Effect of salicylic acid on invasion of human vascular endothelial cells by *Staphylococcus aureus*

Wan Beom Park, Sung-Han Kim, Jae Hyun Cho, Ji Hwan Bang, Hong Bin Kim, Nam Joong Kim, Myoung-don Oh & Kang Won Choe

Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Republic of Korea

Correspondence: Kang Won Choe, Department of Internal Medicine, Seoul National University College of Medicine, 28 Yeongun-dong, Chongro-gu, Seoul, Republic of Korea, 110-744. Tel.: +8 222 072 2212; fax: +8 227 629 662; e-mail: choekw@snu.ac.kr

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Abstract

Invasion of vascular endothelial cells by *Staphylococcus aureus* is associated with diverse complications and recurrent infection. Little is known about the effect of salicylic acid, the major metabolite of aspirin, on the interaction between *S. aureus* and vascular endothelial cells. We examined the adhesion of *S. aureus* strain 8325-4 cultured with or without salicylic acid to human umbilical vein endothelial cells (HUVECs), and the ability of the strain to invade these cells. Strain 8325-4 cells grown in salicylic acid were significantly less adherent to and invasive in HUVECs. Production of cytokine interleukin (IL)-6 was lower from the HUVECs infected with clinical isolates of *S. aureus* cultured in salicylic acid compared with those unexposed to salicylic acid. This study raises the possibility of using salicylic acid as an adjuvant therapeutic agent in the treatment of *S. aureus* bacteremia to prevent its complications or recurrence.

Introduction

*Staphylococcus aureus* is a major cause of severe community-acquired and nosocomial bacteremia. *Staphylococcus aureus* bacteremia is frequently accompanied by complications such as endovascular infection and metastatic infection of other tissues, which are responsible for significant morbidity and mortality. In addition, persistent or recurrent *S. aureus* bacteremia is, despite the use of appropriate antibiotics, another growing problem (Khosrovaneh et al., 2004).

*Staphylococcus aureus* can adhere strongly to vascular endothelial cells and invade them (Hamill et al., 1986). Invasion involves an actin polymerization-dependent zipper-type mechanism and is dependent on fibronectin-binding proteins (FnBP) and host cell α5β1 integrin (Schwarz-Linek et al., 2003). This property of *S. aureus* may be associated with its ability to cause endovascular and metastatic infection. Invasion of vascular endothelial cells by *S. aureus* can induce the expression of adhesion molecule and the production of pro-inflammatory cytokines such as interleukin (IL)-6 by the invaded cells, so contributing to the development of sepsis (Yao et al., 1995). Moreover, intracellular *S. aureus* may be a source of persistent or recurrent infection because the intracellular environment protects it from host defense mechanisms and the bacterial effects of antibiotics (von Eiff et al., 2001). Therefore, it is important to prevent or reduce the invasion of vascular endothelial cells by *S. aureus* without, if possible, promoting the development of antibiotic resistance.

Salicylic acid is the major metabolite of aspirin, which is widely used as a cheap, relatively nontoxic and absorbable compound. There have been several reports that salicylic acid reduces staphylococcal virulence (Farber & Wolff, 1992; Kupferwasser et al., 1999, 2003). A recent study demonstrated that it reduced expression from the FnBP gene promoter as well as binding of *S. aureus* to fibronectin (Kupferwasser et al., 2003). Because FnBP is required for invasion of vascular endothelial cells by *S. aureus* (Peacock et al., 1999; Schwarz-Linek et al., 2003), we hypothesized that salicylic acid might reduce the invasiveness of *S. aureus* for those cells. The aim of the present study was to investigate whether salicylic acid reduces the adhesiveness and invasiveness of *S. aureus* for human vascular endothelial cells, and whether it affects the production of IL-6 by endothelial cells harboring intracellular *S. aureus*.
Experimental procedures

Bacterial strains and cultures

The laboratory strains 8325-4 (FnBP-A⁻B⁺), DU5883 (FnBP-A⁻B⁻), DU5883(pFnBNA4) (FnBP-A⁻B⁺) and DU5883(pFnBB4) (FnBP-A⁻B⁻) of S. aureus were kindly provided by Dr Timothy J. Foster (Department of Microbiology, Trinity College, Dublin, Ireland) (Greene et al., 1995). Five clinical isolates of methicillin-sensitive S. aureus were selected at random from blood culture isolates of patients who had died of S. aureus sepsis. The laboratory strains and clinical isolates were grown at 37 °C in plain brain heart infusion broth (BD Biosciences, Rockville, MD) or in medium containing 30, 50 or 100 μg mL⁻¹ salicylic acid (Sigma Chemical Co., St Louis, MO) for 18 h with constant rotation in air, and stored at 4 °C. Two hours before an experiment, aliquots of the stock cultures were resuspended in fresh brain heart infusion broth containing the same concentration of salicylic acid as the stock cultures, and grown to mid-log phase. Salicylic acid at these concentrations had no effect on the 24-h growth kinetics of S. aureus or the pH of bacteria-salicylic acid cultures (Kuperwasser et al., 2003).

Bacteria were collected by centrifugation, washed three times in phosphate-buffered saline (PBS), and resuspended in antibiotic-free EGM-2 medium (Cambrex, Walkersville, MD). The suspensions were centrifuged at 75 × g for 5 min to remove variable-sized aggregates (Van Belkum et al., 2002), and bacterial concentrations were measured spectrophotometrically and by plating serial dilutions on agar.

Preparation of endothelial cells

Human umbilical vein endothelial cells (HUVECs, Cambrex) were used in these experiments. They were grown to confluence in 24-well plates at 37 °C in antibiotic-free EGM-2 medium containing 5% fetal bovine serum and growth factors, in a humidified incubator with 5% CO₂. Salicylic acid at the concentrations used in this study did not affect the viability of the HUVECs (data not shown). All experiments were performed with cells that had been passaged between four and eight times.

Adhesion of S. aureus to HUVECs

Adhesion of S. aureus to HUVECs was assayed by the method of Peacock et al. (1999) with minor modifications. HUVECs were grown to confluence on 13 mm Thermanox coverslips (Nunc, Rochester, NY) in 24-well tissue culture plates, and washed with antibiotic-free EGM-2 medium. Then 10⁶ CFU of strains 8325-4 and DU5883 were added to the wells and incubated at 37 °C in 5% CO₂ for 1 h. The coverslips were then dip-washed three times in antibiotic-free EGM-2 and once in PBS to remove unbound bacteria. The endothelial cells were fixed with methanol at 4 °C, air-dried and stained with 0.5% crystal violet for 10 min, and the number of bacteria associated with the confluent endothelial cells in one high-power field (×1000 magnitude) was counted using a standard counting procedure (Peacock et al., 1999). Endothelial cell-associated bacteria include both adherent and internalized bacteria, but for simplicity endothelial cell-associated bacteria are referred to as adherent in the remainder of the text. Results are expressed as means ± SEs of means (SEM) of three independent experiments.

Invasion of HUVECs by S. aureus

Invasion assays, as previously described (Ogawa et al., 1985), were performed to determine numbers of internalized bacteria. Each well containing HUVECs was washed with antibiotic-free EGM-2 medium before inoculation with bacteria. A bacterial suspension of 1 mL adjusted by OD to 10⁷ CFU mL⁻¹ was added to each well and incubated for 1 h at 37 °C in 5% CO₂. The HUVECs were then washed twice with antibiotic-free EGM-2 medium and incubated at 37 °C for 20 min in antibiotic-free EGM-2 medium containing 10 μg mL⁻¹ lysostaphin (Sigma) to lyse extracellular staphylococci. They were then detached with trypsin and disrupted in hypotonic solution, and serial dilutions plated on manitol salt agar. Numbers of internalized bacteria are expressed as mean counts ± SEM of five independent experiments performed in duplicate.

IL-6 cytokine assay

Clinical isolates of S. aureus (10⁷ CFU) grown with 100 μg mL⁻¹ salicylic acid were incubated for 1 h with confluent HUVECs in 24-well plates. HUVECs were then incubated for 24 h in antibiotic-free EGM-2 medium containing 10 μg mL⁻¹ lysostaphin, and supernatants were collected and filtered through 0.45-μm filters (Millipore Corporation, Billerica, MA). IL-6 was determined with a sandwich enzyme-linked immunosorbent assay (ELISA; Endogen, Woburn, MA) according to the manufacturer’s instructions. Escherichia coli lipopolysaccharide (0.1 μg mL⁻¹; Sigma) was added to the HUVECs for 24 h as a positive control. Results are expressed as the median values and interquartile ranges of three experiments performed in triplicate.

Statistical analysis

The Mann–Whitney U-test for comparison of unpaired continuous variables, and the Wilcoxon rank-sum test for comparison of paired continuous variables were used. Two-tailed P < 0.05 was considered to be significant. Statistical
analyses were performed with SPSS software (version 12.0; SPSS Inc., Chicago, IL).

**Results**

**Adhesion of *S. aureus* to vascular endothelial cells**

*Staphylococcus aureus* strain 8325-4 that had been cultured in the presence of 50 and 100 µg mL$^{-1}$ salicylic acid was substantially less adhesive to live HUVECs ($P = 0.002$, $P = 0.001$) (Fig. 1). However, bacteria exposed to 100 µg mL$^{-1}$ salicylic acid were no less adhesive than those exposed to 50 µg mL$^{-1}$, and were more adhesive than strain DU5883, which does not express FnBPs ($P < 0.001$).

**Invasion of vascular endothelial cells by *S. aureus***

Pre-exposure to 50 and 100 µg mL$^{-1}$ salicylic acid attenuated the invasiveness of strain 8325-4 for HUVECs ($P = 0.009$, $P = 0.009$) (Fig. 2). As in the adhesion assay, 100 µg mL$^{-1}$ salicylic acid had no greater effect than 50 µg mL$^{-1}$ salicylic acid. Essentially no internalization was detected in the case of strain DU5883, which served as a negative control.

The number of *S. aureus* internalized was proportional to time after inoculation for up to an hour. The effect of salicylic acid on invasiveness was evident early after infection (Fig. 3); pre-exposure to salicylic acid had a significant effect on the number of internalized organisms as early as 10 min after inoculation of the *S. aureus* ($P = 0.019$).

When the invasion assay was performed with strains DU5883(pFNBA4) (FnBP-A$^{-}$B$^{-}$) and DU5883(pFNBB4) (FnBP-A$^{+}$B$^{-}$), we obtained results similar to those with strain 8325-4. However, two other nonsteroidal anti-
inflammatory drugs, acetaminophen and ibuprofen, had no significant effect on the invasion of HUVECs by S. aureus (data not shown).

**IL-6 cytokine assay**

Internalization of all the clinical isolates of S. aureus induced IL-6 production by the HUVECs. Pre-exposure to 100 μg mL\(^{-1}\) salicylic acid significantly reduced the production of IL-6 by endothelial cells harboring intracellular S. aureus; the median level of IL-6 was 320.7 pg mL\(^{-1}\) (interquartile range [IQR], 270.8–382.2) in isolates cultured without salicylic acid, and 204.6 pg mL\(^{-1}\) (IQR, 172.2–232.1) in those cultured with salicylic acid (P = 0.001) (Fig. 4). The average level of IL-6 was 361.6 pg mL\(^{-1}\) in the medium with lipopolysaccharide (0.1 μg mL\(^{-1}\)) and 72.8 pg mL\(^{-1}\) in cell supernatants alone. Salicylic acid did not affect the IL-6 level induced by lipopolysaccharide.

**Discussion**

We have shown that salicylic acid, the major metabolite of aspirin, reduces the adhesiveness and invasiveness of S. aureus for human vascular endothelial cells *in vitro*, and decreases IL-6 production by the endothelial cells. Aspirin has diverse clinical roles including antiplatelet, antianalgesic and anti-inflammatory effects according to increase of its concentration. The concentrations of salicylic acid used in the present study were relatively low and non-toxic (30–100 μg mL\(^{-1}\)), and corresponded to effective antiplatelet and antianalgesic concentrations. Serum levels of 150–300 μg mL\(^{-1}\) salicylic acid are associated with its anti-inflammatory effect, and toxic levels of salicylic acid in serum begin in the range 200–350 μg mL\(^{-1}\) (Needs & Brooks, 1985). We also showed that the effect of increasing concentrations of salicylic acid on adhesion and invasion of S. aureus reaches a ceiling: concentrations of more than 50 μg mL\(^{-1}\) did not further reduce adhesion and invasion.

Farber & Wolff (1992) reported that *Staphylococcus epidermidis* grown in salicylic acid was less adherent to a medical catheter, and Kupferwasser et al. (2003) suggested that S. aureus grown in salicylic acid was less adherent to extracellular proteins such as fibronectin and fibrinogen due to changes of a global regulon including down-regulation of *sarA*. The reduction in adhesiveness of S. aureus cultured with salicylic acid on endothelial cells may involve a mechanism similar to the reduction of adhesion to the extracellular matrix, as adhesion of S. aureus to purified fibronectin is correlated with its ability to bind to endothelial cells (Peacock et al., 1999).

Previous studies have shown that FnBP-deficient mutants of S. aureus do not invade endothelial cells (Peacock et al., 1999). Our results are in accord with that finding. Considering that FnBP is required for the invasion of endothelial cells by S. aureus, the inhibitory effect of salicylic acid may be due to down-regulation of the expression of FnBP (Kupferwasser et al., 2003). The present study showed that salicylic acid also reduced the invasiveness of S. aureus strains expressing only FnBP-A or FnBP-B. This suggests that the mechanism by which salicylic acid reduces the invasiveness of S. aureus can make use of either FnBP-A or FnBP-B.

Since vascular endothelial cells constitute the primary barrier against organisms circulating in the blood, a reduction in the invasiveness of S. aureus for these cells could reduce complications such as endocarditis and metastatic infection. Kupferwasser et al. (1999) demonstrated that salicylic acid reduced bacterial density in vegetation, and hematogenous dissemination of bacteria in an experimental model of S. aureus endocarditis involving cardiac valves injured by a catheter. Our data suggest that salicylic acid could reduce the occurrence of even normal valve endocarditis due to S. aureus, considering that invasion of endothelial cells by S. aureus is a possible mechanism of normal valve endocarditis (Lowy, 1998). Vesga et al. (1996) demonstrated that the intraendothelial cell milieu fosters the formation of small-colony variants, which may be associated with persistent and recurrent infection. Therefore, a reduction of S. aureus invasiveness for endothelial cells could lower the formation of small-colony variants. Salicylic acid is thought to have little effect on the emergence of antimicrobial resistance because it is not an antimicrobial agent, although Gustafson et al. (1999) demonstrated that it slightly increased fluoroquinolone resistance in S. aureus.

Yao et al. (1995) reported that S. aureus infection of endothelial cells resulted in the production of IL-6 and
IL-1β, and that increasing multiplicity of infection increased cytokine production. We chose to examine cytokine IL-6 because the production of IL-1 by endothelial cells is controversial (Strindhall et al., 2005). The present study demonstrated that salicylic acid reduced the production of IL-6 from endothelial cells induced by intracellular S. aureus. A reduction of IL-6 level may help to attenuate progression of sepsis because IL-6 level correlates with disease severity (Casey et al., 1993). The attenuation of IL-6 production by salicylic acid was probably due to the reduced number of internalized S. aureus. Soderquist et al. (2006) recently reported that adhesion to and invasion of S. aureus into endothelial cells are important regulators of cytokine expression. However, it is possible that salicylic acid also influences levels of bacterial products that may contribute to induction of cytokine synthesis.

A limitation of the present study is that under physiological conditions the interaction of S. aureus with endothelial cells takes place in whole blood and under conditions of blood flow involving low bacterial densities. There may also be substantial differences between the microvascular and macrovascular endothelium. Moreover, the limited number of laboratory strains and clinical isolates examined in this study may preclude generalization of the results.

In conclusion, we have demonstrated that salicylic acid reduces not only the adhesiveness but also the invasiveness of S. aureus for human vascular endothelial cells in vitro. The mitigation of invasion into vascular endothelial cells by S. aureus should reduce complications such as endovascular or metastatic infection, and recurrent infection in patients with S. aureus bacteremia. Our findings raise the possibility that salicylic acid could be useful as an adjuvant therapeutic agent in the treatment of S. aureus bacteremia to prevent its complication and recurrence.

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References
