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Original article

Interaction with human plasminogen system turns on proteolytic activity in *Streptococcus agalactiae* and enhances its virulence in a mouse model

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Abstract

Interactions of several microbial pathogens with the plasminogen system increase their invasive potential. In this study, we show that *Streptococcus agalactiae* binds human plasminogen which can be subsequently activated to plasmin, thus generating a proteolytic bacterium. *S. agalactiae* binds plasminogen *via* the direct pathway, using plasminogen receptors, and *via* the indirect pathway through fibrinogen receptors. The glyceraldehyde-3-phosphate dehydrogenase is one of the *S. agalactiae* proteins that bind plasminogen. Presence of exogenous activators such as uPA and tPA are required to activate bound plasminogen. Results from competitive inhibition assays indicate that binding is partially mediated through the lysine binding sites of plasminogen. Following plasminogen binding and activation, *S. agalactiae* is able to degrade *in vitro* fibronectin, one of the host extracellular matrix proteins. Moreover, incubation of *S. agalactiae* with either plasminogen alone, or plasminogen plus fibrinogen, in the presence of tPA enhanced its virulence in C57BL/6 mice, suggesting that acquisition of plasmin-like activity by the bacteria increase their invasiveness.

Keywords: *Streptococcus agalactiae*; Glyceraldehyde-3-phosphate dehydrogenase; Plasminogen; Plasmin; Fibrinogen; Proteolytic activity

1. Introduction

Streptococcus agalactiae, also named group B *Streptococcus* (GBS), is the major cause of bacterial sepsis, pneumonia and meningitis in neonates and has emerged as an increasingly common cause of invasive disease in immunocompromised people [1]. About 50% of infants born from infected women became colonized with *S. agalactiae* during delivery and 1% of the colonized infants develop a severe GBS infection [1]. The main route of neonatal infection is the ascending spread of *S. agalactiae* into the amniotic fluid followed by the aspiration of contaminated amniotic fluid by the fetus. The bacteria can then colonize and infect the lung, resulting in pneumonia. Subsequent transmigration of *S. agalactiae* across the epithelial border allows the bacteria to invade the bloodstream and eventually reach the meninges [1]. Invasion of host cells represents an important mechanism in invasive *S. agalactiae* diseases [2,3].

Numerous human pathogens, including bacteria and viruses, interact with the human plasminogen (Plg) system [4–6]. The importance of these interactions for invasion and dissemination in the infected host has been deduced from *in vitro* analysis [4,6–9] and from *in vivo* studies employing Plg knock-out mice [6,10–13]. In humans, Plg is converted to plasmin (Pl) by Plg activators such as urokinase (uPA) and tissue-type

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plasminogen activator (tPA). However, under normal circumstances, plasminogen activator inhibitors (PAI) inhibit the activation of circulating Plg in blood. Pl is the key enzyme of the Plg system that contributes to the degradation of fibrin and extracellular matrix proteins, like fibronectin, and activates latent collagenases and metalloproteinases [5,6]. Plg activation and Pl formation play an important role in a number of physiological and pathological processes such as fibrinolysis, cell migration, and invasion [6,7,10–15]. Moreover, Plg activation has been suggested as a critical component in establishing invasive bacterial infections in host animals [4,6,10–14]. In particular, Plg binding by *Pseudomonas aeruginosa* resulted in formation of surface-associated plasmin that enhanced bacterial invasiveness [7]. More recently, Plg binding was shown to be essential for the development of cutaneous lesions by *Leishmania mexicana* [12].

In group A streptococci (GAS), Plg binding to bacterial surface can be mediated by specific receptors (direct pathway) or can involve the formation of a fibrinogen (Fg)-containing complex (indirect or fibrinogen-dependent pathway) [9,16]. Direct binding is mediated by the three known surface Plg receptors: plasminogen-binding group A streptococcal M-like protein, α-enolase, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) [5,6,9]. Indirect Plg binding to GAS involves the formation of a trimolecular complex consisting of Plg, streptokinase and Fg that interacts with the cell surface via either Plg or Fg receptors [9,16].

The GBS GAPDH is a moonlighting protein which, when present at the cell surface, interacts with cytoskeleton and extracellular matrix proteins and Plg [17]. This bacterium also expresses a Fg receptor named FbsA that binds to human Fg [18]. Therefore, GBS cells have the potential to bind Plg through the direct and indirect pathways. However, the recruitment of human Plg by GBS and the contribution of this process to the invasive potential or virulence of this bacterium have not been studied. In the present report, we provide the first evidence for the acquisition of surface plasmin-like activity by GBS and we further demonstrate the role of surface-bound Plg in bacterial invasiveness and virulence. We have recently described that GBS GAPDH is a virulence-associated immunomodulatory protein [19] and we show here that this enzyme is also one of the GBS Plg-binding proteins.

2. Materials and methods

2.1. Bacterial isolate and growth conditions

Streptococcus agalactiae NEM316, isolated from a neonatal blood culture [20], was grown in Brain-Heart Infusion (BHI) broth or agar (Difco Laboratories, Detroit, MI, USA) at 37 °C. The complete genome sequence of this strain has been determined [21].

2.2. Plasminogen labeling

Plasminogen (Sigma) was iodinated according to the Iodogen method. Briefly, 200 µl of a plasminogen solution (28 µM in 0.25 M phosphate buffer, pH 7.0) and 0.5 mCi of $Na^{125}I$ (NEN) were added to a reaction tube coated with 500 µg of iodogen (Sigma) and the mixture was incubated in an ice bath for 20 min. Labeled proteins were separated from the iodine in a 5 ml Sephadex G-50 column. Based on the fact that at least 90% of ^{125}I-labeled plasminogen (^{125}I-Plg) were precipitable in 20% trichloroacetic acid, its specific activity was estimated to be approximately 3×10^4 cpm/ng.

2.3. Plasminogen binding by S. agalactiae cells

To assess Plg binding to *S. agalactiae* cells, two binding assays were used.

2.3.1. ^{125}I-Plasminogen binding to S. agalactiae cells in suspension

S. agalactiae cells ($\approx 5 \times 10^8$), previously treated or not with fibrinogen (1 µM) (Sigma), were incubated 2 h at 37 °C with ^{125}I-Plg (2.5×10^5 cpm). The cells were then centrifuged and the unbound ^{125}I-Plg present in the supernatant was removed. The cell pellet was washed with PBS, centrifuged, and the bound ^{125}I-Plg was evaluated by measuring radioactivity in the pellet (gamma counter- Wallac 1470, Perkin Elmer). The binding specificity was determined in a similar assay using a 100-fold molar excess of non-labelled Plg.

2.3.2. Plasminogen binding to S. agalactiae immobilized in microtitre plate wells

S. agalactiae cells ($\approx 2 \times 10^7$ cfu/well) were seeded onto a 96-well microtitre plate (Nunc, Roskilde, Denmark) and incubated 30 min at 37 °C. Plate wells were blocked (1 h at 37°C) with 3% (w/v) BSA in 50 mM carbonate/bicarbonate buffer pH 9.6 (Sigma). After blocking, the immobilized cells were washed with PBS and incubated 1 h at 37 °C with various concentrations of Plg (25, 50, 100, 300, 500, 1000 and 2000 nM) in PBS-BSA 1% or with whole, or PBS-diluted diluted (4% and 50%), human plasma (hP). After washing with PBS, a further 1 h incubation was performed in the presence of mouse anti-human Plg (Calbiochem, Madison, WI, USA) (1:1000) in PBS-BSA 1%. The plates were washed again and incubated 1 h at 37 °C in the presence of peroxidase-labelled goat anti-mouse antibody (Southern Biotechnology Associates, Birmingham, USA). The plates were then revealed with orthophenylenediamine dihydrochloride (Sigma) and H_2O_2 and, after 30 min at room temperature, the reaction was stopped with 10% SDS and the absorbance was measured at 450 nm with a Biotek Chromoscan.

Inhibition of Plg binding was studied in a similar assay where *S. agalactiae* cells were incubated with Plg (500 nM) in the presence of various ε-aminocaproic acid concentrations (ε-ACA: 0.01, 0.1, 1, 10, 100 and 1000 mM) (Sigma).

2.4. Plasminogen activation by S. agalactiae cells

The ability of *S. agalactiae* to activate Plg into plasmin was monitored in a quantitative assay using the chromogenic substrate D-Val-Leu-Lys-*p*-nitroanilide (S-2251) (Chromogenix,

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Milano, Italy). Approximately 5×10^8 *S. agalactiae* cells were washed with PBS, mixed with 100 μl of human Plg (hPlg: 2, 5 and 10 μM) in the presence or absence of human tissue-type plasminogen activator (tPA: 10 nM) (Calbiochem), or with 100 μl of human plasmin (Pl: 1 μM) (Sigma), and incubated 1 h at 37 °C. Similar experiments were performed in the presence of human Fg (1 μM), hPlg (1 μM) and tPA (10 nM) or mouse Plg (mPlg; 1 μM) (Loxo, Dossenheim, Germany) plus tPA (10 nM). After incubation, the cells were centrifuged, washed, and resuspended in 400 μl of chromogenic substrate D-Val-Leu-Lys-*p*-nitroanilide (S-2251: 400 μM). After 12 h incubation at 37 °C, the cells were pelleted and the absorbance of 100 μl of the supernatant fluid was measure at 405 nm in a microplate reader.

To inhibit Plg activation, similar assays were performed where S. *agalactiae* cells were incubated 1 h at 37 °C with Plg (1 μM) plus tPA (10 nM) in the presence of various ε-ACA concentrations (0.01, 0.1, 1, 10, 100 and 1000 mM). The effect of α_2-antiplasmin (α_2-AP 100 nM) (Sigma), the main physiological plasmin inhibitor, was also assessed as follows. Bacteria previously incubated with Plg (1 μM) and tPA (10 nM) were incubated with S-2251 (400 μM) in the presence or absence of α_2-AP (100 nM). Assays where *S. agalactiae* cells were incubated with PBS and soluble plasmin were included as negative and positive controls, respectively.

2.5. Determination of proteolytic activity associated with S. agalactiae *grown in human plasma*

Approximately 5×10^8 *S. agalactiae* cells were pelleted, washed, resuspended in 500 μl of whole, or PBS-diluted (4% and 50%) human plasma, or in 500 μl of plasminogen-depleted human plasma (Calbiochem), and incubated 1 h at 37 °C. In the corresponding inhibition assays, $\approx 5 \times 10^8$ *S. agalactiae* cells were incubated 1 h at 37 °C with 100% human plasma in the presence of various ε-ACA concentrations (0.1, 1, 10, 100 and 1000 mM) (Sigma) or with α_2-AP (100 nM). Bacteria-associated proteolytic activity was determined using the chromogenic substrate S-2251 as described above.

2.6. Degradation of human fibronectin *by* S. agalactiae *cells*

Approximately 1×10^8 *S. agalactiae* cells were washed, mixed with 100 μl of PBS, PBS-containing Plg (1 μM) plus tPA (10 nM), or PBS-containing Fg (1 μM) plus Plg (1 μM) plus tPA (10 nM), and incubated 1 h at 37 °C with gentle shaking. After incubation, the cells were washed with PBS and resuspended in 100 μl Tris buffer [Tris–HCl 50 mM; NaCl, 100 mM, and $CaCl_2$, 5 mM; pH 7.0] containing or not human fibronectin (Fn: 30 μg/ml) (Sigma) and further incubated 40 h at 37 °C with gentle shaking. All samples were centrifuged and 20 μl of supernatant were electrophoresed through a 10% polyacrylamide SDS-PAGE. After electrophoresis, proteins were either silver-stained or transferred to a nitrocellulose membrane and probed with 1:1000 of rabbit anti-human fibronectin antibody (Sigma).

2.7. Plasminogen-binding activity of S. agalactiae *sonicates and rGAPDH*

Enzymatically active His-tagged recombinant GBS GAPDH (rGADPH) was produced from *Escherichia coli* BL21 (λDE3) harbouring pET28aΩ*gap*C as described previously [19]. *S. agalactiae* sonicates, rGAPDH, and Plg (positive control) were subjected to SDS-PAGE (10% polyacrylamide) and transferred to nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). The membrane was blocked with 3% bovine serum albumin (BSA) in Tris Buffered Saline (TBS). After washing in TBST (TBS containing 0.05% Tween 20), the membrane was incubated overnight at 4 °C with hPlg (4 μg/ml) in TBST-BSA 1%. After washing, a further 3 h incubation with mouse anti-human plasminogen (1:1000) (Calbiochem) or anti-rGADPH (1:100) antibodies was performed. The bound antibody was revealed by incubating the blot for 1 h with alkaline-phosphatase-conjugated monoclonal goat anti-mouse antibodies (Southern Biotechnology Associates). Staining was carried out with nitroblue tetrazolium dye (Promega, Madison, WI, USA) and 5-bromo-4-chloro-3-indolyl phosphate (Promega) in AP buffer.

2.8. Mouse anti-rGAPDH IgG antibodies

Antibodies against rGAPDH were obtained from sera of C57BL/6 mice immunized i.p. twice with a 3-week intervening period with 20 μg of rGAPDH in a 1:1 PBS/alum suspension (Aluminium hydroxide Gel; a kind gift of Dr Erik Lindblad, Biosector, Frederickssund, Denmark). Sera of rGAPDH-immunized mice were collected 30 days after the second immunization and pooled for GAPDH detection by western blot analysis.

2.9. Challenge infections and kinetics of the infection

Male C57BL/6 mice were purchase from Gulbenkian Institute of Science (Portugal). Animals were kept at the animal facilities of the ICBAS during the time of the experiments. *S. agalactiae* cells were grown in BHI broth to an OD_{600nm} of 0.120 ($\approx 10^7$ CFU/ml). Aliquots (1 ml) of bacterial suspension were pelleted, washed twice with PBS buffer and resuspended in 100 μl of PBS, PBS-containing hPlg (1 μM) plus tPA (10 nM), or PBS-containing human Fg (1 μM) plus hPlg (1 μM) plus tPA (10 nM), and incubated 1 h at 37 °C. The cells were pelleted, washed and resuspended in 1 ml of PBS. C57BL/6 mice were injected i.p. with 5×10^6 CFU of PBS-treated *S. agalactiae* cells, Plg-treated *S. agalactiae* cells, or Fg- plus Plg-treated *S. agalactiae* cells. One and five days after infection, the livers were aseptically removed, homogenized in PBS, and serially diluted. Bacteria were plated onto BHI plate containing streptococcus selective supplement (Oxoid, Hampshire, England) and *S. agalactiae* CFU were enumerated after 48 h of incubation at 37 °C. The animal experiments were performed according to EEC animal experimentation guidelines Directive of 24 November 1986 (89/609/EEC) and Portuguese rules (DL 129/92).

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2.10. Statistical analysis

The level of significance of the results in all groups of rats was determined by one-way ANOVA, calculated with Microsoft Excel 2000 software. Significance was established at P value of <0.05.

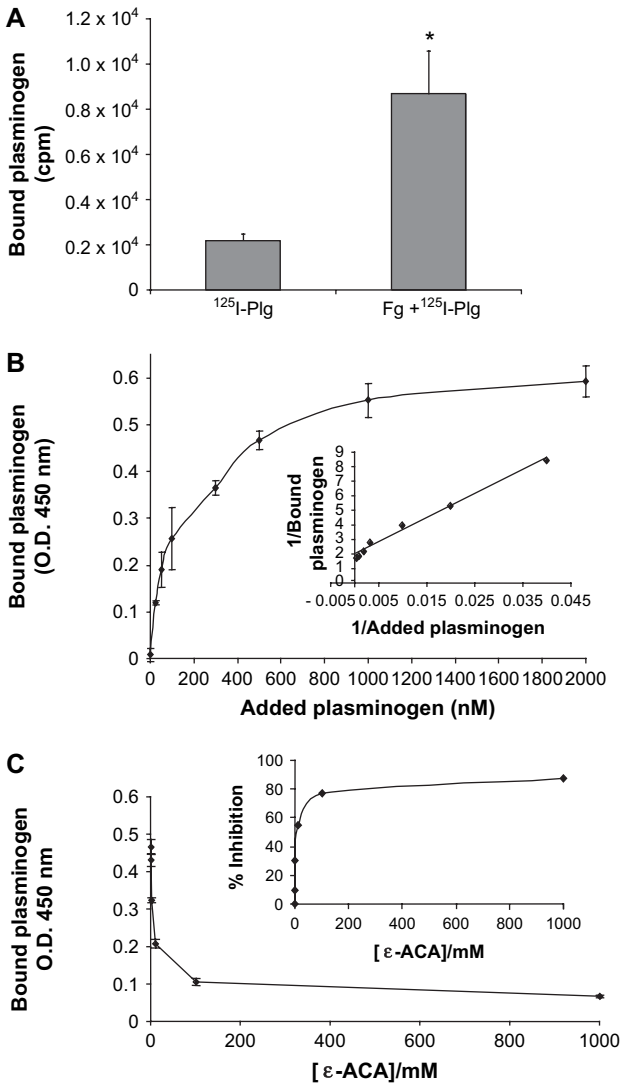

Fig. 1. Binding of plasminogen to *S. agalactiae* cells. Panel A. Binding of ^{125}I-plasminogen (^{125}I-Plg) to *S. agalactiae* cells pre-incubated or not with fibrinogen (Fg + ^{125}I-Plg). *S. agalactiae* cells were incubated with ^{125}I-Plg for 2 h, washed, and subjected to γ-radioactive counting. Data are the means of three replicates ± SD and are representative of three independent experiments. Asterisks denote results with mean differing significantly from control (bacteria incubated with ^{125}I-Plg) experiments: $*P < 0.05$. Panel B. Binding of plasminogen to *S. agalactiae* cells using a microtitre plate method. *S. agalactiae* cells were exposed to plasminogen, incubated with anti-Plg antibody, and revealed with peroxidase-labelled anti-Ig (H + L) antibody. The insert shows a double reciprocal plot of 1/bound against 1/added plasminogen and K_M was −1/x. Data are the mean of three replicates ± SD and are representative of three independent experiments. In this example, the K_M was 87.8 nM with r^2 value 0.99. Panel C. Inhibition of plasminogen binding to *S. agalactiae* cells by the lysine analogue ε-aminocaproic acid (ε-ACA). Binding was measured using various ε-ACA concentrations (0.1; 1; 10; 100 and 1000 mM) using a microtitre plate method, as described above. Data are the mean of three replicates ± SD and are representative of three independent experiments.

3. Results

3.1. Binding of human plasminogen by S. agalactiae cells

The ability of *S. agalactiae* cells to bind human Plg was tested following incubation of bacterial cells with labelled ^{125}I-Plg plasminogen. As shown in Fig. 1A, *S. agalactiae* binds Plg and this interaction is enhanced by pre-incubation of the cells with Fg (≈4 fold of increase) ($P = 0.0042$). Binding of Plg to bacterial cells was characterized in a saturation assay using the microtitre plate method. As shown in Fig. 1B, the binding affinity constant (or K_M) calculated on the basis of double-reciprocal plots was 80.67 ± 10 nM. Moreover, Plg binding to *S. agalactiae* cells can be inhibited in a dose-dependent manner by ε-ACA which reduces the binding by 50% at a concentration of 10 mM (IC_{50}) (Fig. 1C). These results indicate that GBS cells bind Plg specifically.

3.2. Activation of plasminogen by S. agalactiae cells

The ability of *S. agalactiae* cells to bind and activate hPlg was measured using an assay based on the ability of plasmin to cleave the chromogenic substrate S-2251 in a reaction that can be followed spectrophotometrically at 405 nm. In this experiment, the basal level of proteolytic activity was determined using *S. agalactiae* cells treated with PBS only. As shown in Fig. 2, increased proteolytic activities could be detected when bacteria were incubated with hPlg in the presence of human tPA. As expected, a dose-dependent activation of Plg was observed (Fig. 2). Furthermore, the incubation of bacterial cells with Plg plus tPA plus Fg increased twice the proteolytic activity (Fig. 2). These results indicate that *S. agalactiae* Plg binding can be mediated by the direct and indirect pathways. Proteolytic plasmin-like activity was also detected when plasmin was used instead of Plg, indicating that *S. agalactiae* can bind directly the active enzyme (Fig. 2). In contrast, incubation with Plg without a Plg activator did not significantly modify the basal level of proteolytic activity (PBS-treated cells), indicating that *S. agalactiae* did not secrete a Plg activator (Fig. 2). Similarly, no increase in proteolytic activity was obtained when the cells were incubated with a Plg activator alone (data not shown). Furthermore, *S. agalactiae* cells also bind and activate murine Plg (Fig. 2). All these experiments demonstrate that *S. agalactiae* cells have the ability to bind Plg which can be subsequently activated to plasmin.

3.3. Acquisition of proteolytic activity by S. agalactiae after incubation with human plasma

Several microbes acquire proteolytic activity following incubation with human plasma [6,9]. We therefore investigated if *S. agalactiae* acquires proteolytic activity after incubation with human plasma. An increase in proteolytic activity was observed when *S. agalactiae* was incubated with human

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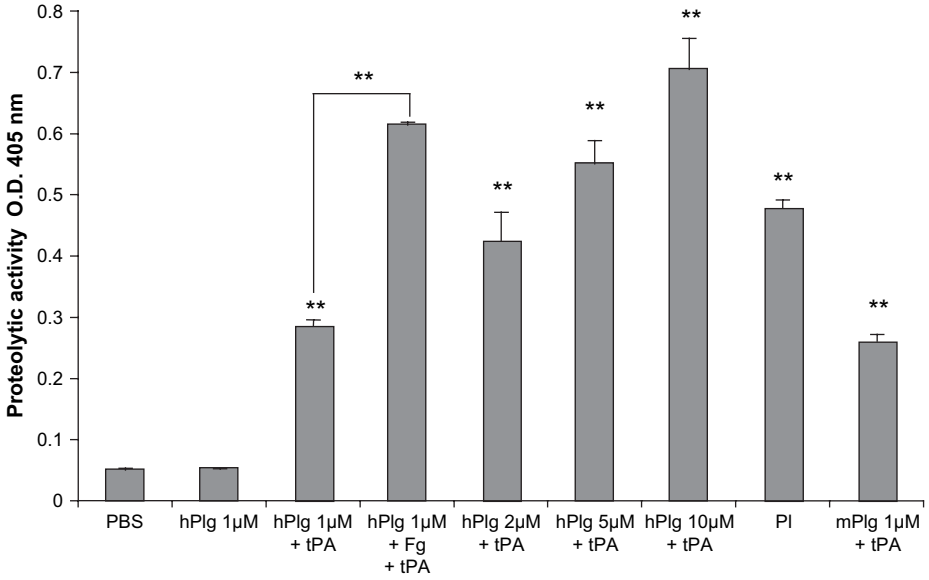

Fig. 2. Surface-associated plasmin activity after incubation of *S. agalactiae* cells with human plasminogen, human plasminogen plus fibrinogen, murine plasminogen or plasmin. *S. agalactiae* cells were incubated with PBS, various concentrations of human plasminogen (hPlg), human fibrinogen (Fg, 1 μM) plus plasminogen (1 μM), murine plasminogen (mPlg, 1 μM) or plasmin (Pl, 1 μM). Human tissue-type plasminogen activator (tPA) was added (10 nM) to all but one tubes containing Plg (negative control). Plasmin activity was measured by incubation of cells with the chromogenic substrate S-2251 and determination of the absorbance at 405 nm. Data represent the mean of triplicate samples ± SD and are representative of three independent experiments. Asterisks denote results with mean differing significantly from control (PBS) experiments: $^{**}P < 0.001$.

plasma as compared with PBS-treated bacteria and this effect was dependent upon the concentration of plasma utilized (Fig. 3A). These results are in agreement with those of the binding assay which showed that incubation of GBS cells with increased concentrations of human plasma have increased Plg binding (Fig. 3B). To demonstrate that the proteolytic activity acquired by the *S. agalactiae* cells after incubation with human plasma is due to binding and activation of Plg, we have similarly assayed GBS cells incubated with plasminogen-depleted human plasma. As shown in Fig. 3A, no increase in proteolytic activity was observed after incubation of GBS cells with plasminogen-depleted human plasma.

3.4. Degradation of human fibronectin by S. agalactiae *cells with a surface proteolytic plasmin-like activity*

The ability of *S. agalactiae* cells previously incubated with Plg (direct pathway) or with Fg plus Plg (indirect pathway) in the presence of tPA to degrade the extracellular matrix protein fibronectin was demonstrated *in vitro* (Fig. 4). Following electrophoresis, fibronectin and fibronectin degradation products were specifically detected with anti-fibronectin antibodies (Fig. 4B, lanes 2, 4, 6, 7 and 8) and by silver staining (Fig. 4A, lanes 2, 4, 6, 7 and 8). In this experiment, human fibronectin was degraded by bacteria incubated with Plg plus tPA (Fig. 4, lane 4), or with Fg plus Plg plus tPA (Fig. 4, lane 6), but

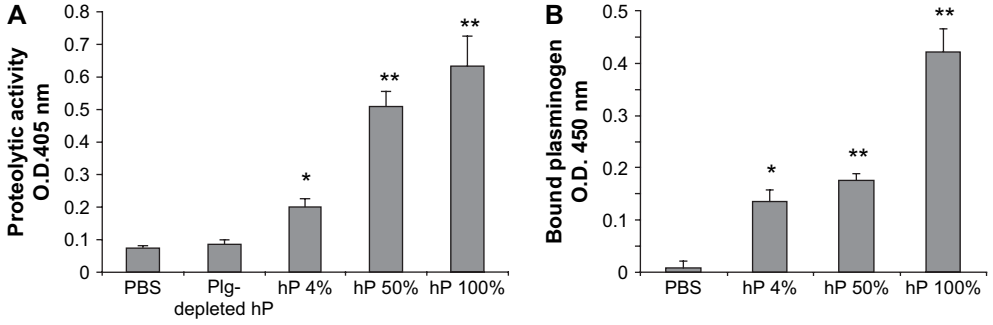

Fig. 3. Surface-associated proteolytic activity (Panel A) and plasminogen binding (Panel B) of *S. agalactiae* cells after incubation with human plasma. Bacteria were incubated with PBS or with different concentrations of human plasma (hP) at 37 °C for 1 h. In Panel A, bacteria were incubated with human plasma or with plasminogen-depleted human plasma (Plg-depleted hp), pelleted by centrifugation, washed, and the enzymatic activity associated with the cell pellet was determined by hydrolysis of S-2251. In Panel B, *S. agalactiae* cells were exposed to human plasma, incubated with anti-Plg antibody, and revealed with peroxidase-labelled anti-Ig (H + L) antibody. Data are the mean of triplicate samples ± SD and are representative of three experiments. Asterisks denote results with mean differing significantly from control (PBS) experiments: $^{*}P < 0.05$; $^{**}P < 0.001$.

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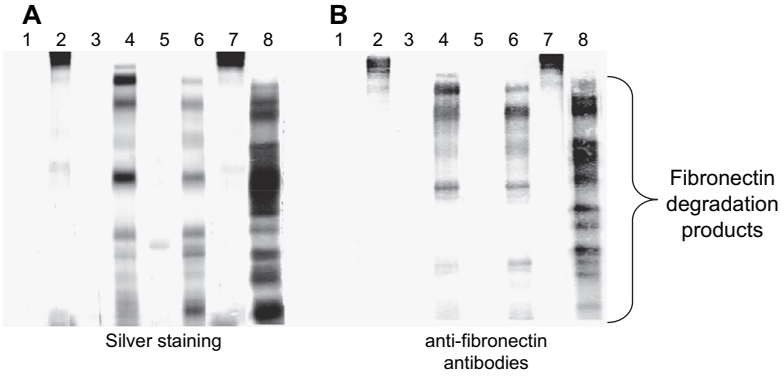

Fig. 4. Degradation of human fibronectin by *S. agalactiae* cells. Bacteria were incubated with: lane 1; PBS; lane 2, PBS plus fibronectin (1 μM); lane 3, plasminogen (1 μM) plus tPA (10 nM); lane 4, fibronectin (1 μM) plus plasminogen (1 μM) plus tPA (10 nM); lane 5, fibrinogen (1 μM) plus plasminogen (1 μM) plus tPA (10 nM); lane 6, fibronectin (1 μM) plus fibrinogen (1 μM) plus plasminogen (1 μM) plus tPA (10 nM); lane 7, fibronectin (1 μM) only; and lane 8, fibronectin (1 μM) plus plasmin (1 μM). After washing with PBS, the samples were incubated (lanes 2, 4, 6) or not (lanes 1, 3, and 5) with fibronectin (30 μg/mL) in buffer (Tris–HCl 50 mM, NaCl 100 mM and $CaCl_2$ 5 mM, pH 7.0). Plasmin plus fibronectin (lane 8) was included as a positive control. Following digestion, the samples were subjected to electrophoresis through a 10% polyacrylamide gel containing SDS and the proteins were transferred to a nitrocellulose membrane and probed with rabbit anti-human fibronectin antibody (Panel B) or were silver-stained (Panel A).

not with cells treated only with PBS (negative control). Our results demonstrate that *S. agalactiae* can interact with plasminogen to increase its surface-associated proteolytic activity although we have shown that this bacterium cannot activate this precursor. It is conceivable that, when bound to the bacterial surface, plasminogen is subject to a conformational change that enables its activation by activators present in plasma such as uPA and tPA.

3.5. Inhibition by ε-aminocaproic acid and α_2-antiplasmin of proteolytic activity acquired by S. agalactiae

Plasminogen kringle domains often mediate interactions with lysine residues of cellular receptors [5]. To evaluate their possible involvement in the interaction of plasminogen with *S. agalactiae*, a binding assay was carried out in the presence of ε-ACA, a lysine analogue, known to inhibit the binding of plasminogen by competing with lysine-binding-sites. We also investigated the effect of α_2-AP, the main physiological inhibitor of plasmin. As shown in Fig. 5, ε-ACA, but not α_2-AP, decreased the proteolytic activity of GBS cells incubated with Plg which indicates that lysine residues are involved in the Plg binding. This inhibition was dose-dependent (data not shown) and reach a maximum of nearly 50% in the presence of 10 mM ε-ACA. To assess the role of these inhibitors in the blood, *S. agalactiae* cells were incubated with hP in the presence of various concentrations of ε-ACA and α_2-AP. Again, a dose-dependent inhibition of proteolytic activity was observed in the presence of increasing concentrations of ε-ACA (data not shown) reaching a maximum of nearly 50% inhibition in the presence of 500 mM of inhibitors (Fig. 5A), but no inhibition was observed with α_2-AP (Fig. 5B). In control experiment, we showed that α_2-AP efficiently inhibited the proteolytic activity of Pl when present in fluid phase (Fig. 5B). The lack of inhibition with α_2-AP observed (Fig. 5B) when *S. agalactiae* cells were pre-incubated with Plg, or with human plasma, might indicate that this broad spectrum proteinase inhibitor did not inactivate plasmin when this enzyme is already bound to bacterial surface, as reported for other pathogens [6,9,15,16].

3.6. Analysis of plasminogen-binding S. agalactiae *proteins*

To demonstrate the binding/interaction of hPlg with *S. agalactiae* proteins, bacterial sonicate and purified rGAPDH were separated by SDS-PAGE and transferred onto nitrocellulose membranes which were incubated in the presence of Plg. Specific interactions of Plg with GBS proteins were detected by immunoblot using mouse anti-human Plg antibody. In this analysis, Plg was used as a negative control.

Several bands were detected in the bacterial sonicate indicating that various *S. agalactiae* proteins specifically interact with Plg (Fig. 6). Interestingly, our analysis demonstrated that rGAPDH bound Plg and, consistently, using anti-GAPDH antibodies, GADPH (45 kDa) was identified on the basis of its immunoreactivity and electrophoretic mobility as one of the GBS proteins present in the bacterial sonicate that interacts with Plg. In control experiments, we demonstrated that Plg antibodies did not react with proteins of GBS sonicate. Thus, as previously shown for Group A streptococci, surface-associated GBS GAPDH can recruit Plg. The fact that the GBS Plg receptors have retained their binding capacity following electrophoresis under denaturing conditions suggests that they are structurally stable.

3.7. Enhancement of S. agalactiae *virulence in C57BL/6 mice after bacterial incubation with human plasminogen or human fibrinogen plus plasminogen*

The ability of bacteria to interact with the human Plg system and to recruit an unregulatable form of plasmin at its surface has been reported to be important for the development of

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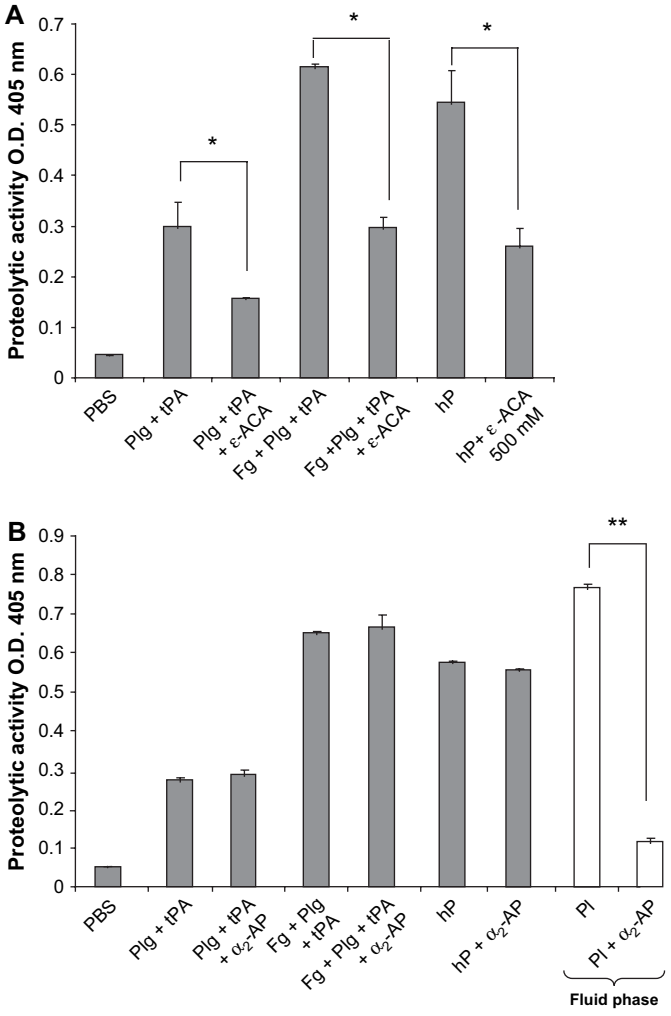

Fig. 5. Inhibition of plasminogen activation by ε-ACA and α_2-antiplasmin. *S. agalactiae* cells were incubated with PBS, PBS-containing plasminogen (Plg, 1 μM) plus tPA (10 nM), PBS-containing plasminogen plus fibrinogen (Plg, 1 μM; Fg, 1 μM) plus tPA (10 nM), or with human plasma (hP). Inhibition was carried out with ε-ACA (Panel A) or α_2-antiplasmin (α_2-AP, 100 nM) (Panel B). In this later panel, fluid phase plasmin (Pl, 1 μM) was included as a positive control. Bacteria were pelleted by centrifugation, washed, and the enzymatic activity associated with the cell pellet was determined by hydrolysis of S-2251. Data represent the mean of triplicate samples ± SD and are representative of three experiments. Asterisks denote results with mean differing significantly from *S. agalactiae* cells incubated with Plg or human plasma: $*P < 0.05$, $**P < 0.001$.

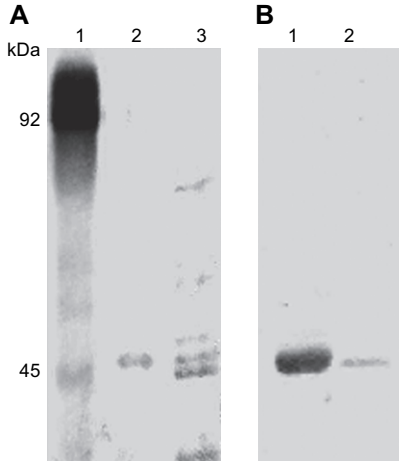

Fig. 6. Binding of *S. agalactiae* sonicate proteins and recombinant GAPDH to human plasminogen. *S. agalactiae* sonicate proteins, recombinant GAPDH (rGAPDH), and plasminogen were electrophoresed through a 10% polyacrylamide gel containing SDS and electroblotted to nitrocellulose membrane.(A) Blot was incubated with human plasminogen and probed with mouse anti-human plasminogen serum (1:1000). Lane 1, plasminogen; lane 2, rGAPDH; lane 3, *S. agalactiae* sonicate. (B) Blot was incubated with mouse anti-GAPDH serum (1:100). Lane 1, rGAPDH, lane 2, *S. agalactiae* sonicate. Both blots were revealed using AP-conjugated monoclonal goat anti-mouse antibodies.

a more invasive phenotype [7,10–14]. In order to evaluate whether *S. agalactiae* cells displaying surface plasmin-like activity were more invasive, groups of C57BL/6 mice (a mouse strain resistant to *S. agalactiae* infection; authors' unpublished data) were inoculated i.p. with 5×10^6 CFU of *S. agalactiae* cells treated with PBS, PBS-containing Plg plus tPA, or PBS-containing Fg plus Plg plus tPA. The numbers of *S. agalactiae* CFU were assessed in the liver of each group of infected mice one and five days after the bacterial challenge. One day after infection (Fig. 7A), the livers of the mice infected with *S. agalactiae* cells pretreated with Plg, or with Fg plus Plg, were more colonized than those of the control group (Fig. 7). Five days after infection (Fig. 7B), all mice from the control group have cleared the bacteria whereas those infected with *S. agalactiae* cells treated with Plg, or with Fg plus Plg, remained heavily colonized. Furthermore, a higher liver colonization was observed in mice infected with *S. agalactiae* cells incubated with Plg plus Fg than in mice infected with bacteria incubated with Fg only (Fig. 7). These results demonstrated that GBS invasiveness is greatly enhanced if bacteria were incubated with Plg prior to infection.

4. Discussion

The ability of invasive pathogens to interact with the host fibrinolytic system through Plg and to capture an unregulable form of plasmin on the bacterial surface is thought to be a common virulence strategy that facilitates invasiveness through tissue barriers [4,6–8,10–14]. Plg binding and its subsequent activation to plasmin turn bacteria into proteolytic organisms. For a wide range of pathogens, the use of in vitro and/or in vivo animal models has demonstrated that Plg binding is critical for the process of invasiveness and establishment of the infection [7,10–14]. In this study, we demonstrated for the first time that *S. agalactiae* can recruit human Plg and that the bound protein can be converted into an active protease by natural plasma activators. Upon activation, surface-bound Plg was shown, in vitro, to be able to degrade fibronectin, one of the main proteins of extracellular matrix. We also demonstrated that *S. agalactiae* cells pre-incubated with Plg or with Fg and Plg in the presence of tPA, were more virulent in an i.p. mouse infection model.

In vitro monitoring of the proteolytic activity at *S. agalactiae* surface revealed an increased activity in bacteria pre-incubated with Plg or Fg plus Plg in the presence of tPA,

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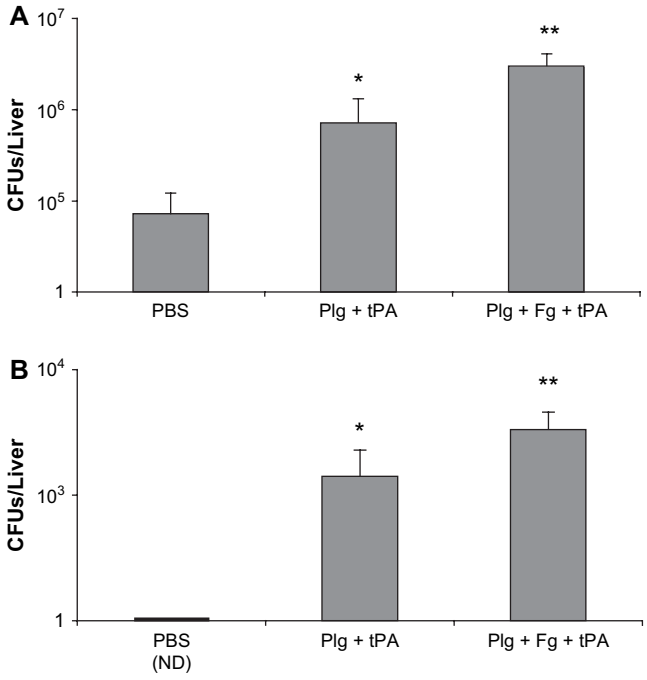

Fig. 7. Enhancement of *S. agalactiae* virulence after incubation with plasminogen plus tPA or with fibrinogen plus plasminogen plus tPA. Colony-forming units (CFUs) in the liver of C57BL/6 mice one (Panel A) and five days (Panel B) after i.p. infection with 5×10^6 *S. agalactiae* cells treated with PBS (Control), plasminogen plus tPA (Plg + tPA) or fibrinogen plus plasminogen plus tPA (Fg + Plg + tPA). Errors bars represent the standard deviation of two independent experiments done with 6 animals for each strain studied. ND, non-detected. $*P < 0.05$, $**P < 0.001$.

human plasma, or plasmin. These results indicate that *S. agalactiae* can recruit Plg in human blood and that the bound enzyme can be then activated to plasmin by blood natural activators. The acquired surface proteolytic plasmin-like activity was higher when *S. agalactiae* cells were incubated with Fg plus Plg plus tPA than with Plg plus tPA, showing the important contribution of the indirect pathway in the acquisition of *S. agalactiae* surface proteolytic activity. This increase in proteolytic activity of GBS cells observed after Fg incubation is in accordance with the results showing that pre-incubation of bacteria with Fg enhances the Plg binding as also described by others [22]. Two pathways for modulation of Plg receptors have been characterized: (i) a protease-dependent pathway based on the ability of the cleaved substrates to expose Plg-binding sites on the cell surface and (ii) a protease-independent pathway that may be initiated by growth factors, chemokines or cytokines that alter the cell membrane and/or cytoskeleton architectures to expose Plg binding sites [23]. It is thus conceivable that Fg binding to GBS can induce conformational changes leading to the exposure of Plg-binding sites that, in turn, enhances Plg binding and increases the Plg binding capacity of the GBS cells.

Like in group A streptococci [6,9,16], we show that GBS recruits Plg using both the direct and the indirect binding pathways. However, unlike GAS, it does not secrete any detectable activator of host Plg. Certain bacteria interact with the host Plg activator system to activate Plg and to acquire a plasmin-like proteolytic activity [6,10]. Moreover, it is worth mentioning that either fibrin or Fg are positive effectors of Plg activation [24]. Our results demonstrate that GBS binds Plg through bacterial cell-surface receptors but the Plg activation pathway(s) used by this bacterium remains to be elucidated.

We have recently showed that GAPDH is a virulence-associated protein [19]. In the present study, we identified this enzyme as one of the GBS plasminogen binding protein. These results are consistent with a previous report [17] demonstrating that the GBS GAPDH is an enzyme that can be present at the bacterial surface to interact in vitro with plasminogen and fibrinogen. GAPDH has also been described as a plasminogen receptor in *Streptococcus suis* [25] and as a surface-displayed plasminogen-binding protein in *Streptococcus pneumoniae* [26]. Recently, Boel et al. constructed a GAS mutant unable to secrete GAPDH, the enzyme being retained in the cytoplasm and/or in the membrane [27]. This mutant strain displayed 5.5-fold less GAPDH activity, bound significantly less plasminogen, adhered poorly to human pharyngeal cells, and lost its innate antiphagocytic activity [27]. Further studies will ascertain whether the *S. agalactiae* GAPDH is involved in the surface acquired plasmin-like activity in vivo. The indirect binding pathway involves the initial binding of fibrinogen at the cell surface and the subsequent recruitment of plasminogen and plasminogen activators to assemble a plasminogen activator complex [9,16]. Thus, all GBS fibrinogen-binding surface proteins might be involved in the indirect binding pathway, including the major GBS fibrinogen binding protein FbsA [18] and the GAPDH [17]. This latter enzyme can therefore mediate the acquisition of proteolytic activity by *S. agalactiae* through both pathways.

Plg kringle domains often mediate interactions with lysine residues in receptors [5]. The interaction of several pathogens with the Plg system is commonly inhibited by ε-ACA, a lysine analog. Our results showed that Plg binding and acquisition of proteolytic activity by *S. agalactiae* by the direct and indirect pathways can be almost entirely inhibited by ε-ACA indicating that Plg binding is mainly mediated through lysine binding sites [24]. We have also shown that the α_2-AP, the main physiological plasmin inhibitor, could not inhibit the proteolytic activity acquired by *S. agalactiae* through direct and indirect pathways or following incubation with human plasma. Immobilization of plasminogen onto lysine-containing surfaces is associated with conformational changes in the molecule making it more susceptible to tPA-mediated activation and more resistant to physiological inhibitors [28]. In this way, our results suggest the acquisition by *S. agalactiae* of an unregulated form of protease activity, a finding already observed with other pathogens [6,9,15,16].

Degradation of fibronectin *in vitro* was observed with *S. agalactiae* cells pre-incubated with Plg plus tPA or with Fg plus Plg plus tPA. Such a high proteolytic activity conferred to GBS may promote *in vivo* alterations of the basal membrane integrity and may enhance bacterial penetration in infected tissues. The degradation of fibronectin observed in our study may

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also have an impact on the inflammatory process as fibronectin-derived peptides are chemotactic for human monocytes [29] and contribute to matrix metalloproteinase production [30]. Consistently, we observed that incubation of *S. agalactiae* with Plg plus tPA or with Fg plus Plg plus tPA enhanced the virulence in an i.p. model of mouse infection. In this experiment, 5 days after infection, the liver colonization was only detected in the mice infected with bacterial cells treated with Plg or with Fg plus Plg whereas a complete bacterial clearance was observed in the mice infected with the PBS treated bacteria. It is noteworthy the observation of a higher colonization when *S. agalactiae* cells were incubated with Fg plus Plg plud tPA in comparison to the one obtained in the Plg plus tPA group. Taken together, these results suggest that acquisition of plasmin-like activity by the bacteria might increase their invasiveness.

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