Expression of Calcitonin Gene-related Peptide in Rat Pulp and Periodontal Tissues by Indirect Immunofluorescence Method

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The aim of this study was to investigate the expression of nerve fibers immunoreactive to calcitonin gene-related peptide (CGRP) in pulp and periodontal tissues of rats. Male Sprague-Dawley rats, aged 6 weeks, were sacrificed, and the jaws were excised, demineralized, and processed for indirect immunofluorescence staining. A considerably higher density of nerve fibers immunoreactive to CGRP was found in the dental pulp and gingiva than in periodontal ligament. The majority of pulpal CGRP immunopositive fibers that were located followed blood vessels parallel to the long axis of the root. A subodontoblastic network of fibers IR to CGRP was found in the coronal pulp in rat molars. In the periodontium, CGRP immunopositive fibers were mainly located in the periapical area and close to the alveolar bone. Gingiva was also well supplied with CGRP-IR nerves.

Introduction

RAT MOLAR DENTAL TISSUES represent a peripheral end organ densely innervated with sensory nerve fibers. The peptidergic sensory nerve fibers take part in nociception and peptide-mediated neurogenic inflammation in normal and injured tissue.^(1–3) The sensory neuropeptides—that is, calcitonin gene-related peptides (CGRP)—are released after sensory nerve stimulation, such as electrical and chemical tooth stimulation or mechanical irritants (tooth movement).^(1,4–11) Many investigations have focused on neuropeptides and their role in regulating pulpal and periodontal blood flow and the development of neurogenic inflammation. Other studies have explored the role that neuropeptides play in traumatic occlusion and during and after orthodontic tooth movement,^(12,13) suggesting the involvement of neuropeptides in pulpal and periodontal tissue injury, as well as in pulpal and periodontal tissue repair.

Investigators have shown that CGRP is a multi-potent neuropeptide, determining regulation of blood flow,^(14,15) modulation of immune cell function,^(16,17) proliferation of endothelial cells,⁽¹⁸⁾ upregulation of endothelial adhesion molecules,⁽¹⁹⁾ inhibitory potency of bone resorption,^(20,21) and stimulation of an osteogenic effect.⁽²²⁾

Although nerve fibers immunoreactive to calcitonin gene-related peptide have been found in dental pulp and periodontium,^(23–25) to explore the regulatory mechanism of CGRP in peripheral tissues, the detailed mapping of nerve fibers immunoreactive to CGRP in pulp and periodontal tissues of rats still requires further investigation.

Materials and Methods

Animals

Six male Sprague-Dawley rats (160–180 g) were housed in separate cages on a standard 12 h light/dark cycle, with water and food pellets available *ad libitum*. All procedures of our experiment were approved by the Committee of Animal Use for Research and Education of the Fourth Military Medical University (Xi'an, China), and all efforts were made to minimize the number and suffering of animals, in accordance with the ethical guidelines for animal research.⁽²⁶⁾ Rats were allowed to acclimatize to the housing conditions for 5 days before being sacrificed.

Experimental procedure

The rats were anesthetized with an overdose of sodium pentobarbital (100 mg/kg i.p.) and transcardially perfused with heparinized phosphate buffer, followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4). The jaws were excised and post-fixed for 1 day in the same fixative as was used for perfusion, and thereafter demineralized for 4 weeks at 4°C in ethylenediaminetetraacetate (EDTA), containing 7.5% polyvinylpyrrolidone (PVP; Aldrich Chemicals, Steinheim, Germany).⁽²⁷⁾ After demineralization the jaws were immersed in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) and serially sectioned sagittal at 30 µm on a freezing microtome.

Immunohistochemistry (indirect immunofluorescence)

For the purpose of immunohistochemical labeling, free-floating sections in tissue culture wells were used, as

FIG. 1. In the root pulp (P), CGRP immunopositive nerve bundles distributed along the long axis of the root with no branching (arrows). D, dentin.

described by Kvinnslund and colleagues.⁽¹³⁾ The sections were alternately incubated for 24 h in CGRP polyclonal antibody (rabbit antibody, 1:2000 dilution, Sigma, St. Louis, MO), followed by incubation with the secondary fluorochrome-conjugated antibody (1:200 dilution, goat anti-rabbit antibody, Sigma). Finally, the sections were coverslipped and analyzed in a light microscope (Olympus BX-60, Tokyo, Japan). The specificity of the immune reaction was tested by omitting the primary antibody and substituting it with PBS. In these sections, specific immunostaining was not observed.

Results

According to the immunohistochemistry results, a considerably higher density of nerve fibers immunoreactive to CGRP was found in the dental pulp and gingiva than in the

FIG. 3. In the mid-section of periodontium, CGRP immunoreactive nerve fibers are sparsely distributed (arrows). AB, alveolar bone; PDL, periodontal ligament; D, dentin.

periodontal ligament (PDL). The majority of pulpal CGRP immunopositive fibers followed blood vessels parallel to the long axis of the root, showing almost no branching (Fig. 1). After entering the coronal pulp, they ramified, forming a subodontoblastic plexus of fine fibers, some of which terminated in the dentinal tubules (Fig. 2). In the periodontium, the CGRP immunopositive fibers were short, mainly located in the periapical area close to the alveolar bone (Fig. 3), although individual fibers extended towards the cellular cementum. In the midroot and cervical parts of the PDL, sparse immunoreactive nerves were found (Fig. 4). Gingiva was also well supplied with CGRP-IR nerves. Most of the nerve fibers IR to CGRP ran parallel to the epithelial surface. Occasionally, fibers were found to penetrate into the epithelium (Fig. 5). In the basal layer of the epithelium, clusters of cell-like structures immunoreactive to CGRP were observed (Fig. 6).

FIG. 2. Distribution of CGRP immunoreactive nerve fibers in the distal pulp horn (P). CGRP containing nerves (arrows) are distributed in the odontoblastic layer (O) with some fibers (small arrows) in dentinal tubules. D, dentin.

FIG. 4. In the periapical periodontium, CGRP immunoreactive nerve fibers (arrows) are closely distributed towards the alveolar bone (AB). C, cementum.

FIG. 5. Nerve fibers IR to CGRP ran parallel to the epithelial surface in gingiva. Some fibers (wide arrows) were found to penetrate into the epithelium (E). CT, connective tissue.

Discussion

The present study showed that an abundant CGRP immunoreactive nerve supply was found in the rat dental tissues. There was a considerably higher density of CGRP immunoreactive fibers in the dental pulp and gingiva than that in the PDL and alveolar bone. The pulp of molars exhibited a relatively dense subodontoblastic network of CGRP-IR fibers, which frequently penetrated the innermost part of dentin. In the periodontium, the CGRP immunopositive fibers were mainly located in the periapical area close to the alveolar bone. Gingiva was also well supplied with CGRP-IR nerves, which ran parallel to the epithelial surface.

FIG. 6. In the basal layer of the epithelium (E), clusters of cell-like structures immunoreactive to CGRP (wide arrows) are observed. CT, connective tissue.

These findings agree with previous results in dental innervation of rat and cat.^(23,28) The wide distribution of CGRP in nerve fibers implies the importance of this neuropeptide in the biology of teeth and their supporting tissues. Additional evidence has suggested that CGRP not only take part in the inflammatory process but also in the healing process. Injury induces a reversible sprouting of nerve fibers adjacent to the injury site, which increases in proportion to the severity of the injury; the significance of this sprouting has not yet been fully determined. Direct stimulation of proliferation of endothelial and smooth muscle cells *in vitro*⁽²⁹⁾ and increased survival of ischemic tissues⁽³⁰⁾ suggest a role in the mediation of tissue healing. Also, the morphological association of CGRP-IR fibers with the edges of healing pulpal lesion and with zones of reparative dentin⁽³¹⁾ suggests a role for these fibers and the neuropeptide CGRP in the healing response of pulpal tissue. Another investigation showed a concentration-dependent stimulation of cell proliferation of human pulpal cells.⁽³²⁾ More recently, dynamic changes in density of CGRP-IR fibers were induced by experimental tooth movement, indicating their involvement in both early stages of periodontal remodeling and later in the regenerative processes of the PDL, generally occurring in concerted action with modulation of blood vessels.^(4,33–35) New data showed CGRP expression in human dental pulp increases when teeth are submitted to severe orthodontic forces.⁽³⁶⁾ CGRP has also been shown to activate adenylate cyclase in different cells.^(18,37) Interaction of CGRP and its receptors is reported to stimulate a c-AMP-mediated pathway. Furthermore, new data indicate that CGRP rapidly activates the MAP kinase signaling pathway, an effect that consequently stimulates the proliferation of gingival fibroblasts.⁽³⁸⁾ Well-designed experimental studies must be implemented to reveal greater detail about the regulatory mechanisms of CGRP in dental and supported tissues.

We also observed clusters of small CGRP-IR cells localized in the base of the epithelial rete pegs in the gingiva, which also agrees with other study findings in cats,⁽²³⁾ humans,^(39,40) and ferrets.⁽⁴¹⁾ Because neither axotomy nor sympathectomy had any effect on their distribution or IR staining, these cells are most likely capable of producing CGRP themselves. Previous investigations^(39,42) have also suggested that the CGRP-IR cells observed in gingiva are Merkel cells, which may be part of a protective inflammatory defense reaction.⁽⁴³⁾ Furthermore, we found that some CGRP-IR nerve fibers were in close contact with these cells, but how these fibers interact with the cells remains unknown.

In conclusion, our study demonstrates that the rat dental tissues have an abundant sensory nerve supply. It is speculated that CGRPs, which are released from peripheral neurons in response to injury, not only contribute to the inflammatory response but also stimulate proliferation of surrounding connective tissue cells and initiate the early stages of healing in dental tissues. However, to confirm this hypothesis, further investigation of the regulatory mechanism of CGRP in peripheral tissues is needed.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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