Articles

Enhancement of antimicrobial activities of antibiotics by combination with epigallocatechin gallate against methicillin-resistant *Staphylococcus aureus*

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Background Methicillin-resistant *Staphylococcus aureus* (MRSA) is the major cause of nosocomial infections. Most MRSA strains are resistant not only to β-lactam antibiotics but also to multiple antimicrobial agents. New therapeutic agents or new approaches are urgently needed for multiple antimicrobial resistant bacteria such as MRSA.

Objective It has been reported that epigallocatechin gallate (EGCg) sensitizes MRSA to β-lactam antibiotics. Based on this observation, we investigated whether EGCg would be able to enhance the antimicrobial activity of β-lactam antibiotics against various microorganisms, and also the appearance frequency of escape mutants of MRSA due to the combination of EGCg with β-lactam antibiotics.

Methods The enhancement effects of the antimicrobial activities of antibiotics in combination with EGCg were tested by using a standard MIC (minimum inhibitory concentration) assay. The proliferation and appearance of escape mutants of MRSA due to the combination of EGCg with antibiotics were investigated for a 3-day cultivation period on the surface of agar plates.

Results Antimicrobial activity of β-lactam antibiotics against MRSA was obviously enhanced by the combination with EGCg. However, the enhancement effect of EGCg was demonstrated to have high specificity to β-lactam antibiotics and to *S. aureus*. The proliferation and appearance of escape mutants of MRSA due to the combination were not observed completely for 3 days.

Conclusion The combined EGCg/β-lactam antibiotics is expected to be a new therapeutic method which possesses high safety and stable effectiveness against infectious diseases caused by MRSA.

Key words methicillin-resistant *Staphylococcus aureus* (MRSA), epigallocatechin gallate, antimicrobial activity, *mecA*

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become the most important causative agent of hospital-acquired (nosocomial) infections in many countries. MRSA has a *mecA* gene, which encodes low-affinity penicillin-binding protein 2' (PBP 2'), and is the most important gene responsible for the resistance to β-lactam antibiotics (Hartman and Tomasz.; 1984, Reynolds and Brown; 1985, Ubukata *et al.*; 1989, Chambers; 1997). Moreover, MRSA has a *blaZ* gene, which encodes the β-lactamase enzyme. This enzyme decomposes the β-lactam ring, and consequently β-lactam antibiotics are inactivated (Brown and Reynolds; 1980). Most MRSA strains have acquired resistance to multiple antibiotics, not only β-lactams, but also aminoglycosides, macrolides and fluoroquinolones (Maple *et al.*; 1989). Therefore, therapeutic agents for MRSA infections are extremely limited. Although vancomycin has been used for treatment of MRSA infection as one of the limited therapeutic agents, the emergence of vancomycin-resistant *S. aureus* (VRSA) has already been reported (Centers for Diseases Control and Prevention; 1997). New therapeutic agents or new approaches are urgently needed for multiple antibiotic-resistant bacteria such as MRSA.

Epigallocatechin gallate (EGCg) is a monomeric polyphenolic constituent of green tea (*Camellia sinensis*), and is responsible for antimicrobial activity (Toda *et al.*; 1991, Ikigai *et al.*; 1993). The structure of EGCg is shown in Fig. 1. It has been reported that EGCg sensitizes MRSA to β-lactam antibiotics (Yam *et al.*; 1998, Zhao *et al.*; 2001, Stapleton *et al.*; 2004), and the combination of EGCg with penicillin takes part in the suppression of β-lactamase activity against β-lactamase-producing *S. aureus* (Zhao *et al.*; 2002). Furthermore, it was suggested that EGCg directly binds peptidoglycan on the cell wall of bacteria, and synergistically enhances the antimicrobial activity of β-lactam antibiotics (Zhao *et al.*; 2001).

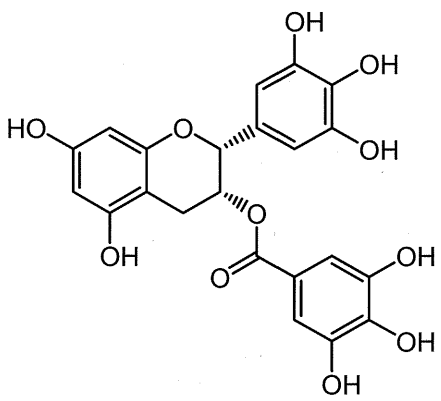

Fig. 1. Structure of (-)-epigallocatechin gallate (EGCg).

Based on these observations, we investigated whether EGCg would be able to enhance the antimicrobial activity of β-lactam antibiotics against the Gram-positive bacteria other than *S. aureus* and Gram-negative microorganisms. Moreover, we investigated the appearance frequency of escape mutants of MRSA due to the combination of EGCg with β-lactam antibiotics.

Materials and Methods

1) Bacterial strains

The bacterial strains used in this study are listed in Table 1. The *S. aureus* IID1678 and IID1679 strains were obtained from International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo. The *S. aureus* SA-12 strain was isolated from a healthy adult volunteer. The *S. aureus* NBRC12732, *Streptococcus mutans* NBRC13955, *Bacillus subtilis* NBRC3134, *Bacillus cereus* NBRC13494, *Escherichia coli* NBRC14237, *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) NBRC3313 and *Pseudomonas aeruginosa* NBRC12582 strains were obtained from the National Institute of Technology and

Table 1 MIC of EGCg against Gram-positive and Gram-negative bacteria

	Bacteria	Strain	MIC of EGCg (μg/mL)
Gram-positive	*S. aureus*	IID1678	25.0
	S. aureus	IID1679	25.0
	S. aureus	SA-12	25.0
	S. aureus	NBRC12732	50.0
	S. mutans	NBRC13955	>200
	B. subtilis	NBRC3134	>200
	B. cereus	NBRC13494	100
Gram-negative	*E. coli*	NBRC14237	>200
	S. enteritidis	NBRC3313	>200
	P. aeruginosa	NBRC12582	>200

Evaluation Biological Research Center.

2) EGCg, antibiotics and susceptibility testing

EGCg, oxacillin (MPIPC), kanamycin (KM) and gentamicin (GM) were obtained from Wako Pure Chemical Industries, Ltd. Benzil penicillin (PCG) and ampicillin (AMP) were obtained from Nacalai Tesque, Inc.

MIC (minimum inhibitory concentration) was determined by a liquid microdilution method in 96-well microtiter plates according to the protocol recommended by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards; 1997). Serially two-fold diluted antibiotics or EGCg were prepared by using the Sensitivity Test (ST) broth (Nissui Pharmaceutical Co., Ltd.) and approximately 5 x 10^4 CFU bacteria were inoculated. When the enhancement effects of antimicrobial activity of the antibiotics used in combination with EGCg were investigated, the ST broth which contained EGCg at 12.5 μg/mL or 50 μg/mL was used for preparation of serially two-fold diluted antibiotics. Cultivation was performed at 35℃ for 24 h under an aerobic condition. The MIC was determined as the lowest concentration of antimicrobial agent at which the bacteria were not able to grow.

3) PCR

PCR primers for the *mecA* gene (Zhao *et al.*: 2001), and for the *blaZ* gene (Okamoto *et al.*: 1996) are described in Table 2. PCR was performed using a DNA thermal cycler model TP600 (Takara Bio Inc.) with 30 cycles of denaturation for 30s at 95℃, annealing for 30s at 62℃, and extension for 30s at 72℃. PCR products were analyzed on 1.2% agarose gels and visualized by SYBR Safe DNA gel staining (Invitrogen). A 313-base-pair DNA fragment of the *mecA* gene and a 325-base-pair fragment of the *blaZ* gene were amplified by using the primers described above.

4) Assay with escape mutants of MRSA due to the combination of EGCg with β-lactam antibiotics on an agar surface

S. aureus IID1678, approximately 1 x 10^6 CFU, were inoculated and spread on Mueller-Hinton (MH) agar (Nissui Pharmaceutical Co., Ltd.) plates (90mm in diameter) which contained 25 μg/mL EGCg, 16 μg/mL PCG, 16 μg/mL AMP, 8 μg /mL MPIPC, 25 μg/mL EGCg + 16 μg/mL PCG, 25 μg/mL EGCg + 16 μg/mL AMP or 25 μg/mL EGCg + 8 μg/mL MPIPC. The plates were cultured at 37℃ for 72h under an aerobic condition.

Results

1) PCR analysis of *mecA* and *blaZ* gene in *S. aureus*

PCR assays employing each primer pair described in Table 2 produced DNA products of the predicted DNA sizes (Fig. 2). DNA fragments of 313 bp of the *mecA* gene were amplified from *S. aureus* IID1678 and IID1679 strains (Fig. 2A). It was estimated that both strains were MRSAs which produce the PBP 2'. On the other hand, DNA fragments of 325 bp of the *blaZ* gene were amplified from *S. aureus* IID1678, IID1679 and SA-12 strains (Fig. 2B). It was expected that those strains would produce the β-lactamase enzyme and be resistant to β-lactam antibiotics. Those DNA

Table 2. PCR primers used for detection of *mecA* and *blaZ* genes

Gene	Primer name	Primer sequence	Positions
mecA	MecF (sense)	5'-AAA CTA CGG TAA CAT TGA TCG CAA C-3'	399-423
	MecR (antisense)	5'-CTT GTA CCC AAT TTT GAT CCA TTT G-3'	712-689
blaZ	BlaF (sense)	5'-ACT CTT TGG CAT GTG AAC TG-3'	5458-5477
	BlaR (antisense)	5'-AAT CCT GCA AGA AGA GTT AG-3'	5172-5153

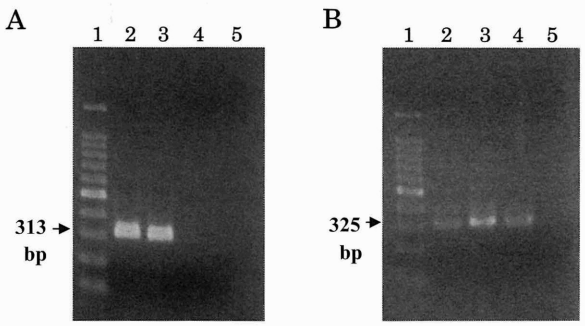

Fig. 2. PCR analysis of (A) *mecA* gene and (B) *blaZ* gene in *S. aureus*. Lane 1, 100-bp DNA ladder (molecular weight marker); lane 2, *S. aureus* IID1678; lane 3, *S. aureus* IID1679; lane 4, *S. aureus* SA-12; lane 5, *S. aureus* NBRC12732 as a control. Expected sizes of PCR products (313 bp, 325 bp) are shown by arrows.

fragments derived from the *mecA* and *blaZ* genes were not amplified from the *S. aureus* NBRC12732 strain. The strain was estimated to be methicillin-sensitive *S. aureus* (MSSA) which is susceptible to methicillin and other β-lactam antibiotics.

2) MIC of EGCg against Gram-positive and Gram-negative bacteria

MICs of EGCg were measured to confirm the antimicrobial activities of EGCg against Gram-positive and Gram-negative bacteria (Table 1). EGCg showed antimicrobial activities against *S. aureus* (MIC: 25～50 μg/mL) and *B. cereus* (MIC; 100 μg/mL). However, antimicrobial activities against *S. mutans*, *B. subtilis* and Gram-negative bacteria (*E. coli*, *S. enteritidis and P. aeruginosa*) were hardly observed (MIC: >200 μg/mL). EGCg was shown to have high specificity to *S. aureus* in its antimicrobial activity. A large difference was not observed in the activities of the strains of *S. aureus*.

3) Enhancement of antimicrobial activity of the antibiotics by EGCg

The enhancement effects of the antimicrobial activity of the β-lactam and aminoglycoside antibiotics against *S. aureus* by combining with EGCg are shown in Table 3. EGCg was used in a concentration by which the proliferation of *S. aureus* was not inhibited (12.5 μg/mL, half of the MIC). The antimicrobial activities of three β-lactams (PCG, AMP and MPIPC) and two aminoglycosides (KM and GM) were not demonstrated to be against the *S. aureus* IID1678 and IID1679 strains. Both strains showed the properties of MRSA which are resistant to multiple antibiotics. On the other hand, the antimicrobial activities of the β-lactam antibiotics against these strains were obviously enhanced by the combination with EGCg (Table 3). However, the activities of the aminoglycoside antibiotics were not enhanced by EGCg. Similar phenomena were observed in the *S. aureus* SA-12 strain, which possesses the β-lactamase (*blaZ*) gene, while the effect of MPIPC was not confirmed because of high susceptibility to MPIPC.

Furthermore, the enhancement effects of the antimicrobial activity of the β-lactam antibiotics (PCG and AMP) by combination with EGCg against Gram-positive bacteria other than *S. aureus* and Gram-negative bacteria were investigated (Table 4). EGCg was used in a concentration of 50 μg/mL which did not inhibit those microorganisms. The enhancement effects were not observed against Gram-positive and Gram-negative bacteria while the effects against *S. mutans* and *B. subtilis* were unclear because they were highly susceptible to the β-lactam antibiotics. The enhanced effect on the antibiotics by EGCg was demonstrated to have high specificity to β-lactam antibiotics and *S. aureus*.

4) Appearance of escape mutants of MRSA due to the combination of EGCg with β-lactam antibiotics

The antimicrobial activity of the β-lactam antibiotics against MRSA used in combination with EGCg was investigated on MH agar plates to confirm the appearance frequency of the escape mutants of MRSA (Fig. 3). Proliferation of *S. aureus* IID1678 (MRSA) was not inhibited on the surface of MH agar plates which contained 25 μg/mL EGCg (Fig. 3A), while the MIC of EGCg against the strain was 25 μg/mL (see Table 1). Similarly, proliferation of the strain was not suppressed on agar plates which contained 16 μg/mL PCG, 16 μg/mL AMP and 8 μg/mL MPIPC (Fig. 3B, C, D). These results of this study support the view

Table 3 Effects of EGCg in sensitizing *S. aureus* to β-lactam and aminoglycoside antibiotics

	MIC (μg/mL)									
						Combination with EGCg (12.5 μg/mL)				
S. aureus	PCG	AMP	MPIPC	KM	GM	PCG	AMP	MPIPC	KM	GM
IID1678	>128	>128	>32	>128	>128	2	4	1	>128	128
IID1679	>128	>128	32	>128	>128	8	8	0.5	>128	>128
SA-12	64	32	≤0.125	1	0.25	≤0.125	≤0.125	0.031	1	0.25
NBRC12732	≤0.125	≤0.125	0.5	0.25	≤0.125	≤0.125	≤0.125	0.25	0.25	≤0.125

PCG, benzil penicillin; AMP, ampicillin; MPIPC, oxacillin; KM, kanamycin; GM, gentamicin

Table 4 Effects of EGCg in sensitizing Gram-positive bacteria other than *S. aureus* and Gram-negative bacteria to β-lactam antibiotics

		MIC (μg/mL)			
				Combination with EGCg (50 μg/mL)	
Bacteria	Strain	PCG	AMP	PCG	AMP
S. mutans	NBRC13955	2	≤0.125	2	≤0.125
B. subtilis	NBRC3134	≤0.125	≤0.125	≤0.125	≤0.125
B. cereus	NBRC13494	>128	>128	128	128
E. coli	NBRC14237	>128	8	>128	4
S. enteritidis	NBRC3313	16	1	16	1
P. aeruginosa	NBRC12582	>128	>128	>128	>128

that the MICs of β-lactam antibiotics against the strain are >128, >128 and >32 μg/mL (see Table 3). On the other hand, the proliferation of *S. aureus* IID1678 was completely inhibited on the surface of agar plates which contained 25 μg/mL EGCg + 16 μg/mL PCG, 25 μg/mL EGCg + 16 μg/mL AMP and 25 μg/mL EGCg + 8 μg/mL MPIPC during the 3-day cultivation period (Fig. 3E, F, G). The proliferation and appearance of escape mutants of MRSA due to the combination of EGCg with β-lactam antibiotics were suppressed completely for 3 days on the agar plates.

Discussion

Extracts of green tea leaves (*Camellia sinensis*) contain many kinds of galloyl catechins such as epicatechin gallate, catechin gallate and epigallocatechin gallate (EGCg), and nongalloylated catechins such as epicatechin and epigallocatechin. EGCg is the main constituent of tea catechins, and has the strongest antimicrobial activity of the catechins. In this study, the antimicrobial activity of EGCg was demonstrated against *S. aureus*, including MRSA and *B. cereus*, but was not observed against other bacteria (Table 1). The high specificity of EGCg to bacteria was demonstrated. It has been reported that EGCg inhibits the synthesis of peptidoglycan on the cell wall of bacteria (Zhao *et al.*; 2001). However, it was not clear that EGCg showed antimicrobial activity against certain limited kinds of bacteria.

MRSA has acquired resistance to many antibiotics among the wide range of antibiotics used to treat infectious diseases caused by *S. aureus* or other microorganisms. In our study, the *S. aureus*

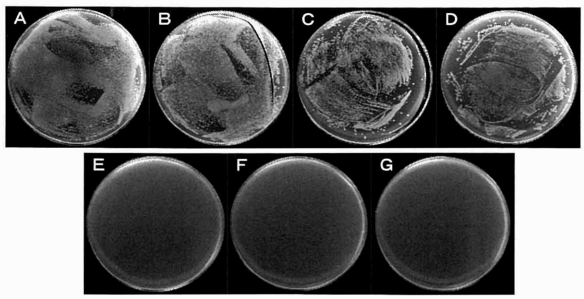

Fig. 3. Assay with the escape mutants of MRSA due to the combination of EGCg with β-lactam antibiotics on the surface of agar plates. *S. aureus* IID1678 was spread on MH agar plates which contain (A) EGCg (25 μg/mL), (B) benzil penicillin (16 μg/mL), (C) ampicillin (16 μg/mL), (D) oxacillin (8 μg/mL), (E) EGCg (25 μg/mL) + benzil penicillin (16 μg/mL), (F) EGCg (25 μg/mL) + ampicillin (16 μg/mL), (G) EGCg (25 μg/mL) + oxacillin (8 μg/mL), and cultured at 37 ℃ for 3 days.

IID1678 and IID1679 strains showed the properties of MRSA that are resistant to β-lactam and aminoglycoside antibiotics (Table 3), and these strains had the *mecA* gene which encodes low-affinity PBP 2' and the *blaZ* gene which encodes β-lactamase (Fig. 2). However, the antimicrobial activities of the β-lactam antibiotics used in combination with EGCg were demonstrated to be enhanced against the IID1678 strain. Although the enhancement effects of EGCg on β-lactam antibiotics have already been reported abundantly (Yam *et al.*; 1998, Zhao *et al.*; 2001, 2002, Stapleton *et al.*; 2004), the specificity of the effects to antibiotics and bacteria have hardly been reported. In this study, the enhancement effects of antimicrobial activities in combination with EGCg were seen in regard to the β-lactam antibiotics and *S. aureus*, the effect was not observed in aminoglycoside antibiotics. There is a possibility that the effect appears in antibiotics which inhibit the synthesis of peptidoglycan on the cell wall because the action of EGCg against microorganisms is similar. Further analyses of the specificity to antibiotics and to bacteria are necessary to clarify the mechanism of enhancement effects in combination with EGCg. Moreover, a new compound which shows the enhancement effect of antimicrobial activity on bacteria other than *S. aureus* might be found by analyzing the mechanism of the enhancement effect.

One of the most important points is to confirm whether the escape mutants appear easily due to the combination of EGCg with β-lactam antibiotics, even though the enhancement effect was observed in the MIC assay. In general, it is known that the escape mutants appear easily on the surface of agar plates compared with microtiter plates containing a liquid medium. In our study, it was shown that the escape mutants of MRSA due to the combined EGCg/β-lactam antibiotics did not appear on the surface of agar plates during the 3-day cultivation period. The enhancement effect of antimicrobial activity by combination with EGCg completely suppressed the proliferation of MRSA and the appearance of resistant bacteria for 3 days. The stability of the enhancement effect by EGCg was certified in this study.

EGCg is the main component of green tea (*Camellia sinensis*). The tea is consumed every day by billions of people in the world and its high safety is a fact. The combined EGCg/β-lactam antibiotics is expected to be a new therapeutic method which possesses high safety and stable effectiveness against infectious diseases caused by *S. aureus*, such as MRSA.

Conclusion

The antimicrobial activities of the β-lactam antibiotics against MRSA and other strains of *S. aureus* were obviously enhanced by the combination with EGCg. However, the activity of the aminoglycoside antibiotics was not enhanced by EGCg. The enhancement effect was hardly observed for Gram-positive bacteria other than *S. aureus* and Gram-negative bacteria. Thus, the effect of EGCg was demonstrated to have high specificity to certain antibiotics and bacteria. Furthermore, the proliferation and appearance of escape mutants of MRSA due to the combination of EGCg with β-lactam antibiotics were not observed at all during the 3-day cultivation period. The combined EGCg/β-lactam antibiotics is expected to be a new therapeutic method.

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(Summary)

没食子酸エピガロカテキンによるメチシリン耐性黄色ブドウ球菌に対する抗生物質の抗菌活性増強効果

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背景　メチシリン耐性黄色ブドウ球菌（MRSA）は、メチシリンに対してだけではなく、β-ラクタム系、アミノグリコシド系、ニューキノロン系など多くの抗生物質に対して耐性を獲得している。医療現場では、このMRSAによる院内感染や日和見感染症が深刻な問題となっており、MRSA感染症に対する新しい治療薬や治療方法の開発が急務となっている。

目的　緑茶由来没食子酸エピガロカテキン（EGCg）による、β-ラクタム系抗生物質のMRSAに対する抗菌活性増強効果については、既にいくつか報告されている。本研究ではこれらの知見を基に、EGCgによるこの増強効果が、どのような種類の細菌に対して現れるか、その細菌種特異性について解析を行った。また、EGCgによるβ-ラクタム系抗生物質の抗菌活性増強効果に対し、耐性を獲得して増殖するMRSAの出現頻度について解析を行った。

方法　EGCgによる抗生物質の抗菌活性増強効果は、EGCg含有液体培地を用いたMIC法（微量液体希釈法）で試験した。また、EGCgによるこの増強効果から、耐性を獲得して増殖するMRSAの出現頻度については、EGCgとβ-ラクタム系抗生物質を含有する寒天培地上で、MRSAを3日間培養することで解析を行った。

結果　MRSAに対してほとんど抗菌活性を示さなかったβ-ラクタム系抗生物質が、EGCgと併用することで顕著な抗菌活性を示した。この抗菌活性の増強効果は、β-ラクタム系抗生物質および黄色ブドウ球菌に対して高い特異性が見られた。EGCgによるβ-ラクタム系抗生物質の抗菌活性増強効果に対し、耐性を獲得して増殖するMRSAの出現は、3日間完全に抑制され、その効果の高い安定性が確認された。

結論　EGCgは緑茶由来であり、その安全性についてはほとんど問題ないものと考えられる。β-ラクタム系抗生物質とEGCgとの組み合わせは、MRSAによる院内感染や日和見感染症に対して、高い安全性と有効性をもった新しい医薬品や治療方法の開発に結びつくものと期待される。

キーワード　メチシリン耐性黄色ブドウ球菌（MRSA）、没食子酸エピガロカテキン、抗菌活性、*mecA* 遺伝子