Isolation and characterization of Streptococcus mutans in heart valve and dental plaque specimens from a patient with infective endocarditis

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Streptococcus mutans, known to be an aetiologic agent of dental caries, also causes infective endocarditis (IE), although a comparison of isolates from the oral cavity and infected heart valve of the same patient has not been reported. In the present study, infected heart valve and dental plaque samples from a patient with IE were analysed. Broad-range PCR with DNA sequencing revealed that 50 clones from the dental plaque isolates were composed of oral streptococci and periodontopathic bacteria, whereas only Streptococcus mutans was detected in 50 clones from the heart valve. Eighteen strains of Streptococcus mutans were isolated from dental plaque and seven from the heart valve, and the biochemical properties of each were in accordance with those of Streptococcus mutans. DNA fingerprinting analysis revealed that all the oral isolates of Streptococcus mutans had similar patterns, which were different from those of the isolates from the infected heart valve. Western blotting using glucosyltransferase (GTF)-specific antiserum showed that the seven strains from the heart valve lacked the three types of intact GTF. In addition, the sucrose-dependent adhesion rates of these isolates were significantly lower than those of the oral isolates $(P < 0.001)$. Furthermore, the isolates from the heart valve were less susceptible to erythromycin and kanamycin. These results indicate that the properties of the Streptococcus mutans strains isolated from the infected valve were different from those of typical oral strains, which may be related to the effects of IE.

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INTRODUCTION

Oral streptococci, which are major members of oral flora, frequently cause bacteraemia and infective endocarditis (IE) (Douglas et al., 1993). In 26 studies published between 1993 and 2003, 3784 cases of IE were presented, in which oral streptococci were considered to be involved in 21 %, second highest in frequency to Staphylococcus aureus (Moreillon & Que, 2004). According to a review of 848 IE cases in Japan, the most common type of micro-organism isolated from patient samples was Gram-positive streptococci (93.1%) , among which oral streptococci were frequently detected at a prevalence of 38.6% (Nakatani et al., 2003). Streptococcus mutans, a major cause of dental caries, has occasionally been

isolated from the blood of patients with IE and several case reports regarding the association of Streptococcus mutans and IE have been presented (Vose et al., 1987; Ullman et al., 1988; Gauduchon et al., 2001).

The complete genome of Streptococcus mutans strain UA159 has been sequenced and was shown to be composed of approximately 2 million base pairs containing nearly 2000 open reading frames (Ajdić et al., 2002). Among them, three types of glucosyltransferase (GtfB, GtfC and GtfD) and protein antigen c (PAc) are known to be major cell surface proteins. In the case of IE, GtfB, GtfC and GtfD are known to induce the production of cytokines, such as interleukin-6 (IL-6) from monocytes (Shun et al., 2005). Several reports have discussed the association of PAc with IE, and PAc antibody titres have been reported to be elevated in IE patients in comparison with healthy subjects (Russell et al., 1992). In addition, results from a recent study suggest that PAc is associated with phagocytosis by human polymorphonuclear

Abbreviations: AP-PCR, arbitrarily primed PCR; GTF, glucosyltransferase; IE, infective endocarditis; IL-6, interleukin-6; MIC, minimum inhibitory concentration; PAc, protein antigen c; RAPD, random amplified polymorphic DNA.

leukocytes, as in an experiment involving rats, PAc-defective mutants were found to survive in larger numbers and for longer durations than the parent strain, which led to more severe levels of systemic inflammation (Nakano et al., 2006).

We have previously reported the biochemical, serological and genetic characterization of four Streptococcus mutans strains identified from a total of 522 streptococcal isolates from the blood of patients with various infectious diseases, including IE (141 isolates from 84 cases), sepsis (85 isolates from 54 cases) and bacteraemia following tooth extraction (15 isolates from 12 cases) (Fujiwara et al., 2001). The Streptococcus mutans blood isolates TW295 and TW871 were found to possess extremely low amounts of glucose side-chain linked to the rhamnose backbone of the serotypespecific polysaccharide and could not be classified into any of the three serotypes $(c, e \text{ or } f)$. Therefore they were designated as belonging to a new serotype, k (Nakano et al., 2004). There are no known studies of Streptococcus mutans being isolated from dental plaque and infected valve samples from the same patient. In the present study, Streptococcus mutans was isolated from dental plaque and the infected heart valve of an IE patient and characterized.

METHODS

Bacterial strains. The Streptococcus mutans strains used in the present study were MT8148 (serotype c) (Ooshima et al., 1983) and its GtfB-, C-, D-defective isogenic mutant BC7s (Ooshima et al., 2001). All strains were grown in Brain Heart Infusion (BHI) broth (Difco) and on Mitis Salivarius (MS) agar (Difco), with the appropriate antibiotic (10 µg erythromycin ml^{-1}) being used when required for selection.

Subject. A 61-year-old male diagnosed with IE was referred by his physician practising in a local hospital in Osaka City to the Department of Cardiovascular Surgery of Osaka Rosai Hospital in December 2004. According to the records, the patient had visited his local hospital in September 2004 with a high fever, and he reported that he had undergone valve replacement surgery in September 2002. The local physician ordered a blood culture examination, which showed a positive reaction, and Staphylococcus aureus was isolated. Cefazolin was prescribed for 1 month. As the patient also had a cerebral haemorrhage, treatment for that was initiated first. In mid-December, the patient was referred to Osaka Rosai Hospital, where a transoesophageal echocardiography examination revealed a vegetation-like mass around the heart valve. Two days prior to heart surgery, the patient visited the Department of Dentistry and Oral Surgery for an oral examination and dental plaque was collected. All of the procedures in the present study, including the collection and use of samples for research purposes, were approved by the Ethical Committee of Osaka Rosai Hospital.

The intraoral examination showed that the patient had 20 teeth, of which 14 were classified as C4, the most severe form of dental caries (tooth crown completely decayed). The remaining six teeth were also affected with dental caries and were classified as C3 (extending into the pulp space). As for his periodontal condition, the periodontal pockets of six teeth were evaluated and the maximum periodontal pocket depth was measured at 5 mm. In addition, the amount of supportive bone was found to be reduced in an orthopantomographic examination,

indicating that those six teeth were affected by periodontitis. It was impossible to evaluate the periodontal condition of the remaining 14 teeth, because of their severely decayed condition.

Isolation of Streptococcus mutans. Prior to the valvereplacement operation, dental plaque was collected from the patient and placed in sterile PBS. This was then diluted and streaked on sheep blood agar (Becton Dickinson) and MS agar plates. At the time of the operation, the extirpated valve was placed into sterile PBS, after being aseptically cut into small pieces, and vortexed vigorously, and the resultant supernatant was streaked on MS agar plates, which were incubated anaerobically at 37 \degree C for 2 days. The colonies from the agar plates were cultured in BHI broth for 18 h and then streaked on MS agar plates containing bacitracin (0.2 U ml^{-1}) ; Sigma–Aldrich) and 15 % (w/v) sucrose (MSB plates). Eighteen strains were isolated from the dental plaque specimens, which were designated DP1–18, whereas seven strains, designated V1–7, were isolated from the heart valve tissue.

Biological properties of strains from dental plaque and heart valve. We sequenced the 16S rRNA genes of the strains isolated (DP1–18 and V1–7) and compared the results with those for the Streptococcus mutans reference strains MT8148 and NCTC 10449^T (Bentley et al., 1991) (GenBank accession no. X58303). Sequencing of the 16S rRNA genes was performed with the primers 8UA (5'-AGAGTTTGATCCTGGCTCAG-3') and 1540R (5'-AAGG-AGGTGATCCAGCC-3'), which encompass the conserved region of the 16S rRNA gene (Fujiwara et al., 2001). Serotyping was done by using an immunodiffusion method, with rabbit antisera specific for Streptococcus mutans serotypes c, e, f and k (Masuda et al., 1985; Nakano et al., 2004). Sugar fermentation profiles were examined using mannitol, sorbitol, raffinose and melibiose (1 % each) in phenol red broth (Difco). The expression of cell-associated or cellfree GTFs and the PAc were analysed using Western blot analysis with antibodies specific for GTF and PAc (Fujiwara et al., 2001). PCR analysis of the gtfB gene was carried out with the primers GTFBF (5'-CAAAGATGAAGAAGCAGTAAT-3') and GTFBR (5'-TTCTATAACAAAAGCACAATG-3') (Mattos-Graner et al., 2004), and primers GTFCF (5'-TTGGAGGAAATATGGAAAAG-3') and GTFCR (5'-GTCTAAAACGAACAGCACT-3') (Mattos-Graner et al., 2004) were used for amplification of the $gtfC$ gene, and GTFDF (5'-TGGGAAGCTCAGTTTCAGCAG-3') and GTFDR (5'-GCCAATA-CGTCTTCCATTGGC-3') for the gtfD gene.

In vitro analysis of caries-inducing capabilities. Sucrosedependent cellular adhesion to a glass surface was analysed using the procedure reported by Kawabata & Hamada (1999). In addition, cellular hydrophobicity was measured using the method described by Rosenberg et al. (1980) and dextran-binding activity was evaluated using the method of Lis et al. (1995). All assays were carried out three times and the mean and SD were calculated.

Antimicrobial susceptibility. Minimum inhibitory concentrations (MICs) for seven antimicrobial agents (erythromycin, kanamycin, vancomycin, gentamicin, penicillin, ampicillin and tetracycline) were determined using a macro-dilution broth method (Clinical and Laboratory Standards Institute, 2006). Nine hundred and fifty microlitres of Mueller–Hinton broth (Difco) supplemented with 5 % sheep blood and containing twofold serial dilutions of the seven antimicrobial agents was placed in sterile 13×100 mm test tubes. The test strain was cultured in BHI at 37° C for 18 h, and then washed and adjusted to 1×10^7 c.f.u. ml⁻¹. Thereafter, 50 µl samples of the diluted test strains $(5 \times 10^5 \text{ c.f.u.})$ were added to the tubes containing the antimicrobial agents, and incubated for 18 h at 37 $\,^{\circ}$ C.

Comparison of genetic properties of oral and blood isolates. Genomic DNA was extracted from the oral and heart valve isolates (DP1–18 and V1–7, respectively). Arbitrarily primed (AP)-PCR

fingerprinting was then performed with the primers OPA-02 (5'-TGCCGAGCTG-3') and OPA-13 (5'-TGCCGAGCTG-3'), as described by Li & Caufield (1998). The PCR assay included 45 cycles of denaturing at 94 °C for 30 s, annealing at 36 °C for 30 s and extension at 72 °C for 1 min. Amplicons were separated by electrophoresis in 1.5% agarose gels.

Random amplified polymorphic DNA (RAPD) analysis was performed using Ready-To-Go RAPD analysis beads and primers (Amersham Biosciences). PCR was performed using five primers (P1, 5'-GGT-GCGGGAA-3'; P2, 5'-GTTTCGCTCC-3'; P3, 5'-GTAGACCCGT-3'; P4, 5'-AAGAGCCCGT-3'; P5, 5'-AACGCGCAAC-3') and comprised 45 cycles of denaturing at 94 °C for 30 s, annealing at 36 °C for 30 s and extension at 72° C for 1 min. Amplicons were separated by electrophoresis in 2 % agarose gels.

Determination of bacterial species by broad-range PCR. Genomic DNA was extracted from dental plaque samples collected in 500 ml sterile PBS as well as the extirpated heart valve specimen, which was aseptically cut into small pieces. Broad-range PCR targeting the 16S rRNA gene followed by direct sequencing was carried out to define the bacterial species in the samples. The 16S rRNA gene was amplified by PCR with AmpliTaq Gold polymerase (Applied Biosystems), using the broad-range 16S rRNA gene primers 536f (5'-CAGCAGCCGGGGTAATAC-3') and 1050r (5'-CACGAGCTG-ACGACA-3') (Rovery et al., 2005). The PCR products were then separated by electrophoresis in a 1.5% agarose gel and the amplified DNA fragments were extracted from the gel using a QIAEX gel extraction kit (Qiagen). The DNA was directly cloned into a pGEM-T Easy vector (Promega), after which 50 clones from each sample were randomly chosen. The nucleotide sequence was determined by using a dye-terminator reaction with a DNA sequencing system (373-18 DNA sequencer; Applied Biosystems) and an ABI PRISM kit. Data analyses were performed with Gene Works software (IntelliGenetics). The 16S rRNA gene sequences obtained were compared with those available in the GenBank/EMBL/DDBJ databases using the gapped BLASTN 2.0.5 program obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih. gov/blast/). Identification to the species level was defined as a 16S rRNA gene sequence similarity of more than 99 % with that of the GenBank prototype strain sequence, whereas identification to the genus level was defined as a 16S rRNA gene sequence similarity of 97 % with that of the GenBank prototype strain sequence.

Statistical analysis. Intergroup differences for various factors were estimated using ANOVA for factorial models. Fisher's protected least-significant difference test was used to compare individual groups. Statistical computations were performed using STAT-VIEW II (Macintosh computer application), with a P value of $\lt 0.05$ being considered significant.

RESULTS AND DISCUSSION

Eighteen strains (DP1–18) were isolated from dental plaque and seven (V1–7) from the heart valve. The colony morphology of the plaque isolates (DP1–18) on MS agar was rough, which was similar to that of strain MT8148. On the other hand, the colony morphology of the valve isolates (V1–7) was large and smooth, which was similar to BC7s, the GtfB-, C- and D-defective MT8148 mutant strain.

The entire length of the 16S rRNA gene sequence of each of the strains isolated (DP1–18 and V1–7) was shown to be 100 % identical to that of Streptococcus mutans NCTC 10449^T (GenBank accession no. X58303). The sugar fermentation profiles for mannitol, sorbitol, raffinose and melibiose for all the strains were positive, which was consistent with those for strain MT8148. Furthermore, all strains isolated from the patient were classified as serotype c. The cellular hydrophobicity and dextran-binding capabilities of DP1–18 were not significantly different from those of V1–7 (data not shown). In contrast, the sucrose-dependent adhesion rate of V1–7 (22 \cdot 8 + 4 \cdot 8 %; range 13 \cdot 4–27 \cdot 1 %) was significantly lower than that of DP1–18 (78 \cdot 7+5 \cdot 5%; 66 \cdot 3–84 \cdot 4%) $(P < 0.001)$.

Western blot analysis for GtfB, GtfC and GtfD revealed that bands corresponding to the molecular masses of these proteins from strain MT8148 were present in strains DP1–18 but not in strains V1–7 (Fig. 1a, b). PCR revealed a 5.2 kb gtfB amplicon in strains MT8148 and DP1–18, whereas no such amplicon was observed in V1–7 (Fig. 1c). For $gtfC$, a 4?3 kb amplicon was detected in MT8148 and DP1–18, whereas there was no band for $gtfC$ in V1–7 (Fig. 1d). On the other hand, PCR for gtfD showed an approximately 4?5 kb band for MT8148 and DP1–18, whereas a band of approximately 5?8 kb was observed for V1–7 (Fig. 1e).

Fig. 1. Identification of expressed GTFs and coding genes. Western blot analyses of whole cells using antisera for cell-associated GTF (a) and cell-free GTF (b) were performed. $gtfB$ (c), $gtfC$ (d) and $gtfD$ (e) genes were amplified by PCR. M, 100 bp ladder. Lanes: 1, strain MT8148; 2, isolate DP1; 3, isolate V1.

A number of different Streptococcus mutans strains have been isolated from the blood of patients with IE (Munro & Macrina, 1993; Shun et al., 2005). In a previous study, four Streptococcus mutans strains isolated from the blood of IE patients were comprehensively characterized biologically, serologically and genetically. Some of these strains showed altered expression of glucan-binding protein C (Nakano et al., 2002), which resulted in resistance to phagocytosis by polymorphonuclear leukocytes (Nomura et al., 2004). In addition, two of the four strains showed an alteration of the serotype-specific polysaccharide, suggesting that changes in antigenicity may contribute to the effects of IE.

The three types of GTF are known to be major virulence factors in dental caries caused by Streptococcus mutans and their absence from strains is associated with an extremely diminished capacity to induce caries (Kuramitsu, 1993; Yamashita et al., 1993). An isogenic mutant strain lacking all three types of GTF (strain BC7s) demonstrated a drastic reduction in sucrose-dependent adhesion properties compared with the parent strain MT8148 (Ooshima et al., 2001). GTFs synthesize glucan using sucrose, which has been demonstrated to increase the incidence of IE in a rat experimental model (Munro & Macrina, 1993). On the other hand, sucrose is generally not present in human blood, suggesting that glucan synthesis may not be an important factor for Streptococcus mutans virulence in the bloodstream.

Recently, GTFs have been reported to be associated with the induction of IL-6 from endothelial cells surrounding infected valves in acute-stage IE patients, whereas the survival rate of mice infected with an isogenic mutant strain lacking all three types of GTF was significantly lower than for those infected with the parent strain and the uninfected control group (Shun et al., 2005). In the present study, none of the three types of intact GTF were expressed in strains V1–7 isolated from the heart valve. Since the incidence of strains deficient in GTF expression is considered to be low in the oral cavity, such a defect may be correlated with the effects of IE caused by Streptococcus mutans. Further research is needed to elucidate this association.

The MICs for the seven antimicrobial agents tested are summarized in Table 1. With regard to erythromycin and kanamycin, the MICs for the two antibiotics for the strains from the infected valve (V1–7) were significantly higher than those for the dental plaque strains (DP1–18). There were no significant differences in the MICs for the remaining five agents between the strains from the infected valve and those from dental plaque. The susceptibility to penicillin by mitis, anginosus, mutans, salivarius and sanguinis streptococci isolated from IE patients between 1994 and 2002 was shown to be lower than that in strains isolated between 1971 and 1986 in a survey performed in the USA (Prabhu et al., 2004). In addition, penicillin-resistant streptococci were reported to be highly prevalent (61.3%) in Japanese children (mean age, 6.7 years) at risk for IE who had not received antibiotics for more than 3 months (Nishi et al., 1999). In the present study, all the strains tested were

Table 1. In vitro susceptibility to antimicrobial agents

Values given are MIC (μ g ml⁻¹).

sensitive to penicillin, whereas the MIC values for erythromycin and kanamycin for the strains isolated from the heart valve were significantly higher than those from the oral cavity (Table 1). A survey performed in Taiwan in 1998 found that the number of erythromycin-resistant streptococci was increasing (Teng et al., 1998). Thus, careful attention is needed regarding the antibiotic susceptibility of micro-organisms that cause IE.

Our AP-PCR results revealed that the fingerprinting patterns for the isolates from dental plaque (DP1–18) were similar, as were those from the infected valve (V1–7), whereas the patterns were significantly different between DP1–18 and V1–7 (Fig. 2a, b). RAPD analysis revealed similar fingerprinting patterns with each of the five primers among all the dental plaque isolates (DP1–18) and heart valve isolates (V1–7) (data not shown). In addition, a comparison of the RAPD patterns of DP1 and V1 showed similar fingerprinting patterns with primers P1 and P2, whereas they were significantly different with primers P3, P4 and P5 (Fig. 2c).

The OPA-02 primer is considered to produce informative and reproducible results regarding amplicons for Streptococcus mutans when performing AP-PCR typing (Li & Caufield, 1998; Li et al., 2001; Nascimento et al., 2004). In our study, a single genotype was observed in the 18 oral isolates, whereas the seven heart valve strains were also of a single, but distinct, genotype. Multiple genotypes are often found in the oral cavity of individual subjects, with a maximum of seven different genotypes having been reported in an analysis of Streptococcus mutans isolated from four teeth (up to ten colonies per tooth) of a subject (Redmo Emanuelsson et al., 2003). Regarding the entry of Streptococcus mutans into the bloodstream, we speculate that a small number of strains similar to those represented by V1–7 may be present in the oral cavities of individuals with bacteria in the blood. It is reasonable that only a single genotype was observed in the present study of 18 oral isolates, given the limited number analysed, and there may have been other genotypes among the oral isolates that were similar to those found in the heart valve samples.

Fig. 2. Genetic analysis of oral and blood isolates using AP-PCR and RAPD. AP-PCR was carried out using primers OPA-02 (a) and OPA-13 (b). RAPD analysis was done using five primers (P1–P5) (c). M, 100 bp ladder. Lanes: 1, DP1; 2, DP2; 3, DP3; 4, DP4; 5, DP5; 6, DP6; 7, V1; 8, V2; 9, V3; 10, V4.

The recent development of molecular techniques has enabled prompt identification of targeted bacterial species in specimens, with significantly improved specificity and sensitivity. PCR methods using primers constructed with a species-specific nucleotide alignment are widely used for the detection of specific species. In addition, a broad-range eubacterial PCR assay with amplification of bacterial DNA and subsequent direct sequencing is considered to be a reliable diagnostic tool (Rovery et al., 2005; Petti et al., 2005). This is the first known study to analyse the bacterial profiles of dental plaque and infected heart valve samples taken from the same patient using PCR.

The nucleotide alignment of all 50 clones amplified from the infected heart valve was consistent with the 16S rRNA gene sequence of Streptococcus mutans (Table 2), indicating that the IE in our patient was caused by Streptococcus mutans. In the dental plaque samples, approximately half of the species determined based on the 16S rRNA gene sequence were oral streptococci, including Streptococcus sanguinis, Streptococcus mutans, Streptococcus mitis, Streptococcus oralis, Streptococcus gordonii, Streptococcus salivarius and Streptococcus pneumoniae, whereas Actinomyces, Neisseria and Capnocytophaga species were also detected. It is interesting to note that Table 2. Specification of isolates from dental plaque and heart valve samples by sequence analysis of fragments amplified using broad-range PCR

there are few reported cases of IE caused by Gram-negative periodontal bacteria, which are oral strains that are often found in elderly patients. Accumulation of data regarding the common bacterial profiles from the dental plaque of IE patients is important to specify the characteristics of oral bacteria in those patients.

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