In Vitro Susceptibility to Thrombin-Induced Platelet Microbicidal Protein Is Associated With Reduced Disease Progression and Complication Rates in Experimental Staphylococcus aureus Endocarditis

Microbiological, Histopathologic, and Echocardiographic Analyses

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Background—Mammalian platelets contain small, cationic, staphylocidal peptides, termed thrombin-induced platelet-microbicidal proteins (tPMPs). Evidence suggests that tPMPs play a key role in host defense against endovascular infections, such as infective endocarditis (IE). In the present study, we evaluated the influence of differences in staphylococcal tPMP-susceptibility profiles in vitro on disease severity in experimental IE.

Methods and Results—Experimental IE was induced in rabbits with either a tPMP-susceptible or an isogenic tPMP-resistant Staphylococcus aureus strain. Vegetation size, left ventricular fractional shortening, and onset of aortic valvular regurgitation were serially assessed by echocardiography over an 11-day postinfection period. In addition, blood cultures were performed daily. Parameters delineated at autopsy included vegetation weights; bacterial densities in vegetations, myocardium, and kidneys; extent of valvular and perivalvular tissue damage; and renal embolization. The following significant differences were observed in animals infected with the tPMP-susceptible versus the tPMP-resistant S aureus strain: substantially lower bacteremia rates (P < 0.02); reduced vegetation growth (P < 0.001) and weight (P < 0.001); a later onset of aortic valvular regurgitation (P = 0.0039); increased preservation of left ventricular function (P < 0.001); reduced valvular tissue damage (P = 0.01) and perivalvular inflammation (P = 0.015); and reduced bacterial densities in vegetations (P < 0.001) and kidneys (P < 0.01).

Conclusions—The in vitro tPMP-susceptibility profile in S aureus substantially affects a number of well-defined cardiac and microbiological parameters related to disease severity and prognosis in IE. These findings underscore the likelihood that platelets mitigate the pathogenesis of endovascular infections via local secretion of antimicrobial peptides.

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Key Words: endocarditis ▪ platelets ▪ peptides

Infective endocarditis (IE) remains a therapeutic challenge, exhibiting high morbidity and mortality rates (≥20%) despite substantial improvements in diagnostic strategies, antibiotic treatment regimens, and surgical techniques.1,2 Acute valvular regurgitation and perivalvular extension of IE (eg, abscess formation) are particularly problematic complications that are potentially fatal and usually require urgent surgical intervention.3 Because of the dire consequences of such complications of IE, it is imperative to delineate both microbial and host factors that contribute to their development.

The key role of endogenous antimicrobial peptides in host defense against a diverse spectrum of infectious diseases has been emphasized recently.4,5 In the context of endovascular infections such as IE, mammalian platelets have been shown to contain small, cationic, microbicidal peptides.6,7 These peptides are secreted after platelet stimulation with thrombin, an agonist abundantly released at sites of endovascular damage or infection,8,9 and exert potent microbicidal activity against many endovascular pathogens, including Staphylococcus aureus.6,10 Such peptides have been termed thrombin-induced platelet microbicidal proteins (tPMPs),6 and analogous polypeptides have been isolated both from rabbit and human platelets.6,7 A growing body of evidence strongly suggests that tPMPs serve an integral role in host defense by limiting the progression of endovascular infections.11-15
However, the overall relationship between microbial tPMP-susceptibility phenotypes and complications in IE has not yet been addressed. In the present study, we evaluated whether microbial differences in tPMP-susceptibility phenotypes in vitro would affect key parameters related to IE severity and prognosis, including the frequency and extent of valvular and perivalvular complications.

Methods

S aureus Strains and General Methods

A well-characterized isogenic S aureus strain pair, derivatives of the parental strain ISP479, was used in the present study. Both strains have been shown to reliably induce experimental IE. ISP479C is a spontaneous, plasmid-cured variant of parental strain ISP479, which is tPMP-susceptible in vitro. ISP479R is a Tn551 transposon mutant of ISP479 that exhibits stable tPMP resistance in vitro. With the exception of their differing tPMP-susceptibility phenotypes, both strains exhibit virtually identical growth kinetics, antibiotics, platelet adherence and aggregation profiles, toxin production, matrix protein adherence phenotypes, and genotypic profiles. Before use, organisms were routinely cultured overnight in tryptic soy broth. After harvest by centrifugation, the staphylococcal cells were washed and resuspended in normal saline, sonicated briefly to ensure single cells, and adjusted by spectrophotometry (optical density of 600 nm) to the desired final inoculum. Such inocula were routinely confirmed by quantitative cultures on tryptic soy broth agar.

Rabbit Model of IE

We used a modification of the well-characterized rabbit model of catheter-induced IE. In brief, anesthetized New Zealand White rabbits (n = 24) underwent transcarotid-transaortic valve catheterization to induce sterile aortic valve and left ventricular vegetations. To mirror native valve IE, the catheter was then removed 6 hours after catheterization. At 24 hours after catheterization, 12 animals each were infected intravenously with the ID50 inoculum of the tPMP-susceptible and tPMP-resistant S aureus strains, respectively (5 × 10^8 colony-forming units [CFU]). To parallel human IE, we chose a prolonged postinfection period of 11 days for analyses. At this time point, animals were euthanized with sodium pentobarbital (100 mg/kg, intravenous push). Animals that died before the scheduled euthanasia date (n = 3) or in which IE was not induced (n = 3) were excluded from analyses. Therefore, the study group consisted of 18 animals infected with the tPMP-susceptible (n = 9) or tPMP-resistant (n = 9) strain. All rabbits were treated in accordance with institutional and Public Health Service guidelines for the humane care and treatment of animals.

Microbiological Parameters

The persistence of bacteremia during the postinfection period is a key marker of disease severity in IE. It reflects both progressive infection in vegetations and extracardiac organs and ongoing microbial reseeding of vegetations. Thus, quantitative blood cultures were obtained daily after S aureus challenge. The frequency of bacteremia was expressed as the proportion of animals in each group with blood-culture negativity.

To assess the extent of infection within target tissues at autopsy, ventricular vegetations were removed and quantitatively cultured as described previously. Bacterial densities within vegetations were determined as log_{10} CFU/gram of vegetation (± SD). To quantify the extent of myocardial infection and hematogenous renal dissemination, samples of macroscopically visible left ventricular lesions and kidney lesions (each ~0.125 cm³) were processed for quantitative culture as described above and expressed as mean log_{10} CFU/gram of tissue (± SD).

Histopathology

Cardiac histopathology was performed to assess the effect of the in vitro tPMP-susceptibility phenotypes on valvular and perivalvular tissue damage. At autopsy, hearts were sectioned along the ventricular septum; after removal of mural vegetations for quantitative culture (see above), hearts were fixed in 10% neutral buffered formalin. The aortic valve and adjacent tissue were evaluated grossly for overt lesions (ie, number of vegetations, valve necrosis, abscess formation, and anuloaortic dehiscence).

For histological assessment, tissues were embedded in paraffin, sectioned at 5 μm, and stained in parallel with either periodic acid-Schiff for general morphology or tissue Gram stain for detection of microorganisms. The sections were then examined in a blinded manner (C.C.N.) for areas of valve necrosis, inflammation (polymorphonuclear and macrophage influx), granulation tissue, karyorrhexis, ulcerations, bacteria, and depth of inflammatory or bacterial penetration into the adjacent tissue. The degree of valvular damage was scored semiquantitatively as indicated in the legend of Figure 2. The incidence and extent of hematogenous embolization and kidney infarction were monitored and quantified by renal histopathology as described previously.

Echocardiography

Transthoracic 2D M-mode and pulsed-wave Doppler echocardiography were performed daily in all animals to serially assess the effect of the in vitro tPMP-susceptibility phenotype on vegetation growth, left ventricular function, occurrence of perivalvular abscess cavities, and aortic valvular regurgitation. A 7.5-MHz transducer linked to an ultrasound unit was used (Hewlett Packard, Sonos Intravascular). The transducer was placed in the third or fourth intercostal space of nonanesthetized animals to achieve a parasternal long-axis view, which was defined as the region of interest for the detection of vegetations or abscess cavities. The settings of the ultrasound unit were identical in all examinations. The presence and maximum diameter of vegetations or abscess cavities were evaluated. The total maximum diameter of vegetations was calculated as the sum of all single maximum vegetation diameters.

To determine the presence or absence of aortic valvular regurgitation, the left ventricular outflow tract was traced by pulsed-wave Doppler echocardiography. When regurgitation was observed, the date of onset of aortic valvular regurgitation during the postinfection period was noted. To evaluate left ventricular function, 2D-directed M-mode echocardiograms of the left ventricle were obtained. Left ventricular internal dimensions at end diastole and end systole were determined, and fractional shortening was then calculated as described previously.

Statistical Analysis

Mean values and SDs were calculated for continuous variables. Differences between groups were analyzed by the appropriate nonparametric tests. A 2-way repeated-measures analysis was performed to test for time- and group-related differences between echocardiographic measurements of left ventricular function and vegetation growth (SAS). A P value of <0.05 was considered significant in all analyses.

Results

Blood Culture Results

Over the 11-day study period, the frequency of blood-culture negativity was significantly higher in animals infected with the tPMP-susceptible strain than in animals infected with the tPMP-resistant strain (Table).

Endocardial Lesions

Macroscopic examination of aortic valves and the periaortic valve circumference revealed a significantly lower mean number of vegetative lesions in animals infected with the tPMP-susceptible strain (1.9 ± 0.8) than in animals infected...
with the tPMP-resistant strain (3.7 ± 1.1; P = 0.028). In addition, vegetations from animals infected with the tPMP-susceptible S aureus strain exhibited significantly lower mean weight and bacterial density than animals infected with the tPMP-resistant strain (Table). Serial echocardiographic examinations demonstrated a rapid increase in mean total vegetation size in both animal groups over the first 3 days after infection, followed by a rapid decrease in vegetation size between days 3 and 4 after infection, which suggests fragmentation of the vegetative lesions and subsequent embolization (Figure 1). However, in animals infected with the tPMP-susceptible strain, there was only a modest increase in echocardiographic vegetation size over the remaining time period (mean increase of 0.03 cm/d). In contrast, in animals infected with the tPMP-resistant strain, there were significantly more rapid and extensive increases in vegetation size both between days 4 and 6 after infection and between days 7 and 9 after infection (mean increase of 0.11 cm/d; P < 0.001 versus animals infected with the tPMP-susceptible strain).

### Aortic Valve Damage

Histopathological analyses allowed for the assessment and semiquantitative grading of the degree of infection-related aortic valve damage. We observed a substantially lower degree of inflammation, karyorrhexis, and valve necrosis, as well as a lesser extent of ulcerations and granulation tissue, in animals infected with the tPMP-susceptible S aureus strain than in those infected with the tPMP-resistant counterpart strain (Figure 2a). Thus, animals infected with the tPMP-susceptible strain demonstrated significantly less extensive aortic valve damage than animals infected with the tPMP-resistant strain (P = 0.01).

### Perivalvular Tissue Damage

Examination of the periaortic valve tissue revealed grossly visible abscess cavities, as well as annuloaortic dehiscence, which occurred only in animals infected with the tPMP-resistant S aureus strain (Figure 2B). Serial echocardiographic examinations revealed that these abscess cavities evolved and reached their maximum diameter between days 3 and 5 after infection (Figure 3). In addition, the penetration of inflammation into the valve ring (P = 0.015) and the presence of bacteria in the adjacent valve tissue (P = 0.057) occurred substantially less frequently in animals infected with the tPMP-susceptible strain than in its isogenic tPMP-resistant counterpart (Figure 4).

### Aortic Valve Regurgitation and Left Ventricular Function

Serial echocardiographic Doppler examinations defined the onset time of aortic valvular regurgitation as a functional marker of disease severity. No aortic valvular regurgitation was detected before infection. Aortic valvular regurgitation was present in all animals infected with the tPMP-susceptible strain at day 9 after infection; in comparison, all animals infected with the tPMP-resistant strain developed aortic valvular regurgitation by postinfection day 4 (P = 0.004).

The hemodynamic effects of aortic valvular regurgitation were demonstrated by the degree of ventricular dilatation and

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**Table:**

<table>
<thead>
<tr>
<th>Blood Culture Negativity, n (%)/d‡</th>
<th>Veg. Weight, mg*</th>
<th>Veg. Bacterial Counts, Log₁₀ CFU/g*</th>
<th>Kidney Bacterial Counts, Log₁₀ CFU/g†</th>
<th>Myocardium Bacterial Counts, Log₁₀ CFU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP479C (n=9) 23±17</td>
<td>0.043±0.008</td>
<td>6.38±0.79</td>
<td>6.26±0.81</td>
<td>4.64±0.87</td>
</tr>
<tr>
<td>ISP479R (n=9) 9±16</td>
<td>0.067±0.01</td>
<td>8.56±0.8</td>
<td>7.7±0.84</td>
<td>5.17±0.8</td>
</tr>
</tbody>
</table>

n indicates number of animals; Veg., vegetation; n (%)/d, number of negative blood cultures per day expressed as percentage of animals; and CFU: colony-forming units.

*P < 0.001; †P < 0.01; ‡P = 0.02.

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**Figure 1.** Echocardiographic parasternal long-axis views of 2 rabbits challenged with either tPMP-resistant strain (left) or tPMP-susceptible strain (right) on day 6 after infection. In both animals, vegetations were detected at aortic valve. Sizes of vegetations in left and right panels were 0.7 and 0.25 cm, respectively. V indicates left ventricle; >, aortic valve vegetation.
deterioration of left ventricular function. Left ventricular fractional shortening decreased \((P<0.001)\) and left ventricular end-diastolic diameter increased \((P<0.001)\) to a significantly lesser degree over the 11-day study period in animals infected with the tPMP-susceptible strain than in those with the tPMP-resistant strain (Figures 5 and 6). Infectious myocarditis was present microbiologically in all animals, with no significant differences between animal groups (Table).

**Kidney Lesions and Embolic Events**

As was observed for vegetation bacterial densities, there were significantly lower bacterial densities within kidney lesions of animals challenged with the tPMP-susceptible strain than within those of animals infected with tPMP-resistant strain (Table). Moreover, a significant reduction in embolic kidney infarction was seen in animals infected with the tPMP-susceptible strain compared with the tPMP-resistant strain \((P=0.0028; \text{Figure 7}).\)

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**Figure 2.** Extent of aortic valvular and perivalvular tissue damage. A, Amount of valve tissue damage was calculated as percentage of entire valvular circumference and scored as follows: I, <10%; II, 10% to 30%; III, 30% to 50%; IV, >50%; and V, >50% and penetration of inflammatory response or infectious process into adjacent tissue. B, Frequency of perivalvular tissue infection, valve ring inflammation, and abscess formation.

**Figure 3.** Echocardiographic parasternal long-axis view with visualization of periaortic abscess cavity. V indicates left ventricle; A, left atrium; O, aorta ascendens; and >, abscess.

**Figure 4.** Representative sections of cardiac valves with underlying valve rings and myocardium from rabbits with \(S\) \(aureus\) endocarditis. A, Infection with tPMP-susceptible \(S\) \(aureus\) strain induced vegetation (arrowhead) with mild inflammation, granulation tissue, adjacent hemorrhage, and intact valve ring (arrow) without inflammation or bacteria. B, Infection with tPMP-resistant \(S\) \(aureus\) strain generated valve ring abscess (arrows). Small portion of infected vegetation (arrowhead) is also apparent. (Hematoxylin and eosin, original magnification \(\times 60\).)
Discussion
Platelets respond rapidly in the setting of damaged vascular and cardiac endothelium and are activated in response to soluble mediators released by these damaged tissues. Moreover, platelets respond to, interact directly with, and are activated by endovascular pathogens themselves, such as S aureus. Because of these rapid responses, platelets accumulate in the setting of endovascular infections such as IE. Thus, platelets represent a significant portion of infected cardiac vegetations and emboli, which has caused them to be interpreted traditionally as contributing to the progression and complications of IE.

However, activated platelets are now known to release a group of microbicidal peptides (ie, tPMPs), which are believed to serve an integral function in antimicrobial host defense. These peptides exert their microbicidal activities both by perturbing the bacterial membrane and by interfering with intracellular macromolecular synthesis. A compelling body of recent evidence has demonstrated that the host defense function of these peptides is particularly relevant to endovascular infections caused by S aureus. For example, among clinical bloodstream S aureus isolates, tPMP-susceptible strains were infrequently associated with human IE compared with tPMP-resistant strains. In addition, bacteremic S aureus strains from patients with IE, resulting from an infected intravascular catheter, tended to be significantly more tPMP-resistant than strains arising from a noncatheter source. Furthermore, multiple studies in experimental S aureus IE have shown that animals infected with tPMP-susceptible strains exhibit reduced bacterial proliferation in vegetations and kidneys and a more rapid response to antimicrobial therapy than animals infected with an isogenic tPMP-resistant counterpart.

Figure 5. M-mode echocardiographic illustration of left ventricular function in 2 animals infected with either tPMP-resistant strain (left) or tPMP-susceptible strain (right). Tall and short bars in left panel indicate end-diastolic and end-systolic time point, respectively. Note increased end-diastolic left ventricular diameter in animal infected with tPMP-resistant strain (1.6 cm) vs tPMP-susceptible strain (0.9 cm). Legends in left panel correspond to respective positions in right panel. V indicates left ventricle; s, left ventricular septum; and p, left ventricular posterior wall.

Figure 6. Left ventricular function as assessed by fractional shortening (FS) expressed in percent (straight line) and left ventricular end-diastolic diameter (broken line) determined during M-mode echocardiography. Dark gray and light gray lines indicate animals challenged with tPMP-1-resistant and -susceptible strains, respectively. Normal value for FS of left ventricle in rabbits is 42.8%, and normal value for left ventricular end-diastolic diameter (EDD) is 6 mm, as determined in population of 25 healthy rabbits. pre-inf indicates before infection.

Figure 7. Extent of renal infarction. Amount of ischemic kidney tissue was calculated as percentage of entire longitudinal section. This was expressed in relative grades assessed in semi-quantitative manner as follows: I, 0% infarction; II, 1% to 4% infarction; III, 5% to 14% infarction; IV, 15% to 40% infarction; and V, >40% infarction.
The present investigation evaluated the effect of the in vitro tPMP-susceptibility phenotype in *S. aureus* on several key parameters of disease severity in IE. Five major findings emanated from this investigation that are indicative of reduced disease severity caused by tPMP-susceptible versus tPMP-resistant isogenic *S. aureus* strains: (1) lower frequencies of bacteremias and degrees of vegetation growth; (2) a lower extent of valve tissue damage and a delayed onset of aortic valvular regurgitation; (3) less extensive perivalvular infection and the absence of perianular abscesses; (4) less deterioration of left ventricular function; and (5) a reduced frequency of extracardiac embolic events.

Specifically, infection with the tPMP-susceptible *S. aureus* strain was associated with reduced structural damage to the aortic valve and perivalvular tissue compared with infection with the tPMP-resistant *S. aureus* strain. Perivalvular abscess cavities were seen exclusively in animals infected with the tPMP-resistant strain, and they evolved early after infection, as determined by serial echocardiography. In this regard, more severe deterioration of left ventricular function in such animals can be interpreted to be a result of left ventricular volume overload due to acute aortic valvular regurgitation rather than to the degree of bacterial myocarditis, which was not significantly different in animals infected with the tPMP-susceptible or tPMP-resistant strains. These data suggest that the balance between tPMP-associated host defenses and the intrinsic tPMP-susceptibility phenotype of the infecting organism may be as important as the duration of the untreated disease in dictating the extent of complications in IE.

The mechanisms by which tPMPs affect disease severity in IE remain to be fully elucidated. However, given our current and prior in vitro and in vivo data, it appears reasonable to hypothesize that the local secretion of tPMPs from activated platelets at sites of vegetation development may limit proliferation of tPMP-susceptible strains. This effect would likely mitigate hematogenous embolic seeding of target organs (eg, kidneys) and reduce the extent of bacteremia, key factors in ongoing microbial seeding of target organs (eg, kidneys) and reduce the extent of extracardiac embolic events.

Such reductions in the degree of intravascular microbial proliferation and bacteremia would also be anticipated to blunt the valvular and perivalvular inflammatory response, resulting in a delayed onset of valvular regurgitation and a lesser degree of left ventricular dysfunction. In contrast, tPMP-resistant strains would be able to circumvent the host defense function of tPMPs and, in turn, exploit platelets at sites of endovascular damage to accelerate the evolution of the vegetation. This event would lead to earlier and more extensive valvular, perivalvular, and target-organ complications of IE.

Conclusions

Data from the present study provide compelling evidence that in *S. aureus* strains, differences in the in vitro tPMP-susceptibility profile are reflected in differences in comparative cardiac functional and anatomic parameters in IE, which are of key relevance to disease severity and prognosis. This further underscores the crucial role of platelets and tPMPs in host defense against endovascular infections. These findings may have significant implications in understanding the pathogenesis of IE, as well as for future improvement in the prevention and therapy of IE.

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References


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