Adaptive Calcified Matrix Response of Dental Pulp to Bacterial Invasion Is Associated with Establishment of a Network of Glial Fibrillary Acidic Protein⁺/Glutamine Synthetase⁺ Cells

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We report evidence for anatomical and functional changes of dental pulp in response to bacterial invasion through dentin that parallel responses to noxious stimuli reported in neural crest-derived sensory tissues. Sections of resin-embedded carious adult molar teeth were prepared for immunohistochemistry, *in situ* hybridization, ultrastructural analysis, and microdissection to extract mRNA for quantitative analyses. In odontoblasts adjacent to the leading edge of bacterial invasion in carious teeth, expression levels of the gene encoding dentin sialo-protein were 16-fold greater than in odontoblasts of healthy teeth, reducing progressively with distance from this site of the carious lesion. In contrast, gene expression for dentin matrix protein-1 by odontoblasts was completely suppressed in carious teeth relative to healthy teeth. These changes in gene expression were related to a gradient of deposited reactionary dentin that displayed a highly modified structure. In carious teeth, interodontoblastic dentin sialo-protein⁻ cells expressing glutamine synthetase (GS) showed up-regulation of glial fibrillary acidic protein (GFAP). These cells extended processes that associated with odontoblasts. Furthermore, connexin 43 established a linkage between adjacent GFAP⁺/GS⁺ cells in carious teeth only. These findings indicate an adaptive pulpal response to encroaching caries that includes the deposition of modified, calcified, dentin matrix associated with networks of GFAP⁺/GS⁺ interodontoblastic cells. A regulatory role for the networks of GFAP⁺/GS⁺ cells is proposed, mediated by the secretion of

glutamate to modulate odontoblastic response. (*Am J Pathol* 2010, 177:1901–1914; DOI: 10.2353/ajpath.2010.100073)

Dentin is a unique calcified tissue matrix formed by odontoblasts that surround the dental pulp. In the mature tooth odontoblasts line the pulp-dentin interface and extend long cellular processes through dentinal tubules toward the dentin-enamel junction. With the primary function as dentin-forming cells,¹ odontoblasts also exhibit features of innate immune cells including the expression of Toll-like pattern recognition receptors.² Accordingly, odontoblasts recognize and react to products of Gram-positive and Gram-negative bacteria by selective engagement of different Toll-like receptors.³ Dental caries can progress to a polymicrobial invasion of dentin where from 7 to 31 bacterial taxa can be detected within a lesion.⁴ Bacterial invasion is associated with degradation of the dentinal matrix and stimulation of odontoblasts. Following bacterial stimulation, odontoblasts undergo major functional adaptations^{5,6} including deposition of intratubular dentin and accelerated dentinal matrix deposition referred to as reactionary dentin.⁷ Despite extensive research, the response of odontoblasts to this microbial ingress remains poorly characterized.⁸

Exposure of differentiated odontoblasts in primary cultures to bacterial products reduces dentin forming capacity.⁹ This contrasts with the observed deposition of reactionary dentin with implications for external regulation of odontoblast function. Transcriptome profiles of primary odontoblast cultures and established lines of odontoblast-like cells exhibit high similarity with the major difference involving expression of regulatory neuropep-

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tides¹⁰ in primary odontoblast cultures with potential involvement in mediation of responses to bacterial challenge. The cross-talk through neurotransmitters could occur between pulpal nerve and odontoblasts or interodontoblastic cells and odontoblasts. Sensory denervation of dental pulp does not alter dentin-forming and immunological properties of odontoblasts,^{11,12} indicating a role for interodontoblastic cells. These cells form gap junction connections with adjacent odontoblasts.¹³ Although the function of these cells is not clearly understood, anatomical proximity and functional coupling through gap junctions implies a regulatory role.¹⁴ Interodontoblastic cells could be involved in the response of odontoblasts to bacterial challenge, and they may be necessary for an integrated response to bacterial invasion.

The *dspp* gene encodes two dentin extracellular matrix proteins, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), implicated in the formation of apatite crystals in the process of dentin mineralization.¹⁵ Products of the *dspp* gene are critical for efficient mineralization during dentinogenesis.¹⁶ The *dmp-1* gene encodes dentin matrix protein-1 (DMP-1) important in formation of the dentin tubular system and in mineralization.^{17,18} Both genes, being active in functional odontoblasts, are important markers of odontoblastic activity. Transforming growth factor- β 1 (TGF- β 1) is an important regulator of extracellular matrix synthesis and has been assigned an essential role in reactionary dentinogenesis.¹⁹ The interplay between these four products is central to the structural response of dental pulp to bacterial insult.

In this paper, we report a high-resolution, quantitative approach to characterize the response of dental pulp to microbial invasion. An unanticipated finding was that altered regulation of critical calcified matrix components in caries was associated with establishment of networks of cells expressing the glial markers glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS).^{20,21} Cells comprising these networks closely associated with odontoblasts, indicating they could play a regulatory role.

Materials and Methods

Materials and Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. The water used throughout the study was treated with diethyl polycarbonate (DEPC) to inhibit contaminating ribonucleases as described elsewhere.²²

Tissues

Healthy noncarious and carious permanent teeth were obtained from male and female patients aged 26 to 42 years attending the dental clinics at the Westmead Centre for Oral Health, Westmead Hospital. The patients were otherwise healthy and did not report a history of diseases known to affect calcified tissues, the vasculature or the immune response. Molar teeth with caries originating as

occlusal lesions with extensions ranging from one- to two-thirds of the dentin, were selected. The teeth were extracted for periodontal or orthodontic reasons. Healthy teeth were anatomically sound without any signs of attrition, abrasion, or erosion leading to dentinal exposure or any associated pathologies affecting the pulp-dentin complex. The ethics committee of Sydney West Area Health Service approved the study, and the guidelines of the National Health and Medical Research Council of Australia were observed. All patients received written information on the research and signed a consent form. Eight healthy and 12 diseased teeth met the inclusion criteria. These teeth were randomly allocated to light and electron microscopic studies.

Processing of Specimens

Pulp tissue was retrieved as previously described,²³ with some modifications. Briefly, immediately after extraction a circumferential, longitudinal groove, was prepared on the extracted tooth using a diamond disk (Thin-Flex; Abrasive Technology, Lewis Center, OH) with ample water cooling and without penetration into the dental pulp. Subsequently, the tooth was split into halves by mechanical leverage. The split tooth half with attached pulp was fixed in 2% paraformaldehyde/5% sucrose in 0.02 M phosphate buffer, pH 7.4 (680 mOsm), for 4 hours at 4°C.²⁴ Then, photographs of fixed split teeth were taken under a dissecting stereomicroscope (Leica MZ8; Leica Microsystems, Wetzlar, Germany). The fixed teeth were demineralized in Morse's solution (22.5% formic acid and 10% sodium citrate)²⁵ for 3 days at 4°C, followed by dehydration through gradual solvent exchange from pure DEPC-treated water to 100% ethanol over 45 minutes at 4°C.²⁶

A commercial glycol methacrylate embedding kit (ImmunoBed; Polysciences, Warminster, PA) was used for embedding according to the manufacturer's protocol. Tissue blocks were stored tightly sealed at 4°C until the time of microtomy. Serial sections of 1- μ m thickness were cut on a Reichert ultracut microtome (Leica Microsystems). The sections were floated on a prewarmed DEPC-treated water bath (37°C), collected using RNase-free-coated glass slides (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany), and allowed to air-dry at room temperature for 4 hours. Slides were stored at -20°C until subsequent use.

Localization of Bacterial Consortia

Acridine orange was used for general tracing of the bacterial consortia within dentinal tubules. The sections were permeabilized using 1% Triton X-100 for 2 minutes. Thereafter, Acridine orange (0.01%) in 20 mmol/L Tris/EDTA buffer, pH 8.0, was applied for 10 minutes, washed thoroughly with DEPC-treated water, and mounted with Aqua-Mount (Lerner Laboratories, Pittsburgh, PA).

Table 1. Oligoprobe Sequences for ISH

Gene	Polarity	Sequence	T _m
<i>β-Actin</i>	Antisense	5'-ATCCATGGTGAGCTGGCGGC-3'	63
	Sense	5'-GCCGCCAGCTCACCATGGAT-3'	63
<i>DSPP</i>	Antisense	5'-CCCTTGATTCTATTCCCTTATCTTGGCTCTTCCC-3'	62.2
	Sense	5'-GGGAAGAGCCAAGATAAGGGAATAGAAATCAAGGG-3'	62.2
<i>DMP-1</i>	Antisense	5'-GTCACTGGGGTCTTCATTGCTTGTTCCTCTGA-3'	62.2
	Sense	5'-GGGAAGAGCCAAGATAAGGGAATAGAAATCAAGGG-3'	62.2

T_m, melting temperature.

Analysis of Calcified Matrix Structure

The hard tissue response pattern to bacterial insult was investigated using Toluidine blue-stained sections. Stained sections were analyzed using ImageJ software (version 1.41o; National Institutes of Health, Bethesda, MD). Density of dentinal tubules and anisotropy of tubular structure was determined to evaluate the capacity of newly formed calcified tissue matrix to limit bacterial invasion toward the pulp. Mean density of dentinal tubules was measured in three random regions of interest (ROI) in physiological and reactionary dentin and a mean ratio calculated. The method of randomization involved random positioning through de-focusing-focusing of the microscope. For calculating the degree of structural anisotropy of tubular dentin in ROI, the degree of anisotropy was calculated based on the following formula: degree of anisotropy = 90/dispersion index, where the dispersion index is the maximum spreading angle of the longitudinal dentinal tubules in each ROI as originally developed for the analysis of bone.²⁷ Dispersion index was determined after noise reduction based on ImageJ protocol.

Expression Profile of Calcified Matrix-Related Genes

In situ hybridization (ISH) was used to identify regions with active formation of reactionary dentin. Synthetic antisense oligonucleotide probes (Invitrogen, Mulgrave, Victoria, Australia), complementary to mRNA sequence of *dspp* and *dmp-1*, were designed to span exon-exon junctions to avoid false-positive results from cross-hybridization with genomic DNA. The sequences and corresponding melting temperatures for oligoprobes are provided in Table 1. Sense probes were designed as complementary to anti-sense probes. Antisense and sense probes were labeled with Alexa 488 at 5' end (Invitrogen).

In Situ Hybridization

Sections were washed in DEPC H₂O (0.1% v/v) for 15 minutes to inactivate intrinsic RNases. After carbethoxylation with DEPC, 0.01 M citrate buffer, pH 6.0, was used as a retrieval solution. The pretreated tissue sections were washed in PBS for 5 minutes. For *dspp* and *dmp-1*, the hybridization buffer included 18% formamide, 2× standard saline citrate, 1× Denhardt's solution, 10% dextran sulfate and 500 μg/ml denatured and sheared

salmon sperm DNA. For *β-actin* the composition of hybridization buffer was similar with the exception that the concentration of formamide was higher (43%). A 25-μl gene frame (ABgene; Thermo Fisher Scientific, Leicestershire, UK) was used for hybridization using 100 ng of each oligonucleotide probe in 25 μl of hybridization buffer and the hybridization was performed in a Hybaid hybridization oven (Thermo Scientific, Billerica, MA) overnight at 37°C. Thereafter, sections were washed for 15-minute periods with 2× standard saline citrate at 37°C, 1× standard saline citrate at 37°C, and 1× standard saline citrate at room temperature. Subsequently, sections were air dried and mounted with Prolong gold anti-fade reagent with 4',6'-diamidino-2-phenylindole (Invitrogen). Several specificity controls were carried out to eliminate the possibility of nonspecific binding.

RNase Digestion

Two sections from each sample were treated for two hours at 37°C with 200 μg/ml DNase-free RNase A (Qiagen, Doncaster, Victoria, Australia) in PBS. Another section was treated with PBS for 2 hours at 37°C to act as a negative control for RNase digestion. One of the RNase-treated sections and the control section were hybridized with *β-actin* probe as described above. The other RNase-treated section was stained with Acridine orange.

DNase Digestion

Two sections from each sample were treated with RNase-free DNase (Qiagen, Australia), 10 Kunitz units/slide, for 2 hours at 37°C. The other DNase-treated section was stained with Acridine orange.

Hybridization with Sense Oligoprobes

Three sections from each sample were hybridized with *dspp*, *dmp-1*, and *β-actin* mRNA sense probes following the same protocol.

Analysis of Calcified Matrix-Related Gene Expression Using Quantitative RT-PCR

To quantify the impact of carious insult on the expression profile of genes of interest, microdissection of the odontoblast layer adjacent to carious dentin (A-site) and non-carious sites remote from caries (R-site) was performed.

The R-site was microdissected from a nonaffected region of the pulp with a safe margin equivalent to the width of the carious site. Odontoblasts from healthy samples were also microdissected from the occlusal site (H-site) equivalent to A-site. Preliminary study did not show any significant differences in *dspp*, *dmp-1*, and *tgf- β 1* expression (also reflected at the protein level) between the occlusal and lateral sites in healthy teeth equivalent to A- and R-site of carious teeth, respectively. Six consecutive 5- μ m sections were used for microdissection. Regions of interest were dissected from the tissue using a PALMs Robot-Microbeam system (Zeiss P.A.L.M. LCM microscope) and collected manually under a stereomicroscope (Leica Microsystems).

RNA was extracted from microdissected sections using RNeasy FFPE kit (Qiagen), according to the manufacturer's protocol. Four sections of 5- μ m thickness were used per preparation and yields were quantified by A260 nm measurement (Nanodrop ND-1000; Thermo Fisher Scientific). Quality of RNA in samples was assessed using Agilent RNA 6000 Pico Chip (Agilent Technologies, Palo Alto, CA). Reverse transcription of extracted RNA was carried out on 4 μ l of total RNA using the Sensiscript reverse transcription kit (Qiagen), according to the manufacturer's protocol. Briefly, reactions containing 2 μ l of Sensiscript Reverse Transcriptase, 2 μ l of 10 $\times$ reverse

transcriptase buffer, 2 μ l of 2'-deoxynucleoside 5'-triphosphate mix (final concentration of 0.5 mmol/L each 2'-deoxynucleoside 5'-triphosphate), 2 μ l of 100 μ mol/L Random nonamer (Geneworks, SA, Adelaide, Australia) (final concentration of 10 μ mol/L), 2 μ l of RNase inhibitor (10 units/reaction), 6 μ l of DEPC-treated water were incubated at 37°C for 60 minutes and then stored at -20°C until the time of use.

Singleplex real-time quantitative PCR for the genes of interest was carried out using 4 μ l of the cDNA in triplicate on a Strategene Mx3005P Real-Time PCR System with the Platinum quantitative PCR Supermix-UDG (Invitrogen). Primers together with TaqMan probes against *dspp*, *dmp-1*, *tgf- β 1*, *ocln* (occludin), *cdh2* (N-cadherin), *gjb2* (connexin-26), *gja1* (connexin-43) were designed to span exon-exon junctions to prevent genomic DNA amplification and are listed in Table 2. The only exception was *gjb1* (connexin 32) with the amplicon designed on exon 2 to detect both transcriptional variants. Transcription profiles of housekeeping genes encoding β -actin, glyceraldehyde-3-phosphate dehydrogenase and 18S RNA were assessed in healthy and carious teeth. The gene encoding β -actin was selected as the most constant endogenous reference control (data not shown). Fluorescence intensities were normalized against a passive fluorophore (ROX) present in the Mastermix and

Table 2. TaqMan Primer and Probe Sets

Gene	GenBank accession no.	Oligos	Primers	Expected amplicon size (bp)
<i>TGF-β1</i>	NM_011577	F-primer R-primer Probe	5'-CTACCATAGCCAACCTTCTGCCTC-3' 5'-GCCAGGACCTTGCTGTACT-3' 5'-CCCTGCCCTACATTTGGAGCCTGGACA-3'	75
<i>DSPP</i>	NM_014208	F-primer R-primer Probe	5'-GCAGAAGGATAGAGAAAGCAAACG-3' 5'-GGGACCCTTGATTTCTATTCCCTTATC-3' 5'-CCAAAGAATCAGAGACACATGCTGTTGGGAAGAGCC-3'	106
<i>DMP-1</i>	NM_001079911 NM_004407	F-primer R-primer Probe	5'-TGTGAACACGAGGGTAGAGG-3' 5'-ACTGGGAGAGCACAGGATAATCC-3' 5'-CACACCCAACATATGAAGATCAGCATCTGCTCATGTTCCCT-3'	92
β -Actin	NM_001101	F-primer R-primer Probe	5'-CCTGACGGCCAGGTCATCAC-3' 5'-GACTCCATGCCCAGGAAGGA-3' 5'-CCGCTGCCCTGAGGCACTCTTCCAG-3'	86
<i>GAPDH</i>	NM_002046	F-primer R-primer Probe	5'-CATCACCATTCTCCAGGAGCAGAT-3' 5'-TGAAGACGCCAGTGGACTCCA-3' 5'-TACTCAGCGCCAGCATCGCCCACTTGA-3'	89
<i>18S RNA</i>	NR_003286	F-primer R-primer Probe	5'-GCCCGTCGCTACTACCGATT-3' 5'-GTCAAGTTCGACCGTCTTCTCA-3' 5'-GGGCCGATCCGAGGGCCTCACTAAACCAT-3'	106
<i>Occludin</i>	NM_002538	F-primer R-primer Probe	5'-GTCTAGGACGCAGCAGATTGGTTT-3' 5'-GGCCTGTAAGGAGGTGGACTTT-3' 5'-AGCTGACCATTGACAATCAGCCATGTCATCCAGGCC-3'	125
<i>N-Cadherin</i>	NM_001792	F-primer R-primer Probe	5'-ACGCCGAGCCCCAGTAT-3' 5'-AGCCGCTTTAAGGCCCTCATT-3' 5'-CCGATCTGCAGCCCCACACCTGGAGAC □3'	86
<i>Connexin-26</i>	NM_004004	F-primer R-primer Probe	5'-CCTCCGACGCAGAGCAAA-3' 5'-CAATGCTGGTGGAGTGTGTTCACA-3' 5'-CGTCTGCAGCGTGCCCCAATCCATCTTCTACTCTGG-3'	96
<i>Connexin-32</i>	NM_000166 NM_001097642	F-primer R-primer Probe	5'-CCAACACAGTGGACTGCTTCGT-3' 5'-GTACACCACCTCGGCCACAT-3' 5'-CCGTCTTCATGCTAGTGCCTCTGGCATCTGCATCATCC-3'	113
<i>Connexin-43</i>	NM_000165	F-primer R-primer Probe	5'-AAGAGTGGTGCCAGGCAAC-3' 5'-CCTCCAGCAGTTGAGTAGGCTTG-3' 5'-GGGTGACTGGAGCGCCTTAGGCAAACCTTGTGACAAGG-3'	85

F-primer, forward primer; R-primer, reverse primer.

converted to absolute quantities using standard curves for each gene. Visual inspection of the amplified products on 2% agarose gel electrophoresis confirmed the presence of a single amplicon. Relative expression of targeted transcripts at each site was expressed as the ratio between the average concentrations of each transcript divided by the average concentration of β -actin transcripts at the same site based on a relative quantification model.²⁸

Immunohistochemistry

Interodontoblastic cells were characterized based on the expression profile of the glial markers GFAP and GS.^{20,21} Immunohistochemical localization of gap junction and tight junction proteins was used to determine the cellular networks and possible routes of communication of cellular elements.

After equilibration in PBS (5 minutes), antigen retrieval was performed with incubation of 1- μ m sections in 1 mmol/L EDTA, pH 8, at 100°C for 30 minutes in a microwave oven. Sections were cooled to room temperature and washed in PBS for 5 minutes before processing for immunohistochemistry. Sections were blocked with 10% horse or goat serum/PBS for 1 hour and primary antibody was applied overnight in 10% fetal calf serum/PBS at 4°C. The primary antibodies included mouse monoclonal anti-DSP (dilution 1/200; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-DMP-1 (dilution 1/200; Santa Cruz Biotechnology), rabbit polyclonal anti-GFAP (dilution 1/500; DakoCytomation, Glostrup, Denmark), rabbit polyclonal anti-GS (dilution 1/500; Sigma-Aldrich), mouse monoclonal anti-vimentin (prediluted; Invitrogen, Zymed Laboratories, South San Francisco, CA), goat polyclonal anti-TGF- β 1 (dilution 1/200; DakoCytomation), mouse monoclonal anti-connexin 26 (dilution 1/200; Invitrogen, Zymed Laboratories), mouse monoclonal anti-connexin 32 (dilution 1/200; Invitrogen, Zymed Laboratories), mouse monoclonal anti-connexin 43 (dilution 1/200; Invitrogen, Zymed Laboratories) and mouse monoclonal N-cadherin (dilution 1/200; Invitrogen, Zymed Laboratories). Sections were also incubated with isotype control antibodies or antibodies of irrelevant specificity to serve as negative controls. After washing with Tris-buffered saline Tween-20 for 30 minutes, fluorochrome-conjugated secondary antibody in 10% fetal bovine serum/PBS, was added and incubated at room temperature for 1 hour. The secondary antibodies were goat anti-mouse IgG Alexa 488 (dilution 1/500; Invitrogen, Zymed Laboratories) or goat anti-rabbit IgG Alexa 488 (dilution 1/200; Invitrogen, Zymed Laboratories). The sections were then washed with Tris-buffered saline (35 minutes) and mounted onto glass slides using ProLong Gold antifade reagent with 4',6'-diamidino-2-phenylindole (Invitrogen, Molecular Probes, Eugene, OR).

For double ISH and immunohistochemistry, consecutive 1- μ m sections were used. After ISH for *dspp* message and immunohistochemistry for connexins 43, 32, and 26 the overlay image was generated. For quantification of fluorescence signal intensity, 13 random regions of interest in the odontoblastic layer were selected begin-

ning from A-site toward R-site. Ten cells were randomly traced in each ROI using Multi_Cell_Outliner plug-in (ImageJ version 1.41o; National Institutes of Health). The mean fluorescent intensity from 10 cells in each ROI was calculated according to the following formula after separation of RGB channels using Measure_RGB plug-in:

$$\text{Intensity} = \frac{\sum_{i=1}^{10} [(\text{Green value} - \text{Red value})_i / A_i]}{10}$$

where Red value is representative of background value and the intensity is normalized against the area (A_i).

Transmission Electron Microscopy

For transmission electron microscopy analysis, carious and noncarious pulp samples were cut into small pieces and fixed in Karnovsky's fixative overnight at room temperature, followed by postfixation in OsO₄ for 1 hour. Preparations were dehydrated in graded alcohols and embedded in low viscosity resin (TAAB Laboratories and Microscopy, Berkshire, UK). Semithin sections were stained in Toluidine blue to facilitate the selection of site and orientation for preparation of ultrathin sections. Sections were mounted on Pioloform/formvar coated slot grids, stained in uranyl acetate and lead citrate and examined in a Phillips CM10 electron microscope. Film negatives were scanned at 100% scale at 4000 pixels/inch using a Nikon Super Coolscan 8000.

Immunoelectron Microscopic Examination

The carious and noncarious pulp samples were cut into small pieces and fixed with 4% PFA/0.1% glutaraldehyde in 0.1 M PBS for 2 hours at room temperature. The tissues were then washed with 0.1 M PBS, dehydrated in graded ethanol and embedded in LRWhite resin (Polysciences, Berkshire, UK).²⁹ Ultrathin sections of embedded materials were cut, mounted on nickel grids and processed for immunohistochemistry.³⁰ Briefly, antigen retrieval was performed by immersion of grids bearing sections in 0.1 M sodium citrate buffer, pH 6.0, at 95°C for 10 minutes. Sections were left to cool to room temperature for 20 minutes in the citrate buffer, jet-washed (deionized water), immersed in drops of 0.5 M NH₄Cl in 0.1 M PBS, pH 7.3, for 20 minutes and then washed for 5 minutes in PBS, pH 7.3, containing 1% bovine serum albumin and 0.1% Tween 20 (washing buffer). After blocking with 10% normal goat serum, sections were incubated at 4°C overnight on a drop of a solution of polyclonal anti-GFAP antibody (Dako) diluted 1/20 with PBS containing 1% ovalbumin. Immuno-Gold labeling was performed using the Leica IGL processor (Leica Microsystems). After washing with PBS, sections were exposed to anti-rabbit IgG antibody conjugated with colloidal gold particles of 10-nm diameter (Biocell International, Medford, MA), diluted with PBS (1/40), for 2 hours at room temperature. Ultrathin sections were stained with uranyl acetate and lead citrate and

Figure 1. **A:** Photograph of a split tooth demonstrating the carious lesion and the regions of pulp adjacent to carious lesion (A-site) and remote from that (R-site). **B:** A-site showing physiological dentin (top) with a regular tubular structure (arrowhead), and reactionary dentin (bottom) marked with irregularity and scarcity of dentinal tubules (Toluidine blue staining). **C:** A-site showing Acridine orange staining of bacteria (orange-red fluorescence) within the dentinal tubules (green staining), demonstrating accumulation at the interface of physiological and reactionary dentin with few penetrating the reactionary dentin. Specificity for bacteria was confirmed by fluorescence ISH staining using a universal bacterial amplicon on an adjacent section.³¹ In addition this pattern of staining was not observed in healthy teeth. **D:** Toluidine blue staining of dental pulp (from A) with the odontoblastic layer, cell-free zone, and central pulp. Note the absence of inflammatory infiltrate. **E:** Toluidine blue staining of odontoblasts in the A-site of the carious tooth (A) demonstrating plump nuclei and interodontoblastic cells (arrowhead) with small nuclei and granular cytoplasm (inset). **F:** Toluidine blue staining of interodontoblastic granular cell extending fine processes (arrowhead). A, A-site; R, R-site; Od, odontoblast; Cf, cell-free zone; Cr, cell-rich zone; Cp, central pulp; Pd, physiological dentin; Rd, reactionary dentin; and Gc, interodontoblastic granular cell.

examined in a Phillips CM10 electron microscope. Specificity controls were carried out by incubating sections with rabbit IgG negative control antibody.

Statistical Analysis

SPSS statistical software (version 16; SPSS, Chicago, IL) was used for the statistical analysis of data. Log transformation of data were performed to normalize the distribution of data. The relative expression levels of *dspp*, *dmp-1*, and *tgf-β1* at A-site, R-site and H-site was compared using univariate analysis of variance. Site-specific intensity of TGF-β1 staining in carious and healthy teeth was compared using the nonparametric Mann-Whitney *U*-test. In the present study, $P < 0.05$ was considered as statistically significant.

Results

Histological Evaluation of Pulp-Dentin Complex

In light microscopic observation, extensive deposition of reactionary dentin by odontoblasts adjacent to the site of carious lesion (A-site) was evident (Figure 1, A and B). Comparison of structure showed distinct differences between physiological and reactionary dentin. Irregular structure was a common feature of reactionary dentin as opposed to the regular tubular structure of the physiological dentin (Figure 1B). The number of tubules in physiological dentin was 20-fold more than reactionary dentin. The degree of anisotropy of the tubular structure of reactionary dentin was 5 times that of physiological dentin (19.17 vs. 4.18). Bacteria were observed traversing the major and accessory dentinal tubules in physiological dentin. Notably, accumulation of bacteria was evident at the border of physiological and reactionary dentin, while few bacteria were detected within the reactionary dentin (Figure 1C).³¹ Of these, none were detected in the inner half of the reaction-

ary dentin and in the vicinity of the odontoblasts. The apparent resistance of reactionary dentin to bacterial insult, reflecting its modified structure, was further investigated. Modulation of calcified tissue matrix was suggestive of radical functional adaptations of odontoblasts. Thus, we sought to determine these adaptations.

Three distinct layers were readily observed at the periphery of the pulp; an intact odontoblastic/subodontoblastic layer, cell-free zone, and a cell-rich zone (Figure 1, D and E). Although there was no evidence of inflammatory infiltrate at this stage of lesion development (Figure 1, D and E) several anatomical features of soft tissue changed at the site of bacterial insult. Odontoblasts adjacent to the carious site displayed irregular nonparallel arrangement. Two different cell types were observed within the odontoblastic layer, odontoblasts with bright non-granular cytoplasm and large nuclei and cells with granular cytoplasm and ramified structure located within the odontoblastic layer (granular cells) (Figure 1E, inset). In the carious site, interodontoblastic granular cells were more dispersed and had migrated toward the dentin extending fine processes to contact adjacent cells (Figure 1F). The disruption of planar polarity of odontoblasts and altered calcified matrix formation by these cells was investigated by molecular techniques.

Profiling of the Genes Involved in Formation of Dentin

DSP (a protein product of *dspp*) and DMP-1 were expressed in odontoblasts of healthy teeth (Figure 2, A and B). In carious teeth, while DSP staining was observed in odontoblasts both in A-site and R-site (Figure 2C), trace staining for DMP-1 was found only in the R-site (Figure 2D).

In ISH assessment of the carious pulp samples, no signal was present for DMP-1 mRNA in either carious or noncarious sites. However, strong expression of DMP-1

Figure 2. **A:** Immunohistochemical localization of DSP (**A**) and DMP-1 (**B**) in odontoblasts of a healthy tooth. (4',6'-diamidino-2-phenylindole, blue fluorescence counterstaining). DSP (**C**) and DMP-1 (**D**) immunostaining in the A-site of a carious tooth. **E:** FISH identification of *dspp* expression in odontoblasts (green fluorescence) also demonstrating nonreactive interodontoblastic cells. Specificity of staining for mRNAs was confirmed by controls (see *Materials and Methods*). **F:** Expansion of inset in **E** indicating odontoblasts (arrow) and interodontoblastic cells (arrowhead). **G:** The relative expression of *dspp* and *dmp-1* in healthy teeth and A- and R-sites of carious teeth. Showing pooled data ($n = 4$). Error bar represents SEM; ** $P < 0.0001$. Od, odontoblast; Cf, cell-free zone; and Rd, reactionary dentin.

mRNA, limited to the odontoblastic layer, was observed in noncarious teeth (data not shown). ISH staining of mRNA from *dspp* demonstrated strong expression in odontoblasts at the A-site (Figure 2E). A weaker signal was present in odontoblasts in the R-site of carious teeth and in healthy teeth. Uniform expression of mRNA from *dspp* was observed in odontoblasts of healthy teeth, whereas interodontoblastic granular cells did not express *dspp* mRNA (Figure 2, E and F). Site-specific difference in expression of *dspp* and *dmp-1* was verified by microdissection and quantitative RT-PCR.

Transcriptional activities of *dspp* and *dmp-1* in microdissected regions were evaluated using real-time RT-PCR. For accurate comparison of *dspp* and *dmp-1* expression levels between A-site, R-site and healthy controls, the housekeeping gene β -actin was used as an internal calibrator gene to control for varying levels of mRNA in each sample. Expression of *dspp* in the odontoblasts of A-sites was 16-fold greater than for R-sites and healthy teeth ($P < 0.0001$) (Figure 2G). Eta for this difference was 0.52, reflecting a large effect size. However, the expression level of *dspp* was not significantly different between odontoblasts in R-sites and healthy teeth ($P = 0.577$). Compatible with ISH findings, expression of *dmp-1* was completely suppressed in all pulpal regions of carious teeth, in contrast to uniform expression of this gene in healthy teeth (Figure 2G). Differential expression patterns for *dspp* and *dmp-1* suggested modulation of regulatory mediators. TGF- β 1 is the best described me-

diator of odontoblast function.¹⁹ Accordingly, the expression and distribution of this mediator was analyzed.

Microanatomical Distribution of TGF- β 1

Histologically, TGF- β 1 was expressed in odontoblasts of carious and healthy teeth. In healthy teeth, expression of TGF- β 1 was observed in odontoblasts as well as central pulp stromal cells and endothelial cells of blood capillaries. However, expression of TGF- β 1 protein was stronger in the odontoblastic layer compared to the central pulp (Figure 3A).

In carious teeth, TGF- β 1 protein showed a gradient mode of expression; weak in the odontoblasts of the A-site and increasing in intensity toward the R-site (Figure 3, A–C). This gradient mode of TGF- β 1 expression was not observed in healthy teeth. Comparison of the average staining intensity between carious and healthy teeth demonstrated significant difference in the A-site, which gradually disappeared toward the R-site (Figure 3, B and C).

At the transcriptional level, expression of *tgf- β 1* in both the A- and R-sites of carious teeth was 12- and sixfold less, respectively, than that of healthy control sites ($P < 0.0001$) (Figure 3D). Eta for this difference was 0.55, a large effect size. The expression of message for this growth factor at the A-site was 14.2% less than for R-sites. However, this difference did not reach statistical significance ($P = 0.2$).

Figure 3. **A:** Immunohistochemical localization of TGF- β 1 in the pulp of a carious tooth. TGF- β 1 is expressed mainly by odontoblasts in a gradient mode decreasing from the R-site to the A-site. **Inset** shows double staining of dotted region for TGF- β 1 and nuclear 4',6'-diamidino-2-phenylindole (blue fluorescence). **B:** Schematic representation of a gradient mode of expression of TGF- β 1 in ROI. **C:** Statistical analysis of TGF- β 1 comparing immunolabeling intensity of healthy versus carious teeth from A-sites to R-sites ($n = 4$). $*P < 0.05$. **D:** The relative expression of *tgfbeta1* in healthy teeth and A- and R-sites of carious teeth showing pooled data ($n = 4$). Error bar represents SEM; $**P < 0.0001$. A, A-site; R, R-site; and cp, central pulp.

Characterization of Interodontoblastic Cells

Expression of vimentin was observed throughout the odontoblastic layer in healthy teeth and carious teeth (Figure 4A). Weak expression of GFAP was observed in granular DSP⁺ cells in healthy teeth with more intense reaction detected in carious teeth (Figure 4B). Expression of GFAP (Figure 4C) was colocalized with GS (Figure 4D) in granular DSP⁺ cells in healthy and carious teeth. In carious teeth, interodontoblastic DSP⁺ granular cells showed expression of GFAP in a gradient mode with stronger signal in A-sites. GFAP and vimentin were not closely colocalized.

Figure 4. **A:** Expression of vimentin in odontoblasts and interodontoblastic cells in the A-site of a carious tooth. Note extension of vimentin filaments into the odontoblastic process within dentin (arrowhead). **B:** GFAP staining of interodontoblastic cells, extending between the odontoblasts in the A-site of a carious tooth. Note the lack of staining in odontoblast process (arrowhead). **C:** Coincidence of staining (arrowheads) for GFAP (C) and glutamine synthetase (D) in a healthy tooth. od, odontoblast; and rd, reactionary dentin.

Histological and Quantitative Study of Junctional Complexes

Disturbed planar polarity and population of the odontoblastic layer by granular cells was indicative of alteration of intercellular attachment complexes. Further investigation of junctional complexes revealed major differences in expression of these proteins in A and R-sites. Occludin was detected between odontoblasts in healthy teeth and in R-sites of carious teeth (Figure 5A). However, in A-sites of carious teeth, occludin was down-regulated suggesting lack of functional tight junctions at these sites (Figure 5B). N-cadherin was expressed in odontoblasts of carious teeth only (Figure 5C). In healthy teeth, moderate staining of connexin 43 was observed (Figure 5D). There was no detectable expression of connexin-32 (Figure 5E) or connexin-26 (Figure 5F) in the odontoblastic layer of healthy teeth. Connexins 26, 32, and 43 exhibited different patterns of expression in the A and R-sites of carious teeth (Figure 5, G–I). In carious teeth, connexin 43 was abundantly expressed and connexins 32 and 26 to a lesser extent (Figure 5, G–I). In carious teeth, connexin 43 expression was observed both in granular (GFAP⁺/GS⁺ that did not express *dspp*) cells and odontoblasts (expressing *dspp*) and was associated with the formation of a network of granular cells (Figure 5, J–L). These findings are summarized in Table 3.

Message for *ocln* (occludin) in the odontoblasts of healthy teeth was threefold greater than for R-sites ($P < 0.001$) (Figure 6). Message for *ocln* was not detected in the A-site. Message for *cdh2* (N-cadherin) was not detected in odontoblasts of healthy teeth. Expression of *cdh2* in the A-site was 1.5-fold greater than for R-site ($P < 0.01$). Expression of *gjb2* (connexin-26) and *gjb1* (connexin-32) was not detected in odontoblasts of healthy teeth. Although there was no significant difference in expression of *gjb1* between odontoblasts in A-site and

Figure 5. **A:** The expression of occludin (arrowhead) in the R-site. **B:** Lack of expression of occludin in A-site. **C:** Peripheral expression of N-cadherin in odontoblasts in the R-site of a carious tooth (arrowhead). **D:** Expression of connexin-43 in the odontoblastic layer of a healthy tooth. Lack of expression of connexin-32 (**E**) and -26 (**F**) in the odontoblastic layer of a healthy tooth. Expression of connexin-43 (**G**), -32 (**H**), and -26 (**I**) in the odontoblastic layer of the A-site of a carious tooth. **J:** After ISH identification of *dspp* expression in odontoblasts and nonexpressing interodontoblastic cells in the A-site of a carious tooth, a region was chosen at random and the outlines of these two cell types overlaid with connexin-43 (black dots), -32 (yellow dots), and -26 (red dots) from sequential 1-μm sections stained for these markers (**K**). Note the presence of connexin-43 in a network of interodontoblastic cells that do not express *dspp* (black arrowheads), odontoblastic-odontoblastic connections (blue arrow) and odontoblastic-interodontoblastic communications (black arrow). **L:** Schematic drawing representing connexin-43 (black dots), -32 (yellow dots), and -26 (pink dots) expression in **K**. Note the networks of GFAP⁺ (pink) cells (black arrowheads). Od, odontoblast; and Cz, cell-free zone.

R-site ($P = 0.448$), *gjb-2* was not detected in the A-site of carious teeth. Expression of *gja1* in odontoblasts of the A-site was 2.3- and 11.6-fold greater than for odontoblasts at R-site and H-site, respectively (Figure 6).

Ultrastructural and Immunoelectron Microscopic Analysis

Semithin sections stained with Toluidine blue indicated structural changes in odontoblastic and interodontoblastic cells in carious teeth. The odontoblasts in the A-site were ensheathed by slender processes of interodontoblastic cells rich in intermediate filaments (Figure 7, A–C). These processes were in close association with odontoblasts through specialized junctions (Figure 7B). Immu-

noreactivity for GFAP was present in granular cell processes surrounding odontoblasts in both carious and noncarious teeth (Figure 7D). However, the labeling density of the processes was higher in carious teeth.

Discussion

Despite the protracted and variable course of caries progression patterns of reactivity observed in this study were consistent and uniform, indicating a strongly imprinted program of adaptation. Using a high-resolution, quantitative approach, it was possible to dissect the characteristic defensive response of this calcified tissue to microbial invasion. The primary indicator was the dep-

Table 3. Summary of Expression of Junctional Complex Proteins in Healthy and Carious Teeth (n = 4)

	Location	Healthy	A-site	R-site
Occludin	Odontoblast	+	–	+
	Interodontoblastic cell	–	–	–
N-cadherin	Odontoblast	–	+	++
	Interodontoblastic cell	–	+	++
Connexin-43	Odontoblast	+	++	+++
	Interodontoblastic cell	+	++	+++
Connexin-32	Odontoblast	–	++	++
	Interodontoblastic cell	–	+	+
Connexin-26	Odontoblast	–	+	+
	Interodontoblastic cell	–	+	+

–, no staining; +, trace staining; ++, moderate staining; and +++, intense staining.

Figure 6. The relative expression of occludin (*ocln*), N-cadherin (*cdh2*), connexin-26 (*gjb2*), connexin-32 (*gjb1*), and connexin-43 (*gja1*) in healthy teeth and in A- and R-sites of carious teeth showing pooled data ($n = 4$). Error bar represents SEM; ** $P < 0.01$.

osition of organic matrix and its subsequent mineralization to an anisotropic structure that effectively limited progression of bacteria toward the pulp. Inherent in this response was a gradient of deposition of reactionary dentin. Granular cells of the odontoblastic layer, which expressed GFAP and GS, demonstrated up-regulation of connexin 43 in carious teeth. Study findings are summarized in Figure 8.³²

Bacterial invasion of dentin is accompanied by the disintegration of dentin matrix and release of bacterial products which stimulate odontoblasts mainly through

Figure 7. **A:** Electron micrograph of the A-site of a carious tooth, demonstrating intimate association of an interodontoblastic process with an odontoblast. **B:** Specialized junction (arrow) between an interodontoblastic process rich in intermediate filaments and an odontoblast. **C:** Interodontoblastic cell with several slender processes rich in intermediate filaments. **Inset** refers to higher magnification of region marked by arrow. **D:** Immunogold labeling of GFAP in interodontoblastic cell processes equivalent to those in Figure 7C. Od, odontoblast; and P, interodontoblastic cell process.

interaction with pattern recognition receptors, for instance Toll-like receptor-2.³³ However, the cascade of events following stimulation of odontoblasts had not been described previously. The relatively atubular and disorganized nature of reactionary dentin appeared to limit bacterial invasion. Furthermore, dentinal tubules in reactionary dentin demonstrate projections of mineralized tissue protruding into the lumen.³⁴ These findings indicate the importance of occluding dentinal tubules to impede bacterial invasion. The mechanism underlying successful adaptation by odontoblasts to the challenge of encroaching bacteria via modulation of dentin structure implicates radical modification of dentin organic matrix.

Although *dspp* expression is substantially up-regulated, *dmp-1* expression is profoundly down-regulated in odontoblasts adjacent to the site of carious insult. This is supported by the findings of McLachlan et al³⁵ who demonstrated increased immunostaining for DSP and DPP (protein products of *dspp*) in carious teeth. An important aspect of successful functional adaptation of odontoblasts to encroaching bacteria, enhanced deposition of dentin barrier, necessitates increased uptake of calcium ions into dentin extracellular matrix through up-regulation of *dspp*. Overexpression of *dspp* promotes mineralization in adipose-derived stromal cells.³⁶ Moreover, overexpression of DSP results in an increased rate of enamel mineralization.³⁷ In dentinogenesis imperfecta type II mutation of *dspp* results in decreased mineralization of tooth structure evident as attrition of teeth.³⁸ DMP-1 can undergo oligomerization in solution and temporarily stabilize newly formed calcium phosphate nanoparticle precursors by sequestration to prevent further aggregation and precipitation.³⁹ This function is important in regulation of microanatomical features of physiological dentin such as maintaining patency of dentinal tubules. Following bacterial insult, down-regulation of DMP-1 could lead to partial or complete obstruction of tubular structure of reactionary dentin and enhanced deposition of apatite, to limit dissemination of bacteria. The relatively disorganized nature of reactionary dentin could be explained by DSP/DPP-mediated acceleration of mineralization and down-regulation of DMP-1 with resultant alteration of anatomical features in reactionary dentin.

Expression of *dspp* and *dmp-1* has been reported in a variety of tissues including but not limited to, bone.^{32,40,41} Products of *dspp* regulate both initial mineralization and remodelling phases in bone.⁴² DMP-1 plays an important role in skeletal mineralization through regulation of osteocyte maturation and phosphate homeostasis.⁴³ It has been demonstrated in *dmp1*-null mice that osteocytes exhibit randomly oriented lacunae with marked abnormalities in the distribution and organization of the osteocyte-lacuno-canalicular system.⁴³ This anisotropic bone formation associated with down-regulation of *dmp-1* is equivalent to anisotropic reactionary dentinogenesis accompanying suppression of *dmp-1* as observed in the present study.

TGF- β 1 has been implicated as a major regulator of reactionary dentinogenesis.¹⁹ Our findings demonstrated significant down-regulation of *tgf- β 1* expression (12-fold) at the A-site of carious teeth. Down-regulation was also

Figure 8. Schematic representation showing response of pulpal cells to invading bacteria. Healthy tooth: processes of the odontoblasts occupy dentinal tubules. Odontoblasts express both *dmp-1* (red granules) and *dspp* (blue granules). There is also strong expression of *tgf-β1* in these cells (black granules). Intercellular connections between odontoblasts include occludin (blue) and connexin-43 (green). Odontoblasts also connect to interodontoblastic cells through connexin-43. No intercellular connexins detected in interodontoblastic granular cells. These cells show moderate reactivity for GFAP (yellow filaments). *A-site*, carious tooth: zone of invasion by bacteria³² and extensive deposition of reactionary dentin (orange). Tubules show retracted odontoblastic processes. Marked upregulation of *dspp*; no detectable expression of *dmp-1*. No detectable staining for occludin. Reactivity in odontoblasts for N-cadherin (red) and connexins 43 (green), 32 (yellow), and 26 (orange). Evidence for intercellular communication in interodontoblastic granular cells mediated by expression of connexin-43. Prominent GFAP⁺ staining (yellow filaments) in interodontoblastic cells. *R-site*, carious tooth: no invasion by bacteria; reduced deposition of reactionary dentin. Expression of *dspp*; no detectable expression of *dmp-1*. Preservation of intercellular adhesion. Evidence for intercellular communication in interodontoblastic granular cells mediated by expression of connexin 43 (green).

evident in *R*-sites of carious teeth, indicating pan-inhibition of TGF-β1 in pulps of carious teeth. There is evidence that TGF-β1 down-regulates *dspp* expression in odontoblasts.⁴⁴ Therefore, there is potential for the observed down-regulation of TGF-β1 to contribute to increased mineralization of dentin extracellular matrix via enhancement of transcriptional activity of *dspp*. Likewise, it has been reported that transgenic mice expressing a cytoplasmically truncated type II TGF-β receptor develop an age-dependent increase in trabecular bone mass.⁴⁵ A lateral gradient of expression throughout the odontoblastic layer was observed for TGF-β1 and *dspp* in carious teeth. This finding reflects a pan-tissue response to encroaching infection. The response was localized to the odontoblastic layer, indicating that a local signal transmission network could be responsible for the gradient of deposited reactionary dentin observed.

The expression of GFAP in Schwann cells and undifferentiated stem cells of the central pulp has been reported previously.⁴⁶ Interodontoblastic granular cells which do not express *dspp* demonstrate low level GFAP expression in healthy pulp. Low expression of GFAP in physiological conditions⁴⁷ and difficulties inherent in detection of the protein⁴⁸ contribute to false negative results. Up-regulated expression of GFAP in granular cells associated with extension of processes of these cells occurred in the context of carious insult. Chen et al⁴⁹ demonstrated that GFAP expression is necessary for the extension of processes of glial cells in central nervous system tissue. Also, GFAP expression is essential to maintain integrity of Müller glial cell processes in retina.⁵⁰ Thus, we hypothesize that up-regulation of GFAP in inter-

odontoblastic cells functions similarly to maintain integrity of elongated processes. Several connexins were identified in odontoblasts and adjacent interodontoblastic granular cells with distinct patterns of expression in carious versus non-carious teeth. There was marked increase in expression of connexins in interodontoblastic cells in carious teeth with connexins 32 and 26 detected as signatures following bacterial invasion. Connexin 43 is expressed during development of human teeth and is subsequently down-regulated.⁵¹ Trauma-induced up-regulation of connexin 43 has also been reported in a variety of other tissues.⁵² Fried et al⁵³ demonstrated that this gap junction protein was confined to sites of contact between adjacent odontoblasts. In addition, coupling of interodontoblastic cells by odontoblasts through gap junctions has been documented by freeze-fracture studies.^{13,54} There is evidence that expression of N-cadherin is associated with up-regulation of connexin 43 and formation of gap junctions⁵⁵ in agreement with findings of the present study.

Connexin 43 is the most abundant gap junction protein in skeletal tissue.⁵⁶ Lack of this protein causes a generalized osteoblast dysfunction, leading to delayed mineralization and skull abnormalities.⁵⁷ Mutation of connexin 43 presents with craniofacial (ocular, nasal, and dental) and limb dysmorphisms, spastic paraplegia, and neurodegeneration characteristic of oculo-dento-digital dysplasia.⁵⁸ Conversely, transplantation of bone marrow stromal cells over-expressing connexin 43 resulted in an increased volume fraction and spatial uniformity of bone *in vivo*.⁵⁹ N-cadherin is likewise involved in osteogenesis through controlling the expression of osteoblast gene

expression and differentiation.⁶⁰ Hence, marked upregulation of connexin 43 and N-cadherin in odontoblasts of carious teeth may function in a similar mode to enhance dentinogenic potential.

A feature of the odontoblastic layer in A-sites of carious teeth was the separation of odontoblasts evident by disrupted planar polarity associated with down-regulation of occludin. This separation could be protective as blocking of gap junctions in neurons has been shown to reduce the size of infarcts after focal ischemia,⁶¹ and down-regulation of occludin desensitizes odontoblasts to proapoptotic stimuli.⁶²

Expression of connexin 43 in a plaque-like pattern suggested functional gap junctions and coupling of odontoblasts, rather than hemi-channel formation.⁶³ In carious teeth, separation of odontoblasts evident as down-regulation of occludin, combined with prominent expression of connexin 43 by the interodontoblastic cells, indicated a potential for transmission of signals through the network. An analogous situation is the expression of this protein in brain where initially glial cells couple to neurons through connexins.⁶⁴ Following injurious stimuli, glial cells express connexin 43 homo-hexamers to form functional gap junctions at the site of injury and disseminate gliotransmitters to adjacent glia.⁶⁵ In a similar manner, Müller glial cells underlying retinal photoreceptors respond to injurious stimuli by forming networks that transmit signals to adjacent glial cells in a gradient mode.^{66,67} Therefore, we postulate that signal transmission occurs through networks of interodontoblastic GFAP⁺/GS⁺ cells mediated via connexin 43 channels to cause modulation of gene expression profiles in odontoblasts in response to infection. In odontoblasts, strong expression of glutamate receptors implicates glutamate as a potential mediator.⁶⁸

In this study evidence is presented for anatomical and functional properties of interodontoblastic GFAP⁺/GS⁺ cells resembling those of Müller glial cells,^{66,69} olfactory ensheathing cells⁷⁰ and supporting cells in the organ of Corti.⁷¹ The current consensus is that glia in these craniofacial sites are derived from neural crest cells.^{72,73} Although these glial cells support sensory structures in neural tissues there is also evidence for a sensory function of odontoblasts.⁷⁴ We propose the term craniofacial, extracerebral, neural crest derivative, sustentacular assemblage to describe glial networks reflecting common developmental, phenotypic and functional properties in these extracerebral craniofacial tissues.

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