Preservation of Bacterial DNA by Human Dentin

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Abstract

Introduction: The capacity of dentin and collagen to bind DNA and protect against spontaneous and nuclease-induced degradation was evaluated individually and by the incubation of DNA with nuclease-producing bacteria in a mixed culture. **Methods:** Extracted *Fusobacterium nucleatum* DNA was incubated with dentin shavings or collagen for 90 minutes. The DNA-bound substrates were incubated in different media (water, sera, and DNase I) for up to 3 months. Amplifiable DNA was released from dentin using EDTA, or from collagen using proteinase K, and evaluated by polymerase chain reaction (PCR). The stability of dentin-bound DNA was also assessed in a mixed culture (*Parvimonas micra* and *Pseudoramibacter alactolyticus*) containing a DNase-producing species, *Prevotella intermedia*. Samples were analyzed for amplifiable DNA. **Results:** In water, dentin-bound DNA was recoverable by PCR at 3 months compared with no detectable DNA after 4 weeks in controls (no dentin). DNA bound to collagen was detectable by PCR after 3 months of incubation in water. In 10% human sera, amplifiable DNA was detectable at 3 months when dentin bound and in controls (no dentin). In mixed bacterial culture, dentin-bound DNA was recoverable throughout the experimental period (3 months), compared with no recoverable *F. nucleatum* DNA within 24 hours in controls (no dentin). **Conclusions:** There is a strong binding affinity between DNA and dentin, and between DNA and serum proteins or collagen. These substrates preserve DNA against natural decomposition and protect DNA from nuclease activity, factors that may confound molecular analysis of the endodontic microbiota yet favor paleomicrobiological studies of ancient DNA. (*J Endod 2014;40:241–245*)

Key Words

Bacterial nucleases, dentin, DNA binding affinity, DNA decomposition, DNA preservation, polymerase chain reaction

Under favorable conditions, host and microbial DNA can be preserved for a long period of time. The durability of teeth and the inherent preservation of dental pulp space integrity have made the dental pulp a target of scientific interest for the recovery of ancient DNA in the study of the etiology of septicemic diseases in ancient remains [1, 2] and forensic applications [3]. The recovery of ancient DNA from dental pulps has been described in 400-year-old specimens (*Yersina pestis*) [4], 1000 year-old specimens (*Mycobacterium tuberculosis*) [5], and 4000-year-old specimens (*Bartonella quintana*) [6]. These studies not only show the resilience of DNA but also highlight the favorable properties of the pulp canal space and surrounding dentin as a unique biological environment for the long-term preservation of DNA.

These environmental characteristics are an advantage for preserving DNA for paleomicrobiological studies; yet, it can be a confounding factor when applying molecular analysis to the study of the endodontic microbiota. Bacterial DNA recovered from the root canal may derive from long-dead cells reflecting the infection history, but it may have limited or no relevance for current disease.

One factor affecting DNA degradation is how cells die. If cells die with their cell membranes predominantly intact, the DNA can be protected from degradative factors and can be recovered and amplified using the polymerase chain reaction (PCR) technique. *In vitro* experiments using dead *Enterococcus faecalis* cells killed by heating to minimize cell lysis have shown that amplifiable DNA is recoverable 1 year after cell death [7], and experiments using dead *E. faecalis* cells inoculated in *ex vivo* teeth have shown recoverable DNA 2 years after cell death [8]. If the bacterial cell wall disintegrates, DNA is released into the adjacent environment (Brundin, Figdor, Sundqvist, Sjögren, manuscript submitted), and the free DNA is subject to decomposition by a variety of spontaneous, ambient, and enzymatic processes [8, 9]. Nucleases rapidly degrade free DNA [8].

Some environmental conditions protect DNA from spontaneous or enzymatic degradation. Hydroxyapatite, the main constituent of dentin, has a specific binding affinity for DNA [10, 11], and the resultant DNA-hydroxyapatite complex confers resistance to spontaneous degradation and protects against breakdown by nucleases [11]. Collagen, the principal organic component of dentin [12], is known to bind DNA [13]. Although the capability of bone [14, 15] and hydroxyapatite [11] to preserve DNA is known, the DNA-binding and protective capability of dentin and its collagen component is a largely unexplored area. Information regarding the DNA preservation capability of these structures is of importance for both endodontic and anthropologic research.

The purpose of this study was to assess the influence of dentin and collagen on the preservation of DNA from spontaneous decay. A further subject of interest was the influence of dentin on DNA preservation when DNA was incubated with nuclease-producing bacteria in a mixed culture *in vitro*.

Materials and Methods

Preparation of Dentin Shavings

Dentin shavings were collected from 3 intact formerly vital teeth. Teeth were stored in sterile saline and processed within 12 hours after extraction. After cleaning the teeth with sterile saline, the teeth were embedded in plaster to the cementoenamel junction. A rubber dam was applied, and the tooth crowns were carefully disinfected with H_2O_2 (30%, 1 minute) followed by iodine tincture (5%, 1 minute). The cervical area at the rubber dam–tooth interface was sealed with glue (Cervitec; Ivoclar Vivadent, Schaan, Lichtenstein). Disinfection was completed with a final iodine tincture wash. A conventional access cavity was prepared, and the pulp tissue was removed with Hedstrom files. Canals were filled with ultrapure water (Sigma-Aldrich, St Louis, MO), and dentin shavings were created by mechanically preparing the canal walls. The shavings were collected by aspirating the canal contents with a syringe and transferring them to a sterile Eppendorf tube. After washing (×3, ultrapure water), the dentin shavings were dried (37°C, 12 hours) and weighed. A suspension (0.1 mg dentin/μL) was prepared by suspending the shavings in ultrapure water. Ultrasonic activation was applied to disaggregate shavings.

As a sterility control, an aliquot of the dentin shavings suspension (35 μL) was plated onto Fastidious Anaerobe Agar (FAA; Laboratory M, Bury, UK) with vitamin K (10 mg/L) for aerobic and anaerobic incubation (37°C, 7 days). Dentin shavings (3 mg) were also treated with EDTA, processed for amplification (PCR), and analyzed for the detection of *Fusobacterium nucleatum* DNA (described later).

Preparation of Bacterial DNA

F. nucleatum (National Collection of Type Cultures 10652) was grown on FAA in an anaerobic box for 4 days. The cells were harvested from plates and suspended in phosphate buffered saline. DNA was extracted from cell pellets with a bacterial genomic DNA kit according to the manufacturer's protocol (Gene Elute, Sigma-Aldrich) and eluted in ultrapure water (200 μL; Sigma-Aldrich). For all the experiments described later, DNA was prepared to a concentration of 44 ng/μL (154 ng in 3.5 μL ultrapure water).

Dentin-DNA Binding, Release, and Analysis by PCR

F. nucleatum DNA (154 ng) was added to the aqueous dentin preparations (3 mg dentin, total preparation = 100 μL) and incubated with slow agitation (22°C, 90 minutes). The DNA-coated dentin was then washed (×3, ultrapure water), and EDTA (80 μL, 250 mmol/L, 15 minutes) was added to release DNA from dentin. The EDTA-containing supernatant was cleaned with the Wizard DNA Clean-Up System (Promega, Madison, WI) before PCR (11). For controls, identical processing was conducted for tubes containing DNA (no dentin) and tubes containing dentin (no DNA).

PCR amplifications were prepared in a 25-μL final reaction volume with 2 μL total DNA template, 12.5 pmol each primer, 200 μmol/L dNTPs (Qiagen, Hilden, Germany), 2.5 μL 10× PCR buffer (Qiagen) containing 1.5 mmol/L $MgCl_2$, 0.6 U HotStarTaq DNA polymerase (Qiagen), and ultrapure water (Sigma-Aldrich) to make up the final reaction volume. Primers used were designed to amplify a 360–base pair amplicon from the *F. nucleatum* 16S ribosomal RNA gene (16). Amplification products (8 μL, 20 cycles) were analyzed by gel electrophoresis (1.5% agarose in Tris-borate-EDTA buffer; Gibco, Invitrogen, Grand Island, NY), stained with ethidium bromide (1 μg/mL, Sigma-Aldrich), and observed under ultraviolet light. A 100–base pair DNA ladder digest (Invitrogen, Fredrick, MD) served as a molecular weight marker. All experiments (including those described later) were repeated at least in triplicate.

Degradation of Dentin-bound DNA in Water and Sera

The degradation of dentin-bound DNA was assessed by incubating DNA (154 ng) bound to dentin (3 mg) in ultrapure water (100 μL) at 37°C for up to 3 months. The effect of serum on DNA degradation was evaluated by incubating dentin (3 mg) with DNA (154 ng, 100 μL) followed by agitation (90 minutes) and washing (×3, ultrapure water) of the dentin preparation. Sera from 4 healthy human adults working at the Department of Odontology, Umeå University, Umeå, Sweden, were pooled, inactivated (56°C, 30 minutes), and then added to the dentin preparations. Samples were analyzed at 2, 4, and 12 weeks. At these intervals, the dentin was washed (×3, ultrapure water) and incubated with EDTA (80 μL, 250 mmol/L, 15 minutes). Samples treated with EDTA were cleaned, processed by PCR (20 cycles), and analyzed as described previously. Controls were tubes containing DNA without dentin.

Dentin-bound DNA in an *In Vitro* Bacterial Community

The stability of dentin-bound DNA was assessed in a bacterial consortium containing DNase-producing species. To confirm bacterial survival over the observation period *Parvimonas micra* (Virginia Polytechnic Institute and State University 5464), *Pseudoramibacter alactolyticus* (Culture Collection, University of Gothenburg 52346), and *Prevotella intermedia* (American Type Culture Collection 25611) were grown individually for 4 days on FAA in an anaerobic box. The cells were harvested and suspended to an optical density of 1–2 in 50% human serum in phosphate buffered saline. Aliquots of the 4 strains were pooled (total volume = 4 mL) and incubated in an anaerobic box for 28 days. At predetermined intervals, samples (2 × 100 μL) were collected and analyzed by cultivation and for amplifiable DNA, respectively. Initial findings showed that the DNase producing *P. intermedia* survived for 2 weeks only. Therefore, in subsequent experiments, a fresh suspension of *P. intermedia* (10^6 cells in 10 μL) was added every other week over the experimental period (3 months).

F. nucleatum DNA (154 ng) bound to dentin (3 mg) was added to 100 μL of the bacterial suspension (*P. micra*, *P. alactolyticus*, and *P. intermedia* suspended and pooled in 50% human serum as described earlier) and incubated anaerobically for 3 months. For controls, extracted *F. nucleatum* DNA (without dentin) and dentin (no DNA) were individually incubated in corresponding bacterial suspensions. At predetermined intervals, samples were collected and analyzed for amplifiable DNA by PCR (30–35 cycles).

Effect of DNase on Dentin-bound DNA

The effect of nucleases on dentin-bound DNA was evaluated by binding DNA (154 ng) to dentin (3 mg) as described earlier. After binding, DNase I (1 U; Invitrogen, Carlsbad, CA) was added, and tubes were incubated with gentle agitation (22°C, 30 minutes). The dentin was washed (×3, ultrapure water), EDTA (80 μL, 250 mmol/L) was added to release the bound DNA, and the tubes were kept on slow rotation (22°C, 15 minutes). EDTA was cleaned from samples with the Wizard DNA Clean-Up System before PCR (30 cycles), and amplification products were analyzed by gel electrophoresis. Positive controls were tubes with dentin and DNA but untreated with DNase I. Negative controls were DNA in water incubated with DNase I (without dentin).

Interaction of DNase with Dentin

The possibility of nuclease binding to dentin was assessed by incubating DNase I (0.5 U) with dentin (3 mg) in water (100 μL) with agitation (30 minutes). For analysis of DNase activity, supernatant was withdrawn (100 μL), and DNA (154 ng) was added and incubated (22°C) for 30 minutes. The DNase reaction was stopped by adding EDTA to a final

concentration of 2.5 mmol/L. As a control, DNA (154 ng) was added to supernatant (100 μL) taken from the incubation of dentin not treated with DNase I. All samples were processed by PCR (30 cycles), and products were analyzed by gel electrophoresis.

Collagen-DNA Interaction

Dentin contains about 18% collagen (12), which may bind DNA. The effect of DNA binding to collagen was assessed in experiments identical to those described previously (but substituting collagen for dentin) to assess degradation in water and the effect of DNase I on collagen-bound DNA. Collagen type I from bovine Achilles tendon (2 mg; Serva, Heidelberg, Germany) was incubated in water (200 μL, Sigma-Aldrich) overnight. After washing (×3, ultrapure water), experiments were performed as described earlier for up to 3 months. DNA was released from the collagen by the addition of proteinase K (50 μL, 100 mg/mL; 55°C, 3 hours; Sigma-Aldrich). Samples (2 μL) were processed by PCR (30–35 cycles) and analyzed by gel electrophoresis as described earlier.

Results

The sterility controls of dentin shavings revealed no viable bacteria and no detectable *F. nucleatum* DNA.

Dentin-DNA and Collagen-DNA Binding

DNA bound to dentin was released by the addition of EDTA. After cleanup, analysis of the supernatant revealed amplifiable DNA in all samples. Collagen-bound DNA was released by the addition of proteinase K, and analysis by PCR of the supernatant showed recoverable DNA in all samples. No DNA was detectable in controls containing dentin or collagen without added DNA.

Degradation of Dentin- and Collagen-bound DNA in Water and Dentin-bound DNA in Sera

In water, DNA was detectable by PCR only up to 4 weeks. In contrast, DNA bound to dentin was recoverable by PCR at 3 months (Fig. 1). DNA bound to collagen was just detectable (as a faint band) by PCR after 3 months of incubation (Fig. 1). In 10% human sera, DNA was detectable at 3 months. Similarly, DNA bound to dentin was recoverable at 3 months after incubation in 10% sera (Fig. 1).
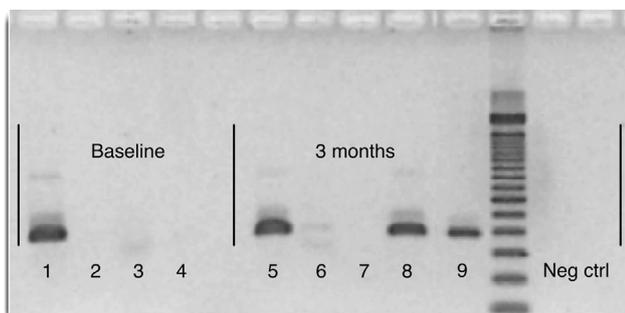

Figure 1. Recovery by PCR of *F. nucleatum* DNA after incubation in water or sera for 3 months. Dentin-bound, collagen-bound, and unbound DNA was released from dentin or collagen by adding EDTA or proteinase K, respectively, and assessed by gel electrophoresis of amplified DNA product (25 cycles). Baseline data: 1, DNA; 2, dentin; 3, collagen; 4, 10% sera in water. Experimental data after 3 months: 5, dentin-bound DNA in water; 6, collagen-bound DNA in water; 7, free (no dentin or collagen) DNA in water; 8, dentin-bound DNA in 10% sera; 9, free (no dentin or collagen) DNA in 10% sera. Negative control: DNA template was replaced with ultra-pure water.

Dentin-bound DNA in an *In Vitro* Bacterial Community

For *P. alactolyticus* and *P. micra,* the initial cell density was 10^8 colony-forming unit/mL, and both species survived in 50% pooled human sera for 28 days. There was a gradual decline in cell density during the observation period. Amplifiable DNA for *P. alactolyticus* and *P. micra* was detectable over the whole test period (data not shown). For *P. intermedia*, the initial cell density was 10^6 colony-forming unit/mL, and it survived for 14 days. Amplifiable DNA was recoverable during the experimental period (28 days, data not shown).

When *F. nucleatum* DNA was inoculated into the mixed bacterial culture containing a DNase producer (*P. intermedia*), there was no recoverable *F. nucleatum* DNA within 24 hours (Fig. 2). In contrast, amplifiable DNA was recoverable throughout the whole experimental period (3 months) when it was dentin bound and inoculated into the same mixed bacterial culture (Fig. 2). When dentin-bound DNA was incubated in the same bacterial culture to which fresh *P. intermedia* was added every other week, DNA was recoverable at the end of the 3-month observation period (Fig. 2).

Effect of Nucleases on Dentin- and Collagen-bound DNA

Dentin-bound DNA was incompletely degraded by DNase I, as shown by detectable DNA after EDTA treatment and cleanup of the supernatant. Similarly, DNA bound to collagen was also detectable after exposure to DNase I. In the control (ie, DNA incubated with DNase I in water [30 minutes]), there was no PCR-detectable DNA.

Interaction of DNase with Dentin and Collagen

DNase activity was evaluated after the incubation of DNase I with dentin and collagen, respectively, by the addition of DNA to their supernatants. Nonrecovery of DNA would indicate unimpaired DNase activity. After treatment, DNA was still detectable, which implies that activity was inhibited after the incubation of DNase with dentin or collagen. In the control (no dentin or collagen), DNA was degraded within 30 minutes as shown by the nonrecovery of DNA.

Discussion

Bone and teeth are generally recognized as favorable sites for preservation and potential recovery of ancient DNA (17). Apart from the physical protection afforded by these hard tissues, the main inorganic component, hydroxyapatite, has a strong binding affinity for DNA (10, 11). It has recently been shown that there is a significant reduction in spontaneous degradation when DNA is bound to hydroxyapatite (11). The present study assessed dentin as a substrate for DNA preservation and the results show that dentin-bound DNA is conserved from spontaneous decomposition when compared with free DNA. Binding to other biomolecules (ie, collagen and serum) was also shown to have a preservative effect against spontaneous decomposition.

When microbes die with their cell walls predominantly intact, it has been shown experimentally that cell-bound bacterial DNA is very stable (7, 8). If the cell wall breaks open, DNA is released (Brundin, Figdor, Sundqvist, Sjögren, manuscript submitted) and is thereby exposed to environmental factors that may accelerate or delay decay (8). Water and nucleases are environmental factors that can induce rapid decomposition of naked DNA molecules within hours to weeks (8, 9).

DNA may remain detectable for tens or hundreds of years (4, 5, 18), which implies that there are favorable ambient conditions that can stabilize the DNA molecule or protect it from degradative factors. DNA can bind to minerals in sand and clay (19, 20) and to hydroxyapatite (10, 11). Laboratory studies show a strong binding affinity of

Figure 2. Recovery by PCR of *F. nucleatum* DNA after coculture in a bacterial consortium (*P. micra*, *P. alactolyticus* and *P. intermedia* in 50% sera) with and without dentin. Analysis by gel electrophoresis of amplified DNA product. Data collected at baseline; 24 hours; and 1, 2, and 3 months. A, Dentin-bound DNA incubated in bacterial mix; B, dentin-bound DNA incubated in bacterial mix with the addition of *P. intermedia* every other week; C, free (no dentin or collagen) DNA incubated in bacterial mix. Controls: positive amplification control (Pos) and negative control; DNA template was replaced with ultrapure water (Neg).

hydroxyapatite for DNA (10, 11). The DNA-mineral complex stabilizes the DNA by changing the charge of the DNA molecule (21). The present study showed that dentin-bound DNA incubated in water was recoverable for 3 months compared with undetectable DNA after 4 weeks without dentin. This finding may seem unsurprising because hydroxyapatite is the main component of dentin (22); yet, dentin also contains a significant quantity of proteins (23), predominantly collagen type I (24), which may influence binding to DNA. Additionally, serum may enter the root canal by diffusion through the apical foramen or via the vessels and bind free DNA (25). Therefore, DNA binding to collagen and serum were assessed as separate biological components.

DNA was shown to bind to collagen, which is consistent with previous reports (13, 26), and the DNA was recoverable after collagen removal by the addition of proteinase K. Collagen-bound DNA was barely detectable after 3 months in water, which suggests a weak binding affinity and a limited protective effect on DNA preservation. Binding to serum had a preservative effect because DNA exposed to sera was recoverable after 3 months. DNA binds to serum proteins (25), in particular to albumin, the most abundant protein, binding DNA between the protein and the G-C bases and backbone PO_2 group of the DNA molecule, resulting in 2 different albumin-DNA complexes with different bonding strengths (25). In a supplementary experiment using collagen-bound DNA incubated in serum for 3 months, there was limited amplifiable DNA associated with the collagen component; yet, the serum supernatant contained ample recoverable DNA (data not shown). This finding implies a variable binding affinity between different tissue proteins and that serum proteins bind more strongly to DNA than collagen. Human sera also contain some DNases (27); yet, these low concentrations (3–23.9 ng/mL) evidently have little impact on DNA degradation when bound to dentin.

In the root canal, it has been suggested that infection and, in particular, the production of innate microbial DNases may contribute to DNA decomposition (28). Previous *in vitro* experiments have shown that free *E. faecalis* DNA is rapidly degraded after incubation in culture with *P. intermedia*; yet, the effect was not observed for cell-bound *E. faecalis* DNA (8). These interactions are undoubtedly complex; yet, the results showing the protective effect of both hydroxyapatite (11) and dentin (this study) together with paleomicrobiological data (4, 6) would suggest that these minerals confer a protective effect against microbial nucleases in root canal infections. Therefore, the effect of dentin on protecting DNA from nuclease-producing bacteria was assessed by incubating DNA in a mixed culture containing *P. micra* and *P. alactolyticus* together with *P. intermedia*, selected for its potent nuclease capability (8, 29, 30).

In mixed bacterial cultures containing dentin, amplifiable DNA was recoverable at 3 months compared with controls (no dentin) with no detectable DNA after 24 hours, which shows a pronounced protective effect of dentin. In some experiments, fresh *P. intermedia* culture was added every second week to ensure that DNase-producing activity was maintained throughout the observation period, and the findings were the same; amplifiable *F. nucleatum* DNA was detected at 3 months. These results show the significance of dentin in both preservation of DNA and mitigating the degradative activity of nucleases in bacterial infection.

The contribution of individual components was assessed in more detail by separately incubating dentin- and collagen-bound DNA with DNase I. Amplifiable DNA was recovered after the incubation of dentin-bound DNA and collagen-bound DNA with DNase I, which shows that the binding of DNA to both substrates individually affords protection from degradation by DNase. These findings are consistent with that seen for hydroxyapatite, which also protects DNA from nuclease activity (11).

The mechanisms for protection against nuclease degradation may include dentin or collagen binding to DNA, making the molecule inaccessible to enzymatic activity, adsorption of the DNase itself to biological substrates, or a combination of both. Hydroxyapatite has been shown to bind DNase I, which contributes to the effectiveness of hydroxyapatite in preserving DNA against nuclease activity (11). In this study, pretreatment of dentin and collagen with DNase I inhibited subsequent nuclease activity. Taken together, the data show that binding to dentin reduces the degradative efficacy of nuclease-producing bacteria and that DNase activity is inhibited individually by hydroxyapatite, dentin, and collagen.

Extracellular DNA is derived from decomposing or disrupted cells or via excretion from live cells (31). Recent studies have shown that excreted extracellular DNA is a natural part of biofilm development and growth (32–34). In the infected root canal, intimate contact of such extracellular DNA on dentin would significantly enhance the preservation of bacterial DNA. It is reasonable to assume that DNA so adsorbed to dentin would then be recoverable a long time thereafter, a factor that should be taken into account when processing samples for molecular analysis.

The results presented herein show a strong binding affinity between DNA and dentin, which is consistent in many ways with previous observations for hydroxyapatite and DNA (10, 11). The resultant dentin-DNA complex preserves DNA against natural decomposition in water and protects DNA against nuclease activity in mixed bacterial culture. These findings help explain why ancient DNA is so well preserved within the pulp space of teeth in centuries-old human remains (1, 3, 6). The binding affinity is variable between different biological substrates; dentin

appears to possess a greater affinity than serum proteins or collagen for DNA. The findings also have implications for research in endodontic microbiology; the limitations must be considered, and special measures may be necessary for root canal samples processed using PCR.

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