Decreased Susceptibility of *Staphylococcus aureus* Small-Colony Variants toward Human Antimicrobial Peptides

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Staphylococcus aureus is a frequent resident of human nose and skin in many individuals, but it is also able to cause a variety of serious infections including those of the skin and soft tissue. There is increasing evidence that particularly persistent, relapsing, and difficult-to-treat infections caused by *S. aureus* are associated with the formation of the small-colony variant (SCV) phenotype. The aim of this study was to investigate the hypothesis that (i) skin-derived antimicrobial peptides (AMPs) exhibit a reduced activity against SCVs and (ii) that switching into the SCV phenotype may endow *S. aureus* with a decreased susceptibility toward the killing activity of human stratum corneum. Here, we show that clinically derived *S. aureus* SCVs are less susceptible to the bactericidal activity of different human skin-derived AMPs as compared with their isogenic corresponding wild-type strains. Similarly, a *S. aureus hemB* mutant displaying the SCV phenotype was less susceptible to the antimicrobial activity of AMPs than its *hemB*-complemented mutant. These findings were accompanied by a higher resistance of SCVs to the killing activity of human stratum corneum. Switching into the SCV phenotype may help *S. aureus* to subvert cutaneous innate defense, thus contributing to the establishment and persistence of infection.

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INTRODUCTION

The Gram-positive opportunistic bacterium *Staphylococcus aureus* is a frequent resident of human skin and nose microbiota in many individuals (Wos-Oxley *et al.*, 2010), but it also has a major impact as a causative agent of a variety of serious infections including those of the skin and soft tissue (Iwatsuki *et al.*, 2006; Miller and Kaplan, 2009). There is increasing evidence that particularly persistent, relapsing, and difficult-to-treat infections caused by *S. aureus* are associated with the formation of the small-colony variant (SCV) phenotype (Proctor *et al.*, 2006; Vaudaux *et al.*, 2006; von Eiff and Becker, 2007). SCVs generally have a longer generation time and are named because of their slow growth leading to small colonies on agar plates. As SCVs often need several days to become visible on agar plates, they can be easily missed by routine microbial diagnostic procedures (Proctor *et al.*, 2006; Vaudaux *et al.*, 2006). Being internalized in the host cell environment, *S. aureus* is able to switch from the wild-type phenotype to a SCV phenotype, thus making it possible to escape host defense responses and to spread the infection (Tuchscherr *et al.*, 2011; von Eiff *et al.*, 2001). The switch to an SCV phenotype is associated with complex physiological and metabolic changes mainly based on defects in electron transport or in biosynthesis of thymidine (Proctor *et al.*, 2006; Kriegeskorte *et al.*, 2011). The phenotype switching enables *S. aureus* to hide inside host cells, leading to escape from immune responses and those antibiotics without intracellular activity (von Eiff *et al.*, 2006; Garcia *et al.*, 2013).

SCVs have been associated with various chronic, recurrent, and persistent infections including soft-tissue and skin infections (von Eiff *et al.*, 2006; Melter and Radojevic, 2010). As the epidermis is the first barrier that *S. aureus* has to overcome in order to enter and persist in the host, we hypothesize that SCVs may subvert cutaneous defense by a decreased susceptibility toward skin-derived AMPs.

RESULTS AND DISCUSSION

The aim of this study was to investigate the hypothesis that SCVs display a higher resistance toward the action of skin-derived antimicrobial peptides (AMPs). To this end, we analyzed the activity of the skin-derived AMPs human beta-defensin (hBD)-2 and -3, RNase 7, and LL-37 against various SCVs. We choose these AMPs because they are all major skin-derived AMPs (Harder *et al.*, 2007; Gallo and Hooper, 2012), and they are all present in the stratum corneum, as demonstrated by immunohistochemistry (Figure 1). In particular, RNase 7 and hBD-3 have been reported to be principle AMPs

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Abbreviations: AMP, antimicrobial peptide; hBD, human beta-defensin; SCV, small-colony variant

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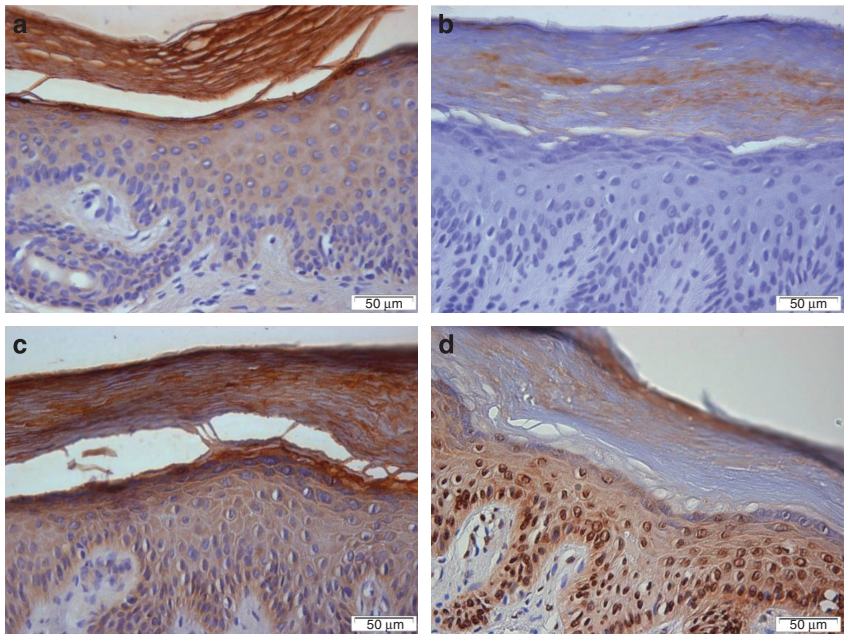

Figure 1. Immunostaining of RNase 7, human beta-defensin (hBD)-2, hBD-3, and LL-37 in the stratum corneum. To demonstrate the presence of the investigated antimicrobial peptides (AMPs) in the stratum corneum, we performed immunohistochemical staining of the AMPs using human skin biopsies derived from the soles of the feet. RNase 7 (**a**), hBD-2 (**b**), hBD-3 (**c**), and LL-37 (**d**) all showed staining in the stratum corneum. Bars = 50 µm.

Table 1. LD_{90} (µg ml^{-1})[1] of the AMPs RNase 7, hBD-2, hBD-3, and LL-37 against SCV and corresponding wild-type strains OM1, A22216, and OM299, as well as against a *S. aureus hemB* mutant and its *hemB*-complemented mutant

	OM1		A22216		OM299		*hemB*	
	Wild-type	SCV	Wild-type	SCV	Wild-type	SCV	Complemented mutant	Mutant
RNase 7	2.5–5	>40	2.5–5	>40	5–10	>40	2.5–5	>40
hBD-2	10–20	>40	10–20	>40	5–10	>40	>40 (10–20)[2]	>40 (>40)[2]
hBD-3	0.6–1.25	2.5–5	0.6–1.25	1.25–2.5	0.6–1.25	1.25–2.5	0.6–1.25	1.25–2.5
LL-37	2.5–5	5–10	2.5–5	10–20	1.25–2.5	10–20	5–10	5–10

Abbreviations: AMPs, antimicrobial peptides; hBD, human beta-defensin; LD, lethal dose; SCV, small-colony variant.
[1]Lethal dose (µg ml^{-1}) to kill 90% of the bacteria.
[2]Lethal dose (µg ml^{-1}) to kill 50% of the bacteria.

released by keratinocytes to control the growth of *S. aureus* (Kisich *et al.*, 2007; Simanski *et al.*, 2010).

We tested these AMPs against three natural SCV isolates recovered from clinical specimens in parallel with their isogenic corresponding wild-type strains. The use of a microdilution assay with different concentrations of AMPs revealed highest activity of hBD-3 against wild-type *S. aureus* followed by LL-37, RNase 7, and hBD-2 (Table 1). In contrast, all SCVs showed a decreased susceptibility toward these AMPs. HBD-3 demonstrated a moderate reduction of its capability to kill SCVs compared with wild-type *S. aureus*. RNase 7 and hBD-2 showed a strongly reduced antimicrobial activity against SCVs. Similarly, LL-37 displayed a decreased activity against all clinical SCVs (Table 1).

It is obvious that hBD-3 killed those isolates that display the SCV phenotype more efficiently than the other AMPs, including its beta-defensin family member hBD-2. It is known that hBD-2 is in general less active against *S. aureus*, and this may be the reason why hBD-2 is also less active against SCVs. It has been suggested that the capacity of hBD-3 to form dimers and its high positive surface charge are responsible for its high activity against S. *aureus* (Schibli *et al.*, 2002). In addition, its special mode of action by interfering with the *S. aureus* cell wall biosynthesis machinery (Sass *et al.*, 2010) may also explain its augmented activity against *S. aureus* and, therefore, also against SCVs. Nevertheless, hBD-3 showed also a decreased activity against all tested SCVs as compared with the corresponding wild-type strains (Table 1).

To further verify the reduced activity of AMPs against SCVs, we used a strain pair representing (i) a genetically defined mutant constructed by interrupting one of the hemin biosynthetic genes, *hemB,* that displays the SCV phenotype, and (ii) its *hemB*-complemented mutant exhibiting the normal phenotype (von Eiff *et al.*, 1997). Except LL-37, all AMPs exhibited