BACTERIAL AND FUNGAL PATHOGENICITY

Human urokinase, a serine proteinase, potentiates the in-vitro growth of micro-organisms which commonly infect burn patients

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Summary. Addition of human urokinase, a serine proteinase, to in-vitro cultures of *Pseudomonas aeruginosa* strain M2 enhanced bacterial growth. The enhancement of growth depended on the dose of urokinase (10–12 500 units) and the enzymic activity of the protein. Other mammalian proteolytic enzymes (trypsin, chymotrypsin, polymorphonuclear leucocyte elastase, thrombin and plasmin) tested did not affect bacterial growth *in vitro*. Experiments with clinical isolates of *Candida albicans*, *Klebsiella pneumoniae* and *Staphylococcus aureus* from burn patients indicated that urokinase could enhance the in-vitro growth of all of these micro-organisms. However, some strain-to-strain variation was noted in the extent of this enhancement. These results indicate that urokinase, which could be released into burn injury sites from either damaged tissues or inflammatory cells, is capable of enhancing the growth of several micro-organisms that commonly infect patients with thermal injuries, particularly under oxygen-limited conditions and when few micro-organisms are present.

Introduction

Burn injuries affect a large number of individuals each year. Patients with third degree burns are at risk from life-threatening infections which develop in a significant proportion of them, even though advances in antibiotic therapy and the nutritional treatments of such patients have been made over the past few decades.^{1–5} Such infections are usually caused by opportunist organisms such as *Pseudomonas aeruginosa*, *Candida albicans*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.^{3,4} These micro-organisms may infect burn victims through the wound site, or possibly *via* the gastrointestinal tract.^{6,7} The susceptibility of such patients to infection by these organisms is believed to be due to multiple factors.^{1–5}

The response of host defence systems to thermal injuries has been studied extensively both in man and in animal models.^{1–3,5,8–13} From such studies, it is apparent that after thermal injury the function of macrophages and polymorphonuclear leucocytes is compromised. These cellular defects probably contribute to the dissemination of infection in burn patients, because their phagocytosis and killing mech-

anisms have been rendered ineffective by the injury. In addition to cellular regulation, burn patients also exhibit alterations in the regulation of many of the plasma protein cascades, such as coagulation, complement and fibrinolysis.^{14–17} Such disruption not only contributes to a loss in the effectiveness of these host defence systems, but their inappropriate activation can also lead to the generation of several proteolytic enzymes that can damage tissues.

Several groups of workers have characterised the microbial virulence factors associated with the ability to colonise and infect animals with thermal injuries.^{9,10,18} In particular, emphasis has focused on pseudomonads, as they are a major pathogenic group in this patient population.^{1–5} Several reports have implicated elastase and alkaline protease as important virulence factors in burns.^{8,9,18–21} Strains defective in proteinase production are considerably less virulent than their counterparts.^{9,10,21,22} The basis for this protease-dependent virulence is not completely understood. However, there is evidence^{8–10,18,19,21–23} that the bacterial proteinases interact with host proteolytic cascades to generate additional activities that are important in the infection process. Interestingly, injection of burned mice with proteinase inhibitors can protect the animals from infection. As some of the inhibitors that are effective in this regard do not inhibit

Fig. 1. Enhancement of the growth of *P. aeruginosa* strain M2 by UK. Strain M2 was cultured for 24 h at 37°C in M9 in the absence or presence of the indicated concentrations of UK (panels a, b, c, three separate experiments), affinity purified UK (panel d) or rh-UK (panel e). The OD₅₅₀ was determined for triplicate samples at each point and the indicated values represent the mean and SD.

Fig. 2. Time dependence of the enhancement of strain M2 growth by low dose UK. Triplicate cultures of *P. aeruginosa* strain M2 were grown in M9 for the indicated times at 37°C in the absence or presence of UK. The indicated values represent the mean OD₅₅₀ and SD of the cultures.

the pseudomonal proteinases, it has been concluded that host enzymes are involved.^{10, 22, 23} In particular, proteinases of the activation cascades, such as Hageman factor activation, kinin generating enzymes and the coagulation (e.g., thrombin) and fibrinolytic

(e.g., urokinase and tissue plasminogen activator) systems, are potential sources of the proteinases which appear to contribute to the infectious process.^{8-10, 18, 21} Holder and Neely^{21, 22} postulate that the activation of host proteinase systems (e.g., Hageman factor ac-

tivation) contributes to infection *via* an effect on the immune system, whereas others have shown that the pseudomonal elastase-induced activation of the Hageman factor system may contribute to the shock response of the host.²⁰

However, the mechanisms by which the host proteinases implicated in the infectious process could potentiate infection have not been completely elucidated. The effects of the proteinases on the organisms involved could be indirect, as indicated above, or they could be direct. This study examined whether urokinase (UK), a plasminogen activator that can be released from damaged cells and from inflammatory cells,²⁴⁻²⁷ could stimulate directly the *in-vitro* growth of micro-organisms that commonly infect burns.

Materials and methods

Microbial strains

The M2 strain of *P. aeruginosa* has been well characterised, particularly in the burned mouse model.^{28,29} The following blood isolates from burn patients were obtained from Dr A. McManus (Fort Sam, Houston, TX, USA): *S. aureus* (920712002, 920919001, 930105001, 930121001 and 930319033) which were designated SA-1-SA-5, respectively; *K. pneumoniae* (901021001, 910405002, 910621003, 920401002 and 920909003) which were designated KP-1-KP-5, respectively; *C. albicans* (901030001, 910213002, 910407002, 920412040 and 920423001) which were designated CA-1-CA-5, respectively. Non-mucoid variants of *P. aeruginosa* (strains PAO and DG-1), were those used previously³⁰ and *P. aeruginosa* strain 44.821 was isolated from the sputum of a patient with cystic fibrosis.

Reagents

Low-mol. wt human urokinase (Abbokinase; UK) and recombinant human urokinase(rh-UK) were supplied by Abbott Laboratories (Abbott Park, IL, USA). For some experiments, the UK was further purified by affinity chromatography on benzamidine-agarose (Pierce Chemical Co.). Diisopropyl fluorophosphate(DFP)-inactivated UK was prepared by incubating the enzyme in 5 mM DFP followed by dialysis.³¹ The enzyme was > 98% inactivated by this procedure. Casamino acids were obtained from Difco Laboratories. Thrombin, chymotrypsin, trypsin, PMN elastase and bovine serum albumin (BSA) were obtained from Sigma. Two different thrombin preparations (product no. T6884, 2000 units/mg of protein and T3010, 4000 units/mg of protein) were used in this study. Recombinant human tissue plasminogen activator (rh-tPA) was a gift from Genentech Inc. (San Francisco, CA, USA). Human plasminogen was prepared as described previously^{25,26,31} by the method of

Deutsch and Mertz.³² *P. aeruginosa* elastase and alkaline proteinase were purchased from Nagase Biochemicals Ltd (Fukuchiyama, Japan). Benza-midine and arginine were obtained from Sigma and leupeptin and antipain from Peninsula Laboratories (San Francisco, CA, USA). The thrombin inhibitor, recombinant hirudin, was the gift of Ciba Geigy Corp. (Switzerland). Medium M9 was formulated as described previously.³³ It contained NH₄Cl 1.0 g, Na₂HPO₄ 3.0 g, KH₂PO₄ 1.5 g, NaCl 2.5 g, MgSO₄ 0.05 g and glucose 0.5% in 1 L of distilled water.

Culture conditions

The micro-organisms were grown overnight in M9 medium in a shaking water bath at 37°C and then inoculated into 2 ml of M9 medium in 17 × 100-mm tissue-culture tubes or 50-ml culture tubes (Falcon). The OD₅₅₀ of the cultures was determined and dilutions to 0.01 OD₅₅₀ were performed in sterile M9 medium. Depending on the experiment and organism investigated, further dilutions (1 in 10–1 in 200) of the 0.01 OD₅₅₀ suspension were used as the starting inoculum of organisms. Additions of proteinases or inhibitors were made before incubating the bacteria. Cultures were set up in duplicate or triplicate for each time point. The cultures were then incubated statically in a water bath at 37°C with the lids of the culture tubes sealed to block air exchange. These oxygen-limited conditions may mimic the *in-vivo* burn environment¹ more closely than vigorous shaking conditions. At the end of the experiments, the OD₅₅₀ was determined and, in most experiments, quantitative bacteriology was performed on serial dilutions by culture on appropriate agar plates. Colonies were counted after incubation for 24–48 h at 37°C.

Statistical analysis

Reported values are expressed as the mean and SD. Statistical analysis of differences between groups was determined by Student's *t* test or ANOVA, with the Epistat software package.

Results

Influence of UK on the growth of P. aeruginosa strain M2

P. aeruginosa strain M2 cultured in M9 medium under static conditions led to only modest growth by 24 h (fig. 1). However, addition of UK to the medium led to a dose-dependent enhancement of growth as assessed by an increase in the OD₅₅₀ of the cultures (fig. 1) and colony counts (data not shown). In the three experiments depicted in fig. 1 (panels a, b and c), addition of 250–12 500 units of UK led to significant enhancement of growth ($p < 0.05$ – $p < 0.001$). This

Table Proteinases and proteinase inhibitors ineffective in modulating the growth of *P. aeruginosa* strain M2

Panel A: Proteinases	Panel B: Proteinase inhibitors*
Units DFP-UK 5000–20000	Benzamidine 0.1–10 mM
Recombinant human tissue plasminogen activator 1–100 µg	Leupeptin 10–100 µg
Human plasminogen (± 5 Units UK) 25–100 µg	Antipain 10–100 µg
Human neutrophil elastase 1–40 µg	Arginine 1–10 mg
Human trypsin 1–40 µg	Hirudin (thrombin inhibitor) 1–50 µg
Human chymotrypsin 1–40 µg	
Human thrombin 1–50 Units	
<i>Pseudomonas</i> elastase 5–50 µg	
<i>Pseudomonas</i> alkaline protease 5–50 µg	

* Preliminary experiments revealed that these inhibitors could inhibit UK or thrombin when incubated in sterile M9 medium.

Fig. 3. Enhancement of the growth of *P. aeruginosa* strains 44.821, PAO and DG-1 by UK. The bacteria were cultured for 24 h at 37°C in medium M9 with or without the indicated concentrations of commercial UK. After 24 h, the OD₅₅₀ was determined. The organisms, 44.821 (panel a), PAO (panel b) and DG-1 (panel c) were cultured in triplicate and the indicated values represent the mean and SD at each point.

range of UK doses was chosen for the initial experiments because similar doses influence in-vivo *P. aeruginosa* infections in a rat chronic pulmonary infection model.³⁰ Enhancement of bacterial growth by both UK purified by affinity chromatography and by rh-UK was also observed (fig. 1, panels d and e).

Further experiments showed that even lower doses of UK were effective in enhancing bacterial growth, particularly when very small inocula were used. As depicted in fig. 2 (panel a), with a small starting inoculum of *P. aeruginosa* strain M2 (1 in 200 dilution of a 0.01 OD₅₅₀ suspension) and only 100 units of UK, enhancement of bacterial growth could be detected

after 24 h. An enhancement of bacterial growth by as little as 10 units of UK could be detected when the time of culture was extended to 30 h (fig. 2, panel a). By 48 h, there was a tendency for the UK enhancement of growth to be less evident, primarily because the growth of the control cultures became more substantial (fig. 2, panel b).

Evidence that the enzymic activity of UK was required for the enhancement of bacterial growth was obtained from experiments with inactivated UK. Diisopropylfluorophosphate-inactivated UK did not stimulate bacterial growth when 5000–20000 units were tested (table). Furthermore, 1–100 µg of rh-tPA,

Fig. 4. Influence of UK on the growth of five clinical isolates of *C. albicans*, CA-1–CA-5, cultured in triplicate with or without (C) 10000 units of UK or 50 units of thrombin (T) for 24 h at 37°C. After 24 h, the OD₅₅₀ of the cultures was determined (panel a) and, for CA-5 (panel b), both OD₅₅₀ and quantitative *C. albicans* counts were determined. The indicated values represent the mean and SD at each point.

Fig. 5. Effect of UK on the growth of five clinical isolates of *K. pneumoniae*, KP-1–KP-5, cultured in triplicate with and without (C) 10000 units of UK for 24 h at 37°C. The indicated values represent the mean OD₅₅₀ and SD at each point.

a fibrinolytic enzyme that is essentially inactive in the absence of fibrin,^{24,27} did not enhance bacterial growth (table). Thus, the enzymic activity of UK was required for the effect on bacterial growth.

Influence of other proteinases on the growth of P. aeruginosa strain M2

Further similar experiments indicated that the obser-

Fig. 6. Influence of UK on the growth of five clinical isolates of *S. aureus*, SA-1-SA-5, cultured in triplicate for 24 h at 37°C with and without (C) 10000 units UK. After 24 h, the OD₅₅₀ of each culture was determined. The indicated values represent the mean OD₅₅₀ and SD at each point.

ved effect of UK on the growth of *P. aeruginosa* was not given by a number of other proteinases (table). These included: plasmin, which is a lysine-specific enzyme generated by the action of UK on plasminogen;^{24, 27, 34} human neutrophil elastase, which is released from activated neutrophils;³⁵ trypsin, which is detected in elevated concentrations in the plasma of burn patients;¹⁵ thrombin, an arginine-specific coagulation enzyme³⁶ whose generation can be elevated during infections with gram-negative bacteria; and chymotrypsin, another pancreatic enzyme that preferentially hydrolyses linkages involving aromatic amino acids. In addition, *P. aeruginosa* elastase and alkaline proteinase, which have been implicated as virulence factors, did not stimulate directly the growth of the bacteria (table). Thus, the effect of UK on bacterial growth was not due to a general proteolysis effect.

Inability of UK inhibitors to influence the growth of P. aeruginosa strain M2

The finding that UK could enhance the growth of strain M2 raised the possibility that the enzyme was performing the function of some endogenous enzyme of similar specificity which was rate limiting under the conditions of growth. There have been reports that *Escherichia coli*³⁷ and *Yersinia pestis*³⁸ express enzymes that can function as plasminogen activators. Therefore, strain M2 was grown in the presence of a number of compounds that can inhibit UK or other arginine-specific proteinases, to determine whether they could affect bacterial growth. The compounds at the concentrations used (table), were without effect on growth

when strain M2 was incubated under either static or vigorous shaking conditions. Therefore, the bacteria do not appear to utilise an enzyme that has exactly the same active site as UK. As the *Y. pestis* plasminogen activator has an active site different from that of UK,³⁸ these findings do not completely eliminate the existence of an analogous UK-like endogenous enzyme.

Influence of UK on the growth of other strains of P. aeruginosa

To determine whether the effect of UK on the growth of strain M2 was unique to this strain or not, the influence of UK on the growth of a clinical isolate (strain 44.821), as well as two other laboratory strains (PAO and DG-1) of *P. aeruginosa* was assessed. As shown in fig. 3, UK enhanced the growth of all three strains of *P. aeruginosa* ($p < 0.01$). Furthermore, UK enhanced the growth of variants of strain PAO (E64 and B1), deficient in alkaline protease and elastase, to the same extent as the parent PAO strain (data not shown). Therefore, the effect of UK on growth was not restricted to strain M2.

Ability of UK to enhance the growth of other micro-organisms

A number of other organisms besides pseudomonads commonly infect burn patients.¹⁻⁵ These include *C. albicans*, *K. pneumoniae* and *S. aureus*. To determine whether UK could also enhance the growth of these species, five isolates of each species from burn

patients were cultured with and without UK. As shown in fig. 4, the addition of 10000 units of UK to the *C. albicans* cultures enhanced the growth of all five strains. Similar experiments with the *K. pneumoniae* (fig. 5) and *S. aureus* (fig. 6) isolates gave a similar pattern of results. The degree of enhancement was variable but the pattern of enhancement was reproducible with these three organisms. The same pattern of stimulation of growth was obtained when these experiments were repeated with purified UK (data not shown). As found previously with *P. aeruginosa*, thrombin did not stimulate growth of *C. albicans* (fig. 4), *K. pneumoniae* or *S. aureus* (data not shown). From these experiments, it would appear that UK can enhance the growth of several organisms which commonly infect burn patients.

Influence of P. aeruginosa proteinases on the in-vitro growth of clinical isolates of C. albicans

Neely and co-workers⁹ reported that *P. aeruginosa* proteinases or pre-infection of burned mice with *P. aeruginosa* increased the susceptibility of such mice to *C. albicans* infection. To determine whether this phenomenon was due to a direct effect of the pseudomonal proteinases on the growth of *Candida* spp., purified enzymes were added to cultures of the clinical isolates of *C. albicans*. In three experiments with 1–50 µg of purified *P. aeruginosa* elastase or alkaline proteinase, there was no detectable effect on the in-vitro growth of the five strains of *C. albicans* (data not shown). This finding was obtained irrespective of whether the organisms were grown under shaking or static conditions. Therefore, these results indicate that the effect of pseudomonal proteinases on candidal infections in burned mice may be indirect.

Discussion

The results of this study support the conclusions of Holder and Neely^{8, 10, 21–23} that host proteinases may contribute to the infectious process that can occur in immunocompromised patients with thermal injuries. They indicate that a specific host proteinase, UK, one of the two primary fibrinolytic enzymes of mammals,^{24, 27, 34} can stimulate uniquely the in-vitro growth of micro-organisms which commonly infect burn patients under oxygen-limited static conditions.

In contrast to tissue plasminogen activator (tPA), which is the primary fibrinolytic enzyme of the vasculature,^{24, 27} UK is the primary plasminogen activator in connective tissues,²⁴ including skin,³⁹ and is expressed by inflammatory cells such as macrophages and polymorphonuclear leucocytes.^{25, 26} Thus, the

enzyme could be released from tissues following thermal injury or as a consequence of inflammatory cells being attracted to the site of injury. As UK does not depend on the presence of fibrin for activity, that present in the wound could influence the growth of a micro-organism in the burn site. The results indicate that UK enhanced the growth of micro-organisms under hypoxic conditions and especially when initially present in low numbers. These are the conditions likely to be present initially at a burn site.¹ Thus, UK in the injury site could contribute to the establishment of the infection locally.

The finding that tPA was without effect in the absence of fibrin supports the conclusion that enzyme activity is required for the effect on the growth of micro-organisms. However, this does not mean that tPA could not contribute to growth *in vivo* when fibrin is present. It is likely that fibrin is deposited in the burn site and, therefore, endogenous tPA could become activated. However, since UK and tPA have some differences in their active sites,⁴⁰ the effect of active tPA on the growth of micro-organisms remains to be confirmed. Irrespective of whether or not tPA can stimulate bacterial growth, the finding that UK uniquely potentiated growth raises some interesting questions regarding the mechanism(s) by which the enzyme exerts this effect.

UK activates plasminogen by cleaving an arg-val bond.³⁴ In addition, the enzyme can cleave arg-ala and arg-thr bonds in fibronectin,⁴¹ as well as activate diphtheria toxin *in vitro* by cleaving an arg-arg bond.⁴² Therefore, it is likely that the protein substrate for UK involves an arg-X bond. The finding that thrombin, another enzyme which cleaves various peptide bonds involving arginine (e.g., arg-val, arg-gly, arg-cys)³⁶ did not affect growth indicates again that the effect of UK is unique and that a specific substrate(s) is involved.

Furthermore, because UK (Abbokinase) has a mol. wt of c. 33 kDa, it is likely that the candidate substrate(s) on the micro-organisms is either exposed on the cell surface or secreted. As UK stimulated the growth of gram-negative and gram-positive bacteria and a yeast, the substrate(s) must be either very similar or common to these micro-organisms or the cleavage site(s) must be conserved. At the present time, we are not aware of any candidate substrate(s) that is involved in cell growth and which would fulfil these criteria. However, investigations are being made to identify such substrates.

The excellent technical assistance of Carol Reno, the secretarial assistance of Judy Crawford, the gift of some of the microbial strains by Dr A. McManus, the supply of UK and derivatives by Dr J. Henkin (Abbott Laboratories; Abbott Park, IL, USA) and the helpful review of the manuscript by Drs I. Holder and A. Neely (Shriners Burn Unit and the University of Cincinnati, Cincinnati, OH, USA) are acknowledged with gratitude. This investigation was supported by grants from Abbott Laboratories and the Canadian Cystic Fibrosis Foundation.

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