Tea Catechin EGCg Suppresses the mgl Gene Associated with Halitosis

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Epigallocatechin gallate (EGCg), the main antimicrobial tea catechin, has been reported to inhibit growth and virulence factors of oral pathogens in vitro. Although the mechanism is unclear, the potential of EGCg in reducing halitosis caused by volatile sulfur compounds (VSCs) has been suggested. This study tested the hypothesis that EGCg reduces VSCs by suppressing \( mgl \), the gene encoding L-methionine-\( \alpha \)-deamino-\( \gamma \)-mercaptomethanelyase, responsible for methyl mercaptan (\( \text{CH}_3\text{SH} \)) production by oral anaerobes. In this study, the effect of EGCg on in vitro growth, \( \text{CH}_3\text{SH} \) production, and \( mgl \) gene expression in \( P.\ gingivalis \) W83 was investigated. EGCg inhibited growth of \( P.\ gingivalis \) W83 (MIC = 97.5 \( \mu \text{g/mL} \)) and was bactericidal (MBC = 187.5 \( \mu \text{g/mL} \)). At sub-MIC levels, EGCg inhibited \( \text{CH}_3\text{SH} \) production, and \( mgl \) mRNA and protein expression (\( p < 0.05 \)). We conclude that EGCg may represent a natural and alternative agent to the antimicrobial chemicals currently available for halitosis control.

**KEY WORDS:** halitosis, \( P.\ gingivalis \), epigallocatechin gallate, volatile sulfur compounds, \( mgl \).

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**INTRODUCTION**

Halitosis (oral malodor) is a common complaint that affects a large portion of the population (Scully and Rosenberg, 2003; Scully and Greenman, 2008). The overgrowth of proteolytic, anaerobic bacteria on the surfaces within the mouth results in microbial degradation of the organic substrates present in saliva, crevicular fluid, oral soft tissues, and retained debris. The major microbial degradation products contributing to halitosis are volatile sulfur compounds (VSCs), including hydrogen sulfide (\( \text{H}_2\text{S} \)), methyl mercaptan (\( \text{CH}_3\text{SH} \)), and dimethyl sulfide \( ([\text{CH}_3])_2\text{S} \) (Kleinberg and Westbay, 1990; Loesche and Kazor, 2002). VSCs have been reported to be cytotoxic to the gingival fibroblasts and epithelial cells in vitro (Ng and Tonzetich, 1984; Johnson et al., 1992). The total amount of VSCs and the increase in the ratio of \( \text{CH}_3\text{SH} \) to \( \text{H}_2\text{S} \) in human gingival crevicular sites are correlated with deeper or bleeding pockets (Coli and Tonzetich, 1992). These findings indicate that VSCs not only contribute to halitosis but also play an important role in the pathogenesis of periodontal disease.

Oral anaerobes, especially those residing on the dorsum of the tongue, possess enzymes that can degrade sulfur-containing peptides and amino acids to produce \( \text{H}_2\text{S} \) and \( \text{CH}_3\text{SH} \) (Nakano et al., 2002a). L-cysteine desulphhydrase (CD) acts on L-cysteine to produce pyruvate, ammonia, and \( \text{H}_2\text{S} \), while L-methionine-\( \alpha \)-deamino-\( \gamma \)-mercaptomethanelyase (METase) produces \( \alpha \)-ketobutyrate, ammonia, and \( \text{CH}_3\text{SH} \) from L-methionine (Pianotti et al., 1986). \( P.\ gingivalis \) produces large amounts of \( \text{CH}_3\text{SH} \) through METase, which is encoded by \( mgl \) (Yoshimura et al., 2000). An \( mgl \)-deficient mutant of \( P.\ gingivalis \) was found to produce significantly less \( \text{CH}_3\text{SH} \) than the wild-type. Mice challenged subcutaneously with the mutant demonstrated a higher survival rate than those challenged by the wild-type. These findings suggested that \( mgl \) was not only responsible for \( \text{CH}_3\text{SH} \) production, but also associated with the virulence of \( P.\ gingivalis \) (Yoshimura et al., 2000).

Various measures have been used to reduce VSCs to ameliorate bad breath, including the use of odor-masking oral hygiene products, mechanical and chemical reduction of micro-organisms and their substrates, and chemical neutralization of odoriferous compounds (van den Broek et al., 2008). Zinc-containing compounds have been commonly used to neutralize oral malodor, possibly because of their antimicrobial and sulfur-affinity properties (Young et al., 2001). However, adverse effects associated with zinc-containing compounds (van den Broek et al., 2008) justify further research and the development of alternative agents.

Public demand for useful natural antimicrobial oral hygiene products is increasing. Various natural substances such as polyphenols and their derivatives have been reported to possess deodorizing properties (Yasuda and Arakawa, 2003).
1995). Among these, tea polyphenols, commonly known as tea catechins, are also well-known for their anti-oxidant and antimicrobial properties (Cabrera et al., 2006). The galloylated catechins act primarily on bacterial membrane, often leading to cell death (Caturla et al., 2003). Epigallocatechin gallate (EGCg), the main antimicrobial tea catechin, has been reported to suppress in vitro growth and various virulence factors of oral pathogens (Wu and Wei, 2002, 2009; Okamoto et al., 2004; Sakanaoka and Okada, 2004). Recently, the potential of tea polyphenols to reduce VSCs in mouth air has been reported (Lodhia et al., 2008), and one in vitro study showed that EGCg removed CH₃SH via a chemical reaction in the presence of atmospheric oxygen (Yasuda and Arakawa, 1995). However, the exact mechanism of inhibition has not been well-elucidated. This study tested the hypothesis that EGCg may reduce CH₃SH production at sub-MIC levels under anaerobic conditions by suppressing the mgl expression in P. gingivalis W83.

**MATERIALS & METHODS**

**Chemicals, Test Bacterium, and Growth Conditions**

Epigallocatechin gallate from green tea (EGCg, 95% HPLC) and all chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Test bacterium P. gingivalis W83 (ATCC BAA-308™) was grown in TSB-yeast extract medium supplemented with 0.05% cysteine hydrochloride, 0.02 µg/mL menadione, 5 µg/mL hemin, and 0.02% potassium nitrate in an anaerobic chamber (37ºC, 10% H₂, 5% CO₂, 0.02% potassium chloride, 0.02 µg/mL menadione, 5 µg/mL hemin, and 0.02% potassium nitrate in an anaerobic chamber (37ºC, 10% H₂, 5% CO₂, and 85% N₂; Forma Scientific, Inc., Marietta, OH, USA).

**Generation of Polyclonal Antibodies**

Rabbit polyclonal antibodies were generated against peptide sequences specific for the METase of P. gingivalis W83 (NCBI accession number: NP_904655) and purified by peptide-affinity column chromatography. A 126-µg/mL stocking concentration of purified antibodies was used for the Western blot assay.

**Bacterial Susceptibility Assay**

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EGCg against P. gingivalis W83 were determined by a micro-dilution method as described previously (Nudera et al., 2007). Growth of P. gingivalis W83 in the presence of sub-MICs of EGCg was also monitored spectrophotometrically (Abₑ₅₄₀nm). Generation time was calculated based on the growth curve obtained according to the formula: \( t_d = \frac{(t_2 - t_1) \ln(2)}{[\ln(OD_2) - \ln(OD_1)]} \), where, \( t_d \) is doubling time, \( t_1 \) and \( t_2 \) are time readings for the beginning and end of the logarithmic growth phase, and \( OD_1 \) and \( OD_2 \) are optical density readings at time-points \( t_1 \) and \( t_2 \) (Khalichi et al., 2004).

**CH₃SH Production Assay**

The production of CH₃SH was determined based on the chemical reaction between the thiol group and 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) to form the 2-nitro-5-thiobenzoate (NTB-), which is yellow (Ellman, 1959). P. gingivalis W83 was grown in 96-well microtiter plates in the presence of sub-MICs of EGCg. After 48 hrs of anaerobic incubation, 10 µL of L-methionine (0.6%, w/v) and 10 µL of DTNB (0.06%, w/v) were added to each well. The plate was further incubated for 12 hrs, and the CH₃SH produced was measured spectrophotometrically at 430 nm and expressed as a percentage of the non-treated control. To determine whether the reduced CH₃SH production at sub-MICs of EGCg was due to growth suppression, we examined the viability of P. gingivalis W83 cells using an XTT (2, 3-bis [2-methyloxoy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide)-based assay (Tunney et al., 2004).

**Protein Extraction and Western Blotting**

P. gingivalis W83 grown in the presence of sub-MICs of EGCg for 20 hrs (late-log phase) was collected, and total protein was extracted with the ReadyPre™ Protein Extraction Kit (BIORAD, Hercules, CA, USA). The protein concentration was determined with the RC DC Protein Assay kit (BIO-RAD). Protein was then separated (50 µg/sample) by 10% SDS-PAGE and subjected to immunoblotting with anti-METase serum (1:3000, BIO-RAD).

**RNA Isolation, Purification, and Reverse Transcription**

P. gingivalis W83 grown in the presence of sub-MICs of EGCg for 20 hrs (late-log phase) was collected by centrifugation, and total RNA was isolated and purified with an RNeasy Protect Bacteria Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instruction. Reverse transcription was then performed by the 1st Strand cDNA Synthesis Kit with random hexamer primers (Invitrogen, Madison, WI, USA).

**Quantitative Real-time PCR**

We used real-time PCR to quantify mgl mRNA expressions with 16S rRNA as an internal control. The mgl (5′-TCGTGCTTATGAGCGGATGTC-3′ and 5′-GGAAATCCTCGTGGAATA-3′) and 16S rRNA (5′-TGTTTTAGGTTCTAGAT-3′ and 5′-CAATCGAGATCTGATGTA-3′) specific primers were used, and amplification was performed with SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on the iCycler iQ detection system (Applied Biosystems). Threshold cycle values \( (C_t) \) were determined and data were analyzed by StepOne™ Software v2.0 (Applied Biosystems) with the 2^ΔΔCt method.

**Statistical Analysis**

All experiments were performed in triplicate and reproduced 3 separate times. We used Student’s \( t \) test to determine the significance of the difference between the experimental group with a given concentration of EGCg and the control group (without EGCg). Significance was set at \( p < 0.05 \).

**RESULTS**

**EGCg Inhibits Growth and CH₃SH Production of P. gingivalis W83**

EGCg inhibited growth of P. gingivalis W83 (MIC = 97.5 µg/mL) and was bactericidal with an MBC of 187.5 µg/mL. At sub-MIC
Inhibition of mgl Gene Expression

levels of EGCg, although the growth rate of *P. gingivalis* W83 was inhibited by 29.1% on average (Fig. 1), the viability of the treated cells was not affected (Fig. 2B, p > 0.05). Meanwhile, the *in vitro* production of CH₃SH was significantly inhibited in the presence of sub-MICs of EGCg, with the most significant reduction (49%) at 31.25 µg/mL compared with the control (Fig. 2A, p < 0.05).

**EGCg at Sub-MIC Levels Inhibits mgl mRNA Expression of *P. gingivalis* W83**

Melt curves revealed the absence of non-specific products in all amplification reactions. The mRNA expression levels of *mgl* were normalized by amplification of the 16S rRNA of *P. gingivalis* W83 as an internal control. EGCg at 31.25 µg/mL significantly inhibited the *mgl* expression by 56% compared with the control group (p = 0.04). A similar down-regulatory effect was also observed at 46.87 µg/mL of EGCg, with 55% inhibition compared with the control group (p = 0.02), while no significant inhibition was observed below the concentration of 23.44 µg/mL (Fig. 3, p > 0.05).

**EGCg at Sub-MIC Levels Inhibits METase Protein Expression of *P. gingivalis* W83**

The specificity of the polyclonal antibodies was confirmed by Western blotting showing a single band at 43 kDa, in agreement with the previously reported molecular weight for METase of *P. gingivalis* W83 (Yoshimura *et al.*, 2000). In agreement with data from real-time PCR, EGCg suppressed METase protein expression by 55.2% at 46.87 µg/mL compared with non-treated control (p = 0.001). At 31.25 µg/mL of EGCg, a 51% reduction in METase expression was also observed (p = 0.007). No significant inhibition was observed when EGCg was below the concentration of 23.44 µg/mL (Fig. 4, p > 0.05).

**DISCUSSION**

Volatile sulfur compounds (VSCs), including H₂S, CH₃SH, and (CH₃)₂S, from the oral cavity are associated with halitosis (Nakano *et al.*, 2002a). The enzyme METase, responsible for the production of CH₃SH from L-methionine, has been reported in various oral and non-oral bacteria, including *Porphyromonas*, *Fusobacterium*, *Pseudomonas*, *Trichomonas*, and *Clostridium* species (Nakano *et al.*, 2002a, b). In *P. gingivalis* and *F. nucleatum*, the deaminating activity of METase has also contributed to the resistance to 3-chloro-DL-alanine, an inhibitor of peptidoglycan synthesis (Yoshimura *et al.*, 2002).

*P. gingivalis* produces large amounts of CH₃SH through the enzymatic action of METase on L-methionine (Yoshimura *et al.*, 2000). Although *P. gingivalis* also produces H₂S from substrates such as L-cysteine (Nakano *et al.*, 2002a), L-cysteine desulfhydrase (*lcd*) has not been reported in this organism. Because CH₃SH is better correlated with oral malodor strength than H₂S (Reingewirtz *et al.*, 1999), we focused our investigation on the
mgl gene only. Two genes (mgI1 and mgI2) have been reported in P. putida and Trichomonas vaginalis (Hori et al., 1996; McKie et al., 1998), but based on data obtained from the GeneBank (NCBI Reference Sequence: NC_002950.2) and other studies (Yoshimura et al., 2000; Nakano et al., 2002b), only one mgl gene with one transcript has been reported in P. gingivalis W83. An mgl-deficient mutant of P. gingivalis was found to demonstrate a significant decrease in CH₃SH production and virulence (Yoshimura et al., 2000), suggesting that specific mgl inhibitors may represent a new class of compounds with potential for reducing halitosis. Because mgl is not found in mammals, its inhibition should have little apparent effect on humans (Nakano et al., 2002a).

Tea, an infusion prepared from leaves of Camellia sinensis, is the most popular and widely consumed beverage in the world today (Wu and Wei, 2002). Green tea or tea catechins have exhibited a potential deodorizing effect against oral malodor, but the exact mechanism is still unclear (Yasuda and Arakawa, 1995; Lodhia et al., 2008). An in vitro study showed that EGCG removed CH₃SH via a chemical reaction by the addition of a methylthiol group to the ortho-quinone generated in the presence of atmospheric oxygen. This reaction was completely suppressed under anaerobic conditions (Yasuda and Arakawa, 1995). Our study demonstrated that the inhibition of P. gingivalis W83 CH₃SH production took place under anaerobic conditions, suggesting mechanisms distinct from the above-described chemical reaction. The observed bacteriostatic and bactericidal effects of EGCG against P. gingivalis support the more general antimicrobial mode of action in reducing VSCs. Because the presence of specific periodontal pathogens on the tongue significantly correlated with the intensity of oral malodor, the reduction of these organisms may improve the condition (Yaegaki and Sanada, 1992; Krespi et al., 2006).

In addition to the antimicrobial mechanism, we suggest a more specific mechanism for CH₃SH inhibition by EGCG at the genetic level, i.e., the suppression of mgl and METase expression by EGCG at sub-MIC levels without affecting cell viability. Although the suppression of transcriptional levels of various quorum-sensing-regulated virulence genes by EGCG has been reported in Escherichia coli 0157:H7 (Lee et al., 2009), studies demonstrating the specific effect of EGCG on halitosis-associated genes of oral anaerobes have not been documented.

The concentration of tea catechins present in a typical cup of tea is approximately 1 mg/mL (Hamilton-Miller, 2001), which is sufficient to reduce VSC production by P. gingivalis through its antimicrobial mode of action. Once the tea has been consumed, a gradual decrease in EGCG concentration to sub-MIC level may occur in the oral cavity, due to dilution by saliva, yet the concentration will still be at the level that promotes the suppression of mgl, leading to a reduction of CH₃SH. Analysis of our in vitro data showed that EGCG was capable of suppressing mgl at 31.25 µg/mL, which is higher than the reported peak salivary EGCG level (4.8–22 µg/mL) in an individual after drinking the equivalent of two to three cups of tea (Yang et al., 1999). However, reports have shown that holding tea infusion in the mouth for a longer period of time or chewing tea leaves may help raise the salivary levels of EGCG (Yang et al., 1999; Lee et al., 2004).

Based on our data, and those of other researchers (Yasuda and Arakawa, 1995; Sakanaka and Okada, 2004; Lodhia et al., 2008; Wu and Wei, 2009), EGCG may represent a natural and alternative agent to the antimicrobial chemicals currently available for halitosis management because: (1) EGCG is a good odor-neutralizing agent through the chemical reaction with the thiol groups of VSCs; (2) EGCG inhibits various virulence factors of periodontal pathogens; (3) EGCG inhibits growth of halitosis-associated oral anaerobes; and (4) EGCG suppresses mgl expression, thus reducing VSC production.

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