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Localization of substance P-induced upregulated interleukin-8 expression in human dental pulp explants

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Abstract

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Aim To localize *ex vivo* expression of interleukin-8 (IL-8) induced by substance P (SP) in human dental pulps.

Methodology Intact caries-free, freshly extracted third molars (n = 20) were collected from patients (15–25 years old). The teeth were split and pulpal tissue was obtained and stored in Dulbecco's modified Eagle medium. Human dental pulp tissue explants were stimulated with SP. Expression of IL-8 in pulp explants was detected and localized by immunohistochemistry.

Results Moderated IL-8 immunoreactivities were detected mainly in the cell-rich zone in pulp tissues 12 h after tumour necrosis factor alpha (TNF-α) stimulation (positive controls), whereas only weak IL-8 expression was observed in tissues stimulated with SP at the same time interval. These data did not differ from those in negative controls. Increased IL-8 expression in pulp explants after 24 h of SP stimulation was noted compared with negative controls and located in fibroblast-like cells, blood vessel-associated cells and extracellular matrix in the central zone and cell-rich zone of pulp explants. Tissues stimulated with TNF-α for 24 h (positive controls) revealed weak IL-8 immunoreactivities with altered cell morphology.

Conclusions Substance P induces IL-8 expression and was located in fibroblast-like pulp cells, blood vessel-associated cells and extracellular matrix of human dental explants. These data support the hypothesis that neuropeptide (SP) coordinates the modulation of pulpal inflammation via up-regulating chemokine IL-8.

Keywords: dental pulp, immunohistochemistry, interleukin-8, substance P, tumour necrosis factor-α.

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Introduction

Neurogenic inflammation is an important feature of pulpal inflammation. Dental pulp tissue is richly innervated by sensory nerve fibres, and it responds to external stimuli such as microbial infection and mechanical or chemical irritation during dental procedures (Jontell *et al.* 1998, Stashenko *et al.* 1998, Byers & Narhi 1999). The influx of leucocytes from the blood stream into the affected tissue compartment is an early sign of a pulpal response to injury (Bergenholtz & Lindhe 1975, Stanley 1977). The release of neuropeptides from the neural components of the pulp leads to increased blood flow and dilatation of blood vessels that

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facilitate the influx of leucocytes into the tissue and the formation of local inflammation; this phenomenon is referred to as neurogenic inflammation. The process of leucocyte migration is highly regulated and involves many factors including chemokines such as interleukin-8 (IL-8), which plays a key role in attracting neutrophils to the site.

Interleukin-8, a potent neutrophil chemoattractant, is frequently expressed in the endothelial cells of inflamed pulps but only rarely expressed in the endothelial cells of normal pulps. This suggests the key regulatory role of IL-8 expressed by endothelial cells in controlling the influx of leucocytes into the pulp tissue.

Various neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP) and neurokinin A are produced in the dental pulp and contribute significantly to the process of inflammation and the transmission of pain sensation (Wakisaka 1990). The vasodilatation caused by the release of these neuropeptides (Heyeraas *et al.* 1994) may increase pulpal blood flow and promote the transendothelial migration of local inflammatory cells (Fristad *et al.* 1997).

There is evidence that neuropeptides stimulate the production of proinflammatory cytokines by various cell types. SP was found to bind specifically to human corneal epithelial cells and to induce IL-8 synthesis, but did not stimulate the synthesis of MCP-1 or RANTES (Tran *et al.* 2000). SP and CGRP upregulated IL-8RA mRNA in an immortalized keratinocyte cell line, but had no influence on IL-8 production (Kiss *et al.* 1999). In addition, CGRP increased IL-6 and IL-8 secretion in primary cultures of the synovial fibroblasts of rheumatoid arthritis patients, but not of osteoarthritis patients (Raap *et al.* 2000). Human dental pulp fibroblasts from orthodontic patients were induced by SP and CGRP to increase the levels of IL-1β, IL-6 and tumour necrosis factor alpha (TNF-α) (Yamaguchi *et al.* 2004), whilst SP and CGRP increased IL-1β and TNF-α production by HSV-infected mouse peritoneal macrophages (Yaraee *et al.* 2003). Similarly, CGRP, NPY, SP and VIP stimulated the production of proinflammatory cytokines and chemokines such as IL-1β, IL-6 and TNF-α by peripheral whole blood cells from patients with rheumatoid arthritis (Hernanz *et al.* 2003), and SP and CGRP increased the production of IL-1β, IL-6 and TNF-α from cultured lymphocyte-enriched mononuclear cells isolated from human peripheral blood (Cuesta *et al.* 2002).

It has been demonstrated previously that in pulp tissues or cultured pulp cells, SP, but not CGRP, increased the production of IL-8 (Patel *et al.* 2003, Park *et al.* 2004). However, technical difficulties prevented accurate localization of the IL-8 in the pulp tissue: the pulp tissue enclosed by mineralized hard tissue (dentine) was too soft to be fixed without damage, and it was difficult to examine the dentine–pulp complexes *in vivo*. Therefore, in the present study, an immunohistochemical approach was employed to locate IL-8 protein *in situ* in SP-stimulated *ex vivo* pulp explants.

Materials and methods

Sample collection

Intact caries-free, freshly extracted third molars ($n = 20$) were collected from the patients (15–25 years old) in the Department of Oromaxillofacial Surgery, Kyung Hee Dental Hospital as described previously (Huang *et al.* 1999a, Patel *et al.* 2003, Park *et al.* 2004). Tooth collection from human subjects followed a protocol approved by the Kyung Hee Dental Institutional Review Board. Immediately after extraction, teeth were stored in phosphate-buffered saline (PBS) and transferred to the laboratory. Each tooth was grooved longitudinally under water coolant with a fissure bur at high speed. The tooth was then split with a screw driver, and the entire pulp (coronal and radicular) was lifted up with cotton pliers so that maximal pulpal tissue was obtained. Some samples were divided into several smaller fragments, washed thrice with PBS and stored in Dulbecco's modified Eagle's medium (DMEM; Life Technologies/Gibco BRL, Gaithersburg, MD, USA) with 10% foetal bovine serum (FBS) before stimulation. Tissue culture media were supplemented with 100 units mL^{-1} penicillin-G, 100 μg mL^{-1} streptomycin and 0.25 μg mL^{-1} fungizone (Gemini Bio-Products, Inc., Woodland, CA, USA).

Stimulation of cultured pulp explants with neuropeptides

Synthetic human SP was obtained from Sigma (St Louis, MO, USA) and dissolved in sterile H_2O with 0.1% low-endotoxin bovine serum albumin (BSA; Sigma). Alpha minimal essential medium containing L-glutamine (Life Technologies) with 1% FBS was used for tissue culture 24 h before stimulation with neuropeptides. This low serum medium was used to minimize elevation of the base-line level of chemokine secretion by serum. Recombinant human (rh) TNF-α, purchased from R&D Systems (Minneapolis, MN, USA), served as a positive control for the induction of IL-8. As a negative

control (mock stimulation), we used concentrations of low-endotoxin BSA equal to those of the neuropeptides in the experimental treatments. SP (10^{-4} mL^{-1}) and TNF-α (final concentration 20 ng mL^{-1}) in 200 μL of tissue culture growth medium were added to the wells and incubation continued for 12 or 24 h at 37 °C, 5% CO_2. After incubation the samples were immediately fixed in 4% paraformaldehyde in PBS (pH 8.0) for 1–3 h at 4 °C, and the fixed tissues were left in 30% sucrose overnight at 4 °C to decrease freezing artefacts. The fixed tissues were then embedded in optimum cutting temperature compound (Tissue-Tek; Miles Laboratory, Elkart, IN, USA), snap-frozen in liquid nitrogen, and stored at −70 °C prior to cryostat sectioning.

Immunohistochemistry

Pulp explants were processed as frozen sections for standard immunohistochemistry to locate the IL-8 using the HRP-Envision system (Dako Corp., Carpentaria, CA, USA,). Cryostat sections (5–8 μm) were mounted on Vectabond pre-coated slides (Vector Laboratories, Burlingame, CA, USA), and immunoperoxidase staining was performed using the HRP-Envision system (Dako Corp.). The sections were dried at room temperature for 12–24 h before staining and washed thrice (for 5 min each) with wash buffer (Dako Corp.) containing 50 mmol L^{-1} Tris–HCl, 150 mmol L^{-1} NaCl 0.05% Tween 20 and a preservative, and incubated in 3% H_2O_2 at room temperature for 5 min to block endogenous peroxidase. The sections were repeatedly washed and incubated at 4 °C overnight with specific rabbit antihuman IL-8 polyclonal antibody (Endogen, Woburn, MA, USA) that had been diluted 1 : 10 in wash buffer. Negative control experiments were performed for each sample by replacing the primary antibody with normal nonimmune rabbit serum. Finally, the sections were incubated with peroxidase-labelled polymer, HRP, at room temperature for 30 min.

After washing, sites of peroxidase activity were visualized by incubation in 3,3′-diaminobenzidine solution for 10 min. Following a further wash, the sections were counterstained with Lillie's modified Mayer's haematoxylin (Dako Corp.), rinsed in H_2O for 5 min and covered with glass cover slips with an aqueous mounting solution (Dako Corp.).

Results

A monospecific IL-8 antiserum was used to determine whether IL-8 protein increased in the pulp tissue explants after *ex vivo* treatment with 10^{-4} mol L^{-1} SP or 20 ng mL^{-1} TNF-α for 12 and 24 h. As shown in Fig. 1(a), relatively weak IL-8 immunoreactivity (brown-coloured precipitates) was observed along the periphery of the pulp tissue in the 12 h mock stimulation (negative control), with little or no IL-8 staining in the central pulp zone. The results of treatment with 10^{-4} mol L^{-1} SP for 12 h were very similar to the control findings (Fig. 1c). The distribution of IL-8 expression in the pulp was similar to that in the mock group (negative control). In addition to staining in the periphery of the tissue, there were some immunoreactivities in the central region of the pulp (Fig. 1c). TNF-α, a strong IL-8 agonist was used as a positive control (Fig. 1e,f) and observed strong IL-8 immunoreactivity in the cell-rich zone of the pulp explants after treatment with this agent for 12 h (Fig. 1f).

The 24 h treatment with SP (10^{-4} mol L^{-1}) led to IL-8 expression in specific areas of the explant tissues (Fig. 2c) with variable staining. Strong staining was seen in some fibroblast-like cells, in blood vessel-associated cells and in the extracellular matrix (Fig. 2c). In the negative control, there was only weak IL-8 staining in the blood vessels of the explanted pulp tissues (Fig. 2a). In contrast to the results for 12 h stimulation with TNF-α, only weak IL-8 staining mainly in the peripheral area of the pulp explants was observed after 24 h of stimulation with TNF-α. In addition, individual cells appeared abnormal and enlarged, and the structure of the pulp tissue was disorganized (Fig. 2e).

Discussion

It has been reported previously that SP induced cultured pulp cells and pulp explants to produce IL-8 (Patel *et al.* 2003, Park *et al.* 2004). The aim of the present study was to locate the IL-8 by *ex vivo* immunohistochemical analysis. The present findings are in agreement with those reported previously; in addition, it was shown that IL-8 was expressed in the cell-rich zone of the pulp tissue and in cells associated with blood vessels. These data support the view that induction of IL-8 by SP in pulp cells occurs *in vivo*.

The sensory nerve fibres that contain SP and CGRP in dental pulps extend from the plexus of Raschkow to the subodontoblast and odontoblast layers (Wakisaka 1990). External irritation may stimulate a sensory response and cause local release of the two neuropeptides. It is likely that when pulp explants are stimulated with SP *ex vivo*, the NK1 receptor on the fibroblasts of

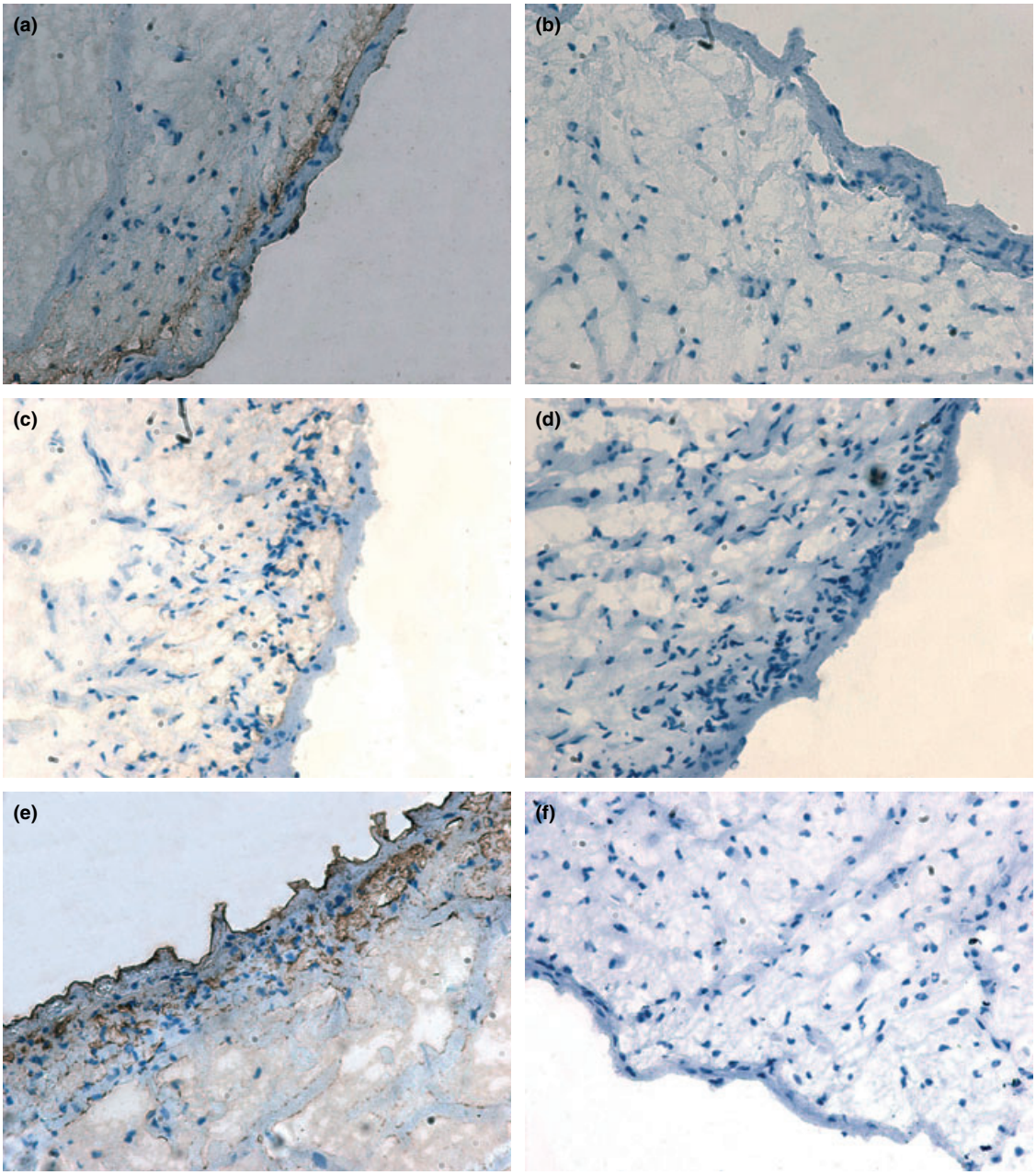

Figure 1 Interleukin-8 (IL-8) immunohistochemical analysis of frozen pulp explant 12 h after mock (a, b), substance P (SP) (10^{-4} mol L^{-1}) (c, d), tumour necrosis factor alpha (TNF-α) (20 ng mL^{-1}) stimulation (e, f). (a) Mock-stimulated pulp explant tissue exhibits IL-8 immunoreactivity only along the border of the tissue (original magnification ×200). Two other pulp explants undergoing the same treatment demonstrated similar results. (b) Nonimmunized normal rabbit serum of the same mock-stimulated sample shows complete negative staining (original magnification ×200). (c) SP (10^{-4} mol L^{-1})-stimulated pulp explant tissue exhibits IL-8 immunoreactivity only along the border of the tissue found in all four samples examined. (d) Nonimmunized normal rabbit serum of the same SP (10^{-4} mol L^{-1})-stimulated sample shows complete negative staining (original magnification ×200). (e) TNF-α (20 ng mL^{-1})-stimulated pulp explant tissue exhibits strong IL-8 immunoreactivity in the cell-rich zone of the tissue which was also observed consistently in two other samples (original magnification ×200). (f) Nonimmunized normal rabbit serum of the same TNF-α (20 ng mL^{-1})-stimulated sample shows complete negative staining (original magnification ×200).

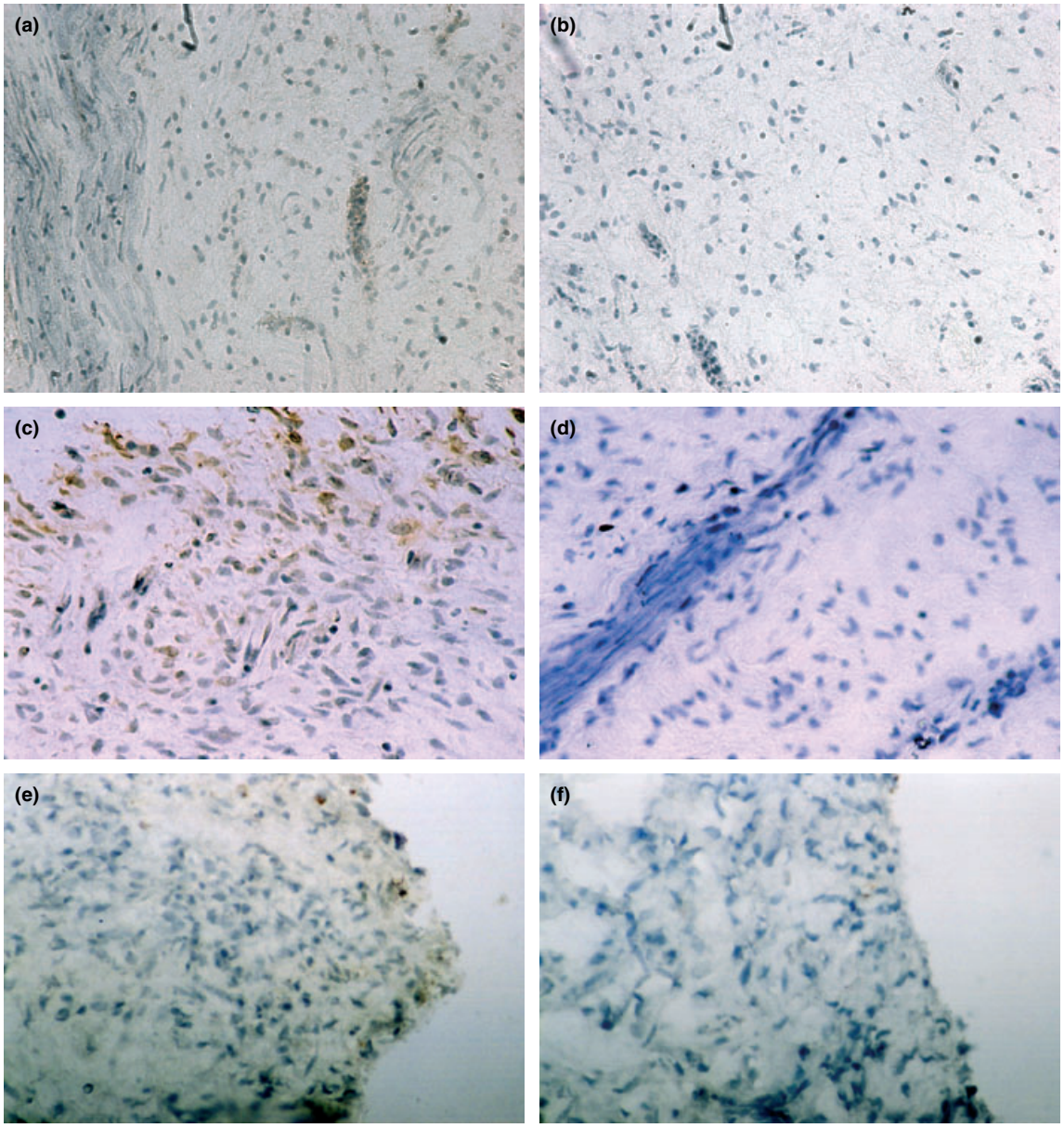

Figure 2 Interleukin-8 (IL-8) immunohistochemical analysis of frozen pulp explant 24 h after mock (a, b), substance P (SP) (10^{-4} mol L^{-1}) (c, d), tumour necrosis factor alpha (TNF-α) (20 ng mL^{-1}) stimulation (e, f) stimulation. (a) Mock-stimulated pulp explant tissue exhibits weak IL-8 immunoreactivity around the blood vessels in the tissue (original magnification ×200). (b) Nonimmunized normal rabbit serum of the same mock-stimulated sample shows complete negative staining (original magnification ×200). (c) SP (10^{-4} mol L^{-1})-stimulated pulp explant tissue exhibits strong IL-8 immunoreactivity in and around the fibroblast-like cells (original magnification ×200). (d) Nonimmunized normal rabbit serum of the same SP (10^{-4} mol L^{-1})-stimulated sample shows complete negative staining (original magnification ×200). (e) TNF-α (20 ng mL^{-1})-stimulated pulp explant tissue exhibits weak IL-8 immunoreactivity only along the border of the tissue in all the three samples tested (original magnification ×200). (f) Nonimmunized normal rabbit serum of same TNF-α (20 ng mL^{-1})-stimulated sample shows complete negative staining (original magnification ×200).

the cell-rich zone and in cells associated with blood vessels in the pulp proper are induced to produce proinflammatory cytokines.

The weak IL-8 staining in the mock treatments could be because of some artefact of the present culture conditions or could be because of the presence of some IL-8 in normal nondiseased pulps (Huang *et al.* 1999a,b). A definite increase of IL-8 staining in the pulp explants was not observed at 12 h, but only at 24 h after SP stimulation. This was different from previous observation (by ELISA) of increased IL-8 production in the supernatants of homogenized pulp explants 36 or 48 h after SP stimulation (Park *et al.* 2004). As previously suggested, it is possible that the diffusion of SP into the pulp tissue required more time so that it took longer to observe increased IL-8 levels in pulp explants than in cultured pulp cells (4–8 h) (Patel *et al.* 2003). Technical difficulties were attempted to simulate exactly the same condition *in situ*, where the expression of SP is mostly restricted to certain areas such as those reached by nerve fibres. In the present study, the entire pulp explants were stimulated with SP, which may probably differ from the *in situ* situation. Nevertheless, based on the current and previous studies (Park *et al.* 2004) using pulp explants and primary pulp cells, respectively, it appears that fibroblast-like cells of pulp tissue are stimulated to up-regulate IL-8 expression by SP, which is released from nerve fibres innervated on the pulp tissue *in situ*.

The finding of IL-8 expression in blood vessel-associated cells (most likely endothelial cells) supports previous observation that SP induced IL-8 expression in cultured endothelial cells (Patel *et al.* 2003).

The pulp cells and extracellular matrix of explants appeared to become disorganized after 24 h of TNF-α stimulation. Others have shown that the TNF-α promotes extracellular matrix remodelling and induces metalloproteinase expression in various tissues (Serandour *et al.* 2005, Little *et al.* 2005, Klooster & Bernier 2005) including dental pulps (Panagakos *et al.* 1996, O'Boskey & Panagakos 1998, Lin *et al.* 2001, Ueda & Matsushima 2001). This may explain why it was not permeated to detect an increase of IL-8 by ELISA in the supernatants of homogenized pulp explants following TNF-α treatment. Preliminary immunohistochemical examination of pulp samples after a longer incubation time (36 or 48 h) with SP and TNF-α revealed that most of the cells in the explant tissues had disappeared and the remaining cells were fragmented (data not shown).

It has been reported that CGRP and SP induced the production of IL-1β, IL-6 and TNF-α in human dental pulp cells in the samples removed from patients during the course of orthodontic treatment (Yamaguchi *et al.* 2004), but our previous data had demonstrated that CGRP did not increase IL-8 in healthy human pulp cells. The released TNF-α may induce IL-8 production in pulp cells in an autocrine or paracrine manner, although such an effect may only occur later as sufficient TNF-α release may not take place until 3–6 h after stimulation with SP and CGRP (Yamaguchi *et al.* 2004). Moreover, it was noted that CGRP did not significantly up-regulate IL-8, suggesting that any autocrine or paracrine effect is minimal (Patel *et al.* 2003).

Furthermore, cultured pulp cells themselves may produce neuropeptides in response to stimulation with bacterial components (Tokuda *et al.* 2004, Tancharoen *et al.* 2005). These workers showed that pulp cells may release SP and CGRP in response to arginine-specific cysteine protease from *Porphyromonas gingivalis*, and demonstrated that *Prevotella intermedia* lipopolysaccharide induced expression of SP and SP-receptor mRNAs in cultured pulp cells, pointing to another possible autocrine or parancrine effect in pulps that may result in the up-regulation of cytokine production. In addition to cytokine production by pulp cells resulting from neuropeptide stimulation, earlier studies revealed an effect of cytokines on the synthesis of neuropeptides in ganglia and spinal cord (Shadiack *et al.* 1993, Ding *et al.* 1995, Malcangio *et al.* 1996, Csillag *et al.* 2004), suggesting potential cross-regulation between neuropeptides and proinflammatory cytokines in inflamed tissues such as pulps, and emphasizing the importance of neurogenic inflammation in host defence against external insults.

Conclusion

In the present study, *in situ* IL-8 formation was revealed in pulp tissue. The data underscore the potential importance of SP in neurogenic inflammation of pulp tissues as an inducer of IL-8 production by pulp cells in the cell-rich zone and in endothelial cells. One role of the IL-8 may be to attract leucocytes. Investigation of the expression of other mediators in the pulp in response to additional neuropeptides is needed.

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