Activation of interstitial collagenase, MMP-1, by *Staphylococcus aureus* cells having surface-bound plasmin: a novel role of plasminogen receptors of bacteria

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Abstract Plasmin, the enzymatically active form of plasminogen, can activate several matrix metalloproteinases (MMPs). In this study, we investigated the activation of MMP-1, one of the major interstitial collagenases, by plasmin which was generated on the surface of *Staphylococcus aureus* cells. Plasmin bound to plasminogen receptors on *S. aureus* degraded the major ^{125}I-labeled 55-kDa proMMP-1 into the 42-kDa form corresponding to the size of active MMP-1. MMP-1 formed by *S. aureus*-bound plasmin was also enzymatically active as judged by digestion of the synthetic collagenase substrate, DNP-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2. The finding that, in MMP-1 molecules generated either by soluble plasmin or by *S. aureus*-bound plasmin, the amino-terminal amino acid sequences were identical indicated that the activation mechanisms of the two plasmin forms do not differ from each other. The present observations emphasise and broaden the physiological importance of bacterial plasminogen receptors. In addition to direct proteolytic effects on components of the extracellular matrix, receptor-bound plasmin is also capable of initiating an MMP-1-dependent matrix-degrading enzymatic cascade.

Key words: Matrix metalloprotease; Collagenase; MMP-1 Activation of MMP-1; Plasminogen; Plasminogen receptor; Bacterial invasion; *Staphylococcus aureus*

1. Introduction

Several Gram-positive and Gram-negative invasive bacteria express specific receptors for plasminogen, the zymogen form of the serine-protease plasmin (for a review, see [1]). These bacteria include the following: *Staphylococcus aureus* [2], group A, B, C and G streptococci [3–6], *Escherichia coli* [7,8], *Salmonella enterica* [9], *Neisseria gonorrhoeae*, *Neisseria meningitidis* [10], *Haemophilus influenzae* [10], as well as *Borrelia burgdorferi* [11,12] and *Helicobacter pylori* [13], the causative agents of Lyme disease and gastritis, respectively. The physiological relevance of plasminogen binding to the receptors on bacterial surfaces is highlighted by findings demonstrating that receptor-bound plasminogen can be activated into plasmin which is protected against the major plasmin inhibitor, α_2-antiplasmin [2,11]. The best characterised bacterial plasminogen receptors are streptococcal enolase and M-like protein on the surface of group A streptococci [14–16], type 1 and type G fimbria and curly structures of *E. coli* and of *S. enterica* [17–19], the aspartase-related 55-kDa protein of *H. influenzae* [20], and the outer surface protein A and the 70-kDa protein of *B. burgdorferi* [11].

It has been shown that *E. coli* and *Salmonella* degrade extracellular matrix (ECM) and penetrate artificial basement membranes at an enhanced rate when these bacteria have receptor-bound plasmin on their surfaces [9,21,22]. Moreover, *B. burgdorferi* with active plasmin bound to their surface plasminogen receptors cause a more severe form of bacteraemia than their counterparts without active plasmin [23]. These findings emphasise the importance of plasmin-mediated proteolytic events in invasion of bacteria through the ECM.

Physiological degradation of ECM is an integral part of tissue remodelling that takes place in both physiological and pathological conditions. ECM is also essential for the maintenance of connective tissue in adult organisms [24]. The degradation of ECM is regulated by the balance between the production of matrix-degrading enzymes and their inhibitors. One of the major matrix metalloproteinase (MMP) involved in the degradation of collagens is the interstitial collagenase or matrix metalloprotease-1 (MMP-1). It cleaves fibrillar type I, II, III, VII and X collagens at a single site [25]. MMP-1 is secreted as a major 53-kDa non-glycosylated and as a minor 57-kDa glycosylated form which can be activated by SH-reacting and denaturing agents, as well as by many proteases such as trypsin and plasmin to produce active 42-kDa and 47-kDa forms, respectively [26]. In this study we show that plasmin on the surface of *S. aureus* is able to activate the major interstitial collagenase, MMP-1, thus enabling more efficient bacterial penetration and invasion of tissues.

2. Materials and methods

2.1. Reagents

Glu-plasminogen, Glu-plasmin and chromogenic substrate S-2251 (H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide hydrochloride) were purchased from Kabi Vitrum. Aprotinin, α_2-antiplasmin, phenylmethylsulphonyl fluoride (PMSF), and 3-cyclohexylamino-1-propane sulfonic acid (CAPS) were from Sigma. Trypsin (sequencing grade) was from Promega. Single-chain tissue-type plasminogen activator (tPA, containing roughly 10% two-chain form) was obtained from American Diagnostics (New York, NY) and Super RX medical X-ray film from Fuji. MMP-1 was purified from phorbol-treated U937

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cells using sequential chromatography on CM-52 and DEAE/red Sepharose as described [27]. Synthetic substrate (DNP-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2) was used for testing of collagenase activity of MMP-1.

2.2. Iodination of MMP-1

MMP-1 was iodinated by the iodogen method [28] as described in detail [2] using an incubation time of 1 min.

2.3. Cultivation of bacteria

S. aureus, strain Newman, was grown in Todd-Hewitt broth for 16–18 h at 37°C on a shaker, collected by centrifugation, washed twice with phosphate-buffered saline (PBS) containing 0.02% (v/w) sodium azide and finally suspended in plain Tris-BSA buffer (50 mM Tris, pH 7.5, 0.1% (w/v) bovine serum albumin) or in buffer additionally containing 5 mM $CaCl_2$ (Tris/CaCl-BSA). The concentration of bacteria was determined spectrophotometrically and adjusted to 2×10^{10} bacterial cells/ml.

2.4. Generation of plasmin on the surface of S. aureus

Surface-bound plasmin on *S. aureus* was generated as described [2]. Briefly, 5×10^9 bacteria were incubated on a shaker for 1.5 h at 20°C in various combinations of 25 μg of Glu-plasminogen, 25 ng of tPA and 2.5 μg of α_2-antiplasmin in a reaction volume of 1 ml of Tris-BSA buffer. After incubation the bacteria were collected by centrifugation at $3800\times g$ for 10 min, washed twice with Tris buffer and finally suspended in 250 μl of Tris-BSA buffer. Formation of active plasmin was tested with a chromogenic assay [2] by incubating the bacteria (1.6×10^9) with 10 μl of 4 mM S-2251 for 2 h at 20°C. Subsequently, optical densities in the supernatants were recorded at 405 nm after removal of the bacteria from the suspensions by centrifugation.

2.5. Processing of MMP-1 by S. aureus-bound plasmin

S. aureus cells (1.6×10^9, 50 μl) having surface-bound plasmin were incubated with 150 pg of ^{125}I-MMP-1 (50 μl) for 4 h at 37°C. The treatment was stopped by adding 50 μl of Laemmli sample buffer containing β-mercaptoethanol [29] and by boiling the samples immediately. Finally, the samples were analysed in 12% SDS-PAGE [29] followed by autoradiography on X-ray film.

2.6. Collagenase activity of MMP-1 modified by S. aureus-bound plasmin

To test the collagenase activity of MMP-1 modified by bacteria-bound plasmin, 1.25 μg of MMP-1 in 250 μl of Tris buffer was first treated with 5×10^9 *S. aureus* cells having surface-bound plasmin for 24 h at 37°C in the presence of 5 μg of α_2-antiplasmin. As positive control the same amount of MMP-1 in 250 μl of Tris buffer was treated with 3 μg of trypsin for 15 min at 37°C. As the negative control, similar incubations were done in the absence of MMP-1 but in the presence of either plain *S. aureus* cells or cells having surface-bound plasmin. The incubations were stopped by adding 10 μl of 57 mM PMSF into the reactions followed by removal of bacteria by centrifugation. Subsequently, collagenase activity in the incubation medium was tested by reaction with the synthetic collagenase substrate, DNP-Pro-Leu-Gly Leu-Trp-Ala-D-Arg-NH_2. Cleavage products were separated by reverse phase chromatography, and quantitated by monitoring the UV absorbance at 214 nm [30,31].

2.7. Cleavage of MMP-1 by S. aureus-bound plasmin

To compare the cleavage mechanism of *S. aureus*-bound plasmin with that of soluble plasmin, proMMP-1 (30 μg) was treated in 100 μl Tris buffer containing 5 mM $CaCl_2$ either with 10^9 *S. aureus* cells having surface-bound plasmin or with 300 pg of soluble plasmin. Samples (60 μl) were taken after 30 and 120 min of incubation at 37°C. After incubation with *S. aureus*, the incubation mixtures were centrifuged to remove the bacteria. 50 μl of each supernatant was mixed with 25 ml of 50% sample buffer [29], boiled immediately and lyophilised. Subsequently, the lyophilised samples were solubilised in 35 ml of H_2O, separated on 15% SDS-PAGE followed by electrophoretic transfer onto ProBlott-PVFD membrane in 10 mM CAPS buffer, pH 11, containing 10% methanol [32]. Proteins on the membrane were visualised by Coomassie brilliant blue staining, and the bands of interest were cut out and subjected to N-terminal sequencing (Procise 494 HT, Applied Biosystems, Perkin Elmer, CA, USA).

3. Results

3.1. Generation of plasmin on the surface of S. aureus and processing of MMP-1 by S. aureus-bound plasmin

Plasmin activity on the staphylococcal surface was measured by reacting *S. aureus* cells with the chromogenic substrate S-2251 after incubation of bacteria with Glu-plasminogen, tPA and α_2-antiplasmin in various combinations. Bacteria alone or in combination with Glu-plasminogen were unable to generate functional plasmin (Fig. 1, upper panel). A strong bacteria-associated plasmin activity was generated after incubation of the staphylococci with Glu-plasminogen and tPA. Presence of the plasmin inhibitor α_2-antiplasmin in the incubation medium did not significantly decrease the enzymatic activity. Processing of ^{125}I-proMMP-1 by bacteria-bound plasmin was detected by SDS-PAGE analysis and subsequent autoradiography. The proMMP-1 which had been incubated with *S. aureus* cells in the absence or presence of Glu-plasminogen migrated as a major 55-kDa band (Fig. 1, lower panel). Incubation of ^{125}I-proMMP-1 under conditions able to generate active plasmin (see upper panel) resulted in conversion of the major 55-kDa species into the 42-kDa one, i.e. the size of active MMP-1. Comparison of the cleavage sites of MMP-1 by soluble plasmin and plasmin associated with *S. aureus* was done by analysing the splitting products

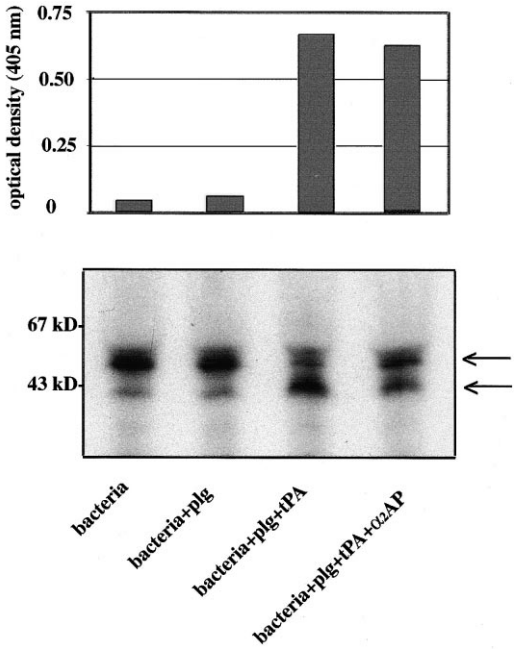

Fig. 1. Upper panel: Plasminogen activation on *S. aureus* surface. *S. aureus* cells were incubated in either the absence or presence of various combinations of Glu-plasminogen (plg), tPA, and α_2-antiplasmin (α_2-AP), as indicated. After incubation for 90 min the bacteria were washed, and plasmin activity associated with bacteria was measured by incubating the bacteria with the chromogenic substrate S-2251, and by monitoring the absorbance at 405 nm. For experimental details, see Section 2. Lower panel: Processing of proMMP-1 by *S. aureus*. ^{125}I-proMMP-1 was incubated with *S. aureus* in various combinations with Glu-plasminogen, tPA and α_2-antiplasmin as above. After incubation for 4 h, the samples were analysed by reducing SDS-PAGE with subsequent autoradiography. Migration of molecular weight markers is shown on the left. The upper and lower arrows indicate the 52-kDa proMMP-1 and the 42-kDa MMP-1, respectively. For experimental details, see Section 2.

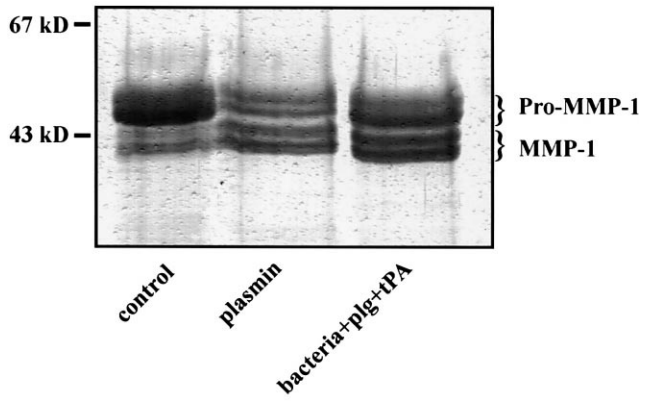

Fig. 2. Separation of proMMP-1 cleavage products for amino-terminal sequencing. ProMMP-1 was incubated in plain buffer, in buffer containing soluble plasmin or in buffer containing *S. aureus* cells having surface-bound plasmin. After incubation for 120 min, the bacteria were removed by centrifugation, and the supernatants were subjected to SDS-PAGE, followed by electrotransfer to PVFD membrane, with ensuing amino-terminal sequencing of the Coomassie blue-stained protein bands. Migration of molecular weight markers is shown on the left. Positions of proMMP-1 and MMP-1 are indicated on the right.

of MMP-1 and by determining the amino-terminal sequences. As shown in Fig. 2, MMP-1 migrated to the same position whether it had been cleaved by soluble or bacteria-bound plasmin. In the two preparations of MMP-1, amino-terminal sequence analyses revealed the sequence Leu-Lys-Val-Met-Lys-Gln-Pro-Arg.

3.2. *Collagenase activity of MMP-1 cleaved by S. aureus-bound plasmin*

To study collagenase activity of the cleaved MMP-1, degradation of the synthetic substrate peptide, DNP-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2, was followed by HPLC analysis after treatment of proMMP-1 with various activators (trypsin served as a positive control). As seen in Fig. 3, proMMP-1 could be activated by the *S. aureus* cells having surface-bound plasmin whereas incubation of proMMP-1 with plain *S. aureus* cells failed to activate. Similarly, activation media lacking proMMP-1 were unable to generate any collagenase activity.

4. Discussion

Activation of MMP-1 is a two-step process involving a primary cleavage of the propeptide region followed by removal of the remaining peptide by self-degradation [25]. Soluble plasmin makes the initial cleavage of proMMP-1 at the Arg^{36}-Asn^{37} bond which is then followed by self-degradation at the bond between Thr^{64} and Leu^{65} [26] leading to the fully converted (42-kDa) active enzyme. In this study our aim was to investigate whether plasmin which is bound to its receptor(s) on the staphylococcal surface can activate MMP-1 like the soluble plasmin.

To generate bacteria-bound plasmin, *S. aureus* cells were incubated with Glu-plasminogen and tPA, and subsequently the surface-associated plasmin activity was determined. Importantly, the presence of α_2-antiplasmin in the incubation medium caused no significant decrease in generated plasmin activity indicating that the plasmin formed was protected against α_2-antiplasmin. This, again, revealed that plasmin was bound to the staphylococcal plasminogen receptors and was not in soluble form in the reaction mixture. The results agree with our previous findings demonstrating that binding of Glu-plasminogen to *S. aureus* enhances its tPA-mediated activation and leads to surface-bound plasmin activity which is protected against α_2-antiplasmin [2].

Processing of proMMP-1 was investigated by incubating ^{125}I-proMMP-1 with *S. aureus* cells together with various combinations of Glu-plasminogen, tPA, and α_2-antiplasmin and by monitoring its digestion by SDS-PAGE analysis and subsequent autoradiography. This experimental design demonstrated clearly that functional plasmin is required to process proMMP-1 into a form corresponding to the size of the active form of MMP-1. By using the substrate peptide DNP-Pro-Leu-Gly-Leu-Trp Ala-D-Arg-NH_2, it could be demonstrated that MMP-1 was indeed enzymically active after having been cleaved by *S. aureus*-bound plasmin.

Processing of the proMMP-1 by soluble or *S. aureus*-bound plasmin appeared to proceed similarly. In SDS-PAGE MMP-1 processed either way migrated to the same position and, moreover, amino-terminal sequence analyses of the two preparations revealed the sequence Leu-Lys-Val-Met-Lys-Gln-Pro-Arg. Thus, both receptor-bound and soluble plasmin had produced the active enzyme where the final processing had occurred between residues Thr^{64} and Leu^{65}. No attempts were made to determine the specific activity of the MMP-1 activated by receptor-bound and soluble plasmin. Neither was the cleavage site of the intermediate form of MMP-1 determined which, in fact, would represent the initial cleavage site by plasmin. However, since the end products had identical amino-termini (Leu^{65}) and MMP-1 did not bind to the staphylococcal surface (data not shown), it is very likely that receptor-bound and soluble plasmin will cleave MMP-1 at the same site in the activation peptide. This is the first time receptor-bound plasmin is shown to have a similar MMP-activating effect as soluble plasmin. One can envisage that solid-phase plasmin has similar properties also on eukaryotic cell surfaces and on other bacteria expressing receptors for plasminogen. The pathophysiological relevance of the present finding is that plasmin activity generated on the staphylococcal surface, if in a tissue environment in which the host cells produce MMP-1,

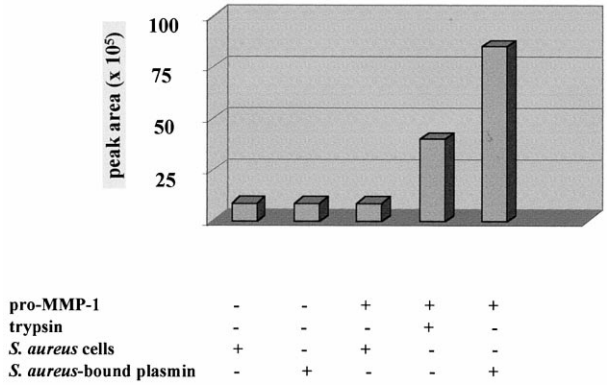

Fig. 3. Collagenase activity of MMP-1 cleaved by *S. aureus*-bound plasmin. Synthetic substrate DNP-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 was reacted with supernatants originating from activation mixtures. Cleavage products of the substrate peptide were analysed by HPLC.

provides *S. aureus* with a powerful additional proteolytic machinery besides the proteases secreted by the bacteria and the direct proteolytic activities of the bacteria-associated plasmin. The collagenolytic activity generated by staphylococci may then help *S. aureus* to penetrate through basement membranes and other structures of the extracellular matrix, and to escape from abscesses.

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