Substance P Expression by Human Dental Pulp Fibroblasts: A Potential Role in Neurogenic Inflammation

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Abstract

Neurogenic inflammation describes the local release of neuropeptides, notably substance P (SP), from afferent neurons and might play a role in the pathogenesis of pulpal disease. The fibroblast is the most numerous cell type in the dental pulp, and recent work has suggested that it is involved in the inflammatory response. Primary pulp fibroblast cell populations were isolated by enzymatic digestion. Whole pulp tissue was obtained from freshly extracted sound (n = 35) and carious (n = 39) teeth. Expression of SP and neurokinin-1 receptor (NK-1) mRNA by pulp fibroblasts was determined by reverse transcriptase polymerase chain reaction (RT-PCR). SP was expressed by pulpal fibroblasts at both mRNA and protein levels. In addition, NK-1 mRNA and protein expression was detected in fibroblast cultures by RT-PCR and Western blotting, respectively. SP levels, determined by radioimmunoassay, were significantly greater ($P < .05$) in carious compared with sound teeth. These findings suggest that pulp fibroblasts play a role in neurogenic inflammation in pulpal disease. *(J Endod 2009;35:73–77)*

Key Words

Caries, dental pulp fibroblasts, neurogenic inflammation, neurokinin-1, substance P

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Neuropeptides released by sensory nerves under physiologic and pathologic conditions can affect or modulate many aspects of dental pulp function such as blood flow and vascular permeability (1). Substance P (SP), an 11 amino-acid peptide, is a member of the tachykinin family of neuropeptides and was the first neuropeptide to be identified in dental tissue (2). It is released on nerve stimulation and induces vasodilation, vascular permeability, mast cell degranulation, and smooth muscle contraction (3). SP is secreted by nerves and inflammatory cells such as macrophages, eosinophils, lymphocytes, and dendritic cells. Elevated levels of SP expression have been reported in pulpitis (4, 5) and correlate with pain experience and caries progression (6, 7). Neuropeptides exert their effects on target tissues via interaction with a membrane-bound receptor. The effects of SP are mediated largely by the neurokinin (NK) receptors, transmembrane G protein–coupled receptors, found in all areas of the central and peripheral nervous systems. There are 3 NK receptors, namely NK-1, NK-2, and NK-3, with the NK-1 receptor having the greatest affinity for SP. NK-1 mediates multiple functions of SP, including a vasoactive function, regulation of vascular permeability, and modulation of pain transmission (8).

Fibroblasts are the major cell type found in the pulp and are responsible for the synthesis and maintenance of extracellular matrix, maintaining structural integrity. Fibroblasts also play a central role in the pathogenesis of pulpal inflammation, producing proinflammatory cytokines including interleukin-1β (IL-1β), IL-6, and IL-8 in response to bacterial stimulation (9, 10). SP also induces pulp fibroblast synthesis of proinflammatory cytokines (11–14). It seems plausible that neuropeptide release from dental pulp fibroblasts could control cytokine expression, lending support to the hypothesis that the pulp fibroblast is central to a bidirectional communication between inflammatory cells and nerve fibers in the pulpal response to caries.

The aim of this study was to further define the interaction between pulp fibroblasts and components of neurogenic inflammation by investigating the expression of both SP and its receptor NK-1 by pulp fibroblasts in vitro. Because expression levels of both IL-1β and transforming growth factor β_1 (TGF-β_1) are increased in the inflamed pulp, the regulation of SP and NK-1 expression by these 2 factors, with opposing effects on pulp fibroblast function (15), was also determined.

Materials and Methods

Derivation of Pulp Fibroblasts

Ethical approval was obtained from the Local Research Ethics Committee, and informed written consent was obtained from all patients. Three dental pulp fibroblast populations (PLP 30, 35, and 41) were used in the study, derived by explant culture from pulps removed from 3 separate healthy teeth from 3 different patients. Briefly, whole pulp was extirpated from extracted teeth under sterile conditions and minced, and the fragments were incorporated into a Type I collagen gel as previously described (16). After significant outgrowth of cells, collagen gels were digested, and fibroblasts were harvested and propagated onto plastic flasks. Fibroblasts were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin. On reaching confluence, fibroblast populations were propagated at a 1:3 split ratio. Only cells between passages 3 and 5 were used in the study.

Reverse Transcriptase Polymerase Chain Reaction

The expression of NK-1 and SP mRNA by pulp fibroblasts was determined by reverse transcriptase polymerase chain reaction (RT-PCR). Pulp fibroblasts from the 3 cell lines (PLP30, 35, and 41) were seeded in 12-well plates at a concentration of 1×10^5 cells/well and grown to confluence. Cells were then washed with Hank's balanced salt solution (HBSS) and incubated for 24 hours in DMEM containing 1% FCS (control medium) to induce quiescence. Cells were then cultured for an additional 48 hours in triplicate in control medium with or without IL-1β (10 ng/mL), TGF-β_1 (10 ng/mL), or SP (10^{-7} mol/L). Total RNA was extracted by using TRIReagent (Invitrogen, Renfrew, UK) according to the manufacturer's instructions. RNA derived from triplicate wells under each culture condition was pooled, and cDNA synthesis was performed by SuperScript II Reverse Transcriptase (Invitrogen). Primers were designed by using the Primer Express (Applied Biosystems, Foster City, CA) software and obtained from Invitrogen Custom Primers (Invitrogen). The upstream and downstream primers were as follows: 5′-CGTCGCAAAAT CCAACATGAAA-3′, 5′-CCTTGATCTGGTCGCTGTCGTA-3′ for SP; 5′-CAATGTGGTGATGGGAAATGAG-3′, 5′-TTGGTAGGA TGGGTGGATTCTC-3′ for NK-1; and 5′-ATCTGGCACCACACCTTCTACAATG-3′ and 5′-TAGCACGCAC TGTAATTCCTCTTCG-3′ for β-actin. PCR amplifications were carried out for 45 cycles under the following cycling conditions: 94°C for 45 seconds, annealing temperature for 45 seconds, and 72°C for 1 minute. A final extension cycle of 72°C for 10 minutes was then performed. All PCR amplifications of cDNA samples were carried out within the exponential range of each product. An appropriate PCR-amplified negative blank (ie, containing no cDNA), positive kit control (Invitrogen), negative RT^- control (without Superscript II RT), and a low molecular weight mass marker (Invitrogen) were used during all gel electrophoresis runs.

All PCR products were identified by size after electrophoresis on a 3% EtBr-stained agarose gel, and the net intensities of bands were measured by using ImageJ Software (http://rsbweb.nih.gov/ij/). Each band was analyzed 3 times, and a mean intensity was calculated. Relative levels of SP and NK-1 mRNA in each sample were calculated to produce expression indices by dividing mean band intensity for SP or NK-1 by the corresponding β-actin band intensity. For individual cell lines the expression indices under each culture condition were normalized to control cultures, where the expression index (EI) for controls = 1.

Radioimmunoassay

Radioimmunoassay was used to quantify levels of SP in fibroblast cultures. Fibroblast populations from the 3 cell lines were grown to confluence in 60-mm dishes, quiesced as before, and cultured for 48 hours in DMEM containing 1% FCS, with or without IL-1β (10 ng/mL), TGF-β_1 (10 ng/mL), or SP (10^{-7} mol/L). The conditioned medium was collected and stored at –20°C before analysis. The cell layer was removed with a cell scraper, washed with HBSS, and centrifuged at 900 rpm for 5 minutes. SP levels in the conditioned medium and cell lysate fractions were determined by radioimmunoassay with antiserum 152(2) (Wellcome Research Laboratories, Queen's University Belfast, UK) and I^{125}-labeled synthetic human SP as radioactive tracer as described previously (7). Each sample was assayed in duplicate.

Western Blotting

NK-1 expression levels on fibroblasts were determined by Western blot. Fibroblast populations were grown to confluence in T75 flasks, washed in HBSS, and then cultured in DMEM containing 5% FCS with or without IL-1β (10 ng/mL) or TGF-β_1 (10 ng/mL) for an additional 5 days. Cell membrane extracts were initially prepared from fibroblast cultures by using the Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce, Rockford, IL) according to the manufacturer's instructions and were further prepared for electrophoresis by using the PAGEPrep Clean Up and Enrichment Kit (Pierce). Samples were electrophoresed on 4%–12% NuPAGE Bis-Tris gels (Invitrogen) by using the XCell SureLock Mini-Cell apparatus (Invitrogen). The blot was blocked for 2 hours in a solution of 4% I-Block (Applied Biosystems, Warrington, UK) in Tris-buffered saline (0. 02 mol/L Tris-HCl buffer, pH 7.4, containing 0.15 mol/L NaCl) and then incubated with polyclonal anti-rabbit NK-1 (Abcam, Cambridge, UK) at a dilution of 1/1000 (1 μL/mL) overnight at 4°C. Subsequent detection of bound primary antibody was achieved by incubation for 2 hours with anti-rabbit immunoglobulin–alkaline phosphatase conjugate (Sigma, Gillingham, Dorset, UK) at a dilution of 1/1000 and detection with the substrates nitro-blue tetrazolium and 5-bromo-4-chloro-indolyl phosphate (Sigma).

Whole Pulp Tissue

Healthy and carious teeth were also used to quantify SP levels and to determine NK-1 expression in dental pulp tissue. In total, 74 teeth, 35 healthy and 39 carious, were collected for SP quantification. Immediately after extraction, teeth were snap-frozen in liquid N_2 and fractured, and the frozen pulp tissue was placed in preweighed Eppendorf tubes and stored at –80°C. SP was initially extracted from whole pulp tissue by boiling in 0.5 mol/L acetic acid (8 mL/g of tissue) for 10 minutes before centrifugation at 2200*g* at 4°C for 20 minutes (7), and SP levels were quantified by radioimmunoassay as described above. NK-1 expression in pooled pulp tissue derived from 2 healthy or 2 carious teeth was determined by Western blot, following the same methods described above.

Statistical Analysis

The effects of the cytokines on SP and NK-1 mRNA by pulp fibroblast expression were analyzed by one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference post hoc test. Comparison of SP expression levels in whole pulp from healthy and carious teeth was performed by the Mann-Whitney *U* test analysis. The level of statistical significance in all cases was set at $P < .05$.

Results

SP Expression

SP mRNA was expressed by all 3 pulp fibroblast cell strains (Fig. 1*A*). IL-1β induced a significant increase in SP mRNA expression ($P < .05$) in all 3 cell lines. TGF-β_1 induced a significant increase in 1 cell strain (PLP35). SP did not affect SP mRNA expression in any of the cell strains.

SP levels were not detected in any conditioned medium fractions except those cells treated by exogenous SP. Levels of SP in the cell lysates of the 3 cell strains are shown in Fig. 1*B*. SP expression was detected in 2 of the 3 unstimulated fibroblast cell populations. IL-1β, TGF-β_1, and SP all significantly increased SP production in the 3 cell populations ($P < .05$).

SP was present in detectable amounts in 68 of the 74 dental pulp tissue samples analyzed. The mean concentration of SP in the pulps of carious teeth (16.03 ± 7.77 ng/g) was significantly greater ($P < .001$) than in noncarious teeth (7.97 ± 4.72 ng/g) (Table 1).

NK-1 Expression

NK-1 mRNA was detected in all 3 pulp fibroblast cell lines. Initial studies indicated detectable levels of NK-1 mRNA after 6 hours in culture, with expression increasing over subsequent time points to a maximum at 48 hours (data not shown). All remaining studies on the effects of cytokines/neuropeptides on NK-1 mRNA expression were undertaken