Role of agr (RNAIII) in Staphylococcus aureus adherence to fibrinogen, fibronectin, platelets and endothelial cells under static and flow conditions

BORIS SHENKMAN, DAVID VARON, ILIA TAMARIN, RIMA DARDIK, MARTHA PEISACHOV*, NAPHTALI SAVION† and ETHAN RUBINSTEIN*

Institute of Thrombosis and Hemostasis, *Infectious Diseases Unit, Sheba Medical Center, Tel Hashomer and †Goldschleger Eye Research Institute, Tel Aviv University, Tel Aviv, Israel

In the present study, the adherence of Staphylococcus aureus (strain 8325-4) and its RNAIII mutant (WA400) to immobilised fibrinogen and fibronectin, and to human endothelial cells (EC), was studied. [3H]Thymidine-labelled bacteria in stationary phase were incubated on the test surfaces for 20 min under static or flow (200/s) conditions. The results showed: (a) increased adherence of the RNAIII mutant to fibrinogen under static conditions, and decreased adherence of the mutant to fibronectin and EC under both static and flow conditions compared with the parental strain; (b) stronger ability of the mutant compared with the parental strain to induce platelet aggregation in suspension; (c) greater adherence of the parental strain and the mutant to EC pre-treated with platelet-rich plasma compared with platelet-poor plasma, and to EC pre-treated with platelet-poor plasma compared with control; (d) increased adherence of S. aureus to EC pre-treated with PMA-activated platelets and decreased adherence to EC pre-treated with tirofiban, a platelet glycoprotein IIb-IIIa inhibitor, which paralleled with increased adherence of PMA-activated platelets and decreased adherence of glycoprotein IIb-IIIa-blocked platelets to EC in the absence of bacteria; and (e) adherence of the mutant was more sensitive to pre-treatment of EC with plasma and PMA-activated platelets. In conclusion, RNAIII down-regulates S. aureus adherence to fibrinogen under static condition and up-regulates S. aureus adherence to fibronectin and EC under both static and flow conditions. The potentiating role of activated platelets in the presence of plasma in S. aureus adherence to EC is down-regulated by RNAIII, probably due to down-regulation of adherence to fibrinogen, the important plasma protein bridging S. aureus, platelets and EC.

Introduction

Adherence of Staphylococcus aureus to the host tissues is dependent on the expression of the bacterial cell wall-associated proteins of the so-called microbial surface components recognising adhesive matrix molecules (MSCRAMM) family [1]. Various bacterial surface proteins associated with adherence have been characterised at the molecular level, such as Cna for collagen, FnbpA and FnbpB for fibronectin, and ClfA and ClfB for fibrinogen [2–4]. Protein A has been found to bind to the Fc domain of IgG and to mediate attachment of S. aureus to von Willebrand factor [5]. S. aureus can also bind to other plasma proteins and extracellular matrix components.

Many of the virulence determinants of S. aureus are regulated by agr and sar global regulatory systems. The agr locus acts at both transcription and translation to regulate the production of numerous toxins, enzymes and cell surface proteins [6, 7]. Agr is composed of two divergent transcripts, RNAII and RNAIII, initiated from P2 and P3 promoters, respectively [8]. A second regulatory locus, sar, is essential for agr-dependent regulation. It is believed that agr activation is partially mediated by the binding of sar gene products to the agr promoter stimulating the transcription of RNAII and RNAIII [9]. Recent studies have shown that sar can bind to the P2 promoter region regulating agr-
mediated exoprotein production [10, 11]. During the exponential growth of bacteria, the sur and agr systems organise cell metabolism to ensure efficient growth rate [12]. At the post-exponential phase, other virulence factors are governed by global regulatory systems. During growth, the actual effector RNAIII significantly increases transcription of various genes [13].

Platelets play an important role in the pathogenesis of infective endocarditis and endovascular infection by providing an adhesive surface for bacterial binding on the vessel wall [14]. S. aureus binds directly to naive platelets causing platelet aggregation that results in formation of infected vegetation [15]. Moreover, in experimental endocarditis, anti-platelet agents cause substantial reduction in vegetation weight [16]. However, platelet aggregation may protect bacteria from exposure to antibiotics or clearance by leucocytes [17]. Stimulated platelets contain an array of microbicidal peptides [18] and generate a profile of molecules that mediate oxidative killing [19]. The role of platelets in S. aureus infection is not fully understood.

The purpose of the present study was to report the role of RNAIII in adherence of S. aureus to immobilised fibrinogen and fibronectin, and to cultured endothelial cells EAhY.926 (EC) under both static and flow conditions (200 s). The promoting role of platelets in bacterial adherence to EC and the contribution of RNAIII to this process were also investigated.

Materials and methods

Bacterial strains

The S. aureus strains used were 8325-4, a laboratory strain and WA400, an RNAIII mutant of this strain in which the RNAIII gene is deleted and replaced by the car86 gene [20]. The RNAIII mutant has a pattern similar to that of an agrA mutant.

Preparation of bacteria

S. aureus was grown overnight at 37°C on Mannitol Salt Agar (BBL/11407), harvested and incubated at 37°C in tryptone soy broth overnight aerobically to stationary phase. For radio-labelling, [methyl-1H] thymidine (1 μCi/ml) (NEN, Boston, USA) was added to S. aureus 1 × 10^8 cfu/ml suspended in tryptone soy broth and incubated overnight. The specific activity of the labelled S. aureus was 12 500 cpm/10^6 cells. Cells were washed three times with phosphate-buffered saline (PBS) (final volume 3 ml) and mixed vigorously. The bacterial count was determined by optical density at 546 nm with a standard suspension (MacFarland 2 = 6 × 10^8 cells/ml) and the bacterial inoculum was confirmed by colony counts. The bacterial count was adjusted to 1.0 × 10^8 cfu/ml and used shortly thereafter. Under these conditions, there was no bacterial champing.

Pre-coating of polystyrene plates with fibrinogen and fibronectin

Human fibrinogen (Sigma) was passed through a gelatine-sepharose column to remove residual fibrinogen [3]. Purified bovine fibronectin was purchased from Biological Industries (Beit Haemek, Israel). The proteins were dissolved in PBS in the presence of bovine serum albumin (BSA; Sigma) 0.1% in the following concentrations: fibrinogen 0.5 and 2.0 mg/ml, and fibronectin 10 μg/ml, as used in a previous study [21]. However, it should be taken into account that the relatively high fibrinogen and fibronectin concentrations reflect the physiological concentrations of the proteins in plasma and are relevant when the proteins are in a complex mixture where the amount of specific protein bound to plastic surface will be much lower than when the purified protein is used in vitro. Protein solutions were exposed to four-well polystyrene tissue-culture plates (Nunc, Roskilde, Denmark) at room temperature for 2 h. After washing with PBS containing BSA 0.1%, plates were further incubated with PBS-BSA 1% for 1 h to block the remaining free non-specific binding sites on the surface. Coated plates were held at 4°C for no more than 7 days before use.

Preparation of endothelial cells

Human endothelial cell line EAhY.926 [22] was cultured in four-well tissue culture plates in Dulbecco's modified Eagle's medium supplemented with fetal calf serum 10%, hypoxanthine-aminopterin-thymidine supplement (100 μM sodium hypoxanthine, 400 nM amionopterin and 16 μM thymidine), 2 mM glutamine, penicillin 100 U/ml, streptomycin 0.1 mg/ml and nystatin 12.5 U/ml. The cells were grown until a confluent monolayer was obtained. Before assay, plates were gently washed twice with PBS. The integrity of the EC monolayer at the end of the experiment was confirmed by inverted phase microscopy. All experiments were conducted at 37°C.

S. aureus adherence to immobilised fibrinogen and fibronectin, and to endothelial cell monolayer

A suspension of 2 × 10^8 cfu [3H]thymidine-labelled S. aureus (total volume 200 μl) was placed on PBS-BSA 0.1% washed plates and incubated for 20 min at 37°C under static (light oscillation) or flow (200/s) conditions. Flow was created by a rotating teflon cone, originally designed to evaluate platelet adhesion and aggregation [22]. After incubation, wells were gently washed twice with PBS. Bound cells were solubilised in SDS 2% and the samples were counted in a β-scintillation counter (Liquid Scintillation Analyser 1600-TR, Packhard, USA). All experiments were conducted in duplicate.
Peripheral vein blood was obtained from healthy volunteers who had not taken medications known to affect platelet function for at least 10 days before blood sampling. The blood was collected in polypropylene tubes (sodium citrate 3.8% anticoagulant:blood ratio 1:9) and centrifuged at 160 \( g \) for 12 min to produce an upper PRP suspension; the suspension was collected. The remaining blood was centrifuged at 1800 \( g \) for 12 min to produce PPP which was also collected. For labelling of platelets, PRP was incubated with \(^{1}H\) adenine 5 \( \mu Ci/mL \) for 30 min at room temperature followed by addition of citric acid to a final concentration of 5 mM, centrifuged at 800 \( g \) for 8 min, washed and resuspended to the original volume in autologous PPP.

**Results**

*S. aureus* adherence to immobilised fibrinogen and fibronectin, and to EC monolayer

Under static conditions, there was a significant increase in adherence of the RNAIII mutant, compared with the parent strain, to fibrinogen used at 0.5 and 2.0 mg/ml for coating the polystyrene surface (by c. 40% and 50%, respectively) (Fig. 1a). However, no difference was found under flow conditions. Decreased adherence to fibronectin of the RNAIII mutant was observed under both static and flow conditions (by 35% and 45%, respectively) (Fig. 1b). No substantial differences were observed in bacterial adherence to both protein ligands under static and flow conditions. A substantial reduction (c. 50%) in adherence to EC monolayer of the RNAIII mutant compared with the parent strain was shown under both static and flow conditions (Fig. 1c). Adherence of both *S. aureus* strains to EC was 2.5-fold higher under static than under flow conditions. The data shown in Fig. 1 suggest that RNAIII decreases bacterial adherence to fibrinogen under static conditions, whereas it increases bacterial adherence to fibronectin and EC under both static and flow conditions.

*S. aureus*-induced platelet aggregation

The ability of *S. aureus* to aggregate platelets in PRP after a 4–8 min lag phase was demonstrated (Fig. 2). The RNAIII mutant had a stronger effect than the parent strain on the length of the lag phase and the extent of maximal aggregation. These data correlate with those in Fig. 1a showing increased adherence of the RNAIII mutant to fibrinogen-coated surface, suggesting that platelet aggregation by *S. aureus* requires binding to fibrinogen [24]. Pre-incubation of PRP with prostaglandin E\(_{1}\) prevented *S. aureus*-induced aggregation (data not shown).

Relative role of plasma ligands and platelets in *S. aureus* adherence to EC

In this assay, EC monolayers were pre-incubated with PBS, PPP or PRP before incubation with *S. aureus*. As shown in Fig. 3, pre-incubation of EC with PPP resulted in an increased binding of both parent *S. aureus* and RNAIII mutant strains compared with their binding to non-treated EC (1.5- and 4.5-fold, respectively). When EC were pre-incubated with PRP, adherence of both types increased further (4- and 6.3-fold, respectively).

In the subsequent experiments, modulation of platelet function (in PRP) was conducted. PGE\(_{1}/IBMX\) was used to inactivate platelets by increasing the intracellular cAMP level [25], PMA as an activator of protein kinase C [26] and tirofiban (a non-peptidic Arg-Gly-Asp analogue) as a blocker of platelet glycoprotein IIb-IIIa [27]. Pre-treatment of EC with PGE\(_{1}/IBMX-\)

**S. aureus*-induced platelet aggregation

Platelet aggregation was monitored by a standard technique in which 225 \( \mu l \) of PRP were incubated at 37°C and stirred at 1000 rpm in a PACKS-4 four-channel aggregometer (Helena Lab., PACKS-4, Beaumont, TX, USA). Aggregation was induced by adding 25 \( \mu l \) of *S. aureus* suspension in PBS (bacteria:platelet ratio 4:1). The change in light transmission was recorded for 20 min. The lag phase and the maximal aggregation extent were considered. In separate experiments, PRP was incubated with 1.0 \( \mu M \) prostaglandin E\(_{1}\) before addition of bacteria.

**Statistical analysis**

Statistical analysis was performed by unpaired two-tailed \( t \) test with \( p < 0.05 \) regarded as significant. GraphPad Prism Software was used.
inactivated platelets did not alter adherence of both S. aureus strains to EC compared to pre-treatment of EC with non-treated PRP (Fig. 3). However, pre-treatment of EC with PMA-activated platelets resulted in substantial enhancement in adherence of both parental strain and RNAIII mutant (1.7- and 4-fold, respectively). In contrast, a blockade of platelet glycoprotein IIb-IIIa receptor with tirofiban resulted in decreased adherence of both S. aureus by 25% and 35%, respectively, as compared with bacterial adherence to non-treated platelets. Thus, plasma ligands and platelets promote S. aureus adherence to EC. Activated platelets further augmented this process.

Adherence of [3H]adenine-labelled platelets to EC

Washed [3H]adenine-labelled platelets were resuspended in autologous plasma and incubated for 20 min with PGE1/IBMX, PMA, or tirofiban. Platelet adherence to EC under flow (200/s) conditions was measured (Fig. 4). Platelet inactivation with PGE1/IBMX did not change platelet binding to EC. However, platelet activation with PMA resulted in an eight-fold increased adherence to EC. In contrast, blockade of platelet glycoprotein IIb-IIIa with tirofiban resulted in a 50% decrease of platelet adherence to EC compared with intact platelets. These data agree with those...
**Fig. 2.** *S. aureus*-induced platelet aggregation. Parental strain (a) or RNAIII mutant (b) *S. aureus*, suspended in PBS, was added to platelet-rich plasma (PRP) (1:10 v:v) at the bacteria:platelet ratio 4:1 in a four-channel platelet aggregometer. Lag phase and change in light transmission were evaluated. Representative picture of three independent experiments.

**Fig. 3.** Adherence of *S. aureus* to endothelial cells pre-treated with PBS-0.1% BSA (●), platelet-poor plasma (PPP; □) or platelet-rich plasma (PRP; ▪). Further PRP samples were pre-treated with PBS, 1 μM prostaglandin E1/100 μM isobutyl-methyl-xantine (PGE1/IBMX; ▲), 1 μM phorbol 12-myristate 13-acetate (PMA; △) or 0.15 μM tirofiban (○) for 20 min at room temperature. Wells with EC monolayer were incubated with blood components for 20 min at 37°C. After washing, [3H]thymidine-labelled *S. aureus* (2 x 10⁶ cfu) were placed on the EC surface and incubated for 20 min at 37°C under flow condition (200/s). Samples were washed and the number of adherent bacteria was measured. Data are mean and SD of 3–5 determinations performed in duplicate. *Compared to EC pre-treated with PBS, # compared to PRP pre-treated with PBS; * or # p < 0.05, ** p < 0.01, *** or ### p < 0.001.
demonstrating *S. aureus* adherence to EC pre-treated with modulated platelets in PRP.

**Discussion**

The role of RNAIII in the regulation of *S. aureus* adherence to the vessel wall, in which flow and static conditions are compared, has not been reported. In the present study, this question was addressed by using the RNAIII mutant, WA400, in comparison to its parent strain, 8325-4, in their stationary phase. Previously it was shown that ligand-mediated binding of bacteria to the vessel wall might differ under flow compared to static conditions [21]. The present results demonstrated increased adherence of the RNAIII mutant to immobilised fibrinogen under static conditions. Similar results, but under flow conditions, were obtained with the *agr* mutant, RN6911 compared to its wild-type, RN6390 [21]. The stronger ability of the RNAIII mutant compared with the wild-type strain to induce platelet aggregation in suspension correlated with increased binding of the mutant to fibrinogen. These results agree with those demonstrating that platelet aggregation by *S. aureus* is mitigated by antibody against staphylococcal fibrinogen-binding protein and is delayed or absent in CIFA-negative strains [24]. It was concluded that *S. aureus* induces platelet aggregation via a fibrinogen-dependent mechanism.

In contrast to the effect of RNAIII mutation on *S. aureus* adherence to fibrinogen, there was a decreased adherence of the mutant to both fibronectin and EC monolayer (under both static and flow conditions). Taken together, these results suggest that RNAIII decreases *S. aureus* binding to fibrinogen and increases *S. aureus* binding to fibronectin and EC. These data agree with the findings of a diminished fibronectin-binding capacity of both *sar* and *agr/sar* mutants of *S. aureus* [21, 28]. Fewer bacteria of the double mutant adhered to valvular vegetations in a rabbit model of endocarditis [28]. Accordingly, inactivation of the *sar* locus resulted in reduced production of both extracellular (haemolysins) and cell wall-binding proteins [29]. Recent studies have highlighted the role of fibronectin-binding proteins (FnBP) of *S. aureus* and EC cell surface fibronectin in bacterial adherence to EC. Both FnBPA and FnBPB are required for bacterial binding and subsequent internalisation by EC [30]. This process is mediated by alpha5-beta1 integrin [31]. The present study showed reduced adherence of the RNAIII mutant to fibronectin that correlated with a reduced adherence of the RNAIII mutant to EC which appeared in the absence of blood components. When bacterial adherence was assayed after pre-treatment of EC with plasma (free from platelets), greater adherence was observed and it was more pronounced with the mutant than the parental strain. This could be explained.
by the increased binding of the mutant to fibrinogen and by the role of fibrinogen as an important bridging molecule for S. aureus adherence to EC [32, 33]. Pre-treatment of EC with platelet-rich plasma further augmented S. aureus adherence to EC. Moreover, pre-activation of platelets with PMA resulted in an enhanced adherence of the parental strain (1.7-fold) and RNAIII mutant (4-fold) to EC, whereas blockade of the Arg-Gly-Asp domain of platelet glycoprotein Ib-IIIa receptor with tirofiban resulted in reduced adherence of both S. aureus types. The modulation of bacterial adherence by platelets can be explained by the change in the number of platelets adhering to EC monolayers. In separate experiments with radiolabelled platelets, an enhanced adherence of PMA-activated platelets and a reduced adherence of glycoprotein Ib-IIIa-blocked platelets to EC was found. Adherence of the RNAIII mutant to EC was more susceptible to pre-treatment of EC with activated platelets than the wild-type strain. These data can be explained by up-regulation and conformational changes of platelet GPIIb-IIIa receptor upon activation leading to fibrinogen binding [34], taking into account the enhanced regulation and conformational changes of platelet GPIIb-IIIa receptor upon activation leading to fibrinogen binding [34], taking into account the enhanced adherence of the RNAIII mutant to fibrinogen. The results of this and the previous study [21] suggest that the promoting effect of platelets on S. aureus adherence to EC is dependent on the number of adherent platelets and available receptors on the platelet membrane. S. aureus may use multiple ligands for binding to both platelets and EC. Deposition of both fibrinogen and fibronectin on the membrane of damaged EC is an important mechanism for tethering circulating platelets to EC. S. aureus adheres to the ligands bound to both platelets and EC. The regulation of adherence properties of S. aureus is multifactorial. As seen from this study, RNAIII decreases S. aureus adherence to fibrinogen under static conditions and increases S. aureus adherence to fibronectin and EC under both static and flow conditions.

We thank Professor S. Arvidson, Microbiology and Tumor-biology Center, Karolinska Institute, Stockholm, Sweden, for providing bacterial strains, and Dr C.J. Edgell, Department of Pathology, North Carolina University, Chapel Hill, NC, USA, for providing human endothelial cell line Eaby-926.

References


