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Short communication

Detection of microbial-derived fatty acids in carious dentin by gas chromatography and gas chromatography–mass spectrometry[1]

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Abstract

The aim of the present study was to analyze the fatty acid content of carious and sound human dentin. Gas chromatography and gas chromatography–mass spectrometry revealed the presence of fatty acids of C_{10}–C_{18} size in the carious dentin, whereas fatty acids of C_{16} size were present in minute amounts in three samples of the corresponding sound dentine controls. No fatty acids were detected in the other sound dentin control samples. The source of fatty acids was considered to be microorganisms invading the dentin during the progression of the caries lesion. The presence of bacterial fatty acids in carious dentin may serve as a marker for the pathological process and thus contribute to the understanding of the mechanisms involved. © 1997 Elsevier Science B.V.

Keywords: Dentin; Fatty acids

1. Introduction

It has been known since 1890 that acid-forming microorganisms colonizing the enamel surface are essential in the pathogenesis of dental caries [1]. The acids formed by microorganisms cause demineralization of the apatite crystals. This in turn provides access for invasion by microorganisms into the dentin, and as invading microorganisms produce both acids and proteolytic enzymes [2], both the apatite crystals and the organic matrix of the tissue are degraded along with the progression of the caries process into the dentin [2].

Carious dentin shows two distinct layers [3–5]. The most superficial layer which is heavily invaded by microorganisms, is decomposed by both acids and proteolytic enzymes. It shows extensive demineralization and degraded collagen and odontoblastic processes. The underlying second layer shows only partial demineralization, sound collagen fibers and intact odontoblastic processes. In contrast to the superficial layer, this layer is assumed to be physiologically remineralizable.

Although the cultivable microflora of carious dentin has been characterized by conventional culture techniques [2,6–13], few efforts have been made to identify bacterial components within this tissue [14,15]. Such identification might provide presumptive diagnosis of the bacterial succession taking place in the lesion as it progresses, as well as explain the

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observed pulpal reactions to caries. Major indicators of bacteria within tissues are specific fatty acids. The objective of this study was to analyze with gas chromatography and gas chromatography–mass spectrometry the content and types of fatty acids in carious and sound dentin.

2. Experimental

2.1. Teeth

Eleven teeth with deep dentin caries were extracted with informed consent from adult patients who had declined the option of tooth restoration. None of the patients reported a history of significant somatic disease or antimicrobial therapy during the last 3 months. Dental plaque overlying the caries lesions was removed and the teeth were rinsed, immediately after extraction, in 0.05 *M* phosphate buffer, pH 7.2, supplemented with 0.02% sodium azide to prevent microbial growth. Using sterile excavators, all softened dentin was meticulously removed as small fragments from the caries lesion. The carious dentin was dried in air, and stored in capped, individual bottles, at 4°C until analyses were performed. Dentin is a solid and degradation of solids is usually a slow process, especially at low temperature. As control samples, small pieces of non-carious dentin from the same teeth were prepared.

Before analysis, all samples were immersed into phosphate buffer (0.05 *M*, pH 7.2, with 0.02% sodium azide), sonicated for 15 min, vacuum dried to constant weight and sonicated in hexane for another 15 min. Thereafter, the samples were air dried and powdered in an agate mortar. The dentin powder was then transferred to the methanolysis tube and 3 ml of 3 *M* HCl in anhydrous methanol was added. The tubes were capped hermetically with a stopper furnished with a teplin liner.

2.2. Methanolysis and derivatization

The powdered dentin (50 mg) was methanolyzed in triplicate at 95°C in 3 ml of 3 *M* HCl in anhydrous methanol for at least 24 h, or until all particles were dissolved [16]. The reaction mixture was cooled on ice, diluted with 5 ml distilled water and extracted twice with 3 ml hexane. The hexane extract was dried with magnesium sulphate and evaporated under N_2 to a volume of 50 μl.

2.3. Reference compounds

Reference fatty acid methyl esters used in this study were 9:0–20:0, purchased from Sigma (St. Louis, MO, USA). Bacterial fatty acid methyl ester mixture CP (catalogue No. 4-7080), gas chromatography standard mixture GLC 70 (catalogue No. 4-7044), American Oil Chemists' Society oil reference mixture, RM-1 rapeseed (catalogue No. 4.7019), National Institute of Health's reference mixtures (catalogue No. A-NHI-C 4-7010, A-NHI-D 4-7011, and A-NHI-F 4-7013) were obtained from Supelco (Bellefonte, PA, USA). 13-Methylmyristic acid methyl ester, 12-methylmyristic acid methyl ester, methyl 15-methylhexadecanoate, mixture FO 1,3-hydroxyhexadecanoic acid, 3-hydroxydecanoic acid, 3-hydroxytridecanoic acid, 3-hydroxytetradecanoic acid methyl ester, 3-hydroxytetradecanoic acid, 3-hydroxypentadecanoic acid, and 3-hydroxyhexadecanoic acid were purchased from Larodan Fine Chemicals (Malmö, Sweden). No. 1200-A calibration standard was obtained from Microbial ID (Newark, DE, USA). Free fatty acids were methylated before assessment of fatty acid recovery.

2.4. Gas chromatography

Gas chromatography (GC) was performed in a model 8600 gas chromatograph (Perkin-Elmer, Norwalk, CT, USA). The column used was a Hewlett-Packard HP U2 cross-linked methyl-phenyl silicone column, 25 m×0.33 μm I.D. Hydrogen was used as carrier gas at 0.5 ml/min. A flame ionization detector which operated at 320°C was used for detection of fatty acids.

The injector operated at 230°C. The sample volume was 2 μl. The temperature program for the oven was as follows: start at 170°C with an increase of 5°C/min up to 310°C, then hold for 1 min. Integration was performed by means of the PE software TorboChrom. The attenuation on the recorder was set to 32 to bring the largest peak into papers size. Each sample was run three times.

2.5. Gas chromatography–mass spectrometry

The instrument used for gas chromatography–mass spectrometry was a Model 4200 gas chromatograph (Carlo Elba, Milan, Italy) equipped with a Model 7072F mass spectrometer (Vg Micromass, Cheshire, UK) and a type 2200 data system (Vg Micromass). The gas chromatograph was equipped with a type HP U2 cross-linked methyl-phenyl silicone column as described above, and the conditions for gas chromatography were also the same. The electron impact ionization spectra were recorded under the following conditions: ionization energy, 70 eV; ionization current, 200 μA; and accelerating voltage, 4 kV. Each sample was run three times.

3. Results and discussion

Derivatization techniques for GC analyses of fatty acids have been well reviewed [16–18]. The most commonly used method include acid-catalyzed esterification, methylation with diazomethane, boron trifluoride or boron chloride in methanol, methylation with hydrochloric acid in anhydrous methanol, and recently also methylation with trimethylanilinium hydroxide [19,20]. None of these techniques exclude the possibility of artifacts being formed from a naturally existing lipid mixture. Many workers consider the presently used methanolysis with hydrochloric acid in anhydrous methanol to be a mild and useful derivatization method in bacteriology [16,21,22].

Fatty acids of C_{10}–C_{18} size were detected in all samples from carious dentin, whereas minute amounts of C_{16} fatty acids, only, were detected in three samples from the sound dentin. The relative distribution of fatty acids is given in Table 1. Representative chromatograms of sound and carious dentin samples are given in Fig. 1a,b, respectively.

In previous studies, conventional culture [2,6–13] or microscopic [23–25] techniques, as well as immunological [26] and histochemical [14,26] techniques have been employed to demonstrate and identify microorganisms and various microbial products in carious dentin. Fatty acids of the C_{10}–C_{18} types are major indicators of microorganisms. Gas chromatography and gas chromatography–mass spectrometry are highly sensitive techniques to identify fatty acids, but to the best of our knowledge this is the first time these techniques have been applied to identify fatty acids in human dentin. The fatty acids identified are given in relative amounts, rather than absolute amounts. This was judged most reasonable, since the amount of uninfected dentin, presumably, varies from sample to sample, thus influencing the total amount of fatty acids.

The most striking feature of the present results was that fatty acids were present in all the samples of carious dentin. These were of the C_{10}–C_{18} size and were considered to be of microbial origin. In three cases, only, were fatty acids detected, in minute amounts, in sound dentin. These were of the C_{16} size, exclusively, and the amounts were far below those found in the carious dentin samples (1/172–1/200, sound/carious). Most likely, these fatty acids were reminiscent of food debris, since no microorganism contains exclusively C_{16} fatty acid. Thus, in none of the sound dentin samples were there signs of microbial-derived fatty acids.

Microbiological techniques have indicated a great variability in the amount and the species composition of the invading microorganisms of carious dentin [2,6–13]. Obligate anaerobes have been claimed to predominate [9], and invasiveness of microorganisms appears to be associated with their proteolytic activities and capacity to survive in an anaerobic environment [2]. The microorganisms most often identified include *S. mutans*, lactobacilli and actinomyces [13]. At present, it is difficult from our data to tentatively identify different microbial species, since the identified fatty acids represent the sum of all acids present. However, since no hydroxy-acids were detected in any of the samples, LPS-producing microbial strains were most likely not present. Furthermore, *porphyromonas* or *prevotella* species (previously *bacteroides* species) which contain hydroxy acids as well as C_{15} and C_{17} iso- and anteiso-fatty acids, were also absent from the present samples. Taken together, these observations indicate that the predominating microorganisms were Gram positive, facultative species.

The most superficial carious dentin layer which is heavily contaminated by microorganisms and which is decomposed by acids and proteolytic enzymes is

Table 1
Distribution (%)[a] of fatty acids in carious and sound dentin (n=3)

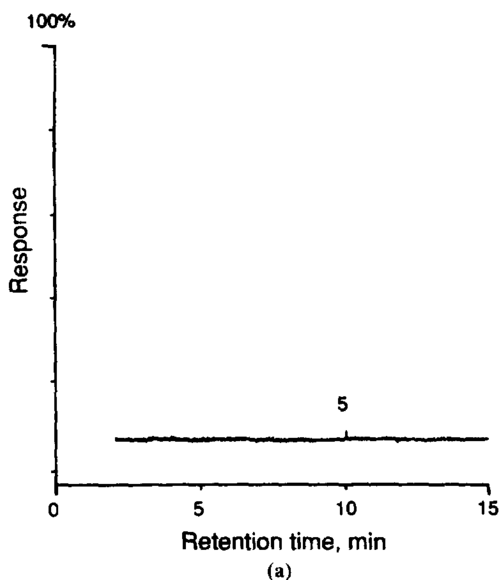
Status	Fatty acid[b]											
	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:1}$	$C_{16:0}$	C^c	$C_{18:2}$	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$	$C_{18:0}$	Ratio[d]
Sample No 1												
carious	0.8	3.0	2.3	0.5	21.5	ND	26.0	25.3	6.6	5.2	8.7	200
sound	ND	ND	ND	ND	100.0	ND	ND	ND	ND	ND	ND	
Sample No 2												
carious	1.5	3.6	3.0	ND	23.0	ND	25.0	25.1	9.6	ND	9.2	
sound	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Sample No. 3												
carious	ND	3.2	13.8	ND	32.9	3.1	12.9	15.9	10.8	ND	6.9	
sound	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Sample No. 4												
carious	0.8	3.3	2.4	0.7	21.9	ND	28.0	26.4	6.4	ND	10.2	196
sound	ND	ND	ND	ND	100.0	ND	ND	ND	ND	ND	ND	
Sample No. 5												
carious	ND	2.5	9.7	0.3	23.0	ND	25.1	19.2	8.1	4.5	7.2	172
sound	ND	ND	ND	ND	100.0	ND	ND	ND	ND	ND	ND	
Sample No. 6												
carious	1.2	2.8	3.6	ND	23.7	ND	27.0	25.1	7.5	ND	8.7	
sound	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Sample No. 7												
carious	0.9	2.8	7.4	ND	19.9	ND	25.5	23.9	6.7	3.7	9.4	
sound	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	

[a]The relative standard deviation is less than 3%.
[b]See Fig. 1b for name of fatty acids.
[c]Non-identified substance.
[c]Ratio carious:sound.

normally not remineralizable [3,4]. The underlying second layer which shows only partial demineralization, sound collagen fibers and intact odontoblastic processes is generally considered to be remineralizable.

From a clinical, therapeutic point of view, it may be important to distinguish between the remineralizable and the non-remineralizable layers of carious dentin, as well as between infected and non-, or lightly infected dentin [10]. Both layers are softened and difficult to distinguish on the basis of resistance to probing [3,5,27]. The use of acid red as a caries-disclosing agent is claimed to reveal the first layer of the carious dentin but not the second layer [3,28–31]. However, the specificity of this staining method has lately been questioned [32–34]. During the preparation of the dentin samples of the present study, removal of soft, carious dentin was continued until the operator considered the remaining dentin to be hard upon probing. No samples were taken from beyond this point which was probably located within the second layer, based on hardness criteria [35]. It was therefore not assessed whether, due to simple diffusion, microbial fatty acids may be found deeper within the dentin. In future studies it would be of interest to do a stepwise excavation in order to determine the penetrability of microbial fatty acids into dentin, since these acids might contribute to inflammatory reactions in the pulp [8,15,26,36,37]. Furthermore, following microbial death and lysis, fatty acids derived from the cell wall of the invading microorganisms come in contact with the dissolving tissue and may bind to apatite crystals. It is conceivable that microbial fatty acids may act as detergents,

and upon binding to calcium in the dentin may contribute to demineralization of the dentin and thus the progression of the caries process.



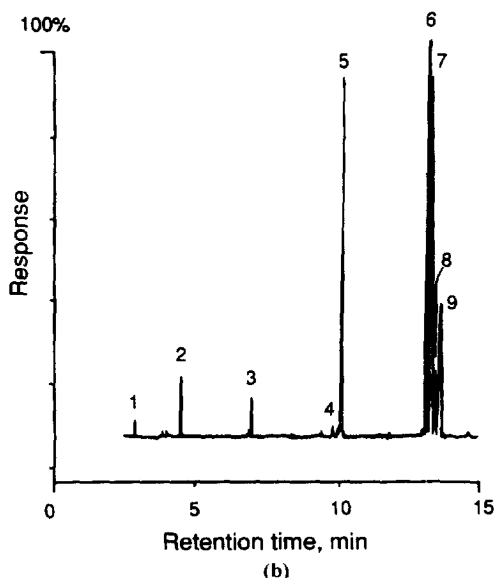


Fig. 1. (a) Cromatogram showing the elution pattern of fatty acids from a sound dentin sample. (5) $C_{16:0}$, hexadecanoic acid. (b) Cromatogram showing the elution pattern of fatty acids from a carious dentin sample: (1) $C_{10:0}$, decanoic acid; (2) $C_{12:0}$, dodecanoic acid; (3) $C_{14:0}$, tetradecanoic acid; (4) $C_{16:1}$, 9-hexadecenoic acid; (5) $C_{16:0}$, hexadecanoic acid; (6) $C_{18:2}$, 9,12-octadecadienoic acid; (7) $C_{18:1(9\ cis)}$, 9-*cis*-octadecenoic acid; (8) $C_{18:1(9\ trans)}$, 9-*trans*-octadecenoic acid; (9) $C_{18:0}$, octadecanoic acid.

Acknowledgments

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