Degradation of fibrinogen and collagen by staphopains, cysteine proteases released from *Staphylococcus aureus*

Takehisa Ohbayashi,1 Atsushi Irie,2 Yoji Murakami,3,4 Magdalena Nowak,5 Jan Potempa,5,6 Yasuharu Nishimura,2 Masanori Shinohara1 and Takahisa Imamura4

Correspondence
Takahisa Imamura
taka@kumamoto-u.ac.jp

Received 11 August 2010
Revised 8 November 2010
Accepted 12 November 2010

1Department of Oral and Maxillo-Facial Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan
2Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan
3Department of Urology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan
4Department of Molecular Pathology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan
5Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland
6Department of Periodontics, Endodontics and Dental Hygiene, University of Louisville School of Dentistry, Louisville, KY 40202, USA

*Staphylococcus aureus* is the most frequently isolated pathogen in Gram-positive sepsis often complicated by a blood clotting disorder, and is the leading cause of infective endocarditis induced by bacterial destruction of endocardial tissues. The bacterium secretes cysteine proteases referred to as staphopain A (ScpA) and staphopain B (SspB). To investigate virulence activities of staphopains pertinent to clotting disorders and tissue destruction, we examined their effects on collagen, one of the major tissue components, and on plasma clotting. Both staphopains prolonged the partial thromboplastin time of plasma in a dose- and activity-dependent manner, with SspB being threefold more potent than ScpA. Staphopains also prolonged the thrombin time of both plasma and fibrinogen, indicating that these enzymes can cause impaired plasma clotting through fibrinogen degradation. Whereas SspB cleaved the fibrinogen Aα-chain at the C-terminal region very efficiently, ScpA degraded it rather slowly. This explains the superior ability of the former enzyme to impair fibrinogen clottability. Enzymically active staphopains, at concentrations as low as 10 nM, degraded collagen with comparable efficiency. These results show novel virulence activities of staphopains in degrading fibrinogen and collagen, and suggest an involvement of staphopains in the clotting impairment and tissue destruction caused by staphylococcal infection.

**INTRODUCTION**

Sepsis is a serious medical condition, in which living bacteria are present in the bloodstream. Shock and disseminated intravascular coagulation (DIC) are common and potentially fatal consequences of sepsis. DIC occurs in as many as 40% of sepsis patients, often leading to multiple organ failure (Levi & ten Cate, 1999), and is directly linked to a high mortality rate. Clinical studies have shown that Gram-positive micro-organisms are as common as Gram-negative bacteria in causing sepsis (Ahmed *et al.*, 1991; Kieft *et al.*, 1993).

*Staphylococcus aureus* is the most frequently isolated pathogen in Gram-positive sepsis (Ahmed *et al.*, 1991; Bone, 1993). In addition, this bacterium is the leading cause of infective endocarditis in many regions of the world (Fowler *et al.*, 2005). The development of endocarditis increases the mortality rate of patients with bacteraemia (Chang *et al.*, 2003) that is due to bloodstream infections (Malani *et al.*, 2008) and/or sepsis caused by *S. aureus*. The pathogenicity of *S. aureus* in causing large numbers of
different clinical manifestations reflects the organism’s ability to produce a variety of virulence factors.

In addition to enterotoxins and haemolysins, *S. aureus* secretes several extracellular proteases, including V8 protease, metalloprotease aureolysin and cysteine proteinases [staphopain A (ScpA) and staphopain B (SspB)], that are considered as putative virulence factors (Potempa & Pike, 2009; Massimi et al., 2002). Proteases can be potentially involved in the pathophysiology of sepsis complications caused by *S. aureus* via interaction with neutrophils and plasma proteins. Indeed, it has been shown that SspB affects the interaction of neutrophils and monocytes with macrophages (Smagur et al., 2009), and arterial injection of staphopains lowers blood pressure through kinin release from plasma kininogens (Imamura et al., 2005). These findings suggest that staphopains contribute to *S. aureus* septic shock, one of the major fatal complications of sepsis.

Clotting induction and a subsequent tendency to bleeding are prominent clinical features of DIC, another lethal outcome of sepsis. Staphopains may participate in the onset of the clotting disorder through activation or inactivation of clotting factors in plasma by proteolytic cleavage. However, the ability of staphopains to affect plasma clotting has not been studied. Invasion of *S. aureus* into the endocardium to cause infective endocarditis requires the degradation of connective tissue including extracellular matrix proteins, and is facilitated by proteases. In this context, staphylococcal proteases can contribute to this process, although little is known about the ability of *S. aureus*-derived enzymes to degrade matrix proteins. The extracellular matrix degradation essential for the pathogenicity of *S. aureus* is inferred from the ability of ScpA to cause widespread plasma leakage induced through bradykinin release by intradermal injection of the protease. This is in stark contrast to the localized plasma leakage induced by injection of bradykinin alone (Imamura et al., 2005). The spreading of leaked plasma is apparently caused by degradation of extracellular matrix proteins, including elastin, that can be cleaved by ScpA (Potempa et al., 1988).

Here we investigated the effect of staphopains on plasma clotting and on collagen (the major component of the extracellular matrix) to determine whether staphopains are associated with the clotting disorder in DIC and cause tissue destruction during *S. aureus* infection.

**METHODS**

**Materials.** Human fibrinogen and plasminogen were purchased from Calbiochem. Human thrombin and Collagenokit (CLN-100) were obtained from Sigma-Aldrich and the Collagen Research Center (Tokyo, Japan), respectively. Other chemicals were purchased from Wako Pure Chemical Industries. Normal human plasma was prepared by centrifugation of a mixture of nine volumes of freshly drawn blood from healthy volunteers and a volume of 4% (w/v) sodium citrate.

**Purification of staphopains and titration of their enzyme activity.** Staphopains were purified from culture supernatants of *S. aureus* strain V8-BC10 or 8325-4, as described previously (Potempa et al., 1988). Purity of staphopains was assessed by SDS-PAGE, MS and N-terminal amino acid sequence analysis. The enzymes were shown to be homogeneous proteins with a molecular mass of 21 kDa (Imamura et al., 2005). The active site concentration of ScpA and SspB was determined by enzyme titration with E-64 and staphostatins, respectively (Potempa et al., 1988; Rzychon et al., 2003). Enzymically inactive staphopains were prepared by boiling for 5 min.

**Clotting time assay.** Clotting time was measured with a KC4d coagulation analyser (Trinity Biotech). For assays of activated partial thromboplastin time (APTT), 90 μl citrated human plasma and 10 μl staphopain were incubated in a plastic cell at 37 °C for 1 min, followed by addition of 100 μl of Automated APTT®/i, incubation at 37 °C for 3 min, and addition of 100 μl 25 mM CaCl2 to initiate clotting (Imamura et al., 1997; Nitta et al., 2007). For assays of thrombin time (TT), 90 μl citrated human plasma or fibrinogen at its plasma concentration (Halkier, 1991; 3 mg ml⁻¹ in Tris-buffered saline) was incubated with 10 μl proteinase at 37 °C for 3 min in a plastic cell, followed by addition of 100 μl human thrombin (5 U ml⁻¹) to initiate clotting (Imamura et al., 1995, 2000, 2008). As a control, 10 mM Tris/HCl pH 7.3, containing 150 mM NaCl (TBS) was used instead of the protease.

**SDS-PAGE.** Five microlitres of staphopain (50 nM) and 15 μl human fibrinogen (3 mg ml⁻¹ in 50 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl) were mixed and incubated at 37 °C. At various time periods, the mixture was withdrawn and boiled for 5 min in the presence of 2-mercaptoethanol, followed by SDS-PAGE on a 10% polyacrylamide gel (Imamura et al., 2008). The gel was stained with Coomassie brilliant blue R-250.

**N-terminal sequence analysis.** To determine the N-terminal sequence of fibrinogen-derived fragments, the mixture was separated by SDS-PAGE and transferred to an Immobilon PVDF transfer membrane (Millipore). The transferred proteins were visualized by staining with Coomassie brilliant blue R-250, excised and subjected to sequence analysis by using protein sequencer (447A, Applied Biosystems).

**Collagenolytic activity assay.** Collagenolytic activity was measured by using FITC-conjugated type I collagen from cow embryo corium (CollagenoKit CLN-100). Type I collagen was purified according to a method described by ChandraRajan (1978). Preparing in this way, collagen maintains the triple-helix conformation and is resistant to degradation by non-collagenolytic proteases. After FITC conjugation, non-collagen proteins were removed by pepsin digestion. The FITC-conjugated type I collagen [0.1 % (w/v) in 0.1 M Tris/HCl, pH 7.5, 0.4 M NaCl, 10 mM CaCl2] was incubated with various concentrations of staphopain at 35 °C for 3 h. Then, 100 μl of the solution was mixed with 100 μl 70% ethanol in 0.17 M Tris/HCl buffer, containing 0.67 M NaCl. After stirring for 20 s, the mixture was centrifuged at 1000 g for 10 min at 4 °C to separate digested collagen in the supernatant from undigested collagen in the precipitate. To assess collagen degradation, 70 μl of the supernatant was withdrawn and fluorescence was measured with a Wallac 1420 ARVO MultiLabel Counter (PerkinElmer) with emission at 520 nm and excitation at 495 nm.

**Statistical analysis.** Statistical differences were determined by using the non-paired Student’s t test.

**RESULTS**

**Effect of staphopains on blood coagulation**

To investigate whether staphopains can affect blood clotting, we first determined the effect of proteases on the APTT of normal human plasma. Incubation of the...
plasma with SspB or ScpA prolonged the APTT in a dose-dependent manner. The effect was significant even at 30 and 100 nM concentrations of SspB and ScpA, respectively. Inactivation of staphopain protease activity abolished the effect, indicating that the prolongation of the APTT was due to proteolytic inactivation of a clotting factor(s) (Fig. 1). Cumulatively, it is clear that staphopains do not induce clotting but are able to impair plasma clotting ability in a proteolytic activity-dependent manner, with SspB being about threefold more potent than ScpA.

**Impairment of plasma clottability through fibrinogen cleavage by staphopains**

To determine whether the prolongation of the APTT by staphopains is caused by loss of fibrinogen clottability, we examined the effect of the proteases on the TT of the plasma. The TT of the plasma incubated with proteolytically active staphopains was prolonged in a dose-dependent manner (Fig. 2a). In accordance with the effect of staphopains on the APTT (Fig. 1), SspB increased the TT about three times more efficiently than ScpA (Fig. 2a). These findings suggest that staphopains prolong plasma clotting time mainly through abolishing fibrinogen clottability and that degradation of other clotting factors is not significant. To confirm that staphopains can strongly affect fibrinogen clottability, we measured the TT after incubating fibrinogen at its plasma concentration (Halkier, 1991) with the staphopains. As expected, the fibrinogen TT was prolonged by incubation with staphopains in a dose-dependent manner. In contrast, inactivated staphopains, even at the highest concentrations investigated, did not affect the fibrinogen TT (Fig. 2b). Altogether, it is clear from these data that both staphopains can impair fibrinogen clottability by cleaving this clotting factor. Because neither of the staphopains can convert plasminogen to plasmin, even in the presence of fibrinogen (data not shown), plasmin-mediated fibrinogen degradation is unlikely to participate in staphopain-induced prolongation of the plasma clotting time. Taking these results together, it is clear that both staphopains can impair plasma clottability by affecting fibrinogen.

**Preferential cleavage of human fibrinogen by staphopains in the C-terminal region of the Aα-chain**

To study the mechanism of impairment of the fibrinogen clottability by staphopains, we analysed staphopain-elicited...
Degradation of fibrinogen and collagen by staphopains

fibrinogen degradation by SDS-PAGE. In comparison with the conditions used in an earlier report that showed total degradation of fibrinogen by SspB (Massimi et al., 2002), we applied a low protease concentration and short incubation periods to monitor initial proteolytic events associated with loss of fibrinogen clottability. At 50 nM, both staphopains cleaved fibrinogen Aα-chain in a time-dependent manner (Fig. 3a, b). Cleavage of the Aα-chain (66 kDa) by ScpA was visible at 2 min and the band of the native Aα-chain disappeared at 8 min. This was accompanied by generation of the 50 kDa fragment just below the γ-chain (53 kDa), followed by accumulation of a 34 kDa fragment (Fig. 3a). The N-terminal sequence of the 34 kDa fragment was identical to that of the native Aα-chain (Watt et al., 1979), indicating that ScpA first cleaved off a 16 kDa fragment from the C-terminal region of the Aα-chain. Subsequent cleavage within the C-terminal region of the 50 kDa fragment of the Aα-chain reduced its molecular mass to 34 kDa (Fig. 3a). The Aα-chain was also targeted by SspB, which generated 41, 37 and 35 kDa fragments derived from this chain. The intensity of the 41 and 37 kDa bands peaked at 8 min, whereas the amount of the 35 kDa fragment continued to increase until 16 min (Fig. 3b). N-terminal sequencing revealed that all three fragments have the N-terminal sequence (ADSGEG) of the native Aα-chain. Apparently, SspB initially released the 41 kDa fragment by proteolysis within the C-terminal region of the Aα-chain. The 41 kDa fragment was further truncated at the C-terminal region, generating 37 and 35 kDa fragments. In contrast to the Aα-chain, Bβ- and γ-chains were not degraded within the first 16 min of incubation with staphopains (Fig. 3a, b). Plasmin degrades all three chains of fibrinogen and it cleaves the Aα-chain at various sites, including peptide bonds at Arg252, Arg424, Arg491 and Lys508 (Henschen, 1983). Proteolysis at these sites generates fragments with molecular masses different from those released from the Aα-chain by staphopains. Therefore, it is clear that staphopains cleave the fibrinogen Aα-chain at sites different from those at which plasmin cleaves.

Collagenolytic activity of staphopains

To study pathogenic activities of staphopains further, we investigated whether these proteases can degrade collagen. We treated fluorescence-labelled type I collagen with staphopain and assayed for the release of fluorescent fragments. Both staphopains degraded collagen in a dose-dependent manner at concentrations as low as 10 nM and inactivation of these proteases abolished their collagenolytic activity (Fig. 4). Although differing in fibrinogenolytic activity (Fig. 2a, b), the two staphopains exerted the same collagenolytic activity (Fig. 4). The ability to degrade collagen strongly suggests that staphopains can participate in tissue destruction by S. aureus.

DISCUSSION

In this report we have unambiguously shown that the fibrinogenolytic activity of staphopains can significantly impair plasma clottability (Fig. 2a, b) and possibly contributes to staphylokinase activity. Staphylocinase is an S. aureus-derived protein that forms a 1 : 1 complex with plasminogen, in which this zymogen gains fibrinogenolytic activity, whereas neither of them has enzyme activity of its own (Bokarewa et al., 2006). In circulation, however, the staphylocinase–plasminogen complex is dissociated by α2-antiplasmin, releasing free staphylocinase (Silence et al., 1993). Therefore, the contribution of staphylocinase to fibrinogenolysis in plasma is questionable. Conversely, the staphopain-induced impairment of plasma clottability through fibrinogen cleavage (Fig. 2a, b) indicates that staphopains can exert fibrinogenolytic activity in vivo.

Thrombin converts fibrinogen to fibrin clot by cutting off fibrinopeptide A from the Aα-chain and fibrinopeptide B from the Bβ-chain of fibrinogen (Blombäck, 1986), which leads to polymerization of generated desAA-fibrinogen into protofibrils and lateral association of protofibrils through binding of the C-terminal domain of the Aα-chain to the two other chains, respectively (Doolittle, 2003). Charged amino acid residues necessary for lateral association are located in an αC domain in the C-terminal two-thirds of

---

**Fig. 3.** Cleavage of fibrinogen by staphopains. Human fibrinogen (3 mg ml⁻¹) was incubated with staphopain (50 nM) for various time periods and 20 μl of the mixture was withdrawn. Samples were analysed by SDS-PAGE under reducing conditions using a 10 % polyacrylamide gel. Coomassie brilliant blue R-250 was used for protein staining. Lanes: a, fibrinogen alone; b–e, fibrinogen incubated for 2, 4, 8 or 16 min, respectively, with ScpA (a) and SspB (b).
the Az-chain (Veklich et al., 1993). The very efficient cleavage of the Az domain by SspB (Fig. 3b) must destroy the interaction necessary for lateral association of protofibrils. ScpA also cleaves fibrinogen at the Az domain and more rapidly than SspB, generating 50 and 34 kDa fragments (Fig. 3a), but cleavage by ScpA seems unlikely to cause a clottability loss of this clotting factor in comparison with cleavage by SspB (Fig. 2b). The difference in cleavage sites explains why SspB impairs fibrin clot formation more efficiently than ScpA.

We have previously reported fibrinogenolytic activity of bacterial proteases that prolong the plasma TT. Although less potent than gingipain K of the major periodontal disease pathogen Porphyromonas gingivalis (Imamura et al., 1995), the fibrinogenolytic activity of SspB is comparable with that of ASP (Aeromonas serine protease) from a gastroenteritis agent, Aeromonas sobria (Imamura et al., 2008).

Expression of MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), clumping factor A (McDevitt et al., 1997) and fibronectin-binding protein A (Wann et al., 2000) by S. aureus makes the fibrinogenolytic activity of staphopains even more significant. These cell wall-anchored molecules bind fibrinogen at the γ-chain C terminus, thereby assembling the clotting factor on the surface of the bacterium. This can potentially facilitate the close interaction of fibrinogen with staphopains secreted from the micro-organism, promoting cleavage of the substrate by the proteases. Although human plasma has some inhibitory activity towards ScpA (Potempa et al., 1988), no effective plasma inhibitor has been reported for SspB. The finding that staphopains impaired plasma clottability and clottability of purified fibrinogen with equal efficiency (Fig. 2a, b) clearly indicates the lack of any efficient proteinase inhibitor in human plasma that can block staphopain fibrinogenolysis in vivo. Therefore, it is likely that staphopains impair the clottability of fibrinogen in plasma in sepsis, thus participating in the induction of the bleeding tendency seen in DIC caused by S. aureus infection.

Adherence of S. aureus to endothelial cells is the initial step in the development of endocarditis. In this process, fibrinogen has been shown to act as a bridging molecule between S. aureus and the cells (Cheung et al., 1991), and the ability of this pathogen to bind to fibrinogen is recognized as an important factor for valve infection and invasion in experimental endocarditis (Que et al., 2005). Thus, interaction of S. aureus with fibrinogen plays a crucial role in the pathogenicity of endocarditis caused by this bacterium. Moreover, a 135 kDa cell surface protein of S. aureus (Switalski et al., 1989) binds specifically to collagen (Speziale et al., 1986; Holderbaum et al., 1987; Naidu et al., 1989; Buxton et al., 1990), constituting another important virulence trait in experimental endocarditis (Hienz et al., 1996).

Here we have described for what is believed to be the first time that staphopains exert collagenolytic activity (Fig. 4). Together with the elastin degradation by ScpA (Potempa et al., 1988), the collagenolytic activity of staphopains could injure the endocardium, causing endocarditis. Binding of S. aureus to collagen (Speziale et al., 1986; Holderbaum et al., 1987; Naidu et al., 1989; Buxton et al., 1990; Switalski et al., 1989) possibly augments the collagenolysis. Besides the collagen-binding protein (Switalski et al., 1989), a staphylococcal surface protein capable of binding to several extracellular matrix glycoproteins (McC Gavin et al., 1993) facilitates adherence of S. aureus to the matrix of various tissues. This possibly enhances collagen degradation by staphopains released in close proximity to their substrate. Furthermore, degradation of cystatins by staphopains (Vin cents et al., 2007) and the lack of efficient staphopain inhibitors in human plasma may further contribute to the damaging activity of staphopains in infected tissues or the bloodstream. Thus, staphopain collagenolytic activity can be implicated in tissue destruction by S. aureus, causing a wide range of infectious diseases such as impetigo, furuncles, carbuncles and abscesses of the skin, endocarditis, pneumonia, meningitis and osteomyelitis (Parsonnet & Deresiewicz, 2001).

In conclusion, staphopains that are secreted from S. aureus in infected tissue or in the circulation are likely to degrade collagen or fibrinogen, respectively. These may represent new virulence mechanisms for this bacterium to cause tissue destruction and induce a tendency to bleeding. Therefore, staphopains may constitute important therapeutic targets to treat S. aureus infectious diseases, and inhibitors of these enzymes could be developed into drugs, particularly against antibiotic-resistant strains (e.g. meticillin-resistant S. aureus).

ACKNOWLEDGEMENTS

This study was supported by the Japanese Society for the Promotion of Science (JSPS), Grant-in Aid for Scientific Research (B) 18390125.
REFERENCES


Degradation of fibrinogen and collagen by staphopains...

(gingipains-R) from *Prophyromonas gingivalis*. *Microb Pathog* 29, 155–163.


JAMA. The Complement System and fibrin. *J Thromb Haemost*


*Edited by:* S. J. Foster