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Enhancement of Tissue Plasminogen Activator-Catalyzed Plasminogen Activation by *Escherichia coli* S Fimbriae Associated with Neonatal Septicaemia and Meningitis

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Summary

The effect of *Escherichia coli* strains isolated from blood and cerebrospinal fluid of septic infants on plasminogen activation was studied. These strains typically carry a filamentous surface protein, S fimbria, that has formerly been shown to bind to endothelial cells and interact with plasminogen. The bacteria effectively promoted plasminogen activation by tissue plasminogen activator (t-PA) which was inhibited by ε-aminocaproic acid. A recombinant strain expressing S fimbriae accelerated t-PA-catalyzed plasminogen activation to a similar extent as did the wild-type strains whereas the nonfimbriate recipient strain had no effect. After incubation with t-PA and plasminogen, the S-fimbriate strain displayed bacterium-bound plasmin activity whereas the nonfimbriate strain did not. Bacterium-associated plasmin generation was also observed with a strain expressing mutagenized S fimbriae that lack the cell-binding subunit SfaS but not with a strain lacking the major subunit SfaA. Both t-PA and plasminogen bound to purified S fimbriae in a lysine-dependent manner and purified S fimbriae accelerated t-PA-catalyzed plasminogen activation. The results indicate that *E. coli* S fimbriae form a complex with t-PA and plasminogen which enhances the rate of plasminogen activation and generates bacterium-bound plasmin. This may promote bacterial invasion and persistence in tissues and contribute to the systemic activation of fibrinolysis in septicaemia.

Introduction

Septicaemia caused by Gram-negative bacteria is commonly associated with activation of the haemostatic and fibrinolytic proteolytic cascades (1). Activation of fibrinolysis is generally thought to be secondary to disseminated intravascular coagulation (2). On the other hand, it was recently shown that endotoxin infusion in humans induces a rapid increase in tissue plasminogen activator (t-PA) activity in plasma which precedes the increase in the concentration of its inhibitor, PAI-1 (3). A corresponding early activation of the fibrinolytic system has also been observed in patients with fulminant meningococcal septicaemia as reflected by low plasma levels of plasminogen and α_2-plasmin inhibitor (4). The critical stage in the activation of fibrinolysis, the t-PA-catalyzed conversion of plasminogen to plasmin, proceeds only slowly in the fluid phase but is remarkably accelerated by complex formation of plasminogen and t-PA with fibrin (5). This is mediated by so-called lysine-binding sites occurring in both plasminogen (6) and t-PA (7, 8). Several Gram-negative bacteria have been recently described to bind plasminogen in a lysine-dependent manner, but it is not known whether this promotes plasminogen activation (9, 10).

To explore the possibility that bacteria causing septic infections might promote plasminogen activation, we have studied the effect of *E. coli* strains isolated from blood and cerebrospinal fluid of septic infants on t-PA-catalyzed plasminogen activation. The plasminogen-binding components identified in *E. coli* are filamentous surface proteins, fimbriae, that are generally thought to mediate bacterial adhesion to host cells and tissues (9). The strains causing septic neonatal infections typically carry S fimbriae (11) that are expressed by the bacteria in circulation during an experimental systemic infection (12) and bind to sialyloligosaccharide structures on vascular endothelial cells (13) and epithelial cells of the choroid plexus and brain ventricles (14). We describe here that these strains effectively enhance t-PA-catalyzed plasminogen activation. This is apparently dependent on complex formation of t-PA and plasminogen with S fimbriae and results to generation of bacterium-bound plasmin activity.

Materials and Methods

Materials

Human Glu-plasminogen, single-chain t-PA, and goat polyclonal t-PA-specific immunoglobulin G (IgG) were from Biopool (Umeå, Sweden). S-2251 was from KabiVitrum (Stockholm, Sweden). CNBr-fragmented fibrinogen was from Technoclone (Vienna, Austria). Rabbit polyclonal plasminogen-specific IgG was from Dakopatts (Clostrup, Denmark). Europium-chelate and Enhancement solution were from Wallac Biochemical Laboratories (Turku, Finland). ε-Aminocaproic acid (EACA) was from Sigma Chemical Co. (St. Louis, MO). Labeling of IgG with europium-chelate was carried out as described before (9).

Bacterial Strains

E. coli strains RK 262, RK 267, RK 304, and RK 327 of the serotype O18ac:K1:H7, RK 308 of the serotype R:K1:H47, and RK 318 of the serotype O6:K^+:H47 isolated from blood or cerebrospinal fluid of septic neonatal infants have been characterized in detail before (11). The recombinant strains harbouring plasmids encoding genes for wild-type S fimbriae [strain HB101 (pANN801-13)], mutagenized S fimbriae lacking the lectin subunit (SfaS$^-$) [strain HB101(pANN801-1321)] or mutagenized S fimbriae lacking the major structural subunit (SfaA$^-$) [strain HB101(pANN801-1)] have also been described before (15). The expression of S fimbriae in the strains was confirmed by immunofluorescence staining (16) and hemagglutination assay (17). The bacteria were cultivated on CFA agar supplemented with appropriate antibiotics in the case of recombinant strains. For plasminogen activation assays, bacteria were suspended in PBS (10 mM phosphate buffer, pH 7.4, 150 mM NaCl) and washed once. S fimbriae were purified by using deoxycholate and concentrated urea as described before (17).

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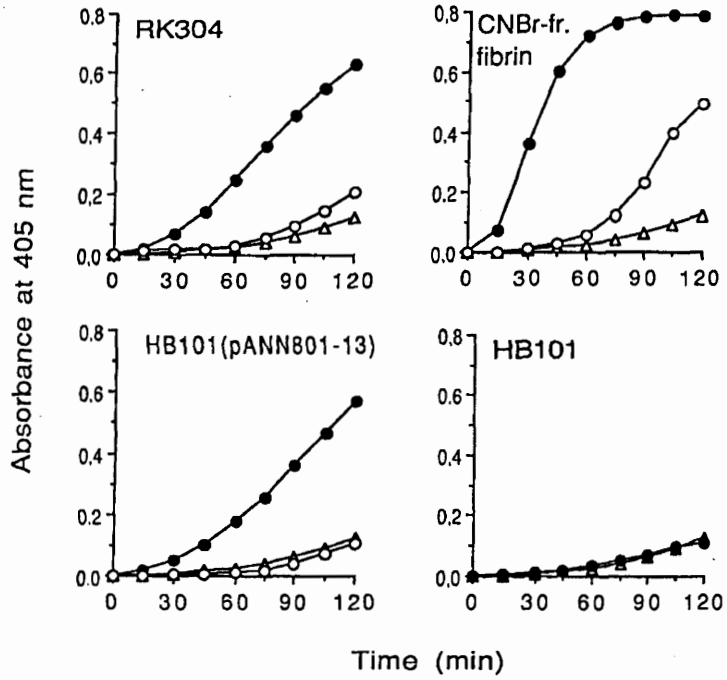

Fig. 1 Effect of *E. coli* strains on t-PA-catalyzed plasminogen activation. t-PA (35 ng/ml), plasminogen (45 μg/ml), and the plasmin substrate S-2251 were incubated with bacteria (10^8/ml) or CNBr-fragmented fibrin (30 μg/ml) in the absence (●) and presence (○) of 2 mM EACA and without any stimulator (△). RK304, an O18ac:K1:H7 wild-type strain; HB101 (pANN801-13), an S-fimbriate recombinant strain; HB101, the nonfimbriate recipient strain

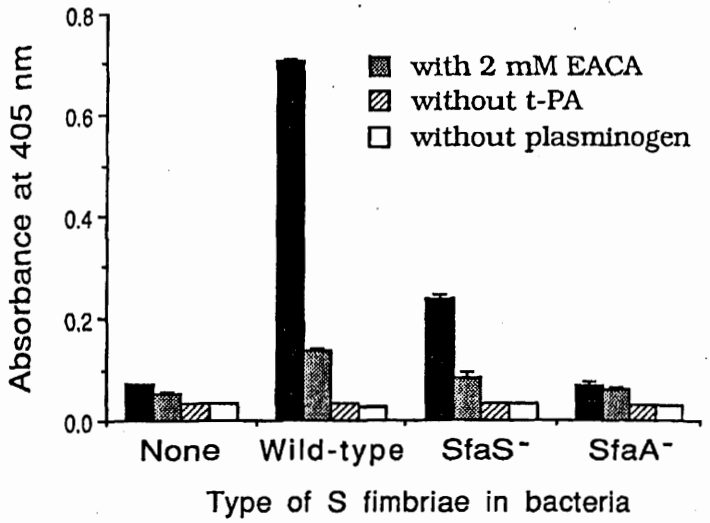

Fig. 2 Generation of bacterium-bound plasmin activity. Bacteria were incubated with t-PA and plasminogen, washed, and incubated with S-2251. Control incubations were carried out with 2 mM EACA, without t-PA, and without plasminogen. The nonfimbriate strain HB101, the strain HB101 (pANN801-13) with wild-type S fimbriae, the strain HB101 (pANN801-1321) with fimbriae lacking the lectin subunit ($SfaS^-$), and the strain HB101 (pANN801-1) lacking the major fimbrial subunit ($SfaA^-$) were studied

Plasminogen Activation Assays

Conversion of plasminogen to plasmin was followed by the plasmin-catalyzed hydrolysis of S-2251, a tripeptide (Val-Leu-Lys) connected at its carboxyl terminus to *p*-nitroaniline. Assays were performed at 37° C in microtitration wells in 200 μl of PBS containing 0.02% Tween 80, 35 ng/ml t-PA, 45 μg/ml plasminogen, and 0.3 mM S-2251 in the presence and absence of bacteria, CNBr-fragmented fibrinogen, or S fimbriae as indicated. Under the initial conditions, ΔA_{405nm} per time squared is directly proportional to the rate of plasmin formation and a straight line is obtained (18). Acceleration rate was calculated as the ratio of the slope ΔA vs. t^2 in the presence of the compound studied over that in its absence. Formation of bacterium-associated plasmin activity was studied by incubating 10^9 bacteria, 20 ng t-PA, and 8 μg plasminogen in 400 μl of PBS containing 0.02% Tween 80 for 2.5 h at 37° C. Separate control incubations were performed in the presence of 2 mM EACA, without t-PA and without plasminogen. The bacteria were sedimentated by centrifugation, washed once with PBS, and suspended in 250 μl of PBS containing 0.2 mM S-2251. After incubation for 60 min at 37° C, bacteria were pelleted and absorbance of the supernatant (200 μl) was measured at 405 nm.

Binding Assays

Purified S fimbriae, 10 μg/ml in PBS, were coated to polystyrene microtitration wells by incubating overnight at 4° C, the wells were saturated with bovine serum albumin (BSA), 1 mg/ml for 2 h at 23° C, washed, and incubated with plasminogen or t-PA in 100 μl of PBS containing 0.05% Tween 20 for 16 h at 4° C. The wells were washed three times and incubated with europium-labeled plasminogen- or t-PA-specific IgG, correspondingly, in 100 μl of 50 mM Tris HCl buffer, pH 7.8, containing 150 mM NaCl, 5 mg/ml BSA, 1 mg/ml bovine gammaglobulin, and 0.05% Tween 20 for 1 h at 23° C with continuous shaking. The wells were washed, 100 μl of Enhancement solution was added, and time-resolved fluorescence was measured after 20 min in an Arcus Fluorometer (Wallac Biochemical Laboratories).

Results

The activation of plasminogen by t-PA was slow in the absence of any promoter whereas CNBr-fragmented fibrinogen at a concentration of 30 μg/ml enhanced it about 50-fold (Fig. 1). At a bacterial density of 10^8/ml, *E. coli* strains isolated from septic neonatal infections caused on an average a 10-fold acceleration of plasminogen activation (Fig. 1). A similar acceleration was observed with four of the six strains studied, whereas two of the strains displayed lower acceleration rates. One of the latter strains expressed also less S fimbriae as indicated by a weak hemagglutination activity, whereas the other strains produced a strong hemagglutination. To find out whether the acceleration of plasminogen activation was mediated by S fimbriae, recombinant bacteria expressing S fimbriae were studied. The recombinant strain HB101(pANN801-13) that expresses wild-type S fimbriae caused a similar acceleration rate as did the wild-type strains, whereas the nonfimbriate recipient strain HB101 had no effect on plasminogen activation (Fig. 1). Similarly to the accelerating effect of fibrin fragments, that of the S-fimbriate bacteria was to a major part inhibited in the presence of 2 mM EACA (Fig. 1). This suggested that the interaction was mediated by lysine-binding sites occurring in t-PA and plasminogen (6–8). If either plasminogen or t-PA was omitted from the activation reaction, no hydrolysis of the plasmin substrate was observed. This indicated that the plasmin activity observed was not due to any endogenous plasminogen activator or plasmin-like activity in *E. coli*.

To study whether the generated plasmin was attached to the bacteria, these were incubated together with plasminogen and t-PA, washed, and then incubated with the plasmin substrate S-2251. The recombinant strains HB101(pANN801-13) with wild-type S fimbriae and HB101(pANN801–1321) with mutagenized S fimbriae lacking the lectin subunit ($SfaS^-$) displayed bacterium-bound plasmin activity (Fig. 2). In contrast, the recombinant strain HB101(pANN801-1) lacking the major fimbrial subunit ($SfaA^-$) and the nonfimbriate strain HB101 exhibited only marginal activity (Fig. 2). The formation of bacterium-bound plasmin activity was to a major part inhibited in the presence of EACA. In

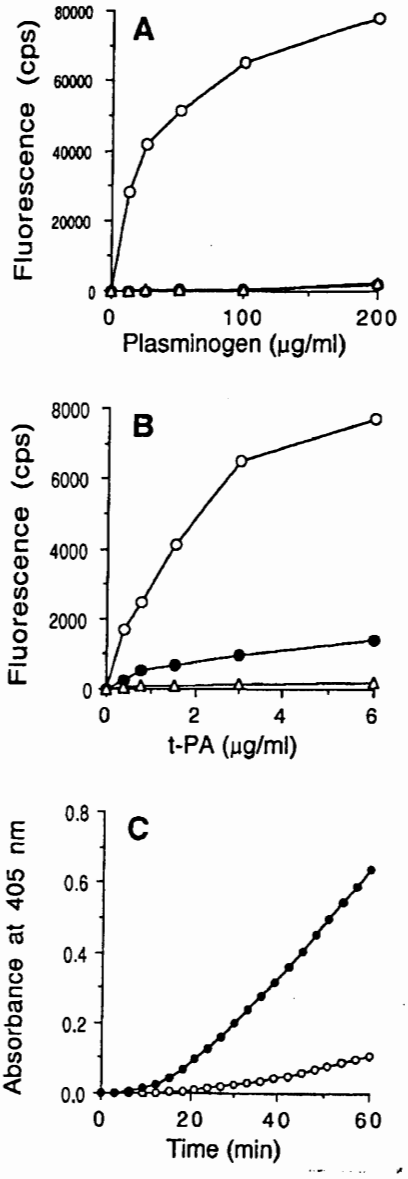

Fig. 3 Binding of t-PA and plasminogen to immobilized S fimbriae and the effect of purified S fimbriae on plasminogen activation. (*A*) Plasminogen and (*B*) t-PA were incubated in microtitration wells coated with purified S fimbriae in the absence (○) and presence (●) of 10 mM EACA and in wells coated with albumin (△). Binding of the proteins was determined by fluoroimmunoassay. (*C*) t-PA (35 µg/ml), plasminogen (45 µg/ml), and S-2251 were incubated with S fimbriae (200 µg/ml) (●) and without them (○)

the absence of either t-PA or plasminogen, no plasmin-like activity was observed (Fig. 2). The plasmin activity on the strain HB101(pANN801-1321) was lower than that on the strain HB101(pANN801-13), but the former strain also expresses significantly less S fimbriae than does the latter (15).

Since the experiments with the recombinant bacteria strongly suggested that the enhancement of plasminogen activation by the wild-type strains was mediated by S fimbriae, these were studied in a purified form for binding of plasminogen and t-PA and for acceleration of plasminogen activation. Both plasminogen and t-PA bound in a concentration-dependent manner to immobilized S fimbriae as detected by europium-labeled plasminogen- and t-PA-specific antibodies, correspondingly (Fig. 3). The binding of plasminogen was almost completely inhibited with 10 mM EACA, and an inverted plot of the binding curve suggested a dissociation constant of about 40 µg/ml (0.4 µM). The majority of the binding of t-PA to S fimbriae was also inhibited with 10 mM EACA, and the apparent dissociation constant of the binding was about 3 µg/ml (40 nM). Purified S fimbriae enhanced t-PA-catalyzed plasminogen activation in a dose-dependent manner, the acceleration rate being about 10-fold at a fimbrial concentration of 200 µg/ml (Fig. 3).

Discussion

The results of the present study indicate the presence of surface components in septigenic *E. coli* that bind both plasminogen and t-PA in a lysine-dependent manner and enhance plasminogen activation. The strains studied had been isolated from neonatal infants with septicaemia and meninigitis and included representatives of serotype O18:K1, which is the major serotype of *E. coli* causing septic neonatal infections (11). The enhancing effect of these bacteria on t-PA-catalyzed plasminogen activation was at least partially mediated by S fimbriae, a filamentous surface protein typically occurring in the O18:K1 strains (11), as indicated by the following findings. Firstly, the recombinant strain expressing S fimbriae accelerated plasminogen activation to a similar extent as did the S-fimbriate wild-type strains, whereas the nonfimbriate recipient strain had no effect. Secondly, purified S fimbriae had an accelerating effect on plasminogen activation. Plasminogen activation evidently takes place on the bacterial surface as purified S fimbriae bound both plasminogen and t-PA in a lysine-dependent manner and, after incubation with t-PA and plasminogen, S-fimbriate bacteria displayed bacterium-bound plasmin activity.

S fimbriae are about 7 nm thick and 0.5–1 µm long protein filaments that consist of a major structural subunit with a molecular weight of 17 kDa (SfaA) and of three minor proteins (SfaS, SfaG, SfaH) (15, 17). The minor subunit SfaS, a 14.9 kDa protein (19), possesses α-sialyl-β-2,3-galactoside-specific binding activity and mediates bacterial binding to host cells (13–15). The findings of the present study that the recombinant strains expressing either wild-type S fimbriae or fimbriae lacking the SfaS protein accelerated plasminogen activation, whereas the strain that lacked the SfaA protein was ineffective, suggest that the SfaA protein is involved in the complex formation with the fibrinolytic components. The possible role of the minor subunits SfaG and SfaH in plasminogen activation remains to be determined. However, our results indicate that S fimbriae are multifunctional proteins and suggest that their pathophysiological function may not be restricted to bacterial adhesion to host cells.

Vascular endothelium is a target tissue for endotoxin effects, either directly or through secondary mediators (20). Endothelial cells also play a crucial part in regulation of coagulation and fibrinolysis by synthetising promoters and inhibitors of both systems and providing binding sites for them (21). In this respect it is of interest that S fimbriae also bind to human endothelial cells by their lectin activity (13). Bacterial adhesion to the endothelium may have pathophysiological importance by resisting clearance of bacteria by the blood flow to organs with reticuloendothelial tissue and by propagating their extravasation. On the other hand, it may also provide S-fimbriate bacteria with high local concentrations of t-PA that is produced as an early effect of endotoxaemia (3).

Bacterium-bound plasmin activity may play a pathogenic role in bacterial invasion and persistence in tissues. Besides fibrin, plasmin effectively degrades various extracellular matrix proteins, such as fibronectin, laminin, and proteoglycan core proteins, and also promotes collagenolysis by activating procollagenase (22). In fact, cell surface-associated plasmin formation is regarded as an important mechanism in pericellular proteolysis of invasive animal cells, particularly in tumor cell invasion (22–24). Occurrence of bacterial surface-associated plasmin generation may therefore suggest that pathogenic bacteria utilize the host fibrinolytic system for tissue invasion. The binding sites of plasmin on the bacteria, fimbrial filaments, are very stable protein assemblies and we have not observed any degradation or loss of their cell-binding function after plasmin treatments (9, J. Parkkinen and T. K. Korhonen, unpublished results). It is also unlikely that plasmin

would deteriorate the polysaccharide shield of encapsulated bacteria.

The observation that a number of Gram-negative bacteria, many of which important human pathogens, bind plasminogen in a lysine-sensitive manner (10) suggests that other pathogenic bacteria may also enhance plasminogen activation similarily to S-fimbriate *E. coli*. This might be a contributing factor in the systemic fibrinolytic state often accompanying Gram-negative septicaemia.

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