ORIGINAL ARTICLE

Tranexamic acid, an inhibitor of plasminogen activation, aggravates staphylococcal septic arthritis and sepsis

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Abstract
Haemostatic balance shifts towards pro-coagulation during infection. Plasminogen, a key molecule of fibrinolysis, may play an important role in the pathogenesis of staphylococcal infections. In the present study, we assessed the impact of inhibition of plasminogen activation by tranexamic acid on the course of staphylococcal sepsis and septic arthritis in mice. We found significantly down-regulated plasmin activity and increased D-dimer levels in the blood from the mice with staphylococcal sepsis. Treatment with tranexamic acid significantly increased the severity and mortality of staphylococcal infection. In addition, tranexamic acid reduced the survival rate in a murine model for staphylococcal enterotoxin A-induced death. The aggravation of diseases by tranexamic acid was due neither to the pro-inflammatory cytokine network, nor to impairment of bacterial clearance. Modulation of fibrinolysis, either by supplement of fibrinolytic molecules (tissue plasminogen activator or plasmin) or by fibrinogen depletion, did not reduce the mortality of staphylococcal sepsis. In conclusion, we report that treatment with tranexamic acid led to distinct aggravation of staphylococcal septic arthritis and sepsis in mice, suggesting the clinical importance of fibrinolytic balance in staphylococcal infection.

Introduction
During systemic infection there is a regulatory cross-talk between the haemostatic and immune systems. Activation of pro-inflammatory cytokines (e.g., tumour necrosis factor α (TNF-α) and interleukin (IL)-6) up-regulates the synthesis of tissue factor, which mediates thrombin generation, and leads to activation of coagulation and subsequent fibrin deposition [1]. Simultaneously, the fibrin removal mechanism, i.e. fibrinolysis, is markedly impaired [2]. We recently showed dramatically elevated plasminogen activator inhibitor type-1 (PAI-1) levels in both the circulation and local tissues during staphylococcal sepsis [3], suggesting fibrinolysis is down-regulated not only in Gram-negative bacterial infections, but also in staphylococcal sepsis.

Plasminogen, a key molecule of fibrinolysis, is converted to the active enzyme plasmin by plasminogen activators, e.g. tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Several lines of evidence indicate that plasmin(ogen) plays a role in inflammatory/infectious diseases. It has been shown that plasmin and plasmin-derived fibrin(ogen) degradation products are chemoattractants for leukocytes in vitro [4]. Additionally, plasmin regulates activation of transforming growth factor β (TGF-β) [5], and activates pro-inflammatory signalling networks, leading to phosphorylation and activation of p38 MAPK and JAK/STAT signalling pathways [6]. During the inflammatory response, leukocyte migration is clearly reduced in plasminogen-deficient mice [7]. Importantly, it has been demonstrated that plasminogen is required for efficient dissemination of Borrelia in ticks and for enhancement of spirochtemia in mice [8,9]. Recently, Guo and co-workers in Sweden found impaired activation of inflammatory cells in plasminogen-deficient mice, which led to more severe disease in a model of local Staphylococcus aureus arthritis, suggesting that plasmin(ogen) may play a protective role in S. aureus-induced arthritis [10]. However, very few data are available on the role

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of plasminogen activation in the induction of septic arthritis and sepsis by S. aureus.

Would intervention on plasminogen activation change the outcome of staphylococcal infection? Tranexamic acid, a synthetic anti-fibrinolytic substance, has been widely used to prevent and treat blood loss, with good efficacy, in an array of clinical situations, e.g. perioperative blood loss and excessive menstrual bleeding. The mechanism of the anti-fibrinolytic effect of tranexamic acid is to competitively block the lysine binding sites of plasmin(ogen), thereby preventing plasminogen activation and binding of plasmin to fibrin [11]. In the present study we assessed different methods of modulating fibrinolysis in our murine model of staphylococcal septic arthritis and sepsis: either inhibiting plasminogen activation by tranexamic acid or enhancing fibrinolysis by supplement of exogenous plasminogen activator or plasmin.

Methods

Mice

Female NMRI and BALB/c mice were purchased from B&K Universal AB (Sollentuna, Sweden) and maintained in the animal facility of the Department of Rheumatology and Inflammation Research, Göteborg University, Sweden. They were housed up to 10 animals in each cage under standard conditions of light and temperature, and were fed standard laboratory food and water ad libitum. Animals were used at 6–8 weeks of age. All animal experiments were approved by the Animal Ethics Committee of the University of Göteborg.

Bacterial strains and reagents

We used S. aureus AB-1 [12] in the animal model for staphylococcal sepsis and LS-1 [13] in the animal model for staphylococcal septic arthritis. One characteristic of AB-1 is the production of large amounts of staphylococcal enterotoxin A (SEA). S. aureus LS-1 was originally isolated from the swollen joint of a spontaneously arthritic NZB/W mouse. Both AB-1 and LS-1 do not code for staphylokinase, which might interfere with mouse fibrinolytic molecules. The bacterial strains were prepared as previously described [3]. After each inoculation procedure, viable counts were performed in the leftover suspension to confirm the actual number of administered bacteria.

Todd–Hewitt broth (THB) and horse blood agar were obtained from Difco (Boule Nordic, Huddinge, Sweden). Tissue plasminogen activator (tPA) was purchased from Boehringer Ingelheim GmbH (Stockholm, Sweden). Human plasminogen, human plasmin, and plasmin-specific substrate S-2251 were purchased from Haemochrom Diagnostica AB (Mölndal, Sweden). Ancrod, a thrombin-like enzyme from Agkistrodon rhodostoma venom, SEA from S. aureus, Escherichia coli O55:B5 lipopolysaccharide (LPS), Bovine serum albumin (BSA) fraction V, and dimethylsulfoxide (DMSO) were purchased from Sigma Chemicals (St. Louis, MO, USA). Tranexamic acid (100 mg/ml) was purchased from Pfizer AB (Sollentuna, Sweden).

Experimental protocols for staphylococcal sepsis

Five separate in vivo experiments were performed (n=9–12/group) to assess the impact of tranexamic acid on the outcome of staphylococcal sepsis. Experiments differed with respect to bacterial load and the duration of infection. In all experiments the NMRI mice were injected intraperitoneally with 0.2 ml of tranexamic acid, corresponding to 800 mg/kg body weight, every 8 h starting 1 day before inoculation of bacteria and continuing until the experiments were terminated. Mice injected intraperitoneally with 0.2 ml of PBS served as controls. All mice were inoculated intravenously into the tail vein with a suspension of staphylococcal strain AB-1 in a volume of 0.2 ml. In a first experiment 10 mice per group were inoculated with a dose of $2\times 10^8$ S. aureus per mouse, a dose known to induce severe sepsis. In a second experiment, 23 mice were inoculated with a dose of $1\times 10^8$ staphylococci per mouse. In a third experiment 9 mice per group were inoculated with a dose of $1.4\times 10^7$ staphylococci each. During the course of experiments the mortality was recorded at frequent intervals. In a fourth experiment 20 mice were inoculated with a dose of $1\times 10^8$ staphylococci each. Ten mice were treated intraperitoneally with tranexamic acid as described previously, and another 10 mice were given intraperitoneal PBS as controls. Twenty-seven hours after inoculation, the lungs and kidneys were collected for later histopathology and immunohistochemistry. In a fifth experiment we wanted to assess the kinetics of plasmin activity during staphylococcal infection. Twenty-two mice were inoculated with a dose of $1\times 10^8$ staphylococci each. Mice were sacrificed and plasma was collected after 2 h ($n=7$), 8 h ($n=7$), and 27 h ($n=8$), respectively.

The impact of exogenous plasmin/tPA supplement was studied in mice with staphylococcal sepsis. Eight mice were injected intravenously with 0.2 ml of plasmin (5 mg/kg) or tPA (4 mg/kg) directly, 12 h after intravenous inoculation of S. aureus AB-1 ($1\times 10^8$ colony-forming units (cfu)/mouse). The treatment was repeated after another 12 h and thereafter every 24 h until the experiment was terminated 3 days after bacterial inoculation. Ten mice treated intravenously with 0.2 ml of PBS served as controls.
Experimental protocols for staphylococcal enterotoxin-induced shock

BALB/c mice were challenged with an intraperitoneal injection of 10 µg of SEA from S. aureus (product No. S9399, Sigma), followed 4 h later by a further intraperitoneal injection of 170 µg of E. coli O55:B5 LPS (product No. L2880, Sigma), and the number of deaths was recorded at frequent intervals. The procedures regarding the induction of enterotoxin-triggered death, including doses of SEA and LPS, were adopted from previous studies [14]. Neither SEA nor LPS given alone was sufficient for lethal toxicity at these doses.

Two separate in vivo experiments were performed (n=8–10/group) to assess the impact of tranexamic acid on the mortality of SEA-induced shock. Eighteen mice were injected intraperitoneally with 0.2 ml of tranexamic acid (800 mg/kg) every 8 h, starting 1 day before SEA challenge and continuing until the experiment was terminated. Seventeen mice challenged with SEA were injected intraperitoneally with 0.2 ml of PBS as controls. To check the side effects of tranexamic acid, 4 mice were injected intraperitoneally with only 0.2 ml of tranexamic acid (800 mg/kg) every 8 h for 40 h.

Blood was collected for analyses of serum alanine aminotransferases and urea.

To study whether fibrinogen depletion abrogates the deteriorative effect of tranexamic acid on SEA-challenged mice, 3 units of ancrod in 50 µl of PBS were injected subcutaneously into 10 mice every 12 h, starting 2 h before SEA challenge and continuing until the experiment was terminated. In the control group, 10 mice received the same volume of PBS. The protocols of SEA challenge and tranexamic acid treatment were as described above.

Experimental protocols for staphylococcal septic arthritis

Twenty NMRI female mice received S. aureus LS-1 (4×10^6/mouse) intravenously. They were divided into 2 distinct groups: (1) 10 mice in the control group without treatment; (2) 10 mice received tranexamic acid in drinking water (around 700 mg/kg/day) starting 1 day before inoculation of bacteria. During the course of experiments mice were regularly weighed and examined for arthritis by an observer blinded to inoculation groups. Kidneys were examined for bacterial load by determination of cfu in serial dilutions of homogenate.

Clinical evaluation of arthritis

All 4 limbs of each mouse were inspected visually. Arthritis was defined as erythema and/or swelling of the joints. To evaluate the severity of arthritis, a clinical scoring system of 0–3 for each limb was used, as described previously [15].

Histopathology

Following staphylococcal sepsis, the lungs of each mouse were processed, embedded in paraffin, and sectioned at 3–4 µm. Haematoxylin and eosin (H&E) staining of tissue was performed to identify the tissue and to detect alterations in cellular architecture.

Bacteriological examination of infected animals

The kidneys were aseptically removed, homogenized, diluted serially in PBS and transferred to agar plates containing 5% v/v horse blood. Bacteria were grown for 24 h and then counted as cfu.

Plasma isolation procedures

Blood was collected from mice into 1/10 vol. of sodium citrate (110 mmol/l) as an anticoagulant. The collected blood samples were centrifuged at 800×g for 20 min, aliquoted and stored frozen at −70°C until use.

Plasmin activity assay

Plasmin activity was determined by hydrolysis of a specific plasmin substrate, S-2251 (H-D-Val-Leu-Lys-pNA.2HCl). Briefly, 25 µl of mouse plasma samples were diluted 1:4 in a Tris buffer (0.1 M, pH 7.4). The active plasmin was measured by hydrolyzation of S-2251 (4×10^{-4} M) to form free pNA. The formation of free pNA was measured by colour development at 405 nm and was proportional to the enzymatic activity of plasmin.

Analyses of serum IL-6, IL-1β, TNF-α, and IL-10 levels

A bioassay method using the murine hybridoma cell line B9, which is dependent on IL-6 for growth, was used to detect the serum levels of IL-6, as previously described [16].

Levels of IL-1β, TNF-α, and IL-10 were quantified using DuoSet ELISA Development Systems (R&D Systems, Abingdon, UK) according to the manufacturer protocols.

Analyses of alanine aminotransferases and urea

The liver enzymes serum alanine aminotransferases (s-ALAT) and serum urea were analyzed by spectrophotometric methods (Roche, Stockholm, Sweden).

Statistical analysis

Statistical evaluation was done using the Mann–Whitney U-test, Chi-square test, and log-rank...
Results

Decreased plasmin activity in plasma during staphylococcal sepsis

The plasmin activity in plasma decreased significantly during staphylococcal sepsis (Figure 1). There was a small peak of plasmin activity in plasma from mice 3 h after S. aureus inoculation. Thereafter plasmin activity started to decline, and after 27 h the plasmin activity decreased to around 50% of the levels before infection ($p<0.05$).

Tranexamic acid aggravated staphylococcal sepsis

Three doses of S. aureus AB-1 were used to investigate the impact of tranexamic acid in staphylococcal sepsis (Figure 2). At the highest dose ($2\times10^8$ cfu/mouse), more than 80% of mice died within 50 h with no difference between tranexamic acid and control groups (Figure 2(a), not significant). However, when S. aureus doses were reduced to $1\times10^8$ cfu/mouse, the median survival of animals in the tranexamic acid group was significantly shorter than in the control group (median survival 31 h vs 67 h, $p<0.001$, Figure 2(b)). Results were similar at a dose of $1.4\times10^7$ cfu/mouse (median survival 74 h vs 156 h, $p<0.001$, Figure 2(c)), demonstrating that tranexamic acid aggravates the outcome of staphylococcal sepsis.

Figure 1. Kinetics of plasmin activity in the blood from NMRI mice after a single intravenous injection of $1\times10^8$ cfu of Staphylococcus aureus AB-1 per mouse. Plasma samples were collected 3 h ($n=7$), 8 h ($n=7$), and 27 h ($n=8$) post-injection. The plasmin activity is represented as the absorbance at 405 nm; plasma from healthy mice ($n=10$) was assigned 100% plasmin activity. Data are presented as mean±SEM. $^*^*^*p<0.01$ by Mann–Whitney U-test.

Figure 2. Cumulative survival of NMRI mice (9–12 mice/group) inoculated intravenously with 3 different septic doses of Staphylococcus aureus AB-1: (a) $2\times10^8$ cfu/mouse, (b) $1\times10^8$ cfu/mouse, and (c) $1.4\times10^7$ cfu/mouse. All mice were treated intraperitoneally with 0.2 ml of the tranexamic acid (100 mg/ml) or 0.2 ml of phosphate-buffered saline (PBS), every 8 h starting on day 1 before inoculation of bacteria and continuing until the experiments were terminated. ns=not significant. $^*^*^*^*p<0.001$ by Kaplan–Meier log-rank test.
Tranexamic acid aggravates staphylococcal septic arthritis

To evaluate the impact of tranexamic acid on staphylococcal arthritis, NMRI mice were inoculated with a low dose (4×10^6 cfu/mouse) of the arthritogenic S. aureus LS-1, and treated with per oral tranexamic acid. During the early stage of infection (days 3–6), mice treated with tranexamic acid tended to develop more pronounced arthritis than the controls (not significant, Figure 3 (a)). Importantly, there was more pronounced weight loss % in the tranexamic acid group than in controls at day 6 after inoculation (p<0.05, Figure 3(b)).

Tranexamic acid had no impact on the release of pro- or anti-inflammatory cytokines in sepsis

Table I shows the dynamic levels of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) and anti-inflammatory cytokine IL-10 in the plasma from septic mice treated with tranexamic acid. The production of IL-1β, TNF-α, IL-6, and IL-10 started to increase early after inoculation of S. aureus. However, no clear differences were observed between the tranexamic acid group and the control group, suggesting that the cytokine network did not contribute to more severe disease in the tranexamic acid-treated mice.

Increased D-dimer levels under staphylococcal sepsis

In staphylococcal sepsis, D-dimer levels started to increase at 8 h following bacterial injection, and were 10-fold higher at 27 h than the levels in PBS-injected controls (p<0.001, Table I), indicating an ongoing pro-coagulation process. However, there was no difference in D-dimer levels between the tranexamic acid group and the control group.

Exogenous tPA or plasmin did not rescue mice from staphylococcal sepsis

To investigate the impact of enhanced fibrinolysis on staphylococcal sepsis, we supplied extra tPA or plasmin to the mice with staphylococcal sepsis (Figure 4). Mice receiving an intravenous injection of tPA had the same median survival as animals in the control group (Figure 4(a)). In contrast, there was a tendency towards a shorter median time of survival in mice treated with plasmin than in control mice (48 h vs 60 h, p=0.088, Figure 4(b)).

Tranexamic acid did not impair bacterial clearance in the host

We compared the density of bacteria in blood and kidneys of the tranexamic acid treated group and the control group (Table I). Kidneys from septic mice treated with tranexamic acid contained a similar amount of bacteria compared with the controls (2.3±0.7×10^8 compared to 2.7±0.7×10^8 cfu/kidney, difference not significant). Similarly, no differences were observed in the bacterial density in blood between the tranexamic acid group and the control group, suggesting that tranexamic acid had no impact on bacterial clearance.

Tranexamic acid did not affect extravasation in the lungs of septic mice

At 27 h following intravenous injection of S. aureus AB-1 (1×10^8 cfu/mouse), lungs were sliced and stained with H&E. All animals in both the PBS and tranexamic acid groups had hyperemia. Erythrocytes were observed in the alveolar lumen in half of the animals from both the tranexamic acid group and the

Figure 3. Clinical signs of staphylococcal septic arthritis in NMRI mice (10 mice/group) inoculated intravenously with 4×10^6 cfu of the arthritogenic Staphylococcus aureus LS-1. Mice were provided with either tranexamic acid in drinking water (700 mg/kg/day) or water alone, starting 1 day before inoculation of bacteria and continuing until the experiments were terminated on day 6. (a) Development of clinical arthritis. (b) Changes in body weight. Results are presented as mean±SEM. ns=not significant. *p<0.05 by the Mann–Whitney U-test.
control group. There was no difference regarding percentage of abnormal area (oedema and collapsed area) between groups (data not shown). In contrast, neither hyperemia nor alveolar bleeding was observed in the lungs of non-infected animals.

Tranexamic acid aggravated staphylococcal enterotoxin-induced shock

Intraperitoneal injection of BALB/c mice with 10 µg of SEA caused death within 2 days. Figure 5(a) shows that treatment with tranexamic acid in mice challenged with SEA led to a significantly lower median survival rate than in controls ($p < 0.001$). Ninety-five percent of tranexamic acid-treated animals died within 20 h post-SEA challenge, whereas more than 80% of controls were still alive at 40 h and subsequently recovered completely. All 4 mice receiving only tranexamic acid survived. Analysis of s-ALAT and s-urea in these mice showed normal levels, indicating that the dose of tranexamic acid used did not have nephrotoxic and hepatotoxic effects (data not shown).

To deplete fibrinogen in blood, ancrod was subcutaneously injected into mice following a previously described method [17]. Depletion of fibrinogen led to significantly shorter median survival in mice receiving both enterotoxin and tranexamic acid (2.5 h vs 7.5 h, $p < 0.01$, Figure 5(b)), suggesting that fibrinogen may play a protective role in SEA-induced shock.

Discussion

In this study we have shown that plasmin activity in the circulation was distinctly decreased in mice with staphylococcal sepsis. Tranexamic acid, an inhibitor of plasminogen activation, aggravated staphylococcal septic arthritis and sepsis by down-regulating fibrinolysis. Importantly, we also found aggravation of
Tranexamic acid aggravates staphylococcal infections

Micro-thrombosis and higher mortality. Importantly, supplementation of extra plasmin may induce rapid degeneration of TFPI, consequently leading to more severe fibrinolytic complications of sepsis [22]. Therefore, potentiation by plasmin [21]. It has been shown that (TFPI) can be degraded with loss of functional potency by plasmin [20]. Additionally, tissue factor pathway inhibitor (TFPI) can be degraded with loss of functional potency by plasmin [21]. It has been shown that down-regulation of TFPI underlies the widespread thrombotic complications of sepsis [22]. Therefore, supplement of extra plasmin may induce rapid degradation of TFPI, consequently leading to more severe micro-thrombosis and higher mortality. Importantly, we found systemic fibrinogen depletion by anord to significantly aggravate SEA-induced shock in mice, indicating that hypofibrinogenemia should be avoided in the clinical management of staphylococcal sepsis. We conclude the host resistance during staphylococcal infection to be severely impaired by disruption of the fibrinolytic balance by either inhibition of plasminogen activator or addition of plasmin.

Staphylococcal components are known to induce excessive production of pro-inflammatory cytokines, e.g. TNF-α, IL-1β, and IL-6, in human monocytic cells. Administration of proinflammatory cytokines, e.g. TNF-α, induces systemic inflammatory response syndrome, characterized by bowel necrosis, liver damage, and severe hypotension, leading to death [23]. Neutralization of TNF-α and IL-1β prevents the development of septic shock in animal models [24]. In our study, the cytokine network, including pro- and anti-inflammatory cytokines, was unaltered after treatment of tranexamic acid in staphylococcal sepsis, indicating that aggravation of disease by tranexamic acid is not mediated by the cytokine network. In addition, the absence of increased bacterial counts in kidneys and in blood from the tranexamic acid-treated septic mice suggests that tranexamic acid did not deteriorate the host immune defence. D-dimer, the fibrin degradation product, is a specific marker for increased procoagulatory activity, as well as fibrinolysis. Elevated D-dimer levels have been shown to have a negative correlation to survival in patients with disseminated intravascular coagulation (DIC) accompanying severe sepsis [25]. In the present study, D-dimer levels were indeed significantly elevated after staphylococcal inoculation in mice, reflecting an imbalanced homeostasis in infection. However, it remains unexplained why D-dimer levels were almost identical in the septic mice that received protease inhibitors. It is however known that fibrinolytic agents in patients with major blood loss due to DIC [11]. However, there is still a lack of clinical and experimental data to support this.
recommendation. The present distinct aggravation of staphylococcal septic arthritis and sepsis by tranexamic acid provides experimental support for the recommendation that extreme caution should be taken in the use of anti-fibrinolytic agents in systemic staphylococcal infection, even in the early stages of disease.

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References