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RESEARCH REPORTS
Biological

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Tea Catechin EGCg Suppresses the *mgl* Gene Associated with Halitosis

ABSTRACT

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-α-deamino-γ-mercaptomethane-lyase, responsible for methyl mercaptan (CH_3SH) production by oral anaerobes. In this study, the effect of EGCg on *in vitro* growth, CH_3SH production, and *mgl* gene expression in *P. gingivalis* W83 was investigated. EGCg inhibited growth of *P. gingivalis* W83 (MIC = 97.5 μg/mL) and was bactericidal (MBC = 187.5 μg/mL). At sub-MIC levels, EGCg inhibited CH_3SH 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 (H_2S), methyl mercaptan (CH_3SH), and dimethyl sulfide [$(CH_3)_2S$] (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 CH_3SH to H_2S 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 H_2S and CH_3SH (Nakano *et al*., 2002a). L-cysteine desulfhydrase (CD) acts on L-cysteine to produce pyruvate, ammonia, and H_2S, while L-methionine-α-deamino-γ-mercaptomethane-lyase (METase) produces α-ketobutyrate, ammonia, and CH_3SH from L-methionine (Pianotti *et al*., 1986). *P. gingivalis* produces large amounts of CH_3SH through METase, which is encoded by *mgl* (Yoshimura *et al*., 2000). An *mgl*-deficient mutant of *P. gingivalis* was found to produce significantly less CH_3SH 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 CH_3SH 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 odorous 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,

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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; Sakanaka 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 CH_3SH *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 CH_3SH 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% H_2, 5% CO_2, and 85% N_2; 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 (Ab_{600nm}). Generation time was calculated based on the growth curve obtained according to the formula: $t_d = (t_2-t_1)\ln(2)/[\ln(OD_2)-\ln(OD_1)]$, 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).

CH_3SH Production Assay

The production of CH_3SH 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 CH_3SH produced was measured spectrophotometrically at 430 nm and expressed as a percentage of the non-treated control. To determine whether the reduced CH_3SH 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-methyloxy-4-nitro-5-sulfophenyl]-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 (BIO-RAD, 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 (1:1000) and horseradish-peroxidase-conjugated secondary antibodies (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'-TCGTGCTTA TGAGCGATGTC-3' and 5'-GGAAGTCACCCTCGTGGATA-3') and *16S rRNA* (5'-TGGGTTTAAAGGGTGCGTAG-3' and 5'-CA ATCGGAGTTCCTCGTGAT-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 $2^{-\Delta\Delta C_T}$ 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 CH_3SH 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

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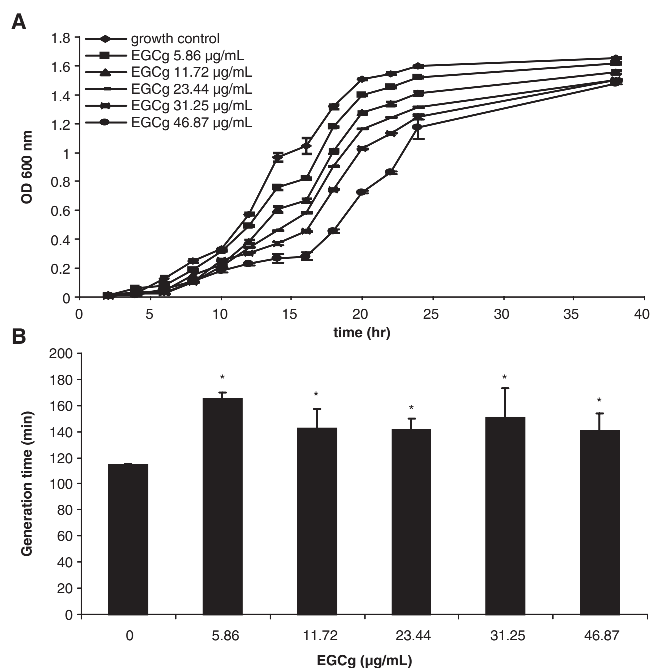

Figure 1. Effect of EGCg on the growth of *P. gingivalis* W83. **(A)** The growth curve of *P. gingivalis* W83 monitored over 38 hrs in the presence of sub-MICs of EGCg. **(B)** Generation time calculated based on the growth curve. Data represent mean ± SD of 3 independent experiments. *Significant difference was observed compared with the growth control (EGCg = 0 µg/mL).

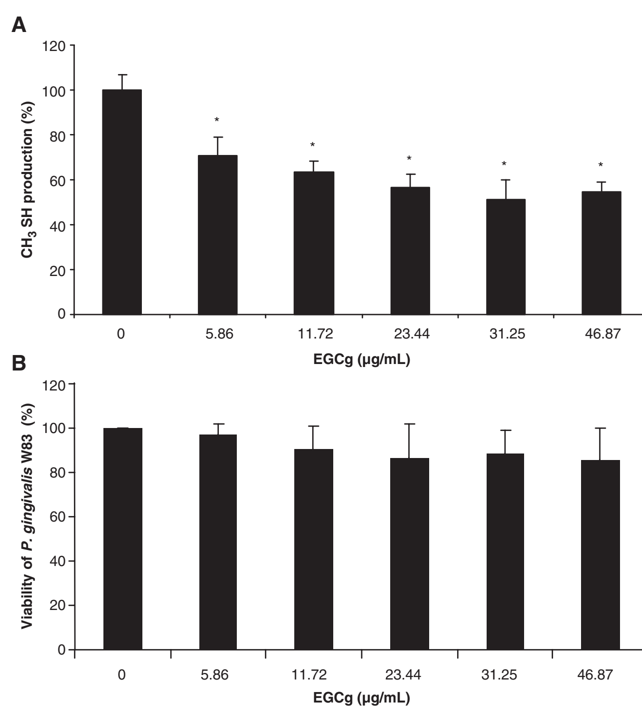

Figure 2. Effects of increasing concentrations of EGCg on the CH_3SH production by *P. gingivalis* W83. **(A)** *In vitro* CH_3SH production in the presence of sub-MICs of EGCg. **(B)** Viability of *P. gingivalis* W83 growing in the presence of sub-MICs of EGCg determined by XTT assay. Data represent mean ± SD of 3 independent experiments. *Significant inhibition was observed ($p < 0.05$).

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 CH_3SH 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 H_2S, CH_3SH, and $(CH3)_2S$, from the oral cavity are associated with halitosis (Nakano *et al*., 2002a). The enzyme METase, responsible for the production of CH_3SH 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 CH_3SH through the enzymatic action of METase on L-methionine (Yoshimura *et al*., 2000). Although *P. gingivalis* also produces H_2S from substrates such as L-cysteine (Nakano *et al*., 2002a), L-cysteine desulfhydrase (*lcd*) has not been reported in this organism. Because CH_3SH is better correlated with oral malodor strength than H_2S (Reingewirtz *et al*., 1999), we focused our investigation on the

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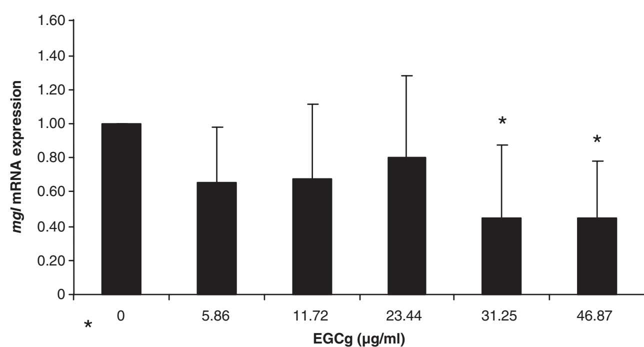

Figure 3. Effects of increasing concentrations of EGCg on the mRNA expression of *mgl* in *P. gingivalis* W83. The expression of *mgl* mRNA (treated *vs.* non-treated control) is shown as mean ± SD of 3 independent experiments. *Significant inhibition was observed at 31.25 μg/mL and 46.87 μg/mL ($p < 0.05$).

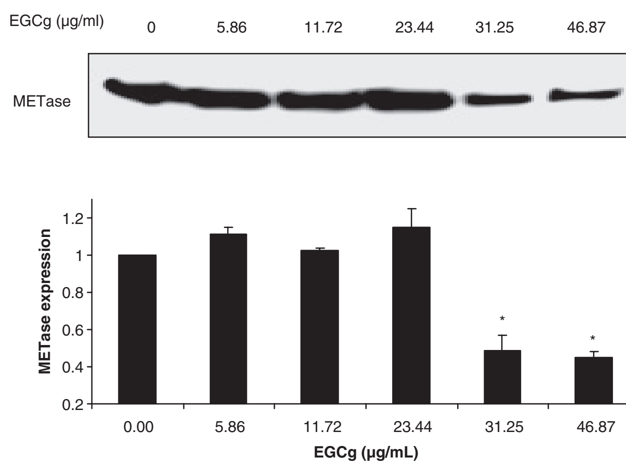

Figure 4. Effects of increasing concentrations of EGCg on METase protein expression in *P. gingivalis* W83. METase expression was determined by Western blotting, and a representative image is shown. The expression of METase (treated *vs.* non-treated control) is shown as mean ± SD of 3 independent experiments. Asterisk indicates significant inhibition ($p < 0.05$).

mgl gene only. Two genes (*mg11* and *mg12*) 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 CH_3SH 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 CH_3SH *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 CH_3SH 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 CH_3SH 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 CH_3SH. 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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