Role of substance P and calcitonin gene-related peptide in the regulation of interleukin-8 and monocyte chemotactic protein-1 expression in human dental pulp

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Abstract

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Aim To determine whether leucocyte infiltration during neurogenic inflammation in the pulp is regulated by neuropeptides via inducing the release of proinflammatory chemokines interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) from human dental pulp.

Methodology Cultured primary pulp cells and pulp tissue explants were stimulated with substance P (SP) and/or calcitonin gene-related peptide (CGRP). IL-8 or MCP-1, secreted from cultured cells or produced in pulp explants, was analysed by enzyme-linked immunosorbent assay.

Results Substance P induced IL-8 secretion from cultured pulp cells (approximately threefold increase over control, $P < 0.05$) and from pulp tissue explants (two- to three fold). SP only minimally to moderately induced MCP-1 (approximately two fold) in cultured pulp cells. While MCP-1 induction in cultured pulp cells was detected after 24 h of SP stimulation, no induction was observed in pulp tissue. CGRP did not induce IL-8, but moderately increased MCP-1 production (approximately three fold) in cultured pulp cells. There was no synergistic induction of MCP-1 by SP plus CGRP stimulation of pulp cells.

Conclusions Substance P is a stronger inducer of IL-8 production in dental pulp than CGRP. IL-8 is more strongly induced than MCP-1 by SP, suggesting a more important role for IL-8 than MCP-1 in leucocyte infiltration during neurogenic inflammation in dental pulp.

Keywords: calcitonin gene-related peptide, dental pulp, interleukin-8, monocyte chemotactic protein-1, substance P.

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Introduction

Dental pulp is vulnerable to irritation from caries invasion and mechanical or chemical treatment during dental procedures. Neurogenic inflammation plays an important role in pulpal inflammation (reviewed by Jontell *et al.* 1998, Stashenko *et al.* 1998, Byers & Närhi 1999). The richly innervated dental pulp expresses neuropeptides, the release of which (e.g. following injury) causes increased blood flow, plasma extravasation and leucocyte accumulation, leading to local inflammation. Leucocyte migration across the blood vessel wall into local tissue is a highly regulated process (Muller & Randolph 1999). Local release of chemokines interleukin-8 (IL-8), a potent neutrophil chemoattractant, and monocyte chemotactic protein-1 (MCP-1) plays a key role in attracting leucocytes to the sites of tissue injury.

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Substance P (SP) and calcitonin gene-related peptide (CGRP) are two important sensory neuropeptides expressed in the dental pulp (reviewed by Wakisaka 1990). Although the primary function of these two neuropeptides, upon release, is to induce vasodilatation and pulpal blood flow (Wakisaka 1990, Heyeraas *et al.* 1994), they may play a more direct role in initiating the local inflammatory cell infiltration (Fristad *et al.* 1997). Substantial evidence has shown that neuropeptides can stimulate the production of proinflammatory cytokines by epithelial cells or fibroblasts from different tissues. SP or CGRP induces corneal epithelial cells to secrete IL-8 (Tran *et al.* 2000a,b) and bronchial epithelial cells to synthesize and release IL-6, IL-8 and tumour necrosis factor α (TNFα) (Veronesi *et al.* 1999). In addition, SP induces IL-8 production by fibroblasts in patients with osteoarthritis, while CGRP increases IL-8 and IL-6 secretion from fibroblasts in patients with rheumatoid arthritis (Raap *et al.* 2000).

Cultured human pulp cells have been reported to increase IL-8 secretion in response to SP stimulation (Patel *et al.* 2003). In the present study, the aims were to determine (i) whether another important proinflammatory chemokine MCP-1 is up-regulated by SP and/or CGRP in cultured pulp cells; and (ii) whether induction of IL-8 or MCP-1 in pulp tissue can be detected using *ex vivo* pulpal explants exposed to neuropeptides in culture.

Materials and methods

Sample collection and cell culture

Caries-free, intact, freshly extracted third molars ($n = 26$) were collected and processed according to procedures described previously by Huang *et al.* (1999) and Patel *et al.* (2003). Teeth collection from human subjects followed a protocol approved by the UCLA Medical Institutional Review Board. Pulpal tissue was obtained from teeth and divided into several fragments approximately 1 mm × 1 mm × 2 mm in size each. Pulp cells outgrown from the fragments were subcultured and grown to confluence in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies/Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% foetal bovine serum (FBS). Subcultures were successively passed at 1 : 2 ratio until used for experiments (passages 3–8). These cells showed a fibroblast-like phenotype. Cell 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).

Neuropeptide stimulation of cultured pulp cells and explants

Synthetic human SP, spantide I (SP receptor antagonist), and α-CGRP (Sigma, St Louis, MO, USA) were dissolved in sterile H_2O with 0.1% low-endotoxin bovine serum albumin (BSA; Sigma). Alpha minimal essential medium (MEM) containing L-glutamine (Life Technologies) with 1% FBS was used for cell culture 24 h before stimulation with neuropeptides. This low serum medium was used to minimize the serum effect, which elevates the base-line level of chemokine secretion from these cells. Recombinant human (rh) TNFα, (R&D Systems, Minneapolis, MN, USA), served as a positive control for the induction of IL-8 and MCP-1. For negative control (mock stimulation), corresponding concentrations of low-endotoxin BSA to those present in the experimental groups were used. Pulp cells were seeded into 48-well plates and grown to confluence. Neuropeptides or TNFα in 200-μL volumes of cell culture growth medium was added into each well and after various incubation periods, the supernatant was collected for enzyme-linked immunosorbent assay (ELISA) described below. For stimulation of pulp tissue, pulp explants divided into approximately 1 mm × 2 mm × 2 mm size were incubated in alpha MEM without FBS for 2 days to allow adaptation to the culture condition before stimulation with SP. The size of the pulp fragments for these experiments were determined based on the requirement that each fragment should contain detectable amount of IL-8 by ELISA in our experimental settings. Each pulp fragment was placed in a well of 96-well plates and stimulated with the BSA control solution (mock) or SP in 200-μL volumes of alpha MEM. At the end of the stimulation period, the pulp fragments were weighed and homogenized in the 200-μL medium using Micro Centrifuge Sample Pestle (Scienceware; distributed by Fisher Scientific, Pittsburgh, PA, USA). The dispersed tissue was centrifuged at 10 000 ***g*** for 5 min and the supernatant was collected for ELISA. The relative IL-8 or MCP-1-value of each tissue sample is presented as picograms per millilitre per microgram of the sample weight to normalize the amount of pulpal tissue.

Enzyme-linked immunosorbent assay for IL-8 and MCP-1

Standard ELISA for IL-8 was performed as described previously by Huang *et al.* (1999) using 2 μg mL^{-1}

of polyclonal goat antihuman IL-8 (R&D Systems); as capturing antibodies, 1 μg mL^{-1} polyclonal rabbit antihuman IL-8 (Endogen, Inc., Cambridge, MA, USA) as detecting antibodies and 0.1 μg mL^{-1} horseradish peroxidase (HRPO)-labelled polyclonal goat antirabbit immunoglobulin G (Biosource International, Camarillo, CA, USA) as a secondary antibody. Subsequently, fresh developing buffer containing substrate of optimal concentrations of 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma, St Louis, MO, USA), H_2O_2 and sodium acetate (pH 6.0) was added, and the developing reaction was stopped with sulfuric acid. Absorbance at 450 nm was determined with a microplate reader (Bio-Tek Instrument, Inc., Laguna Hills, CA, USA) and the concentrations were derived using the DELTA SOFT III software (Bio-Tek Instrument, Inc.). Known concentrations of rhIL-8 (Endogen, Inc.) was used to establish a standard curve for determining the concentrations of the experimental samples.

ELISA for MCP-1 was similar to that for IL-8. The following antibodies were used: monoclonal mouse antihuman MCP-1 (R&D Systems) as capturing antibodies, polyclonal rabbit antihuman MCP-1 (Cell Sciences, Inc., Norwood, MA, USA) as detecting antibodies and HRPO-labelled polyclonal goat antirabbit immunoglobulin G as a secondary antibody. Known concentrations of rhMCP-1 (R&D Systems) was used to establish a standard curve.

Statistical analysis

Student's *t*-test was used to determine whether SP or CGRP induced significant amount of IL-8 or MCP-1 from cultured pulp cells or explants at the 0.05 significance level.

Results

Increased IL-8 secretion from cultured pulp cells or tissue explants in response to substance P stimulation

A statistically significant induction (approximately three fold increase; *t*-test, $P < 0.05$) of IL-8 in pulp cells was observed after SP stimulation as shown in Fig. 1. Induction of IL-8 by SP was inhibited by the antagonist spantide I, whereas induction by TNFα was not affected, indicating specificity of SP induction. The data further validate our previous findings on the IL-8 induction in pulp cells by SP (Patel *et al.* 2003).

Increased IL-8 production (two- to threefold) was detected in pulp explants 36 or 48 h after SP stimulation, as shown in Fig. 2. A high concentration (10^{-4} mol L^{-1}) of SP was used to maximize the induction. No IL-8 induction was observed in pulp explants at 24 h after SP stimulation (data not shown). The specificity of this IL-8 induction in pulp explants by SP was verified by spantide I, the presence of which lowered the IL-8 induction. A significant difference was found between the mock (control) and stimulated groups ($P < 0.05$) at 36 h, but not at 48 h (Fig. 2A) because of great sample variations of IL-8 level from the SP-stimulated pulp fragments. The base-line (mock-stimulated) levels of IL-8 shown in Fig. 2(B) are higher than those in Fig. 2(A). This could be because of the variations among different independent experiments.

MCP-1 induction in cultured pulp cells or in pulp tissue explants by substance P

Dose–response studies, presented in Fig. 3, show approximately two fold increase of MCP-1 in pulp cells

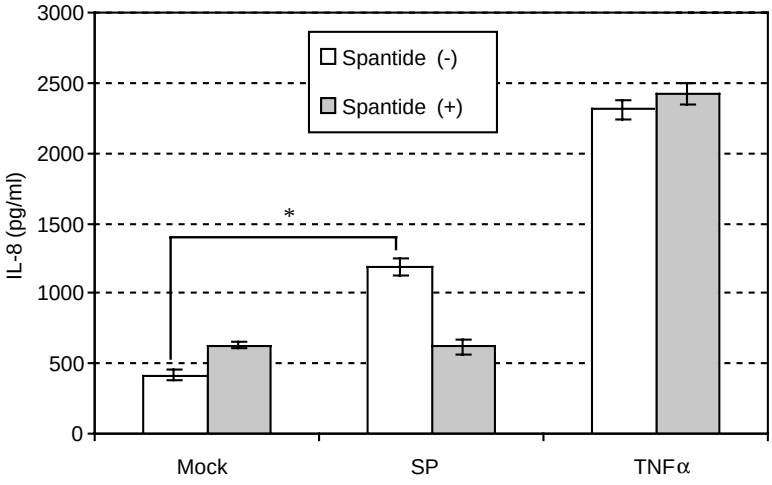

Figure 1 Interleukin-8 secretion from pulp cells in response to 12 h of SP stimulation. Data are mean ± SEM from one representative experiment performed in duplicate assays. Spantide (10^{-6} mol L^{-1}) was added in 200-μL volumes into confluent cells 15 min prior to stimulation. TNFα (20 ng mL^{-1}) was used in parallel cultures as a positive control. Mock stimulation is the negative control. Statistically significant (*$P < 0.05$).

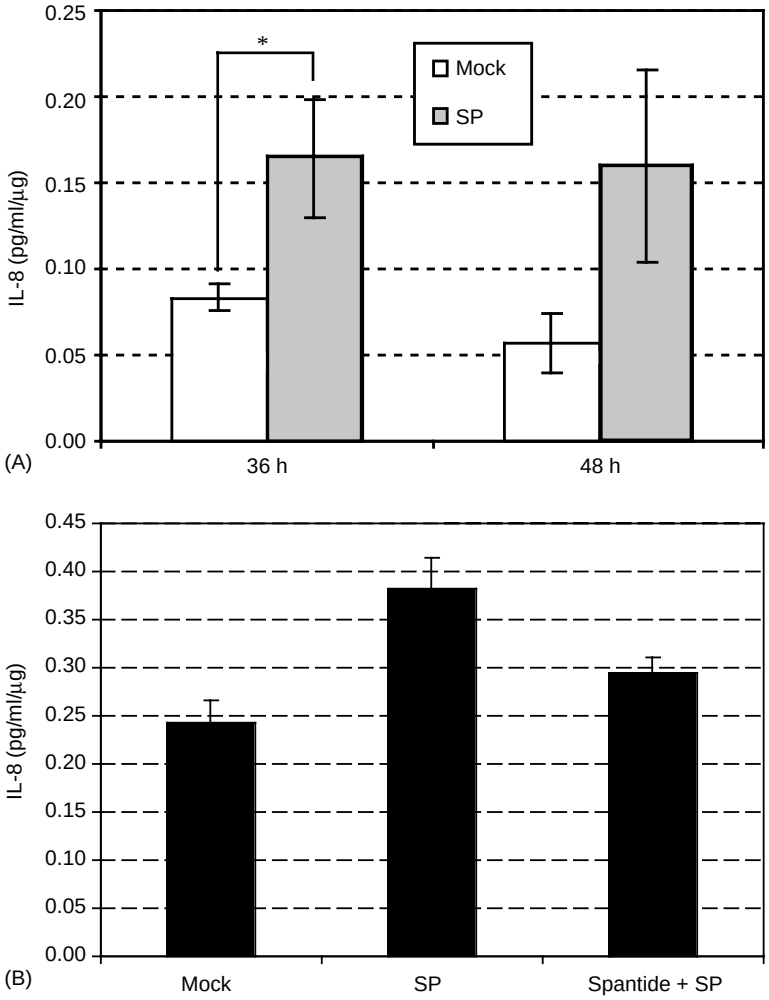

Figure 2 Interleukin-8 secretion from pulp explants following SP (10^{-4} mol L^{-1}) stimulation. (A) Data are mean ± SEM of six independent experiments of 1–6 sets of pulp fragments (each set was obtained from one tooth). The fragmented pulp tissue from each tooth was divided into different groups (mock- or SP-stimulated; each has 36 or 48 h stimulation period). Statistically significant ($^{*}P < 0.05$). (B) Result of another independent experiment showing mean ± SEM from six sets of pulp fragments (from six teeth). Only one stimulation period, 36 h, was tested and the concentration of spantide was 10^{-5} mol L^{-1}.

after 12 h stimulation with SP (10^{-4} mol L^{-1}). This mild induction was blocked by spantide I, indicating specificity of the response to SP (data not shown). A time-course plot showing the kinetics of MCP-1 production up to 24 h of SP stimulation is presented in Fig. 4. When pulp cells were exposed to SP only during the first 4 h, MCP-1 increased and reached the peak at 4–8 h after SP stimulation, followed by a decrease to a level just

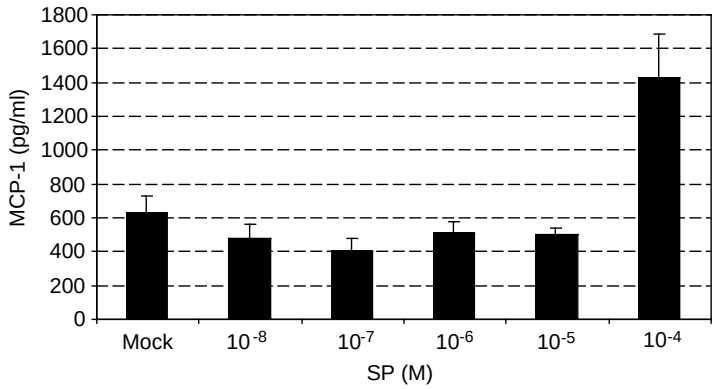

Figure 3 Dose–response of MCP-1 secretion from pulp cells in response to 12 h of SP stimulation. Data indicate mean ± SEM from two independent experiments in duplicate or triplicate assays. Lower concentrations of SP (10^{-9}, 10^{-10}, 10^{-12} and 10^{-16} mol L^{-1}) not presented here also showed no induction of IL-8.

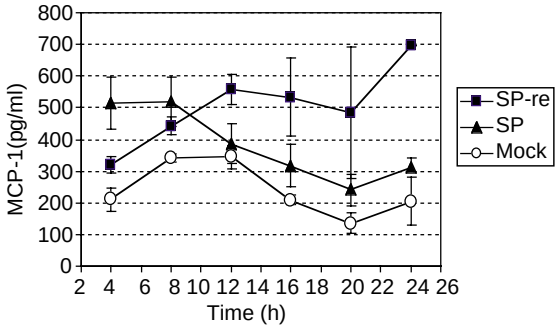

Figure 4 Time course of MCP-1 secretion from pulpal cells following SP (10^{-4} mol L^{-1}) stimulation. Mock group: 0.1% BSA was added into the cultures. SP group: SP was only added at the time 0. After supernatant collection at the 4-h time point, only 0.1% BSA in fresh medium was added for rest of the time points. SP-re (replenished) group: after collecting the supernatant from the well, fresh medium containing SP was replenished at every time point. Supernatants collected at each time point represent a 4-h interval of MCP-1 accumulation in cultures. Data indicate mean ± SEM from three independent experiments in duplicate or triplicate assays except the 24-h time point (from one experiment in duplicate).

slightly higher than the base-line level. When SP was present during the entire course, i.e. fresh medium containing SP added at every time point after collection of the supernatant (SP-replenished group), MCP-1 secretion appeared to increase over time and remained high up to 24 h after SP stimulation. We next determined whether an increase of MCP-1 could be detected in pulp explants exposed to SP. After up to 48 h of SP (10^{-4} mol L^{-1}) stimulation, no significant MCP-1 increase was observed in pulp explant samples (Fig. 5).

IL-8 and MCP-1 induction in pulp cells by CGRP and SP plus CGRP

Various doses of CGRP, as demonstrated in Fig. 6, were used to stimulate the pulp cells. No or negligible IL-8 induction was observed even at the highest dose of CGRP (10^{-4} mol L^{-1}). Similarly, CGRP, at concentrations between 10^{-9} and 10^{-5} mol L^{-1}, only minimally induced MCP-1 production, whereas there was an approximately threefold increase of MCP-1 when 10^{-4} mol L^{-1} of CGRP was used (Fig. 7A). Minimal or no further increase of MCP-1 was observed when pulp cells were exposed to SP and CGRP simultaneously compared with individual neuropeptide stimulation (Fig. 7B).

Discussion

The present study sheds light on the role of neuropeptides SP and CGRP in regulating the expression of the chemokines IL-8 and MCP-1 in dental pulp tissue. Although CGRP is highly expressed in the human dental pulp, it does not appear to play a significant role in inducing the expression of IL-8 and MCP-1 in dental pulp cells. While SP at the physiological level (10^{-10}–10^{-8} mol L^{-1}; Hargreaves *et al.* 1994, Awawdeh *et al.* 2002, Bowles *et al.* 2003) does not appear to induce MCP-1, it shows a strong potential to induce IL-8 in human dental pulps. The data support the possibility that IL-8 induced by SP plays a more important role than MCP-1 in the early response of dental pulp to irritations by establishing local inflammatory cell infiltration.

It has been found previously that IL-8 induction in cultured pulp cells can be observed at concentrations between 10^{-12} and 10^{-4} mol L^{-1} of SP stimulation (Patel *et al.* 2003). Our present studies further demonstrated that IL-8 levels in pulp tissue explants increased following SP stimulation. The induction of IL-8 observed in pulp explants required a longer time (36 h) to occur (Fig. 2) in contrast to induction in cultured pulp cells (within 4 h). It is possible that the diffusion of SP into the pulp tissue required more time and, therefore, it took longer to observe the increased IL-8 level in the pulp. The variation of IL-8 levels among pulp tissues from different teeth, as well as within the same tooth, makes the difference of IL-8 level between the mock- and SP-stimulated groups difficult to measure. Nevertheless, mean values were consistently higher in the SP-stimulated groups. Although SP appears to be a potent inducer of IL-8 in pulp cells, it requires a high dose (10^{-4} mol L^{-1}) to mount a mild MCP-1 induction. In comparison, CGRP at a high concentration of 10^{-4} mol L^{-1} induced negligible IL-8, while inducing a moderate MCP-1 production in pulp cells.

Taken together, considering the physiological levels of SP and CGRP that are found in the human dental pulp (10^{-10}–10^{-8} mol L^{-1}; Awawdeh *et al.* 2002, Bowles *et al.* 2003), CGRP is not likely to play a significant role in initiating the inflammatory cell infiltration in dental pulp through inducing IL-8 or MCP-1. However, CGRP may participate in the process by inducing chemotactic response of T-cells or dendritic cells (Foster *et al.* 1992, Dunzendorfer *et al.* 2001). In contrast, SP plays an important role in neurogenic inflammation because of its direct chemotaxis effect on neutrophils (Roch-Arveiller *et al.* 1986) along with its potential to induce IL-8 production in pulp.

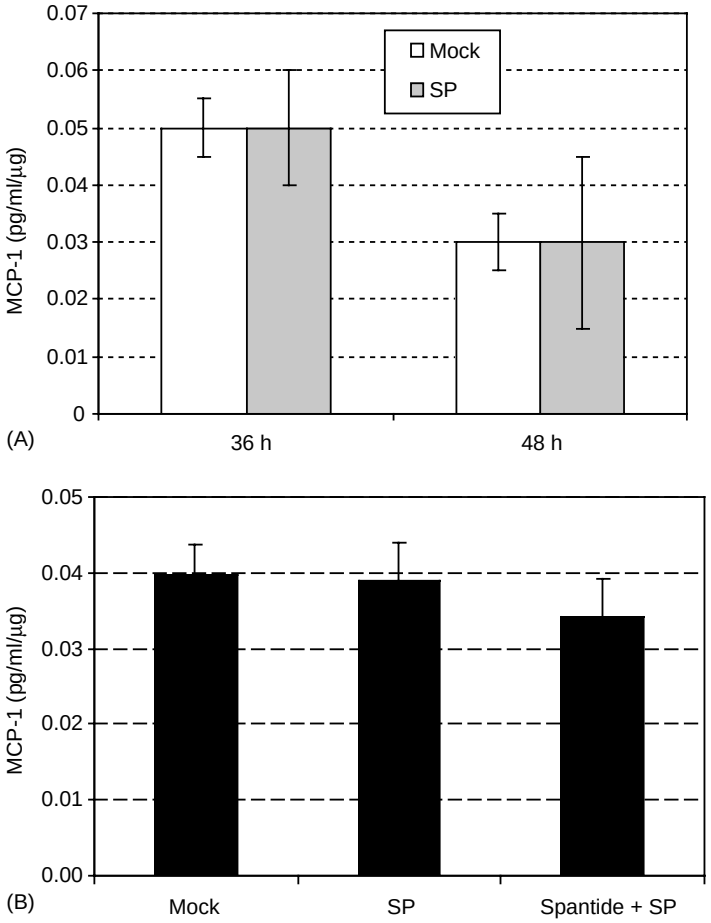

Figure 5 Monocyte chemotactic protein-1 secretion from pulp tissue fragments following SP (10^{-4} mol L^{-1}) stimulation. (A) Data are mean ± SEM of one independent experiment of three sets of pulp fragments (from three teeth). The experimental procedures are the same as for IL-8 measurement presented in Fig. 2(A). (B) Same samples for measuring IL-8 as presented in Fig. 2(B).

Local accumulation of neutrophils in the pulp underneath the invading caries or the dentine cavity filled with bacterial factors has been clearly demonstrated (Bergenholtz & Lindhe 1975). Bacterial components can serve as a potent chemoattractant to neutrophils. However, in a situation where bacteria may not be present and the dentine is only damaged mechanically, such as cavity or crown preparation, neutrophils egress from blood vessels and accumulation in the local pulp tissue underlying the damaged dentine can also be observed

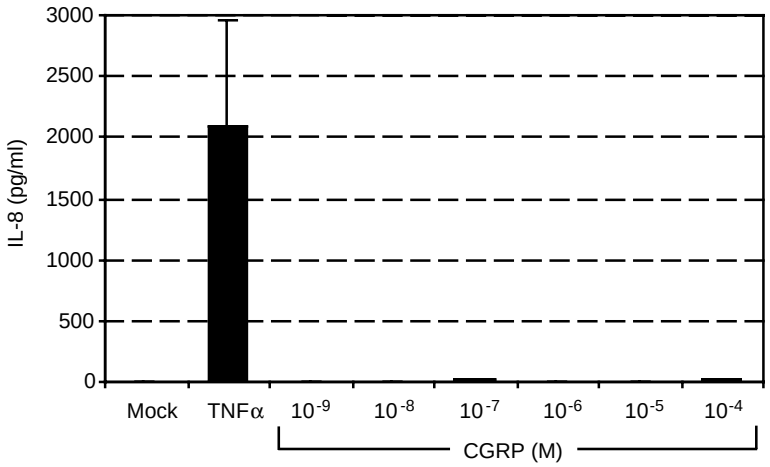

Figure 6 Interleukin-8 secretion from pulp cells following 12 h of TNFα (20 ng mL^{-1}) or various doses of CGRP stimulation. Data are mean ± SEM from two independent experiments in duplicate or triplicate assays.

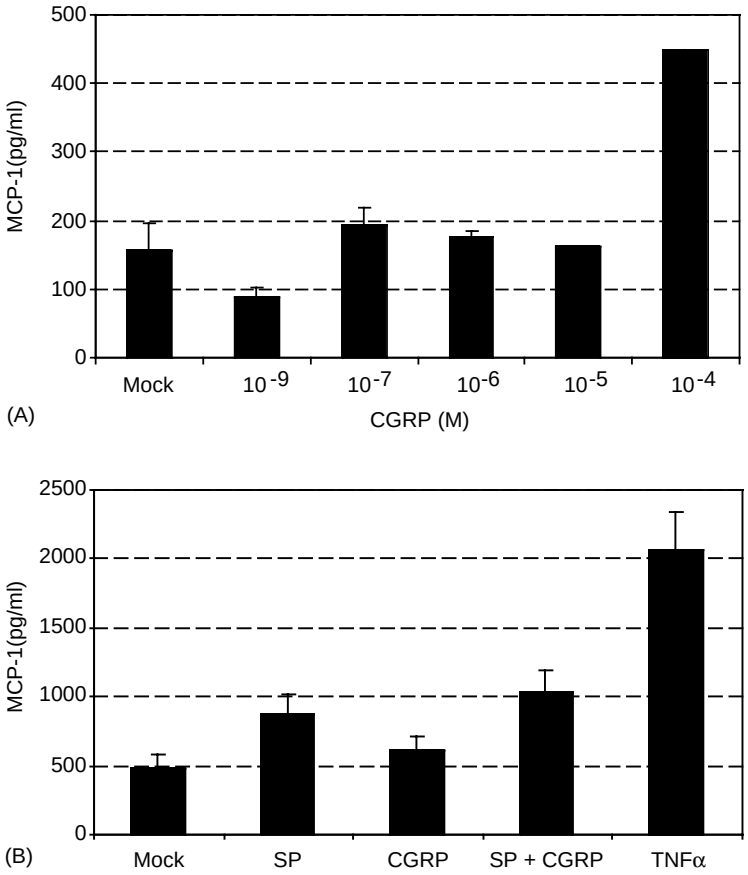

Figure 7 Dose–response of MCP-1 secretion from pulp cells in response to 12 h of CGRP stimulation. (A) Data indicate mean ± SEM from two independent experiments in duplicate assays except CGRP groups (10^{-4} and 10^{-5} mol L^{-1}) which were from one experiment in duplicate assays. (B) Pulp cells were incubated with SP (10^{-5} mol L^{-1}), CGRP (10^{-5} mol L^{-1}), or SP + CGRP (both 10^{-5} mol L^{-1}) for 12 h, and the supernatants were collected for analysis. Data indicate mean ± SEM from three independent experiments in duplicate or triplicate assays.

(Takahashi 1990). SP receptor neurokinin-1 (NK1) is expressed in cells of the cell-rich zone beneath the odontoblast layer (Fristad *et al.* 1999), suggesting that when SP is released from the excited sensory fibres, these cells may be activated by SP to produce IL-8.

It seems logical to link SP, IL-8 and neutrophils as three important early-responsive players following imposition of the external stimuli on the pulpodentine complex. The fast-responding neural system releases SP rapidly upon stimulation, inducing local tissue cells to produce and secrete IL-8, which, in turn, attracts neutrophils to migrate towards the irritated sites. This sequence of events leads to the formation of neutrophil infiltration in the microenvironment – a localized acute inflammatory response. In contrast to the role of IL-8, which preferentially attracts and stimulates neutrophils, MCP-1 attracts and stimulates mainly monocytes (Van Damme 1994). Therefore, the finding of no significant MCP-1 induction by either neuropeptides appears to fit into the scheme of the early stage of inflammation in the pulp that more neutrophils, than macrophages, are recruited into the localized area subjacent to the irritated dentine.

Conclusion

Substance P, but not CGRP, significantly induces IL-8 expression in human dental pulp, suggesting an important role of SP in initiating the accumulation of neutrophils in localized pulp tissue. Neither SP nor CGRP significantly induces MCP-1 in the pulp, therefore MCP-1 appears to be less involved in the early establishment of pulpal inflammation in response to irritation, such as mechanical insult of dentin.

Acknowledgements

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