogenesis was analyzed by examining the presence of GFP+ expressing cells (originating from the donor GFP+ mice and circulating through the common blood circulation to the recipient GFP– animal) in and around the new reparative dentin formed at the site of the pulp exposure in the recipient GFP– mice after 4 and 8 weeks.

Materials and Methods

Transgenic Mice

All animal procedures performed in this study were in accordance with Animal Care Committee guidelines from the University of Connecticut Health Center, Farmington, CT. Wild-type C57BL/6 (GFP–) and C57BL/6-TgN(ACTbEGFP)10sb/J transgenic mice (GFP+) were obtained from Jackson Laboratory (Bar Harbor, ME). C57BL/6-TgN (ACTbEGFP) 10sb/J is a transgenic mouse in which the expression of EGFP is under the control of a chicken β -actin promoter and a cytomegalovirus enhancer. In this transgenic mouse, the EGFP is expressed uniformly in all cells except in erythrocytes and, therefore, it can be used to track the fate of various cells including the hematopoietic cells and the nonresident progenitor cells (21).

Parabiosis

Twelve- to 16-week-old animals were paired as described previously (22). Briefly, 2 mice (a GFP+ and a GFP– as shown in Fig. 1) with the average weight of 20 g were anesthetized with 100 mg/kg ketamine (Midwest Veterinary Supply Inc, Burnsville, MN) and 10 mg/kg xylazine (Midwest Veterinary Supply Inc, Burnsville, MN). The surgical site of each mouse was shaved and sterilized with 5% povidine-iodine solution. Skin incisions from the hip to the angle of the mandible were made in both mice. Animals were aligned, and the connective tissue and the skin edges were sutured with absorbable silk suture material and

joined with 9-mm wound clips at the dorsal side. Buprenorphine hydrochloride (0.2–0.5 mg/kg) (Midwest Veterinary Supply Inc) was administered daily for the first 2 days after surgery. The wound clips were removed after 2 weeks.

Pulp Exposure

To stimulate the reparative dentinogenesis, 2 weeks after parabiosis, dental pulps of the maxillary first molars of the GFP⁺ mice were exposed as described earlier (23, 24) (Fig. 1). Briefly, mice were anesthetized, and a cavity was prepared with a carbide burr (diameter = 0.40 mm) (Komet, Paris, France) on the mesial occlusal half of the first maxillary molar until the pulp was visible through the cavity floor. A pulp exposure of approximately 150 μ m was created mechanically through the floor of the cavity using an endodontic hand file (0.15-mm tip diameter with a 2% taper). Exposed pulps were capped using mineral trioxide aggregate (MTA Angelus; Angelus S/A, Paraná, Brazil) mixed with sterile water following the manufacturer's recommendations. Mineral trioxide aggregate was placed in contact with the pulp using a probe tip and condensed gently with a sterile paper point # 35 (Dents-ply Maillefer, Ballaigues, Switzerland). Subsequently, the cavity was sealed with light-cured composite resin (Z100; 3M, São Paulo, SP, Brazil) using a 2-step adhesive system (Clearfill SE Bond; Kuraray, Okayama, Japan) (Fig. 1).

Flow Cytometry

To verify cross-circulation between the parabionts, blood was collected from the GFP⁺ mice 2 and 10 weeks after parabiosis (Fig. 1) and analyzed by flow cytometry (fluorescence-activated cell sorter[FACS]) as described earlier (25, 26). Briefly, blood samples were collected from the tail of the GFP⁺ mice into FACS tubes containing 500 μ L 8 μ mol/L EDTA in phosphate-buffered saline (PBS). The collected blood was mixed with 2 mL dextran (2%) and centrifuged to collect the red blood cells. The blood cells were lysed with 0.5 mL ammonium chloride (ACK Lysing Buffer; In-vitrogen, Grand Island, NY), washed with 500 μ L of staining medium, and stained first with 5 μ g/mL propidium iodide (24) and then with CD45-APCcyan (1:100) antibody for 30 minutes at 4°C. Between 100,000 to 300,000 cells were used for analysis using a BD-LSR II multicolor analyzer (Becton Dickinson, San Jose, CA). Data were processed using FlowJo 7.6 software (Tree Star Inc, Ashland, OR) in the Institutional Flow Cytometry Facility at the University of Connecticut Health Center.

Tissue Isolation and Analysis

To analyze the sequence of reparative dentinogenesis and the contribution of GFP⁺ cells to this process, animals were euthanized by intracardiac perfusion with 10% buffered formalin 4 and 8 weeks after pulp exposures (Fig. 1). After perfusion, maxillary arches were isolated, cleaned from soft tissue, trimmed, and fixed in 10% formalin solution for additional 24 hours. Samples were decalcified for 7 days in 15% EDTA and 0.5% formalin (pH = 7.5) at 4°C, dehydrated through ascending gradations of ethanol and xylene, and embedded in paraffin. Serial cross-sections of 7 μ m were placed onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA), deparaffinized, rehydrated, washed in distilled water, and mounted with glycerol/PBS (50%:50%). The fluorescence signal was examined in at least 20 sections through the region of injury and repair from each tooth using an Axio Observer.Z1 for epifluorescence microscope (Carl Zeiss, Thornwood, NY) using GFPtopaz and GFPmcherry filters. The images were overlaid to eliminate tissue autofluorescence using a user-defined computation program AxioVision Rel 4.7 software (Carl Zeiss, Thornwood, NY). After epifluorescence analysis, the same sections were washed in PBS, processed for hematoxylin-eosin staining using standard protocols, and analyzed by light microscopy. The number of mice analyzed at each time point is shown in Table 1.

Tartrate-resistant Acid Phosphatase Staining

Odontoclast/osteoclasts were identified using tartrate-resistant acid phosphatase (TRAP) staining using the naphthol alkaline system BI (1.1'-bi-2-naphthol) phosphate method as described previously (27). Sections were incubated in the staining solution for 1 hour at 37°C, washed with water, and then counterstained with hematoxylin-eosin.

Results

GFP Expression in Transgenic Mice

Histologic and epifluorescence analysis of molars from 12-week old GFP+ mice showed high levels of expression of EGFP in the dental pulp cells and in the entire layer of a fully differentiated odontoblast and odontoblastic process (Fig. 2A–C). These observations confirmed the expression of EGFP in all cells including odontoblasts in these animals and the feasibility of using the C57BL/6-TgN (ACT-bEGFP) 10sb/J transgenic mice animal model for tracking cells giving rise to odontoblasts.

Analysis of the Contribution of Circulating Progenitor Cells to the Reparative Dentinogenesis

We found that parabiosis was a challenging surgical procedure and was associated with high mortality rates. We observed a 70% mortality rate resulting in the analysis of only 6 pairs of parabionts. Flow cytometry analysis of peripheral blood from the GFP– parabiont showed the presence of approximately 54.3% and 59.8% of GFP+ cells after 2 (n = 4) and 10 weeks (n = 2) of parabiosis, respectively (Table 1), confirming the cross-circulation between the parabionts. A high percentage of the donor-derived GFP+ cells expressed the hematopoietic antigen CD45 (Table 1), indicating that the majority of GFP+ cells were of a hematopoietic origin (Table 1).

The analysis of sections through maxillary molars of the recipient GFP– parabiont 4 weeks after pulp exposure (6 weeks after parabiosis) showed the presence of dentin chips at the periphery of the exposure site (Fig. 3A) and no evidence of calcified dentin bridge formation (Fig. 3A). Epifluorescence analysis of the same sections showed an influx of donor-derived GFP+ cells into the pulp through the apical foramen (Fig. 4). Scattered GFP+ cells were detected in the pulp and the surrounding alveolar bones (Fig. 4). The analysis of sections through the injury and repair site showed the presence of a few large GFP+ cells in close association with dentin chips (Fig. 3B and C).

Clear evidence of reparative dentinogenesis at the exposure site was observed 8 weeks after injury (10 weeks after parabiosis) (Fig. 3D). At this point, the atubular reparative dentin extended into the pulp (Fig. 3D and G) and was surrounded by a thin layer of less calcified matrix resembling predentin (Fig. 3D and G). Epifluorescence analysis showed a large number of GFP+ cells around and in close vicinity of the reparative dentin (Fig. 3E and H). The relatively large size of GFP+ cells in the close vicinity of the reparative dentin suggested they had an osteoclast/odontoclast phenotype. TRAP staining showed very few, if any, osteoclasts/odontoclasts in the GFP+ cells around the reparative dentin (Fig. 5A–C). On the other hand, TRAP staining was detected in the GFP+ cells in the alveolar bone around the bone marrow lacunae (Fig. 5D–F).

Discussion

Parabiosis is a surgical union of 2 mice so that they share the same cross-blood circulation. Parabiosis using a GFP+ parabiont partner has been used to investigate various contributions of circulatory cells including dermal fibroblast/myofibroblast progenitors (25), non–bone

marrow progenitors (19), endothelial progenitors (17), and bone marrow stem cells (18). In the present study, we used parabiosis to evaluate the contributions of nonresident progenitors/stem and hematopoietic cells in the circulating peripheral blood to reparative dentinogenesis. In these experiments, reparative dentinogenesis was stimulated by pulp exposure in the GFP⁻ parabiont, and the contribution of the circulatory cells to reparative dentinogenesis was evaluated by examining the presence of GFP⁺ expressing cells to reparative dentin formed at the site of the pulp exposure in the recipient GFP⁻ mice.

FACS analysis showed that parabiosis resulted in a blood chimerism between 2 parabionts at 2 weeks and was maintained up to 10 weeks, which is in agreement with previous observations (28, 29). Our observations also showed that the majority of the GFP⁺ cells were of a hematopoietic origin (CD45⁺ cells). Epifluorescence analysis of molars with pulp exposures in the recipient parabiont GFP⁻ mice 4 and 8 weeks after pulp exposure showed an influx of GFP⁺ cells through the apical foramen.

Our observations showed the absence of dentin bridge formation at 4 weeks after pulp exposure and the presence of a few GFP⁺ cells scattered in the pulp chamber and in close association with dentin chips. Analysis of sections 8 weeks after pulp exposure showed clear evidence of reparative dentin formation surrounded by a predentin-like tissue that is in agreement with our previous study (23). At 8 weeks after pulp exposure, there were numerous GFP⁺ cells in close contact with the newly synthesized matrix. The location and the size of the GFP⁺ cells suggested that these cells might represent odontoclast/osteoclasts cells that are derived from the mononuclear phagocyte lineage. The mononuclear phagocyte lineage includes the premonocytes, monocytes, macrophages, osteoclasts, and odontoclasts derived from mononuclear hematopoietic cells (26, 30–33). Odontoclasts are mononucleated or multinucleated cells that are mainly involved in the resorption of dental hard tissues through their ruffled border (31, 32, 34). On the other hand, macrophages are mononuclear cells committed to phagocytosis of bacteria and cellular matrix debris and play important roles in tissue repair (35–37).

The lack of TRAP staining, a specific histochemical marker for odontoclast/osteoclasts cells (26, 38), in the majority of the GFP⁺ cells excluded the possibility of their odontoclast identity, suggesting that the GFP⁺ cells associated with reparative dentin might represent other cells such as macrophages within the mononuclear phagocyte lineage originating from the hematopoietic system of the GFP⁺ donor animals (33). Macrophages are present in pulpitis during intense inflammatory infiltrate (39) and in the initial steps of pulp healing (40).

It is also possible that the GFP⁺ cells around the reparative dentin represent new odontoblast-like cells derived from either a small population of mesenchymal stem cells (MSCs) (CD45⁻ of approximately 4.3%) or hematopoietic cells (CD45⁺ of approximately 54%) in the circulating peripheral blood. Further immunocytochemical experiments with dentin sialophosphoprotein antibody are necessary to verify the odontoblast phenotype of the GFP⁺ cells around the reparative dentin. However, the lack of contribution of the hematopoietic cells to the regeneration of the cells in skeletal tissues (25, 26) suggests the GFP⁺ cells around the reparative dentin most likely do not represent odontoblasts derived from hematopoietic cells.

The possibility that GFP⁺ cells represent odontoblast-like cells that originated from the small population of MSCs in the CD45⁻ fraction of the peripheral blood needs further characterization for GFP⁺ cells including the expression of known MSCs markers including CD73, CD90, and CD105 (41, 42). The contributions of MSCs from peripheral blood to the regeneration of skeletal tissues are controversial. Previous studies have shown that despite

their low percentage, allogeneic MSCs from peripheral blood enhance bone regeneration in rabbit bone defects (43) and contribute to the formation of bone-like tissue in the dental pulp (44).

On the other hand, a recent study (45) showed that the evoked bleeding of the periapical tissues resulted in the accumulation of MSCs expressing CD73 and CD105. The significantly higher levels of the expression of markers of MSC in the blood isolated from the root canals after evoked bleeding compared with levels found in the systemic blood collected from the same patients suggested that MSCs did not originate from the systemic circulation and most likely originated from local tissues adjacent to the apex including the apical papilla, bone, periodontal ligament, dental follicle, and dental pulp (45).

The roles of progenitor/MSCs in dental pulp in reparative dentinogenesis are supported by our recent study (23) using pulp exposure and capping in pOBCol3.6GFP transgenic mice in which GFP expression is under control of the rat 3.6-kb collagen type I promoter fragments and is expressed in the early stages of polarization and fully differentiated odontoblasts (46). Our study showed odontoblast-like cells expressing high levels of 3.6-GFP participating in the reparative dentinogenesis processes. These 3.6-GFP odontoblast-like cells were lined underneath the new dentin bridge participating in the secretion of the new dentin matrix. In conclusion, in the present study, we have provided evidence of the participation of hematopoietic cells and nonresident progenitors/stem in the reparative dentinogenesis.

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Figure 1.

A schematic drawing showing the outline of the experiments in this study. (A) Parabiosis between GFP+ (*green mice*) and GFP- (*black mice*). (B) A collection of a blood sample from the tail and a pulp exposure on the maxillary first molars of recipient GFP- mice after 2 weeks. (C) Four and 8 weeks after pulp exposures, animals were separated, blood samples were collected from the GFP- mice, animals were sacrificed, and maxillary molars were isolated and processed for histologic and epifluorescence analyses.

Figure 2.

Images of sections through an intact first maxillary molar of C57BL/6-TgN(ACTbEGFP)10sb/J transgenic (GFP+ mice). (A) The odontoblast layer (*dashed arrow*) and pulp cells (*solid arrow*) in hematoxylin-eosin staining. (B) The expression of GFP is detected in the intact odontoblast layer (*dashed arrow*) and pulp cells (*solid arrow*). (C) An overlay of hematoxylin-eosin and epifluorescence images. d, dentin; p, pulp. Scale bars = 100 μ m.

Figure 3.

Histologic and epifluorescence analyses of sections through the first maxillary molar of the C57BL/6 recipient GFP⁻ mice (A–C) 4 weeks and (D–H) 8 weeks after pulp exposure. (A, D, and G) Hematoxylin-eosin staining and (B, E, and H) epifluorescence images of adjacent sections. (A) At 4 weeks after pulp exposure, the pulp contains dentin chips (*) surrounded by elongated cells (arrows). At this time point, there is no dentin bridge. (B) Note the GFP⁺ cells (arrows) around the dentin chips. (C) An overlay of images A and B. (D) Note the formation of reparative dentin (dashed lines) surrounded by predentin-like tissue and cells (arrows). (E) Note the presence of GFP⁺ cells (arrows) around the regenerative dentin. (F) An overlay of images D and E. G and H represent a higher magnification of images D and E, respectively. (G) Note the predentin-like tissue (arrowheads) surrounding the reparative dentin and cells (arrows) in close contact with it. (H) Also note the numerous GFP⁺ cells (arrows) in close contact with the predentin-like tissue. Star, pulp exposure site; d, dentin; p, pulp. Scale bars = 100 μ m.

Figure 4.

Images of sections through the first maxillary molar from the GFP- mice 4 weeks after pulp exposure (*star*). Note the GFP+ cells entering the pulp through the apical foramen (*arrows*). Also note GFP+ cells in the dental pulp and in the surrounding alveolar bone. b, alveolar bone; d, dentin; p, pulp. Scale bar = 100 μ m.

Figure 5.

Images of sections through the upper first molar from the GFP+ mice 8 weeks after pulp exposure. (A–C) The reparative matrix is indicated by dashed line. (A) GFP+ cells from peripheral blood lining the reparative dentin (arrows). (B) Note the lack of TRAP staining GFP+ cells surrounding the reparative dentin (arrows). (C) An overlay of both images A and B. D through F are images of sections through the alveolar bone from the GFP+ mice 8 weeks after pulp exposure. (D) Note the GFP+ cells in bone marrow and large and elongated cells lining the bone marrow space (arrows). (E) TRAP staining (arrows) is detected in large and elongated cells lining the bone marrow space. (F) An overlay of both images D and E. Star, pulp exposure site; b, alveolar bone; d, dentin; p, pulp. Scale bars = 100 μ m.

TABLE 1

The Number of Animals Analyzed at Each Time Point and the Percentage of GFP+ and CD45+ in Peripheral Blood of the Wild-Type GFP- Recipient Mice Determined by FACS at Different Time Points after Parabiosis

Weeks after parabiosis	% GFP-	% GFP+	% CD45-	% CD45+
4 (n = 4)	45.7 ± 2.1	54.3 ± 4.1	3.5 ± 0.8	96.5 ± 0.8
10 (n = 2)	40.2 ± 3.0	59.8 ± 2.1	4.3 ± 0.8	95.8 ± 0.8

Values represent means ± standard error from at least 2 to 4 individual experiments.