Histologic and Immunohistochemical Findings of a Human Immature Permanent Tooth with Apical Periodontitis after Regenerative Endodontic Treatment

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Abstract
Specimens of human immature permanent teeth after regenerative endodontic treatment (RET) are sparse. This case report describes the histologic and immunohistochemical findings of tissue formed in the canal space of a human immature permanent tooth with apical periodontitis after RET. A patient presenting with immature human permanent tooth #29 with apical periodontitis underwent RET. At the 10-month follow-up visit, radiographic examination revealed complete resolution of the periapical lesion, marked narrowing of the apical foramen, increased thickness of the canal walls, and minimal lengthening of the root. Notably, the tooth regained pulp sensibility. Tooth #29 was extracted for orthodontic reasons and processed for histologic and immunohistochemical examination. The canal space was filled with newly formed cementumlike tissue, bonelike tissue, and fibrous connective tissue. The apical closure, thickness, and length increment of the root were caused by the deposition of cementumlike tissue without dentin. Furthermore, neurons and nerve fibers were observed in the canal space; this observation was confirmed by immunohistochemistry. Based on the findings in the present case, after RET, the newly formed tissues in the canal space of the human immature permanent tooth with apical periodontitis were primarily fibrous connective tissue, cementumlike tissue, and bonelike tissue. Nerve regeneration was identified. (J Endod 2015;41:1172–1179)

Key Words
Apical periodontitis, cementumlike tissue, human immature permanent tooth, nerve regeneration, regenerative endodontic treatment

The interest in regenerative endodontics has increased because of case reports with successful outcomes (1, 2). A number of case reports and case series regarding the regenerative endodontic treatment of human immature permanent teeth with pulp necrosis or apical periodontitis have been published. Radiographically, many of these cases have shown favorable results as evidenced by the resolution of apical radiolucency, root lengthening, apical closure, and hard tissue deposition on the canal walls (3, 4). In addition, the recovery of pulp sensibility has been observed in a few cases (1, 2, 5–8). The newly formed soft tissue might be regenerated by pulp tissue or periodontal ligament (PDL) tissue, whereas the hard tissue deposition could be caused by the ingrowth of dentin, cementum, or bone (9). Histologic studies in animal models (10–12) showed that the tissues growing in the canal space were cementumlike or bonelike hard tissue and PDL-like connective tissue. However, because only human histologic studies could directly answer the question of tissue identity after regenerative endodontic treatment in patients, samples obtained at rare opportunities are valuable for accumulating evidence of tissue identity. Recently, 5 case reports described histologic findings after successful regenerative endodontic treatment (RET) in humans (13–17). These reports showed that the tissue growing in the pulp space was different (Table 1). Moreover, where these tissues come from and whether nerve regeneration in the pulp space occurs after RET remain unknown. The aim of this study was to describe histologically and immunohistochemically a human immature permanent mandibular premolar that initially had apical periodontitis and then regained sensibility after RET. To our knowledge, this is the first histologic and immunohistochemical study of a human immature permanent tooth after RET that has positive responses to cold and pulp vitality tests similar to those of the adjacent tooth.

Case Report
A 10-year-old girl was referred by a general dentist to the Department of Endodontics and Operative Dentistry, School and Hospital of Stomatology, Fujian Medical University, Fuzhou, Fujian, China. The patient’s chief complaint was the presence of pain during mastication for 2 weeks. The patient suffered from pain for 2 weeks before visiting her general dentist. The general dentist made an opening to access tooth #30 based on the diagnosis of irreversible pulpitis. However, because the pain persisted, the dentist referred this patient to our hospital. Clinical examination revealed extensive caries in tooth #30, and the access point was kept open with a cotton pellet. Tooth #30 was not sensitive to percussion and palpation and did not have mobility. Tooth #29 was
First Treatment Visit

No anesthesia was administered initially to evaluate whether vital tissue was present in the root canal of tooth #29. When the access cavity was made under rubber dam isolation, a purulent hemorrhagic exudate was discharged from the pulp chamber. Observation with a Zeiss surgical microscope (Carl Zeiss Meditac Inc, Dublin, CA) confirmed the absence of vital tissue in the pulp chamber and canal. Without mechanical instrumentation, the pulp chamber and canal were gently irrigated with 20 mL 1% sodium hypochlorite. Then, the canal was dried with sterile paper points. Subsequently, a triple antibiotic paste that consisted of a powder of 100 mg each of ciprofloxacin, metronidazole, and cefaclor mixed with 1 mL sterile water was placed into the apical portion of the canal and filled up to the level directly below the cemento-enamel junction. A sterile moist cotton pellet was applied with gentle pressure apical tissue into the canal up to 3 mm below the cemento-enamel junction. A sterile moist cotton pellet was applied with gentle pressure.

Second Treatment Visit

The patient returned to the clinic 4 weeks later. The tooth was asymptomatic with intact temporary fillings. Tooth #29 was not tender to percussion or palpation. No mobility was noted. Local anesthesia was administered with 2% lidocaine without a vasoconstrictor. After isolation with a rubber dam, the temporary filling was removed, and no sign of inflammatory exudate was observed. Then, the antibiotic paste was gently flushed out of the canal with copious amounts of sterile normal saline. Next, the canal was irrigated with 10 mL 17% EDTA solution and dried with sterile paper points. Under a surgical microscope, a sterile #35 K-file was introduced into the canal through the apical foramen with a push and pull motion to provoke bleeding from the periapical tissue. Then, the canal was filled with a layer of CollaPlug (Zimmer Dental, Carlsbad, CA) and then by a 3-mm thickness of a ProRoot mineral trioxide aggregate (MTA) mixture (Dentsply Tulsa Dental Specialties, Tulsa, OK). A moist

### TABLE 1. Current Published Histologic and Immunohistochemical Reports of Regenerative Endodontic Treatment in Humans

<table>
<thead>
<tr>
<th>Authors</th>
<th>Diagnosis</th>
<th>Scaffold</th>
<th>Post-treatment vitality responses</th>
<th>Histologic findings</th>
<th>Immunohistochemical findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torabinejad et al, 2012 (17)</td>
<td>Pulp necrosis and symptomatic apical periodontitis</td>
<td>Platelet-rich plasma</td>
<td>Yes</td>
<td>Collagen fibers, cells, and blood vessels in pulplike connective tissue. No odontoblastlike cells could be observed in the canal (only soft tissue in the canal).</td>
<td>NA</td>
</tr>
<tr>
<td>Shimizu et al, 2012 (16)</td>
<td>Symptomatic irreversible pulpitis</td>
<td>Blood clot</td>
<td>No</td>
<td>Connective tissue, odontoblastlike cells and epithelial-like HERS. No nervelike fibers and hard tissue was formed in the canal.</td>
<td>Strol-1–positive cells were observed in the connective tissue near the apical foramen.</td>
</tr>
<tr>
<td>Martin et al, 2013 (14)</td>
<td>Pulp necrosis and symptomatic apical periodontitis</td>
<td>Platelet-rich plasma</td>
<td>NA</td>
<td>Cementoid/osseoid tissue and uninfamed fibrous connective tissue. No HERS or odontoblastlike cells could be observed in the canal.</td>
<td>NA</td>
</tr>
<tr>
<td>Shimizu et al, 2013 (15)</td>
<td>Pulpal necrosis and chronic apical abscess</td>
<td>Blood clot</td>
<td>NA</td>
<td>Connective tissue similar to that in the periodontal ligament and cementumlike or bonelike hard tissue. No tubulilelike structures of mineralized tissue or odontoblastlike cells could be observed in the canal.</td>
<td>NA</td>
</tr>
<tr>
<td>Becerra et al, 2014 (13)</td>
<td>Pulpal necrosis and a chronic apical abscess</td>
<td>Blood clot</td>
<td>NA</td>
<td></td>
<td>Positive immunoreactivity for BSP was observed, whereas DSP and neurofilament immunoreactivity were negative.</td>
</tr>
</tbody>
</table>
A cotton pellet was placed over the MTA, and the access cavity was sealed with Cavit.

Third Treatment Visit

One week later, the tooth was asymptomatic. Cavit was removed and replaced with a bonded resin restoration (Filtek Z350 XT; 3M ESPE Dental Products, St Paul, MN). Follow-up visits were scheduled at 3, 6, 9, 12, 18, and 24 months.

Follow-up Visits

At the 6-month follow-up visit, tooth #29 was asymptomatic and not sensitive to percussion or palpation. The radiographic examination revealed a slight increase in the thickness of the root canal walls and a narrower apical foramen (Fig. 1B). When testing sensibility, EPT (Parkell Inc, Farmingdale, NY) gave a positive response, whereas no reaction was obtained with the cold test.

At the 10-month follow-up visit, tooth #29 remained asymptomatic and was not sensitive to percussion or palpation. Marked narrowing of the apical foramen and increased thickness of the canal walls of tooth #29 were observed radiographically. A slight lengthening of the root was also noticed (Fig. 1C). In addition, notably, a positive response was elicited in tooth #29 by either the ice stick test or EPT, similar to the responses of teeth #28 and #20. At this period, for orthodontic reasons, the mandibular second premolars were required to be extracted by the treating orthodontist after a complete case study. With the permission of the patient’s parents, we were allowed to process the extracted teeth for histology. After extraction, teeth #29 (Fig. 1D) and #20 (used as the control) were immediately fixed in 10% neutral buffered formalin solution for histologic and immunohistochemical processing.

Histologic Procedure

After fixation, the teeth were demineralized in 10% EDTA (pH = 7.4) for 6 months at room temperature. Subsequently, the samples were rinsed under running water for 4 hours followed by dehydration with ascending concentrations of ethanol. Then, the teeth were deparaffinized in xylene, infiltrated, and embedded in paraffin. With the microtome set at 4 μm, longitudinal serial sections were cut on a buccolingual plane until the specimen was exhausted. Every fifth slide was stained with hematoxylin-eosin and Masson trichrome for screening purposes and for assessing the tissues formed in the canals. The slides were observed under a light microscope.

Immunohistochemical Procedure

To detect the presence of odontoblasts and neural cells, antibodies for nestin (Clone 2C1.3A11 [ab27053; Abcam, Cambridge, UK]) and protein gene product (PGP) 9.5 (ab27053, Abcam) were used. PGP 9.5 is a neuron-specific protein that is widely distributed in both central and peripheral neurons (18). Immunohistochemical staining was performed using the streptavidin-biotin system (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer’s protocol. In brief, the sections were cleared with xylene and then dehydrated in ethanol. After antigen retrieval with 10 mmol/L sodium citrate buffer solution, endogenous peroxidase activity was blocked by 3% H2O2. The sections were incubated with 10% normal goat serum and then with mouse anti-nestin monoclonal antibody or rabbit anti–PGP 9.5 polyclonal antibody overnight at 4°C. Samples of tooth #20 were used as positive controls, whereas sections incubated with phosphate-buffered saline served as negative controls. Then, the sections were incubated with secondary biotinylated goat anti-mouse or anti-rabbit immunoglobulin G and washed with phosphate-buffered saline. Finally, staining was completed.
by incubation with 3,3’-diaminobenzidine substrate (Vector Laboratories Inc, Burlingame, CA) for 5 minutes.

**Histologic and Immunohistochemical Observations**

Longitudinal sections of tooth #29 revealed that the tissues in the canal space consisted of newly formed calcified tissue and fibrous connective tissue interspersed with blood vessels (Fig. 2A–G). The new tissue filled the canal space up to the coronal MTA (Fig. 2C). The newly formed mineralized tissue on the canal walls was both cellular and acellular cementumlike tissue (Fig. 2D), and many cementoblasts were present in the cellular cementumlike tissue (Fig. 2D). The demarcation between the cementumlike tissue and canal dentin could be easily recognized by the absence of dentinal tubules in the former (Fig. 2D). Bone-like tissue with osteocyte-like and osteoblast-like cells (Fig. 2E) formed mineralized tissue islands in the middle portion of the canal space (Fig. 2A and B). The canal dentin appeared to connect directly to the cementumlike tissue (Fig. 2F). At the border of the uninfamed fibrous connective tissue, which was characterized primarily by spindle-shaped fibroblasts and collagen fibers (Fig. 2C), collagen bundles were inserted into the cementumlike (Fig. 2F) and bone-like tissue (Fig. 2M) at right angles. The fibrous connective tissue in the apical canal appeared to be an extension of the periodontal ligament (Fig. 2L). Neurons and nerve fibers were observed in the newly formed tissue (Fig. 2H): this observation was confirmed by PGP 9.5 immunoreactivity (Figs. 2I and 34–C). In contrast to the control group (tooth #20) (Fig. 2G), no odontoblast-like cells could be observed histologically and immunohistochemically in tooth #29 (Figs. 2D and 3E–G).

**Discussion**

The results of the present case showed that the tissue formed in the canal space after RET consisted primarily of cementum-like tissue, bone-like tissue, and fibrous connective tissue. The narrowing of the apical closure and the increased thickness and length of the root were caused by the deposition of cementum-like tissue. Based on the absence of a tubule-like structure and negative immunoreactivity to nestin, the newly formed mineralized tissue was not dentin. These findings were consistent with those observed in animal studies (10–12) and previous human case reports (13, 15). The cells that are able to promote continued root growth include PDL stem cells (19), stem cells from the apical papilla (SCAPs) (20), the Hertwig epithelial root sheath (21), and bone marrow mesenchymal stem cells transplanted into the canal space (22). Histologically, the fibrous connective tissue in the apical canal appeared to be an extension of the PDL. Additionally, PDL-like fibers were inserted into the cementum-like and bone-like tissue at right angles as Sharpey’s fibers. Therefore, in this case, the cementoid/osteoid tissue in the pulp space might be generated by cementoblasts/osteoblasts that differentiated from the PDL. Because no nestin immunoreactivities or nestin-positive cells were identified in the canal space, pulp tissue regeneration was not present. Our observation of the absence of pulp tissue is in agreement with most previous studies. However, recent histologic evidence from 2 case reports suggested that regenerated pulp-like tissue might be in the canal space after RET (16, 17). Theoretically, the infection duration and severity, the involved microbial species, the RET variables, the host immunity, and the open apex size all may play a role in the outcome of tissue regeneration.

PGP 9.5 is advantageous for immunohistochemical analyses when studying the innervation of hard tissues because it is not affected by demineralization procedures (23). Dense PGP 9.5 immunoreactivity has been reported in the distribution of nerve fibers in human radicular dental pulp, and the usefulness of the antibody directed against PGP 9.5 for the identification of nerve fibers in human teeth has been confirmed (24).

To our knowledge, this is the first histologic and immunohistochemical evidence in the dental literature that shows that nerve tissue can be regenerated in a human immature permanent tooth with previous necrotic pulp and apical periodontitis. Our clinical examination revealed continuous recovery of the sensibility of the tooth after RET. Positive responses to tooth sensitivity tests have been reported by a few previous case studies (1, 2, 5–8). In these cases, tooth sensibility recovery was observed from 5 to 6 months to 2 years postoperatively. A histologic study was conducted in only 1 of these cases without findings of regenerated nerve tissue (17). In this case, the presence of nerve tissue was identified by histologic observation as well as immunohistochemical investigation with PGP 9.5. The results confirmed the existence of regenerated neurons and nerve fibers in the canal space and apical region, indicating the feasibility of nerve regeneration after RET. Nerve regeneration may be attributed to many mechanisms.

One possible mechanism postulates that stem cells might differentiate from the PDL. Human PDL stem cell subpopulations have been reported to express the markers of undifferentiated neural crest cells (nestin, Slug, and p75) and to exhibit the potential to differentiate into neurogenic lineages (25–27).

Another hypothesized mechanism depends on the survival of dental pulp stem cells (DPSCs) from residual vital apical pulp tissue. In mature teeth, some vital pulp tissue might remain despite the presence of a periradicular lesion (28). DPSCs have the potential to differentiate into neuronal cells in vitro (29) and can induce axon guidance (30). In addition, DPSCs can produce a series of neurotrophic factors, including nerve growth factor, glial cell line–derived neurotrophic factor, and brain-derived neurotrophic factor (31–33). Human DPSCs that were implanted into the developing brain of embryonic chicken exhibited neuronal morphology and were positive for neuronal markers (29). All these facts support the idea that DPSCs might have initiated the regeneration of nerve tissue.

The third possible mechanism relies on SCAPs. In the case of an immature tooth with apical periodontitis, SCAPs residing in the apical papilla likely survived the infection (34). Moreover, SCAPs have been shown to stain positive for several neural markers (35). SCAPs might be derived from neural crest cells or at least associated with neural crest cells analogous to dental stem cells such as DPSCs and stem cells from human exfoliated deciduous teeth (SHED) that have been shown previously to possess neurogenic potential (36, 37).

The fourth possible mechanism relies on bone marrow mesenchymal stem cells. During the induction of periapical bleeding by irritating the periapical tissues, mesenchymal stem cells from the bone marrow may be transplanted into the root canal. Bone marrow mesenchymal stem cells can differentiate into neurons and astrocytes under appropriate conditions (38). It has been shown that transplanted bone marrow mesenchymal stem cells into the injured spinal cord of rats could differentiate into neurons and astrocytes and repair spinal cord ischemia injury (39).

The fifth possible mechanism is that the pulp canal likely received a collateral reinnervation by the sprouting or ingrowth of neighboring ipsilateral nerves, similar to the reinnervation of replanted and autotransplanted immature teeth after surgery. Several studies have reported that the reinnervation, tooth sensitivity restoration, and prognosis of the replanted teeth are favored if the tooth infection is controlled with an open apex and rapidly regained revascularization (40–42). In addition, nerve fibers are known to regenerate after the transection of the inferior alveolar nerve and to grow into the tooth pulp by the sprouting and ingrowth of intact nerves in adjacent tissues (43, 44). However, the underlying mechanism of this action needs further investigation.
Figure 2. (A and B) The section passing approximately at the center of the root canal of tooth #29; the newly formed tissue consists of connective tissue, mineralized tissue deposited on the canal walls, and mineralized tissue islands in the canal space (hematoxylin-eosin; original magnification, ×40). (C) A detailed view of
Different concentrations of NaOCl ranging from 1.25%–5.25% were used in previous case studies (45). However, high concentrations of NaOCl could have a profound negative effect on the survival and differentiation of stem cells (46, 47). Several studies have shown that dentin conditioning with 5.25% NaOCl prevented the differentiation of SHED and DPSCs into odontoblastlike cells (48, 49). In addition, previous studies have found no significant difference in the antibacterial effects of 1%, 2.5%, and 5.25% NaOCl (50, 51). Thus, the ususal concentration of NaOCl in RET is rational. Moreover, a previous study found that EDTA could effectively release growth factors from human

Figure 3. Immunohistochemical staining for PGP 9.5 and nestin. (A) A low-magnification view of tooth #29; positive immunoreactivity for PGP 9.5 is observed in the newly formed connective tissue (original magnification, ×100). (B and C) A high-magnification view of the area indicated by the rectangle in A (original magnification, ×400). (D) Section with PGP 9.5 immunoreactivity similar to A in the positive control group (tooth #20) (original magnification, ×200). (E) A low-magnification view of tooth #29; no nestin immunoreactivity is detected (original magnification, ×40). (F) A detailed view of the right rectangle in E (original magnification, ×200). (G) A high-magnification view of the area indicated by the rectangle in E (original magnification, ×400). (H) Nestin immunoreactivity in the positive control group (tooth #20); intense immunoreactivity for nestin is exhibited in odontoblasts (original magnification, ×400).

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dentin (52–54), which might promote the development of odontoblast proliferation after RET (55, 56). Therefore, in this case, we used 1% NaOCl followed by EDTA for irrigation to preserve potential stem cells.

**Conclusion**

Based on the findings in the present case, after RET, the newly formed tissues within root canals of the human immature tooth with api-
cal periodontitis were fibrous connective tissue, cementumlike tissue, and bonelike tissue. The continued root development was caused by the deposition of cementumlike tissue, and nerve regeneration was identified.

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The authors deny any conflicts of interest related to this study.

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