Treatment of *Streptococcus mutans* with antisense oligodeoxyribonucleotides to *gtfB* mRNA inhibits GtfB expression and function

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Abstract

We examined the effects of phosphorothioate-modified antisense oligodeoxyribonucleotides (PS-ODN) targeted to mRNA transcribed from *gtfB*, which encodes synthesis of water-insoluble glucans in *Streptococcus mutans*. Treatment of *S. mutans* with 10 μM antisense PS-ODNs inhibited *gtfB* mRNA transcription, GtfB expression and water-insoluble glucan synthesis. The architecture of biofilms formed by antisense PS-ODNs-treated *S. mutans* showed reduced biomass, more dispersed distribution with enlarged interspaces and fewer layers of attached cells. PS-ODN treatment had no effect on the growth of *S. mutans*. Our results indicated that it might be feasible to use antisense PS-ODN as a novel agent in caries prevention.

Introduction

*Streptococcus mutans* is the principle etiological agent of dental caries. Colonization of the cariogenic pathogens on tooth surfaces is considered to be the first step in the development of dental caries (Hamada & Slade, 1980). Bacterial cell-associated proteins that mediate the interaction between *S. mutans* and the host are therefore often targeted as vaccine candidates (Smith et al., 2003; Rhodin et al., 2004). Most strains of *S. mutans* harbor three distinct *gtf* genes, *gtfB*, *gtfC* and *gtfD*, expressing GtfB, GtfC and GtfD, respectively. They mediate the firm attachment of *S. mutans* to tooth surfaces, and its accumulation, by synthesizing water-insoluble glucan and water-soluble glucan (Munro et al., 1991; Yamashita et al., 1993). Sequence analysis demonstrated that these three *gtf* genes and their products exhibited high levels of nucleotide and amino acid sequence homology (Fujiwara et al., 1998). It was reported that the decrease in the cariogenicity of *gtf*-deficient strain was related to the decreased adherence of *S. mutans* to tooth surfaces (Fukushima et al., 1992). Fujiwara et al. (1996) inactivated *gtfB* and/or *gtfC* of *S. mutans* MT8148 through insertional mutagenesis by allelic exchange and found that sucrose-dependent adherence of these mutants was significantly lower than that of their parental strain. Reintroduction of *gtfB* and/or *gtfC* into corresponding deficient mutants could mostly recover the sucrose-dependent adherence ability of these strains. The inhibition of Gtf activity may have potential use in the prevention of dental caries (Younson & Kelly, 2004).

Selective disruption and/or downregulation of gene expression are widely used tools for elucidating information about genes which are essential for bacterial growth, pathogenicity, and gene therapy (Geller, 2005). Antisense technology has been shown to be an effective means of controlling gene expression and has been used effectively to downregulate eukaryotic gene expression in a variety of systems (Donk et al., 2006). The antisense phenomenon occurs naturally in bacteria but antisense technology is seldom used in bacteria. The major hurdle of utilization of antisense oligonucleotides is the inefficient uptake rate of oligodeoxyribonucleotides (ODNs) into targeted bacteria cells due
to restriction imposed by the cell wall barrier. Various methods have been developed to overcome this membrane barrier resistance, among them, cationic polymer was one of the most widely used. Recent reports demonstrated that an antisense approach using various synthetic or expressed oligomers might be a promising means of developing new chemotherapeutic agent against infectious diseases (Harth et al., 2000; Wang & Kuramitsu, 2003; Tan et al., 2005). Furthermore, the antisense strategy may enable genes with multiple copies or gene homologs to be targeted (Bennett & Cowsert, 1999). This work was designed to determine the feasibility of introducing gtfB-specific antisense oligonucleotides into S. mutans, and to observe the effects of antisense oligonucleotides on gtfB mRNA transcription, GtfB expression and activity. The influence of phosphorothioate (PS)-ODN on biofilm formation was also observed.

Materials and methods

Bacteria strain and culture conditions

Streptococcus mutans GS-5 was grown as a standing overnight culture at 37 °C anaerobically in Todd Hewitt broth (THB, Difco), stored in Brain Heart Infusion (BHI) (Difco) supplemented with 25% sterile glycerol at −70 °C. BHI containing 1.5% agar was used for bacteria recovery. THB supplemented with 10% heat-inactivated (56 °C, 30 min) horse serum (Gibco, NY) was used in the preparation of genetically competent cells (THBS). Streptococcus mutans cells were maintained in THB supplemented with sucrose for scanning electron microscopy examination. For isolation of cell-associated protein and total RNA, S. mutans were grown in THBS supplemented with 0.3% yeast extract.

PS-ODN selection and preparation

Two target sites for the binding of the antisense ODN were chosen. One site was located near the initial code region (ODN1: 5′-agcagcctttgctcat-3′), the other in the catalytic domain (ODN2: 5′-accaggctggatatctc-3′), corresponding to 709–726 bp and 3479–3497 bp of the gtfB gene (GenBank accession number: M17361), respectively. A mismatched ODN was designed (ODN3: 5′-agcagcctttgctcat-3′) and used as a control. No apparent similarity between PS-ODNs and gtfC and gtfD was found by aligning the DNA sequence using bl2seq (http://www.ncbi.nlm.nih.gov). All intranucleotide linkages were phosphorothioates. PS-ODNs were synthesized by Shanghai Songon. PS-ODN stock solution was prepared with sterile double distilled water (DDW) and stored at −20 °C. Immediately before use 2 × THB was added to the solution (1:1, v/v). Finally So-FastTM (Taiyangma, Xiamen, China), a transfection reagent composed of cationic polymer which had been shown to notably improve the penetration of PS-ODN into S. mutans cells in our preliminary study, in THB (1:20, v/v) was added to PS-ODN solution and incubated at room temperature for 20–30 min.

Transformation of S. mutans

Transformation of S. mutans was performed as previously reported by Perry et al. (1983). PS-ODNs were added to the competent cultures respectively. So-FastTM or THB of equal volume was added as controls. Unless otherwise noted, PS-ODNs were added at a final concentration of 10 μM.

RNA isolation

Aliquots were removed at 1, 2 and 8 h intervals from resultant cultures. Total RNA was isolated from S. mutans for subsequent reverse transcription-PCR (RT-PCR) using a modified one-step method. Briefly, bacterial cells were harvested by centrifugation (4300 g for 5 min at 4 °C) and washed once with 0.1% diethylpyrocarbonate (DEPC)-treated water (Sigma). Lysis of the bacteria was carried out as described previously (Shiroza & Kuramitsu, 1993). RNA was extracted with Tri reagent (Molecular Research Centre) following the manufacturer’s instructions. The RNA was dissolved in sterile DEPC-treated water, checked for integrity and purity by 1.2% agarose electrophoresis and quantified by an Ultrrospec 3300 pro spectrophotometer (Amersham Biosciences, Sweden) at 260 nm.

RT-PCR

RT-PCR was used to characterize the transcription of gtfB mRNA of S. mutans. Specifically, a first-strand cDNA synthesis kit (MBI, Fermentas, Life Science, Lithuania) was used to amplify 2 μg DNase-treated total RNA isolated from resultant cultures in accordance with the recommendations of the supplier. PCR was performed with the primer set of gtfB/F1 (5′-acccagtcagccattacct-3′) and gtfB/R1 (5′-agcagctctttcttatcaat-3′), which was designed to anneal to an internal coding region of gtfB (903–1247 bp). The primer set of recA/F1 (5′-ccggaatcttctggtaag-3′) and recA/R1 (5′-taaatccacctgtacgag-3′), corresponding to the recA gene of S. mutans, was cited as internal control (Fujiiwara et al., 2002). The expected size for each PCR product from gtfB and recA was 345 and 692 bp, respectively. The primers, at a final concentration of 0.5 μM, were added to each reaction mixture of 2 × Taq PCR MasterMix (Tian Wei Shi Dai, China) following the manufacturer’s instructions. The reaction mixtures were subjected to PCR amplification with the following thermal cycling protocol: 95 °C for 5 min for initial denaturation, followed by 35 cycles of three steps consisting of 95 °C for 45 s, 56 °C for 45 s, 72 °C for 45 s and a final extension at 72 °C for 10 min in a thermocycler (GeneAmp PCR system 9700, Applied Biosystem,
100% ethanol and incubated overnight at 4 °C. The resulting lysates were cleared by centrifugation (10,000 g, 10 min, 4 °C). Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard.

Cell-associated protein isolation

The resulting S. mutans cells grown for 8 h were harvested by centrifugation (4300 g, 5 min, 4 °C) and resuspended in 2 × cracking buffer as described by Hazlett et al. (1998). The resultant lysates were cleared by centrifugation (10,000 g, 10 min, 4 °C). Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and determination of GtfB activity

Streptococcus mutans cell-associated proteins (16 µg per lane) were resolved on a denaturing gradient gel (5%–7.5%) by SDS-PAGE at 4 °C in duplicate (Mini. VE Hoefer, Amersham Biosciences, Sweden). One gel was stained with 0.1% Coomassie Brilliant blue (CBB) (Sigma) and the band intensity was measured by a scanning densitometer (Docol 2000, BioRad). The other gel was used to determine GtfB activity. The gel was eluted with 50 mM Tris-HCl (pH 7.5), incubated in 50 mM sodium phosphate buffer containing 1% sucrose, 1% Triton-X 100% and 0.04% dextran T-10 (pH 6.5) at 37 °C for 18 h. It was subsequently soaked in Schiff’s reagent for 60 min (Hung et al., 2002) and photographed against a white background. The above experiments were repeated three times.

Isolation and analysis of water-insoluble glucans

After growing for 8% h, 50% sterile sucrose was added to the recipient S. mutans to a final concentration of 1% and the bacteria were allowed to grow for another 1 h. Cells were collected and washed three times with sterile DDW. The precipitate was resuspended in 0.5 M NaOH and incubated at 37 °C with rotation at 250 r.p.m. for 5 min. After centrifugation (10,000 g, 4 °C, 10 min), the supernatant was collected. This procedure was repeated three times and the supernatant was pooled. The insoluble glucans in supernatants were isolated by the addition of 3 volumes of cold 100% ethanol and incubated overnight at 4 °C. The mixture was centrifuged at 12,000 g, for 5 min at 4 °C and the precipitate was dissolved in 0.5 M NaOH. The total amount of insoluble glucans were measured by the phenol-sulfuric acid method (Dubois et al., 1956) at 488 nm with glucose as the standard. Each experiment was carried out in triplicate and repeated three times.

Morphological observation of S. mutans biofilm

The architecture of S. mutans biofilm was observed by a scanning electron microscope (SEM, GSE-80, Japan). Biofilm growth was initiated on the bottom of a 24-well plate (Costar) as previously described (Loo et al., 2000). Free PS-ODNs, So-FastTM and a combination of PS-ODNs with So-FastTM were added to different wells respectively, and incubated at 37 °C. At 2 h of incubation, sterile 50% sucrose was added to each well to a final concentration of 1%. After 18 h incubation, the biofilms were washed once with 10 mM phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde in 10 mM PBS at room temperature overnight. The biofilms were examined with a SEM.

Growth assay of S. mutans

The growth of the bacteria was determined by measuring the optical densities (OD) at 540 nm with a spectrophotometer. OD was monitored at 1-h intervals over a period of 24 h. The growth experiment was conducted in triplicate and the values represented the average of three determinations.

Statistical analysis

Data analysis was performed by ANOVA using SPSS 11.0. Differences were considered to be statistically significant when a value of P ≤ 0.05 was obtained.

Results

Effect of PS-ODNs on gtfB transcription

The effects of PS-ODNs on gtfB transcription are shown in Fig. 1. No significant difference of gtfB mRNA transcription was observed among different treatment groups at 1 h (Fig. 1a) and 2 h (Fig. 1b). At 8 h, PS-ODN1 (lane 1) and PS-ODN2 (lane 2) greatly inhibited gtfB transcription, whereas the mismatched PS-ODN3 (lane 3) only showed slightly inhibitory effect. However, So-FastTM (lane 4)
exerted no inhibition on \textit{gtfB} transcription (Fig. 1c). No PCR signal was detected in the absence of the reverse transcription reaction (data not shown).

**Effect of PS-ODNs on GtfB expression**

The intensities of GtfB bands of different treatment groups were revealed by SDS-PAGE (Fig. 2a) and scanning densitometry. The mean pixel values of GtfB bands as determined by scanning densitometer were shown in Table 1. PS-ODN1, PS-ODN2 and PS-ODN3 inhibited GtfB expression by 41.32%, 41.94% and 17.88% respectively. The differences between antisense and mismatched PS-ODNs were statistically significant ($P < 0.01$). However, PS-ODN3 only showed little inhibitory effect ($P > 0.05$). Similar inhibitory effects were observed between PS-ODN1 and PS-ODN2 groups ($P > 0.05$).

**Effect of PS-ODNs on GtfB activity**

By \textit{in situ} cell-associated Gtf activity determination, both GtfB and GtfC bands were found in PS-ODN3, So-Fast\textsuperscript{TM} and control groups (Fig. 2b, lane 3, 4,5), whereas only the GtfB band was found in PS-ODN1- and PS-ODN2-treated \textit{S. mutans} (Fig. 2b, lane 1, 2). In addition, the GtfB activity bands in antisense PS-ODN groups were weaker than that in other groups (Fig. 2b). Quantitative analysis revealed that both PS-ODN1- and PS-ODN2-treated \textit{S. mutans} synthesized much lower levels of water-insoluble glucans compared with PS-ODN3, So-Fast\textsuperscript{TM} and control groups ($P < 0.05$). However, similar levels of water-insoluble glucans synthesis were observed between PS-ODN1 and PS-ODN2 groups ($P > 0.05$) (Table 2).

**Effect of PS-ODNs on biofilm formation**

SEM examination demonstrated that the architectures of the biofilms were different among PS-ODNs-treated, PS-ODNs with So-Fast\textsuperscript{TM}-treated, So-Fast\textsuperscript{TM}-treated and control groups (Fig. 4). The thickness, biomass and density of the biofilm formed by free antisense PS-ODNs-treated \textit{S. mutans} were decreased. A combination of antisense PS-ODNs with So-Fast\textsuperscript{TM} exerted an even greater inhibitory effect on biofilm formation (Fig. 3).

**Effect of PS-ODN on the growth of \textit{S. mutans}**

The growth of PS-ODNs- and So-Fast\textsuperscript{TM}-treated \textit{S. mutans} was assessed in an independent growth study. Figure 4 showed that different treatments of \textit{S. mutans} did not affect the growth of the bacteria over a period of 24 h.

**Discussion**

The Gtfs are composed of four distinct structural domains. Their N-terminal begins with a signal peptide, followed by a

![Fig. 2. Electrophoresis profile of cell-associated proteins of \textit{Streptococcus mutans}. Positions of GtfB bands and GtfB activity bands were indicated by arrows. (a) Equal amounts of cell-associated proteins were resolved by SDS-PAGE and stained with CBB. (b) \textit{In situ} GtfB activity determination. The gel was incubated with sucrose and Triton X-100 buffer and finally by Schiff’s reagent. After incubation the gel was photographed against a white background. Lane 1. PS-ODN1; Lane 2. PS-ODN2; Lane3. PS-ODN3; Lane 4. So-Fast\textsuperscript{TM}; Lane 5. Control; M. protein molecular mass standards (TaKaRa, Japan).](image-url)
region which is highly variable. They present a highly conserved core region, namely a catalytic domain, which is capable of binding and cleaving sucrose. The C-terminal is composed of a series of tandem repeats and constitutes the glucan-binding domain (Monchois et al., 1999).

Based on the available nucleotide sequence of \textit{gtfB} of \textit{S. mutans}, two target sites were selected with Vector NTI 5.0. Antisense oligonucleotide design principles were observed and the relationship between Gtf structure and function was also considered when designing the antisense ODN. An abundance of literature indicates that phosphorothioate oligonucleotides not only serve as efficient substrates (when coupled with RNA) for RNase H enzymes, but also enhance its cellular uptake rate and stability (Baker & Monia, 1999). Consequently, PS-ODNs were used in this work.

In the present study, it was found that antisense PS-ODNs inhibited \textit{gtfB} mRNA transcription, GtfB expression and activity, and biofilm formation of \textit{S. mutans}. The effects of the antisense PS-ODNs were highly specific, because mismatched PS-ODN showed no effect on \textit{S. mutans} under the same conditions. Our results implied that PS-ODNs could penetrate into \textit{S. mutans} cells. The mechanism by which the antisense PS-ODNs inhibited the expression of GtfB and subsequent water-insoluble glucan synthesis probably involved: (1) binding of their cognate targets, therefore blocking RNA processing and translation; (2) being a substrate for RNase H when the DNA-RNA duplex was formed; (3) repressing gene transcription via triple-helix formation between the oligomer and double strands (Stein \textit{et al.}, 2005).

Our results demonstrated that the effects of the two antisense PS-ODNs on \textit{S. mutans} were similar, supporting Wang & Kuramitsu’s (2005) suggestion that it is the size, but not the relative position, of an antisense DNA fragment that is important in mediating the antisense phenomenon. The inhibitive effect of antisense PS-ODNs on GtfB activity was confirmed by \textit{in situ} cell-associated Gtf activity determination and water-insoluble glucan measurement. The decrease in enzyme activity correlated with the reduced GtfB level as revealed by SDS-PAGE and scanning densitometry. It was interesting to note that both GtfB and GtfC bands were

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{control.png}
\caption{Scanning electron micrographs of biofilms formed by differently treated \textit{Streptococcus mutans}. The pictures were magnified at \times 1000.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{growth_curve.png}
\caption{Growth of \textit{Streptococcus mutans} in THB medium with different treatments. \textcolor{red}{\textbullet} PS-ODN1; \textcolor{blue}{\textbullet} PS-ODN2; \textcolor{green}{\textbullet} PS-ODN3; \textcolor{black}{\times} So-Fast\textsuperscript{TM}, \textsuperscript{\textplus} Control. The absorbance of the culture was monitored at 540 nm with a spectrophotometer.}
\end{figure}
observed in PS-ODN3, So-Fast™ and control groups, whereas only the GtfB band was observed in PS-ODN1 and PS-ODN2 groups. We are not able to explain this phenomenon at present and it is necessary to address this problem in the future.

Dental plaque is a biofilm of adherent bacteria cells separated by fluid-filled spaces and imbedded in a capsular polysaccharide matrix (Li et al., 2002). Li et al. (2001) reported that S. mutans cells growing in biofilms were able to incorporate foreign DNA much more efficiently than their free-living counterparts. The natural transformation frequencies of biofilm-grown cells were 10- to 600-times higher than those of planktonic cells. In this study, both free PS-ODNs and a combination of free PS-ODNs with So-Fast™ were used to observe their differential effects on biofilm formation. The result showed that free antisense PS-ODNs exhibited relatively weaker inhibitory effects when compared with the combination of free antisense PS-ODNs with cationic polymer So-Fast™. The potential explanation for this phenomenon might be that free antisense PS-ODNs could not be taken up efficiently by the bacterial cells. However, the use of high concentration of PS-ODNs (> 10 μM) is not feasible because of the risk of non-sequence-specific interaction with the proteins and nucleic acids (Harth et al., 2000). It was argued that positively charged polymers can neutralize negatively charged PS-ODNs and also provide sufficient residual charge for interaction with cell membrane, therefore able to facilitate the internalization of PS-ODN (Chirila et al., 2002). In our study, it was found that free PS-ODN, when combined with So-Fast™, exhibited stronger inhibitory effect on S. mutans biofilm formation. Our results supported Chirila et al.'s view. Since it appears that Gtf transport is dependent on normal proton motive force (Markevics & Jacques, 1985), it is possible that the combination of PS-ODNs with So-Fast™ might be responsible for reduction of translation of gtfB and disruption of normal membrane function.

Previous studies suggested that GtfB might play certain roles in sucrose-dependent adherence and affect the density and cohesiveness of S. mutans in biofilm ( Munro et al., 1991; Fukushima et al., 1992; Fujiwara et al., 1996; Wang & Kuramitsu, 2005). Alternation in biofilm architecture by antisense PS-ODN treatment, including reduction in biofilm thickness, is possible that the combination of PS-ODNs with So-Fast™ might be responsible for reduction of translation of gtfB expression and activity, and biofilm formation of S. mutans. Antisense PS-ODN might be used as a novel reagent against S. mutans cariogenesis. However, the effects of antisense PS-ODNs on animal models need to be studied in order to elucidate the anticariogenic roles of the altered biofilm architecture.

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