

Expression of Pituitary Adenylate Cyclase-Activating Peptide (PACAP) and PAC1 in the Periodontal Ligament After Tooth Luxation

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Abstract Pituitary adenylate cyclase-activating peptide (PACAP) is widely distributed throughout the nervous system. PACAP not only acts as a neurotransmitter but also elicits a broad spectrum of biological action via the PACAP-specific receptor, PAC1. However, no studies have investigated PACAP and PAC1 in the periodontal ligament (PDL), so we aimed to perform this investigation in rats after tooth luxation. In the PDL of an intact first molar, there are few osteoclasts and osteoblasts. However, at days 3 and 5 after luxation, large PAC1-positive cells, thought to be osteoclasts because of their expression of the osteoclast marker, tartrate-resistant acid phosphatase, were detected in appreciable numbers. Osteoblast numbers increased dramatically on day 7 after luxation, and PAC1-positive mononuclear small cells were increased at day 14, many of which expressed the osteoblast marker, alkaline phosphatase. PACAP-positive nerve fibers were rarely detected in the PDL of intact first molars, but were increasingly evident at this site on days 5 and 7 after luxation. Double-immunofluorescence analysis demonstrated the relationship between PACAP-positive nerve fibers and PAC1-positive osteoclasts/blasts in the PDL. At 5 days after luxation, PACAP-positive nerve fibers appeared in close proximity to PAC1-positive osteoclasts. At 7 days after luxation, PACAP-positive nerve fibers appeared in close proximity to PAC1-positive osteoblasts. These results suggest that PACAP may have effects on osteoclasts and osteoblasts in the PDL after tooth luxation

and thus regulate bone remodeling after these types of injury.

Keywords PACAP · PAC1 · Osteoclast · Osteoblast · Luxation

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a vasoactive intestinal peptide (VIP)-like peptide originally isolated from ovine hypothalamus (Miyata et al. 1989) that exists mainly in a 38-amino acid form in most tissues (Harmar and Lutz 1994). PACAP is widely distributed in the central and peripheral nerve systems (Arimura 1992; Fahrenkrug 2001; Vaudry et al. 2000), and is known to stimulate adenylate cyclase in rat anterior pituitary cells (Arimura 1992). Although PACAP is classically viewed as a neurotransmitter, it is capable of eliciting a broad spectrum of other biological actions (Vaudry et al. 2000). Two VIP receptors, termed VPAC1 and VPAC2, are known to exhibit high affinity for both VIP and PACAP (Harmar et al. 1998), and a third receptor, PAC1, exhibits some specificity for PACAP, having only low affinity for VIP (Harmar et al. 1998). Many reports indicate that PACAP, mainly via PAC1, regulates neural cell proliferation, survival, axon regeneration and oligodendrocyte progenitor proliferation and maturation (Botia et al. 2007; Somogyvari-Vigh and Reglodi 2004; Waschek et al. 1998; Armstrong et al. 2008; Lee et al. 2001).

It has been reported that several neuropeptides exist in nerve fibers in the skeleton, particularly in areas with high metabolic activity such as the growth plate (Bjurholm et al. 1988; Strange-Vognsen et al. 1997). Neuropeptides also exist in the callus of fractures (Li et al. 2007). Some

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functional receptors for neuropeptides are expressed in both osteoclasts and osteoblasts, and regulate bone resorption and formation (Lerner and Persson 2008). Neuropeptides have also been found in many other tissues. Several studies have demonstrated the existence of neuropeptides such as CGRP, Substance P, galanin, and VIP in the periodontal ligament (PDL) of teeth (Nagayama et al. 2012; Kook et al. 2009; Deguchi et al. 2003; Kato et al. 1990). Furthermore, occlusal loading appears to induce VIP expression in osteoblastic layers of the PDL during alveolar bone remodeling (Barros et al. 2007), suggesting that the neuropeptides may act as biochemical bone regulators.

To understand the reaction of the PDL to traumatic injury, experimental rat models have been developed to analyze luxation injuries in the periodontal tissues around molar teeth (Miyashin et al. 1990, 1991). After luxation of rat molars, irregular periodontal fibers, degenerate cells, hemorrhage, and edema are detected in the PDL (Miyashin et al. 1990, 1991), and alveolar bone resorption occurs, after which the bone gradually repairs. These results suggest that luxation trauma results in active remodeling of the alveolar bone. However, the distribution and role of neuropeptides and their receptors in the PDL after tooth luxation has not been studied. This study therefore aimed to characterize the distribution of PACAP and PAC1 in the PDL of luxated rat teeth.

Materials and Methods

Animals

Six-week-old male Sprague–Dawley rats were purchased from CLEA Japan, Inc. (Tokyo, Japan) for use in this study. The protocols for all animal procedures were in accordance with Tohoku University regulations.

Tooth Luxation

For partial extraction of the upper first molar tooth, its distal edge was raised by 0.5 mm using an elevator with a curved tip (thickness = 1 mm, length = 3 mm) under general anesthesia (intraperitoneal administration of 40 mg/kg pentobarbital sodium).

Histological Observation

Unoperated and operated animals were re-anesthetized and transvascularly perfused with Zamboni fixative. The maxillae were dissected, and these samples were treated with 4.13 % ethylene diamine tetraacetic acid (EDTA) in 0.1 M phosphate buffer (pH 7.4) for 3 weeks at room temperature before soaking in phosphate-buffered 20 % sucrose

overnight. For PACAP immunohistochemistry, samples were frozen-sectioned into 40 μ m slices. For hematoxylin-eosin (HE) staining or PAC1 immunohistochemistry, samples were frozen-sectioned into 8 μ m slices.

For determination of PACAP expression, we performed the ABC (avidin–biotin–horseradish peroxidase complex) method. Sections were incubated with rabbit anti-PACAP antibodies (1:100,000; Peninsula Laboratories, San Carlos, CA, USA), biotinylated barbula anti-rabbit IgG and ABC reagent (Vector Laboratories, Burlingame, CA, USA). Following the diaminobenzidine reaction, sections were dehydrated in a graded series of alcohols, and cleared in xylene. For determination of PAC1, the sections were incubated with rabbit anti-PAC1 sera (1:750; Novus Biologicals, Littleton, CA, USA), then incubated in a mixture of Alexa-conjugated anti-rabbit IgG (1:100; Jackson ImmunoResearch Labs, Bar Harbor, ME, USA). Sections were stained for tartrate-resistant acid phosphatase (TRAP) activity, counterstained with hematoxylin for analysis of osteoclast formation, and further counterstained for alkaline phosphatase (ALP) activity (ALP staining kit; Muto Chemical Co., Tokyo, Japan) for analysis of osteoblast formation. For quantitative structure analysis of PACAP-positive nerve fibers in the PDL, the length of these fibers was measured in every third section of each tooth using Image J software (<http://rsbweb.nih.gov/ij/>). Osteoclasts, osteoblasts, and PAC1-positive cells were counted in every fourth section of each tooth.

For simultaneous localization of PACAP and PAC1, a double-immunofluorescence method was performed. Frozen sections were incubated for 24 h at room temperature with a mixture of rabbit anti-PAC1 (1:100; Novus Biologicals, San Carlos, CA, USA) and guinea pig anti-PACAP (1:500; Peninsula Laboratories, Littleton, CA, USA) antibodies. The frozen materials were incubated in a mixture of DyLight488-conjugated anti-guinea-pig IgG (1:100; Jackson ImmunoResearch Labs, Bar Harbor, ME, USA) and Alexa-conjugated anti-rabbit IgG (1:200; Jackson ImmunoResearch Labs, Bar Harbor, ME, USA). Immunofluorescent sections were studied with a laser scanning microscope (Nikon, Japan) at a slice thickness of 0.5 μ m.

Statistical Analysis

All data are expressed as mean \pm standard deviation (SD). Statistical analysis was performed using ANOVA with Bonferroni post-tests for multiple comparisons.

Results

PACAP-positive nerve fibers and PAC1-positive cells were detected in the PDL of intact teeth (Fig. 1a, b). On

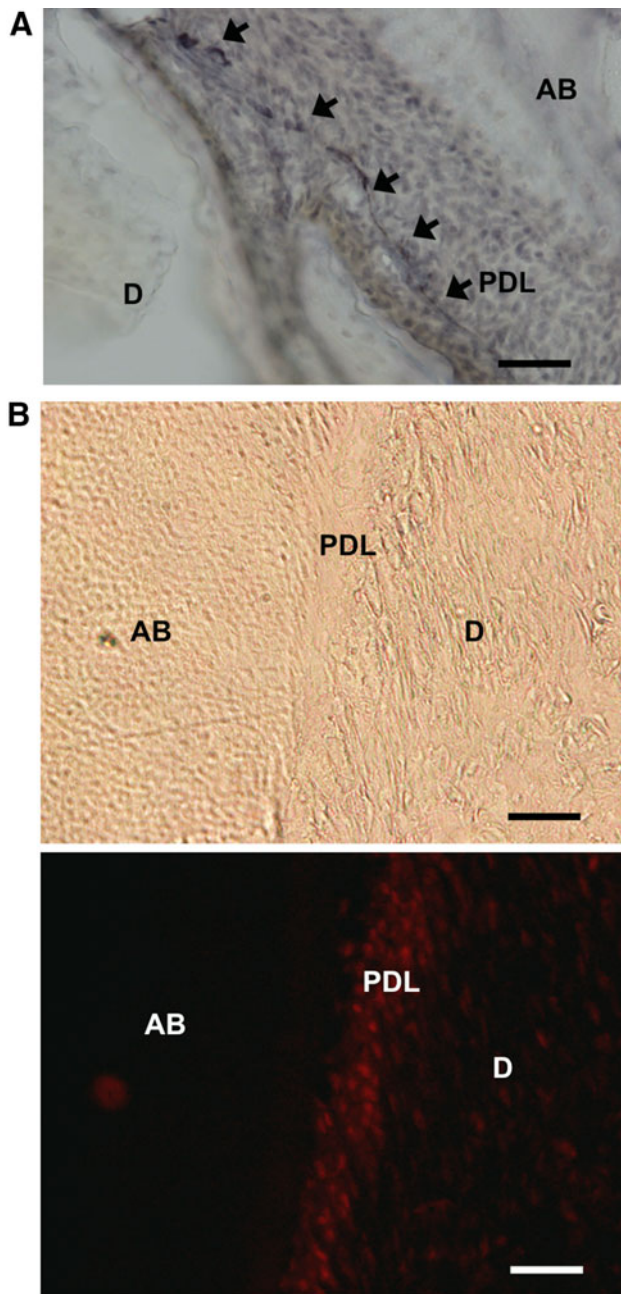


Fig. 1 PACAP-positive nerve fibers and PAC1-positive cells were detected in the PDL of intact teeth. Histological sections of PDL were obtained from intact rat first molar teeth. Sections were incubated with antibodies specific for PACAP and counterstained with hematoxylin (a) and PAC1 (b), followed by detection with ABC reagent and Alexa-conjugated secondary antibodies, respectively. PACAP-positive nerve fibers are indicated by arrows. AB alveolar bone, PDL periodontal ligament, D dentin. Scale bar 50 and 20 μm

performing quantitative morphometric analysis of periodontal PACAP-positive nerve fibers, however, we found that these were rarely detected in the periodontal ligament of intact first molars and day 0 (Fig. 2a, b, d), but that they were increased at days 5 and 7 after luxation (Fig. 2c, d).

We also performed analyses of bone remodeling cells. There were few osteoclasts or osteoblasts in the PDL of intact first molars (Fig. 3a), but osteoclasts (and resorption cavities) became evident at the alveolar bone surface at day 5 after luxation (Fig. 3b). Furthermore, many osteoblasts appeared on the alveolar bone surface at day 7 after luxation (Fig. 3c). Osteoclasts were stained red by TRAP staining (Fig. 3d). Osteoblasts were detected by ALP staining (Fig. 3e).

Morphologically, the PAC1-positive cells were divided into large and small types (Fig. 4a, c). The diameter of large PAC1-positive cells was 15.2–51.4 μm (mean \pm SD = $24.9 \pm 0.5 \mu\text{m}$). By contrast, small PAC1-positive cells were 6.3–16.1 μm (mean \pm SD = $10.3 \pm 0.2 \mu\text{m}$) in diameter and had oval cell bodies. Many of the large cells were positively stained for TRAP (Fig. 4b), whereas small cells were ALP-positive (Fig. 4d). On days 1, 3, and 5 after luxation, the numbers of PAC1-positive osteoclasts were increased (Fig. 4e). Similarly, PAC1-positive osteoblast numbers were increased on day 14 after luxation (Fig. 4f).

A double-immunofluorescence analysis demonstrated the relationship between PACAP-positive nerve fibers and PAC1-positive osteoclasts/blasts in the PDL. At 5 days after luxation, PACAP-positive nerve fibers appeared in close proximity to PAC1-positive osteoclasts (Fig. 5a–c), and at 7 days after luxation, PACAP-positive nerve fibers appeared in close proximity to PAC1-positive osteoblasts (Fig. 5d–f).

Discussion

The distribution of VIP-containing nerve fibers has been investigated using immunofluorescence, with results demonstrating VIP-containing nerve fibers in the PDL surrounding the roots of molar teeth (Kato et al. 1990). In this study, we have extended these findings to illustrate PACAP and PAC1 expression in the PDL, despite PACAP-positive nerve fibers being rarely detected in the PDL of intact first molars. We believe that this is the first time that PACAP and PAC1 expression in PDL has been documented.

PACAP-reactive cells are found in various tissues associated with the immune system, including lymph nodes, thymus, spleen, and bone marrow (Gaytan et al. 1994). PACAP increases cell survival in thymocytes (Tatsuno et al. 1991a; Delgado et al. 1996) and up-regulates interleukin-6 (IL-6) transcription and release through its interaction with PAC1 (Martinez et al. 1998). PACAP also affects lymphocyte maturation indirectly by stimulating the release of IL-6 (Tatsuno et al. 1991b), and also induces IL-6 production in astrocytes (Gottschall et al. 1994) and IL-10 expression in lymphocytes (Martinez et al. 1996). Thus, PACAP modulates immunological responses. The luxation

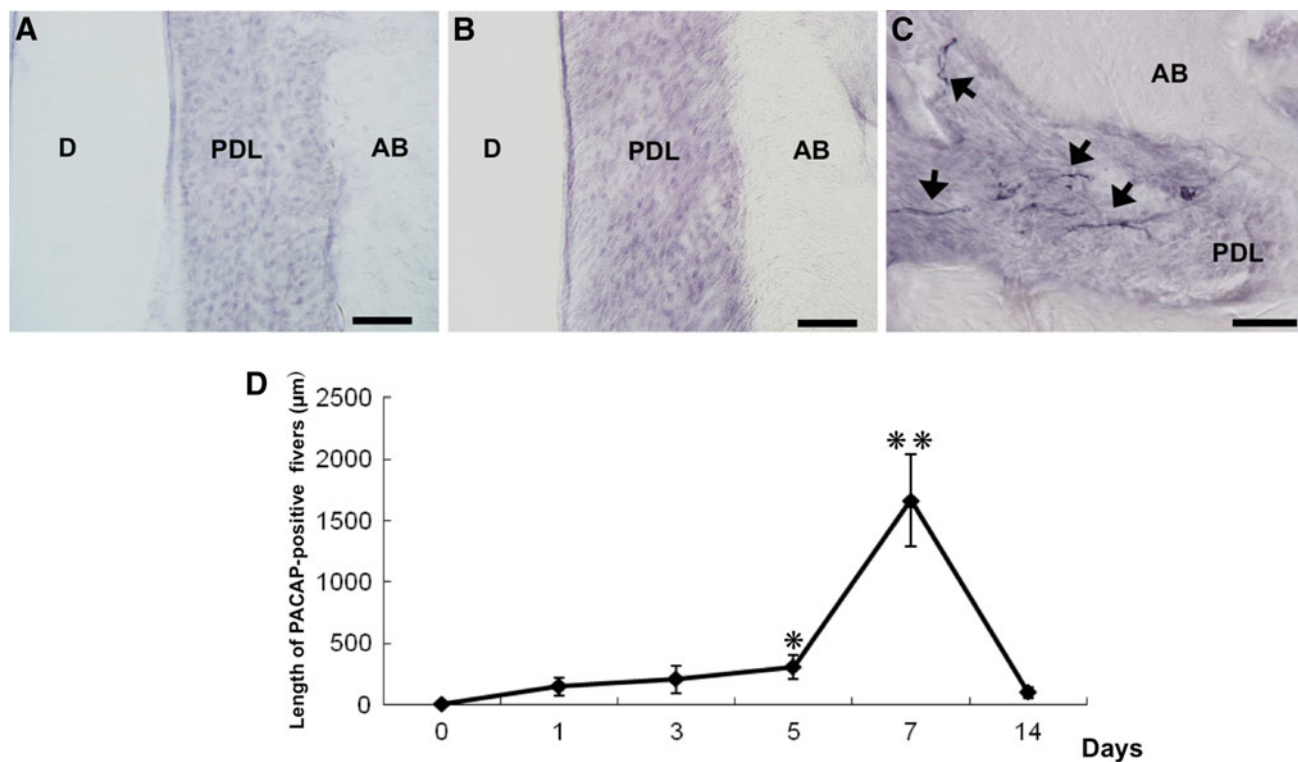


Fig. 2 PACAP-positive nerve fibers increased in the periodontal ligament after luxation. Histological sections of PDL from intact teeth (a), sectioned at day 0 (b) and at day 5 (c) after teeth luxation were labeled with antibodies specific for PACAP and counterstained with hematoxylin. Image d shows increased PACAP-positive nerve fibers

on days 5 and 7 after luxation (d). PACAP-positive nerve fibers are indicated by *arrows*. *AB* alveolar bone, *PDL* periodontal ligament, *D* dentin. Scale bar 50 μm. * $p < 0.05$, ** $p < 0.01$ by ANOVA and Bonferroni's multiple comparison test

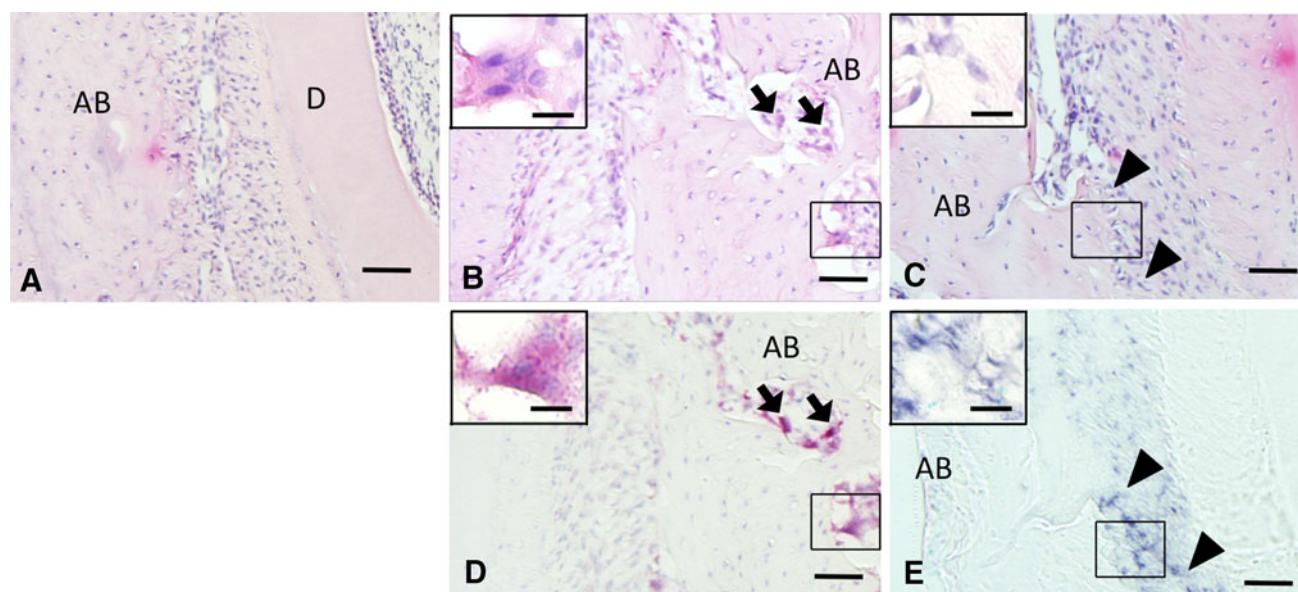


Fig. 3 Osteoclasts and osteoblasts appear at the surface of alveolar bone after luxation. Histological sections of PDL from teeth before (a) and 5 days (b) and 7 days (c) after luxation was stained with hematoxylin-eosin. TRAP staining was for the detection of osteoclast

(d). ALP staining was for the detection of osteoblast (e). Osteoclasts are indicated by *arrows* and osteoblasts are indicated by *arrowheads*. *AB* alveolar bone, *D* dentin. Scale bars 50 and 20 μm

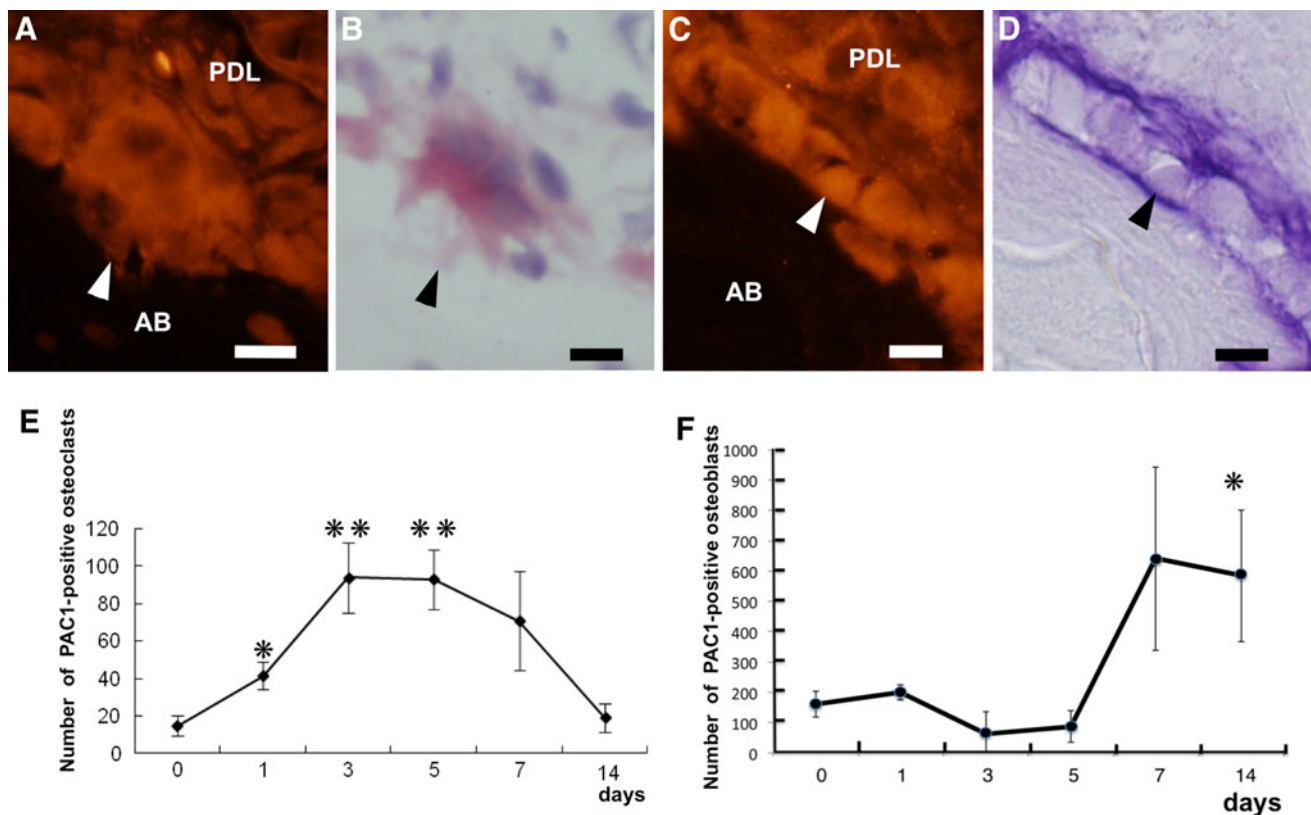


Fig. 4 Numbers of PAC1-positive osteoclasts and osteoblasts increased after luxation. Sections of PDL were immunohistologically stained with antibodies specific for PAC1. Images show the two characteristic PAC1-positive cell types: large (a), which stained positively for tartrate-resistant acid phosphatase (TRAP) (b), and

small (c), which stained positively for alkaline phosphatase (ALP) (d). Arrowheads indicated these cells. Images e and f show increases in PAC1-positive osteoclasts on days 1, 3, and 5 after luxation (e), and PAC1-positive osteoblasts on day 14 (f). * $p < 0.05$, ** $p < 0.01$ by ANOVA and Bonferroni's multiple comparison test. Scale bar 10 μ m

trauma in this study induces an immunological reaction in the PDL (Mackie 2003), and our results suggest that PACAP has some immunological role in the area of traumatic injury. We found that PACAP-positive nerve fibers increased in the periodontal ligament after luxation injury, which may act as the source of PACAP mediating this immunological response.

Indeed, PACAP release into an area of injury has been shown in a peripheral nerve injury model (Reimer et al. 1999), and PACAP is known to enhance neuron survival in the injured brain (Takei et al. 2000), and regulate axonal regeneration after facial nerve injury (Suarez et al. 2006). Furthermore, PACAP has growth factor-like actions on cells following injury (Vaudry et al. 2000; Waschek et al. 1998). Thus, PACAP appears to be important in preventing neuron damage following injury. We show here that although PACAP-positive nerve fibers were rarely detected in the periodontal ligament of intact first molars, their frequency increased after luxation injury. We propose that PACAP may protect and help to restore PDL neurons damaged by luxation.

Tooth luxation causes tissue damage and reactions in the PDL. The development of periodontal traumatic injury models in rats has been described previously (Miyashin et al. 1990, 1991), and these models reveal severe alveolar bone resorption after injury followed by gradual bone repair and remodeling. Bone resorption is controlled by osteoclasts differentiated from hematopoietic stem cells. Mature osteoclasts are multinuclear giant cells formed by fusion of mononuclear cells, and release hydrogen ions through the ruffled border that dissolve mineral (e.g., calcium ions) from the bone matrix. They also express cathepsin K and matrix metalloproteases, which digest organic components of the bone matrix (Teitelbaum 2000). Conversely, osteoblasts have important functions in creating bone and skeletal structure, and often become trapped in the bone matrix as osteocytes (Mackie 2003). We show here that many osteoclasts and resorption cavities appear at the alveolar bone surface after luxation, and then disappear, to be replaced by osteoblasts. These results demonstrate that a normal process of bone remodeling occurs after luxation (i.e., bone resorption by osteoclasts followed by bone formation by osteoblasts in the PDL).

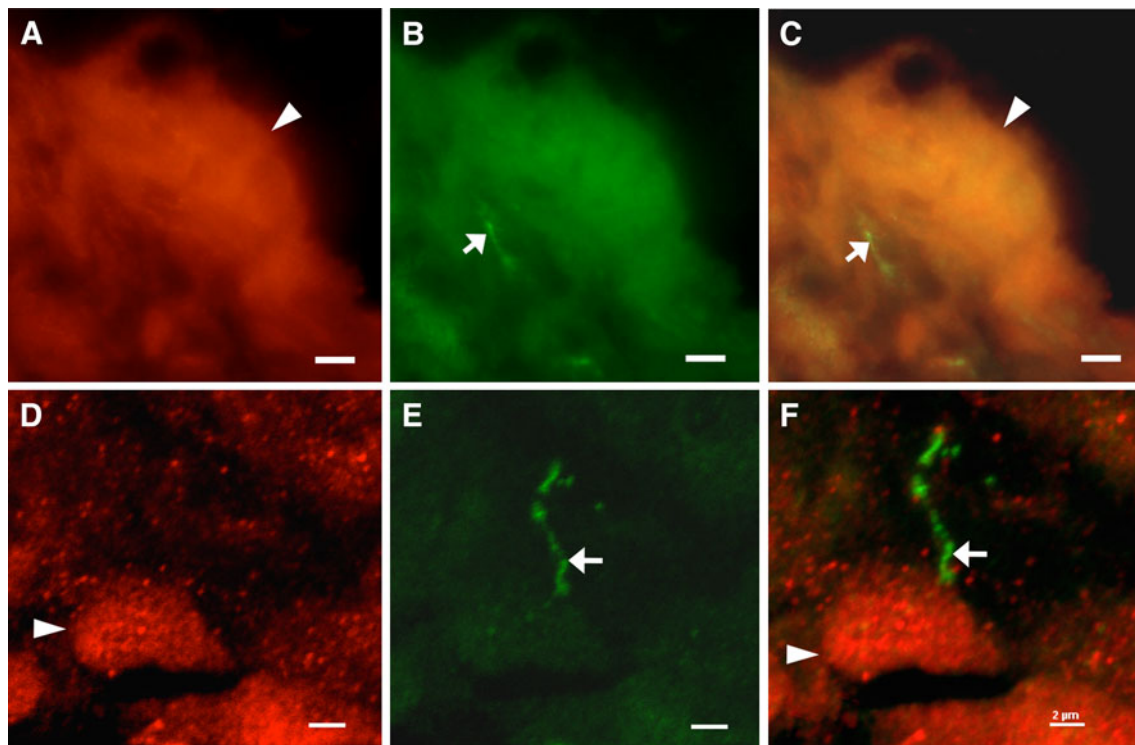


Fig. 5 Double-immunofluorescence analysis of the relationship between PACAP-positive nerve fibers and PAC1-positive osteoclasts/blasts in the PDL. Sections of PDL were immunohistologically stained with antibodies specific for PAC1 (**a** and **d**) and PACAP (**b** and **e**). Also shown are merged images of PAC1- and PACAP-

labeled cells (**c** and **f**). Labeling was performed at either 5 days (**a–c**) or 7 days (**d–f**) after luxation. PACAP-positive nerve fibers are indicated by *arrows*. PAC-1 positive osteoclasts/blasts are indicated by *arrowheads*. Scale bars 5 μm (**a–c**) and 2 μm (**d–f**)

It has been reported that skeletal tissue contains a network of nerve fibers expressing several neuropeptides, including CGRP, Substance P, galanin, VIP, and PACAP (Strange-Vognsen et al. 1997). VIP and PACAP regulate osteoclast formation and activity (Winding et al. 1997), and in vitro experiments have shown that VIP-1 receptors and PAC1 are expressed by osteoclasts in mouse bone marrow cultures and isolated osteoclasts (Ransjo et al. 2000). VPAC1 and VPAC2 mRNA have also been detected in osteoblasts (Nagata et al. 2009; Lundberg et al. 2001; Togari et al. 1997). It has been reported VIP stimulates ALP activity and calcium accumulation in mouse calvarial osteoblast (Lundberg et al. 1999). VIP enhanced formation of cAMP was demonstrated in osteoblastic cell line MC3T3-E1 (Bjurholm et al. 1992). PACAP also stimulated cAMP formation in intact bone, primary osteoblast and osteoblastic cell line (Lerner et al. 1994; Suzuki et al. 1994; Kovacs et al. 1996). In the present study, the number of large multinuclear PAC1-positive cells increased after luxation, and many stained positively for TRAP, suggesting that osteoclasts express the PACAP receptor, PAC1. Conversely, there are also many PAC1-positive, oval, mononuclear cells, suggesting that osteoblasts also express PAC1. Therefore, both osteoclasts and osteoblasts may be able to respond to

PACAP. We demonstrated the relationship between PACAP-positive nerve fibers and PAC1-positive osteoclasts/blasts in the PDL using double-immunofluorescence. The close proximity of PACAP-positive nerve fibers to PAC1-positive osteoclasts at 5 days after luxation, and to PAC1-positive osteoblasts at 7 days after luxation, suggest temporal coordination of PACAP regulation of PAC1-positive osteoclasts/blasts at the luxation site.

In this study, we found that luxation injury causes significant up-regulation of PACAP and its receptors in the PDL and its retinue of cells and neurons. Given that the PACAP-PAC1 axis is known to have roles in immunological reactions and bone remodeling, we propose that luxation injury in rat first molars precipitates a similar reaction in the PDL, with PACAP and PAC1 playing a central role in the repair and restoration of function of the periodontium by modulating and coordinating the activities of osteoclasts and osteoblasts. More studies will be necessary to further define the role and mechanism of action of PACAP in the PDL.

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Conflict of interest All authors declare that they have no conflict of interest.

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