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A study of the interaction between *Helicobacter pylori* and components of the human fibrinolytic system

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Abstract

The interaction of plasminogen, tissue plasminogen activator (t-PA) and urokinase with a clinical strain of *Helicobacter pylori* was studied. Plasminogen bound to the surface of *H. pylori* cells in a concentration-dependent manner and could be activated to the enzymatic form, plasmin, by t-PA. Affinity chromatography assays revealed a plasminogen-binding protein of 58.9 kDa in water extracts of surface proteins. Surface-associated plasmin activity, detected with the chromogenic substrate CBS 00.65, was observed only when plasminogen and an exogenous activator were added to the cell suspension. The two physiologic plasminogen activators, t-PA and urokinase, were also shown to bind to and remain active on the surface of bacterial cells. ε-Aminocaproic acid caused partial inhibition of t-PA binding, suggesting that the kringle 2 structure of this activator is involved in the interaction with surface receptors. The activation of plasminogen by t-PA, but not urokinase, strongly depended on the presence of cells and a 25-fold enhancer effect on the initial velocity of activation by t-PA compared to urokinase was established. Furthermore, a relationship between cell concentration and the initial velocity of activation was demonstrated. These findings support the concept that plasminogen activation by t-PA on the bacterial surface is a surface-dependent reaction which offers catalytic advantages.

Key words
- *Helicobacter pylori*
- Plasminogen
- t-PA
- Urokinase
- Plasminogen activation

Introduction

Plasminogen, a 92-kDa glycoprotein present in plasma and extracellular fluids, is the main component of the fibrinolytic system (1). It circulates in plasma as an inactive zymogen which, under different conditions, could be activated to the active form, plasmin, by plasminogen activators (PA) such as the tissue-type PA (t-PA) and urokinase. The activation reaction involves the hydrolysis of a single Arg-Val peptide bond (2), converting the zymogen to active two-chain plasmin, a broad spectrum serine protease, which degrades fibrin clots, as well as other proteins (3).

Plasminogen activation has been demonstrated in various biological processes such as trophoblast implantation (4), wound repair and tissue remodeling (5), tumor cell invasion and metastasis (6). Assembly of plasminogen and PAs on the surface of nu-

merous cell types and fibrin clots facilitates plasmin generation, resulting in an enhancement of the catalytic efficiency (7,8); furthermore, surface plasmin cannot be regulated efficiently by host inhibitors such as α_2-antiplasmin (9).

It has been recently established that binding and activation of human plasminogen on the surface of bacterial cells may be a common mechanism used by invasive bacteria to facilitate movement through normal tissue barriers (10,11). Several species generate their own plasminogen activators: this is the case of group A streptococci and *Staphylococcus aureus*, which secrete streptokinase and staphylokinase, respectively, two bacterial PAs widely used in the therapy of acute myocardial infarction (12). Other pathogens, not known to produce a PA, may be capable of using host PAs to generate surface plasmin activity, a mechanism which is predicted to be less efficient (11). These organisms can possibly express surface receptors for eukaryotic activators which enable them to acquire plasmin-like enzymatic activity in the human host (10).

Helicobacter pylori is a gram-negative human pathogen that colonizes the gastric mucosa and is associated with various stomach and duodenal disorders such as active chronic gastritis, peptic ulceration and possibly gastric carcinoma (13). Like other bacterial pathogens, *H. pylori* expresses surface proteins with affinity for several human proteins, components of the mammalian extracellular matrix such as laminin, vitronectin, collagen types I and IV and plasminogen (14-18). Plasminogen binding to the surface of *H. pylori* has been shown to be inhibited by lysine, lysine analogues and miniplasminogen (fifth kringle and catalytic domain (1)), suggesting an important role of the fifth kringle structure of the zymogen which possibly interacts with two surface proteins of 42 and 57 kDa (19).

In the present study we have examined the binding of plasminogen and its activation by t-PA and urokinase on the surface of an *H. pylori* clinical isolate. The effect of bacterial cells on plasminogen activation was studied. In addition, the capability of *H. pylori* to bind other components of the plasminogen system such as t-PA and urokinase was also explored.

Material and Methods

Bacterial isolates

A clinical *H. pylori* isolate was obtained from a patient with gastric ulcer at the Hospital Universitario de Los Andes (HULA), University of Los Andes, Mérida, Venezuela. Bacteria were recovered from gastric biopsies collected by endoscopy. The isolate represents a single colony obtained from a biopsy, which was processed for Gram staining, urease test and histology as well as *H. pylori* culture, as previously described (20). In some experiments, comparisons were made among different clinical isolates obtained as previously described.

In order to prepare cell suspensions, *H. pylori* cells incubated for 6-7 days under microaerophilic conditions on blood-agar plates were collected in sterile PBS (0.14 M NaCl, 0.06 M sodium phosphate, pH 7.2), washed twice and resuspended in the same buffer.

Reagents

The synthetic substrate for plasmin, methylmalonyl-4-hydroxyprolyl-arginyl-paranitroanilide (MM-Hyp-Arg-pNA or CBS 00.65) was purchased from Diagnostica STAGO (Asnières, France). All other reagents, unless specified, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Human plasminogen was purified on lysine-Sepharose 4B (Pharmacia, Uppsala, Sweden) from blood of human donors, according to the method of Deutsch and Mertz (21). Briefly, proteins bound to the column were

eluted with 50 mM ε-aminocaproic acid (EACA) and plasminogen-containing fractions were further purified by filtration chromatography. t-PA was from Biopool (Umeå, Sweden). Urokinase was purchased from Sanofi Winthrop (Gentilly, France).

Plasminogen binding and activation assays

H. pylori (10^8 cells) were incubated with plasminogen (0-0.4 µM) in 0.1 ml PBS containing 1% BSA for 1 h at 25ºC under continuous mixing. At the end of the incubation period, the cells were pelleted. Bacteria were washed with 10 volumes of PBS containing 0.1% Tween 20 followed by washing in 50 mM Tris HCl, pH 7.4 (assay buffer), and finally resuspended in assay buffer. Bound plasminogen was activated with t-PA and the resultant plasmin activity monitored in the mixture at 405 nm with the chromogenic substrate CBS 00.65 (0.5 mM) after 30 min incubation at 37ºC.

Plasminogen activation by t-PA was studied in the presence or absence of *H. pylori* cells. For this purpose, 1.5 µM plasminogen, 2.1 nM t-PA, 0.5 mM CBS 00.65 and increasing cell concentrations from 10^7 to 10^9/ml were mixed in 0.1 ml assay buffer. The A_{405} of the mixture was monitored continuously at 37ºC. Initial velocities of activation were calculated from plots of A_{405} versus t^2, according to Wohl et al. (22).

Detection of cell-associated t-PA and urokinase activity

Urokinase and t-PA were tested for their ability to bind to the surface of *H. pylori* cells. For this, increasing concentrations of both activators (0-14 nM t-PA; 0-50 nM urokinase) were incubated with 10^8 *H. pylori* cells as described above. Washed cells were incubated with plasminogen (1.5 µM) in the presence of CBS 00.65 (0.5 mM). The A_{405} of the mixture was recorded at different time intervals, and plotted against the PA concentration.

Affinity chromatography assays

Human plasminogen was covalently coupled to NHS-activated Sepharose (HiTrap; Pharmacia) according to the manufacturer's instructions. *H. pylori* protein fractions were batch adsorbed to the plasminogen-Sepharose resine in PBS for 1 h at room temperature under continuous shaking. Following extensive washing with PBS, bound proteins were eluted with either 50 mM EACA or 50 mM glycine, 0.1 M NaCl, pH 2.7, and further analyzed by SDS-PAGE.

Electrophoresis

SDS-PAGE was performed according to Laemmli (23). *H. pylori* total cell extracts, surface proteins extracted with water, both obtained in the presence of 0.2 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine, as well as proteins eluted from plasminogen-Sepharose assays, were separated on 10% acrylamide gels under reducing conditions. The proteins were not boiled prior to electrophoresis and were silver stained.

Results

Plasminogen binding and activation on the surface of *H. pylori*

Purified human plasminogen was tested for its ability to bind and be activated on the surface of a clinical isolate of *H. pylori*. Figure 1 shows that t-PA is able to generate a surface plasmin activity to an extent which is strongly dependent on the initial concentration of the plasminogen allowed to bind to the bacteria. The acquisition of enzymatic activity was absolutely dependent on the presence of plasminogen; furthermore, the isolate tested did not exhibit surface plasmin activity in the absence of an exogenous activator (data not shown).

H. pylori proteins involved in the interaction with human plasminogen were isolated by affinity chromatography. This assay revealed the presence of one polypeptide of 58.9 kDa in water extracts of surface proteins (Figure 2). This protein was eluted with 50 mM EACA; however, the same result was obtained when glycine buffer, pH 2.7, was used in the elution step.

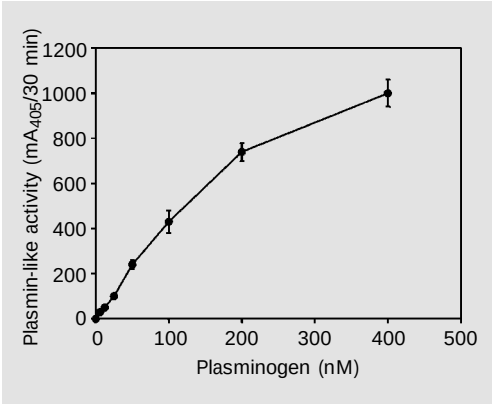

Figure 1 - Activation of plasminogen to plasmin on the surface of *H. pylori*. Cells (10^8) were incubated with different amounts of human plasminogen. After extensive washing, surface-bound plasminogen was activated with 2.1 nM t-PA. Plasmin activity was detected by digestion of the chromogenic substrate CBS 00.65 after 30-min incubation at 37ºC (circles). Data are reported as the mean change in milliunits of absorbance at 405 nm obtained from at least three determinations. Standard errors are shown.

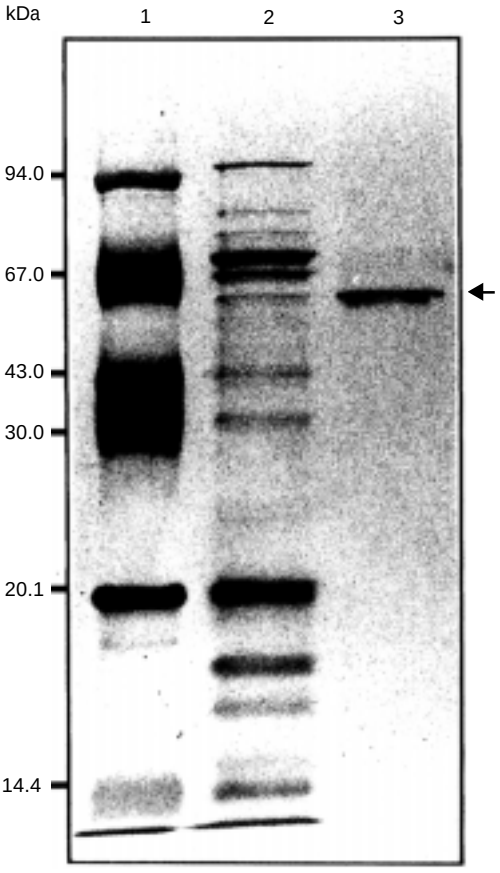

Figure 2 - Plasminogen-Sepharose affinity purification of plasminogen-binding protein. *H. pylori* water extracts of surface proteins were allowed to bind to plasminogen-Sepharose for 1 h. After extensive washing, bound proteins were eluted with 50 mM EACA and further analyzed by SDS-PAGE under reducing conditions. *Lane 1*, Molecular mass standards; *lane 2*, water extract of surface proteins; *lane 3*, purified proteins after elution from plasminogen-Sepharose affinity matrix. The arrow indicates the position of the 58.9-kDa plasminogen-binding protein.

Enhancement of t-PA-catalyzed plasminogen activation by *H. pylori* cells

The effect of *H. pylori* cells on plasminogen activation rate by t-PA and urokinase was explored. When t-PA was the activator used, as shown in Figure 3, the addition of cells induced a ≈25-fold higher amidolytic activity (expressed in mA_{405}/min^2) compared with the activity in the absence of cells. In contrast to t-PA, when bacteria were incubated with plasminogen and urokinase under conditions identical to those used in the t-PA experiment, only a two-fold increase in plasminogen activation was observed (Figure 3). It thus appears that *H. pylori* enhancement of plasminogen activation is more specific with t-PA.

It was also observed that the initial rate of plasminogen activation (expressed as mA_{405}/min^2) increased significantly with cell concentration (Figure 4). A definite effect was seen at bacterial concentrations as low as 5 x 10^7 cells/ml.

Binding of t-PA and urokinase to *H. pylori*

To assess whether *H. pylori* cells may express surface receptors for physiological plasminogen activators, a binding assay was developed. In both cases, an enzymatic activity proportional to the initial concentration of the activator was observed (Figure 5A and B), showing that both activators bind to the surface of *H. pylori* cells in a concentration-dependent manner and are able to activate plasminogen. These results suggest that the activators remain active following immobilization on the surface of bacterial cells. The interaction between t-PA and *H. pylori* cells was inhibited by addition of the lysine analogue EACA, as shown in Figure 5A (inset).

Discussion

Plasminogen binding and activation on the surface of pathogenic bacteria have been implied in the invasion of tissues, hydrolysis of host immunoglobulins as well as availability of peptides from proteins for bacterial growth (11,24,25). Several mechanisms are used by these organisms to activate host plasminogen. These include the expression of membrane-anchored activators, the secretion of soluble activators and/or the presence of receptors for both plasminogen and its physiological activators on the surface of bacteria (10). *H. pylori* has been recently added to the list of such organisms due to its ability to bind plasminogen, a zymogen which may be subsequently activated by host activators (19).

In the present study, we demonstrated that a clinical isolate of *H. pylori* is able to bind not only human plasminogen but also the two physiological activators, t-PA and urokinase. Binding of t-PA to *H. pylori* was inhibited by the lysine analogue EACA, suggesting an important role of the lysine-binding sites located in the activator kringle 2 structure (12). We also demonstrated that, once bound, these proteins remain active and may be used by the cells to express a surface plasmin activity. This strategy appears to be a common and efficient mechanism shared by invasive pathogens (11). It has already been observed that *Borrelia burgdorferi* expresses a receptor for urokinase in addition to binding plasminogen and that *Escherichia coli* and *Salmonella enteritidis* bind both t-PA and plasminogen, suggesting that organisms which bind plasminogen but do not produce their own plasminogen activators may acquire surface plasmin activity through host activators (10). *H. pylori* can be now included in this class of pathogens which share an alternative strategy to efficiently acquire cell-surface, unregulatable, plasmin activity under physiological conditions. Although we believe it is likely that specific

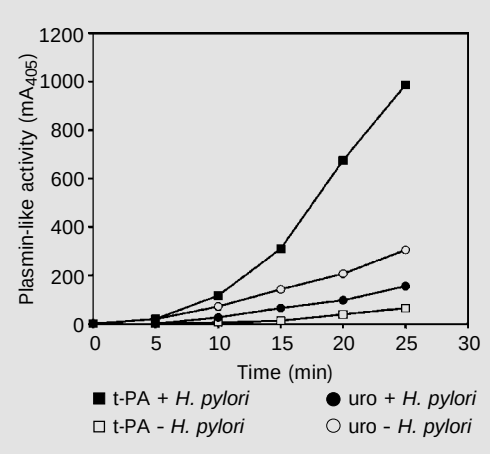

Figure 3 - Effect of *H. pylori* cells on t-PA- and urokinase-catalyzed plasmin formation. Human plasminogen (0.4 µM) was activated by 2.1 nM t-PA (squares) or 0.2 nM urokinase (circles) in the presence (filled symbols) and absence (open symbols) of 10^8 *H. pylori* cells. The formation of plasmin activity was measured using a chromogenic substrate.

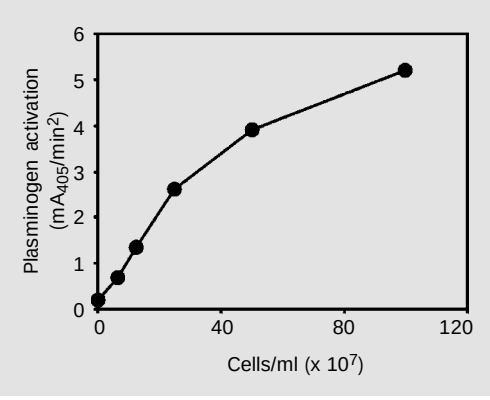

Figure 4 - Effect of cell density on t-PA-catalyzed plasminogen activation. Plasminogen (1.5 µM) was activated with 2.1 nM t-PA in the presence of increasing concentrations of *H. pylori* cells. The resultant plasmin activity was measured with the chromogenic substrate CBS 00.65 at different time intervals and the initial rates of plasmin generation determined. Each point represents the mean value of at least three determinations.

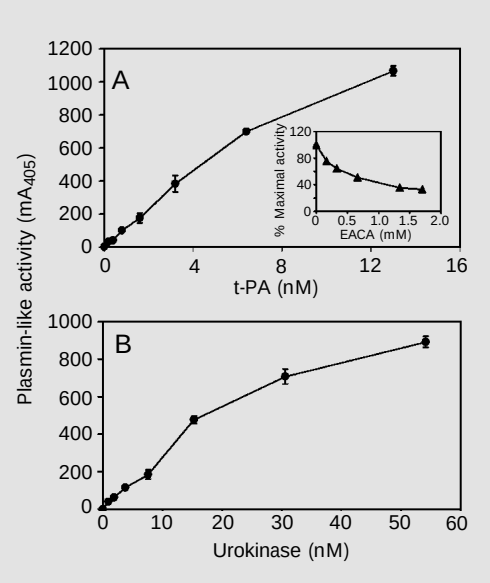

Figure 5 - t-PA and urokinase binding to the surface of *H. pylori*. Different amounts of t-PA (A) and urokinase (B) were allowed to interact with 10^8 *H. pylori* cells. Following extensive washing, plasminogen (1.5 µM) was added and the resultant plasmin activity recorded with CBS 00.65 after 5-min (urokinase) or 15-min (t-PA) incubation at 37ºC. Each point represents the mean value of at least three determinations. Standard errors are shown. *Inset*, Inhibition by ε-aminocaproic acid (EACA) of the binding of t-PA (14 nM) to *H. pylori* cells.

receptors for both t-PA and urokinase may be present on the surface of *H. pylori* cells, their identity remains obscure and needs further experiments to be elucidated.

The receptors for human plasminogen in several pathogenic bacteria have been characterized (10). In the case of group A streptococci, such proteins appear to play a significant biological role since almost four different proteins are produced by these species (26). In the particular case of *H. pylori*, two proteins of 42 and 57 kDa have been recently demonstrated to be involved in plasminogen binding, using immunoblot procedures (19). In the present study, with a different experimental approach, affinity binding assays of water extracts of surface proteins allowed the detection of a plasminogen-binding polypeptide of 58.9 kDa, a protein of a molecular mass similar to that of one of the proteins found in the other strain of *H. pylori* already studied (19). This protein was eluted with EACA, indicating specific binding through lysine-binding sites in the plasminogen molecule. The identification of this protein will allow to determine whether it is structurally related to other proteins which have been identified as plasminogen receptors (27,28).

A significant enhancement of plasminogen activation by t-PA in the presence of *H. pylori* cells has been clearly established; when urokinase was used, only small differences were seen. These results are not surprising since others have observed the same behavior in the presence of platelets (7). It is well known that plasminogen activation *in vivo* is a surface-dependent reaction that offers a catalytic advantage for enzyme complex formation and that assembly of plasminogen and plasminogen activators on many cell types facilitates plasmin generation (reviewed in 29). Molecular assembly of t-PA and plasminogen on the surface of fibrin or cells results in a ternary complex that leads to a profound enhancement of the reaction rate (29). In the case of platelets, this enhancement appears to be particularly efficient when t-PA is the activator, resulting in a 5- to 8-fold increase in catalytic efficiency; however, when either urokinase or streptokinase is used, no enhancement is observed (7). Furthermore, enhancement of plasminogen activation by a number of bacteria expressing receptors for t-PA has been reported (10,30). These observations correlate with the requirement of a third macromolecular component for the formation of a complex between plasminogen and t-PA. Fibrin, casein, denatured proteins, aggregated IgG and the eukaryotic cell surface are among the factors which enhance the activation of plasminogen (31,32). In agreement with this, it is tempting to speculate that the cell surface of *H. pylori* may also promote complex formation between plasminogen and t-PA through specific receptors, leading to the pronounced rate of activation observed. From a biological perspective, our data suggest that t-PA, instead of urokinase, might be the physiological activator of plasminogen on the *H. pylori* surface. However, a decrease in t-PA and an increase in urokinase levels in *H. pylori*-associated gastritis have been previously reported (33). The activator responsible for the *in vivo* plasmin formation on the *H. pylori* surface is still to be elucidated.

The biological role of the interaction of *H. pylori* cells with components of the fibrinolytic system remains unclear, since this pathogen has not been demonstrated yet to invade host tissues, like other pathogenic bacteria (34). Nevertheless, plasmin-like surface activity may be used by these bacteria to degrade host proteins, like immunoglobulins (24), to obtain low molecular weight peptides for growth and survival (25) and/or to activate latent metalloproteinases (35).

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