Immunoelectron Microscopic Study of CGRP-Immunoreactive Nerve Terminals in Wound Healing and Dentin Bridge Formation after Pulpotomy in Rat Molar

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The purpose of the present study was to investigate the relationship between the neuropeptide calcitonin gene-related peptide-immunoreactive (CGRP-IR) nerves and the differentiation of undifferentiated mesenchymal cells into fibroblast-like cells and odontoblasts during the healing process after a pulpotomy. The first maxillary molars from 56-day-old Wistar rats (n=60) were used. The rats were sacrificed to undergo an immunoelectron microscopic examination at 1, 3, 7, 14, and 28 days, postoperatively. In 1 to 3 postoperative days, numerous unmyelinated degenerated CGRP-IR nerve terminals were observed. In 7 postoperative days, regenerated terminals were found to contain numerous large granular vesicles, small clear vesicles, a few mitochondria and a labeled organelle. Certain terminals were found to be attached with the cell bodies of fibroblast-like cells and their processes at the fibrous matrix layer of the dentin bridge during healing process following a pulpotomy. In 14 to 28 postoperative days, CGRP-IR nerve terminals had come into contact with the differentiating odontoblasts at the odontoblast layer of the dentin bridge. These findings demonstrate strong evidence that CGRP may be related to both the proliferation and cytodifferentiation processes as well as to the active function of the renewed odontoblasts in dentin bridge formation.

Key words: calcitonin gene-related peptide, immunoelectron microscopy, nerve terminals, pulpotomy, dentin bridge formation

I. Introduction

The immunohistochemical identification of nerve fibers has been used to evaluate sensory neuropeptides such as CGRP and substance P [5, 8, 10, 13]. Although the pulpal nerve fibers that contain CGRP have recently been shown to play an important role in the pulp response to inflammation and wound healing [10, 14], no studies have yet elucidated the relationship between the CGRP-IR nerve terminals and the differentiation of undifferentiated mesenchymal cells into fibroblast-like cells and odontoblasts during the healing process after a pulpotomy with the use of immunoelectron microscopy. Our preceding paper described the relationship between CGRP-IR innervation and dentin bridge formation following a pulpotomy in the rat molar based on light microscopic immunohistochemistry [17]. In the present study, we attempt to further clarify whether the neuropeptide CGRP-IR nerve terminals are related to both the proliferation and cytodifferentiation processes and also the active function of renewing and regenerating odontoblasts in dentin bridge formation during the pulp healing using the calcium hydroxide method by immunoelectron microscopy.

II. Materials and Methods

Male Wistar rats (n=60), 56 days of age, body weight, 280–300 g, were used, and they were anesthetized by intraperitoneal injection of sodium pentobarbiturate (0.1 ml/100 g body wt.). According to the pulpotomy method [17], using a dental electric rotator with a sterilized water coolant, the left first maxillary molar was ground off with a diamond point bur and then the coronal pulp was removed with steel round burs (numbers 1/2 and 1) and cut with a spoon excavator at the coronal orifice of the root pulp. Thereafter, while alter-
nately using 3% H$_2$O$_2$ and 5% sodium hypochlorite solution, the residual pulp was rinsed 3 times to clean the wound surface, and a sterile cotton pledget pellet was replaced at the wound surface for both hemostasis and drying. Next, the wound surface was properly covered with calcium hydroxide paste, zinc phosphate cement as the base lining and light-cured composite resin as a permanent filling. The right first maxillary molar was used as a control.

For ultrastructural immunohistochemistry, on 1, 3, 7, 14 and 28 postoperative days, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbiturate (0.1 ml/100 g body wt.), and were killed by transcardiac perfusion with 250–300 ml of 4% paraformaldehyde plus 0.2% picric acid and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB, PH 7.4) at room temperature over 15–20 min with the use of perfusion pump. Following perfusion, the left and right upper jaws were immediately removed and immersed in the same fixative for 24 hr at 4°C. Decalcification in daily changes of 5% EDTA-4Na in PB at 4°C required 3 weeks. Subsequently, the tissue specimens were saturated with cold 30% sucrose in PB overnight.

The specimens were serially sectioned at 40 μm on a freezing microtome and incubated with anti-CGRP (1:4000; Amersham UK) for 72 hr at 4°C and then with biotinylated anti-rabbit IgG (1:300; DAKO Denmark) and avidin-biotin complex (ABC: 1:100; DAKO Denmark) for 3 hr at room temperature, respectively. Peroxidase was visualized using 3,3’-diaminobenzidine (DAB substrate kit; Vector Laboratories, Burlingame, CA, USA). The sections were postfixed in 1% OsO$_4$ in 0.1 M phosphate buffer for 1 hr at 4°C and stained with 2% uranyl acetate in distilled water for 1 hr, dehydrated through a graded alcohol series and embedded in Spurr’s resin. Ultrathin sections were contrasted with lead citrate and examined with a JEM-1200EXII electron microscope.

### III. Results

#### Control groups

CGRP-IR positive nerve fibers were easily identified in the pulp by electron microscopy. The major populations of the CGRP-IR nerve fibers were unmyelinated, while only a few were thinly myelinated. In the radicular pulp, the nerve fibers contained both unmyelinated and myelinated nerve fibers that were often found close to blood vessels. In the coronal pulp, numerous unmyelinated CGRP-IR nerve terminals were characterized by containing few mitochondria. Some unmyelinated CGRP-IR nerve terminals were observed to contact with odontoblasts in the odontoblast layer (Fig. 1A). A few CGRP-IR nerve terminals were also found to penetrate in the predentin (Fig. 1B).

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![1A](image1a.png) ![1B](image1b.png)

**Fig. 1.** Immunoelectron micrograph of CGRP-IR nerve distribution in a control tooth. **A:** In the coronal, the CGRP-IR nerve terminal (arrow) is observed to come in contact with the odontoblast (OB) in the odontoblast layer. **B:** The CGRP-IR nerve terminal (arrow) also penetrates the predentin (PD). Bars=1 μm (A), 2 μm (B).
Experimental groups
1–3 day group

There were three distinct zones in the pulpotomized lesion including: 1) a necrotic zone at superficial layer; 2) a calcified demarcation zone; and 3) a residual pulp zone with a slight degree of inflammatory cell infiltration. Adjacent to the zone of calcified demarcation in the residual pulp, degeneration changes were found to have occurred most frequently in unmyelinated CGRP-IR positive nerve fibers. Unmyelinated CGRP-IR terminals showed scattered small and large clear vesicles, and the mitochondria of terminals swollen than in the controls could be seen (Fig. 2A). Especially during the first 3 postoperative days, many undifferentiated mesenchymal cells or fibroblast-like cells proliferated

Fig. 2. Immunoelectron micrograph of CGRP-IR terminals 1–3 day after a pulpotomy. A: Some CGRP-IR terminals show degeneration with swollen mitochondria (arrowheads) and scattered small and large vesicle structures (*) under the demarcation zone. B: Many undifferentiated mesenchymal cells or fibroblast-like cells proliferation and differentiation in the residual pulp (RP) and a few regenerated CGRP-IR terminals (arrows) are found to be in close contact with the either the cell bodies (Fc) or their processes (OP) of the fibroblast-like cells. Bars=0.5 μm (A), 1 μm (B).
and differentiated in the residual pulp. A few unmyelinated CGRP-IR fibers sprouted in the residual pulp, and the regenerated terminals came in contact with the cell bodies of the undifferentiated mesenchymal cells and the fibroblast-like cells and their processes could be seen (Fig. 2B).

7-day group

The fibrous matrix zone (FM) of the dentin bridge formed under the calcified demarcation zone. Fibroblast-like cells existed in the cellular layer under the FM. Numerous unmyelinated CGRP-IR fibers were observed sprouting up from a beaded-like structure (Fig. 3A). The regenerated CGRP-IR terminals in the residual pulp contained numerous large granular vesicles, small clear vesicles, a few mitochondria and some CGRP-positive granular vesicles (Fig. 3B). Many unmyelinated regenerated CGRP-IR nerve terminals were revealed to come in contact with the cell bodies of the fibroblast-like cells and their processes in the fibroblast layer (Fig. 3C) and were also embedded in the FM. No specialized synaptic structures were displayed between the terminals and either the cell bodies or their processes of fibroblast-like cells.

14-day group

Progressive healing was found in the osteodentin-like layer of the dentin bridge and odontoblasts were developed to line this layer. Some unmyelinated regenerated CGRP-IR nerve terminals were surrounded by cell bodies of the differentiating odontoblasts and their processes, and they were also surrounded by newly formed collagen fibers of the dentin bridge. No specialized synaptic structures were observed between the terminals and either the cell bodies or their processes of the differentiating odontoblasts (Fig. 4).

28-day group

The osteodentin layer and tubular dentin layer were both observed to have formed at 28 days after the pulpotomy. Only a few CGRP-IR nerve terminals attached to cell bodies of the odontoblasts in the odontoblast layer of the dentin bridge. In addition, no specialized synaptic structures were observed between the terminals and the odontoblasts (Fig. 5A, B).

IV. Discussion

Few immunoelectron microscopic studies for CGRP-IR nerve terminals have described the CGRP-IR nerve terminals and their relation with dentin bridge formation in the pulp tissue during degeneration and regeneration processes. This may be due to the disadvantages in using hard tissue specimens that make it difficult to decalcify them with suitable preservation of both ultrastructure and immunoreactivity. The present study was conducted to investigate the changes which occur in the CGRP-IR nerve terminals at the immunoelectron microscopic level and to estimate the relationship between the CGRP nerve terminals and both proliferation and cytodifferentiation process of odontoblasts during wound healing, including dentin bridge formation following a pulpotomy.

In our present study, 1 to 3 days after a pulpotomy, CGRP-IR nerve terminal changes were developed in numerous CGRP-IR unmyelinated nerve terminals in the residual pulp. Based on our immunoelectron microscopic observation, the finding as mitochondria swollen, scattered clear vesicles appearance was characteristiclly seen. These changes are similar to the axonal changes that occur during Wallerian degeneration [11]. This phenomenon suggested that the CGRP-IR nerve terminals degenerated due to mechanochemical injury at an early stage after a pulpotomy.

At the dentin bridge formation, a close contact between fibroblast-like cells or differentiating odontoblasts and CGRP-IR nerve terminals at the dentin bridge front were observed in the healing process after a pulpotomy (Figs. 2B, 3C, 4, 5A, 5B). Our findings showed strong evidence that the regenerated CGRP-IR nerve terminals may thus be related to both the proliferation and cytodifferentiation processes of the renewed odontoblasts for dentin bridge formation.

Pulp nerve fibers are generally closely related to odontoblasts both functionally as well as morphologically [6]. Sensory nerve fibers play a possible role both in regulating the differentiation and proliferation of pulpal cells during the inflammation or healing processes following pulpal exposure or cavity preparation [1, 3, 9, 10, 13]. Sensory denervation showed a significant reduction in dentin formation at the pulp horn [8]. These findings support our opinion that the expression of CGRP plays a biological role in regulating the differentiation and function of odontoblasts during pulp wound healing and dentin bridge formation.

The mechanism of action regarding how CGRP regulates odontoblasts is still unclear. Sakakura [12] reported that calcitonin accelerated the development of tooth germs and such suggested that calcitonin affected the odontoblasts promoting the differentiation of odontoblasts and predentin formation. Trantor, et al. [15] reported that CGRP causes a significant stimulation of pulp fibroblasts proliferation in vitro. In addition, CGRP has been shown to activate adenylate cyclase in different cells [4]. This finding was confirmed by [7], who demonstrated increased formation of cyclic AMP (c-AMP) after application of CGRP. Calland et al. [2] reported that CGRP stimulation of pulp cells in injured pulp tissue in culture causes an increase in BMP-2 expression and is also associated with pulpal repair and may such lead to tertiary dentin formation.

In another hand, recently study for the existence of mRNA for the CGRP1 receptor in human pulp tissue has been well established [16]. However, the exact localization of the receptors on the membrane of differentiating odontoblasts has yet to be demonstrated.

In the present electron immunohistochemical study, we demonstrated that the regenerated CGRP-IR nerve terminals come in contact with newly developed fibroblasts-like cell or odontoblasts after a pulpotomy, thus suggesting that CGRP may possibly play a role in regulating the differentiation and function of odontoblasts during wound healing.
Fig. 3. Immunoelectron micrograph of CGRP-IR nerve terminals 7 days after a pulpotomy. A: The CGRP-IR nerve terminal (arrows) sprouts from the beaded-like structure (growth cone) in the residual pulp (RP). B: Many regenerated CGRP-IR terminals (N) are observed containing numerous granular vesicles (LGV), small clear vesicles (SCV), a few mitochondria (M) and some CGRP-positive granular vesicles (arrow). C: Some regenerated CGRP-IR terminals (N) can be seen to come in contact with the processes of the differentiating odontoblast (OP). Bars=1 μm (A), 0.2 μm (B), 0.5 μm (C).
Fig. 4. Immunoelectron micrograph of CGRP-IR nerves 14 days after a pulpotomy. Two CGRP-IR terminals (N) are surrounded by cell body (OB) of the differentiating odontoblasts and their processes (OP), and they are also surrounded by the newly formed collagen fibers (arrowheads) in the fibrous matrix layer of the dentin bridge. Bar=0.5 μm.

Fig. 5. Immunoelectron micrograph of CGRP-IR nerve terminals 28 days after a pulpotomy. A: A few regenerated CGRP-IR terminals (N) are also observed to come in contact with the cell bodies of the odontoblasts (OB). B: A high magnification of (A) shows no specialized synaptic structures between the terminal (N) and the cell bodies (OB) of the odontoblasts. (PD: Predentin) Bars=1 μm (A), 0.2 μm (B).
However, no specialized synaptic structures were observed between the CGRP-IR terminals and either cell bodies or processes of differentiated odontoblasts. Further characterizations based on more detailed studies are therefore needed.

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VI. References