doi:10.1111/j.1365-2591.2007.01250.x

Vasoactive intestinal peptide receptor expression in chronic periapical lesions

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Abstract

Caviedes-Bucheli J, Azuero-Holguín MM, Moreno GC, González IL, Mateu E, Salazar JF, Munoz HR. Vasoactive intestinal peptide receptor expression in chronic periapical lesions. *International Endodontic Journal*, **40**, 521–525, 2007.

Aim To use radioreceptor analysis for evaluating whether vasoactive intestinal peptide (VIP) receptors are present in chronic periapical lesions and to determine whether differences in its expression are found according to the size of the lesions.

Methodology Twelve periapical lesions were obtained from teeth diagnosed with chronic apical periodontitis and indicated for endodontic surgery; they were classified according to the size of the lesion in two groups of six samples (lesion size greater or smaller than 5 mm), and then processed and labelled with ^{125}I-VIP. Binding sites were identified by ^{125}I-VIP and standard VIP competition assays. Mann–Whitney's test was used to establish statistically significant differences in the VIP receptor expression between groups.

Results Vasoactive intestinal peptide receptor expression was found in all periapical lesion samples. There was a statistically significantly higher expression in periapical lesions <5 mm ($P < 0.001$).

Conclusion Vasoactive intestinal peptide receptors were expressed in chronic periapical lesions with levels inversely proportional to lesion size.

Keywords: neuropeptides, periapical lesion, vasoactive intestinal polypeptide, VIP-receptor.

Received 12 April 2006; accepted 14 December 2006

Introduction

Chronic periapical lesions develop as an inflammatory response to pulp tissue necrosis to prevent dissemination of bacteria and their toxins infection send towards the periapical tissues. Although a chronic periapical lesion is a radiographic finding, histologically they are classified as granulomas and periapical cysts (Marton & Kiss 1993, Rodini & Lara 2001, de Oliveira Rodini *et al.* 2004).

Periapical lesions, such as granulomas and cysts, are densely innervated (Leonardi *et al.* 2003), comprising an inflammatory exudate with abundant macrophages, lymphocytes, polymorphonuclear leukocytes and plasma cells (Marton & Kiss 1993). Nervous and immune systems become intimately related during periapical tissue inflammatory responses (Segura *et al.* 1996). These interactions are facilitated by anatomical connections and chemical mediators such as neuropeptides and cytokines released and recognized by both systems (Gozes *et al.* 1999).

Vasoactive intestinal peptide (VIP) has been associated with the development of chronic periapical lesions suggesting that it could participate in their growth and maturation (Azuero-Holguin *et al.* 2003). VIP is a parasympathetic neuropeptide consisting of 28 amino acids, distributed throughout the central and peripheral nervous systems (Gozes *et al.* 1999). This neuropeptide has a vasodilator effect and is expressed in healthy and inflamed human dental pulps (Caviedes-Bucheli *et al.*

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2006). The presence of VIP in periapical lesions has been reported with an inversely proportional relationship between lesion size and neuropeptide concentration (Azuero-Holguin *et al.* 2003). However, its role in the development in chronic periapical lesions remains unclear.

It has been demonstrated that immune system cells such as macrophages and lymphocytes express VIP receptors (Sakakibara *et al.* 1994, Delgado *et al.* 1999, Delgado & Ganea 2000). These cells are predominant in chronic periapical lesions, releasing inflammatory mediators and cytokines regulating the inflammatory process (Marton & Kiss 1993, Metzger 2000). Macrophages also play an important role in the development and perpetuation of chronic inflammatory reactions by activating humoral and cellular immunological responses (de Oliveira Rodini *et al.* 2004), which could also be regulated by VIP (Sakakibara *et al.* 1994, Delgado *et al.* 1999, Pozo *et al.* 2000).

As well as macrophages, T-CD4-and CD8-lymphocyte populations are also present in early and late stages of periapical lesions (Cymerman *et al.* 1984, Stashenko & Yu 1989, de Oliveira Rodini *et al.* 2004). The function of these cells can also be regulated by VIP (Metwali *et al.* 1996).

Given the actions of this neuropeptide over predominant immune cells, it is hypothesized that VIP participates in regulating periapical lesion growth. However, to determine whether VIP contributes in the development of chronic periapical lesions, the presence of VIP receptors in such lesions must be ascertained. Although the presence of VIP in periapical lesions has already been established, its participation in the growth or development of periapical pathology is not clear. This study is therefore aimed to establish VIP receptor expression in chronic periapical lesions and differences in its expression related to lesion size.

Materials and methods

A descriptive comparative study was performed according to the Colombian Ministry of Health recommendations regarding ethical issues in research with human tissues. Written informed consent was obtained from each patient in the study. Twelve samples of chronic periapical lesions were obtained from teeth with clinically necrotic pulps and radiographically diagnosed with chronic apical periodontitis. All of these teeth were indicated for endodontic surgery from different human adult donors (20–45 year old) of both sexes, nonsmokers and systemically healthy.

Digital periapical radiographs (Image Works; Digital Dental Systems, Olympia, WA, USA) were taken for every tooth using a standardized paralleling technique to allow calibration of the measurements taken and establishment of the longest diameter of the lesion. The periapical lesions were classified according to their size as being >5 mm or <5 mm in diameter.

Surgical sites were anaesthetized using 4% prilocaine by infiltration injection in the maxillary teeth and by inferior alveolar nerve block injection for mandibular teeth. Surgical flap and osteotomy were performed to gain access to the periapical lesion, which was obtained by enucleation. Samples were then fixed in 4% paraformaldehyde and stored at −70 °C until use.

After all the samples had been obtained, they were unfrozen at room temperature, before adding 200 μL of 4% sucrose solution in Tris–HCl buffer with protease inhibitors (0.1% EDTA, 0.1% PMFS) and 0.01% bovine serum albumin (BSA). Each sample was disaggregated in an ice bath at 0 °C using a tissue homogenizer (Wheaton Dounce, Milville, NJ, USA).

Samples were then centrifuged at 500 *g* for 5 min at 4 °C (IEC Centra GP6/GP6R; Thermo Electron Corporation, Waltham, MA, USA); obtaining precipitate P1 and supernatant S1. As much as 500 μL of 4% sucrose buffer was added to precipitate P1; the spin was repeated, obtaining precipitate P2 and supernatant S2. Another 500 μL of 4% sucrose buffer was added to precipitate P2 which was then subjected to 10 s pulses (10% intensity) using a Branson Digital Sonifier S-450D (Branson Ultrasonic Corporation, Danbury, CT, USA). Each sample was centrifuged (IEC Centra) at 500 *g*, obtaining precipitate P3 and supernatant S3. The mixture of supernatants S1, S2 and S3 was centrifuged at 51400 *g* for 3 h at 4 °C (RC5C Centrifuge; Sorvall, Wilmington, Delaware, USA). Moreover, 600 μL Tris–HCl buffer (0.05 mol L^{-1} and 7.5 pH) was added to the precipitate to obtain a suspension containing cell membrane fractions.

About 50 μL of suspension from each disaggregated sample was mixed with 150 μL detergent solution (5% CHAPS). This mixture was vigorously shaken in a vortex agitator (IKA Werke GmbH & Co, Staufen, Germany) to solubilize the proteins present in the cell membrane suspension. They were then centrifuged (Sorvall) at 29 300 *g* for 3 min at 4 °C. Twenty μL phosphate buffer saline (PBS) 7.4 pH and 100 μL reagent were added to an aliquot containing 80 μL supernatant for protein quantification (Micro-BCA Protein Assay Reagent Kit; Pierce, Rockford, IL, USA). After homogenization, the mixture was

incubated at 37 °C for 2 h. Aliquot absorbency for each sample was read on a spectrophotometer (MultiSkan; Thermo Electron Corporation) at 570 nm. Each sample's protein concentration was calculated by interpolating the absorbency read on a standard calibration curve (absorbency cf. BSA concentration) previously done in the same conditions as the assay. Determinations were done in duplicate.

Radioreceptor assay

Standard VIP and ^{125}I-VIP were obtained from Phoenix Peptide Pharmaceutical laboratories (Ref. 061-05 and T061-10; Belmont, CA, USA). Standard VIP was reconstituted with distilled water and serially diluted with HSA/Tris–HCl buffer solution to obtain different reagent concentrations. ^{125}I-VIP was reconstituted with distilled water and diluted until there were 10 000 counts per minute (cpm) in 100 μL reagent.

Each cell membrane suspension sample was submitted to competition assays with ^{125}I-VIP in the absence (total binding) or presence (nonspecific binding) of standard VIP and left incubated for 12 h at 4 °C. The reaction mixture was then passed through a 60 : 40 dibutilphthalate-dioctylphthalate cushion (d = 1.015 g mL^{-1}) to separate bound from free VIP (Suarez *et al.* 2001). After centrifuging (Sorvall) at 28 400 *g* for 3 min, peptide bound to the cells was quantified by measuring the pellet radioactivity on a Gamma counter (Gamma Assay LS 5500; Beckman, Fullerton, CA, USA).

Free peptide (F) was quantified by measuring the difference between added cpm and bound peptide at each point. The maximum quantity of bound peptide was extrapolated from the Rosenthal graph: B/F cf. B.

Statistical analysis

The values obtained are expressed in pmol of bound peptide (VIP-receptor) per 100 μg tissue protein. The mean and standard deviation were calculated for each group. The Mann–Whitney's U test was performed to establish significant differences between the values obtained for both groups ($P < 0.05$). Pearson correlation was also calculated to determine whether there was a correlation between apical lesion size and VIP receptor expression.

Results

The average lesion diameter for each group was 4.5 ± 0.24 mm for periapical lesions <5 mm and 8.24 ± 1.47 mm for those >5 mm. VIP receptor expression was found in all chronic periapical lesions. Mean receptor expression for the group of periapical lesions <5 mm was 12.96 ± 3.26 pmol/100 μg protein. Mean expression for the group of lesions >5 mm diameter was 4.79 ± 1.19 pmol/100 μg protein (Table 1). There was 2.7 times more VIP receptor expression in lesions <5 mm than in the larger ones.

Mann–Whitney's U test returned $P < 0.001$, confirming that the difference in VIP receptor expression was significantly greater in chronic periapical lesions <5 mm compared with those >5 mm.

Pearson correlation also revealed a −0.76 inverse relationship which is significant at 0.01 level, confirming the existence of an inverse correlation between apical lesion size and VIP receptor expression (Fig. 1).

Discussion

It has been hypothesized that VIP could have an important role in regulating the inflammatory phenomenon in the development of periapical lesions via cellular receptors. Therefore, the purpose of this study was to establish VIP receptor expression in chronic periapical lesions and differences in its expression related to lesion size.

Vasoactive Intestinal Peptide expression in chronic periapical lesion has been established before, being inversely proportional to lesion size, using a 5 mm diameter as a cut-off point to classify lesions as smaller than and greater than 5 mm (Azuero-Holguin *et al.* 2003). However, to validate VIP participation in growth and maturation of periapical lesions, the appropriate receptor should be found in the tissue.

Table 1 Vasoactive intestinal peptide (VIP) receptor expression in chronic periapical lesions according to size

Type of lesion	*n*	Mean lesion size (mm)	Mean VIP-receptor expression*	SD	Minimum	Maximum
<5 mm diameter	6	4.50	12.96	3.26	10.06	18.91
>5 mm diameter	6	8.24	4.79	1.19	3.43	6.84

*Values are given in pmol of bound VIP per 100 μg of tissue protein.
Differences between groups are statistically significant ($P < 0.001$).

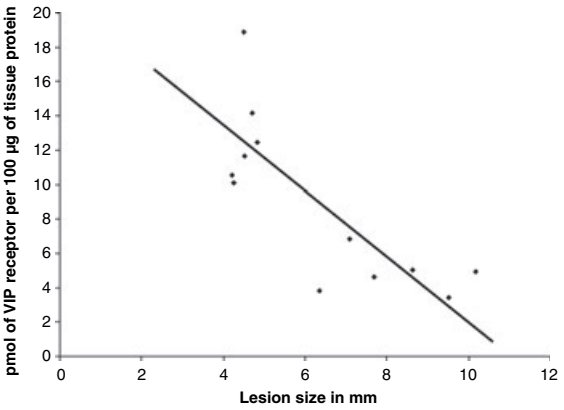

Figure 1 Correlation between periapical lesion size and vasoactive intestinal peptide (VIP) receptor expression.

Therefore, the same parameter for lesion sizes was used in the present study to allow a better correlation between results, showing also an inversely proportional expression of VIP receptors to lesion size, supporting the hypothesis that VIP may play a role in lesion development and enlargement.

It is widely accepted that periapical lesions result from continuous antigenic stimulation from infected root canals combined with the toxic effects of microbial products and the immune response of the host (Torabinejad *et al.* 1985, Nair 1998). *Ex vivo* and *in vivo* antigenic cell stimulation have shown to be effective in inducing VIP release. Due to its short half-life in extracellular environment, VIP rapidly activates immune system cells via receptors on their cell membrane, playing an important role as endogenous modulator of immune response (Delgado *et al.* 1999). It has been shown that VIP is a complex pluripotential neuropeptide and its action depends on type, differentiation stage and the state of cell activity. It is also known that VIP exerts its functions in a concentration-dependent manner (Delgado & Ganea 2000).

Macrophages regulate inflammatory response in the presence of bacteria in infected root canals (Marton & Kiss 1993, Lin *et al.* 2000, Rodini & Lara 2001). It has been stated that VIP exerts an effect on macrophages blocking the production of TNF-α, interleukin 6 and 12 when they are stimulated by lipopolysaccharides, consequently reducing inflammation (Delgado *et al.* 1999, Pozo *et al.* 2000).

As well as macrophages, T-CD4- and CD8-lymphocytes populations are also present in periapical lesions (Cymerman *et al.* 1984, Stashenko & Yu 1989). Previous studies have shown that there are more CD4 lymphocytes in relation to CD8 during the initial stage of a periapical lesion and that this relationship becomes inverted during later stages when lesions become stable (Stashenko & Yu 1989, Sakakibara *et al.* 1994, Lara-Marquez *et al.* 2001). This could explain why there is a significantly higher expression of VIP and its receptor on smaller periapical lesions, as it has also been demonstrated that VIP participates in T-lymphocyte apoptosis, protecting CD4 and stimulating CD8 apoptosis (Zheng *et al.* 1995).

On the basis of the results from the present study, it could be suggested that in small periapical lesions (<5 mm diameter), VIP provides greater control and modulation over the growth process due to its influence via receptors on different cell types. However, further studies are needed to ascertain this. Future research should be carried out to determine VIP receptor localization by immunohistochemistry to evaluate the role and importance of VIP and its receptor in periapical pathology.

Conclusion

Vasoactive intestinal peptide receptors are expressed in chronic periapical lesions; its levels are inversely proportional to lesion size.

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