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Bacterial Plasminogen Receptors: In Vitro Evidence for a Role in Degradation of the Mammalian Extracellular Matrix

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The potential of bacterium-bound plasmin to degrade mammalian extracellular matrix and to enhance bacterial penetration through basement membrane was assessed with the adherent strain SH401-1 of *Salmonella enterica* serovar Typhimurium. Typhimurium SH401-1 was able to bind plasminogen and to enhance the tissue-type plasminogen activator-mediated activation of the single-chain plasminogen to the two-chain plasmin. The end product, the enzymatically active, bacterium-bound plasmin activity, was also formed in a normal human plasma milieu in the presence of exogenous tissue-type plasminogen activator, indicating that plasmin was protected from the plasminogen activator inhibitors and plasmin inhibitors of plasma. Plasmin bound on Typhimurium cells degraded ^{125}I-labeled laminin as well as ^{3}H-labeled extracellular matrix prepared from the human endothelial cell line EA.hy926. The degradations were not seen with Typhimurium cells without plasminogen and were inhibited by the low-molecular-weight plasmin inhibitor aprotinin. Plasmin bound on Typhimurium cells also potentiated penetration of bacterial cells through the basement membrane preparation Matrigel reconstituted on membrane filters. The results give in vitro evidence for degradation of the mammalian extracellular matrix by bacterium-bound plasmin and for a pathogenetic role for bacterial plasminogen receptors.

Plasminogen is a single-chain glycoprotein that is abundant in human plasma and extracellular fluids. It is converted to the active, proteolytic form plasmin by eukaryotic activators such as tissue-type plasminogen activator (tPA) and urokinase as well as by prokaryotic activators such as staphylokinase and streptokinase. tPA is the principal activator in plasma and intercellular fluid, and its action is due to a proteolytic cleavage at an Arg-Val peptide bond of the plasminogen molecule. Thus, plasmin formed by tPA is composed of two polypeptides (heavy and light chains; Pla_H and Pla_I) held together by disulfide bonds (6). Furthermore, plasmin is able to modify itself by cleaving an amino-terminal, 8-kDa activation peptide, thus converting the so-called Glu-plasmin into the Lys form.

Plasminogen activation by tPA proceeds poorly in solution but is enhanced dramatically by immobilization of plasminogen on fibrin or eukaryotic tissue surfaces. Immobilization of plasminogen is mediated by five kringle domains which bind to lysine-containing domains on the target molecules. In addition to the plasminogen activator tPA, human plasma contains inhibitors of plasminogen activation as well as of proteolytic plasmin activity. Immobilization of plasminogen onto lysine-containing surfaces is associated with dramatic conformational changes in the molecule (27), which now is more susceptible to tPA-mediated activation and more resistant to the physiological inhibitors. Thus, the activation mechanism generates targeted, localized, and transient proteolytic activity.

Plasmin is a trypsin-like serine protease with a broad substrate specificity. It has a well-documented physiological function in the degradation of fibrin (fibrinolysis), but it also degrades noncollagenous glycoproteins of mammalian extracellular matrices (ECM) and basement membranes (BM) such as laminin. Local plasminogen activation has many functions in eukaryotic cells in, e.g., embryonal development and penetration of BM by metastatic cancer cells (for reviews, see references 6, 29, and 37).

Bacteria intervene in plasminogen activation at various stages. Endothelial cells secrete tPA into plasma (30); however, the concentration of tPA in human plasma is far below the concentration of its inhibitor, PAI-1, which rapidly inhibits tPA in plasma. Endotoxin infusion in humans induces a rapid increase in tPA activity in plasma, which precedes the increase in the concentration of PAI-1 (41). A similar early activation of the fibrinolytic system has been observed in patients with meningococcal septicemia (3). It is likely that the transient increase in tPA secretion results from the action of endotoxin on endothelial cells. On the other hand, several gram-positive and gram-negative invasive bacterial pathogens have recently been shown to express plasminogen receptors (for reviews, see references 17 and 25). These pathogens include *Staphylococcus aureus*, group A, C, and G streptococci, *Salmonella enterica* serovar Enteritidis, and meningitis-associated *Escherichia coli*, *Haemophilus influenzae*, and *Neisseria meningitidis* (16, 18, 32, 33, 38, 42–44). Only a few bacterial plasminogen receptors have been characterized on the molecular level. In *E. coli* and *S. enterica*, certain fimbrial and flagellar antigens serve as plasminogen receptors (21, 32, 33, 38). Very recently, the M53 protein of group A streptococci was found to have plasminogen-binding characteristics (2). In addition, a receptor binding plasmin, but not plasminogen, has been reported to exist on group A streptococci (4, 5). This molecule was identified recently as glyceraldehyde-3-phosphate dehydrogenase, a major surface antigen with multiple adhesive properties (24, 31).

The fact that the adhesive filaments fimbriae also function as plasminogen receptors suggests a functional link between bacterial adhesion and generation of localized proteolysis on host tissues. Since many of the identified bacterial plasminogen

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receptors enhance plasmin formation by tPA, it has been hypothesized that they promote bacterial invasiveness in tissues (13, 25), especially at the ECM, which is also recognized by a number of bacterial adhesins (46). To date, however, degradation of mammalian tissue by bacterium-bound plasmin has not been documented, in part because of a lack of suitable in vitro models. In this communication, we report the development of such assays. We also show that plasmin activity associated with a bacterial surface is able to degrade laminin as well as mammalian ECM and can potentiate in vitro bacterial penetration through a reconstituted BM.

MATERIALS AND METHODS

Bacteria. The adherence properties of strain *Salmonella enterica* serovar Typhimurium SH401 have been described (15). The strain was first described mistakenly as *S. enterica* serovar Enteritidis. For the degradation and penetration experiments, plasmid pBR322 was transformed into the strain, and the resulting ampicillin-resistant strain was named SH401-1. Bacteria were cultivated in static Luria broth, supplemented with ampicillin in the case of strain SH401-1.

Activation and conversion of plasminogen. Kinetic measurements of plasminogen activation were performed as described previously (21, 32). The bacterial densities were 2×10^8 and 4×10^8/ml; plasminogen (Biopool, Umeå, Sweden) was tested at 20 μg/ml, tPA (Biopool) was tested at 50 ng/ml, the chromogenic substrate S-2251 (Kabivitrum, Stockholm, Sweden) was tested at 0.45 mM, and ε-aminocaproic acid (EACA; Sigma Chemical Co., St. Louis, Mo.) was tested at 1 mM, in a test volume of 200 μl. Cell-bound plasmin activity was determined essentially as described in Parkkinen et al. (32). Bacteria (1.5×10^9 cells) were incubated with 4 or 8 μg of plasminogen in 200 μl of phosphate-buffered saline (PBS) containing 0.02% Tween 80 (PBS-Tween), with or without 2 mM EACA, at 37°C for 3 h with slow rotation. Bacteria were washed twice with PBS-Tween and then incubated for 80 min with tPA (100 ng/ml) and 0.45 mM S-2251 in a total volume of 250 μl. The cells were pelleted, and the A_{405} of the supernatant (200 μl) was measured. Formation of cell-bound plasmin activity in human plasma was tested (16, 44) by incubating 6×10^8 bacteria and 100 ng of tPA in 200 μl of citrated human plasma obtained from a healthy volunteer. The tests were carried out with or without 1 mM EACA. After incubation for 2.5 h, the bacteria were washed with PBS-Tween and incubated with 0.45 mM S-2251 for 1 h. The bacteria were pelleted, and the A_{405} of the supernatant was measured. The results of the activation assays are given as a representative assay with duplicate independent samples; the range of individual test results was within ±10% of the mean.

Conversion of radiolabeled plasminogen into plasmin was assessed as described previously (16). Briefly, Glu-plasminogen was labeled with ^{125}I by the Iodogen method (28), and 9 ng of ^{125}I-Glu-plasminogen (2×10^6 cpm/μg of protein) was incubated for 2 h at 4°C with 10^9 SH401-1 cells in 0.5 ml of PBS containing 0.1% (wt/vol) bovine serum albumin (BSA) in the presence or absence of 10 ng of tPA. To monitor the conversion of bound Glu-plasmin to Lys-plasmin, incubations were carried out in the presence or absence of 100 KIU of aprotinin (Sigma) ml^{-1}. After incubation, the bacteria were washed and suspended in 400 μl of sample buffer (19). Supernatants and controls without bacteria were diluted 1/10 with 50% sample buffer. After boiling, 40 μl of each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% acrylamide), and the peptides were visualized by autoradiography.

Degradation and penetration assays. To obtain a maximal amount of plasminogen bound on SH401-1 cell surfaces, 10^{10} bacterial cells were incubated with 100 μg of plasminogen in 1 ml of PBS, with or without 500 KIU of aprotinin, at 37°C with gentle agitation for 4 h. The cells were washed twice with PBS and suspended in 1 ml of PBS or PBS containing 500 KIU of aprotinin, and 500 ng of tPA was added to the suspensions. An aliquot (100 μl) of the suspensions was used to measure plasmin activity on the bacterial cells, and aliquots of the remaining suspensions were used immediately, after dilution in PBS if necessary, in the degradation or penetration assays described below.

For the degradation assays, laminin (Collaborative Research, Bedford, Mass.) was labeled with ^{125}I (Amersham, Buckinghamshire, United Kingdom) by the Iodogen method (28). The activity obtained was 4×10^6 cpm/μg of laminin. For coating, Lab-Tek Chamber slides (Nunc, Roskilde, Denmark) were incubated with laminin (5×10^5 cpm in 150 μl of PBS per well) overnight at room temperature. The slides were washed three times with PBS, and bacterial suspensions (1.5×10^9 cells in 200 μl of PBS or PBS-aprotinin) were added to the wells. The slides were incubated at room temperature, and 20-μl samples were taken at time intervals to measure radioactivity in an LKB 1272 Clinigamma Counter (Wallac, Turku, Finland). Selected samples from the slides were subjected to SDS-PAGE in 5 to 18% slab gels, and the peptides in the samples were visualized by autoradiography.

Radiolabeled subendothelial ECM for degradation studies were prepared with the continuous human endothelial cell line EA.hy926 (7). EA.hy926 cells were cultivated to subconfluence on 24-well tissue culture plates (Nunc) in RPMI 1640 medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% (vol/vol) fetal calf serum (Gibco), 2 mM L-glutamine (Nordcell), 100 IU of penicillin ml^{-1}, and 100 μg of streptomycin ml^{-1}. The cells were washed with PBS, and then glucose-free medium (minimal essential medium) with 2 mM L-glutamine and 0.2% (wt/vol) BSA was added. After incubation for 1 h at 37°C with 5% CO_2, the medium was replaced with a new one containing 2.5 μCi of D-[2-^{3}H]mannose (Amersham) ml^{-1}. After incubation for 24 h, ECM was prepared by detergent treatment as detailed previously (9). After the procedure, the absence of endothelial cells on the bottom of the wells was checked by microscopy. The presence of laminin in the ECM preparation was confirmed by indirect immunofluorescence with antilaminin antibodies (45), and adherence of SH401-1 to the ECM was assessed as detailed previously (15). Bacteria (10^{10} cells in 1 ml of PBS or PBS-aprotinin) were added to the wells, and 100-μl samples were taken from the suspension at time intervals to measure the radioactivity released from the surface. Radioactivity was measured in an LKB 1215 Rackbeta liquid scintillation counter (Wallac). Results of the degradation assays are given as a representative assay done with duplicate independent samples; the range of individual test results was within ±15% of the mean.

The BM preparation Matrigel (Collaborative Research) was dialyzed against ice-cold PBS (to remove gentamicin). For penetration assays, Matrigel was diluted 1/5 (in some experiments, 1/3) in ice-cold PBS, and 50 μl was pipetted on a 3-μm membrane in Transwell cell culture chamber inserts (Costar, Cambridge, Mass.). Matrigel was kept for 30 min at 4°C and then gelled for 1 h at 37°C and air dried in a laminar-flow hood for 10 h at room temperature. In the penetration assay, bacterial suspensions (10^9 cells in 100 μl of PBS or PBS-aprotinin) were added to the upper well; the lower well contained 600 μl of PBS. The culture chambers were incubated at 37°C, and 10-μl samples were taken at time intervals and plated on Luria agar plates containing ampicillin.

RESULTS

We have shown earlier (15) that strain SH401 of *S. enterica* serovar Typhimurium adheres efficiently to laminin and to mammalian ECM. This finding together with the efficient plasminogen-activation-promoting effect displayed by the strain (see below) led us to use strain SH401 in testing in vitro the degradation and penetration of mammalian ECM by an invasive bacterial pathogen. Since some of the tests were performed with nonsterilized material, we transformed the plasmid pBR322 to SH401; the resulting Ap^r strain was named SH401-1. The transformation did not cause detectable changes in the capacity of SH401 to enhance plasminogen activation (details not shown).

Immobilization and conversion of plasminogen on the surface of SH401-1 cells. We first studied the ability of strain SH401-1 to bind plasminogen and to enhance the tPA-induced formation of plasmin, the active proteolytic form. This was first assessed by incubating ^{125}I-labeled Glu-plasminogen with SH401-1 cells and by analyzing the labeled products both from the bacterial cells and the corresponding test supernatants by SDS-PAGE and autoradiography. The tests were performed in the absence or presence of tPA and of aprotinin, an inhibitor of plasmin proteolytic activity. The proportion of the bound ^{125}I-plasminogen varied considerably in different cultures; in a representative assay with 10 ng of plasminogen and 8×10^8 cells, 2.8% of the label was bound. In the presence of EACA, a lysine analog that prevents immobilization of plasminogen on its targets, the level of binding was 0.7%.

Autoradiographic analyses also showed that ^{125}I-plasminogen was bound onto SH401-1 cells (Fig. 1A, lane 1) as was ^{125}I-plasminogen in the presence of aprotinin, an inhibitor of plasmin activity (Fig. 1A, lane 2). Analysis of the bound label revealed no evidence of a conversion of the cell-bound plasminogen molecule into the two-chain plasmin form. In contrast, incubation of bacteria with tPA and ^{125}I-plasminogen resulted in the formation of plasmin composed of heavy (Pla_H) and light (Pla_L) chains on SH401-1 cells (Fig. 1A, lane 3). When tPA was added with aprotinin (which prevents plasmin-induced cleavage of the 8-kDa activation peptide from the amino terminus of the Pla_H chain), the formed Pla_H migrated slightly slower, indicating that the activation peptide had not

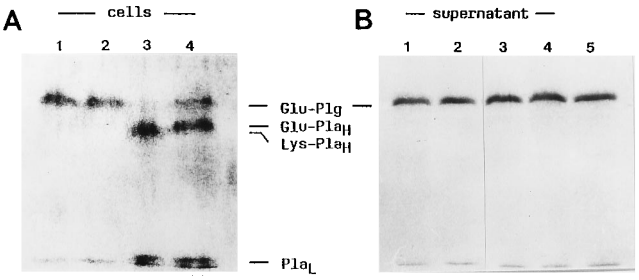

FIG. 1. Conversion of plasminogen into Pla_H and Pla_L on the surface of *S. enterica* serovar Typhimurium SH401-1 cells. SH401-1 cells were incubated with ^{125}I-plasminogen in PBS-BSA (lanes 1) or in PBS-BSA containing 100 KIU of aprotinin (lanes 2), 10 ng of tPA (lanes 3), or both aprotinin and tPA (lanes 4). The bacteria were removed, and the cells (A) and the corresponding supernatants (B) were analyzed by SDS-PAGE and autoradiography. Lane 5 shows the ^{125}I-Glu-plasminogen preparation used in the assays. Note that Pla_H and PLa_L chains are formed efficiently only in the presence of tPA and SH401-1 cells and that aprotinin prevents the cleavage of Glu-PLa_H.

been released (Fig. 1A, lane 4). We analyzed both the cells and the supernatants from each test combination (Fig. 1). It is important to notice that under these test conditions, no plasmin formation was detected in the supernatants of the test suspensions (Fig. 1B) and that no plasmin conversion took place in the absence of bacterial cells (data not shown). This supports the hypothesis that immobilization of ^{125}I-plasminogen on the SH401-1 cell surface is a prerequisite for an efficient conversion of plasminogen to plasmin.

Formation of proteolytic activity on SH401-1 cells. We next tested whether the immobilization and the tPA-mediated conversion of plasminogen on *Salmonella* strain SH401-1 cells produced functionally active enzyme capable of breaking down the synthetic chromogenic plasmin substrate S-2251. Kinetic measurements in which plasminogen and tPA were incubated with bacterial cells (Fig. 2A) revealed a cell-number-dependent enhancement of plasmin formation by tPA. The formation of plasmin activity was greatly inhibited in the presence of

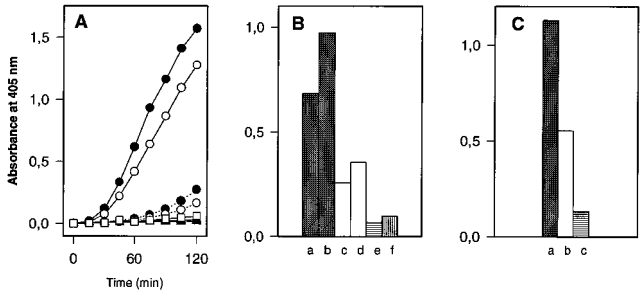

FIG. 2. Formation of plasmin activity on *S. enterica* serovar Typhimurium SH401-1 cells. (A) Kinetic measurement of plasmin formation in the presence of SH401-1 cells (2×10^8 [open symbols] or 4×10^8 [closed symbols] cells ml^{-1}). The tests were performed in the presence (dashed lines) or absence (solid lines) of 1 mM EACA. Symbols: △ and ▲, plasmin activity when tPA or plasminogen was omitted; □, plasmin activity in the absence of bacterial cells. (B) Formation of cell-bound plasmin. SH401-1 cells were incubated with tPA and plasminogen in the absence (bars a and b) or presence (bars c and d) of 2 mM EACA, washed thoroughly, and then incubated with the chromogenic plasmin substrate S-2251. Plasminogen was used in two concentrations, 20 $\mu g\ ml^{-1}$ (bars a and c) and 40 $\mu g\ ml^{-1}$ (bars b and d). Control tests were performed without tPA (bar e) or without plasminogen (bar f). (C) Formation of bacterium-bound plasmin activity in human plasma. SH401-1 cells were incubated in citrated plasma with (bars a and b) or without (bar c) exogenous tPA as well as in the absence (bars a and c) or presence (bar b) of 1 mM EACA. The bacteria were washed, and the cell-bound plasmin activity was measured with the chromogenic plasmin substrate S-2251.

EACA, which further emphasized the importance of plasminogen being bound to the bacterial surface prior to activation. It is noteworthy that very little, if any, plasmin activity was detected in the test suspensions if tPA was omitted from the test (Fig. 2A), indicating that strain SH401-1 does not express an active endogenous plasminogen activator. Again, no functionally active plasmin was detected if plasminogen was incubated with tPA but without bacterial cells (Fig. 2A).

The formation of cell-bound plasmin activity was further assessed (Fig. 2B) by incubating SH401-1 cells in the presence of plasminogen, washing them thoroughly, and finally incubating them in the presence of tPA and the substrate S-2251. A dose-dependent formation of SH401-1 cell-bound plasmin activity was observed; this activity was decreased to 40% in the presence of 2 mM EACA (Fig. 2B).

Formation of surface-associated plasmin activity in human plasma. Human plasma contains an excess of inhibitors for both plasminogen activation and plasmin activity. Key issues of surface-associated plasmin formation are the enhancement of tPA-mediated activation and the protection of the formed plasmin activity against the physiological inhibitors (16, 44). To investigate the possible in vivo significance of SH401-1 plasminogen receptors, we tested the formation of bacterium-bound plasmin activity in normal human plasma (Fig. 2C) containing both plasminogen and the various inhibitors.

Cell-bound plasmin activity was formed on *Salmonella* strain SH401-1 when the bacteria were incubated in normal human plasma with exogenous tPA (Fig. 2C). Formation of plasmin activity was decreased to 50% in the presence of 1 mM EACA, and only marginal plasmin activity was detected when no exogenous tPA was added (Fig. 2C).

Degradation of ECM material by the plasmin bound on Typhimurium SH401-1 cells. Laminin, a major glycoprotein of BM, is a target for the proteolytic activity of plasmin (8, 29) as well as for adhesion of *S. enterica* serovar Typhimurium SH401 (15). Our hypothesis was that the plasmin-SH401-1 complex, through bacterial adhesion, directs the proteolysis at the ECM and subsequently promotes bacterial penetration through the damaged ECM. We first tested whether the SH401-1 cells having plasmin on their surface are able to degrade laminin as well as ECM secreted by a human endothelial cell line. Control tests included plain bacterial cells (with no cell-bound plasmin) as well as the bacterium-associated plasmin in the presence of aprotinin. The effect of aprotinin on bacterium-bound plasmin activity is shown in Fig. 3A; the amount of 500 KIU completely inhibited the plasmin activity formed on SH401-1 cells.

In the degradation experiments, glass slides were coated with ^{125}I-laminin, bacteria were incubated on the coated glass surfaces, and the release of radioactivity from the glass surface was monitored at time intervals (Fig. 3B). When SH401-1 cells alone were added to the immobilized ^{125}I-laminin, no significant release of radioactivity was observed. However, when an equal number of SH401-1 cells containing surface-bound plasmin were incubated, a substantial amount of radioactivity was released into the incubation buffer. This release was inhibited completely by the presence of aprotinin (Fig. 3B), indicating that the release was due to the cell-bound plasmin activity. Autoradiographic analyses revealed that the laminin released from the glass slides by the plasmin-SH401-1 complex was a lower-molecular-weight species than the laminin molecules originally used to coat the glass surface (Fig. 4). Control assays with plasmin revealed an essentially similar breakdown of laminin (details not shown).

We next tested whether plasmin bound to the SH401-1 cells also degrades a more complex ECM preparation. The tests

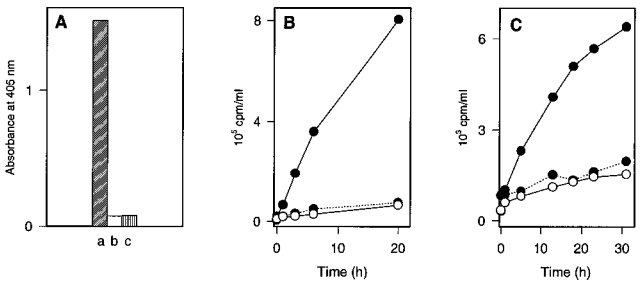

FIG. 3. Degradation of ECM material by the plasmin-SH401-1 complex. (A) Comparison of the plasmin activity of the three SH401-1 cell suspensions used in the analysis. Bars: a, plasmin generated by tPA on SH401-1 cells; b, cell-bound plasmin activity in the presence of aprotinin (500 kIU); c, plasmin activity on SH401-1 cells without added tPA or plasminogen. Note that aprotinin completely prevents plasmin activity on SH401-1 cells. (B) Release of radioactivity from glass slides coated with ^{125}I-laminin. Bacterial suspensions were added to coated glass surfaces, and the release of ^{125}I from buffer was measured as a function of time. Symbols: ●——●, plasmin-SH401-1 complex; ●----●, plasmin-SH401-1 complex in the presence of aprotinin; ○——○, SH401-1 cells. Note that radioactivity was released efficiently only by the plasmin-SH401-1 complex. (C) Degradation of ECM of human endothelial cells by the plasmin-SH401-1 complex. Endothelial cells were cultured with [^{3}H]mannose, and ECM was prepared on the culture wells by detergent treatment. Bacterial suspensions were added, and the release of ^{3}H from the ECM was monitored as a function of time. Symbols: ●——●, plasmin-SH401-1 complex; ●----●, plasmin-SH401-1 complex in the presence of aprotinin; ○——○, SH401-1 cells alone. Note that ^{3}H was released efficiently in the presence of the plasmin-SH401-1 complex and that it was inhibited by aprotinin.

were carried out as described above but with a ^{3}H-labeled ECM from the human endothelial cell line EA.hy926 (Fig. 3C). The ECM from this cell line reacted with antilaminin antibodies, as tested by indirect immunofluorescence, and SH401-1 cells adhered efficiently to it (data not shown). SH401-1 with bound plasmin efficiently released radioactivity from the ECM preparation, and the release was inhibited by aprotinin to the level seen with the SH401-1 cells alone (Fig. 3C).

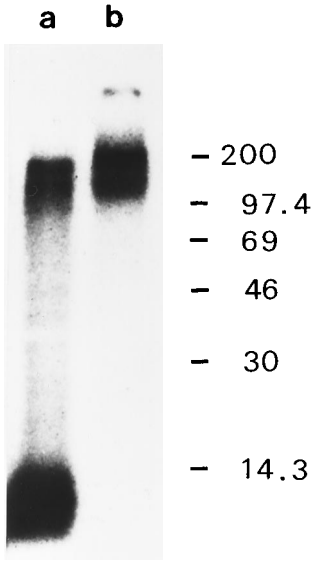

FIG. 4. Comparison of the ^{125}I-laminin molecules released by the plasmin-SH401-1 complex (lane a) and the ^{125}I-laminin molecules used to coat the glass (lane b). The laminin preparations were subjected to SDS-PAGE and analyzed by autoradiography. The migration distance of molecular mass markers (size in kilodaltons) are shown on the right.

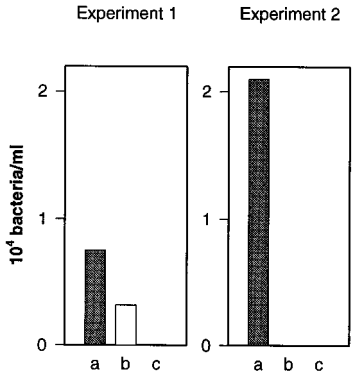

FIG. 5. Penetration of bacteria through reconstituted BM. The BM preparation Matrigel was reconstituted on 3-μm membranes in cell culture chamber inserts, and bacterial penetration through Matrigel was assessed as viable counts from the buffer in the lower well. The results from two separate experiments and for samples taken after a 21-h incubation are shown. SH401 cells with bound plasminogen were added with tPA to the upper well in the absence (bar a) or presence (bar b) of aprotinin. Bar c shows lack of penetration by SH401 cells with no bound plasminogen.

Penetration of bacteria through reconstituted BM. We tested whether the plasmin formed on SH401-1 cells potentiates bacterial penetration through Matrigel, a reconstituted BM preparation used in studies of metastasis of cancer cells (1, 10, 34, 35) and a target for adherence by strain SH401-1 (15). Matrigel was reconstituted on 3-μm membranes in Transwell cell culture chamber inserts, bacteria were added to the upper well, and their appearance in the lower well buffer was monitored. Controls included penetration experiments in the presence of aprotinin or with the SH401-1 cells without bound plasminogen.

The SH401-1 cells alone did not penetrate through Matrigel into the lower chamber (Fig. 5). In contrast, when SH401-1 cells with bound plasmin were used, more than 10^4 bacteria per ml were detected in the lower chamber after a 21-h incubation. This penetration into the lower chamber was inhibited by the plasmin inhibitor aprotinin (Fig. 5). In these assays, we did not detect any significant bacterial penetration by 15 to 20 h.

DISCUSSION

The recent demonstrations of plasminogen receptors and formation of plasmin on the surface of invasive bacterial pathogens have given grounds to the assumption that plasminogen receptors enhance bacterial virulence. The focus of this study was to assess whether plasmin generated on a bacterial plasminogen receptor is able to degrade mammalian ECM and BM and to promote bacterial migration through these mechanical tissue barriers. Our hypothesis (13) was that bacterial adherence and plasminogen receptors contribute to the creation of a localized, bacterium-bound proteolytic activity at mammalian ECM. This localized proteolysis may damage mechanical tissue barriers and thus promote bacterial invasiveness in tissues. Such invasiveness might be important in bacterial penetration through subepithelial and subendothelial BM into circulation and subsequently into secondary infection foci. Testing this hypothesis required the development of suitable in vitro test systems. Our ongoing work (20) shows that the in vitro assays described here are suitable for functional analysis and comparison of other prokaryotic plasminogen receptors as well, such as those on *H. influenzae* and *S. aureus*.

The strain SH401-1 of *S. enterica* serovar Typhimurium expressed a plasminogen receptor(s). This was evidenced by the

binding of Glu-plasminogen to the bacteria, by the tPA-induced conversion of the bacterium-bound single-chain ^{125}I-Glu-plasminogen to the two-chain plasmin, and by the ability of the cell-bound plasmin to modify itself by releasing the 8-kDa activation peptide. By use of a chromogenic plasmin substrate, we also demonstrated the enhancement of plasmin formation in the presence of SH401-1 cells as well as the formation of cell-bound plasmin activity. Surface-associated plasminogen activation was greatly inhibited by the lysine analog EACA. EACA also inhibited the binding of plasminogen to SH401-1 cells, and our results strongly suggest that the immobilization on SH401-1 cells involved the lysine-binding kringle domains of plasminogen. Formation of plasmin on SH401-1 cells also took place in the presence of plasma when exogenous tPA was added. This strongly favors the idea that the plasminogen receptors of SH401-1 also are functional under physiological conditions, i.e., in the presence of inhibitors of plasminogen activation and of plasmin activity. We have identified a gene cluster encoding a type 1 fimbrial antigen (14) of SH401 that functions as a plasminogen receptor but have not yet determined how common this fimbrial variant is among salmonellae. Strain SH401-1 does not express thin aggregative fimbriae that have been indicated as plasminogen receptors (38); it thus seems that, as detected in *E. coli* (33), many fimbrial types of salmonellae function as plasminogen receptors.

We could not detect any significant conversion or activation of plasminogen unless tPA was included in the assays, which indicates that Typhimurium SH401-1 lacks a functional plasminogen activator of its own. *E. coli* possesses an outer membrane protease, OmpT (36), which has been suggested to be a plasminogen activator since outer membrane vesicles from an OmpT-positive *E. coli* strain activate plasminogen (22). Lundrigan and Webb (26) detected that among 282 clinical *E. coli* isolates, *ompT* had a frequency of 77% but the OmpT protease activity was expressed by only 12% of isolates. In particular, Lundrigan and Webb (26) detected plasminogen activator activity in only 1 of the 282 *E. coli* isolates, which is in accordance with our previous finding that meningitis-associated *E. coli* isolates do not express endogenous plasminogen activator activity (32). OmpT has sequence similarity with the plasminogen activator (Pla) surface protein of *Yersinia pestis* and PrtA of *Salmonella* spp. (39). OmpT, Pla, and PrtA degrade certain outer membrane proteins, and Pla was shown to increase the invasiveness of *Y. pestis* (40), either through its own proteolytic activity or through plasmin formation. Plasmin activated by Pla is sensitive to the plasmin inhibitors of plasma (40), which indicates that it was not in a bound form. Thus, two systems for plasminogen activation seem to exist in enteric bacteria, one generating soluble plasmin with a surface protease involved in processing of outer membrane proteins and the other involving plasminogen receptors and requiring a heterologous plasminogen activator. It is interesting to note that Pla has also been reported to exhibit adhesive properties (11), which may increase its effectiveness in creation of localized proteolysis and ECM degradation.

Metastatic cancer cells use plasminogen activation to degrade the laminin network and to activate collagenases; both processes are believed to potentiate penetration of tumor cells through ECM. The methods that we used to assess in vitro the pathogenetic potential of a bacterial plasminogen receptor, i.e., the release of radioactivity from a labeled, immobilized ECM component and from metabolically labeled ECM formed by cultured endothelial cells as well as bacterial invasion through the reconstituted ECM Matrigel, were developed from the current methodology for tumor invasion studies (for a critical review of these methods, see reference 29). We demonstrated degradation of ^{125}I-labeled laminin by plasmin on Typhimurium SH401-1 cells. The release of radioactivity from immobilized laminin was not seen with SH401-1 cells lacking bound plasminogen and was abolished in the presence of aprotinin, which strongly suggested that we were measuring laminin degradation. Indeed, the laminin molecules released by the plasmin-SH401-1 complex were a smaller-molecular-weight species than those coated onto the surface. In similar experiments, we demonstrated release by the plasmin-SH401-1 complex of ^{3}H from the metabolically labelled ECM of the human endothelial cell line EA.hy196. The time scale in the degradation assays was significantly longer than that in the activation assays. This was to be expected since the activation assays involved the soluble low-molecular-weight substrate S-2251, whereas the targets in the degradation assays were high-molecular-weight proteins immobilized on a surface to which the bacterium-plasmin complexes first had to sediment and adhere.

We used the BM preparation Matrigel to assess the penetration potential of Typhimurium SH401-1 through a BM. Matrigel preparation contains laminin, type IV collagen, heparan sulfate proteoglycan, and entactin and forms, under physiological conditions, a gel-like structure resembling the ultrastructure of the lamina densa zone of BM (12). Matrigel is a commonly used tool in the analysis of tumor cell penetration into BM (1, 10, 34, 35), and it also has biological activity in stimulating the growth and differentiation of eukaryotic cells. Plasmin bound on Typhimurium SH401-1 potentiated bacterial penetration through a Matrigel layer reconstituted on a membrane filter. The penetration was inhibited by aprotinin and not exhibited by the intact SH401-1 cells, strongly suggesting that it was due to degradation of Matrigel by the bacterium-bound plasmin. It should be noted that in these experiments, the only plasminogen that was added to the test systems was bound on the Typhimurium SH401-1 cells. Plasmin degrades laminin efficiently but the collagen network of BM only poorly, if at all (8, 29). On the other hand, plasmin activates procollagenases, which leads to further degradation of the ECM barrier function and further promotes metastasis of tumor cells. Our results suggest that, because of their small size, bacterial cells are able to penetrate through the type IV collagen network in Matrigel even in the absence of induced collagenase activity.

The penetration of SH401-1 cells with bound plasmin through Matrigel became dramatically evident after a ca. 15- to 20-h incubation; the time required for the collapse of the penetration barrier function of Matrigel varied from one experiment to another. The latter is most likely due to physical variability in the BM reconstituted at different dates. We constructed the assay so that the plain leakage of bacteria through Matrigel was minimized. It is difficult to compare the thickness and penetration capacity of the reconstituted Matrigel with that of the BM present in tissues. Furthermore, our ongoing experiments (20) show that the numbers of living cells in the lower chamber follow complex kinetics and are controlled by three factors, penetration of bacteria through Matrigel, growth of bacteria on glycopeptides released from Matrigel, and killing of stationary-phase cells by activated plasmin. Hence, the penetration assay as described here is more qualitative than quantitative in nature.

As a next step, the role of plasminogen receptors in bacterial metastasis should be tested in vivo in animal models. Typhimurium cells and meningitis-associated *E. coli* both express multiple plasminogen receptors in the form of specific flagellar and fimbrial antigens (14, 20, 21, 33, 38), which have other

functions (motility and adhesion) affecting the pathogenetic processes. Constructing specific mutants impaired only in plasminogen binding and not in other functions requires a detailed knowledge of the plasminogen-binding regions in these surface proteins. These factors will complicate the in vivo evaluation of enterobacterial plasminogen receptors. It can be speculated that plasminogen receptors enhance ECM degradation by potentiating the action of proteases, such as PrtA, on the surface of *Salmonella* cells. Considering the very high in vivo concentration of plasminogen (ca. 200 μg/ml of serum) and hence potentially of plasmin as well, such an indirect effect is unlikely to have a major role in ECM degradation.

In summary, we present herein in vitro evidence for a pathogenetic function of plasminogen receptors in promoting tissue degradation and penetration by an invasive bacterial pathogen. Adherence to laminin and localized plasminogen activation are believed to be crucial in the metastasis of tumor cells (23, 29); the same characteristics are exhibited by a number of bacterial pathogens. Our results give support for a novel mimicry in cellular metastasis, i.e., prokaryotic and eukaryotic cells may utilize similar principles to invade mammalian tissue barriers.

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