Intracellular Persistence of *Staphylococcus aureus* Small-Colony Variants within Keratinocytes: A Cause for Antibiotic Treatment Failure in a Patient with Darier’s Disease

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Intracellular persistence assays were performed with small-colony variants (SCVs) derived from a patient with Darier’s disease from whom different phenotypes and genotypes of *Staphylococcus aureus* were isolated over a 28-month period; the assays revealed that >100-fold more SCV cells persisted intracellularly relative to the normal phenotype. The presence of intracellular *S. aureus* SCVs may protect against host defenses and antibiotic therapy and thus may have contributed to this patient’s very prolonged skin infection.

*Staphylococcus aureus* small-colony variants (SCVs) are a naturally occurring subpopulation of *S. aureus* that were first described >80 years ago. These variants grow slowly and produce small colonies on standard media. They also demonstrate a number of other characteristics that are atypical for *S. aureus*, including reduced α-toxin production, delayed coagulase activity, and failure to use mannitol [1–3]. Recently, the significance of SCVs as causative organisms in chronic, recurrent, and antibiotic-resistant infections, particularly in patients with chronic osteomyelitis or cystic fibrosis, has been demonstrated in prospective studies [4, 5].

Biochemical characterization of the large majority of clinically isolated SCVs suggests specific defects in electron transport. Clinical and laboratory-generated SCVs are frequently auxotrophic for menadione or hemin, compounds required in the biosynthesis of the electron transport chain components menaquinone and cytochromes, respectively. Electron transport-deficient *S. aureus* SCVs were found to persist inside endothelial cells in culture, which might shield the SCV from the host immune response and the action of antibiotics [6–8].

Darier’s disease, also known as keratosis follicularis, is an autosomal, dominantly inherited genodermatosis with a wide variation in clinical severity. The etiology is unknown, but electron microscope studies suggest that the essential abnormality is a defect in the synthesis, organization, or maturation of the tonofilament-desmosome complex, which leads to abnormal (premature and faulty) keratinization and a loss of cohesion of keratinocytes in the lower epidermis. Although there may be fluctuation in severity, usually the disease becomes progressively more chronic. The disease is often exacerbated by pyogenic infection [9].

Here we describe the first known case of persistent and antibiotic-resistant skin infection with different phenotypes and genotypes of *S. aureus*, including clonally different *S. aureus* SCVs, in a patient with Darier’s disease. This is also the first demonstration of intracellular persistence within keratinocytes.

**Materials and methods.** *S. aureus* isolates were recognized and characterized as SCVs if they had the following characteristics: pinpoint colonies (incubation on Columbia agar, brain-heart infusion agar with 5% NaCl, and Schaedler agar for 48–72 h), mostly decreased pigment formation, reduced hemolytic activity, and low coagulase activity. Very small colonies on Columbia agar were suspected of being SCVs if they grew nearly normally on Schaedler agar [4]. In addition, all isolates were confirmed as *S. aureus* by testing for the *S. aureus*-specific *nuc* and *coa* genes by PCR [10, 11].

Auxotrophy for hemin (Unipath) was tested by use of standard disks and for thymidine (Fluka Chemie) and menadione (Sigma Aldrich Chemie) by inoculating disks with 15 μL of thymidine at 100 μg/L or menadione at 10 μg/mL. Test isolates were inoculated on chemically defined medium agar as described elsewhere [4].

Small digests of total bacterial DNA were resolved with use of pulsed-field gel electrophoresis (PFGE), and strains were considered clonal if <2 bands varied on a gel [4, 12]. In addition, arbitrarily primed PCR was used as typing method, as described elsewhere [13].

Gentamicin and oxacillin MICs were determined by use of the microdilution method, and results were determined after 48 h of incubation at 36°C. Methicillin resistance, also tested
by use of the microdilution method, was confirmed by testing for the mecA gene by means of PCR [14].

The intracellular persistence assay was done with the HaCaT cell line, a spontaneously transformed human keratinocyte cell line from adult skin that maintains full epidermal differentiation capacity [15]. We used 2 clonally different strains of the SCV phenotype (hereafter referred to as “SCV1” and “SCV2”) and their corresponding parent strains of the normal phenotype (hereafter referred to as “NP1” and “NP2”), all isolated from the patient with Darier’s disease. In addition, we tested an isogenic normal strain and an SCV strain of S. aureus that were recovered from a patient with cystic fibrosis.

The assay was performed according to the method used for the phagocytosis assay for bovine aortic endothelial cells, with minor modifications [7]. Briefly, HaCaT cell monolayers were grown in Dulbecco’s modified Eagle medium (DMEM; Sigma) with 10% bovine calf serum to confluence (cells) in 52 minor modifications. HaCaT cell monolayers were recovered from a patient with cystic fibrosis.

The assay was performed according to the method used for the phagocytosis assay for bovine aortic endothelial cells, with minor modifications [7]. Briefly, HaCaT cell monolayers were grown in Dulbecco’s modified Eagle medium (DMEM; Sigma) with 10% bovine calf serum to confluence (2 × 10^6 cells) in 24-well tissue culture plates. The numbers of washed bacteria were adjusted to be nearly equal for each strain and were added to the washed monolayers. The infected monolayers were incubated for 3.5 h at 37°C to allow adhesion and phagocytosis of the bacteria. The monolayers were then washed 3 times with DMEM and 10% bovine calf serum to remove nonattached organisms; next, 1 mL of medium containing 10 μg of lysostaphin/mL was added, which effectively eliminated extracellular staphylococci. Incubation in the presence of lysostaphin was continued for 30 min, 24 h, and 48 h. At these time points, the monolayers were washed 3 times with DMEM to remove lysostaphin, followed by addition of 1 mL of sterile water to disrupt epithelial cells and to release intracellular organisms. Serial dilutions were made in sterile water.

The number of intracellular cfu was determined by plating 100-μL aliquots on trypticase soy agar in duplicate. The detection limit was 10 cfu. The number of intracellular cfu at each time point was determined in duplicate or triplicate, and the mean number ± SD from 3 experiments was calculated.

After incubation of the infected HaCaT cells in the presence of lysostaphin for 30 min and 48 h, the cells were washed twice in PBS, dehydrated in ethanol, fixed in glutaraldehyde and osmium tetroxide, and embedded in Epon. Ultrathin sections were counterstained with lead citrate and uranyl acetate and examined with use of a Philips EM10 electron microscope.

S. aureus strains were derived from a 39-year-old patient who was hospitalized several times during the last 3 years because of continuous worsening of his skin condition. At the age of 22, the first typical skin lesions appeared, and the diagnosis of Darier’s disease was confirmed by histological examination. His mother and 2 brothers show the same disorder but in a milder form; 1 sister is healthy.

In the last 4 years, the patient had severe recurring herpes simplex virus infections and recurring purulent infections. In 1999, his skin condition dramatically worsened, with moist and fetid skin lesions all over the body. Because skin care could not be done at home, the patient was again admitted to the hospital. Although he received different combinations of antibiotics, the skin condition continuously worsened. Because methicillin-resistant S. aureus was isolated from skin and anterior nares, antimicrobial agents, such as vancomycin, rifampicin, and clindamycin, were given iv for 4 weeks. In addition, a topical mupirocin ointment for the nasal mucosa was given for 2 weeks. However, the skin condition did not improve significantly. Additional therapeutic approaches included several topical treatments with steroids and antiseptics: povidone-iodine, chlorhexidine, and chlorquininaldol.

Results. SCVs of S. aureus and strains with normal colony size isolated from simultaneous or sequential cultures were recovered over a period of 28 months. All together, 119 isolates were derived from 53 different clinical specimens, which were predominantly obtained from different areas of the affected skin (on swabs or from needle biopsy) and from the anterior nares.

Phenotypic characterization of the isolates showed that different S. aureus strains with normal colony size as well as hemin-auxotrophic SCVs were associated with the infection of the skin. In contrast to S. aureus that exhibited typical colony size, pigmentation and hemolysis on Columbia agar, S. aureus SCVs grew as nonhemolytic, nonpigmented, and very small colonies. However, in addition to these strains, we isolated S. aureus that exhibited typical colony size (but not pigmentation) and hemolysis as well as SCVs that grew as pinpoint colonies but exhibited pigmentation) and hemolysis. Moreover, methicillin-resistant S. aureus strains were found in isolates of both the normal and the SCV phenotype. In comparison with clonal identical strains of the normal phenotype, SCVs had up to 32-fold higher gentamicin MICs.

With use of PFGE and arbitrarily primed PCR, molecular typing revealed 7 genotypes involved over this 28-month period, including 4 genotypes of SCVs. PFGE-revealed bands of S. aureus and SCVs strains with the same profile were considered to be clonal. One clone, growing on one occasion as a pinpoint colony and another as S. aureus with the normal colony size, persisted over a period of 18 months; the other clones were isolated during periods of 1 week and 5, 7, 13, and 16 months. One clone was detected only once in a single specimen. One of the 2 SCVs selected for the intracellular persistence assay belonged to the clone that persisted in the infected patient for 18 months.

Assays of S. aureus SCVs were done to determine whether these variants could persist intracellularly within keratinocytes more efficiently than could the corresponding parent strains that were of the normal phenotype. Because a keratinocyte cell line from a patient with Darier’s disease was not available, we
used the HaCaT cell line for the intracellular persistence assays. This spontaneously transformed human keratinocyte cell line from adult skin has been shown to be an appropriate model to study keratinocyte biology [15]. After initial coincubation for 3.5 h to allow uptake of *S. aureus* SCVs by HaCaT cells, lysostaphin treatment revealed that >100-fold more SCV cells than cells of the normal phenotype persisted intracellularly after 24 or 48 h of incubation (figure 1). The intracellular survival of SCVs within the HaCaT cell line was studied by use of 2 clonally different SCVs in comparison with *S. aureus* strains of the normal phenotype isolated from the patient with Darier’s disease. The difference in intracellular persistence was not due to differential susceptibilities of the strains to lysostaphin, which was maintained in the culture medium during the assay to eliminate extracellular organisms. Comparable results for SCVs that persisted intracellularly were also found when we studied a clinical isogenic normal strain and an SCV strain of *S. aureus* that were recovered from a patient with cystic fibrosis (data not shown).

Ultrastructural examination of the HaCaT cells confirmed persistence of *S. aureus* SCVs within the epithelial cells. After 30 min and 48 h, staphylococcal SCVs appeared to be within the cytoplasm (figure 2a and figure 2b, respectively). The epithelial cells appeared viable and showed no signs of degeneration. Likewise, the corresponding parent strain of the normal phenotype was incorporated after 30 min by intact HaCaT cells (figure 2c), but after 48 h of incubation, most of the epithelial cells underwent severe lytic degeneration and release of bacteria (figure 2d). The very few remaining cells were vacuolated and showed no *S. aureus* when the parent strain was used (figure 2d), whereas the SCVs showed many intracellular bacteria at 48 h (figure 2b).

**Discussion.** In this study, the in vitro persistence of SCVs in keratinocytes relative to their clonally identical parent strains of the normal phenotype is presented. This ability of SCVs to persist intracellularly combined with the relatively low virulence of SCVs before they revert to the rapidly growing form helps to explain why *S. aureus* seems to be eradicated, only to recur weeks to months later [3, 5, 6]. All *S. aureus* strains tested were derived from a 39-year-old patient with Darier’s disease, in whom for the first time *S. aureus* SCVs combined with other phenotypes of *S. aureus* were shown to cause a chronic and persisting skin infection. The patient demonstrated poor clinical and bacteriologic response to antimicrobial agents active against these pathogens in vitro. In the past, this phenomenon has been attributed to infections caused by *S. aureus* SCVs, particularly in patients with osteomyelitis [5]. In addition, the methicillin resistance of one of the clones might have contributed to the recurrence of the infection, because vancomycin is not very efficient in treating skin infections.

*S. aureus* SCVs can be selected by antimicrobial agents, as shown by exposure of *S. aureus* of the normal phenotype to subinhibitory concentrations of gentamicin [3]. In vivo, emergence of SCVs has been associated with antibiotic use; however, SCVs can also be recovered after prolonged antibiotic-free intervals [1, 5]. Whether the isolation of SCVs from our patient was due to the use of a specific antimicrobial agent remains uncertain, because the patient was treated with various combinations of antibiotics.

All together, 7 different genotypes were involved in recurrent infections over a 28-month period. Although the patient acquired clonally different *S. aureus* strains over this period, isolated uniquely or during a period of >1 year, recovery of *S. aureus* SCVs in consecutive specimens from a patient during the extended time span also indicates the persistence of clonally identical strains. Furthermore, parallel or consecutive cultures yielded phenotypically different *S. aureus* populations despite the clonal identity of the isolates [4, 5].

SCVs are rapidly overgrown in liquid media and form pinpoint colonies on agar and, thus, are easily missed when rapidly growing organisms are present. Considering the special phenotypic SCV characteristics, it is therefore not surprising that SCVs have been cultivated, in particular, from specimens of soft tissue abscesses or osteomyelitis and samples of sterile body fluids. On skin or mucous membranes or in complex specimens, such as bronchial fluid, SCVs are difficult to detect,
Figure 2. Electron micrographs of keratinocyte HaCaT cells that were infected with isolates of clinical isogenic normal *Staphylococcus aureus* and *S. aureus* small-colony variants. After incubation of infected HaCaT cells in presence of lysostaphin for 30 min or 48 h (analogous to an intracellular persistence assay), cells were washed, dehydrated, and embedded in Epon. Ultrathin sections were counterstained and examined by electron microscopy. 

a, b, Intracellular persistence of SCVs (SCV1) within viable HaCaT cells after (a) 30 min or (b) 48 h of incubation. Epithelial cells appear viable and show no signs of degeneration (original magnification, ×3400).  
c, d, *S. aureus* of the normal phenotype (NP1) is incorporated after 30 min by intact HaCaT cells (c); however, after 48 h of incubation, most epithelial cells show severe lytic degeneration and release of bacteria (d; original magnification, ×3400).

despite their high prevalence, as has been shown in bronchial secretions of patients with cystic fibrosis [4]. This report suggests that in cases of chronic exacerbating skin diseases, such as Darier’s disease, in which cultures of specimens obtained under routine conditions are negative for *S. aureus*, this pathogen must be actively sought using appropriate selective media and growth conditions. In addition, the decreased susceptibility of *S. aureus* SCVs against antimicrobial agents that are typically used to treat *S. aureus* infection requires the identification of these variants, even if normal *S. aureus* are present in the specimens [3, 6, 16].

In summary, *S. aureus* SCVs may persist in chronic skin infection, probably because they persist within keratinocytes, as shown in vitro with the human keratinocyte cell line HaCaT. When an infection is particularly resistant to therapy, persists for a long period, or fails to respond to apparently adequate therapy, one should consider special efforts to search for SCVs. Different SCV phenotypes and genotypes may be found in chronic infection in a single patient.

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References