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Borrelia burgdorferi Induces Secretion of Pro-Urokinase-Type Plasminogen Activator by Human Monocytes

HANS FUCHS,[1] MARKUS M. SIMON,[2] REINER WALLICH,[1] MICHAEL BECHTEL,[1] AND MICHAEL D. KRAMER[1]*

Institute for Immunology, Laboratory for Immunopathology, University of Heidelberg, 69120 Heidelberg,[1] and Max Planck Institute for Immunobiology, 79108 Freiburg,[2] Germany

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***Borrelia burgdorferi* is transmitted by infected ticks and causes Lyme disease. To infect distant organ sites, *B. burgdorferi* spirochetes must disseminate from the site of the tick bite. During dissemination from the dermal tissue, they breach tissue barriers, probably by proteolysis. The previous findings that spirochetes bind serum-derived plasminogen and that plasmin favors spirochetal invasiveness and infectivity suggested a role for plasmin in the pathogenicity of *B. burgdorferi*. Binding of plasminogen to spirochetes and activation into plasmin is favored in a microenvironment that is rich in plasminogen and plasminogen activators. Plasminogen is abundant in plasma and interstitial fluids, and it is increased in inflammatory exudates. Since *B. burgdorferi* does not express endogenous plasminogen activators, the conversion of spirochete-bound plasminogen depends on host-derived plasminogen activators. In this report, we show that both intact *B. burgdorferi* organisms and its recombinant outer surface lipoprotein A induce human monocytes to express and secrete urokinase-type plasminogen activator in its zymogen form (pro-uPA). Moreover, we demonstrate that the presence of *B. burgdorferi* accelerates the interaction between (pro-)uPA and plasmin(ogen), leading to spirochete-bound plasmin. In a pro-uPA–serum mixture, spirochete-bound plasmin activity is generated. Taken together, the data suggest that *B. burgdorferi* may induce pro-uPA in a monocyte-containing inflammatory site and that the spirochetal surface provides an appropriate milieu for subsequent interactions between (pro-)uPA and plasmin(ogen), which result in spirochete-bound plasmin even in the presence of inhibitors for plasminogen activators and plasmin.**

In humans, the spirochete *Borrelia burgdorferi* causes a multisystem disorder (Lyme borreliosis) which involves inflammatory manifestations in the skin, joint, heart, and the central and peripheral nervous system (31). During their blood meal, infected ticks form a feeding cavity in the dermal tissue in which they deposit *B. burgdorferi* organisms. Concomitantly, an inflammatory response characterized by exudation of plasma constituents and the infiltration of mononuclear cells, primarily monocytes/macrophages, is induced in the dermis (references 28 and 38 and our observations). Therefore, the first encounter of the pathogen with host tissue occurs in a hemorrhagic and inflammatory microenvironment. The tick-transmitted *B. burgdorferi* organisms disseminate into the connective tissue of the skin itself (38) and/or invade distant organs after a period of spirochetemia (10). In analogy to what has been found for other bacteria (for a review, see reference 21), the penetration of spirochetes through the extracellular matrix of the skin or through vascular basement membranes may be facilitated by proteolytic degradation of matrix proteins (7, 9).

It has been shown that *B. burgdorferi* is able to bind plasminogen via various binding structures, including the outer surface protein A (OspA) (9), a 70-kDa protein (13), and several low-molecular-weight proteins (7). In the presence of urokinase-type plasminogen activator (uPA), the bound plasminogen is processed to surface-bound plasmin, which is enzymatically active and refractory to inhibition by α_2-antiplasmin, the major plasmin inhibitor in serum and interstitial fluids. Spirochete-bound plasmin degrades high-molecular-weight glycoproteins (9) and increases the spirochete's potential to traverse endothelial cell monolayers (7). Moreover, Klempner and coworkers found that spirochetes that have been loaded with uPA have a higher infectivity than nontreated spirochetes (15). In analogy to previous findings with nonspirochetal bacteria (3, 16–19, 35, 36; for a review, see reference 21), these findings were taken to suggest that the interaction of *B. burgdorferi* with the host plasminogen activator system facilitates the dissemination of spirochetes within the infected host (7, 9, 15).

Conversion of spirochete-bound plasminogen into plasmin is favored in a microenvironment that is rich in plasminogen and uPA. Plasminogen is synthesized in the liver, and it is abundant in plasma and interstitial fluids; its concentration is increased in inflamed tissues by exudation from the vascular system. The amounts of uPA in plasma and interstitial fluids are low to undetectable (2, 8) but may be increased by induction and secretion from activated inflammatory cells, in particular monocytes (11, 12, 22). In this study, we show that intact *B. burgdorferi* organisms or its purified recombinant lipidated OspA are able to induce the production and secretion of (pro-)uPA [the term used when the method employed, e.g., detection by antibodies, cannot discriminate between pro-uPA and uPA; similarly, plasmin(ogen) is used when the method employed cannot discriminate between plasmin and plasminogen] from monocytes in vitro. Moreover, we show that *B. burgdorferi* facilitates the conversion of pro-uPA to uPA in a pro-uPA–plasminogen mixture, which finally results in the generation of plasmin at the spirochete surface.

* Corresponding author. Mailing address: Institute for Immunology, Laboratory for Immunopathology, University of Heidelberg, Im Neuenheimer Feld 305, 69120 Heidelberg, Germany. Phone: 49-6221-564015. Fax: 49-6221-564030.

MATERIALS AND METHODS

Reagents. Dextran 500, neutral red, *o*-phenylenediamine dihydrochloride, and EDTA were from Sigma Co. (Deisenhofen, Germany). Bovine serum albumin

was from Serva (Heidelberg, Germany). Fluorescein isothiocyanate-labeled mouse monoclonal antibodies for fluorescence-activated cell sorting (FACS) analysis were from Becton Dickinson (San Jose, Calif.) or Dianova (Hamburg, Germany). RPMI 1640, fetal calf serum (FCS) and L-glutamine were from Biochrom (Berlin, Germany); NycoPrep 1.068 was from Nycomed Pharma AS (Oslo, Norway). The chromogenic substrate S-2251 was from Chromogenix (Mölndal, Sweden). High-molecular-weight uPA was from Serono (Freiburg, Germany).

Bacteria and recombinant OspA. *B. burgdorferi* ZS7 and B31 were used (29). Bacteria were grown in BSK medium to about 5×10^7 cells per ml. After centrifugation (10,000 × *g*, 20 min) and three washes in phosphate-buffered saline (PBS), the bacteria were either used directly as intact spirochetes or used after disintegration by sonication. Recombinant lipidated OspA (lip-OspA) and methionine-aspartate-proline-OspA (MDP-OspA) were prepared as previously described (34) and were kindly provided by SmithKline Beecham (Rixensart, Belgium).

Preparation of human monocytes and U937 cells. Peripheral blood monocytes were isolated from venous potassium-EDTA blood derived from healthy donors. Ten parts of EDTA-blood was mixed with 1 part of 6% (wt/vol) dextran 500 in 0.9% (wt/vol) NaCl to settle the erythrocytes. Five milliliters of the leukocyte-rich plasma was layered on top of 3 ml of NycoPrep 1.068 solution and centrifuged at 600 × *g* for 15 min. The monocytes at the plasma-Nycoprep interphase were removed and washed twice with 0.9% (wt/vol) NaCl–0.13% (wt/vol) EDTA. Platelets were removed by layering the cell suspension on top of platelet-free autologous EDTA-plasma and centrifugation at 50 × *g* for 10 min. After being washed in Ca^{2+}- and Mg^{2+}-free Hanks solution, the cells were resuspended in RPMI 1640–5% (wt/vol) FCS at a concentration of 1.8×10^6 cells per ml. The purity of the cell preparation was estimated by FACS analysis using fluorescein isothiocyanate-labeled anti-CD2, anti-CD14, anti-CD16, anti-CD22, and anti-CD64 MAbs. About 80% of the cells stained for CD64, and about 85% stained for CD14. The enriched monocytes were allowed to adhere to 96-well microtiter plates (Becton Dickinson) at 0.36×10^6 cells per well for 2 h in a humidified atmosphere of 5% CO_2 in air at 37°C. Nonadherent cells were removed by three washes with RPMI-1640 at 37°C. Under these conditions, >90% of the adherent cells were monocytes, as estimated by immunocytological analysis. Alternatively, we used cells of the myelocytic/monocytic cell line U937. The cells were cultivated in RPMI 1640–10% (vol/vol) FCS. After centrifugation (100 × *g*, 10 min), cells were washed twice in RPMI 1640 and then used for the experiments.

Activation of monocytes and preparation of supernatants and lysates. The adherent monocytes (0.36×10^6 per well) were incubated with the indicated stimuli for 42 h in RPMI 1640 supplemented with 2 mM glutamine at 37°C in humidified air containing 5% CO_2. Medium was collected. Cell lysates were prepared by scraping and resuspending the cell monolayers in 0.1% Triton X-100 in 10 mM Tris HCl (pH 7.5).

U937 cells (1.5×10^6/ml) were incubated with the indicated stimuli for 18 h in RPMI 1640 supplemented with 2 mM glutamine at 37°C. After centrifugation (100 × *g*, 10 min), the supernatant was harvested.

Cell viability was assessed by a dye uptake assay. The cells were washed with PBS (37°C) and incubated with 50 μg of neutral red per ml for 3 h at 37°C. After extensive washing with PBS, 50% ethanol–1% acetic acid was added to extract the color, which was quantified by measuring the A_{562} in an automated enzyme-linked immunosorbent assay (ELISA) reader. Neither intact spirochetes, sonic extracts, nor lip-OspA caused a reduction of the cell viability compared with nonstimulated cells (data not shown).

ELISA for quantification of (pro-)uPA. The ELISA for quantification of (pro-)uPA was performed as previously described (5). Flat-bottom microtiter plates were coated with polyclonal goat anti-uPA immunoglobulin G (IgG; 4 μg/ml). After washing and blocking of unspecific binding sites with PBS–0.2% (wt/vol) gelatin, the test samples diluted in PBS–0.05% Tween 20 were added for 1 h. After washing, the anti-(pro-)uPA monoclonal antibody HD-UK1 (1 μg/ml) was added for 1 h. After washing, a peroxidase-labeled goat anti-mouse IgG (Fc-specific) antibody (catalog no. 115 035 071; Dianova) diluted 1:5,000 was added for 1 h. After washing, bound peroxidase was detected by using 1 mg of *o*-phenylenediamine per ml and 1 μg of H_2O_2 per ml in 0.1 M KH_2PO_4 buffer (pH 6.0). The reaction was stopped after 5 min by adding 100 μl of 1.3 N H_2SO_4 per well, and the reaction product was quantified by measuring the $A_{492/405}$ in an automated ELISA reader.

Detection of uPA- and GAPDH-specific mRNAs. The uPA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene probes (catalog no. 57090 and 57328; American Type Culture Collection, Rockville, Md.) were separated from vector DNA by restriction enzyme digestion and preparative agarose gel electrophoresis. The insert DNA fragments were then purified by using a Geneclean kit (catalog no. 1001-400; Bio 101, La Jolla, Calif.) according to the manufacturer's instructions. U937 cells grown in RPMI 1640–10% FCS were washed in RPMI 1640 and then seeded in six-well plates at a density of 4×10^6 cells per ml in RPMI 1640. After 0, 4, 6, 7, 7.5, and 8 h, lip-OspA was added to the cells at a final concentration of 1 μg/ml. At 8 h, the cells were harvested, spun down at 1,000 × *g* at 4°C, and washed in cold RPMI 1640. Total cellular RNA was then prepared by acid guanidine thiocyanate-phenol-chloroform extraction (6). The extracted RNA was quantified by measuring the A_{260}. RNA (5 to 10 μg per lane) was size fractionated on 1.2% agarose–2.2 M formaldehyde gels and transferred to nylon membranes (catalog no. RPN 203N; Amersham, Braunschweig, Germany) by capillary or vacuum blotting. RNA was cross-linked to the nylon membrane by exposure to a UV light source (catalog no. 40072; Stratagene, Heidelberg, Germany).

Nylon membranes were prehybridized in Church buffer (0.5 M NaP_i [pH 7.2], 0.5 M EDTA, 7% sodium dodecyl sulfate [SDS]) for 4 h. The DNA probes were labeled with [α-^{32}P]dCTP by using a random primed labeling kit (catalog no. 1004760; Boehringer, Mannheim, Germany) and hybridized to the immobilized RNA in Church buffer at 67°C overnight. The membranes were washed at high stringency (2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS; 0.2× SSC–1% SDS) and then exposed to Kodak XAR film (catalog no. 1651454; Kodak, Rochester, N.Y.) at −70°C with an intensifier screen. Autoradiograms were scanned with an Image Master scanning system (Pharmacia, Freiburg, Germany). Densitometric measurements for uPA bands were normalized to the measurement for the corresponding GAPDH band.

Chromogenic substrate assay for plasmin or plasminogen activators. The assays were performed in 30 mM Tris HCl–60 mM NaCl (pH 7.4). To analyze plasmin activity in 96-well flat-bottom microtiter plates, 50 μl of the chromogenic substrate S-2251 (0.8 mg/ml) in assay buffer was added to 50 μl of the sample diluted in PBS or RPMI 1640–2 mM glutamine without phenol red. To analyze plasminogen activator activity, 0.5 U of plasminogen per ml was added to the mixture. The change in absorbance at 405 and 650 nm (ΔA) was monitored directly in the plate. Plasmin activity is given as ΔA per minute, and plasminogen activator activity is given as ΔA per minute squared.

RESULTS

Induction of (pro-)uPA in human monocytes. Freshly isolated peripheral blood monocytes or cells of the myelocytic/monocytic cell line U937 were incubated under serum-free conditions with intact *B. burgdorferi* organisms, spirochetal lysates, purified lip-OspA, or MDP-OspA. After 42 h, the supernatants (Fig. 1 and 2A) and cell lysates (Fig. 2B) were analyzed for (pro-)uPA by using an ELISA. With the exception of MDP-OspA, all spirochetal preparations induced the secretion of (pro-)uPA in a dose-dependent manner in both freshly isolated blood monocytes (Fig. 1A) and the myelocytic/monocytic cell line U937 (Fig. 1B). Moreover, a time-dependent increase of (pro-)uPA was observed in supernatants and lysates, as shown in Fig. 2 for isolated monocytes stimulated with lip-OspA. The fact that peripheral monocytes and U937 cells responded similarly suggested that the U937 cell line is suitable to study the mechanism of (pro-)uPA induction in myelocytic/monocytic cells. MDP-OspA, in which the 18 N-terminal amino acids are replaced by a methionine-aspartate-proline tripeptide to prevent lipidation, was unable to induce (pro-)uPA (Fig. 1). This result indicated that either the lipidation or the missing amino acids of MDP-OspA are critical for the stimulatory activity of OspA.

To analyze the expression of uPA at the transcriptional level, U937 cells were incubated with 1 μg of lip-OspA per ml for various intervals of time and the uPA-specific mRNA was determined. A significant increase of uPA-specific mRNA was first seen after 2 h, reaching an optimum at 4 h (eightfold increase above uPA mRNA levels of unstimulated cells) and subsequently declining (Fig. 3).

Characterization of secreted (pro-)uPA. Next we analyzed whether (pro-)uPA released from stimulated peripheral blood monocytes was present as the enzymatically inactive precursor form, i.e., pro-uPA. Pro-uPA can be activated to uPA by plasmin treatment. Plasmin-treated and nontreated monocyte-conditioned media were analyzed for uPA activity (Table 1). The uPA activity was enhanced ≈7-fold after plasmin treatment, while uPA activity was not enhanced when the plasmin inhibitor aprotinin was present during the plasmin treatment. The data show that a large fraction of uPA present in the supernatants of lip-OspA-stimulated peripheral monocytes was present in its precursor form, i.e., pro-uPA.

Functional consequences of the interaction between *B. burgdorferi*, plasminogen, and pro-uPA. Given the previous observations that *B. burgdorferi* binds plasmin(ogen) (7, 9, 13, 15)

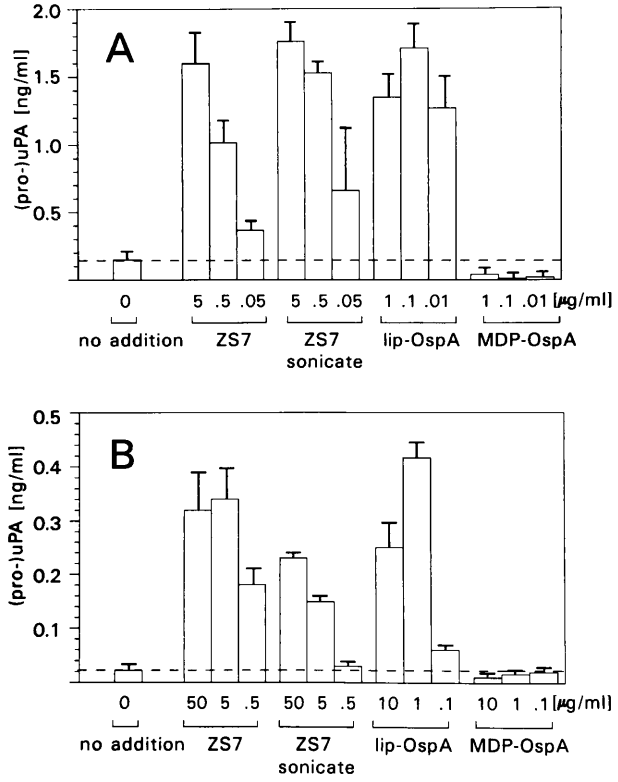

FIG. 1. (pro-)uPA production upon stimulation with different *B. burgdorferi* and OspA preparations. (A) Normal human monocytes (0.36×10^6 per well) were incubated with the indicated stimuli for 42 h at 37°C under serum-free conditions. The (pro-)uPA content in the conditioned medium was analyzed by using a (pro-)uPA-specific ELISA. (B) Cells of the myelocytic/monocytic cell line U937 (1.5×10^6/ml) were incubated with the indicated stimuli for 18 h at 37°C. The conditioned medium was analyzed for (pro-)uPA by using a (pro-)uPA-specific ELISA. Mean values and standard deviations of quadruplicate determinations are given.

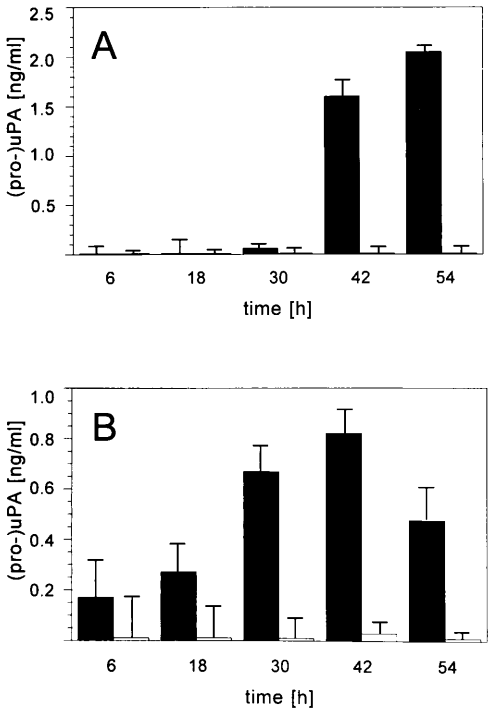

FIG. 2. Time dependence of (pro-)uPA induction in monocyte conditioned medium and cell lysates. Normal human monocytes (0.36×10^6 per well) were incubated with 1 μg of lip-OspA per ml for different intervals of time at 37°C. (A) The conditioned medium was analyzed in a (pro-)uPA-specific ELISA. (B) After being washed with PBS, the monocytes were lysed by using 0.1% Triton X-100 in 100 mM Tris (pH 7.0). After centrifugation, the lysates were analyzed for (pro-)uPA by using a (pro-)uPA-specific ELISA. Closed bars, Lip-OspA-stimulated monocyte-conditioned medium; open bars, control medium of unstimulated monocytes. Mean values and standard deviations of triplicate determinations are given.

and (pro-)uPA (15), we examined whether the interaction between spirochetes, pro-uPA, and plasminogen would accelerate plasmin generation. Pro-uPA and plasminogen were mixed in the absence and presence of spirochetes, and the generation of plasmin was monitored. Plasmin generation was ≈80-fold faster in the presence than in the absence of spirochetes (Table 2). Generation of plasmin was blocked either by anticatalytic polyclonal goat anti-uPA IgG or by the plasmin inhibitor aprotinin. Moreover, no plasmin was generated in the presence of the lysine analog tranexamic acid (Table 2), which is known to inhibit plasmin(ogen) binding to spirochetes (9). Since tranexamic acid does not influence uPA-mediated plasminogen activation in solution (27), the data indicate that the binding of plasmin(ogen) to the surface of the spirochetes is required for the enhancement of plasmin formation in a pro-uPA–plasminogen mixture.

Generation of spirochete-bound plasmin in the presence of pro-uPA and plasminogen. Spirochetes were incubated with 3 μM plasminogen, which corresponds to the concentration in human serum, and with pro-uPA (2 ng/ml). After 4 h of incubation at 37°C, the spirochetes were washed and surface-associated plasmin activity was analyzed by using the plasmin-specific chromogenic substrate S-2251. Spirochetes incubated with pro-uPA showed ≈48-fold higher surface-associated plasmin activity than spirochetes incubated without pro-uPA (Table 3). Again the effect of pro-uPA was blocked by anticatalytic goat anti-uPA IgG and tranexamic acid (Table 3).

Generation of spirochete-bound plasmin in the presence of pro-uPA and serum. Previous studies have shown that soluble plasmin is inhibitable by α_2-antiplasmin whereas spirochete-bound plasmin is protected from inhibition (9). We examined whether pro-uPA would lead to the generation of spirochete-

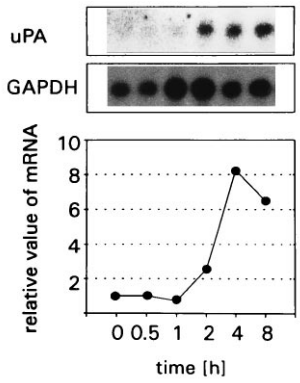

FIG. 3. Induction of uPA-specific mRNA. uPA-specific mRNA was analyzed in lip-OspA-stimulated U937 cells by Northern (RNA) blotting. Total RNA was prepared as described in Materials and Methods from U937 cells that had been incubated for the indicated time with 1 μg of lip-OspA per ml. The blots were hybridized with [^{32}P]uPA cDNA, cleaned, and rehybridized with [^{32}P]GAPDH cDNA. The autoradiograms of a representative Northern blot are shown. The line graph was derived from the uPA and GAPDH densitometric measurement for each time point. The data are expressed relative to the value for GAPDH.

TABLE 1. Characterization of pro-uPA secreted by lip-OspA-stimulated monocytes[a]

Prepn	$\Delta A/\text{min}^2$ (10^{-6})	Relative activity
Supernatant	1.32 ± 0.13	0.14
Supernatant + plasmin	9.38 ± 1.38	1.00
Supernatant + plasmin + aprotinin	1.25 ± 0.03	0.13
RPMI 1640 + plasmin	0.75 ± 0.00	0.08

[a] Supernatants of lip-OspA (0.1 μg/ml, 42 h, 37°C) stimulated human monocytes were added into microtiter plates coated with goat anti (pro-)uPA IgG. After washing, the bound compounds were treated with 100 μl of a solution of 0.02 U plasmin per ml for 30 min at 37°C. The plasmin activity was then blocked by adding 100 μl of the plasmin-specific inhibitor aprotinin (100 U/ml). As a control, aprotinin was added during the incubation period with plasmin. After washing, the plasminogen activator activity was analyzed by using plasminogen (0.5 U/ml) and the chromogenic plasmin substrate S-2251 (0.4 mg/ml). Mean values and standard deviations of triplicate determinations are given.

associated plasmin in the presence of serum, which contains not only plasminogen but also abundant amounts of inhibitors for plasmin and plasminogen activators. Spirochetes were incubated in 50% (vol/vol) human AB^+ serum in the absence or presence of pro-uPA (2 ng/ml) for 4 h at 37°C and then washed, and the spirochete-associated plasmin activity was determined by using the chromogenic plasmin substrate S-2251. Spirochetes incubated with pro-uPA contained much higher amounts of plasmin activity than the control spirochetes (Table 4) that had been incubated in serum but in the absence of pro-uPA. The induction of spirochete-associated plasmin was inhibitable by goat anti-uPA IgG, by tranexamic acid and by aprotinin (Table 4). The findings that aprotinin as well as tranexamic acid prevented the pro-uPA-mediated generation of spirochete-associated plasmin activity suggested that plasmin activity is required during the preincubation period, most likely to convert pro-uPA to uPA. Moreover, the bound plasmin was inhibitable by aprotinin and could be eluted from the spirochetes by tranexamic acid (data not shown).

Generation of spirochete-bound plasmin in the presence of plasminogen and monocyte-conditioned medium. Next we analyzed whether the supernatants of stimulated monocytes induced spirochete-bound plasmin. Spirochetes were mixed with concentrated conditioned medium and plasminogen and incubated for 4 h and then washed, and spirochete-associated plasmin activity was determined by using the chromogenic plasmin substrate S-2251. Spirochetes incubated with conditioned medium of lip-OspA-stimulated monocytes had bound ≈15-fold-higher plasmin activity on their surface when compared with spirochetes incubated in control medium (Table 5). Again the generation of surface-associated plasmin was inhibited by the anticatalytic goat anti-uPA IgG and tranexamic acid.

TABLE 2. Accelerated plasmin generation in a pro-uPA–plasminogen mixture in the presence of intact spirochetes[a]

Mixture component(s)[b]	$\Delta A/\text{min}^2$ (10^{-4})
B. burgdorferi + plasminogen	0.000 ± 0.009
B. burgdorferi + pro-uPA + plasminogen	1.413 ± 0.030
B. burgdorferi + pro-uPA + plasminogen + tranexamic acid	0.026 ± 0.002
B. burgdorferi + pro-uPA + plasminogen + goat anti-uPA	0.004 ± 0.002
B. burgdorferi + pro-uPA + plasminogen + aprotinin	0.000 ± 0.002
B. burgdorferi + pro-uPA	0.000 ± 0.004
Pro-uPA + plasminogen	0.016 ± 0.008

[a] The effect of *B. burgdorferi* on plasmin generation in a chromogenic substrate assay was tested by mixing the plasmin-specific substrate S-2251 (0.4 mg/ml), plasminogen (0.5 U/ml), pro-uPA (1 ng/ml), and spirochetes (150 μg/ml). Moreover, either tranexamic acid (15 mM), anticatalytic goat anti-uPA IgG (100 μg/ml), or aprotinin (100 U/ml) was added. Mean values and standard deviations of triplicate determinations are given.

[b] In addition to S-2251.

TABLE 3. Generation of spirochete-bound plasmin in the presence of pro-uPA and plasminogen[a]

Pretreatment of *B. burgdorferi*	$\Delta A/\text{min}$ (10^{-3})
Plasminogen	0.300 ± 0.067
Pro-uPA + plasminogen	14.422 ± 1.296
Pro-uPA + plasminogen + tranexamic acid	1.989 ± 2.522
Pro-uPA + plasminogen + goat anti-uPA	1.744 ± 0.107

[a] Spirochetes (600 μg/ml) were incubated with plasminogen (3 μM) and pro-uPA (2 ng/ml), tranexamic acid (15 mM), or anticatalytic goat anti-uPA IgG (100 μg/ml) for 4 h at 37°C. After extensive washing, the cell-bound plasmin was determined by adding the chromogenic plasmin substrate S-2251. Mean values and standard deviations of triplicate determinations are given.

DISCUSSION

Here we report that *B. burgdorferi* and its purified outer surface lipoprotein A induce pro-uPA in isolated peripheral blood monocytes. Moreover, we show that *B. burgdorferi* organisms accelerate the generation of plasmin in a pro-uPA–plasminogen mixture, finally resulting in spirochete-associated plasmin. The significance of these findings is indicated by the previous observations that (i) *B. burgdorferi* organisms bind plasminogen and uPA (7, 9, 13, 15); (ii) uPA converts spirochete-bound plasminogen into active plasmin, and plasmin-loaded spirochetes degrade the high-molecular-weight matrix glycoprotein fibronectin (9); (iii) plasmin-coated spirochetes show an enhanced ability to penetrate endothelial cell monolayers grown on connective tissue substrates compared with untreated controls (7); and (iv) in a mouse model of infection, uPA-loaded *B. burgdorferi* organisms are more infectious than control spirochetes (15).

Although we found that large amounts of uPA antigen (up to 2 ng/ml) are present in the cell culture supernatants (Fig. 1A) of stimulated monocytes, no uPA activity was demonstrable. uPA activity in the supernatants was, however, demonstrable upon plasmin treatment of the supernatants (Table 1). This result indicates that at least part of the uPA was present in its single-chain form (pro-uPA) that could be converted into the active form by plasmin-mediated proteolysis.

Given this finding, the second part of our study was focused on the consequences of the interaction between spirochetes,

TABLE 4. Generation of spirochete-bound plasmin in the presence of pro-uPA and serum[a]

Pretreatment of *B. burgdorferi*	$\Delta A/\text{min}$ (10^{-3})
Serum	0.000 ± 0.100
Pro-uPA + serum	1.288 ± 0.251
Pro-uPA + serum + tranexamic acid	0.000 ± 0.058
Pro-uPA + serum + goat anti-uPA	0.000 ± 0.236
Pro-uPA + serum + aprotinin	0.050 ± 0.083

[a] Spirochetes (600 μg/ml) were incubated with 50% human AB^+ serum and pro-uPA (2 ng/ml), tranexamic acid (15 mM), an anticatalytic goat anti-uPA IgG (100 μg/ml), or aprotinin (100 U/ml) for 4 h at 37°C. Unbound enzyme was removed by extensive washing of the spirochete. Afterwards, cell-bound plasmin was determined by using the chromogenic substrate S-2251. Mean values and standard deviations of triplicate determinations are given.

TABLE 5. Generation of spirochete-bound plasmin in the presence of plasminogen and monocyte-conditioned medium[a]

Pretreatment of *B. burgdorferi*	ΔA/min (10^{-3})
RPMI + plasminogen	0.072 ± 0.053
Conditioned medium + plasminogen	1.078 ± 0.210
Conditioned medium + plasminogen + tranexamic acid	0.333 ± 0.009
Conditioned medium + plasminogen + goat anti-uPA	0.125 ± 0.082

[a] Conditioned medium of lip-OspA (0.1 μg/ml, 42 h, 37°C)-stimulated monocytes was concentrated 20-fold by using the Centricon 30 ultrafiltration system. The concentrated medium, spirochetes (600 μg/ml), and plasminogen (3 μM) were incubated for 4 h at 37°C together with tranexamic acid (15 mM) or an anticatalytic goat anti-uPA IgG (100 μg/ml). After washing, spirochete-associated plasmin activity was analyzed by using the plasmin-specific chromogenic substrate S-2251. Mean values and standard deviations of triplicate determinations are given.

plasminogen, and pro-uPA. We found that the spirochetal surface provides an appropriate template to accelerate plasmin generation in a microenvironment that is rich in pro-uPA and plasminogen, even when plasmin and plasminogen activator inhibitors are present in excess, as in serum (compare Table 4).

Plasmin is one of the most potent pro-uPA-activating enzymes (2). It has been suggested that trace amounts of plasmin initiate the activation reaction that is further amplified by reciprocal (pro-)uPA–plasmin(ogen) interactions, in particular when both compounds are colocalized at appropriate surfaces (2). On the other hand, pro-uPA has little though significant activity (23). It has been found that in HT-1080 osteosarcoma cells, plasminogen bound to the cell surface can be activated by pro-uPA that is bound to its specific cell surface receptor (CD87). The newly formed plasmin can, in turn, activate single-chain uPA to the two-chain form with largely increased enzymatic activity. This provides a mechanism for the feedback amplification of plasminogen activation at cellular surfaces (23). In view of the previous findings that spirochetes bind plasmin(ogen) (7, 9, 13, 15) as well as uPA (15), we speculate that the spirochetal surface provides a similar mechanism for the acceleration of plasminogen activation that could be operative in a microenvironment that is rich in pro-uPA and plasminogen. Several candidate molecules that can serve as spirochetal plasmin(ogen) receptors have been identified. The (pro-)uPA binding structures (15), however, remain to be characterized in detail.

It is to be expected that the spirochetal plasmin(ogen) binding sites are occupied especially in inflammatory lesions, when a plasma-derived plasminogen-rich exudate is formed. It may thus be the level of monocyte/macrophage-derived pro-uPA that regulates the local generation of spirochete-associated plasmin. This view is supported by the finding that pro-uPA leads to the generation of spirochete-associated plasmin even in the presence of serum which contains excessive amounts of inhibitors for plasmin and plasminogen activators (Table 4). In a local inflammatory environment, this process may be even more enhanced, since the natural plasmin inhibitor, α_2-antiplasmin (24), as well as a major plasminogen inhibitor, PAI-1 (1), are functionally inactivated by reactive oxygen intermediates (4, 32, 33) that are also generated by activated monocytes/macrophages.

The natural history of a *B. burgdorferi* infection involves a local inflammatory lesion in the skin beginning at the site of the tick bite (termed erythema migrans). The inflammatory infiltrate consists predominantly of mononuclear cells with a great percentage of monocytes/macrophages (29, 30, 38). The interaction between *B. burgdorferi* organisms and monocytes/macrophages leads to the induction of proinflammatory cytokines (interleukin-1 and tumor necrosis factor alpha) (14, 25, 26, 37). It has previously been shown that proinflammatory cytokines such as interleukin-1 and tumor necrosis factor alpha induce the production and secretion of uPA in monocytes/macrophages (11, 12). It remains undetermined whether pro-uPA is induced directly or via these cytokines. Finally, the induction of uPA may not be restricted to monocytes/macrophages but may also involve local tissue constituent cells, such as fibroblasts and endothelial cells, that have also been shown to secrete pro-uPA upon appropriate stimulation (20; for a review, see reference 8).

We have presented evidence that *B. burgdorferi* may induce pro-uPA in its inflammatory microenvironment and that the spirochetal surface provides an appropriate template for subsequent interaction between (pro-)uPA and plasmin(ogen) leading to efficient uPA-dependent plasmin generation. These mechanisms may favor spirochetal dissemination and pathogenicity.

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