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Inflammatory mediators in fluid extracted from the coronal occlusal dentin of trimmed teeth

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Abstract

Background—Chemokines and cytokines may occur in dentinal fluids in response to local infection and inflammation. To test this hypothesis, we assessed the presence and concentration of inflammatory mediators in fluid extracted from the coronal occlusal dentin of trimmed teeth.

Design—Freshly extracted sound, carious, and restored molars were trimmed through the enamel to expose the underlying dentin, etched with 35% phosphoric acid, and rinsed. Fluid was extracted from the coronal occlusal dentin of these trimmed teeth by centrifugation at $2,750 \times g$ for 30 minutes.

Results—When assessed by MALDI-TOF, fluid extracted from the coronal occlusal dentin from 16 molars contained at least 117 peaks with different masses suggesting that this fluid was rich

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Competing interests

None.

Ethical approval

Informed consent was obtained from each subject in accordance with guidelines established by the University of Iowa Institutional Review Board.

with molecules within the appropriate mass range of potential mediators. Indeed, when assessed for chemokines and cytokines, fluid extracted from the coronal occlusal dentin from 25 extracted molars with caries lesions, 10 extracted restored molars with occlusal amalgam, and 77 extracted sound molars contained IL-1 β , TNF- α , IL-6, IL-8, IL-12(p70), and IL-10. A significant elevation was found for TNF- α (p=0.041) in extracted fluid from teeth restored with amalgam fillings.

Conclusions—Overall, fluid extracted from the coronal occlusal dentin of trimmed teeth may be useful in identifying proteins and other molecules in dentin and pulpal fluids and determining their role as mediators in the pathogenesis of oral infection and inflammation.

Keywords

Dental fluid; Chemokines; Cytokines; MALDI-TOF

1. Introduction

Dentin has a blood supply to maintain its physiological needs and such supply ends in the pulp chamber. However, this lack of supply is compensated by the fluid flow transport mechanism orchestrated by odontoblasts that perfuses the dentin with dentinal fluid and delivers essential nutrients generated from the pulpal tissue metabolism¹. Therefore, dentinal fluid is the transudate of extracellular fluid that appears on the dentin surface of a freshly non-cut, cut, or fractured tooth. As the 'lymph' or fluid of dentin, it originates from pulpal blood vessels and moves from the dental pulp via the dentinal tubules. An extensive number of early and recent studies have assessed dentinal fluid composition *in vitro* and *in vivo*²⁻¹⁴. The outward/inward flux of dentinal fluid¹⁵⁻¹⁷, and the association of changes in dentinal fluid contents following odontoblast aspiration and transmission of pain-producing stimuli through the dentin have been well documented¹⁸. Dentinal fluid is generally considered to be a plasma-derived fluid containing serum proteins and immunoglobulins^{14, 19, 20} and therefore likely contains a number of important mediators, including chemokines, cytokines, and defensins^{19, 21, 22}. Since enamel is permeable^{23, 24}, it is reasonable to assume that such a fluid can be considered as a tooth self-defense mechanism against external injury. However, the presence and concentration of inflammatory mediators have been scantily presented in the literature.

In this study, we sought to determine the presence and concentration of inflammatory mediators in the fluid extracted from the coronal occlusal dentin of trimmed teeth. The presence of these mediators may be useful in identifying their role in the pathogenesis of oral infection and inflammation.

2. Materials and Methods

2.1. Subject recruitment

One hundred-forty nine molars were collected from male and female subjects that had their third molars extracted as part of their routine dental treatment in the Oral Surgery Department at the College of Dentistry of the University of Iowa. All subjects were systemically healthy and had not taken antibiotics during the six months prior to the extraction. Informed consent was obtained from each subject in accordance with guidelines established by the University of Iowa Institutional Review Board.

Extracted sound molars from 21 subjects were used in a preliminary study to extract fluid from the coronal occlusal dentin of trimmed teeth. Fluids extracted from an additional 16 sound molars were examined by Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) to determine the presence of molecules within the appropriate mass

range of potential mediators. Finally, fluids extracted from 25 molars with incipient or occlusal caries lesions (Group A), 10 restored molars with occlusal amalgam (Group B), and 77 sound molars (Group C) were assessed for the presence of chemokines and cytokines using a commercial multiplexed fluorescent bead-based immunoassay.

2.2. Extraction of fluid from the coronal occlusal dentin of trimmed teeth

In a preliminary study, fluid was extracted from 21 sound third molars. The occlusal crown and the roots of extracted molars were trimmed in an orthodontic model trimmer (Whip Mix Corp., Louisville, KY) to expose the coronal occlusal dentin (Figure 1a). The coronal occlusal dentin was treated with phosphoric acid (35%) for 5 seconds to open dentinal tubules and facilitate outward flow, rinsed for 10 seconds, and dried with air. The trimmed molar was placed with the coronal occlusal dentin surface down in a 15 ml conical centrifuge tube and centrifuged at $2,750 \times g$ for 30 minutes at 4°C (Figure 1b). The molar was then removed and discarded. The fluid extracted from the coronal occlusal dentin of trimmed teeth pooled in the bottom of the tube and was easily collected using a Pipetman P10 pipettor (Gilson, Middleton, WI). Simultaneously during collection, the volume was determined.

2.3. MALDI-TOF

MALDI-TOF was determined as previously described²⁵ on fluid extracted from 16 sound third molars. Briefly, cyano-4-hydroxycinnamic acid (CHCA, 20 mg/ml) was dissolved in a mixture (1:1) of isopropanol:acetone containing cellulose nitrate (10 mg/ml) and added directly to the MALDI plate to create a thin, base layer. The purpose of this layer is to increase the sensitivity. The entire volume of fluid extracted from the coronal occlusal dentin of trimmed teeth or standards were mixed with CHCA (in 1:1 ratio of acetonitrile and 0.1% trifluoroacetic acid, 20 mg/ml) and spotted on the top of the thin layer of CHCA in isopropanol:acetone and cellulose nitrate. Angiotensin II (1,046.54 m/z) and ACTH (18-39) (2,465.198 m/z) were used as standards to calibrate the instrument. Spots were air dried and analyzed in a Biflex III MALDITOF (Bruker Daltonics Inc., Billerica, MA USA). This matrix generates a plethora of lower mass ions. Therefore, the lower mass range was gated from 500 m/z. The upper limit was near 22,172.311 m/z.

2.4. Chemokine and cytokine determination

The entire volumes of fluid extracted from the coronal occlusal dentin of trimmed teeth of molars in groups A, B, and C were mixed into 300 μl of 0.01 M sodium phosphate buffer, pH 7.2 containing 0.14 M sodium chloride (0.01 M PBS, pH 7.2). This procedure is comparable to our previous method where gingival crevicular fluid collected on periopaper for 30 seconds was placed in 300 μl of 0.01 M PBS, pH 7.2 and the results reported as pg/30 seconds^{26, 27}. Concentrations of pro-inflammatory cytokines IL-1 β , TNF- α , and IL-6; chemokine CXCL8 (IL-8); Th1 cytokine IL-12(p70); and Th2 cytokine IL-10 were determined using a commercial multiplexed fluorescent bead-based immunoassay (Millipore, Billerica, MA) in the Luminex 100 IS Instrument (Luminex, Austin, TX) as previously described^{26, 27}. Briefly, 50 μl of 0.01 M PBS, pH 7.2 containing extracted fluid were incubated with anti-human multi-cytokine beads at 4°C for 18 hours. Unbound material was removed by filtration. Anti-human multi-cytokine biotin reporter was added, and reactions were incubated at room temperature for 1.5 hours in the dark. Streptavidin-phycoerythrin then was added, and the plates were incubated at room temperature for an additional 30 minutes. Stop solution was added, and the plates were read in the plate reader (Luminex model 100 IS, Austin, TX). Standard curves for each cytokine were prepared from 2.3 to 5,000 pg/ml. Concentrations of chemokines and cytokines in each sample were interpolated from standard curves with Beadview software (Millipore, Billerica, MA).

Results were reported as pg/ml of chemokine or cytokine in the 300 μ l of 0.01 M PBS, pH 7.2.

2.5. Statistical analysis

A log transform was applied to the cytokine amounts in subject extracted fluid samples. Differences among chemokines and cytokines in fluid extracted from the coronal occlusal dentin of trimmed teeth from 25 molars with incipient or occlusal caries lesions (Group A), 10 restored molars with occlusal amalgam (Group B), and 77 sound molars (Group C) were compared using a one-way ANOVA and Kruskal-Wallis test. A difference was declared statistically significant if the corresponding p-value was less than the 0.05 level of significance.

3. Results

3.1. Volume of extracted fluid

In the preliminary study, the volume of the extracted fluid (n=21) was $1.1 \mu\text{l} \pm 0.1 \text{ SEM}$ (range 0.2 - 2.0 μ l). These volumes were similar to the volumes of collected gingival crevicular fluid and allowed us to use previously developed methodologies^{26, 27} to determine the presence and concentration of chemokines and cytokines in the fluid extracted from the coronal occlusal dentin of trimmed teeth.

3.2. MALDI-TOF profiles

To determine the diversity of components, we examined the fluid extracted from 16 sound molars by MALDI-TOF. MALDI-TOF uses desorption ionization to produce intact molecular ions (often $M + H^+$ or $M + Na^+$) of large biological compounds. Extracted fluids contained at least 117 peaks with different masses from 566.744 to 22,172.311 m/z, with many common peaks among the samples. For example, 5 extracted fluids had less than 10 peaks (Figure 2a); 8 extracted fluids contained 11-20 peaks; 2 extracted fluids contained 21-30 peaks; and one extracted fluids contained greater than 31 peaks (Figure 2b). Fluid extracted from the coronal occlusal dentin of trimmed teeth had many components of similar masses. For example, 4 extracted fluids had a component of 566.744-566.965 m/z; 9 extracted fluids had a component of 649.258-654.963 m/z; and 6 extracted fluids had components with masses of 864.839-865.414 m/z, 1,075.653-1,076.440 m/z, 1,286.131-1,287.401 m/z, and 1,496.358-1,498.383 m/z. Similarly, 7 extracted fluids had a component of 1,048.656-1,050.500 m/z; 8 extracted fluids had a component of 1,612.008-1,618.357 m/z; 4 extracted fluids had a component of 3,182.774-3,183.610 m/z; 4 extracted fluids had a component of 3,443.367-3,444.410 m/z; and 7 extracted fluids had a component of 6,365.742-6,368.748 m/z.

3.3. Chemokine and cytokine concentrations

In the preliminary study, we added the fluid extracted from the coronal occlusal dentin of trimmed teeth ($1.1 \mu\text{l} \pm 0.1 \text{ SEM}$, range 0.2 - 2.0 μ l) directly to 300 μ l 0.01 M PBS, pH 7.2. This was a step that worked well when determining chemokines and cytokines in 1-2 μ l of gingival crevicular fluid in our prior studies^{26, 27}. In the extracted fluids from 21 sound molars, we detected pro-inflammatory cytokines IL-1 β ($3.4 \text{ pg/ml} \pm 0.5 \text{ SEM}$, range 2.2-12.7), TNF- α ($8.3 \text{ pg/ml} \pm 0.1 \text{ SEM}$, range 7.2-9.0), and IL-6 ($9.5 \text{ pg/ml} \pm 1.8 \text{ SEM}$, range 1.3-34.3); chemokine CXCL8 (IL-8) ($18.0 \text{ pg/ml} \pm 7.2 \text{ SEM}$, range 7.4-147.3); Th1 cytokine IL-12(p70) ($18.2 \text{ pg/ml} \pm 0.2 \text{ SEM}$, range 17.1-20.5); and Th2 cytokine IL-10 ($10.8 \text{ pg/ml} \pm 0.1 \text{ SEM}$, range 10.3-12.1).

To test the hypothesis that fluid extracted from the coronal occlusal dentin of trimmed teeth from subjects with carious lesions or existing restorations would have different levels of

inflammatory mediators, we expanded our study and examined fluid extracted from an additional 112 molars. Test of normality of the cytokine data showed evidence that the cytokine values do not have a normal distribution. Applying the log transform to the data removed excess positive skewedness, but did not eliminate other irregularities (e.g. the appearance of bimodal distribution) for most variables. Since normality assumption for use of one-way ANOVA was not met, comparison of cytokine levels among extracted fluid from 25 upper molars with incipient occlusal caries lesions (Group A); 10 molars with occlusal amalgam restorations (Group B); and 77 sound molars (Group C) were compared using Kruskal-Wallis test. The results of these comparisons for each of the 6 cytokines in extracted fluid are shown in Table 1. A significant difference was found for TNF- α ($p = 0.042$). Pairwise comparison based on Kruskal-Wallis rank-sums showed the extracted fluid in Group B (extracted restored molars) had significantly higher TNF- α levels compared to extracted fluid samples in Group C (extracted sound molars, $p = 0.049$).

4. Discussion

The absolute certainty of the origin of dentinal fluid has not been reached^{28, 29}. Generally, the odontoblast cell layer is suggested to form a functional barrier which mostly restrains the passage of fluid, ions and other molecules along the extracellular pathway, so that at least in intact teeth, dentinal fluid content would be strictly under odontoblast control³⁰⁻³². Most of the studies measuring dentinal fluid flow and consistence are based on cavity preparation, causing disruption in the tight junctions between odontoblasts^{30, 31, 33} and fluid from the pulp – either from the pulp tissue extracellular space, from the blood vessels, or both – may diffuse into dentin. Then the fluid would be a physiologic response to trauma^{30, 33-35}, and such dentinal fluid would probably originate from pulpal tissues³⁶. Dentin also contains several serum proteins, at least albumin, IgG, transferrin, fetuin-A and superoxide dismutase 3 (SOD3)²⁹, and the genome-wide database of mature human odontoblast gene expression profile (Gene Expression Omnibus database # GSM215142)³⁷ demonstrates only the expression of transferrin. Therefore, serum proteins most likely have a passage to dentinal fluid even in intact teeth. However, the presence of SOD3³⁸, the significantly higher concentration of fetuin-A in dentin compared to serum (up to 100 to 200-fold)³⁹, and the studies disturbing microtubule-guided transcellular transport⁴⁰ and with calcium antagonists⁴¹, strongly indicate that active transport systems by the odontoblasts are involved for the serum proteins.

In this study, fluid was extracted from the coronal occlusal dentin of trimmed teeth. For this, the occlusal crown and the roots of extracted third molars were trimmed in an orthodontic model trimmer to expose the coronal occlusal dentin. The coronal occlusal dentin was then treated with phosphoric acid (35%) for 5 seconds, rinsed for 10 seconds, and dried with air. The trimmed molars were placed with the coronal occlusal dentin surface down in a conical tube and centrifuged at $2,750 \times g$ for 30 minutes at 4°C. The molars were then removed and discarded. The volume of fluid extracted from the coronal occlusal dentin of trimmed teeth ranged from 0.2 to 2.0 μ l. Although we believe that this fluid is primarily dentinal fluid, we cannot exclude the fact that this technique may also extract some pulpal fluid with it. Thus, fluid extracted from the coronal occlusal dentin of trimmed teeth may be contaminated to a lesser degree with pulpal fluid.

In this study, fluid extracted from the coronal occlusal dentin of trimmed teeth contained at least 117 peaks with different masses, with many common peaks among the extracted fluids. Although the MALDI-TOF instrumentation used could not identify the components present, it did suggest that mediators might be present. For example, pro-inflammatory cytokines IL-1 β , TNF- α , and IL-6 have masses of 17, 17.5, and 20.3 kDa respectively. Chemokine CXCL8 (IL-8) has a mass of 8.4 kDa, Th1 cytokine IL-12(p70) has a mass of 75 kDa, and

Th2 cytokine IL-10 has a mass of 18.5 kDa, all within the range of the MALDI-TOF analysis of this study (with the exception of IL-12(p70)). Defensins, examined recently under similar conditions by our laboratory have observed masses of 3,446.973 m/z (HNP-1), 3,934.616 m/z (HBD1), 4,115.205 m/z (HBD2) and 5,167.895 m/z (HBD3)²⁵.

In this study, fluid extracted from the coronal occlusal dentin of trimmed teeth contained cytokines and chemokines. We detected IL-1 β , IL-6, IL-8, IL-10, IL-12(p70), and TNF- α . Bletsa and co-workers measured IL-1 β , IL-6, and TNF- α in rat pulp interstitial tissue and serum, and found markedly higher levels than those detected in the present study⁴². They found that during inflammation, there were cytokines locally produced in the pulp and were not serum derived. This suggests that there may be active transportation from serum to dentinal fluid or, alternatively or in addition, the synthesis of cytokines by odontoblasts. As mentioned earlier, this also suggests that a permeable barrier, such as an odontoblast layer, might be present. Also, an injury at the enamel/dentin level, may increase the presence of inflammatory mediators that eventually are transported to the dentinal tubules. Indeed, odontoblasts can be induced to express or increase the expression of cytokines and chemokines by TGF- α or by bacterial toxins⁴³. Interestingly, there was a high concentration of IL-8 in some subjects suggesting there could be a local microbial infection in the enamel or inflammation in the pulp. While conflicting data exists of the expression of IL-8 in human odontoblasts⁴³⁻⁴⁵, it is clearly inducible by growth factors or bacterial toxins⁴³⁻⁴⁵ and has been suggested as one of the key cytokines in odontoblast-mediated immune response⁴³. Thus the results of this study support the hypothesis that extracted fluids from the dentin of teeth have detectable levels of inflammatory mediators.

Dental pulp contains other innate immune factors like human neutrophil peptide (HNP) α -defensins and human β -defensins (HBDs)^{19, 21, 22}. HNP-1, -2, -3, and -4 contain 29-34 amino acid residues and vary in mass from 3,374.52 to 3,827.83 m/z. HBD1, 2, 3, and 4 contain 36-49 amino acid residues and vary in mass from 3,931.78 to 5,855.87 m/z. Both HNPs and HBDs are expressed in oral tissues, salivary glands, salivary secretions, gingival crevicular fluid, and gingival tissue and likely may be present in fluid extracted from coronal occlusal dentin. However, this will have to be determined. Defensins have broad antimicrobial activities against gram-negative and gram-positive bacteria, fungi, and viruses. Since they serve as a first line of defense against microbial infections, it is possible that they are up-regulated to respond to infections of the tooth and may occur in dentinal and pulp fluids.

In summary, we observed inflammatory mediators in the fluid from the coronal occlusal dentin of trimmed teeth. We recovered 0.2 to 2 μ l of fluid, which contained cytokines and chemokines in sufficient amounts to differentiate among subjects with basal levels of cytokines and chemokines and those that appear to have localized infections or inflammation. Additional studies are now possible to determine the vast diversity and concentrations of mediators in dentinal fluids.

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Figure 1.

Image of an extracted molar after the occlusal crown and the roots were trimmed away using an orthodontic model trimmer (Whip Mix Corp., Louisville, KY) to expose the occlusal dentin (a). The coronal occlusal dentin surface was placed upside down in a 15 ml conical centrifuge tube and centrifuged at $2,750 \times g$ for 30 minutes at 4°C to extract the fluid extracted from the coronal occlusal dentin (b).

Figure 2. Mass spectrograms showing the range of molecules detected by MALDI-TOF in fluid extracted from the coronal occlusal dentin of one trimmed molar, which only contained a minimum of 6 peaks above 0.3 a. u. fractions ranging from 612.7406 m/z to 858.4988 m/z (**a**) fluid extracted from the coronal occlusal dentin of another trimmed molar, which contained a maximum of 31 peak fractions ranging from 653.673 m/z to 9954.282 m/z (**b**).

Table 1

Comparison of chemokine and cytokine concentrations in 112 fluids extracted from the coronal occlusal dentin of trimmed teeth fluids from the coronal occlusal dentin of extracted third molars.

Cytokine	Group A (Caries, n=25)	Group B (Restored, n=10)	Group C (Sound, n=77)	p-value
IL-1 β				0.391
Median	1.8	1.0	2.0	
25 th -75 th	1.0-4.0	1.0-2.0	1.0-2.3	
percentile	3.2 (3.6)	2.2 (2.3)	2.0 (1.4)	
Mean (SD)				
IL-6				0.345
Median	17.0	17.0	16.4	
25 th -75 th	15.3-18	17.0-18.0	15.0-17.0	
percentile	16.7 (1.9)	17.2 (3.1)	16.6 (2.2)	
Mean (SD)				
IL-8				0.724
Median	4.0	5.0	4.0	
25 th -75 th	2.0-9.0	2.0-41.0	2.0-6.0	
percentile	15.0 (37.7)	34.7 (56.1)	8.8 (16.7)	
Mean (SD)				
IL-10				0.077
Median	2.0	2.0	2.0	
25 th -75 th	2.0-4.9	2.0-2.0	2.0-5.1	
percentile	3.0 (1.5)	2.0 (0.4)	3.3 (1.6)	
Mean (SD)				
IL-12p70				0.359
Median	5.0	5.0	5.0	
25 th -75 th	4.0-6.9	5.0-5.0	5.0-7.1	
percentile	5.4 (1.6)	5.0 (1.1)	5.6 (1.4)	
Mean (SD)				
TNF- α				0.042
Median	6.0	7.0	6.0	
25 th -75 th	4.4-7.0	6.0-7.0	4.1-7.0	
percentile	5.9 (1.5)	6.8 (1.0)	5.5 (1.5)	
Mean (SD)				

Values are mean pg/ml.